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Award Number: DAMD17-99-1-9260

TITLE: The Importance of ATM Mutations and Polymorphisms in Breast Cancer and Radiation Sensitivity

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REPORT DATE: October 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2003	Annual Summary (1 Oct 1999 - 30 Sep 2003)

4. TITLE AND SUBTITLE
The Importance of ATM Mutations and Polymorphisms in Breast Cancer and Radiation Sensitivity

5. FUNDING NUMBERS
DAMD17-99-1-9260

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The objective of my 4-year Career Development Award was to determine whether ATM heterozygosity contributes to breast cancer development and radiation injury. We sequenced the ATM cDNA of 93 breast cancer patients and found 4 repetitive single-base genetic variants in the ATM cDNA. We then compared the frequency of these variants to that of a control set of samples from 996 individuals without cancer. We found that a Ser49Cys variant was more commonly represented in the breast cancer patients (6.7% vs 1.3%, p=0.006). In addition, we found that a Pro1954Arg variant was more common in a group of 27 Caucasian patients who experienced a significant normal tissue injury after radiation treatments compared to the Caucasian controls(18.5% vs 6.6%, p=0.037). We also developed an in vitro assay that isolated protein complexes that bind double-strand breaks in order to study the role of ATM protein in double-strand break repair. We identified that ATM and at least ten other proteins make up a clinically relevant protein complex. We found that the amount of this complex in both normal and tumor cells strongly correlated with their radiosensitivity. We feel this assay may prove to have significant clinical relevance as a predictor of both tumor and host radiosensitivity.

14. SUBJECT TERMS
ATM mutations, breast cancer development, radiosensitivity

15. NUMBER OF PAGES
364

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Overview of the Impact of the Career Development Award

The stated purpose of the Career Development Award is to help junior investigators, without a proven track record in breast cancer research, develop productive careers focused on breast cancer research. Philosophically, the four-year support of my Career Development Award enabled me to achieve this goal. Specifically, the support of this award allowed my department to provide me with time protected from administrative and clinical responsibilities to begin a career track focused exclusively on breast cancer treatment, education and research. On October 1, 1999, at the time when my Career Development Award funding began, I was an Assistant Professor in Radiation Oncology with 8 peer-review publications that focused on breast cancer. Over the four-year award period, I was able to publish or co-author 54 peer-review breast cancer research articles, 9 invited breast cancer research articles, 38 breast cancer editorials/commentaries, and 5 breast cancer book chapters. In addition, over this four-year period, either myself or a trainee under my mentorship presented original breast cancer research at the following national/international meetings: American Society of Therapeutic Radiology and Oncology annual meeting (8 presentations), the American Radium Society Annual Meeting (7 presentations), American Society of Clinical Oncology (3 presentations), San Antonio Breast Cancer Symposium (2 presentations), Radiation Research Society (1 presentation), ECCO11 European Cancer Conference (1 presentation), First International Congress on Translational Research in Radiation Oncology (1 presentation), Department of Defense Era of Hope Meeting (1 presentation). In addition, during the period of my award, I was also invited as a breast cancer research speaker at a number of national/international breast cancer conferences including Radiological Society of North America, and breast cancer symposia in Hiroshima, Kyoto, Austin, Houston, Bali, Taiwan, Steamboat Springs, Istanbul, Mexico City, Rovigo, Seoul, Corpus Christi, and Banff.

My breast cancer research efforts during the period of my funding have led to a number of awards. In the second year of the award I was promoted to Associate Professor with tenure and awarded a travel grant from the First International Congress on Translational Research in Radiation Oncology. In the third year of the award, I received the annual Faculty Scholar Award of the University of Texas M. D. Anderson Cancer Center. I was the first faculty member of our department to ever receive this recognition. Trainees who I have mentored in research projects during this period have won research awards from the American Society of Therapeutic Radiology and Oncology, the American Radium Society, the American Society of Clinical Oncology, and the San Antonio Cancer Breast Symposium.

My career interest in breast cancer research, education, and treatment have become more solidified over the past four-years. I was recently appointed as program director and section chief of breast radiation oncology. The breast cancer radiation oncology program at M. D. Anderson currently treats more breast cancer patients than any other single-institution in the United States. Currently, 100% of my clinical and research efforts remain committed to breast cancer. I spend approximately 45% of my effort in clinical care, 30% on clinical and translational research, and 25% on education and administration.

My future career goals are to continue to contribute to breast cancer research and further develop the clinical and translational breast cancer research program within our institution. I am currently being considered for promotion to Professor and have been challenged by my department chair to begin to help mentor more junior faculties research efforts in breast cancer. Our clinical volume is projected to significantly increase over the next five years and currently are recruiting to add a 5^{th} and 6^{th} faculty member whose clinical and research efforts will be exclusive to breast cancer radiation oncology. As leader of this service, my efforts will increasingly be to help facilitate the academic growth of our new faculty. As such, our service will have increased opportunities to significantly impact breast cancer treatment and research.

In addition to the goals I have to contribute within our own department, my future efforts will include continuing to contribute to multidisciplinary research efforts in breast cancer research. I am a member of the

M. D. Anderson breast cancer research steering committee and together we are working towards an application for a SPORE grant in this upcoming year.

The funding and support of the Career Development Award were critical in allowing me to focus my research efforts exclusively in breast cancer and help to facilitate my achievements to date. Furthermore, the award helped to solidify my research interests in breast cancer and provided me with a foundation on which to further grow.

Body

The major focus in the statement of work was to investigate whether ATM heterozygosity was associated with breast cancer development. The rationale for such a study was based on epidemiological evidence suggesting that obligate ATM heterozygosity may increase the relative risk of breast cancer.[1-4] Subsequent to cloning of the ATM gene in 1995, a number of groups have tested for an association between ATM heterozygosity and breast cancer development with direct sequencing studies. Thus far, these studies have produced contradictory results.[6-15] The discrepancy in these reports is difficult to interpret, in that each involved a different subset of breast cancer patients, most had small sample sizes, and many different assays were used among these studies to detect ATM mutations. The methodology used to detect ATM mutations is of particular importance, with studies that have used a protein truncation assay failing to find an association between ATM mutations and breast cancer.[13,15] These studies suggest that deletions or major frameshifts mutations in ATM are not significant contributors to breast cancer risk. However, other studies that sequenced ATM have reported that a significant percentage of patients have single base change variants.[6,9-12, 14] The biological significance of these changes remains largely unknown and it is unclear whether these variants directly contribute to breast cancer risk. In the work supported by my Career Development Award, we were able to identify a single base change variant that was significantly more frequent in the breast cancer patients compared to controls. A manuscript detailing the results of this component of the study is included as Appendix 1. This manuscript is currently under consideration for publication in Cancer.

To briefly summarize the results of this study, we found that a Ser49Cys genetic variant was more common in breast cancer patients and a subgroup of patients with bilateral breast cancer than in a large sample size of normal controls with no personal cancer history. The details of these data are shown in the

table below. For further details concerning the methodology and results, please see the full manuscript (Appendix 1).

Table 1, Frequency of genetic variants between breast cancer cases and controls

Site	Genotype	Frequencies (%) in		P-value
		Cases	Controls	
Asp1853Asn*	Asp/Asp	39/58 (67.2%)	394/528 (74.6%)	P=0.807
	Asp/Asn	17/58 (29.3%)	119/528 (22.5%)	
	Asn/Asn	2/58 (3.4%)	15/528 (2.8%)	
Pro1954Arg*	Pro/Pro	58/61 (95.1%)	476/510 (93.2%)	P=0.841
	Pro/Arg	3/61 (4.9%)	33/510 (6.5%)	
	Arg/Arg	0/61 (0%)	1/510 (0.2%)	
Ser49Cys	Ser/Ser	70/75 (93.3)%	928/940 (98.7%)	P=0.006
	Ser/Cys	5/75 (6.7%)	12/940 (1.3%)	
	Cys/Cys	0/75 (0%)	0/940 (0%)	

*represent frequencies only in Caucasian cases and controls

This component of our research is the first report associating breast cancer with the Ser49Cys genetic variant. These data justify further study to determine whether the Ser49Cys variant may confer an increased risk for developing breast cancer.

A second aim was to determine whether there was an association between ATM heterozygosity and the development of a normal tissue injury after ionizing radiation treatment. The rationale to study this aim was previously published data that suggested that normal skin fibroblasts from individuals with a

heterozygous ATM mutation have in vitro evidence of cellular radiosensitivity compared to controls.[16] To evaluate this aim, we sequenced ATM cDNA from 45 individuals with significant radiation injuries (RTOG grade III or IV). As radiation complications are relatively uncommon, we elected to not limit our cases to breast cancer patients. Similar to our breast cancer development study, the data from our radiosensitivity study also found a positive correlation. We are finalizing further details concerning these data and plan to submit a manuscript describing our findings in the near future. This manuscript will acknowledge the support provided by the Career Development Award. A summary of our results showed:

Table 2, Frequency of genetic variants between radiation injury breast cancer cases and controls

Site	Genotype	Frequencies (%) in		P-value
		Cases	Controls	
Pro1954Arg*	Pro/Pro	22/27 (81.5%)	494/528 (93.6%)	P=0.030
	Pro/Arg	5/27 (18.5%)	34/528 (6.4%)$^{+}$	

* represent frequencies only in Caucasian cases and controls

$^{+}$ includes one case with a Arg/Arg homozygous genotype

In total, we successfully sequenced the Pro1954Arg region of ATM in 112 cancer patients and indentified 7 patients with the Pro1954Arg variant. As noted above, 5 of these individuals were Caucasians who had a significant normal tissue complication after radiation treatments. One the two other individuals also experienced a complication but was not included in the statistics above because he was of Arabic decent. The final individual elected not to be treated with radiation. The rates of radiation-induced complications

between the irradiated patients with Pro/Arg variant and those with a Pro/Pro haplotype are shown in the table below.

Site	Genotype	Frequencies (%) in		P-value
		Cases – Injury	Cases – No Injury	
Pro1954Arg*	Pro/Pro	26/119 (21.8%)	93/119 (78.2%)	P<0.001
	Pro/Arg	6/6 (100%)	0/6 (0%)	

This component of our research provided the first reported evidence that a Pro1954Arg variant may be associated with a radiation injury. If confirmed by other studies, this finding would have significant clinical relevancy for the practice of Radiation Oncology, in that a simple allele specific test could be developed to screen for patients about to receive radiation in order to stratify their risk of injury.

A final aim of our research was to develop a functional assay to study ATM and radiosensitivity. We developed and patented an electrophoretic mobility shift assay (EMSA) and identified an ATM-containing molecular complex involved in the repair of DNA double-strand breaks. Human cells with homozygous ATM mutations lost the particular band of interest and anti-ATM antibodies altered the mobility of the bands. Our first objective was to identify the protein complexes present in this band. We have recently submitted a manuscript describing the data from these investigations to Clinical Cancer Research. This paper is included as Appendix 2. In summary, we found that the band contained ATM, Ku70, DNA Ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51 and p53.

We also discovered that the density of the band correlated with cellular radiosensitivity, as defined by a clonogenic survival assay (SF2 – surviving fraction at 2 Gy). However, the intranuclear concentrations of the proteins we found to be present in the complex did not correlate with either the SF2, which may suggest

that radiosensitivity resulted from a post-translational modification of EMSA band components. Finally, the band density also correlated with the presence or absence of a BRCA-1 or BRCA-2 mutation. These data are important in that they suggest that band intensity may predict both cellular radiosensitivity and also predict BRCA mutation status.

Finally, we have conducted further studies concerning the relationship of the EMSA band to both normal and tumor cellular radiosensitivity. These data have been accepted for publication in a separate manuscript to Clinical Cancer Research. This manuscript is included in Appendix 3. In this work, we correlated the band density and SF2 in 21 primary human fibroblast cultures and 15 tumor cell lines and found a significant correlation for both the fibroblasts and tumor cell lines. These data indicate that EMSA analysis may be a practical, clinically relevant method to predict both tumor and primary cell radiosensitivity. Confirmation of such a finding would have a significant impact on the practice of radiation treatments for breast cancer.

Key research accomplishments

- Established a control bank of DNA from 996 individuals without a cancer history for population comparison studies
- Developed an allele specific oligonucleotide assay for 3 repetitive single nucleotide base changes in the ATM gene
- Established a collaborative effort that demonstrated the feasibility of using haplotype association studies to detect single nucleotide changes in the ATM gene.
- Demonstrated bilateral breast cancer patients have an increased number of radiation-induced chromatid breaks compared to control showing the feasibility of a phenotype rather than genotype assay to predict breast cancer risk.
- Identified that breast cancer patients are more likely to have a Ser49Cys genetic variant compared to controls
- Identified that the Ser49Cys variant is also more common in patients with bilateral breast cancer compared to controls
- Identified that patients who develop radiation injuries are more likely to have a Pro1956Arg variant than controls.
- Identified that the rate of significant radiation injuries in treated patients with a Pro1956Arg variant was 100%, and statistically much higher than the normal Pro1956Pro haplotype.
- Demonstrated that the ATM protein is present in an EMSA molecular complex
- Identified that this complex also contains the following proteins: Ku70, DNA Ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51 and p53.
- Showed the density of this complex strongly correlates with cellular radiosensitivity in both normal fibroblasts and tumor cell lines

- Showed the density of this complex correlates with the presence of a germline mutation in BRCA1 or BRCA2.

Reportable Outcomes

- manuscripts:

Submitted Articles In Which the Support from the Career Development Award Was Acknowledged
*** Denotes the article in which the award was acknowledged (these articles are included in Appendix with the acknowledgement of the Award highlighted)**

1. **Buchholz TA**, Weil MM, Ashorn CL, Strom EA, Sigurdson A, Bondy M, chakraborty R, Cox JD, McNeese MD, and Story MD: A Ser49Cys Variatn in the Ataxia Telangiectasia, Mutated (ATM) Gene that is More Common in Breast Cancer Patients Compared to Population Controls. Cancer, 2003 (submitted) (included as Appendix 1).*

2. Ismail SM, **Buchholz TA**, Story M, Brock WA, and Stevens CW: DNA end-binding complex density correlates with SF2 but not with the protein level of band components. Clin Cancer Res, 2003. (peer-review article, submitted) (included as Appendix 2).*

3. Ismail SM, Puppi M, Prithivirajsingh S, Munshi A, Raja U, Meyn R, **Buchholz TA**, Story M, Brock WA, Milas L, Stevens CW: Predicting radiosensitivity using DNA end-binding complex analysis. Clin Cancer Res, 2003. (peer-review article, in press) (included as Appendix 3).*

Published Articles During the period of the Career Development Award.
*** Denotes the article in which the award was acknowledged (these articles are included in Appendix 4 with the acknowledgement of the Award highlighted)**

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– patents and licenses applied for and/or issued:

1. StevenS CW, Buchholz TA, Ismail S, Story M, Brock W: Electrophoretic assay to predict risk of cancer and the efficacy and toxicity of cancer therapy, U.S., MDA02-014, pending

– degrees obtained that are supported by this award: none

– development of cell lines, tissue or serum repositories

1. established a control bank of DNA from 996 individuals with no cancer history to serve for population frequency testing

– informatics such as databases and animal models, etc:

1. established a database for enrolled patients and controls that cover patient demographics, cancer history, and toxicity from radiation treatment.

- funding applied for based on work supported by this award: none

- employment or research opportunities applied for and/or received on experiences/training supported by this award: none.

Conclusion

Over the period of the Career Development Award, we have been successful in sequencing ATM cDNA in breast cancer patients and patients with radiation injury and found two potentially clinically relevant genetic variants in ATM. The first is a single-base change that leads to a Ser49Cys substitution. This variant is more common in breast cancer patients and patients with bilateral breast cancer compared to controls. These data indicate that this variant may be associated with breast cancer development and warrant further studies into the possible functional consequence of this base change in ATM. The second genetic variant, Pro1946Arg, was more common in individuals with a significant radiation injury compared to controls. These data suggest that this variant may increase cellular radiosensitivity.

Establishing a relationship between ATM and breast cancer development and normal tissue toxicity following breast cancer radiation treatment would be a significant contribution to breast cancer research. If confirmed, our data could prove to be useful to further stratify an individual's risk of developing breast cancer. In addition, patients who were to receive radiation can be tested for this genetic variant and modifications of the therapeutic strategies may be adopted to avoid significant radiation injury.

Finally, we have been successful in identifying a potentially clinically relevant predictive assay for both tumors and normal tissue. This assay measures the density of a protein complex in which ATM is a part. We have shown that band density significantly correlates with radiosensitivity of tumor cell lines and normal tissues in vitro. We now plan to move to assess response of human tumors and also assess the value of this assay in predicting radiation injury in treated patients.

The support from the Career Development Award has provided me with the opportunity of launching a career dedicated to breast cancer research and treatment. With the support from this grant, I have been able to achieve my stated goals and build a foundation of skills on which to build a successful academic career. Thank you for this support.

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Appendices 1-29

APPENDIX 1

A Ser49Cys Variant in the Ataxia Telangiectasia, Mutated (ATM) Gene that is More Common in Breast Cancer Patients Compared to Population Controls

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Running title: Genetic variant in ATM and breast cancer

Number of pages: text – 17, tables - 3, figures – 0

This work and Dr. Buchholz were supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154, and the Kleberg Fund for New and Innovative Research (MDS). We wish to acknowledge Scott Manatt for his contribution in collecting samples for the control population.

Condensed Abstract

The cDNA of the ATM gene was sequenced in 91 breast cancer patients for mutations or polymorphisms. A single oligonucleotide genetic variant causing a Ser49Cys change in the protein was present in 6.7% (5/75) versus a frequency of this variant of 1.3% (12/940) in a control population, (P=0.006, by Fisher's 2-sided exact test). These data warrant further investigations to determine whether this genetic variant in ATM increases the risk for breast cancer development.

Abstract

Background: Mothers of children who have ataxia telangiectasia have been reported to be at increased risk for breast cancer development. To test whether sequence variants in the ataxia telangiectasia (ATM) gene are associated with breast cancer, we compared the frequency of ATM cDNA sequence changes in breast cancer patients and controls.

Methods: We sequenced ATM cDNA in 91 breast cancer patients and compared sequence changes in these patients to the frequency of these variants in a control set of 940 individuals with no cancer history.

Results: Thirty-five breast cancer patients had one or more single base changes in ATM. Three genetic variants were found in at least two patients. These variants resulted in Asp1853Asn, Pro1054Arg, or Ser49Cys amino acid substitutions in the ATM protein. The Ser49Cys variant was more common in the breast cancer patients than the controls, with respective frequencys of 6.7% (5/75) and 1.3% (12/940), (P=0.006, by Fisher's 2-sided exact test). The subgroup of patients with bilateral breast cancer had a Ser49Cys

frequency frequency of 11.8% (2/17), which again was significantly different from the control group (P=0.024, by Fisher's 2-sided exact test). The allele frequencies of the other two variants were not different between cases and controls.

Conclusions: Breast cancer patients, particularly those with bilateral disease, were more likely to have a variant in the ATM gene that resulted in a Ser49Cys change in the protein. These data raise the question of whether the Ser49Cys change may have functional consequences and justify further studies to determine the relevance of this variant in breast cancer development.

Key Words: Breast cancer, ATM gene, variants, polymorphisms

Introduction

The genetic determinants of breast cancer risk remain elusive for the majority of patients. However, some recent evidence suggests that abnormalities in the ATM gene (ataxia telangiectasia, mutated) may be a contributor to breast cancer risk. Individuals with homozygous ATM mutations develop the rare autosomal recessive disease ataxia telangiectasia (AT). A germline mutation in one ATM allele (ATM heterozygosity) does not produce a recognizable phenotypic change, but epidemiological evidence suggests that ATM heterozygosity may increase the relative risk of breast cancer.[1-4] The first evidence linking ATM heterozygosity and breast cancer came from studies of AT families. From these studies, it was estimated that 6% - 8% of breast cancers in the United States occur in ATM heterozygotes[1-5], which would make this condition a more common genetic contributor to breast cancer than the combined number of patients with a mutation in BRCA1 or BRCA2.

The ATM gene was cloned in 1995 and since then a number of groups have undertaken molecular epidemiology studies to test for an association between ATM heterozygosity and breast cancer development. Thus far, these screening studies have produced contradictory results.[6-15] The discrepancy in these reports is difficult to interpret, in that each involved a different subset of breast cancer patients, most had small sample sizes, and many different assays were used among these studies to detect ATM mutations. The methodology used to detect ATM mutations is of particular importance. Mutations in patients with AT lead to protein truncations in approximately 80% of the cases, which led some investigators to use a protein truncation assay for ATM

heterozygosity studies. In general, the studies using such assays have failed to find a association between ATM mutations and breast cancer.[13,15] These studies suggest that deletions or major frameshifts mutations in ATM are not significant contributors to breast cancer risk. What remains less clear is whether some ATM missense mutations are associated with a significant risk for breast cancer development.

Based on direct sequencing of the ATM gene in breast cancer patients, a number of studies have reported that a significant percentage of patients have single base change variants.[6,9-12, 14] However, the biological significance of these changes remains largely unknown and it is unclear whether these variants directly contribute to breast cancer risk.

In this study, we set out to determine the relevance of single base changes to the risk of breast cancer. We sequenced the ATM cDNA from breast cancer patients and studied the frequencies of identified variants in a large control population. One of the variants we identified was significantly more frequent in the breast cancer group.

Methods

Study population

This study was conducted on protocols approved by the surveillance committee of The University of Texas M. D. Anderson Cancer Center. All participants provided written informed consent.

A total of 111 breast cancer patients, recruited during1997– 2000, participated in the study. In 20 patients, the sequencing of ATM was unsuccessful, thus leaving 91 participants. Most of the patients were enrolled at the time of radiation treatments for

breast cancer. Breast cancer patients with previous cancer histories were specifically recruited for the study, although all women with a breast cancer history were eligible.

Normal controls.

Blood samples from 940 normal controls were obtained during institutional-sponsored community-based blood drives. Normal control participants did not have a personal history of cancer. Family history information and other specific data were not obtained.

ATM analysis

Consenting participants donated 20 ml of blood. Total RNA was extracted from lymphocytes isolated by centrifugation on Ficoll-Hypaque. Total lymphocyte RNA was prepared by the method of Chomcznski[16] using commercially available reagents (RNAzol B, Tel-Test, Friendswood, TX). Total RNA was then reverse transcribed, and the ATM cDNA subsequently amplified by PCR. The PCR primer sets were designed to amplify ATM cDNA as eight overlapping products ranging in size from 1200 to 1600 base pairs. RT-PCR products were purified by agarose gel electrophoresis and sequenced using commercially available cycle sequencing methodology and ^{33}P-labeled chain terminators (Amersham Inc., Piscataway, NJ). The PCR primers doubled as sequencing primers. A Genomyx-LX sequencing apparatus was used for electrophoretic resolution of the sequencing products. Each reaction was assayed on two gels, the first designed to resolve from the primer to 350 bases and the second designed to resolve out to 800 bases. Following electrophoresis, the dried sequencing gel was exposed to x-ray film and the

radiograph was analyzed for genetic variants in the ATM cDNA. We defined ATM genetic variants as any sequence other than the normal ATM sequence defined in GenBank (reference sequence, HSU33841). We confirmed the validity of our cDNA sequencing assay through analysis of two obligate AT heterozygotes. Figure 1 shows a schematic of the ATM protein and describes the primers used for the sequencing.

Allele-specific oligonucleotide assay

We used an allele-specific oligonucleotide (ASO) assay to screen DNA samples from the control populations for selected sequence variants originally identified in breast cancer patients. We did not sequenced the entire ATM gene in any controls. The result of the ASO assay was a simple plus/minus determination of a single base change at the location of interest and a determination of whether one or both alleles are affected.

The assay we developed was based upon the assay designed by the Baylor Institute for Molecular Genetics (Houston, TX), DNA Diagnosis Laboratory for analysis of the CF gene.[17] Hybridization probes were designed to avoid G:T or G:A mismatches that would result in false-positive results. Blot signal intensity was quantified with storage phosphor technology. Ninety-six samples were examined at a time.

Statistical Methods

Any variant in ATM that occurred in at least two breast cancer patients were considered to be of interest. For these variants, an ASO assay was developed, and the frequency of these variants was tested in the control population. The frequencies of these variants were then compared between cases and controls. Because normal gene

polymorphisms can have different frequencies across ethnic groups, we first compared the frequency of each variant across the different ethnic groups in the control population. If no difference was noted, then the frequency of the particular genetic variant was compared in the entire cohort of patients and the entire control population. If a difference was noted across ethnic groups, we compared the frequency of the particular genetic variant between Caucasian cases versus Caucasian controls, since they constituted the largest subgroup. For one identified variant (Asp1853Asn), two individuals were homozygous for the variant (Asn) allele. Therefore, for this variant we compared the allele frequencies among cases and controls. All comparisons were made with a Fisher's exact tests, and two-sided p values < 0.05 were considered significant.

Results

Of the 111 breast cancer patients that enrolled, the sequencing assay was unsuccessful 20 individuals. For the remaining 91 patients, the full-length of the cDNA of the ATM gene was successfully sequenced in 67. In the other cases, one or more of the 8 overlapping regions of the cDNA (see methods) were not successfully sequenced. The region not sequenced varied amongst individuals. No mutation that would lead to protein truncation were identified. However, it is possible that a truncation or deletion mutation was missed because of gel purification of PCR products or nonsense mediated decay of the mRNA.

Table 1 displays the demographic and patient characteristics of the breast cancer subjects studied. The median age of the participants was 55 years. The majority (75%) of patients were Caucasian (all ethnic information was self-reported). Forty-nine of the 91 patients had a history of a second cancer independent from their breast cancer, the most common type being a second breast cancer (25%). Other types included lymphoma, soft tissue sarcoma, leukemia, melanoma, and endometrial, anal, colon, thyroid, lung, ovarian, and gastric carcinoma. In the patients treated with radiation, normal tissue complications were recorded. For the control subjects, 56% were Caucasian, 23% were Hispanic-American, 15% were African-American, 4% were Asian-American, and 2% were other ethnicities (ethnic information self-reported); 52% were women and 48% were men.

Several single base change variants were identified in the breast cancer cases. Fifty-six patients (62%) had the reference sequence. Twenty–four (26%) had one sequence variant indentified and the remaining 11 (12%) had two or more variants.

Four genetic variants were found in three or more cases. These variants caused the following amino acid substitutions in the ATM protein: Asp1853Asn, Pro1054Arg, Ser49Cys, and Pro1526Pro. The Asp1853Asn was the most common variant, with two homozygotes and 17 heterzygotes for the variant (Asn) allele. Neither of the homozygotes had been diagnosed with AT or had clinical findings associated with the disease. The Ser49Cys was the next most common variant followed by the Pro1054Arg and Pro1526Pro variants. No patient was homozygous for these three variants. Two additional variants were found in two cases; Asp126Glu and Asp1853Val.

The development of an ASO assay was successful for the Asp1853Asn, Pro1054Arg, and Ser49Cys variants, but not for the Pro1526Pro variant.

Table 2 shows the allele frequency of the three studied genetic variants in the controls and the inferred haplotypes and their frequencies classified by ethnicity. Within the control population, there were ethnic differences in the allele frequencies at the Asp1853Asn site and thePro1054Arg site. Therefore, the case-control frequency comparsions were done using only Caucasians. In contrast, no ethnic or sex difference of allele frequencies was detected for the Ser49Cys site, and hence the freqeuency of the variant at this site was compared across the entire set of control samples.

Table 3 shows the comparison of the frequency of these genetic variants in the cases and controls. As shown, there were no statistical differences in frequencies in the Asp1853Asn and Pro1054Arg variants. However, the Ser49Cys variant was found in 6.7% (5/75) of the breast cancer patients compared to 1.3% (12/940) of the control group (P=0.006, by Fisher's 2-sided exact test). Subjects carrying the Cys variant allele at this site are 5.52-fold at highre risk of breast cancer as compared to the Ser/Ser homozygotes (OR = 5.52; 95% CI: 1.89-16.12). Interestingly, the subgroup of patients with bilateral breast cancer had a frequency frequency of 11.8% (2/17), which again was significantly different from the control group (P=0.025, by Fisher's 2-sided exact test). Only 1 of the 5 breast cancer patients with the Ser49Cys variant had a family history of breast cancer. The odds ratio for bilateral breast cancer for the Cys-variant carrying individuals was 10.31 (95% CI: 2.12 – 50.13), compared with homozygote Ser/Ser individuals. Nine of the patients developed a clinically relevant complication after radiation treatment and none of these patients had the Ser49Cys variant.

Discussion

In this study, we identified a variant in the ATM gene that resulted in a Ser49Cys substitution in the protein product. This specific single nucleotide change was more common in breast cancer patients than in the control population. While a number of other investigators have suggested that single-base changes in the ATM gene may be associated with an increase in breast cancer risk, to our knowledge this is the first report associating breast cancer with the Ser49Cys genetic variant. Our study had a relatively small sample size and thus should be considered as preliminary, hypothesis-generating data that requires validation. However, a strength of this study was the large control group, which enabled us to determine the allelic frequencies of these variants in a control population. For example, two of the three genetic variants that were present in two or more cases were equally represented in individuals with no cancer history.

The interest in the relationship between ATM and breast cancer began after the observation that mothers and grandmothers of children with AT (obligate heterozygotes) had a 5.1-fold increased risk of developing breast cancer.[3] This original study predated the cloning of the ATM gene, but the observation was reconfirmed in a study of 50 nordic families with AT.[4] In that study, the standard incidence ratio for the development of breast cancer was 7.1 in obligate heterozygotes (95% CI = 2.3 to 17). In addition, other studies have confirmed the increased breast cancer risk in obligate heterozygotes.[5]

The cloning of the ATM gene not only allows genotype-based assays of breast cancer patients, such as the one we report, but it also permits new laboratory methods to

investigate the relationship between ATM and breast cancer. For example, a recent study found that mice genetically engineered to be heterozygous for a 7636del9 ATM truncating mutation developed cancers at an increased frequency, likely due to the production of an abnormal protein that acts as a dominant negative.[18] In contrast, knock-out heterozygous ATM mice did not have increased susceptibility.[18] This same study also found evidence of an association of the 7636del9 mutation and breast cancer development in human patients.[18] These data suggest that not all ATM mutations will have the same affect on cellular phenotype and cancer development risk. Specifically, for ATM heterozygosity to affect cancer risk, it is possible that the specific mutation must produce a dominant negative protein product, a hypothesis that is further supported by other preclinical work.[19] However, this hypothesis is inconsistent with the aforementioned epidemiological data indicating that obligate heterozygotes, who most commonly have a mutation that truncates the ATM protein, have an increased breast cancer risk.

The result of a dominant negative protein may be one explanation why the data associating genetic changes in ATM and cancer risk have been inconsistant. Table 4 provides an overview of the recently published series that investigated the relationship between ATM genetic variants and breast cancer development risk.[6-15] As shown, a number of studies have now suggested that single-base oligonucleotide changes may increase the risk of breast cancer development, but further laboratory studies are needed to clearly show that these variants lead to a change in cellular phenotype. There are no previous published articles investigating whether the specific Ser49Cys variant affects

cellular phenotype. Furthermore, the Ser49Cys position in the ATM protein is not recognized as being part of a structural or functional protein domain.

The novel findings in this study suggesting a relationship between Ser49Cys variant in ATM and breast cancer risk needs to be independently validated. Our case sample size was relatively small, and only 5 cases had this sequence variant. None the less, the difference in the frequency of this variation in breast cancer patients versus controls was highly significant. The higher frequency frequency of the Ser49Cys variant in the women with bilateral breast cancer adds support to the postulate that the variant affects risk.

The Ser49Cys variant has been reported by other authors, although this is the first report to find this variant more often in breast cancer patients than in controls. Vorechovsky et al. were the first to report this variant and considered it to be a rare polymorphism, present in only 1 of 49 breast cancers tumors or cell lines.[20] Subsequently, Izatt et al. reported this variant in 1/100 breast cancer patients under the age of 40 compared to a frequency of 1/50 in a control set.[14] Finally, Dörk et al. found the Ser49Cys variant in 3 of 192 unselected breast cancer cases.[10] It is unclear why we found a higher frequency of the Ser49Cys variant in our patient population compared to previous reports. One reason may be due to differences in the study population. For example, the study by Dörk et al studied a predominantly German population extraction and our population had a higher percentage of patients with bilateral breast cancer and this variant was seen in 11% of this subgroup. Of course, the difference may also be due to chance.

One method to validate the relevance of the Ser49Cys variant to breast cancer development would be to test for this specific variant in a large independent dataset. This methodology has been done previously for other genetic variants in ATM. For example, after Broeks et al. reported in a relatively small study that the IVS10-6T-->G may contribute to breast cancer development [12], Chenevix-Trench et al. tested specifically for this variant (without complete gene sequencing) in a much larger case-control study and confirmed that this variant was over-represented in families with multiple breast cancers.[8] A second method to test the relevance of single-base changes found in association studies is genetically engineer cells or mice to have the relevant genetic variant. This method, which has been successfully used to evaluate other single base variants of ATM,[18] allows for testing of whether the specific change leads to the production of a dominant-negative protein that can affect cellular phenotype.

In conclusion, we found that a single base genetic variant in the ATM gene that leads to a Ser49Cys change in the protein product was statistically overrepresented in a breast cancer population compared to population controls. These results are useful as hypothesis-generating data that justify further study to determine whether this variant may confer an increased risk for developing breast cancer.

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Table 1, Characteristics of the breast cancer cases

Characteristic		**Number (Percent)**
Age	>40	10 (11%)
	40 – 49	19 (21%)
	≤50	61 (68%)
Ethnicity	Caucasian	68 (75%)
	Hispanic-American	10 (11%)
	African-American	8 (9%)
	Asian-American	3 (3%)
	Other	2 (2%)
History of Second Cancer	None	42 (46%)
	Second Breast	23 (25%)
	Other	26 (29%)
Family History of Breast Cancer	First Degree Relative	75% (n=68)
	Other Relative	11% (n=10)
	Negative	9% (n=8)
	Unknown	3% (n=3)
Stage	Ductal carincoma in situ	4% (n=4)
	I	31% (n=32)
	II	44% (n=45)
	III	21% (n=22)
Type of Surgery	Breast Conservation	38% (n=38)

	Mastectomy	62% (n=63)

Table 2, Allele frequency of the genetic variants in the control population

Variant	Caucasian	African-American	Hispanic-American	Asian-American	Other	Homogeneity (P value)
Asp1853Asn						<0.00005
wildtype	907 (86%)	278 (98%)	396 (93%)	83 (99%)	32 (100%)	
variant	149 (14%)	6 (2%)	28 (7%)	1 (1%)	0 (0%)	
Pro1054Arg						0.0473
wildtype	1021 (97%)	282 (99%)	416 (98%)	84 (100%)	31 (97%)	
variant	35 (3%)	2 (1%)	8 (2%)	0 (0%)	1 (3%)	
Ser49Cys						0.3634
wildtype	1046 (99%)	284 (100%)	422 (99.5%)	84 (100%)	32 (100%)	
variant	10 (1%)	0 (0%)	2 (0.5%)	0 (0%)	0 (0%)	

Table 3, Frequency of genetic variants between cases and controls

Site	Genotype	Frequencies (%) in		P-value
		Cases	Controls	
Asp1853Asn*	Asp/Asp	39/58 (67.2%)	394/528 (74.6%)	P=0.807
	Asp/Asn	17/58 (29.3%)	119/528 (22.5%)	
	Asn/Asn	2/58 (3.4%)	15/528 (2.8%)	
Pro1954Arg*	Pro/Pro	58/61 (95.1%)	476/510 (93.2%)	P=0.841
	Pro/Arg	3/61 (4.9%)	33/510 (6.5%)	
	Arg/Arg	0/61 (0%)	1/510 (0.2%)	
Ser49Cys	Ser/Ser	70/75 (93.3)%	928/940 (98.7%)	P=0.006
	Ser/Cys	5/75 (6.7%)	12/940 (1.3%)	
	Cys/Cys	0/75 (0%)	0/940 (0%)	

* represent frequencies only in Caucasian cases and controls

Table 4, Series investigating the role of the ATM gene and breast cancer

First Author	Method	Case Sample Size	Control Group	Conclusion
Thorstenson[6]	DNA sequencing	270	Yes (n=122)	L1420F variant more common in high-risk population Other variants also identified
Offit[7]	cDNA sequencing	37	No	ATM unlikely a factor in breast cancer development after radiation for Hodgkin's Disease
Chenevix-Trench[8]	Mutation-specific assay	525 or 262*	Yes (n=381 or 68)*	T7271G and IVS10-6T-->G variants were increased in familial breast cancer
Sommer[9]	DNA sequencing	43	Yes (n=43)	A variety of single-base changes more common in breast cancer patients, specific changes not compared
Dörk[10]	DNA sequencing / mutation specific assay	192 / 1000+	Yes (n=500)	T7271G and IVS10-6T-->G variants were more common in breast cancer patients
Teraoka[11]	cDNA	258	Yes	Single base changes more

	sequencing		(n=81)	common in cases with young age or positive family history
Broeks[12]	DNA sequencing	82	No	Single base changes, including IVS10-6T-->G may contribute to breast cancer development
Shafman[13]	cDNA truncation assay	57	No	Truncation mutations do not contribute to the incidence of a second breast cancer after radiation for the primary breast cancer
Izatt[14]	DNA sequencing	100	Yes (n=106)	Germline mutations rare in breast cancer patients under 40 years old
FitzGerald[15]	protein truncation assay	400	Yes (n=202)	Truncation mutations not associated with breast cancer risk
Current series	cDNA sequencing	91	Yes (n=940)	Ser49Cys more common in breast cancer patients

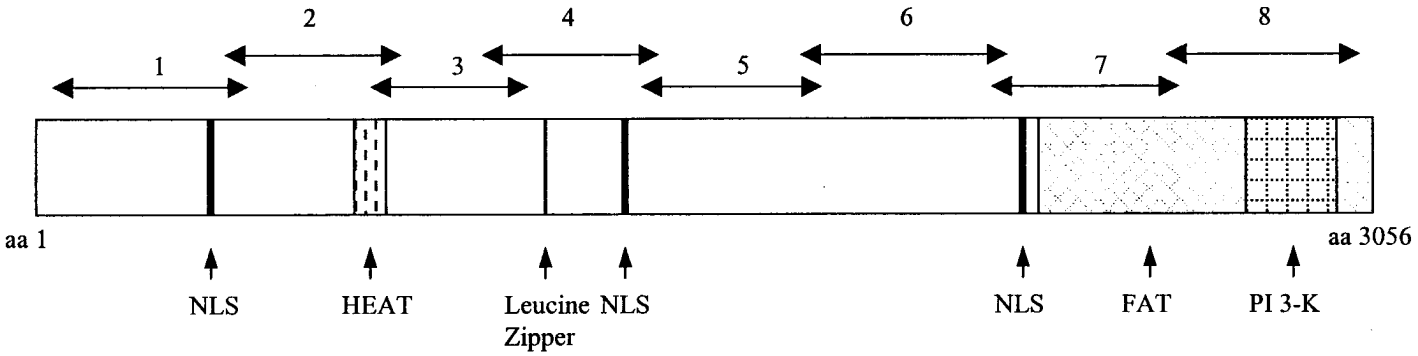
* The T7271G variant was examined in 525 cases and 381 controls and the IVS10-6T-->G 525 variant was examined in 262 cases and 68 controls.

+ 192 cases had sequencing of the ATM gene, 1000 cases had only the region of the gene specific to the variants examined.

Figure 1. Schematic display of representative domains of the ATM protein.

Domains are listed as follows: NLS, nuclear localization sequence; HEAT, sequence elements conserved in the following proteins: (H) Huntington's protein; (E) elongation factor 3; (A) protein phosphatase 2a; (T) Tor 1p; FAT, conserved region in FRAP, ATM, and TRRAP proteins, including a highly conserved 30 aa residue tail; and PI3-K, the C-terminal kinase. Also shown is the Leucine zipper region. Horizontal arrows indicate overlapping regions of cDNA sequenced as outlined below.

Region	Primer pairs		ATM cDNA region sequenced (nt)
1	tgaaattgtgaaccatgagtc	ttggggtagaagctgagatag	177 - 1504
2	gcaaaaggaagaaaatagaac	ctcaagcaacgtgtacatagc	1340 - 2532
3	ctgttacatgggtgtaatagc	atccaaagtttcagggttctc	2376 - 3604
4	ggctgcagagtcaatcaatag	ggagaagctacgtaatgacac	3453 - 4645
5	taaaaagtggcttaggaggag	aacatgtgtagaaagcagatt	4547 - 5767
6	agttcgatcagcagctgttac	ttcagagagttgtctatgtgt	5373 - 6768
7	cagccttgagtctgtgtattc	tttaggcacatttttagttat	6672 - 7936
8	gtttattatactggccttagc	tgagatttttggggtctatgg	7860 - 9305


1
2
3
4
5
6
7
8
aa 1
aa 3056
NLS
HEAT
Leucine Zipper
NLS
NLS
FAT
PI 3-K

APPENDIX 2

DNA end-binding complex density correlates with SF2 but not with the protein level of band components

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Key Words: ATM, BRCA1, BRCA2, radiosensitivity, haploinsufficiency

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Acknowledgements
The authors would like to thank Dr. Jinsong Liu for providing the homozygous BRCA -/- cell line and Ms. Cora Bartholomew for her assistance with the preparation of this manuscript. This work was supported by grant numbers CNE-98699 from the American Cancer Society (C.W.S.), BC980154, a USAMRMC Breast Cancer Research Program Career Development Award (T.A.B.), and CA06294 (M.S.).

ABSTRACT

Purpose: We previously determined that the density of a rapidly migrating DNA end-binding complex (termed "band-A") predicts radiosensitivity of human normal and tumor cells. The goal of this study was first to identify the protein components of band-A. Then, using a panel of fibroblasts expressing BRCA1 mutations but with a spectrum of radiosensitivities, to determine if the protein levels of band-A components would correlate with band-A density and radiosensitivity.

Experimental Design: DNA-EBC protein components were identified by adding antibodies specific for a variety of DNA repair-associated proteins to the DNA-EBC reaction and then noting which antibodies super-shifted various DNA-EBC bands. Band-A levels were correlated with SF2 for a panel of primary human fibroblasts heterozygous for sequence-proven mutations in BRCA1 or BRCA2. The nuclear protein levels of band-A components were determined in each BRCA1 heterozygote by western hybridization.

Results: DNA-EBC analysis of human nuclear proteins revealed 10 identifiable bands. The density of the most rapidly migrating DNA-EBC band correlated closely with both BRCA-mutation status and radiosensitivity (r^2=0.85). This band was absent in cells with homozygous mutations in their ATM genes. This band was also completely supershifted by the addition of antibodies to ATM, Ku70, DNA Ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51 and p53. However, the intranuclear concentrations of these proteins did not correlate with either the SF2 or DNA-EBC density. The addition of antibodies the to BRCA1, BRCA2, RAD50, c-abl, mre11, NBS1, and poly(ADP-ribose) polymerase (PARP) to the DNA-EBC reaction did not result in supershifting

***Conclusions*:** DNA-EBC analysis of human nuclear extracts resulted in 10 bands, at least six of which contained ATM. The density of one of the DNA-EBCs predicted the radiosensitization caused by BRCA haploinsufficiency, and this band contains Ku70, ATM, DNA ligase III, Rpa32,

Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51, and p53. The density of this DNA-EBC was independent of the nuclear concentration of any of its known components, suggesting that band-A density (like radiosensitivity) can be modified by post-translational modification of band-A components.

INTRODUCTION

Predicting normal tissue and tumor radiosensitivity have been desirable, but elusive, goals in radiobiology (Recently reviewed in 1). Current radiation oncology practice guidelines assume that the risk of complications in an individual can be predicted by the complication rates seen in similar populations. However, this line of reasoning (which treats all patients the same regardless of susceptibility to radiation damage) limits the dose that is delivered to relatively radioresistant resistant patients while providing a relatively high risk of complications to others. Thus, it would be desirable to have a practical assay to estimate the relative sensitivity of normal tissues. Even stratifying patients into three risk group could significantly impact on clinical outcomes (2).

Towards that end, we recently demonstrated that an analysis of proteins that bind DNA double strand breaks (the lethal lesion caused by radiation (3-7)) would predict radiosensitivity (manuscript submitted). DNA end-binding protein complexes (DNA-EBCs) were compared for many normal and tumor cell lines. We found that human cell nuclear extracts resulted in 10 identifiable bands, in contrast with a single DNA-EBC from rodent nuclear extracts (8). Also, the density of a DNA-EBC (termed "band-A") correlated strongly (r^2=0.85) with the surviving fraction after 2 Gy (SF2). The density of other bands, or total end binding activity, did not correlate with SF2. This strong correlation may have clinical importance, but it is of fundamental importance to understand the mechanism by which the density of a DNA-EBC can predict/influence radiosensitivity. We chose to study this mechanism in primary cells with BRCA mutations because of the strong association between BRCA1 and DNA repair.

The gene products of BRCA1 and BRCA2 play an important role in preserving genomic integrity. Data suggest that mutations in both alleles of BRCA1 or BRCA2 result in a radiosensitive phenotype, probably owing to a dysfunction in double-strand break repair. For

example, previous studies have demonstrated that cells with a homozygous BRCA1 mutation display diminished oxidative damage repair in the transcribed strands of DNA (9) and have a diminished capacity for DNA end-rejoining (10). In addition, homozygous mutations in the BRCA2 gene impair Rad51-mediated homologous recombination by interfering with BRCA2-Rad51 binding and nuclear translocation, resulting in radiosensitivity (11, 12). There has been some question as to whether BRCA heterozygocity results in radiosensitization; our results with the human cell strains used in this study, however, clearly demonstrate relative radiosensitivity compared with controls (13).

The mechanism by which BRCA genes affect radiosensitivity and DNA repair is not clear, but BRCA proteins associate with a number of DNA-repair proteins. Both BRCA1 and BRCA2 colocalize with Rad51 (11, 12, 14) in a protein complex that is important for the recognition, processing, and repair of double-strand DNA breaks. In addition, DNA damage promotes localization of BRCA1 on proliferating-cell nuclear antigen-(PCNA) positive replicating structures, implying that BRCA is involved in a checkpoint response (15). It was recently reported that BRCA1 joins histones H2A and H2AX at DNA break sites within minutes of damage and that this association forms independently of Rad50 and Rad51 (16). Furthermore, a recent report described the binding of BRCT domains of BRCA1 directly to double-strand breaks in DNA (17). Finally, BRCA1 associates with and is phosphorylated by ataxia-telangiectasia-mutated protein (ATM) (18). The ATM gene plays a critical role in double-strand break repair and mutations in ATM result in profound cellular radiosensitivity (19, 20). Both ATM and BRCA1 have been shown to be present in a large complex of repair proteins that may have a role in the sensing and processing of DNA damage (21). Therefore, we hypothesized that BRCA1 mutation would lead to altered intranuclear levels of band-A components.

In this study we identified eleven protein components of band-A in normal human

fibroblasts. We then compared the nuclear protein levels of each of these proteins in seven primary cell lines from patients with inactivating mutations in BRCA1. There was no correlation between the intranuclear concentration of any single DNA-EBC component and either band-A density or SF2. This analysis led us to conclude that a BRCA mutation most likely affects band-A density via post-translational modification of the DNA-EBC components.

MATERIALS AND METHODS

Cell culture and radiosensitivity determination. Primary fibroblast cultures derived from BRCA heterozygotes were previously described (13). The human breast carcinoma cell line HCC1937 was kindly supplied by Dr. Jinsong Liu (The University of Texas M. D. Anderson Cancer Center). AT mutant cell lines were obtained from the Coriell Cell Repository. Cells were cultured at 37°C in Alpha MEM supplemented with 20% fetal bovine serum in humidified 5% CO_2/95% air. All cultures were fed twice weekly, and the initial primary culture was harvested after 2-3 weeks. Cellular radiosensitivity was measured by at least three independent clonogenic assays, as we have previously described (13).

Statistical methods. The surviving fraction of fibroblasts at 2 Gy (SF2) were determined using data from a minimum of three (range 3–9) replicate experiments. Experiments were repeated until there was a good agreement of the data. A random-effects regression analysis using the "xtreg" routine from the Stata statistical software (StataCorp 2001, Stata Statistical Software, Release 7.0; Stata Corporation, College Station, TX) was performed. The random-effects model is a way to use all the data (replicates), while taking into account that patient-to-patient differences in SF2 values exist.

Nuclear extract preparation. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), harvested by scraping with a rubber policeman, and centrifuged at 800 rpm for 10 min at 4°C. The cells were resuspended in 400 µl of cold lysis buffer (10 mM HEPES pH 7.9, 10 mM

KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM diethylthreitol (DTT), 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl [PMSF]). After a 10-min incubation on ice, 12.5 μl of 10% Nonidet P-40 (NP-40) was added, and the mixture was vortexed for 5 sec. The lysate was centrifuged for 5 min at 4°C (14,000 rpm). The supernatant was removed and stored as cytosolic extract. The pellet was resuspended in 30 μl of extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM PMSF), vortexed thoroughly, and incubated on ice for 30 min. Every 10 min, the preparation was vortexed. At the end of 30 min, it was centrifuged for 10 min at maximum speed (14,000 rpm) at 4°C. The supernatant, designated as nuclear extract, was divided into aliquots, and stored at -70°C (22).

Detection of DNA-EBCs, and supershifts. The DNA-EBC assay, which is a modified electrophoretic mobility shift assay, was performed as previously described for rodent nuclear extracts (23). Briefly, nuclear extracts (0.5-1.0 μg) were incubated with 0.5 ng of labeled probe for 20 min at room temperature. The plasmid DNA, pUC18 (1 μg of closed circular DNA) was used as a nonspecific competitor in a final volume of 20 μl of binding buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, and 10% [v/v] glycerol). The technique has been widely described by other authors to analyze DNA end binding proteins (24, 25) The electrophoretic mobility of the protein-DNA complexes was analyzed in 5% polyacrylamide gel electrophoresis (PAGE) gel at 20-25 mA in TBE buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1mM EDTA). The dried gel was subjected to autoradiography.

pUC18 plasmid was digested with Pvu II and EcoR I to generate a 144-bp probe. The probe was purified by 8% PAGE and electroeluted in 10 mM Tris, pH 8.0, and 0.1 mM EDTA. The eluted DNA fragments were concentrated in a Qiaquick Gel Extraction Column (Qiagen, Valencia,

CA). The probe was ^{32}P-labeled using the Klenow fragment of DNA polymerase I in the presence of [^{32}P] deoxyadenosine triphosphate (dATP) (Dupont, NEN), and the unincorporated nucleotides were removed by chromatography on Sephadex G-50 spun columns (26).

Supershift assays were performed in essentially the same manner as the DNA-EBC reactions were, except that after 20 min of incubation (nuclear extract plus labeled oligonucleotide), antibodies were added at a concentration equivalent to 20 times that normally used in western blot and incubated for an additional 10 min. The samples were then electrophoresed in a 5% polyacrylamide gel.

Antibodies and western blot analysis. The monoclonal antibodies used in this study were: anti-Ku 80 (Sigma, St. Louis, MO), anti-ATM (Genetex, San Antonio, TX), anti-DNA ligase III and IV (Genetex), and anti-Rad50 (Upstate Biotechnology, Waltham, MA). The following polyclonal antibodies were used in this study: anti-Ku80 (Serotec, Raleigh, NC); anti-Ku70, anti-WRN, and anti-BLM (Santa Cruz, Santacruz, CA); anti-p95/Nbs1 (Oncogene Science, Boston, MA), anti-Mre11 (Oncogene, Boston, MA), anti-Xrcc4 and anti Ku70 (Genetex); anti-PARP (Oncogene); and anti-DNA ligase IV (kindly supplied by Dr. Thomas Stamato). The following monoclonal anti-BRCA1 antibodies were used: C20 (Santa Cruz), AB-1 (Oncogene); MS-BRCA 14 (Genetex); and polyclonal Ab-2 (Neomarker, San Francisco, CA). The following monoclonal anti-BRCA2 antibodies were used: H300 (Santa Cruz); 2b6 (Genetex); and polyclonal antibodies BRCA2-AB-1 and BRCA AB-2 (Oncogene).

Western blot analysis was performed as previously described (23). Briefly, actively growing cells were harvested on ice, and nuclear extracts were prepared. Total protein was estimated using the BioRad Protein Assay (BioRad laboratories, Hercules, CA). The antigen–antibody reaction was visualized using ECL an electrochemiluminescence western blot analysis detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The relative protein levels were

determined by densitometry of each band, corrected for the β-actin intensity found on that same blot.

RESULTS

Radiosensitivity of primary BRCA heterozygote fibroblasts correlated with Band A levels. The band-A density was measured in seven primary fibroblast strains developed from patients heterozygous for mutations in BRCA1 (C44, C46, C51, C63, C75, C76) or BRCA2 (C49), and a BRCA sequence-normal control (C80). Fig. 1*A* demonstrated that the intensity of the DNA-EBC band labeled Band A was dramatically reduced in BRCA heterozygotes compared with that in the normal controls, though was still detectable in all samples. Note that the samples were loaded in the order of SF2 (derived from the radiation clonogenic cell-survival curve), as shown at the bottom of Figure 1*A*. DNA-EBC analyses were then performed in triplicate. A human breast cancer cell line with a homozygous BRCA1 mutation (HCC1937) was included as an additional control. The band-A density relative to C80 controls ± SD was plotted vs. SF2 (Fig. 1*B*). There was a very good correlation between SF2 and band-A density (r^2=0.85).

Identification of ATM as a component of Band A. Because ATM phosphorylates BRCA1, we hypothesized that AT cells might also have reduced amounts of band A. To test this hypothesis, the DNA-EBC pattern was analyzed in fibroblasts with ATM mutations in both alleles (Fig. 2*A*, lanes 2 and 3). Three major (A, B, and C) and three minor (small arrows, left) bands were missing in the ATM mutants but were present in the controls (Fig.2*A*, lane 1). One unique band was observed in the ATM cells (Fig. 2A, double arrow). To determine whether ATM is a component of band A or simply affects the DNA-EBC pattern by another mechanism, a supershift analysis was performed. Anti-ATM antibody was found to supershift each of the six bands missing from the ATM mutant cells (Fig. 2*B*). This demonstrated the presence of ATM protein within several bands, including band A.

Determination of other Band A components. Supershift analyses were then performed using a variety of antibodies to proteins known to be important for DNA repair or phosphorylated by ATM. Figs. 3*A*, 3*B*, and 3*C* demonstrate the presence of Ku70, DNA ligase III, DNA ligase IV, XRCC4, RPA32, RPA14, p53, Rad51, BLM, and WRN within band A. Ku80, BRCA1, BRCA2, Rad50, c-abl, NBS1, Mre11 and PARP were not demonstrated to be present by supershift analysis (Figs. 3*A* and 3*D)*. This does not rule out the presence of these proteins because the relevant epitopes could be blocked by other proteins. However, because of these potential epitope-recognition issues, BRCA1 and BRCA2 supershift analyses were each performed with four antibodies from several manufactures and gave identical negative supershift results. Also, band A never partially supershifted, suggesting that band A is a single complex. Unfortunately, the complex pattern of supershifting obscured the pattern of more slowly migrating bands, which prevented a comprehensive analysis. Despite this complexity, it should be noted that, for most of the lanes in which band A is supershifted, there are other bands which do not supershift. This suggests specificity of the antibody in question.

Band-A density is independent of the protein levels of known band components. One possible explanation for a reduction in band-A density is that a BRCA mutation reduces the level of key band components. As these components are reduced, the band density might decrease as well. To test this hypothesis, western blot analysis was performed on nuclear extracts from the control cell line C80 and each of the BRCA1 heterozygote cell lines (C46, C63, C76, C75, C51, C44) with antibodies to each of the proteins identified (Fig. 3) within band A (Fig. 4). Densitometry was performed on each band, corrected for β-actin loading, and the results were plotted against SF2 values for each protein. There was no correlation between SF2 and the levels of any protein found in band A (ATM, Ku70, DNA-PK ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51, or p53). The r^2 values ranged from < 0.001 to 0.49, in contrast with the r^2 value for

band-A density, which was 0.85. Importantly, there was no correlation with BRCA1 or ATM protein levels and band-A density seen in Fig. 1.

DISCUSSION

Band-A density predicts SF2 extremely well for a wide variety of human primary and tumor cell lines, including those with mutation in BRCA1 (Figure 1) and ATM(Figure 2). There are a number of possible explanations for this predictive power. First, it could be chance that SF2 correlated with band-A density. However, the "p" value of 0.00001 makes that very unlikely. Second, it could be that the DNA-EBCs form only *in vitro* and that the DNA-EBC pattern is an artifact of the assay. However, some components of band-A have been previously shown to associate *in vivo.* In particular, ATM has been shown to phosphorylate or associate with RPA32 (27, 28), BLM (29), p53 (30, 31), and RAD51 (32). However, this is the first study to demonstrate an association between ATM and DNA ligase IV, XRCC4, DNA ligase III, and RPA14. Using the preliminary consensus sequence generated from p53 and PhasI (33) others have identified that WRN is a putative substrate for ATM. In this study, we show for the first time that WRN can physically associate with ATM at sites of DNA double-strand breaks. Although ionizing radiation can increase Ku70 by an ATM-dependent mechanism (34), this study is the first to demonstrate a stable physical interaction between Ku70 and ATM. Thus, some band-A components have been previously shown to associate with ATM and others have not. Our findings do, however, suggest that such associations should be investigated *in vivo* .

Irrespective of whether the complex forms *in vivo*, the predictive power of band-A density must be explained mechanistically. The observation that band *density* rather than apparent complex size can be affected by BRCA mutations has mechanistic implications. Our initial hypothesis was that the band density would fall with the level of key band components.

We initially thought that the most likely affected components would be the Ku proteins because they have been shown to nucleate the binding of DNA-PK and other DNA-repair proteins to the site of DNA breaks (35). However, intranuclear Ku70 levels did not change in a BRCA-dependent way. Although it is possible that BRCA mutations resulted in the reduction of some yet-to-be-identified protein component, there is certainly no evidence from our studies that BRCA haploinsufficiency systematically alters levels of any band-A components. This raises the possibility that BRCA mutations affect post-translational modification of one or more band components, possibly through an ATM-dependent phosphorylation, although other mechanisms are certainly possible.

The association between BRCA1 and ATM is well known (18, 21, 27). Because of this direct association of BRCA1 with ATM, we hypothesized that ATM might be a key component of the DNA-EBC that was most affected by BRCA mutation status (band A). Although ATM was clearly present within band A, BRCA proteins could not be detected. Despite using several anti-BRCA1 antibodies, a number of which were polyclonal, we were not able to demonstrate the presence of BRCA1 or BRCA2 in band A by supershift. This suggests that either all of the relevant epitopes are blocked or neither BRCA1 nor BRCA2 is present in band A.

Our current data suggests that band A represents a single complex. First, band A is not partially supershifted by any antibody, as might be expected if many different complexes of similar molecular weight migrated together by chance. Second, band A is not supershifted by anti-Ku80 antibodies, although all nine other bands are supershifted. This is unusual and suggests either a complete Ku80 epitope blockade (since polyclonal antibodies were used in the supershift analysis) or the absence of Ku80 from band A. Both of these suggest that band A is distinct from the other bands and is most likely a single complex.

Thus far, we have identified 11 proteins within band A. Large complexes such as this

have previously been reported to play an important role in genome surveillance. For example, human BRCA1 has been shown to be part of the BASC complex, which includes putative DNA damage sensors such as ATM, Rad50, Mre11, Nbs, MSH2/6, MLH2, and BLM (21). BASC has been hypothesized to play an important role as a protein scaffold that orchestrates repair or signaling pathways, depending on the type of DNA lesion encountered. It is likely that band A is not BASC, because the RAD50/MRE11/NBS1 heterotrimer and BRCA1 are absent from band A. Also, the technique by which BASC was identified was not based on DNA or DNA ends, so it is not surprising that our assay detected complexes somewhat different from those detected by Wang et al. (21).

Unfortunately, the specific BRCA sequence information is not available for our cell lines and can be recovered only by resequencing. This precludes a correlation between specific BRCA mutations and either DNA-EBC patterns or protein densities as part of this initial study. The functional significance of different BRCA mutations may explain the heterogeneity of SF2 values seen described in Fig. 1*B*. In the future, we plan to determine the effect of defined BRCA mutations on DNA-EBC patterns, and expression of DNA-EBC components.

In conclusion, we have demonstrated that the DNA-EBC pattern of human nuclear extract results in 10 bands, at least six of which contain ATM. The most prevalent ATM-containing complex (band A) also contains Ku70, DNA ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51, and p53. This is the first report to demonstrate a stable interaction between ATM and DNA ligase IV, XRCC4, DNA ligase III, RPA14, WRN, and Ku70. The density of band A strongly predicts radiosensitivity (SF2), whereas the protein levels of individual band components do not. DNA-EBC pattern may also be useful as a screening tool for predicting BRCA mutation status and breast cancer risk.

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Figure legends

Fig. 1. *A*, DNA end-binding complexes from BRCA heterozygotes and control cells. Band A density is markedly reduced in the BRCA heterozygotes. The SF2 for each cell line is shown at the bottom of the figure. *B*, DNA-EBC density was determined in triplicate relative to the normal C80 control. Band-A density correlated with SF2 (r^2=0.85). All data shown are the means of at least three replicate measurements ± one SD.

Fig. 2. *A*, DNA-EBC from a normal (C29) and two AT (GM05849B and AT5BiSV40) cell lines. Three major bands (labeled "A," "B," and "C") and three minor bands (thin arrows, left) are missing from both AT cell lines, whereas four prominent bands (thin arrows, right) are present in both normal and AT extracts. A novel band (double arrow, right) is seen in the AT cells but not the normal control. *B*, Anti-ATM antibody supershifts the missing bands from panel A in a cell line with normal ATM. Arrows at left mark the bands shown in panel *A*.

Fig. 3. *A*, Band A was supershifted by the addition of antibodies to Ku70 but not to Ku80. *B*, Band A was supershifted by the addition of antibodies to DNA ligase III, XRCC4, RPA32, and RPA14. *C*, Band A was supershifted by the addition of antibodies to p53, DNA ligase 4, RAD51, BLM, and WRN. *D*, Band A was not supershifted by antibodies to BRCA1, BRCA2, Rad50, c-abl, mre11, NBS1, or PARP.

Fig 4. Nuclear protein levels of band-A components determined by western analysis. Nuclear extracts of the indicated cells were loaded in order of SF2 (bottom), and the blots probed with the

indicated antibody. Densitometry was performed, and corrected for loading by normalization with β-actin .

Figure 1a

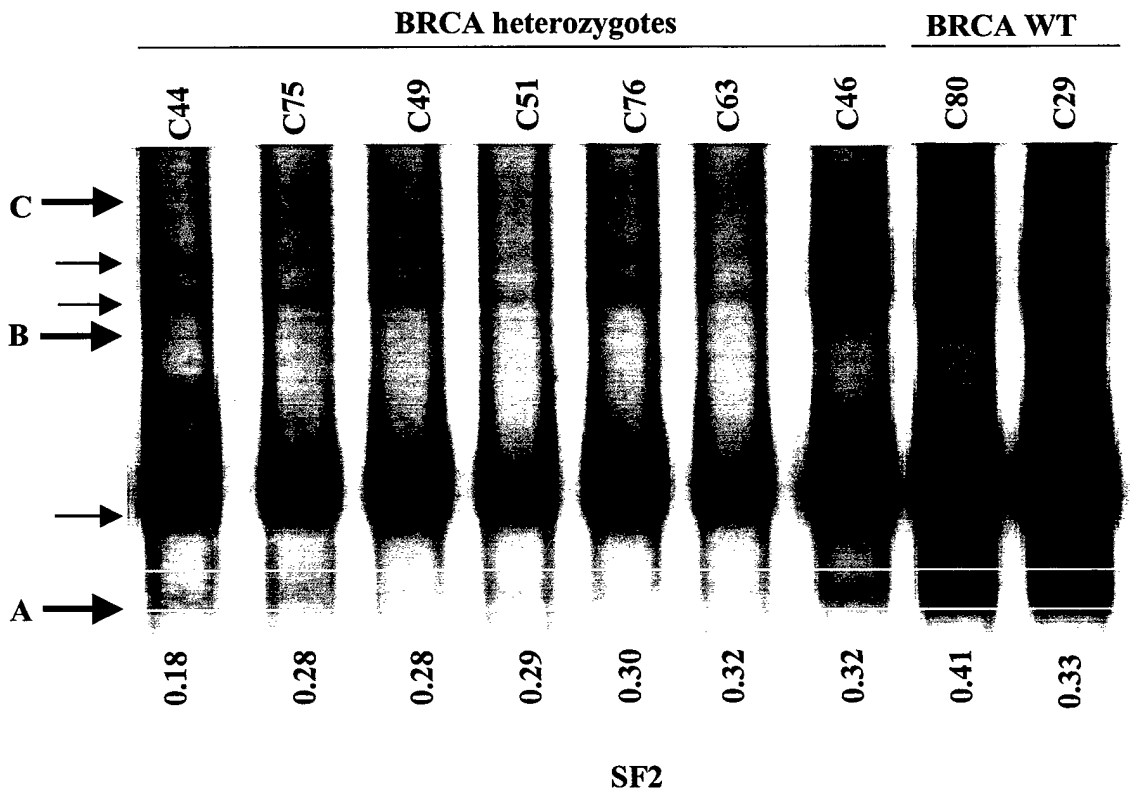

BRCA heterozygotes
BRCA WT
C44
C75
C49
C51
C76
C63
C46
C80
C29
C
B
A
0.18
0.28
0.28
0.29
0.30
0.32
0.32
0.41
0.33
SF2

Figure 1b

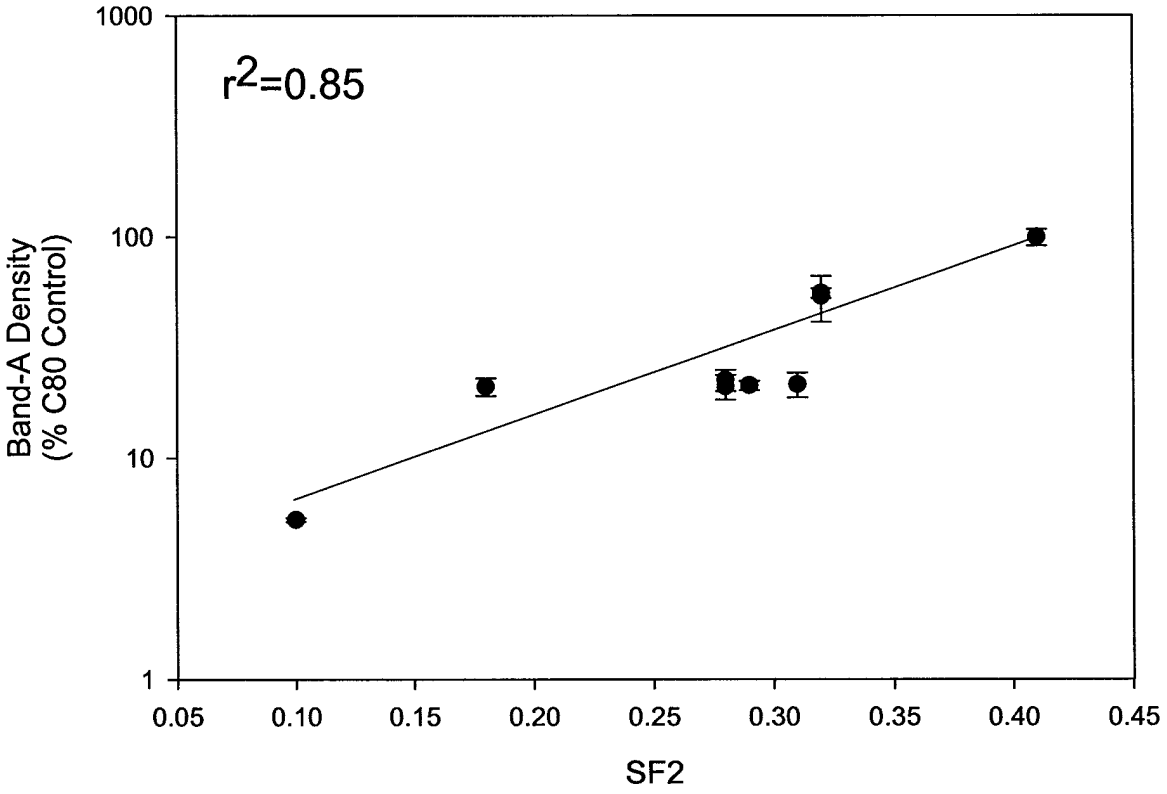

1000
100
10
1
r^2=0.85
Band-A Density
(% C80 Control)
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
SF2

Figure 2a

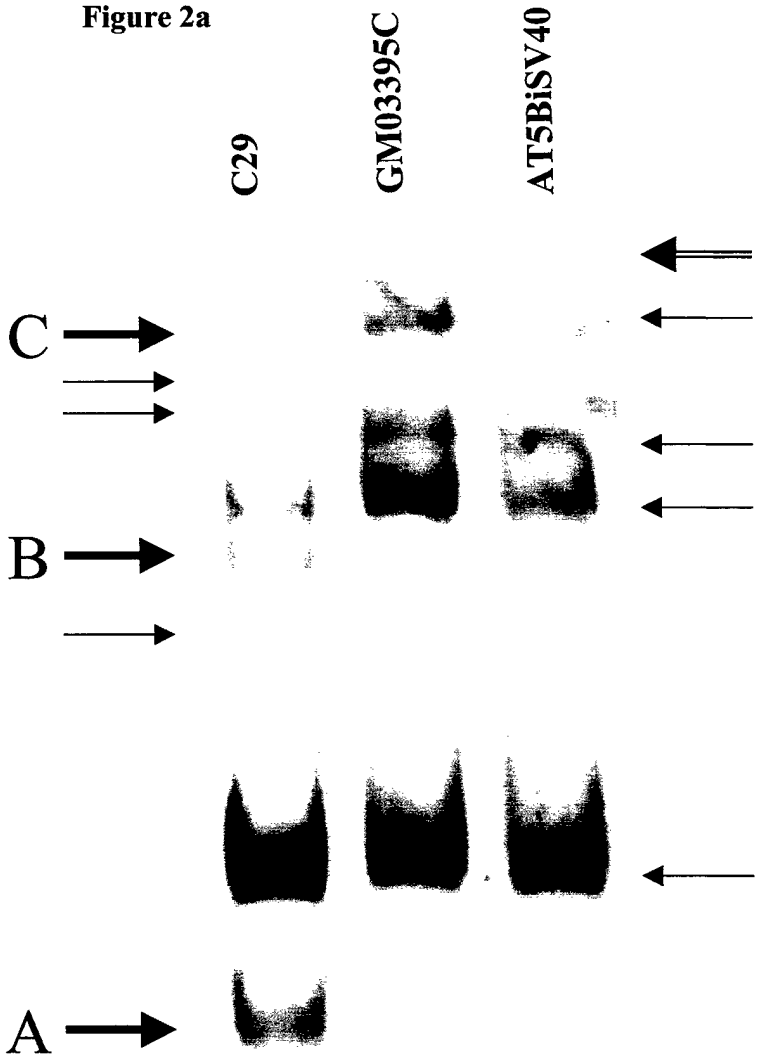


Figure 2b

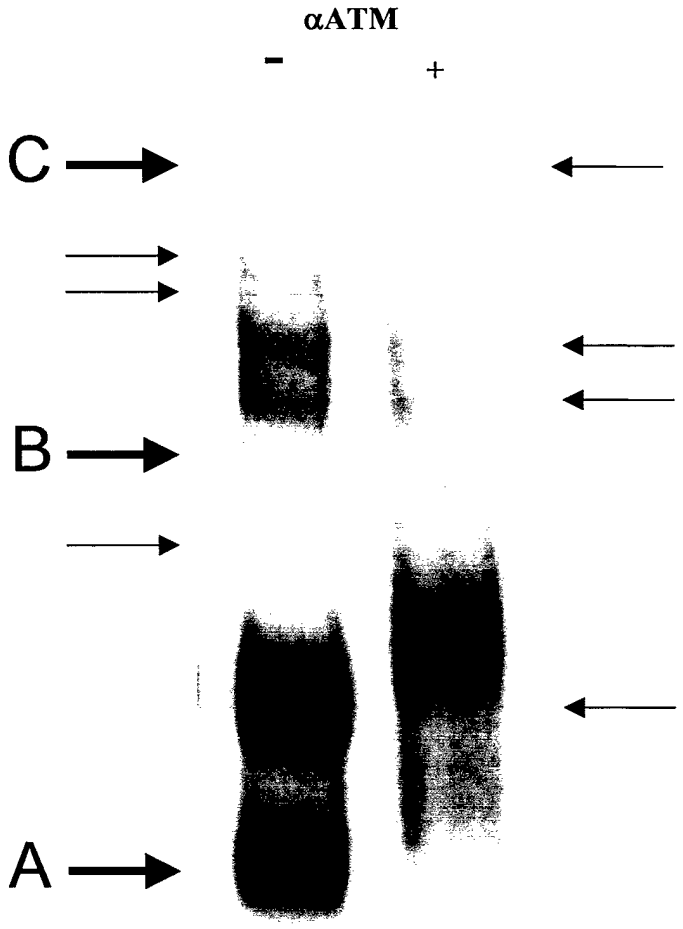


Figure 3a

Figure 3b

Figure 3c

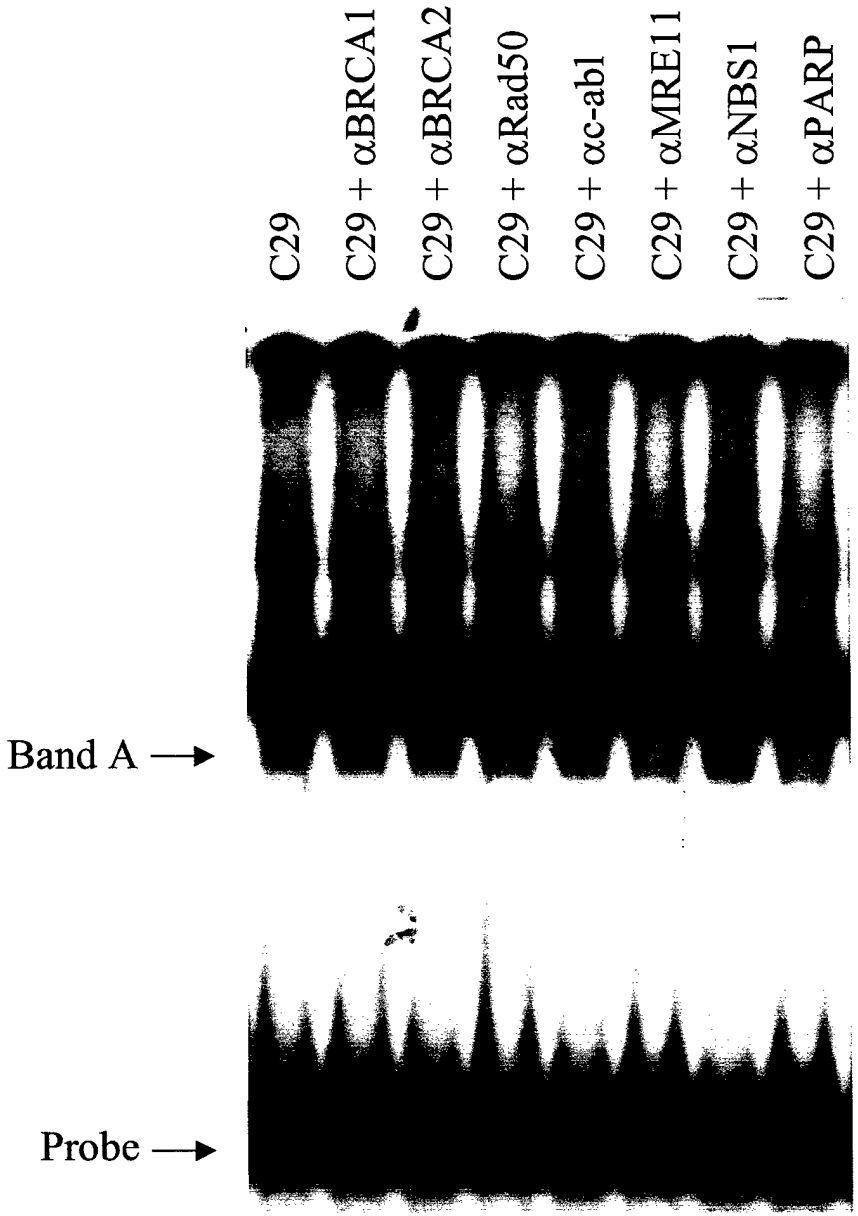
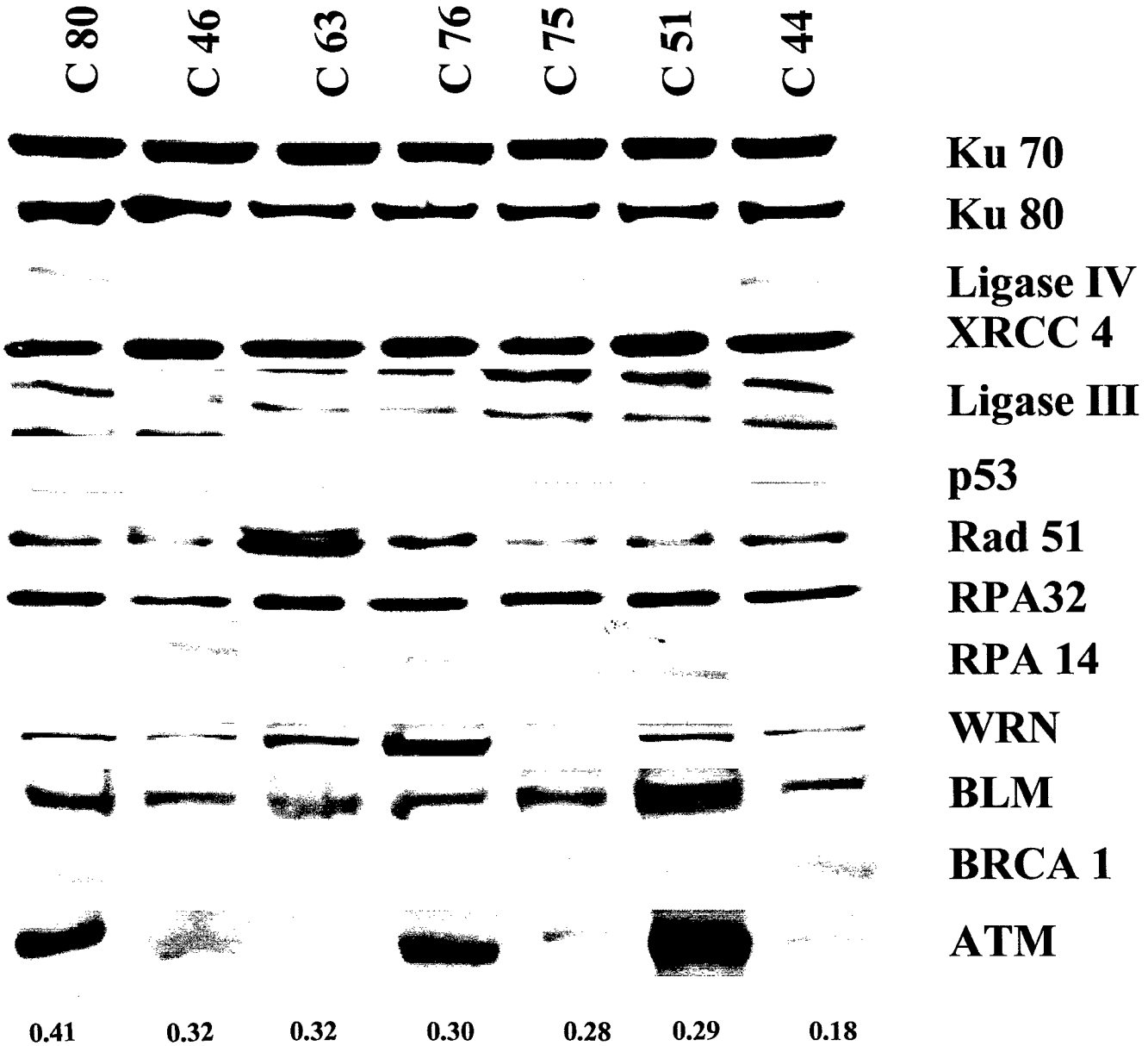

C29
C29 + αBRCA1
C29 + αBRCA2
C29 + αRad50
C29 + αc-abl
C29 + αMRE11
C29 + αNBS1
C29 + αPARP
Band A
Probe

Figure 3d

Figure 4


C 80
C 46
C 63
C 76
C 75
C 51
C 44
Ku 70
Ku 80
Ligase IV
XRCC 4
Ligase III
p53
Rad 51
RPA32
RPA 14
WRN
BLM
BRCA 1
ATM
0.41
0.32
0.32
0.30
0.28
0.29
0.18
SF2

APPENDIX 3

Predicting Radiosensitivity using DNA End-Binding Complex Analysis

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This work was supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154 (TAB); National Institutes of Health Research Grants CA-06294 (AM, UR, REM, MDS, and LM)

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The abbreviations used are: TCP, tumor control probability; NTCP, normal tissue complication probability; SF2, Surviving fraction after 2Gy; DNA-EBC, DNA end-binding complex.

Running Title: DNA-end binding predicts radiosensitivity

Key Words: DNA repair, predictive assays, radiation.

Abstract

Previous reports have suggested that measuring radiosensitivity of normal and tumor cells would have significant clinical relevance for the practice of radiation oncology[redacted]e hypothesized that radiosensitivity might be predicted by analyzing DNA end-binding complexes (DNA-EBCs) which form at DNA double-strand breaks, the most important cytotoxic lesion caused by radiation. To test this hypothesis, the DNA-EBC pattern of 21 primary human fibroblast cultures and 15 tumor cell lines were studied. DNA-EBC patterns were determined using a modified electrophoretic mobility shift assay and were correlated with radiosensitivity, as measured by SF2. DNA-EBC analysis identified a rapidly migrating ATM-containing band (identified as "band-A") whose density correlated with SF2 ($0.02 \leq SF2 \leq 0.41$) in primary fibroblasts ($r^2=0.77$). The DNA-EBC pattern of peripheral blood lymphocytes was identical to that of fibroblasts. In addition, band-A density correlated with SF2 ($0.35 \leq SF2 \leq 0.80$) in 15 human tumor cell lines ($r^2=0.91$). Densitometry of other bands, or total DNA-EBC binding, correlated more poorly with SF2 ($r^2<0.45$). These data indicate that DNA-EBC analysis may be a practical, clinically relevant predictor of tumor and primary cell radiosensitivity.

INTRODUCTION

Predicting normal-tissue and tumor radiosensitivity has been the subject of intensive investigation, but has yet to be routinely integrated into radiotherapy (reviewed in Ref. 1). Currently, complication risks for an individual irradiated patient can be predicted only by the complication rates seen in similar populations. This assessment fails to account for variation in the DNA repair capacity of the individual. Thus, the "standard" dose for a population may be inappropriately low for some patients whose tumors are resistant, while this same dose may carry a relatively high risk of complications for others whose normal tissues are sensitive. Modeling data from our group has previously shown that, if the most radiosensitive patients could be identified by a predictive assay, the remaining patients could be safely treated with higher doses (2). Similarly, those predicted to have the greatest risk of complications because of unusual radiosensitivity can have relatively complex/expensive treatment techniques employed in an effort to reduce toxicity. In lung cancer radiotherapy, this might involve the use of respiratory gating, using time-consuming but more reliable patient set-up techniques and perhaps the use of radioprotectors such as amifostine. As more resource-intensive, highly conformal therapies become available (e.g., intensity modulated radiotherapy, proton therapy, etc.), they could be first applied to patients at greatest risk for side effects. Thus, the development of a good predictor of normal-cell radiosensitivity is becoming increasingly important.

Similarly, predicting tumor radiosensitivity has significant clinical applicability. If such prediction could be done accurately, radiation doses could be tailored to the radio-

curability of individual tumors. In addition, such an assay could be helpful in determining the optimal doses and schedules of biological and chemotherapeutic radiosensitizers. Several groups have published modeling data demonstrating the clinical value of predicting normal tissue and tumor radiosensitivity (3-10). These data indicate that both tumor control probability (TCP) and normal tissue complication probability (NTCP) can potentially be improved by individualizing treatment according to the results of predictive assays. The benefits were dependent on the predictive power of the assay, but clinically meaningful benefit could be demonstrated even if the correlation between test results and TCP or NTCP is within the range of 0.4 and 0.6. Even if the assay only stratifies patients and tumors into only 3 risk categories (low, medium, and high), the potential gain in TCP was predicted to be between 22% and 33%.

Radiosensitivity of cells *in vitro* has been shown to be predictive of *in vivo* radiosensitivity in both normal and tumor tissue. Radiosensitivity has been most often measured by determining the surviving fraction after 2 Gy (SF2). This dose has historically been chosen because curative radiotherapy is typically delivered using a daily fraction size of 1.8 to 2 Gy. However, the quantification of SF2 is time consuming, requiring weeks to grow explants and determine radiosensitivity, and expensive. These problems significantly limit the clinical applicability of the assay. In principal though, if SF2 could be accurately and reproducibly determined, radiation dose could be individualized to both increase TCP and decrease NTCP. Also by combining tumor and normal radiosensitivity, tumor hypoxia, and proliferative potential measured, a very accurate TCP/NTCP model could be constructed (1).

Radiotherapy kills cells primarily through DNA damage, and DNA double-strand breaks are thought to be the lethal lesion caused by radiation (11-15). The presence of DNA double strand breaks activates a variety of signal transduction cascades, such as ATM and DNA-PK pathways, that alter cell functions (e.g., cell cycle) to allow time for DNA repair. Both of these proteins can be found at DNA double strand breaks, and inactivation of either kinase results in profound radiosensitivity (16-19). Obviously, DNA-repair enzymes must also be present at DNA breaks for repair to occur. Thus, we hypothesized that an analysis of DNA-EBCs would be predict radiosensitivity.

[redacted] The pattern/[redacted] of DNA-EBCs was compared in primary and tumor cells with a variety of known radiosensitivities. This approach does not require that cells grow *in vitro*, nor does it require radiation. Several practical aspects of DNA-EBC analysis were also explored.

MATERIALS AND METHODS

Cell Culture. The cell lines used in this study and their radiosensitivities are shown in Table 1. Cells were grown as monolayers in Dulbecco's modified Eagle's medium and maintained as logarithmically growing cultures. Primary fibroblast cultures were supplemented with 20% fetal bovine serum, whereas tumor cell cultures were supplemented with 10% fetal bovine serum. The primary human fibroblast cell lines were obtained on protocols approved by our Institutional Review Board. One protocol

involved the acquisition of primary fibroblasts from patients with abnormally severe radiation reactions; the other involved the generation of primary fibroblast cultures from patients heterozygous for BRCA mutations (20) but no diagnosis of cancer. Five "normal" cell lines without known mutations in DNA repair/signaling genes were also included (C42, C74, S23, C37 or C80).

SF2 Determination. SF2 was determined for each cell line by methods previously described (20). Radiosensitivity was measured by at least 3 independent clonogenic assays. These were performed within ~5-10 population doublings of the population used for DNA-EBC analysis. This was done to minimize possible senescence-related changes in SF2 in primary cells and any possible genetic drift in tumor cell populations. Our data generally agree with the published SF2 values for these cell lines.

Nuclear Extract Preparation. Cells were rinsed twice with ice-cold phosphate-buffered saline, harvested by scraping with a rubber policeman, and centrifuged at 800 rpm for 10 min at 4°C. The cells were resuspended in 400 μL of cold lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM diethylthreitol [DTT]); 2 μg/mL leupeptin; 2 μg/ml aprotinin; and 0.5 mM phenylmethylsulfonyl [PMSF]). After a 10-min incubation on ice, 12.5 μL of 10% Nonidet P-40 (NP-40) was added, and the mixture was vortexed for 5 sec. The lysate was centrifuged for 5 min at 4°C (14,000 rpm). The supernatant was removed and stored as a cytosolic extract. The pellet was resuspended in 30 μL of extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 2 μg/mL leupeptin; 2 μg/mL aprotinin; and 0.5 mM PMSF) and extracellular membrane disruption confirmed microscopically.

Samples were then vortexed thoroughly, and incubated on ice for 30 min. Every 10 min, the preparation was vortexed. After 30 min, the extract was centrifuged for 10 min at maximum speed (14,000 rpm) at 4°C. The supernatant, designated as nuclear extract, was divided into aliquots and stored at -70°C (21). Because SF2 can be dependent on the length of time in culture due to genetic drift of the population, it was considered important to obtain nuclear extracts for cells that were as near as possible in passage number to those used for SF2 determination. Nuclear extracts were made from peripheral blood lymphocytes using a slight modification of the above-described procedure.

Detection of DNA-EBCs. The DNA-EBC assay, which is a modified electrophoretic mobility shift assay, was performed using the method we previously described for rodent nuclear extracts (22). Briefly, nuclear extracts (0.2-1.0 μg) were incubated with 0.5 ng of ^{32}P-labeled oligonucleotide probe (144-bp fragment of pUC18) for 20 min at room temperature. Plasmid DNA (1 μg of closed circular pUC18 DNA) was used as a nonspecific competitor in a final volume of 20 μL of binding buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 150 mM NaCl; 1 mM DTT, 1 mM PMSF; and 10% [v/v] glycerol). [redacted] d identical amounts of protein were loaded into each lane, and the electrophoretic mobility of the protein-DNA complexes was analyzed in 5% polyacrylamide gel electrophoresis (PAGE) gel at 20-25 mA in TBE buffer (45 mM Tris-HCl, pH 8.0; 45 mM boric acid; and 1 mM EDTA). The dried gel was subjected to autoradiography. The technique has been widely described by other authors as a way to analyze DNA end binding proteins (23, 24).

pUC18 plasmid was digested with PvuII and EcoRI to generate a 144-bp probe. The probe was purified by 8% PAGE and electroeluted in 10 mM Tris, pH 8.0; and 0.1 mM EDTA. The eluted DNA fragments were concentrated in a Qiaquick gel extraction column (Qiagen, Valencia, CA). The probe was ^{32}P-labeled using the Klenow fragment of DNA polymerase I in the presence of [^{32}P] deoxyadenosine triphosphate (dATP) (Dupont, NEN, Boston, MA), and the unincorporated nucleotides were removed by chromatography on Sephadex G-50 spun columns (25).

DNA-EBC analysis was done at least in triplicate (typically using a phosphoimager [Typhoon 9400, Amersham Biosciences, Piscataway, NJ] and ImageQuant image analysis software [Sunnyvale, CA]), and densitometry was performed. The density of each band was corrected for the density of the corresponding lane in a region below band-A far from a DNA-EBC. It was not possible to run all samples on a single gel. Comparison between gels was accomplished by normalizing all band densities to a normal control (usually C80) that was present on all gels.

[redacted]

Correlations between band-A densities and SF2 were calculated using linear regression, along with their significance expressed as a p-value. We considered other types of regression analyses, since there was no biological reason to assume that the relationship between band-A density and SF2 should be linear. The r^2 value was slightly better (0.85) for an exponential fit for primary cells than for a linear regression (0.77). However, when

the data from primary and tumor cells were pooled as shown in Fig. 6, a linear regression was a better fit. For consistency, therefore, we chose linear regression analysis for all results.

RESULTS

DNA-EBC Pattern Predicts SF2 of Primary Fibroblasts.

A representative DNA-EBC analysis is shown in Fig. 1A. We noted that there were at least 10 bands present in DNA-EBC gels from normal primary human fibroblasts, but the relative intensity of each band could vary significantly from cell line to cell line. It was noted that the relative intensity of the band labeled "band A" decreased as SF2 decreased. Densitometry was performed and the relative density of band A was plotted vs SF2 (Fig. 1B). This analysis demonstrated a strong statistical correlation between SF2 and band-A density (r^2=0.77, $p<0.000005$). This analysis included cell lines with marked radiosensitivity such as ATM mutants and BRCA1 homozygous mutants, intermediate radiosensitivity such as BRCA heterozygotes and some cell lines from patients with marked radiation reactions, and normal radiosensitivity in 2 unrelated normal lines (Table 1).

Because AT cells are particularly radiosensitive and because the ATM protein was thought to bind at sites of DNA breaks, it was hypothesized that ATM might be an important component of band A. To test this hypothesis we determined the DNA-EBC pattern of 2 cell lines derived from patients with AT. As can be seen in Figure 2, band A was essentially undetectable (lanes 2 and 3) in AT cells. In fact, [redacted]nd 4 minor (thin arrows on the left) bands were missing in both ATM mutants compared with a normal control (Fig. 2, lane 1). One unique band was observed in the ATM cells

(double arrow, right). Also, the relative intensity of bands was different in AT cells compared with controls, with some bands relatively more intense and others less intense. This suggested that mutations in ATM cause widespread changes in the complexes that form at DNA double-strand breaks.

DNA-EBC Analysis of Lymphocytes is Similar to That of Fibroblasts.

To implement DNA-EBC analysis easily in the clinic, the assay would be better done on more readily available samples than fibroblasts, such as peripheral blood lymphocytes. As a first step in developing a lymphocyte-based DNA-EBC analysis, we compared the DNA-EBC pattern in 2 primary fibroblast lines (Fig. 3A, lanes 1 and 2) with the DNA-EBC patterns from 4 peripheral blood lymphocytes samples from unrelated individuals (Fig. 3A, lanes 3-6). The DNA-EBCs from fibroblasts and lymphocytes were indistinguishable. Fibroblasts and lymphocytes from 2 individuals were also obtained. One patient (C84, Fig. 3B left) was heterozygous for an inactivating mutation in ATM, and the other patient heterozygous for a deletion in BRCA1 (C85, Fig. 3B right). The DNA-EBC pattern was found to be similar for lymphocytes and fibroblasts derived from these patients. There are also unusual bands (arrows) in each lymphocyte/fibroblast pair that are not seen in the C80 control. While the biological significance of these bands is unclear, these data suggest that DNA-EBC analysis of lymphocyte nuclear extracts can predict SF2 of fibroblasts.

DNA-EBC Pattern Predicts SF2 of Human Tumor Cell Lines.

It might be expected that the genetic variability of human tumors, or their genetic divergence from primary cells, could complicate DNA-EBC analysis. Also, the SF2 of tumor cells is often much higher than that of primary cells, which might exceed the

functional range of the assay. Therefore, the DNA-EBC pattern of human tumor cell lines was determined for lines with a wide variation in SF2 (0.35 to 0.80). Figure 4A shows representative DNA-EBC analysis. Significant variability was seen in the DNA-EBC pattern of these cells in the bands above band A. However, the band A density continued to correlate strongly with SF2. When the densitometry of band-A was plotted against SF2 (Fig. 4B), a very strong correlation with band A density was seen (r^2=0.91, $p<0.000005$). These data demonstrate that band A density was a good predictor of SF2 in tumor cell lines as well as normal tissues.

It was possible that either total DNA end binding capacity (i.e., the total density of a lane), or the density of another DNA-EBC might correlate well with SF2 in a manner similar to that found for band-A. To test this hypothesis, densitometry was performed on either the entirety of the lane [redacted], or bands that were easily seen and separated from neighbors (band-B or band-D as shown in Figure 2) on each of the three DNA-EBC analyses from tumor cell lines. The results of the densitometry correlated poorly with SF2 for any of these parameters, with the correlation coefficients (r^2) for linear regression of 0.18, 0.44, and 0.40 for total, band-B and band-D respectively (data not shown). We concluded that band-A density correlated better than any other DNA-EBC component with SF2.

DNA-EBC Pattern is Accurate Despite Contamination with Cells of Different SF2.

Determining DNA-EBC patterns in tumors is likely to be more complex than for cell lines. *In vivo*, tumors contain both malignant and normal cells. In preclinical animal models, there will also be contamination with normal rodent cells. Therefore, it was important to determine the sensitivity of the assay to contamination with normal cells

because very small changes in DNA-EBC density could lead to a relatively large error in predicted SF2. For this reason, nuclear extracts from normal human fibroblasts (SF2=0.4) were mixed with nuclear extracts from cells with a homozygous mutation in ATM and DNA-EBC analysis performed on the mixture (Fig. 5A). Densitometry demonstrated that the predictive power of DNA-EBC analysis was proportional to the level of contamination. Thus, if both the SF2 and proportion of normal cells within a tumor specimen is known, the SF2 of the tumor cells can be accurately predicted. Similar to the human study, the density of band-A was proportional to the level of contamination with rodent cells (Fig. 5B) as might be encountered in the study of xenografts. Note that rodent nuclear extracts demonstrate only a single DNA-EBC as we have previously reported (22). Perhaps most interesting was the result of mixing mouse nuclear extracts with those from AT cells (Fig. 5C). Mouse extracts have no effect on band A at any mixing ratio.

DISCUSSION

Our data demonstrate that the density of band A is an excellent predictor of primary fibroblast SF2 over a range of radiosensitivities ($0.02 \leq SF2 \leq 0.41$). While the number of samples is small, analysis of Figure 1B demonstrates that band-A density analysis can distinguish between three groups: low SF2 ($SF2 \leq 0.1$), intermediate SF2 ($0.15 \leq SF2 \leq 0.33$), and high SF2 ($SF2 > 0.33$). Combining Figures 1B and 4B (Figure 6) also clearly demonstrates the predictive power of band-A density. This suggests that band A density can be used as an intermediate marker for patient intrinsic radiosensitivity. Previous reports have shown that SF2 of patient fibroblasts is predictive

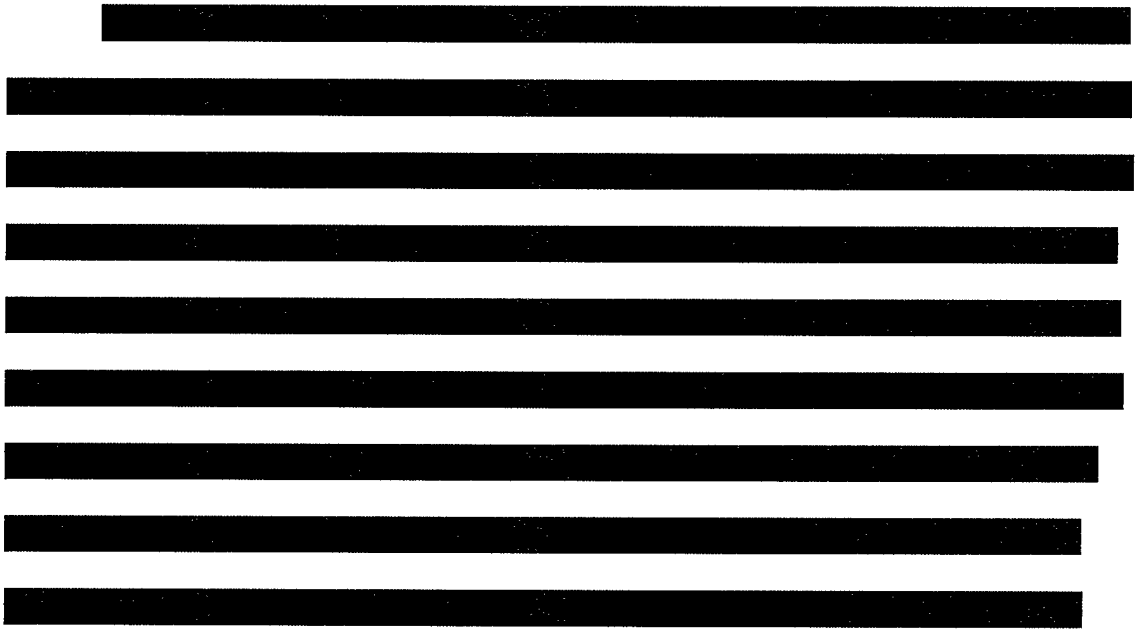
of radiation-induced late toxicity. It remains to be determined how well DNA-EBC analysis will predict toxicity; however, these observations and the ease of the assay (particularly when done on lymphocytes) suggests that a large scale study could be undertaken to determine the predictive power in a much larger sample and correlate this with complication rate.

Using a clonogenic survival assay of dermal fibroblasts, previous reports have correlated SF2 with the degree of skin fibrosis after breast radiation (27). However, acute reactions and skin erythema were not predicted by this assay. Another study (28) also demonstrated that fibroblast SF2 correlated with the maximal toxicity grade for patients irradiated for breast cancer. In selected cases, patients with severe DNA repair deficits such as Ataxia Telangiectasia can have their treatment tailored to their intrinsic radiosensitivity with good results. In one report, a patient with an inherited defect in DNA repair (ataxia telangiectasia) with medulloblastoma was treated with 0.6 Gy fractions to 15 Gy, based on the measured SF2 of his fibroblasts (29). This patient's sibling had severe toxicity when treated with standard radiotherapy doses, whereas the patient with individualized therapy had good local control and no severe side effects after 9 months. This demonstrates that, at least in rare patients with severe repair deficits, treatment might possibly be individualized based on SF2.

A study by [redacted] (30) compared SF2 of tumor cells and local-regional control. In 38 patients, tumors were biopsied, explants cultured in soft agar, and SF2 determined. They found no correlation between SF2 and loco-regional control for these patients treated with radiation alone. Interestingly, they also found no correlation between tumor cell SF2 and fibroblast SF2, suggesting that these are

independent parameters. One caveat of their observation was that their plating efficiency was extremely low (only 1/38 was above 1%, and seven were about 0.01%), suggesting that they may have measured SF2 only on a small subset of tumor cells. In fact, five tumors had SF2 of 1.00, yet two of these patients had local control! Therefore, SF2 of small tumor subsets must not be representative of the entire tumor population.

A larger study (31) of 84 curatively treated patients with head & neck cancer did demonstrate a significant correlation between tumor SF2 and local control ($p=0.036$), but not survival. These patients were treated with a variety of radiation-containing regimens, and some patients received surgery or chemotherapy. The median follow-up was 25 months in this study, in contrast with the Overgaard study which had a median follow-up of only 14 months. They did not discuss the plating efficiency for the tumors in their study. Thus SF2 is predictive of local control in head & neck cancer in the largest study with the longest follow-up.



[redacted]

Band A density also correlates very well with SF2 of tumor cell lines (r^2=0.91), and the analysis of Figure 4B suggests that band A density can at least dichotomize between tumors with SF2 above and below 0.60. This is potentially important because other investigators have shown that tumor cell SF2 can predict outcome, particularly for head and neck and cervical cancers, but not gliomas (30-32, 37, 38). DNA-EBC analysis has a marked advantage over other assays in that tissue culture is not required. Figure 7 is a composite of figures 1B and 4B and shows that band-A density is extremely predictive of SF2 over the entire range of SF2 studied ($0.02 \leq SF2 \leq 0.80$; r^2=0.89, $p<0.000005$). Thus band-A density is predictive of SF2 over the range of radiosensitivities likely to be encountered in clinical practice.

The mixing studies provide additional support for the idea that DNA-EBC analysis of tumors will be practical. Band A density can be reproducibly and accurately measured when samples are minimally contaminated with cells of different SF2 or from other species. This is in contrast with PCR-based techniques that are extremely contamination sensitive. Interestingly, since rodent ATM and human ATM are highly conserved structurally, mixing nuclear extracts from these cell types might have resulted in the restoration of band-A, especially when mixed 50:50. However, our observations that the presence of rodent proteins have no impact on human band-A density suggest that ATM *activity* may be required for band A assembly. ATM probably does not simply play a structural role in band-A.

The mechanism by which band A density relates to SF2 is unclear at present. The observation that band *density* rather than band migration speed (or perhaps broadness of the band) predicts SF2 has potential mechanistic implications, but these cannot be fully explored until all of the band components have been identified. However, in a companion paper, Ismail *et al.* (39), we demonstrated that band A contains at least ATM, Ku70, DNA ligase III, Rpa32, Rpa14, DNA ligase IV, XRCC4, WRN, BLM, RAD51, and p53. However, at least for the BRCA1 heterozygotes, band A density did not correlate with the nuclear levels of any of these proteins, suggesting that band A assembly may depend on post-translational modification(s) of its components. This suggests that DNA-EBC analysis may provide *functional* information about many proteins simultaneously, in contrast with genomic or proteomic approaches that assess only mRNA or protein levels. This may also allow the detection of post-translational modifications that are important for DNA repair. It will also be important to determine which DNA repair deficits can be predicted by DNA-EBC, and this work is underway.

Several other bands also seemed to decrease with decreasing SF2, in particular those shown by arrows to the left of Fig. 2, all of which are absent from AT cells. We chose to study band A for several reasons. First, the other bands were either very faint or migrated very close to bands that did not have SF2-dependent density. Second, the analysis of band components is easier for more rapidly migrating bands (particularly when using supershift analysis, see the companion paper). Finally, the density of band-A correlated better with SF2 than the density of any other easily measured band.

In summary, band A density was predictive of SF2 over a wide range, though it seemed somewhat more predictive at higher SF2s. Band A density of lymphocytes may

provide an easily accessible source for SF2 prediction and so possibly predict normal-tissue toxicity from radiation. Band-A density was also very predictive of tumor cell SF2, with the potential to stratify individual tumors based on radiosensitivity. DNA-EBC analysis may serve as an important intermediate marker for radiosensitization of tumors by many otherwise nontoxic radiosensitizers. This may allow more rational dose selection for these agents, and prediction of tumor radiosensitization by a particular agent before completing the course of treatment. Finally, the relative insensitivity of band A density to contamination may make DNA-EBC analysis more practical than other approaches.

Acknowledgments

The authors would like to thank Ms. Cora Bartholomew for her assistance with the preparation of this manuscript.

TABLE 1. Characteristics of cell lines used in this project

Cell Line	Characteristics/Mutations	SF2
GM03395C	Fibroblast, Ataxia-Telangiectasia;	.02
AT5BiSV40	Fibroblast, SV40 transformed, Ataxia – Telangiectasia; (GM05849B).	.04
HCC1937	Breast carcinoma, homozygous for 5382C mutation in BRCA1	.10
C84	Primary skin fibroblast, ATM heterozygote	.17
C42	Primary skin fibroblast, multiple cancers.	.17
C85	Primary skin fibroblast.BRCA1 mutation.	.18
C44	Primary skin fibroblast, BRCA1 mutation.	.18
C74	Primary skin fibroblast acute XRT reaction.	.18
GM03396A	Fibroblast, ATM heterozygote;	.22
S23	Primary fibroblast line derived from a patient with breast cancer.	.25
C75	Primary skin fibroblast, BRCA1 mutation.	.28
C49	Primary skin fibroblast, BRCA2 mutation.	.28
C83	Primary skin fibroblast, BRCA1 mutation.	.29
C51	Primary skin fibroblast, BRCA1 mutation.	.29
C19	Primary skin fibroblast, patient had breast cancer, no known BRCA mutation.	.30
C76	Primary skin fibroblast, BRCA1 mutation.	.31
C63	Primary skin fibroblast, BRCA1 mutation.	.32
C46	Primary skin fibroblast, BRCA1 mutation.	.32
C29	Primary skin fibroblast	.33
T47D	Breast carcinoma	.35
PC3	Prostate carcinoma	.38
C37	Primary skin fibroblast.	.39
A375	Malignant melanoma.	.41
C80	Skin fibroblast, sequence-normal daughter of C75.	.41
MeWo	Malignant melanoma.	.43
U251	Glioblastoma	.44
U87MG	Glioblastoma	.45
SW620	Colon adenocarcinoma	.47
MIA PaCa-2	Pancreatic adenocarcinoma	.48
CAPAN-1	Pancreatic adenocarcinoma, BRCA-2 mut.	.62
T98G	Glioblastoma	.67
A549	Lung adenocarcinoma	.68
A431	Epidermoid carcinoma	.74
NIH/3T3	Mouse embryo fibroblast.	.74
HN-5	Head and neck, squamous cell carcinoma	.75
H1299	Non-small cell lung carcinoma	.75
HT29	Colon adenocarcinoma	.80

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Figure Legends

Figure 1. DNA EBC analysis predicts radiosensitivity of normal fibroblasts. (A) Representative DNA-EBC analysis of primary human fibroblasts with a variety of radiosensitivities. Note that not all primary cell lines described in Table 1 are included in this example. (B) densitometry was performed on DNA-EBCs in triplicate, normalized to that of the C29 strain, and plotted vs SF2. The correlation coefficient for linear regression was 0.77. Data points represent the mean of at least 3 determination of both SF2 and DNA-EBC density.

Figure 2. Comparison of DNA-EBC pattern from normal and AT cells. DNA-EBC pattern of normal human fibroblasts (C29), and fibroblasts from patients with Ataxia telangiectasia. Those bands marked at the left were absent from ATM mutants. Bands [redacted]re major absent bands. The bands marked at the right were not affected by ATM mutation. Band-D was the major band that was unaffected by ATM mutation. The double arrow (right top) demonstrates a band present in ATM mutant cells but absent in normal controls.

Figure 3. The DNA-EBC pattern of lymphocytes is similar to that of fibroblasts. (A) Comparison of DNA-EBC pattern from fibroblasts (lanes 1 and 2) and lymphocytes from unrelated individuals (lanes 3-6). (B) DNA-EBC pattern of lymphocytes and fibroblasts from a patient heterozygous for ATM mutation (left panel), and a patient heterozygous for an inactivating mutation in BRCA1 (right panel) compared to normal control. An

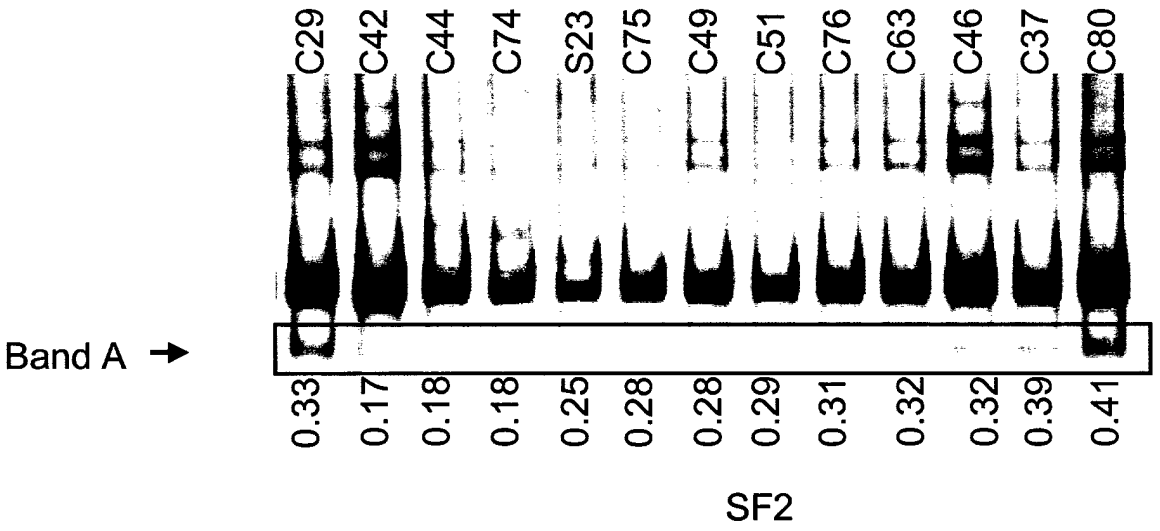
arrow indicates bands seen in both lymphocyte/fibroblast pairs that are not seen in the C80 control.

Figure 4. DNA EBC analysis predicts radiosensitivity of tumor cell lines. (A) DNA-EBC pattern of 15 human tumor cell lines, loaded in order of SF2. (B) band A density predicts SF2 of tumor cell lines (r^2=0.91, P<0.0001).

Figure 5. DNA-EBC pattern is relatively insensitive to contamination with cells of different SF2. (A) Band A density of AT cells (which is very low) is stable until it is contaminated by more than 20% with proteins from normal human cells (C80). (B). Band A density of C80 normal human fibroblast extracts is stable when less than 10% contaminated with rodent proteins. (C). Rodent nuclear extracts do not affect the band A density of AT cell extracts. The difference in quality of panel A from panels B and C reflect a departmental change from X-ray film to digital imaging.

Figure 6. DNA-EBC density vs SF2 for all human cell line. A composite of Figures 1B and 4 demonstrating the correlation between DNA-EBC and SF2 is quite good (r^2=0.85, p<0.000005)

A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C42
C44
C74
S23
C75
C49
C51
C76
C63
C46
C37
C80
Band A
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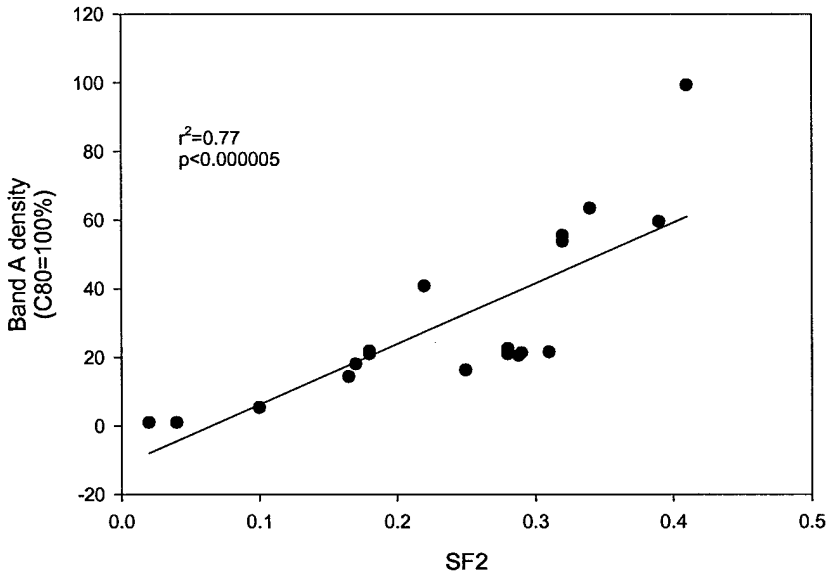
0.17
0.18
0.18
0.25
0.28
0.28
0.29
0.31
0.32
0.32
0.39
0.41
SF2

B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100
80
60
40
20
0
-20
r^2=0.77
p<0.000005
Band A density
(C80=100%)
0.0
0.1
0.2
0.3
0.4
0.5
SF2

Figure 1

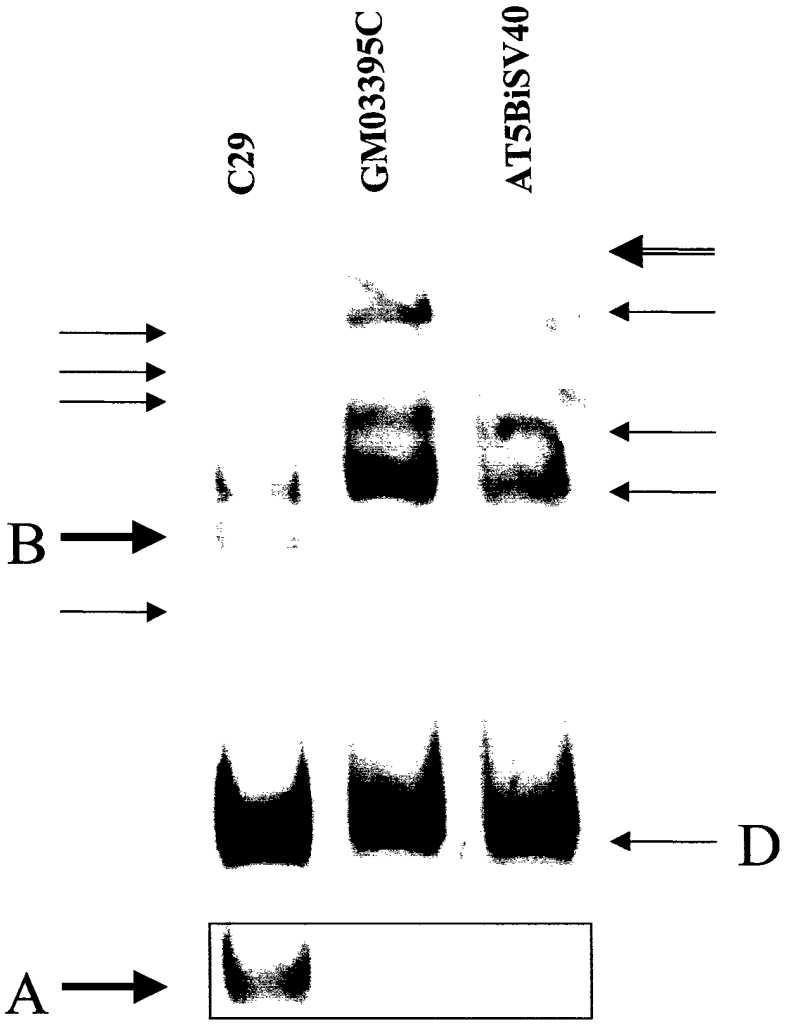
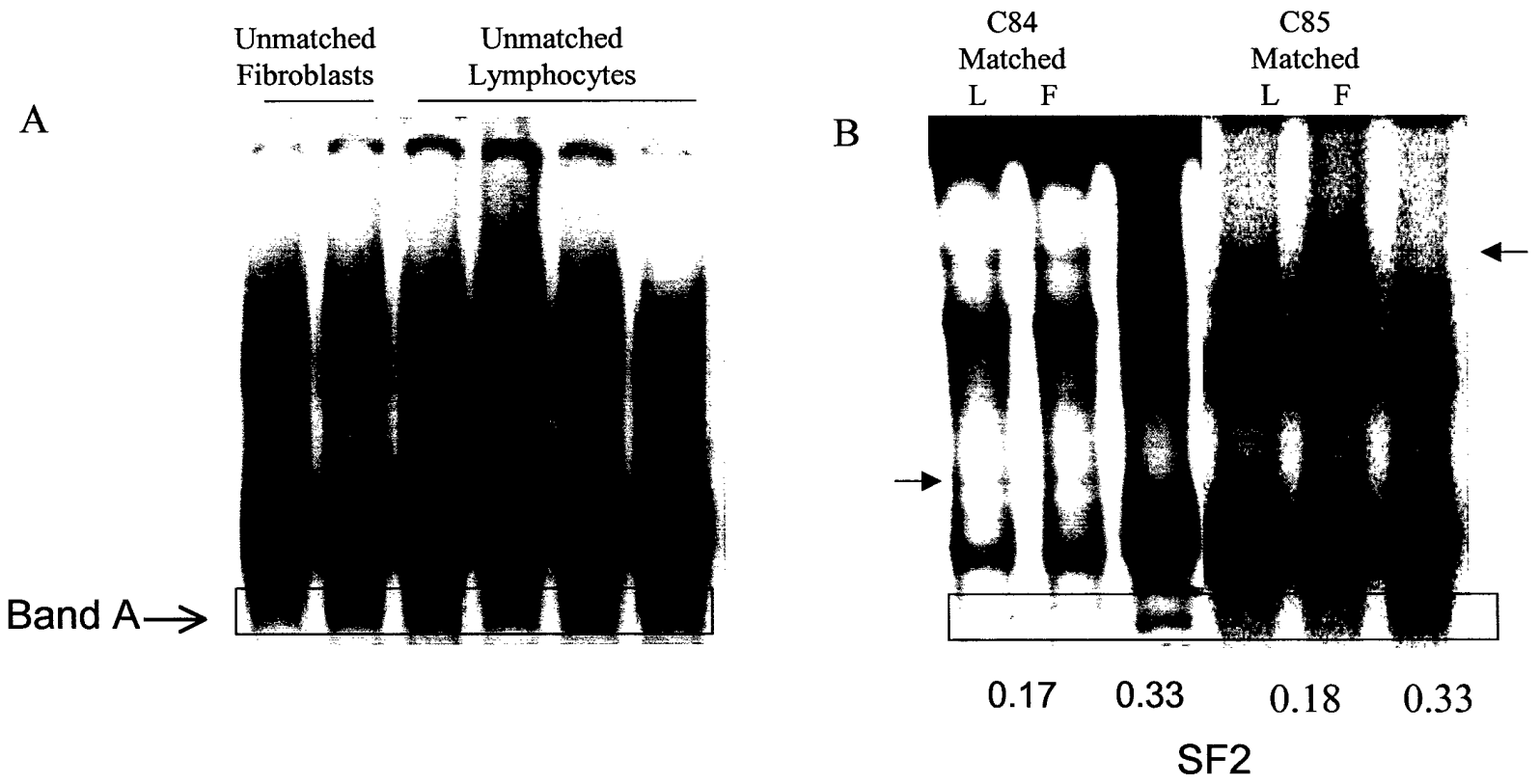


Figure 2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Unmatched
Fibroblasts
Unmatched
Lymphocytes
Band A
B
C84
Matched
L F
C85
Matched
L F
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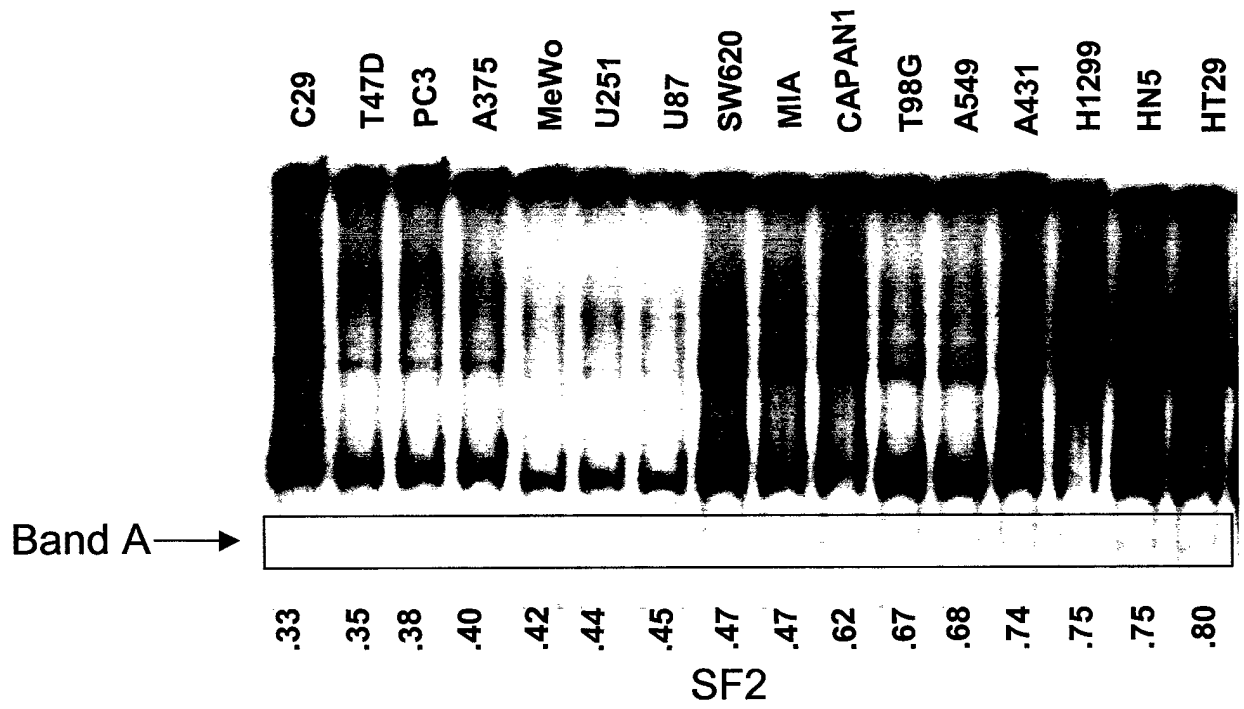
SF2

Figure 3

A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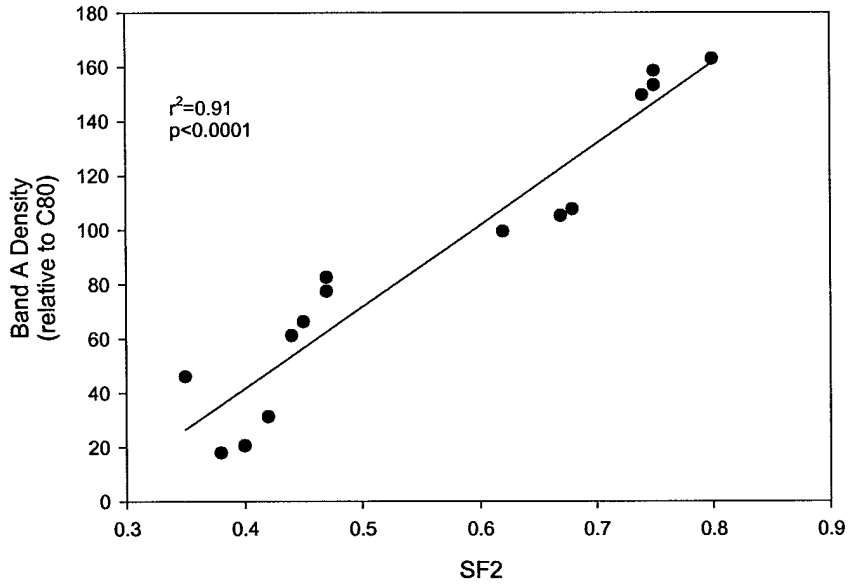
SF2

B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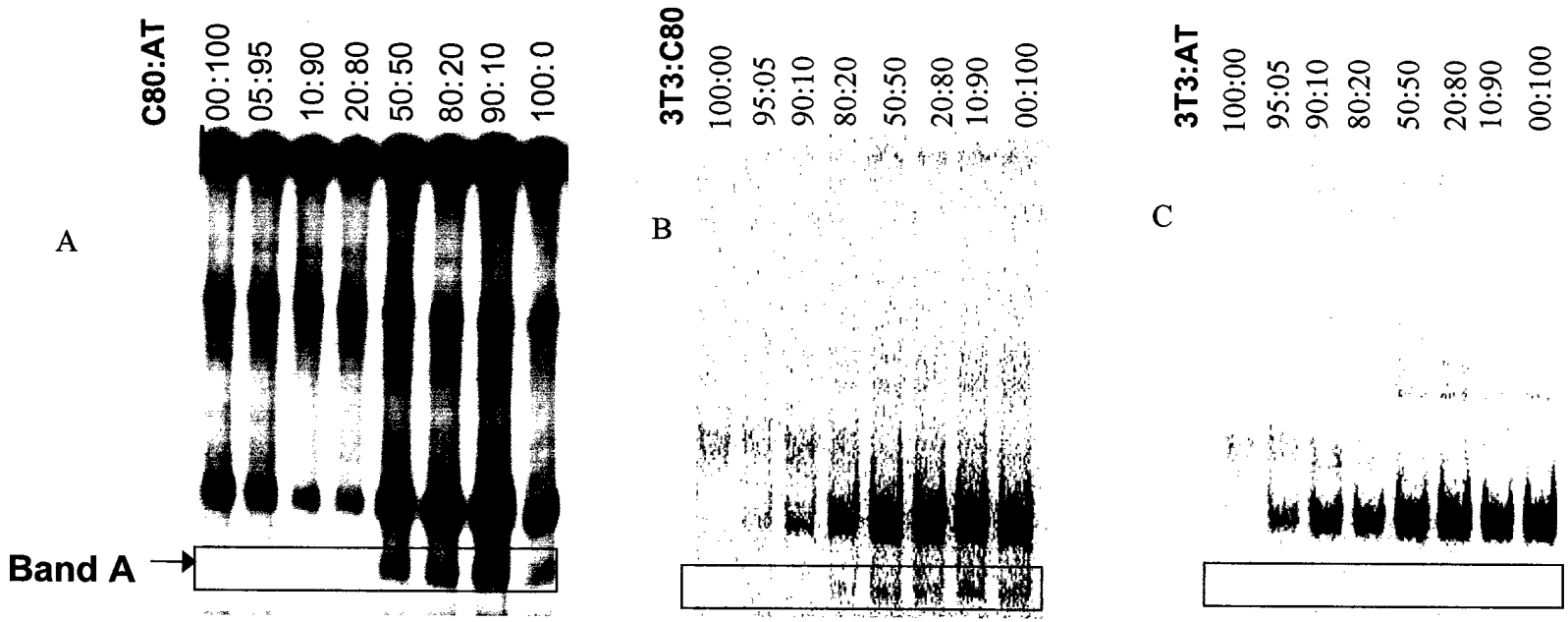
p<0.0001
Band A Density
(relative to C80)
180
160
140
120
100
80
60
40
20
0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
SF2

Figure 4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C80:AT
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10:90
20:80
50:50
80:20
90:10
100:0
Band A
B
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100:00
95:05
90:10
80:20
50:50
20:80
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00:100
C
3T3:AT
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90:10
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Figure 5

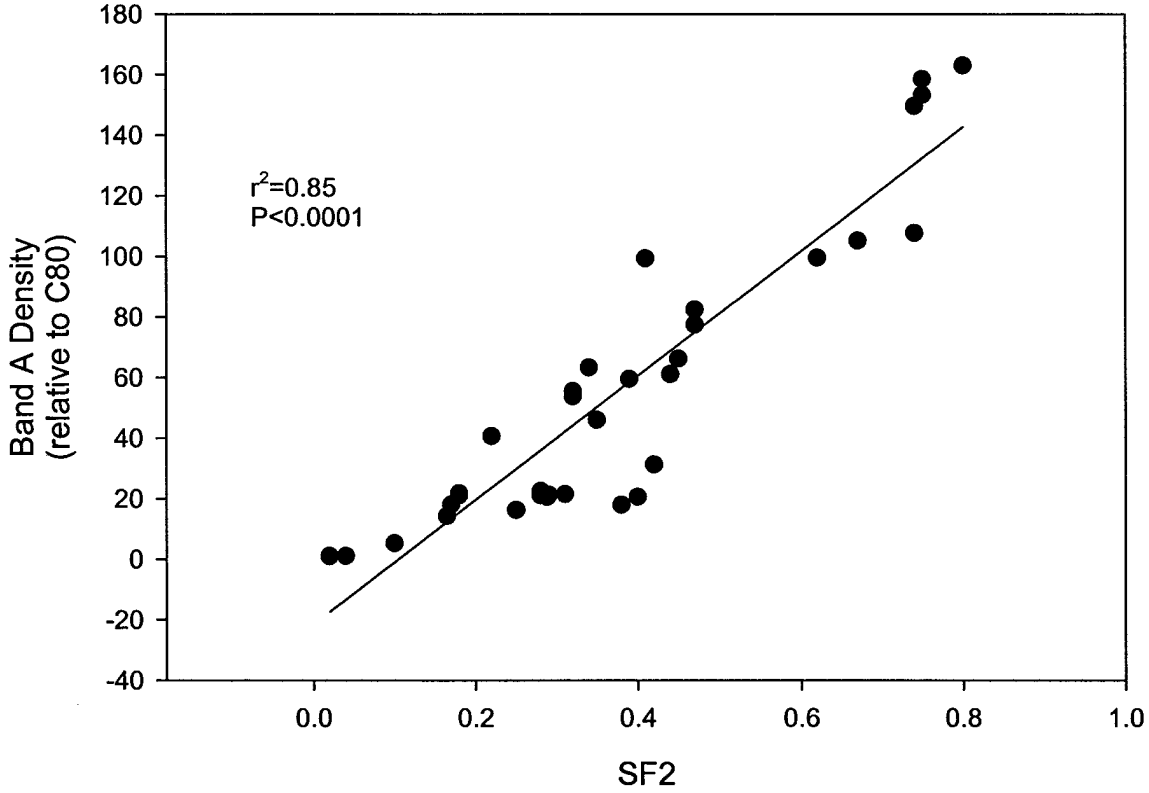
180
160
140
120
100
80
60
40
20
0
-20
-40
Band A Density
(relative to C80)
r2=0.85
P<0.0001
0.0
0.2
0.4
0.6
0.8
1.0
SF2

Figure 6

APPENDIX 4

SURGICAL
CLINICS OF
NORTH AMERICA

Surg Clin N Am 83 (2003) 911–930

Radiation therapy as an adjuvant treatment after sentinel lymph node surgery for breast cancer

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The development of sentinel lymph node surgery has been the most significant recent advance in the local-regional management of early-stage breast cancer. Compared with a standard Level I and II dissection, this surgical technique results in fewer problems with range-of-motion of the shoulder, less sensory loss in the axilla and upper brachium, and lower rates of chronic edema [1,2]. Sentinel lymph node surgery also allows for serial sectioning and immunohistochemical staining of lymph nodes most likely to harbor micrometastatic disease, something that is not feasible for all lymph nodes removed in a standard axillary dissection because of the time and expense this would require.

The Internet and news coverage by the lay press have allowed most breast cancer patients to be well informed of these potential advantages. Correspondingly, it is not uncommon for patients with newly diagnosed breast cancer to seek out surgeons who perform this technique. This consumer demand has led to a marked increase in the use of sentinel lymph node surgery in recent years. The widespread use of sentinel lymph node surgery, however, has preceded scientific evidence regarding the safety and efficacy of the procedure.

Doctor Buchholz was supported by a USAMRMC Career Development Award from the Breast Cancer Research Program (BC980154).

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doi:10.1016/S0039-6109(03)00048-3

There are two ongoing multicenter randomized prospective clinical trials in the United States that are designed to evaluate the effect of sentinel lymph node surgery on staging, morbidity, local-regional control, and survival. The National Surgical Adjuvant Breast and Bowel Project (NSABP B-32 trial) randomizes patients with pathologically-negative sentinel lymph nodes to sentinel lymph node biopsy alone versus a standard Level I/II dissection. The second study, the American College of Surgeons Oncology Group (ACOS-OG Z-0011 trial), randomizes patients with positive sentinel lymph nodes to observation versus a standard axillary dissection.

Ideally, these two trials should have been completed and analyzed before the adoption of sentinel lymph node surgery as a standard therapeutic option. Consumer demand, however, makes this option impractical. Unfortunately, this early adoption carries potential risks, including the potential for overtreatment when patients with biologically-irrelevant cells detected by immunohistochemistry study of sentinel lymph nodes receive systemic treatment, and the potential for undertreatment (and consequent axillary recurrences) when patients with false-negative sentinel lymph node surgery do not have the involved lymph nodes resected. This second risk, arising from leaving microscopically positive lymph nodes undissected, will be explored in greater detail in this article, as radiation treatments may be able to obviate this risk.

Is the false-negative rate of sentinel lymph node surgery a clinically relevant problem?

A false-negative sentinel lymph node surgery is defined as a case in which all sentinel lymph nodes are negative but disease is present in a nonsentinel lymph node. False-negative rates are calculated by dividing the number of false-negative cases by the total number of cases with positive axillary lymph nodes. The incidence of false-negative sentinel lymph nodes surgery can be determined by performing completion axillary dissection after sentinel lymph node surgery in a series of patients. A number of single institutional studies, multicenter studies, and large registry studies have reported false-negative rates using this method (Table 1). The rates have ranged from 6.7% to 13.4% [1,3–11].

False-negative rates are highly correlated with the experience of the surgeon performing the procedure. In a multicenter center reported by Krag et al, false-negative rates for 10 surgeons ranged from 0% to 28.5% [7]. In a another multicenter study, Tafra et al reported that surgeons who performed the procedure in more than 30 cases had a false-negative rate of 15.5% compared with a rate of 4% in surgeons who performed it in 30 or fewer cases [10]. Furthermore, in this study the false-negative rate after surgeons had performed in a minimum of 30 cases was 0%. As shown in Table 1, Giuliano et al reported an 11.9% false-negative rate in their initial 42 patients with positive lymph nodes, but in a subsequent publication of

Table 1
False-negative rates in series with sentinel lymph node surgery followed by completion axillary dissection

Series	Total no. of cases	No. of cases with +LN	False-negative rate
Krag [7]	443	114	11.4%
Tafra [10]	535	140	13%
Veronesi [11]	376	180	6.7%
McMasters [8]	2148	Not reported	8%
Begkvist [3]	450	184	11%
O'Hea [9]	60	23	13%
Dupount [4]	555	114	4%
Hill [6]	458	47	10.6%
Giuliano			
Early series [1]	174	42	11.9%
Later series [5]	107	42	0%

Abbreviations: No, number; +LN, positive lymph nodes.

42 more recent patients with positive lymph nodes, there were no false-negative cases [1,5]. Finally, in a large registry of community and academic surgeons (over 2000 cases), McMasters reported that the identification rate and false-negative rate improved after a surgical experience of 20 cases [12].

It is generally accepted that when a surgeon experienced in the technique performs sentinel lymph node surgery, the false-negative rates should be 5% or less. When surgeons are learning the technique, however, the false-negative rate can be as high as 30%. It seems likely that the rates of false-negative surgeries across the United States are higher than those reported in the literature. These data are significantly subject to publication biases, in that surgeons with high-false negative rates would be less likely to submit their data for peer-reviewed presentations or publications than surgeons with low false-negative rates.

The American Society of Breast Surgeons and the ACOSOG standards currently recommend that all surgeons who perform sentinel lymph node surgery undergo a training set of 20 cases in which they perform the procedure with a concomitant axillary dissection [13,14]. This number of cases was selected as a benchmark based on a number of published reports concerning the learning curve. One consideration often overlooked in determining experience, however, is the time over which the experience is achieved. The data suggesting a 20- to 30-case benchmark came from studies in which surgeons performed a high volume of sentinel lymph node surgeries. It is intuitive that surgeons who infrequently perform sentinel lymph node surgery may require more cases to become proficient. In addition, there currently are few data assessing how the number of cases per unit time affects false-negative rates or whether the continued use of the technique is needed to maintain surgical skills. There is data; however, to suggest that the frequency index affects sentinel lymph node identification rates [4].

According to the ACOSOG standards, the false-negative rate for an individual surgeon (as defined during a 30-case training set) must be below 10% for the surgeon to begin performing the procedure as a stand-alone procedure. Most patients with clinically negative-lymph nodes who undergo sentinel lymph node surgery, however, have no disease in the axilla. Therefore, within any study case set, it is possible that the denominator for the false-negative rate calculation would be very low and the statistical uncertainty about the rate therefore would be very high.

Another source of data suggests that false-negative surgery may be a clinically relevant issue in US practices. In a recently published survey of the practice patterns of 1000 randomly selected Fellows of the ACOS, 77% of the respondents performed sentinel lymph node surgery for breast cancer patients, which again suggests that this technique has been widely adopted into most surgical practices [15]. It is of concern that 28% of the surgeons who performed the procedure began performing sentinel lymph node surgery without completion axillary dissection after 10 or fewer cases. This percentage is also likely to be an underestimation, because survey subjects with more favorable statistics were more likely to respond.

In addition to the experience of the surgeon, other factors can influence the rates of false-negative events. Patients treated with neoadjuvant chemotherapy before surgery may have selective sterilization of disease in the sentinel lymph nodes with residual disease remaining in a second echelon draining lymph node. Also, it is possible that lymphatic drainage patterns may change after neoadjuvant chemotherapy. Breslin et al reported a 12% false-negative rate in a group of 51 patients treated with neoadjuvant chemotherapy, which is a higher rate than that previously reported from the same institution for patients treated with surgery first [16]. Furthermore, 2 of the 3 patients with false-negative results had histological changes consistent with previous metastatic involvement, suggesting that sterilization of disease within the sentinel lymph node does not guarantee sterilization of all involved lymph nodes. Evidence of multifocal or multicentric disease may also affect false-negative rates. In a multicenter Swedish study of 491 patients, the false-negative rate was 33% when multiple foci of disease were found versus 9% for unifocal disease ($P < 0.01$) [3]. This study also suggested that a higher S-phase fraction was associated with higher false-negative rates (20% versus 6%, $P = 0.01$). Tumor location may also affect false-negative rates, particularly if radiocolloid is used to identify sentinel lymph nodes. McMasters reported the outcome of 2148 patients treated on a multi-center trial and found that false-negative rates were higher in patients with upper outer quadrant tumors, possibly because of high radioactivity background counts in the axillary region from primary tumor sites in the upper outer quadrant [8,12]. The data associating tumor location with false-negative surgery have been inconsistent, however. Specifically, Tafla et al found that the false-negative rate was higher in patients with inner quadrant tumors compared with the rate in patients with tumors located in other regions of the breast [10].

In summary, false-negative rates after sentinel lymph node surgery are low when performed by academic and community physicians with sufficient experience in this technique and the multidisciplinary infrastructure required to support this procedure. False-negative rates, however, may be high enough to be clinically relevant for less-experienced surgeons and for selected subpopulations of patients, including those treated with neoadjuvant chemotherapy and those with multicentric or multifocal disease.

Can a false-negative event affect breast cancer survival?

Whether local-regional therapies affect breast cancer survival has been a controversial question for many decades. One of the first randomized prospective trials addressing how management of the axilla affects outcome was performed by the NSABP in the 1970s [17,18]. In this study, patients with clinically negative lymph nodes were randomized to radical mastectomy (surgical treatment of the axilla), total mastectomy (no axillary treatment), or total mastectomy plus postmastectomy radiation to the chest wall and draining lymphatics. No axillary treatment was associated with higher rates of axillary recurrences, but there was no difference in survival between the groups. These data led many to believe that prevention of regional recurrences through intervention would be unlikely to improve cure rates. Data from more recent trials, however, have suggested that prevention of axillary recurrences can improve survival. Two randomized prospective trials investigating postmastectomy radiation found that reductions in local-regional recurrence improved overall survival [19,20]. Furthermore, in the patients randomized to the no-radiation arm in whom a local-regional recurrence developed, the axilla was a component of local regional-failure in 45% [21]. These data suggest that axillary recurrences may seed new distant sites and compromise survival. Additional evidence regarding the importance of avoiding axillary recurrence came from a meta-analysis of six studies investigating prophylactic axillary node dissection [22]. This meta-analysis reported that axillary dissection provided an approximately 5% survival advantage.

From these data, we conclude that prevention of axillary recurrences is a worthy objective and that efforts should be made to avoid false-negative sentinel lymph node surgeries or prevent its consequences.

Is radiation therapy an effective axillary treatment?

Radiation treatment of axillary lymph nodes may be one way to prevent the consequences of false-negative sentinel lymph node surgery. Doctors Montague and Fletcher from the M.D. Anderson Cancer Center began investigating primary radiation as an alternative treatment to surgery for patients with clinically negative axillary lymph nodes during the 1970s, well

before the advent of sentinel lymph node surgery. The NSABP B-04 trial was the first randomized trial to directly compare primary radiation and axillary lymph node dissection [17,18]. In patients with clinically negative lymph nodes, the axilla was a component of first site of failure in only 1.4% in those with primary surgical treatment compared with 3.1% in those with primary radiation treatment. In addition to the randomized data, a number of institutions that have investigated management of the axilla with primary radiation. A summary of these data is provided in Table 2 [23–30].

These data together suggest that radiation treatment of micrometastatic disease present in axillary lymph nodes is a highly effective therapeutic modality. Correspondingly, radiation treatment of the axilla may minimize the consequences of a false-negative sentinel lymph node surgery, provided treatment fields are designed to encompass the axillary lymph nodes.

Optimizing radiation techniques to treat the axilla

For women with early-stage breast cancer treated with radiation after breast-conserving surgery, standard radiation treatment fields are designed to cover the ipsilateral breast as the primary treatment volume. The fields typically are of oblique angles designed to tangentially traverse the anterior thorax, thus minimizing dose to the underlying intrathoracic structures such as the heart and lungs. For patients with early stage disease, radiation field design for axillary treatment has historically focused on the addition of a third field, matched above the tangent fields. This field was added for patients requiring treatment of the axillary apex and supraclavicular fossa. Our group and others, however, have shown most of the Level I and II axilla are below (caudal to) the match line of the tangent fields and the supraclavicular/axillary apex field [31–34]. Therefore, effective nodal radiation treatment of patients who have had a false-negative sentinel lymph node surgery is more dependent on the proper design of the tangent breast fields than any field or fields added above the cranial edge of the tangents for those patients with early stage breast cancer.

Table 2
Efficacy of radiation in preventing axillary recurrences for patients with clinically negative axilla

Series	Number of patients	Radiation of level III/SCF	Follow-up	Regional recurrence rate
Haffty [23]	327	Yes	5-year rate	3%
Recht [27]	9	Yes	77 months	2.1%
Wazer [28]	73	Yes	54 months	1%
Wong [29]	92	No	50 months	1%
Halverson [24]	75	Varied	Not provided	2.7%
Zurrida [30]	221	Yes	42 months	0.5%
Hoebers [25]	105	Yes	5-year rate	2%
Kuznetsova [26]	456	Yes	52 months	0%

Traditionally, axillary anatomy is not considered in the design of the tangent breast fields; however, this issue has clinical relevance for selected patients in whom sentinel lymph node surgery rather than axillary dissection is performed. We recently reviewed anatomical landmarks that can be used during fluoroscopic simulation of radiation fields in an effort to define field borders to help optimize radiation management of the undissected axilla. [34]. We found that tangent fields with a nondivergent cranial border within 2 cm of the ipsilateral humeral head and a deep field edge 2 cm within the chest wall/lung interface permitted coverage of over 80% of the Level I and II axilla. In addition, these fields covered the sentinel lymph node dissection region in over 95% of the cases. Other investigators have also reported that the majority of the Level I and II axilla is located within tangent fields used for breast cancer treatment [31–33]. If the axillary anatomy is not specifically considered during the treatment planning process, however, the Level I and II axilla frequently can extend deep to the field border.

Three-dimensional computed tomography (CT) treatment planning of radiation fields is a much more precise method for covering axillary structures within radiation fields. For this type of treatment planning, patients undergo a CT scan in the treatment position and immobilization cradle used for the daily treatments. The treatment fields are then virtually designed on the CT data set. For patients at higher risk of having a false-negative sentinel lymph node surgery, the axilla can be contoured as a target volume on sequential CT slices. This three-dimensional contoured structure is then visualized during the virtual planning of the tangent radiation fields, and the field borders can be designed to include the volume at risk. As these fields often require a deeper field border near the cranial edge of the tangent fields, a custom block in the lower half of the field is often needed to minimize the volume of heart and lung that is irradiated.

For patients at low risk for disease in the infraclavicular or supraclavicular lymph node basins, we treat with a technique commonly called "high tangents" because the cranial edge of the field is raised up toward the humeral head to include as much of the axillary contents as feasible. Radiation fields diverge as they leave the treatment machine. This divergence can be eliminated at the cranial edge of the field by rotating the patient position relative to the machine by the same angle as that of the beam divergence. Fig. 1 shows an example of a high-tangent radiation field that includes the contoured low axillary structures.

Selected patients may be at risk for disease in the infraclavicular and supraclavicular fossae. For these patients, tangent fields with nondivergent cranial borders are matched to a third field. The height of the match line is less important in this scenario, in that axillary structures extending above the tangent fields are covered in the matched third field. An example of a matched pair of fields is shown in Fig. 2.

In addition to greatly facilitating treatment field design, three-dimensional CT-based treatment planning also allows for accurate calculation of

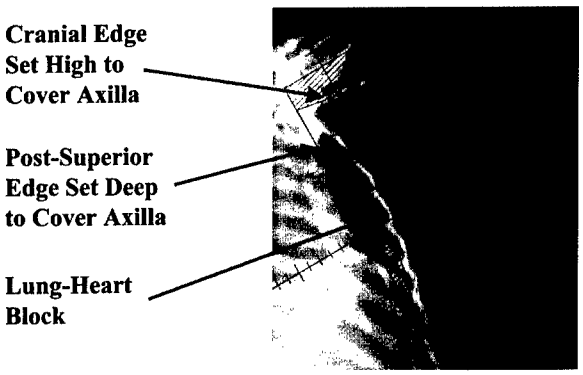


Fig. 1. A beam's-eye view of a high tangent radiation treatment field designed to cover the breast and low axilla. The cranial (top border) is raised close to the humeral head and the treatment couch is rotated to eliminate beam divergence along this field edge. The deep border is extended with the thorax in the upper half of the field to assure coverage of the axilla. A custom block is used to minimize radiation dose to the lung and heart in the lower half of the field. The reconstructed contours of the tumor bed and low axillary lymph nodes are shown.

radiation dose delivered to the axilla. During a course of radiation, not all points within the treatment volume receive the prescribed dose of radiation. For example, in areas in which the radiation beams must reach a greater depth, dose falloff can lead to a lower total dose. We recently reported that with conventional treatment planning, the sentinel lymph node dissection region may receive as little as 77% of the prescribed dose [34]. Three-dimensional treatment planning can improve dose compensation and help avoid underdosing or overdosing specific regions within the treatment field.

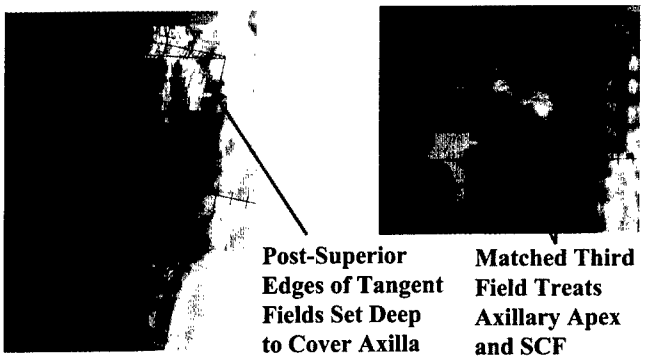


Fig. 2. A three-field radiation technique used to comprehensively treat the axilla and supraclavicular fossae in addition to the intact breast. The third field is geometrically matched to the cranial edge of the tangent fields and encompasses the high Level II and level III axilla. For this arrangement, the cranial edge of the tangent fields can be lower than when tangent fields are used alone to treat the axilla. The reconstructed contours of a large postoperative tumor bed and the low axilla are shown.

For example, a small supplemental dose can be given to the small volumes of low dose regions in either the tangent fields or in the third matched field, and selective shielding can be applied to areas receiving dose in excess of that prescribed. These techniques assure not only that the axillary lymph nodes are within the treatment fields but also that they receive an adequate radiation dose.

The relationship of radiation field design to axillary recurrences has relevance for the two ongoing national sentinel lymph node trials. The NSABP B-32 trial, investigating sentinel node resection alone for patients with pathologically negative sentinel lymph nodes, includes both patients treated with breast conservation therapy and patients treated with mastectomy. Correspondingly, it will be interesting to compare axillary recurrence rates in patients treated with mastectomy and sentinel lymph node surgery (who do not receive radiation) with those in patients treated with radiation as a component of breast conservation therapy. Similarly, all the patients in the ACOSOG Z0011 trial investigating sentinel lymph node surgery alone for patients with positive sentinel lymph nodes (defined by hematoxilin and eosin staining) receive breast-only radiation as a component of their management. Our data indicate, however, that in most patients, radiation treatment fields designed to treat the breast will coincidentally cover a significant portion of the lymph node region at risk. It is likely that this will affect the outcome of the ACOSOG trial.

In fact, incidental axillary radiation during breast treatment may have affected a recently reported randomized prospective trial that compared "no axillary treatment" with axillary radiation for women with small primary tumors and clinically negative axillary lymph nodes [30]. In this study, 435 patients with early stage breast cancer were randomized to breast-conserving surgery and radiation without axillary treatment versus breast-conserving surgery and radiation with axillary radiation (presumably meaning the addition of a third field matched above the tangent fields). At 5 years, rates of axillary recurrences were below 3% in both arms. Other data also support the concept that tangent fields alone can be used as primary axillary management. Wong et al reported an axillary failure rate of only 1% in 92 patients with clinically negative lymph nodes treated without axillary dissection and tangent radiation fields alone (median follow-up 50 months) [29].

Giuliano et al published one of the first series to quantify axillary recurrences for patients with pathologically negative lymph nodes from sentinel lymph node surgery without axillary dissection. In a group of 67 patients (over 95% of whom were treated with a breast conservative surgery and radiation), there were no axillary recurrences after a median period of 39 months [35]. From these data it cannot be determined whether the false-negative rate was 0% or whether the breast radiation was therapeutic in cases of false-negative surgery. In addition, longer follow-up is needed for all of these studies, as breast cancer patients may develop nodal failures many years after primary treatment.

Radiation management of patients with positive sentinel lymph nodes

The standard management for all patients with positive sentinel lymph nodes is a completion axillary dissection. As shown in Table 3, these patients have high rates of additional axillary lymph node disease [7,11,36–38]. This risk varies according to a number of factors, however, including the size of the metastasis in the sentinel lymph node, the size of the primary tumor, the location of the primary tumor, the number of involved sentinel lymph nodes, the presence of lymphovascular invasion, and the number of sentinel lymph nodes resected. Some patients with factors predicting a low risk for additional axillary disease elect to forgo completion axillary dissection.

The size of the sentinel lymph node metastasis is the one discriminator of risk of residual axillary disease. In a study initially reported by Chu et al but updated by Turner et al, 26% of the 93 patients with a micrometastases ($\leq$2 mm) had additional disease in a non-sentinel lymph node [38,39]. Reynolds et al reported a 22% rate of additional axillary disease in 27 patients with micrometastases, and Veronesi et al found additional axillary disease present in 53% of the 51 patients in their series with a sentinel lymph micrometastasis [11,37].

It is likely that some patients with micrometastases and small primary tumors have a low risk of additional disease. Both Chu et al and Reynolds et al reported that the size of the primary tumor independently correlated with the risk of nonsentinel lymph node involvement [37,39]. In the Chu et al study, the risk of additional disease for patients with a micrometastasis in a sentinel lymph node was 0% for T1a and T1b tumors, 6% for T1c tumors, 10% for T2 tumors, and 33% for T3 tumors [39]. Similarly, Reynolds et al found that none of the 18 patients with primary tumors under 2 cm and micrometastatic sentinel lymph node disease had additional axillary disease [37]. In the update of the Chu study, Turner et al found that lymphovascular space invasion and the presence of extranodal hilar disease also predicted for rates of involvement of nonsentinel lymph nodes. In this study, the 57 patients with T1 or T2 tumors and a micrometatases with neither of these two features had a rate of nonsentinel lymph node involvement of 12% [38]. Finally, in a study of 131 patients with positive sentinel lymph nodes who underwent completion lymph node dissection, Hwang et al also found that

Table 3
Likelihood of additional axillary disease in patients with positive sentinel lymph nodes

Series	Number of patients with positive sentinel lymph nodes	Percentage of these patients with additional axillary disease
Turner [38]	194	45%
Reynolds [37]	60	47%
Krag [7]	101	40%
Veronesi [11]	168	58%
Hwang [36]	131	41%

primary tumor sizes under 2 cm, lymph node metastastses measuring 2 mm or less, and absence of lymphovascular space invasion independently predicted for low risk of nonsentinel lymph node involvement. In addition, these authors found that as the number of sentinel lymph nodes resected increased the rate of involvement of nonsentinel lymph nodes decreased [36].

It is unclear what the optimal radiation fields should be for patients with positive sentinel lymph nodes who elect not to undergo a standard axillary dissection. It is likely that the area of greatest risk is within the Level I and II axilla. In fact, the studies that have suggested significant rates of residual disease have only examined the Level I and II nodes. As previously mentioned, the majority of the Level I and II axilla is below (caudal to) third fields historically used to treat the axillary contents. Correspondingly, proper design of the breast tangent fields to include the axillary structures remains the most important component of axillary radiotherapy for these patients. The risk of Level III or supraclavicular disease for patients with positive sentinel lymph nodes is poorly defined. Following a standard Level I/II dissection, the Level III axilla and supraclavicular fossa are thought to be at risk when four or more axillary lymph nodes are involved or when primary tumors are greater than 5 cm [40]. For these patients, matched radiation fields are considered to be an important component of radiation treatments. For patients with one to three positive lymph nodes, it is controversial whether the added morbidity (arm edema, small risk of injuring the brachial plexus, and a small increase in the risk of lung injury) is warranted, given the low likelihood of an added benefit.

There are few data concerning features of sentinel lymph node surgery that predict for the presence of four or more positive lymph nodes. In one of the few published papers addressing this issue, Krag et al found that the incidence of four or more positive lymph nodes after axillary dissection was dependent on the number of involved sentinel lymph nodes [7]. The rates were 16% for patients with a single involved sentinel lymph node, 30% for patients with two involved sentinel lymph nodes, and 60% for patients with three involved sentinel lymph nodes. Therefore, even if tangentially designed breast fields effectively treated the low axilla, a fair percentage of patients with two or more involved sentinel lymph nodes would be at risk for disease in areas beyond the tangent field.

There are no reports of studies specifically testing the efficacy of radiation as an alternative to axillary lymph node dissection for patients with positive axillary lymph nodes. Recently however, Galper et al reported that regional nodal failure as an isolated event occurred in just 1.4% of patients treated with comprehensive nodal radiation after a positive lymph node was found on axillary sampling [41]. These data and the data regarding the efficacy of primary radiation treatment (see Table 2) suggest that radiation is likely to be an effective treatment.

For patients with positive sentinel lymph nodes, our standard is to recommend participation in the ACOSOF Z-0011 trial or to proceed with an

axillary dissection. We discuss the data regarding risk of residual axillary disease with all patients. For those with a single micrometastasis and a T1 tumor who elect not to undergo axillary dissection, we favor treatment with high tangent fields alone. For all other patients who forgo an axillary dissection, it is our practice to add match fields to cover the axillary apex and supraclavicular fossa.

When should the internal mammary lymph nodes be irradiated?

Sentinel lymph node surgery has led to a renewed interest in radiation treatment to the internal mammary chain (IMC) of lymph nodes. The use of lymphoscintigraphy as a component of sentinel lymph node surgical planning has proven that the IMC is a primary channel of lymphatic drainage for up to 20% of patients with early stage breast cancers. Table 4 shows the rates of drainage to the IMC and also provides data that indicate that the incidence of drainage is dependent on the location of the primary tumor within the breast, with central and medial tumors having higher rates of IMC drainage than outer quadrant tumors [42–48]. The rate of drainage to the IMC may also be dependent on the injection technique used. For example, it is less common to find drainage to the IMC with subdermal injections compared with parenchyma injections.

Most of the data concerning the risk of IMC involvement come from series predating sentinel lymph node surgery that used IMC dissection as a component of a mastectomy [49–52]. How relevant these data are to current patients with mammographically detected early stage disease can be questioned, but the data are useful in highlighting the potential risks of IMC involvement. The data from these series suggest that patients with central or medial tumors with positive axillary lymph nodes have IMC rates of involvement ranging from 50% to 65% [49–52]. For patients with central

Table 4
Frequency of lymphoscintigraphy-demonstrated drainage to the internal mammary lymph nodes

Series	Number of cases	Tumor location	Percentage that drain to the IMC
Uren [47,48]	159	Overall	45%
	16	Inner quadrant/central	44%
Johnson [44]	80	Overall	12%
	32	Inner quadrant/central	12%
Byrd [42]	220	Overall	17%
	61	Inner quadrant/central	17%–29%
Haigh [43]	76	Overall	20%
Laronga [45]	331	Overall	22%
	105	Inner qudrant/central	24%
Smitt [46]	89	Overall	18%

and medial tumors and axillary lymph node negative disease, rates of IMC involvement run from 12% to 14% [49–52]. How the use of systemic therapy affects these percentages is unknown.

The largest experience to date regarding dissection of IMC lymph nodes as a component of sentinel lymph node surgery was reported from Milan, Italy [53]. Twelve out of 122 cases (10%) with evidence of drainage to the IMC chain on lymphoscintigraphy from inner quadrant tumors had pathologically involved IMC lymph nodes. In all cases the axilla was also dissected and if the nodes were positive, the IMC was positive 18% of the time (8/45). If the axillary nodes were negative, the IMC was only positive in 5% (4/77).

Data from the Moffit Cancer Center on 36 resected sentinel internal mammary lymph nodes showed a positivity rate of 14% [54]. Two of these five cases had IMC lymph node involvement only, with the 3 other cases also having axillary disease. Two of the five cases had multiple involved lymph nodes in the IMC chain.

Finally, in a much smaller series, 80 patients had lymphoscintigraphy, and 10 were found to have drainage to the IMC [44]. Metastatic disease was present in the IMC chain in 3 of the 10 cases. All 3 also had disease in the axilla. Interestingly, 2 of the 3 had outer quadrant tumors.

Whether to treat the IMC remains one of the more controversial areas of breast radiation. It is natural to speculate that for some tumors, IMC lymph nodes are analogous to the axilla and that treatment of a primary lymph node basin in addition to treatment of the primary is appropriate. Whether treatment of the IMC containing micrometastatic disease lengthens survival, however, remains uncertain. One argument against treatment of the IMC is that this site is a very unusual location for an isolated regional recurrence. There are many potential explanations for this, foremost of which is that IMC lymph nodes are intrathoracic structures and early recurrences cannot be detected by physical examination. Furthermore, screening studies are not routinely performed to evaluate this region for first-event recurrences, so IMC recurrences may remain undetected. Regardless, the more appropriate endpoint to support the use of IMC irradiation is an improvement in overall survival rather than a reduction in the incidence of isolated IMC recurrence. Current data comparing IMC therapy to no-IMC therapy are inadequate to reach firm conclusions. Given the limited nature of the available data, the strongest support for the use of IMC irradiation has come from a multivariate analysis of 1195 patients treated at the Institut Gustave-Roussy [55]. For patients with medial or central primary tumors who also had positive axillary lymph nodes, IMC treatment was independently associated with a reduction in the risk of distant metastases ($P = 0.02$). In the 607 patients with tumors lateral to the areola, there was no clear benefit for IMC treatment.

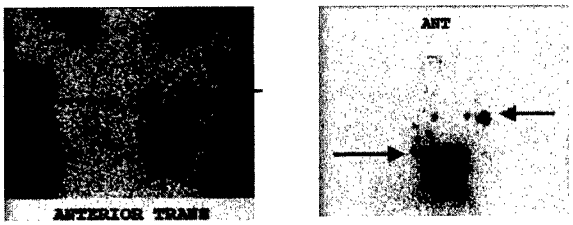
Additional information that may have relevance with respect to IMC treatment is the finding that patients with early stage medial tumors have a lower survival rate than lateral tumors. In a study of 2396 patients treated

with breast conservation and no directed therapy to the IMC nodes, a 30% increase in the rate of distant metastases was noted for patients with medial/central tumors compared with those with lateral tumors [56]. These data have recently been supported by another study using the SEER database, which showed that in 96,543 patients with lymph node negative disease, patients with medial tumors had significantly poorer outcome than patients with lateral tumors ($P = 0.0001$) [57].

To more appropriately address the benefit of IMC radiation, two ongoing randomized trials are currently underway in Canada and Europe. The beginning of these trials predate the routine use of sentinel lymph node surgery, so it would be appropriate to conduct a randomized Phase III trial comparing IMC treatment versus no treatment exclusively in breast cancer patients with lymphoscintigraphic evidence of IMC drainage.

Given the aforementioned data, our philosophy has been to consider treating the IMC chain for selected patients with primary drainage to these lymph nodes. We attempt to include the IMC in those patients with intermediate stage breast cancer with drainage patterns to both the IMC and axilla who have positive axillary sentinel lymph—balancing this against the potential compromise of primary tumor bed coverage, excessive normal tissue effects, and impaired cosmetic outcomes. We routinely treat the IMC chain in the context of locally advanced or recurrent breast cancer.

Treatment of the IMC lymph nodes with radiation is technically challenging. Often treatment of the IMC necessitates accepting an increase in the volume of heart or lung that are irradiated. Most patients with IMC drainage also have medial primary tumor beds. It is this area of the breast, therefore, that has the greatest risk of local recurrence. The imprecision of the radiation dose delivery at the match line between two fields causes us to be hesitant to use a separate electron field to treat the IMC, because this choice often places the match line over the tumor bed. The alternative to matching a separate field with the breast tangents is to use a deeper tangent field that includes the primary tumor bed, upper IMC, and undissected axilla. We prefer this approach for patients with upper IMC lymph nodes because custom blocking can minimize heart/lung dose in the lower half of the tangent field. Based on surgical dissection series, the primary lymph nodes in the IMC chain at risk for involvement are within the first three or four intercostal interspaces. More recent lymphoscintigraphic data, however, suggest that drainage to lower interspaces is not infrequent, particularly for patients with lower quadrant tumors [46]. Fig. 3 shows an example of two patients with IMC drainage seen on lymphoscintigraphy, one with the more common drainage to the upper three interspaces and one with drainage to the lower interspaces. For some cases, particularly those with left-sided low IMC drainage and medial tumor beds, there are no field arrangements that would provide adequate coverage of the target volumes while still achieving an acceptable volume of heart and lung dose. In these patients, we often elect not to include the IMC.



Dual IMC/Axillary Drainage with Drainage to Upper IMC

Dual IMC/Axillary Drainage with Drainage to Lower IMC

Fig. 3. Lymposcintrigraphies of two breast cancer patients showing dual drainage to the axilla and internal mammary chain (IMC). The scan on the left drains to IMC in the area of the upper three intercostal spaces. The scan on the right is from a patient with a lower central tumor that had drainage to the low IMC region.

IMC radiation requires CT-treatment planning to assure adequate target volume coverage while minimizing the risk of normal tissue injury. The IMC volume can be contoured by outlining the internal mammary vein and artery, which are easily identifiable on CT. Typically, the IMC lymph nodes lie medial to the vessels in the first two interspaces and then cross lateral to the vessels in the third interspace. The contoured volumes can then be visualized during field design. Fig. 4 shows an example of treatment planning of fields used to include the IMC.

Future radiation techniques may be able to overcome some of the limitations of IMC radiation. For patients with drainage to left-sided lower IMC lymph nodes (see left panel of Fig. 3), it is frequently difficult to deliver therapeutic doses of radiation to these volumes without simultaneously irradiating the left atrium, anterior left ventricle, and the left main and left anterior descending coronary arteries. One strategy that is being investigated to overcome this limitation is to gate the delivery of radiation to specific periods of the respiratory cycle. This technology allows the radiation beam to be on only during maximum inspiration. Preliminary studies have indicated that during maximum inspiration, the heart falls away from the chest wall, permitting a greater separation between the targeted IMC volume and the heart [58,59]. A second new technology that may enable better dose conformality around the IMC lymph nodes is proton radiation. Protons have a sharper dose falloff than conventional radiograph beams, allowing for a greater degree of dose-shaping. Finally, three-dimensional intensity modulated electron beam therapy may also be a future method of delivering tightly conformal radiation to the IMC.

Summary

In this article, we have suggested that radiation can be an important adjuvant to sentinel lymph node surgery. Combining radiation with sentinel

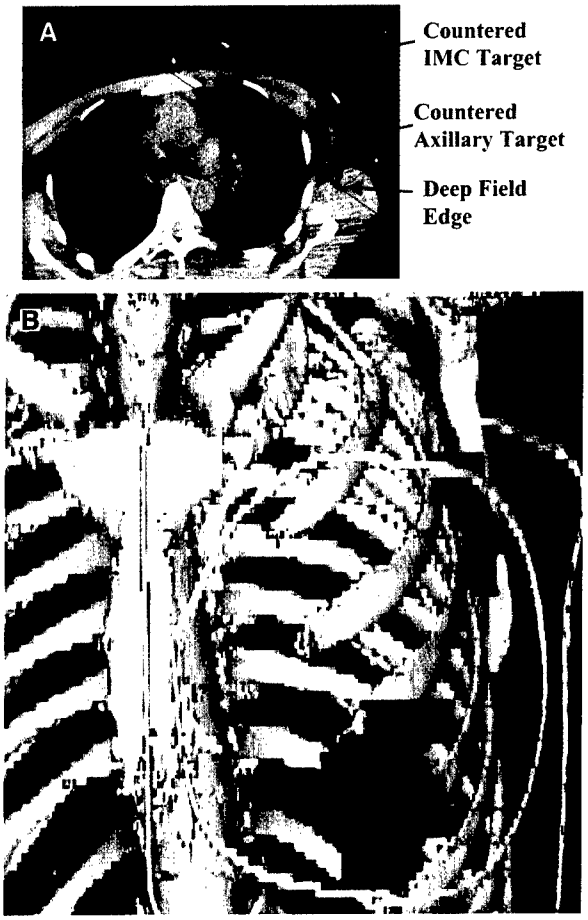


Fig. 4. Method of irradiating the upper internal mammary chain (IMC) lymph nodes and low axilla using three-dimensional treatment planning. (*A*) The IMC lymph nodes and axilla are contoured in sequential computed tomography slices. (*B*) An anterior projection of the reconstructed contoured IMC, axilla, and tumor bed volume. The circular volume is from radio-opaque wires placed around the palpable breast tissue at the time of treatment planning simulation. (*C*) A beams-eye view of a high tangent radiation treatment field designed to cover the breast, low axilla, and upper IMC. The reconstructed contours of a tumor bed, upper IMC and the low axilla are shown.

lymph node surgery has the potential to minimize the risk and consequences of false-negative surgery and can be used in selected cases as the definitive axillary treatment after finding a positive sentinel lymph node. In addition, radiation can be used as definitive therapy for patients at risk for IMC involvement.

The radiation treatment fields after sentinel lymph node surgery need to be individualized, depending on the characteristics of the case. In general,

Fig. 4 (*continued*)

we use standard tangent beams with the breast as the target volume for patients with unifocal breast cancer and negative sentinel lymph nodes treated initially by an experienced surgeon. We include the breast and the majority of Level I and II axilla within the irradiated target volume for patients with negative sentinel lymph nodes who have one of the following features: (1) unknown or inexperienced surgeon, (2) multifocal breast cancer, or (3) neoadjuvant chemotherapy. We include this target volume by contouring these structures on a three-dimensional treatment planning system and designing our tangent fields with a raised and nondivergent cranial field edge and a field edge deep enough to cover the low axilla.

For patients with positive sentinel lymph nodes, we recommend axillary dissection. For patients who do not undergo a dissection, we use the high tangent fields described above if the primary disease is under 2 cm and the axillary disease is 2 mm or less and present in a single lymph node, provided there is no lymphovascular space invasion or extracapsular disease. For all other sentinel lymph node-positive patients, we match fields above the tangent fields to treat the axillary apex and supraclavicular fossa. The upper IMC lymph nodes are contoured and included in tangent fields for patients with primary drainage to this site or dual drainage to this site with a positive axillary sentinel lymph node. If too much lung or heart is included in these

fields, alternative field arrangements are sought, but if none are feasible, the IMC is left untreated. The radiation dose is calculated for all contoured nodal structures, and supplemental fields are used to assure that the regions are treated to the prescribed dose.

These treatment policies are based more on scientific rationale rather than scientific outcome data. Clearly, more research is needed to assess the value of radiation as an adjuvant modality for regional treatment after sentinel lymph node surgery.

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EDITORIAL

Lung Carcinoma Development after Radiotherapy for Breast Carcinoma

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Treatment-induced cancer is one of the greatest fears of patients receiving radiotherapy for breast carcinoma. The majority of the literature concerning radiation-induced malignancies in patients with breast carcinoma published to date has focused on the small but real risk of developing a posttreatment sarcoma.[1] However, more recent data suggest that radiotherapy also may increase the risk of developing lung carcinoma. During radiotherapy for breast carcinoma, a small percentage of the ipsilateral lung receives some radiation dose. Previously, when radiation was used to treat ankylosis spondylitis or Hodgkin disease and a portion of the lung was coincidentally treated, the long-term risk of lung carcinoma development was found to be increased.[2,3] In addition, Japanese survivors of the atomic bomb were found to have an increased relative risk of developing lung carcinoma.[4]

In a very important article in this issue of *Cancer*, Deutsch et al. report rates of lung carcinoma development in 3515 breast carcinoma patients treated on the National Surgical Bowel and Breast Project (NSABP) B-04 and B-06 trials.[5] These data are particularly valuable in that both trials tested radiation as a randomized variable and both studies now have 20-year outcome information. In their study, Deutsch et al. report that radiation use was associated with a small increase in the development of lung carcinoma. Furthermore, the increased risk of developing lung carcinoma was noted exclusively in those patients randomized to undergo radiotherapy in the B-04 trial but not those treated with radiation in the B-6 trial. The likely reason for this difference is that in the NSABP B-04 trial, radiation fields included both the chest wall and the regional lymph nodes whereas in the B-06 trial the radiation fields included only the breast. Correspondingly, the volume of normal lung incidentally included in the radiotherapy volume was much higher in the NSABP B-04 trial compared with the B-06 trial.

For the study by Deutsch et al., it was not possible to retrospectively quantify the volumes of normal lung included within the irradiated treatment volumes for each patient. Such data would be useful in helping to define parameters that are associated with the risk of lung carcinoma development. Modern three-dimensional treatment planning tools now permit the precise quantification of lung volumes irradiated. For example, Das et al. estimated the volume of lung

See referenced original article on pages 1362–8, this issue.

Dr. Buchholz is supported by Grant No. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

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Received May 27, 2003; accepted June 25, 2003.

DOI 10.1002/cncr.11657

included in breast fields by studying treatment planning computed tomography scans of 108 patients.[6] Using data from the study by Das et al. and assuming an average of 2 cm of lung in the tangent field, the estimated percentage of lung irradiated in the radiation field for the NSABP B-06 study would be 10% for carcinomas of the left breast and 12% for those of the right breast. Because postmastectomy radiotherapy typically requires more circumferential coverage of the anterior thorax compared with intact breast treatment, the lung volumes in the chest wall fields used in the NSABP B-04 trial were likely a little higher than the above estimates for patients in the B-06 trial. In addition, the B-04 trial used a separate field to include the axillary apex and supraclavicular fossa. The data from the study by Das et al. estimate that the use of this field increases the irradiated lung volume an additional 12%, essentially doubling the volume of irradiated lung.[6] The data from this study also provide an example of the magnitude of interindividual variation. For example, the range of percent lung volume included in supraclavicular field irradiation ranged from a low of 5% to a high of 26%.[6]

The percentage of patients who were treated with postmastectomy radiotherapy in the B-04 trial and who developed lung carcinoma was approximately twice the percentage for those who were not treated with radiotherapy. These results are remarkably consistent with a similar study, also recently published in *Cancer*.[7] This second study, by Zablotska and Neugut, used data from the Surveillance, Epidemiology, and End Results (SEER) program and examined the development of lung carcinoma after treatment with lumpectomy (n = 65,560) or mastectomy (n = 194,981). The relative risk of developing lung carcinoma for patients treated with postmastectomy radiotherapy compared with those treated with mastectomy without radiotherapy was 2.06 (95% confidence interval [95% CI], 1.53–2.78) 10–14 years after treatment and 2.09 (95% CI, 1.50–2.90) for ≥ 15 years after treatment.[7] There was no apparent increase in the relative risk of developing lung carcinoma within the first 10 years after radiotherapy, reconfirming the long latency period required for radiation-induced second malignancies. Finally, similar to the study by Deutsch et al.,[5] there was no reported increase in the risk of developing lung carcinoma in those patients treated with radiotherapy after lumpectomy, again suggesting that the volume of lung irradiated is an important variable in risk determination.

The volume component of risk determination is an extremely important consideration. One of the most significant advances in the practice of radiation oncology has been the development and implementation of intensity-modulated radiation therapy (IMRT). IMRT typically improves the conformality of radiation treatments, allowing for the high-dose region to be more selectively delivered to the targeted volume of interest. This permits the normal structures that anatomically approximate the target volume to be preferentially spared the high radiation doses. However, this improvement in the shaping of high-dose radiation regions often comes at the expense of treating a larger volume of normal tissue with low radiation doses. The carcinogenic risks associated with increasing the volume of lung and other normal tissues exposed to low doses of radiation currently are unknown, but may be a relevant concern.[8] Furthermore, if an increase in second tumors results from this treatment strategy, it will not be clinically evident for another decade.

It is critical that the risk of developing lung carcinoma that may be associated with postmastectomy radiotherapy be appreciated in the context of the benefits of radiation. Postmastectomy radiotherapy has been shown consistently to reduce the probability of a locoregional recurrence after mastectomy by approximately 67%.[9] More important, by reducing the rate of locoregional recurrence, radiotherapy has been found to improve the overall survival of selected breast carcinoma patients. In these randomized trials, the absolute improvement in overall survival at 10-years was 9%.[10–12] In comparison, the absolute increased risk for lung carcinoma development after 20 years in the NSABP B-04 trial was approximately 1%. Furthermore, a meta-analysis of 20,000 breast carcinoma patients treated on radiotherapy trials found no evidence of increased deaths due to second malignancies in those patients randomized to receive radiotherapy.[9] It also should be recognized that other breast carcinoma treatments also increase the risk of second malignancies. In a recent article from the NSABP, the risk of acute myeloid leukemia and myelodysplastic syndrome was found to be increased in patients treated with dose-intensive doxorubicin and cyclophosphamide.[13] In addition, a study of 1500 patients with breast carcinoma who were treated on clinical trials at the University of Texas M. D. Anderson Cancer Center found that the use of radiotherapy after mastectomy was not associated with an increased risk of second tumors but that the use of > 10 cycles of an alkylator-containing chemotherapy regimen increased the risk of hematologic malignancies.[14]

The study by Deutsch et al.[5] and the recently published study from Zablotska and Neugut[7] provide very important data regarding the incidence of lung carcinoma development after radiotherapy for breast carcinoma. These data should not discourage the rou-

tine use of radiotherapy for patients at moderate risk for recurrence after mastectomy because the benefits of this treatment clearly outweigh the risks. However, this risk becomes more relevant in clinical circumstances in which the risks of recurrence are lower and also is relevant when considering the use of supraclavicular radiation fields in instances in which the risk of disease recurrence in this region is low. Finally, although not addressed specifically in either the study by Deutsch et al.[5] or Zablotska and Neugut,[7] other data suggest that radiotherapy and cigarette smoking may have a synergistic relation in the development of lung carcinoma after breast carcinoma treatment.[15] Therefore, all breast carcinoma patients receiving radiotherapy should be discouraged from smoking.

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T3 disease at presentation or pathologic involvement of four or more lymph nodes predict for local-regional recurrence in stage II breast cancer treated with neoadjuvant chemotherapy and mastectomy without radiation

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Dr. Buchholz is supported by Department of Defense Breast Cancer Research Program Career Development Award BC980154.

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Running head: Local recurrence after neoadjuvant chemotherapy and mastectomy

Abstract

Purpose: To help define the clinical and pathologic predictors of local-regional recurrence (LRR) in breast cancer patients treated with neoadjuvant chemotherapy and mastectomy without radiation for early stage disease.

Material and Methods: We retrospectively reviewed the outcomes of all 132 patients with stage I or II breast cancer treated in prospective institutional trials with neoadjuvant chemotherapy and mastectomy without radiation between 1974 and 2001. Clinical stage (AJCC 1988) at diagnosis was I in 5%, IIA in 46%, and IIB in 49% of patients. The median age at diagnosis was 49 years. All patients were treated with either a doxorubicin-based neoadjuvant regimen or single agent paclitaxel. Rates of total LRR were calculated by the Kaplan Meier method and comparisons were made with two-sided log-rank tests. The median follow-up was 46 months.

Results: The actuarial LRR rates at 5 and 10 years were both 10%. Factors that correlated positively with LRR included clinical stage T3 N0 disease ($P = 0.0057$), 4 or more positive lymph nodes at surgery ($P = 0.0001$), age ≤ 40 at diagnosis ($P = 0.0001$), and no use of tamoxifen. In the patients who did not receive tamoxifen, estrogen receptor-positive disease positively correlated with LRR ($P = 0.0067$). The 5-year LRR rate for the 42 patients with clinical T1 or T2 disease and 1 - 3 positive lymph nodes at surgery was 5% (only 2 events).

Conclusion: For patients with clinical stage II breast cancer, having T3 primary disease, 4 or more positive lymph nodes after chemotherapy or being 40 years old or younger predict for LRR. For most patients with clinical T1 or T2 disease with 1 - 3 positive lymph nodes, the 5-year risk for LRR is low and the routine inclusion of postmastectomy radiation does not appear to be justified.

Key words: Neoadjuvant chemotherapy, mastectomy, tamoxifen, local-regional recurrence

Introduction

Radiation treatment after mastectomy is an important contributor to cure for selected patients with breast cancer who have disease features associated with a 20% to 30% risk of local-regional recurrence (LRR) (1-4). For patients with this degree of risk, randomized trials have shown that the use of radiation prevents the rate of LRR by two thirds (1) and in doing so, improves overall survival (2-4). Radiation likely reduces recurrences by effectively treating microscopic residual tumor after mastectomy, and these trials suggest that this benefit may also reduce secondary dissemination (5).

Historically, pathologic assessment of primary disease and the extent of lymph node involvement have been the most consistent variables used to define an individual's risk for LRR after mastectomy. Clinical features of the disease at presentation have been used less often, mainly because clinical assessment is often imprecise at quantifying disease extent, especially in identifying the number of involved lymph nodes. Indeed, both The American Society for Therapeutic Radiation and Oncology (ASTRO) and The American Society of Clinical Oncology (ASCO) consensus statements on the use of radiation after mastectomy (6, 7) defined their recommendations on the basis of pathologic extent of primary and nodal disease.

The use of neoadjuvant (initial, primary) chemotherapy has complicated the selection criteria for use of postmastectomy radiation. Neoadjuvant chemotherapy is becoming an increasingly popular treatment strategy for breast cancer. In addition to allowing the assessment of tumor resistance to specific chemotherapy regimens and providing early treatment of potential micrometastatic disease, it may provide selected patients with advanced disease the option of breast-conserving treatment (8, 9). A

consequence of neoadjuvant therapy is that it changes the pathologic extent of disease in 80% to 90% of cases (10, 11). Therefore, indications for radiation that are based on pathologic features may be different for this group than for patients treated with surgery first.

In a previous study, we addressed the risk of LRR in patients treated with neoadjuvant chemotherapy and mastectomy in a cohort of patients with relatively advanced disease at presentation (10). We found that advanced clinical stage at presentation and pathologic extent of residual disease were independent predictors of LRR. In addition, we found that patients with locally advanced breast cancer had a clinically relevant risk of LRR even after a favorable response to chemotherapy. Our previous study had limited data on LRR patterns among patients with early-stage disease (stage I-II), and additional data concerning LRR patterns among such patients are needed. Few data exist concerning whether patients with stage II breast cancer and 1 - 3 positive lymph nodes after chemotherapy have a high, low, or moderate risk of LRR after treatment with mastectomy. This issue is extremely important in that neoadjuvant chemotherapy is used with increasing frequency for patients with early-stage disease. For example, the most recent National Surgical Adjuvant Breast Project (NSABP) randomized prospective clinical trials for patients with early-stage breast cancer (NSABP B-27) included neoadjuvant chemotherapy in all treatment groups.

The purpose of this study was to investigate risk factors for LRR after neoadjuvant chemotherapy and mastectomy for patients with stage I or II breast cancer. We included updated information from the cohort of patients from our previous study and

added information from additional patients, thereby providing the largest dataset currently available to address this clinically important question.

Methods

Data from six consecutive prospective clinical trials investigating the use of neoadjuvant chemotherapy for the treatment of non-inflammatory breast cancer between 1974 and 2001 were reviewed retrospectively. These trials were conducted at The University of Texas M. D. Anderson Cancer Center, and the primary aims of the trials focused on chemotherapy-related questions. In these studies, the use of radiation was not a randomized variable. The purpose of our study was to assess risk factors predictive of LRR in patients with stage I or II breast cancer treated with neoadjuvant chemotherapy and mastectomy without radiation. Therefore, we reviewed the outcomes of the 132 out of 1228 patients within these protocols who were treated in this manner (see Table 1).

This study population included updated outcome data for the 66 patients from our previous study (10) who had stage I or II breast cancer, combined with data from 66 additional patients who were treated in a trial that was ongoing at the time of our previous work. The details concerning chemotherapy from the earlier trials have been published (10, 12-14). In the most recent clinical trial, 258 patients with operable breast cancer were randomized to receive paclitaxel either weekly or every 3 weeks (standard). Doses of weekly paclitaxel varied according to clinical lymph node status, with patients with negative lymph nodes receiving 80 mg/m^2/wk for 12 weeks and patients with positive lymph nodes receiving 150 mg/m^2/wk for 3 weeks followed by a 1-week break (constituting one cycle) for 4 cycles. The dose of paclitaxel in the standard dose group

was independent of node status and consisted of a 24-hour continuous infusion of 225 mg/m^2 every 3 weeks for 4 cycles. In both groups, patients were subsequently treated with 4 cycles of neoadjuvant 5-fluorouracil, doxorubicin, and cyclophosphamide (FAC).

All patients in the present study underwent modified radical mastectomy with complete clearance of all gross disease. The median number of recovered lymph nodes from the axillary dissection was 15, with a range of 0—49 (only 1 patient had no lymph nodes recovered).

Local-regional control and survival curves were generated by the Kaplan and Meier method (15). Local-regional control was defined as any recurrence in the skin or soft tissue over the chest wall or a recurrence in regional lymphatic sites (axilla, internal mammary, infraclavicular, supraclavicular). All LRR were scored as events independent of whether they occurred after a distant metastasis. Event and follow-up times were established from the date of diagnosis, and two-sided log-rank tests were used to assess significant differences in time to recurrence or time to death. A multivariate analysis was not performed because of the low number of events.

Results

Clinical and pathologic characteristics of the 132 patients included in this study are listed in Table 2. The median age was 49 (range 29—73), with 28 patients (21%) being 40 years old or less. Fifty-one percent of the patients had stage I or IIA disease, and the rest had stage IIB disease (49%). The median follow-up of alive patients was 46 months (range 9—312 months).

Twenty-five patients (19%) achieved a complete clinical response to chemotherapy (absence of residual tumor by imaging and physical exam). Ninety-six patients (73%) achieved a partial clinical response (residual tumor $\leq$ 50% of its original size per imaging or clinical examination), and 7 patients (6%) had a minimal response (> 50% of initial tumor size) or no response (no change). Two patients showed evidence of progressive disease during the neoadjuvant chemotherapy but were still able to undergo mastectomy.

The median size of residual primary tumor was 1.5 cm with a range of 0 to 7.5 cm. Fifty-seven patients (43%) had residual tumors $\leq$ 2 cm, 34 (26%) had residual tumors > 2 cm, and 25 patients (19%) had no invasive residual disease at the primary site. Forty-eight patients (36%) had at least 1 positive lymph node at pathologic examination (range 0 - 9 lymph nodes). The median size of the largest lymph node recovered was 1.7 cm, with a range of 0 to 4.5 cm. Of the 57 patients who presented with clinically involved lymph nodes, 36% had no pathologic evidence of axillary disease after chemotherapy. Of the 75 patients who presented with clinical N0 stage, 17% had axillary disease identified in the pathology specimen.

Nineteen patients (14%) had disease recurrence as LRR or DM. LRR developed in 10 patients (8%), 9 of which LRR was an isolated first event and 1 with LRR following distant metastasis (DM). DM occurred in 15 patients (11%), with 6 patients (4%) having DM following LRR, and 9 patients (7%) having DM alone. The site of LRR in all 10 patients was the subcutaneous tissue overlying the chest wall, with 1 of these patients also experiencing a simultaneous recurrence in the supraclavicular lymph node

region. For all 132 patients, the 5-year actuarial LRR rate was 10%, the 5-year actuarial DM rate was 16% and the 5-year actuarial overall survival rate was 91%.

The actuarial LRR-free survival curves according to initial clinical stage are shown in Figure 1. The LRR rate was higher in the 19 patients (14%) with T3N0 disease than in those with T2N1 disease or T1-2N0 disease (29% [95% confidence interval {CI} = 7% to 50%] versus 11% [95% CI = 1% to 22%] versus 2% [95% CI = -1% to 5%] respectively, P = 0.0057). The actuarial LRR-free survival curves according to number of positive lymph nodes are shown in Figure 2. Eighty-four patients (64%) had no involved lymph nodes after neoadjuvant chemotherapy and surgery, 42 patients had 1 - 3 positive lymph nodes, and only 6 patients (4%) had 4 or more positive lymph nodes. Despite the small number, having 4 or more positive lymph nodes was associated with a significantly higher rate of LRR ($P < 0.0001$), with an actuarial 5-year LRR rate of 67% (95% CI = 29% to 104%).

We also analyzed patients with clinically negative lymph nodes at presentation (n = 75) according to whether they were subsequently found to have pathologic involvement of 1 or more lymph nodes. Sixty-two of these patients (83%) had both clinically negative and pathologically negative lymph nodes and 13 patients (17%) had clinically negative lymph nodes with at least 1 positive lymph node on pathologic analysis. The actuarial 5-year LRR rate among this latter group was 48% (4 failures in 13 patients) as compared with a rate of 5% among patients with pathologically node-negative disease ($P < 0.0001$).

Forty-two patients (32%) had clinical T1 or T2 disease and 1 - 3 positive lymph nodes after surgery. Only 2 LRR events took place in this group, giving a 5-year LRR rate of 5%.

With regard to tumor response, the 5-year actuarial LRR rates for patients who achieved a partial or complete response to chemotherapy were lower (9%) than for those who did not achieve at least a partial response (19%), but this apparent difference was not significant (P = 0.2483). However, only 1 out of 25 patients with a complete response recurred, compared to 6 out of 95 patients with a partial response, and 2 out of 9 patients with minimal, no response or progressive disease.

The actuarial LRR-free survival curves according to age are shown in Figure 3. Being age 40 or less was associated with a significantly higher rate of LRR than being older than 40 years (31% [95% CI = 49% to 89%] versus 4% [95% CI = 0% to 8%], P = 0.0001). Of the 7 patients with LRR who were age 40 or less, none had stage I disease, 3 had T3N0, 1 had T2N0, and 3 had T2N1 disease. Furthermore, 4 of these patients had at least 1 positive lymph node at surgery (2 with $\geq$ 4 positive lymph nodes and 2 with 1 - 3 positive lymph nodes), 2 had tumors with lymphovascular space invasion (LVSI), 3 had tumors that were estrogen receptor (ER)-positive, none had extracapsular extension (ECE) of disease, and none received tamoxifen.

Actuarial 5-year survival according to adjuvant tamoxifen use is shown in Figure 4. Although 91 patients (69%) had ER-positive tumors, only 67 patients (51%) received adjuvant tamoxifen. Twenty-four patients (18%) with ER-positive tumors did not receive adjuvant tamoxifen (it should be noted that before 1995, patients < 50 years did not receive adjuvant tamoxifen regardless of receptor status). Patients with ER-positive tumors who did not receive tamoxifen had significantly higher 5-year LRR rates (27% [95% CI = 6% to 48%]) than those receiving tamoxifen (2% [95% CI = -2% to 4%]) or

those who had ER-negative tumors and did not receive adjuvant hormone therapy (9% [95% CI = -3% to 22%]) (P = 0.0067).

Other pathologic variables analyzed included the presence of lymphovascular space invasion (LVSI), extracapsular extension (ECE), clinically multifocal or multicentric disease, and positive surgical margins. Patients with LVSI (n = 19) had a 5-year LRR rate of 23% versus 7% for those without LVSI or no mention of it in the pathology report (P = 0.1462). Eight patients had ECE and those that did had a 5-year actuarial LRR rate of 29% versus 9% for those without ECE or no mention of it in the pathology report (P = 0.0834). Eighty-nine patients (67%) had single masses on initial clinical assessment by imaging and physical examination, and 43 patients (32%) had multifocal disease (more than 1 mass in close proximity within breast) or multicentric disease (more than 1 mass dispersed within breast). The corresponding 5-year LRR rates for those with single, multifocal, and multicentric disease were 9%, 18%, and 6% (P = 0.9289). Only 3 patients had positive surgical margins, and none had LRR.

Discussion

In this study we have showed that for patients with clinical stage I or II breast cancer treated with neoadjuvant chemotherapy and mastectomy without radiation, having T3 primary disease at presentation or pathologic involvement of 4 or more lymph nodes was associated with a clinically relevant risk for LRR. Our findings further suggest that most patients with clinical T1 or T2 disease who have 3 or fewer positive residual lymph

nodes were at low risk for LRR at 5 years. The small number of LRR events in this subgroup (n = 2) precluded analysis of whether specific cofactors such as age, the presence of LVSI or ECE, tamoxifen use, and ER status affect recurrence rates within this subgroup. Our findings also indicated that tamoxifen use in patients with ER-positive disease was associated with a lower risk of LRR. The lack of tamoxifen use by patients with ER-positive disease was associated with a higher LRR risk than that seen for patients with ER-negative disease. Finally, early age at diagnosis was associated with higher rates of LRR, but several of the younger patients with recurrent disease also had other high-risk features such as T3 primary size (n = 3) or involvement of 4 or more lymph nodes (n = 2).

It should be noted that all patients in this trial had standard a modified radical mastectomy with complete axillary level I/II dissection. In addition, all patients were treated with doxorubicin, a taxane, or both. Finally, patients with bulky palpable adenopathy (3 cm or greater) have historically been classified in our institution as N2 disease, because of the high likelihood that this represents an aggregate of multiple matted lymph nodes. These details are important to consider in putting our low LRR rates in their proper context.

In a previous article examining all stages of breast cancer, we showed that advanced clinical stage was an independent predictor for LRR among patients treated with neoadjuvant chemotherapy and mastectomy (10). Our previous study however, had insufficient data to determine which subsets of patients with clinical stage I or II breast cancer are at high risk for LRR. This issue is particularly relevant in that the use of neoadjuvant chemotherapy for women with stage II breast cancer is increasing. Of

particular interest is the cohort of women with clinical T1 or T2 primary disease who have 1 - 3 positive lymph nodes after chemotherapy. One accepted threshold for using postmastectomy radiation in patients with stage II disease is the presence of 4 or more lymph nodes. Because determining the number of positive lymph nodes can be difficult by clinical examinations and radiographic studies, it has been unclear which patients with 1 - 3 positive lymph nodes after chemotherapy had 4 or more involved lymph nodes before chemotherapy. However, we have previously shown that most patients with positive lymph nodes before chemotherapy continue to have positive lymph nodes after chemotherapy. Specifically, a previous study from our group indicated that neoadjuvant chemotherapy converted FNA-positive axillary lymph nodes to pathologically negative axillary lymph nodes in only 23% of patients (16), a result supported by a later report as well (17). In addition, we have reported that for any given pathological size of the primary tumor, the associated rate of LRR was higher for patients treated first with chemotherapy compared to those treated with surgery first. This finding was most likely explained by the fact that tumor size so frequently changes with chemotherapy treatment. In contrast to primary tumor size, the LRR rate associated with having no involved lymph nodes or 1 - 3 positive lymph nodes was very similar in patients treated with neoadjuvant chemotherapy compared to those treated with surgery first. These data again can be interpreted as suggesting that chemotherapy treatment changes the number of positive lymph nodes much less frequently than it changes primary tumor size.

The data presented in this paper further support the hypothesis that most patients have the same number of positive lymph nodes before and after chemotherapy. Specifically, we found a low LRR rate for patients with stage II disease and 1 - 3 positive

lymph nodes. The 5-year rate of 5% is similar to the 5-year rate we previously reported for patients with stage II disease and 1 - 3 positive lymph nodes who were treated with surgery first (18). Within this cohort, in a previous publication we identified three features that conferred a higher LRR risk. Specifically, tumor size over 4 cm, the presence of ECE measuring 2 mm or greater, and resection of 10 or fewer lymph nodes conferred a LRR risk for which postmastectomy radiation should be considered. In a later study, we also identified close or positive margins, multifocal or multicentric disease, and LVSI as being associated with higher risk of LRR (19). Finally, in this current study we found that young age may also be an important consideration, although too few young patients with stage II disease and 1 - 3 positive lymph nodes were included to study the effect of age in this cohort.

Some limitations of this study should be noted when interpreting our results. First, the follow-up was short, and thus the LRR rates reported here underestimate the lifetime LRR rates of this group. A previous report from our group showed that 21% of the total number of LRRs after mastectomy and adjuvant chemotherapy developed after 5 years of follow-up (18). Furthermore, while only 1 patient in our study developed LRR after DM, it is possible that some LRR occurring after DM may not have been recorded. Although larger than the previously analyzed cohort, this study is still based on a modest number of patients. In addition, the number of patients with early-stage breast cancer reviewed here yielded a relatively small number of LRR events (n = 10), potentially masking significant variables. Despite these limitations, we believe that these data are important because they represent the only data currently available regarding LRR after mastectomy for patients with early-stage disease treated with neoadjuvant chemotherapy.

In conclusion, we showed that in early-stage breast cancer, clinical T3 primary disease at presentation and the presence of 4 or more positive lymph nodes after neoadjuvant chemotherapy predict for LRR and warrant the addition of radiation to the postmastectomy treatment regimen. In contrast, most patients with stage II disease and 1 - 3 positive lymph nodes are at low risk for LRR at 5 years. However, within this subset, postmastectomy radiation may be appropriate for those aged 40 or less, those with fewer than 10 recovered lymph nodes (or 20% positive lymph nodes), ECE of 2 mm or greater, LVSI, or positive or close surgical margins. These results, considered with our previous findings, indicate the importance of both clinical and pathologic factors in the assessment of LRR risk for such patients. It should be noted, however, that follow-up periods of our studies were limited, and as more information becomes available in the future, the role of adjuvant radiation for patients undergoing neoadjuvant chemotherapy and mastectomy will become clearer.

Table 1. Neoadjuvant Chemotherapy Treatment Trials Reviewed for this Analysis

Protocol	Years of Study	Chemotherapy	No. of Patients Analyzed of Total No. of patients in Original Study
Advanced Primary	1974—1985	FAC	3 of 191
85-01	1985—1989	VACP	6 of 200
89-007	1989—1991	FAC	5 of 203
91-015	1991—1994	FAC or dose-escalated FAC	3 of 202
94-002	1994—1998	FAC alone or FAC plus paclitaxel	49 of 174
98-240	1998—2001	Weekly paclitaxel and FAC	23 of 258
98-240	1998—2001	q3-week paclitaxel and FAC	43 of 258
Total	1974—2001		132 of 1228

Abbreviations: FAC, fluorouracil, doxorubicin, and cyclophosphamide; VACP, vincristine, doxorubicin, cyclophosphamide, and prednisone; q3-week, every 3 weeks.

Table 2. Patient Clinical and Pathologic Characteristics

Characteristic	No. of Patients of Total in Group	%
Age		
≤ 40 years	28 of 132	21
41 - 50 years	40 of 132	30
51 - 60 years	37 of 132	28
≥ 61 years	27 of 132	21
Race		
Asian	5 of 132	4
Black	8 of 132	6
Spanish/Hispanic	20 of 132	15
White	94 of 132	72
Other	5 of 132	3
Clinical stage at presentation		
T1 N0	6 of 132	5
T1 N1	11 of 132	8
T2 N0	50 of 132	38
T2 N1	46 of 132	35

T3 N0	19 of 132	14
Adjuvant hormone therapy		
Tamoxifen	67 of 132	51
Other	4 of 132	3
None	58 of 132	44
Unknown	3 of 132	2
ER status		
Negative	36 of 132	27
Positive	91 of 132	69
Unknown	5 of 132	4
PR status		
Negative	51 of 132	39
Positive	66 of 132	50
Unknown	15 of 132	11
Her-2/NEU status		
Negative	43 of 132	33
Positive	15 of 132	11
Unknown	74 of 132	56

Clinical MF/MC disease

Single mass	89 of 132	68
Multifocal	26 of 132	19
Multicentric	17 of 132	13

Pathologic size of tumor

0.1 - 1 cm	13 of 132	10
1.1 - 2 cm	44 of 132	33
2.1 - 3 cm	22 of 132	17
3.1 - 4 cm	6 of 132	4
4.1 - 5 cm	4 of 132	3
> 5 cm	2 of 132	2
no residual	25 of 132	19
unknown	16 of 132	12

Clinical response to chemotherapy

Complete	25 of 132	19
Partial	96 of 132	73
Minimal or none	7 of 132	6
Progressive disease	2 of 132	2

No. of positive lymph nodes

0	83 of 132	63

1 - 3	42 of 132	32
4+	6 of 132	4
Unknown	1 of 132	1

Pathologic size of largest node

≤ 1 cm	19 of 132	14
1.1 - 2 cm	23 of 132	17
2.1 - 3 cm	15 of 132	11
>3 cm	1 of 132	1
No measurement recorded	74 of 132	56

Clinical N0 with no. of involved nodes

0	62 of 75	83
1 - 3	9 of 75	12
4+	4 of 75	5

Clinical N1 with no. of involved nodes

0	21 of 57	36
1 - 3	34 of 57	60
4+	2 of 57	4

Lymphovascular space invasion

Present	19 of 132	14
Absent or not mentioned	113 of 132	86
Surgical margins		
Positive	3 of 132	2
Negative	123 of 132	93
Unknown	6 of 132	5
Extracapsular extension		
> 2 mm	2 of 132	2
>0 - 2 mm	6 of 132	5
none or not mentioned	123 of 132	93

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; MF/MC, multifocal or multicentric

Figure Legends

Figure 1. Actuarial 5-year LRR-free survival according to disease stage at presentation.

Figure 2. Actuarial 5-year LRR-free survival according to number of lymph nodes remaining positive after neoadjuvant chemotherapy.

Figure 3. Actuarial 5-year LRR-free survival according to patient age at presentation.

Figure 4. Actuarial 5-year LRR-free survival according to use of adjuvant hormone treatment and estrogen receptor status.

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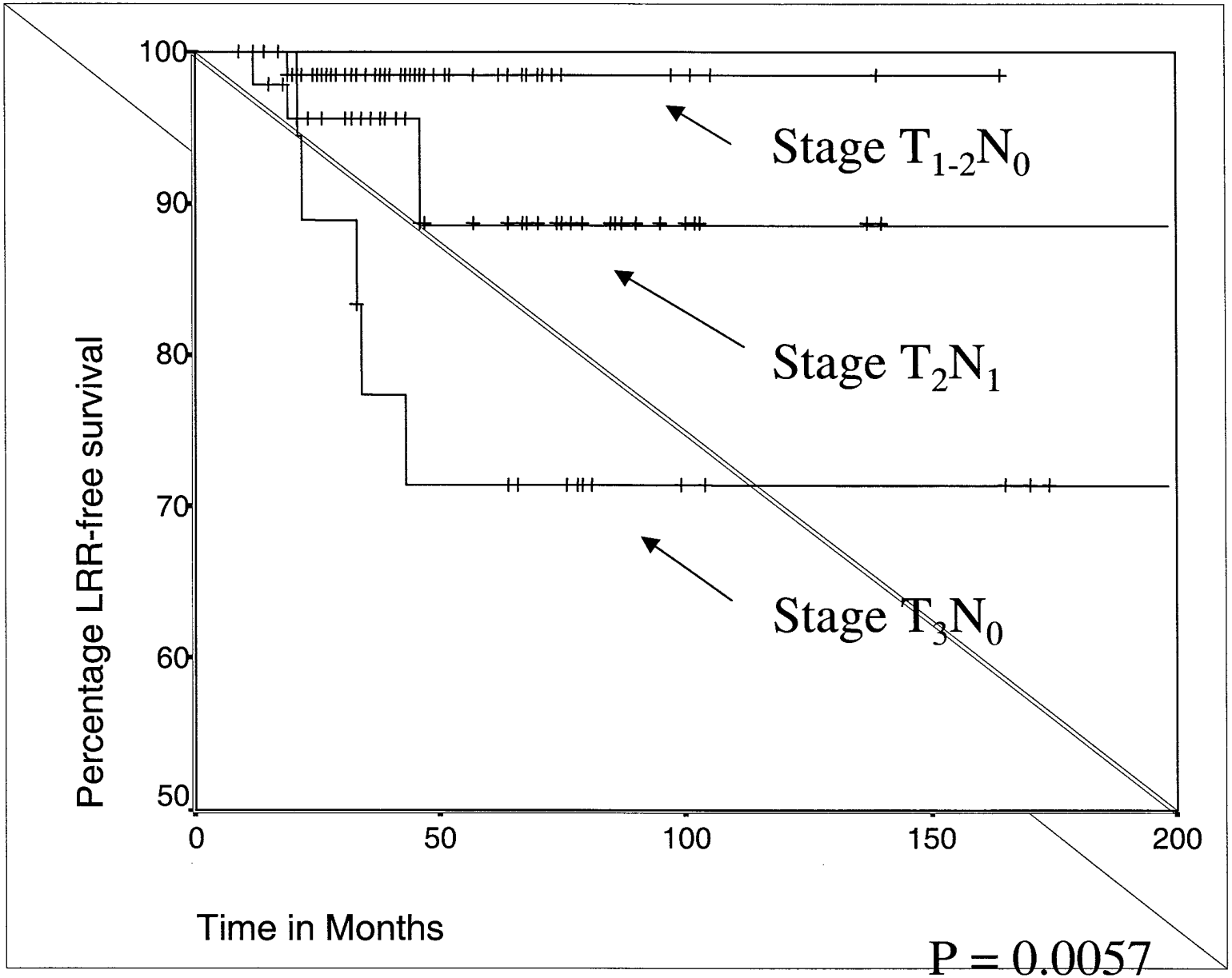
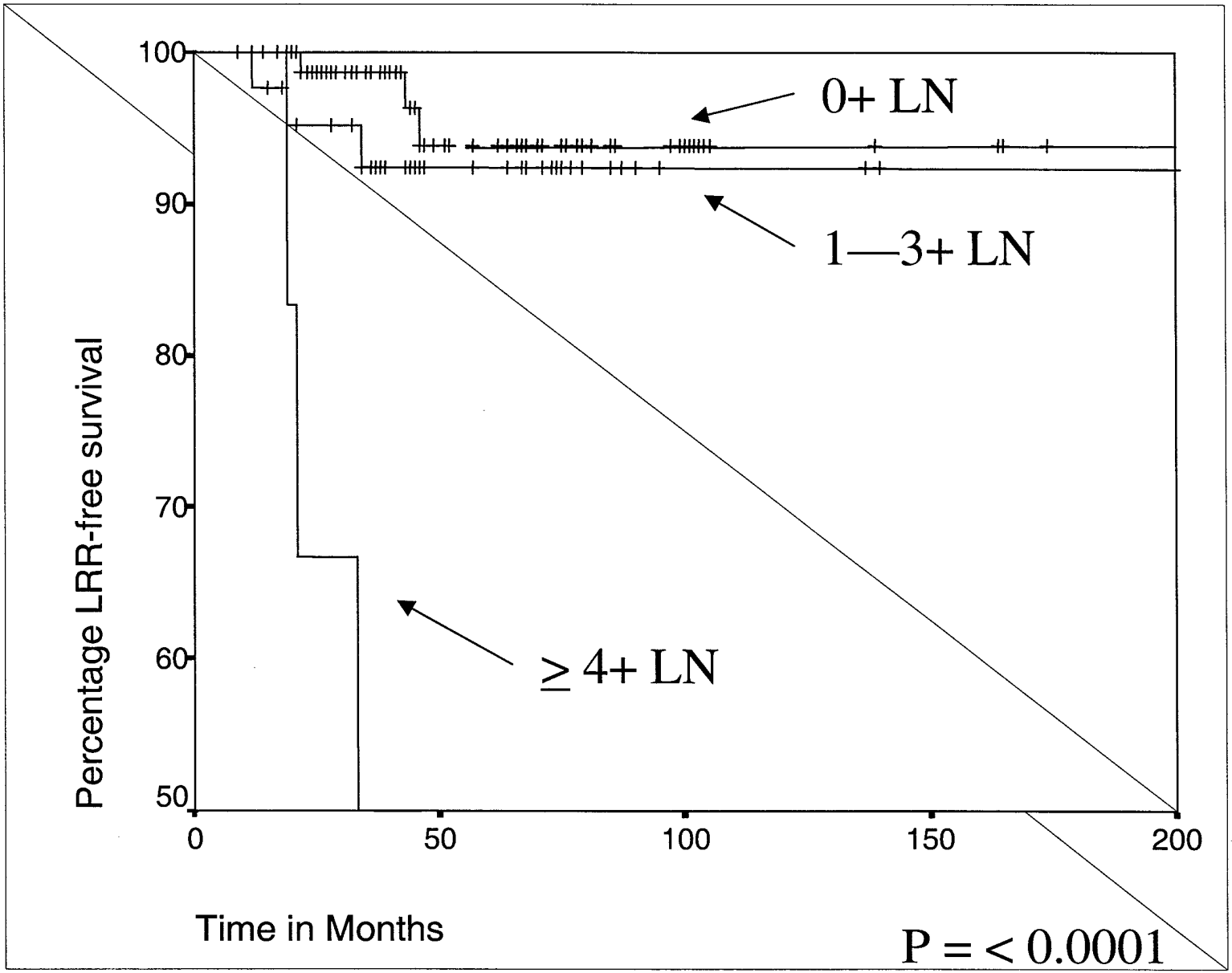
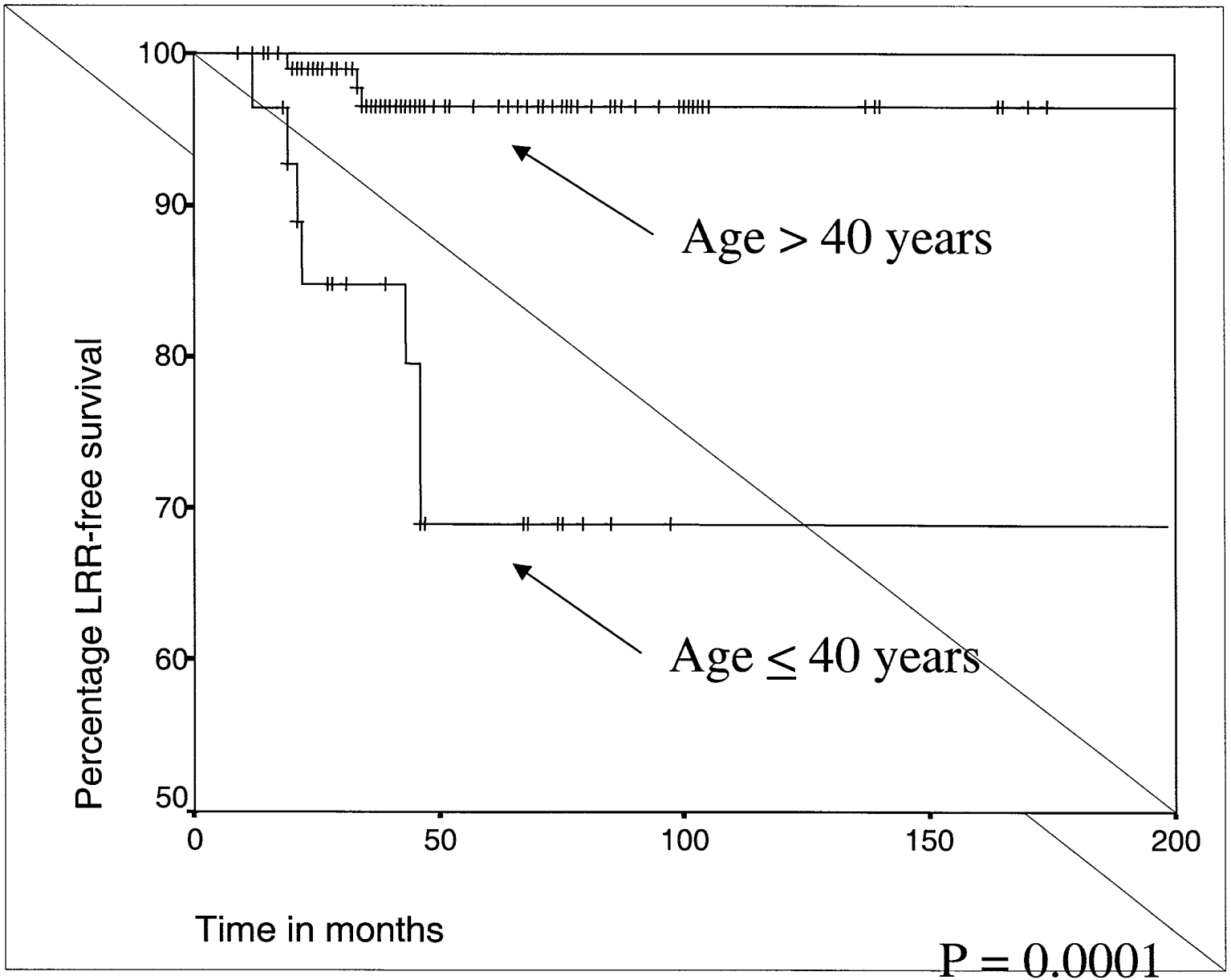
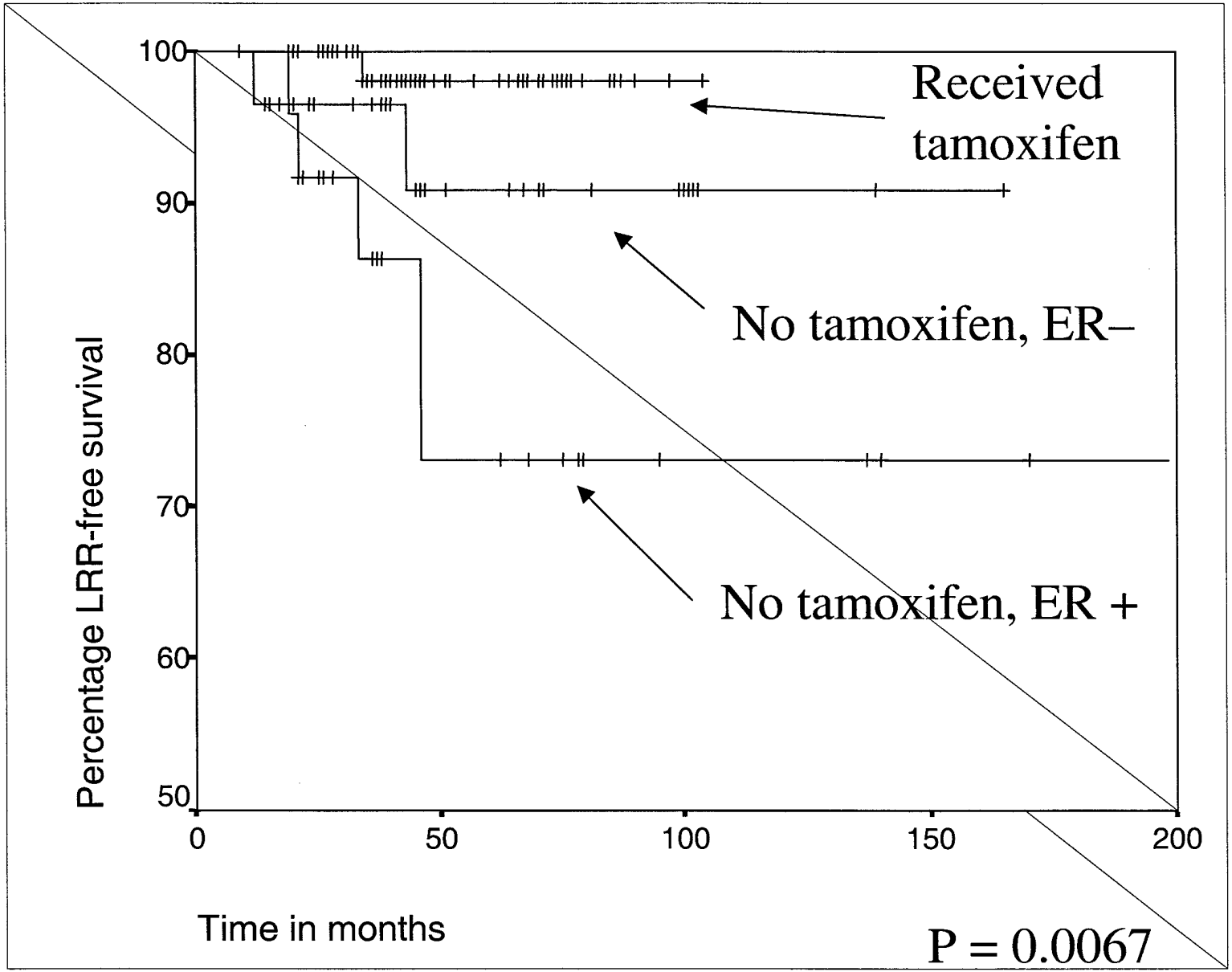

100
90
80
70
60
50
Percentage LRR-free survival
0
50
100
150
200
Time in Months
Stage $T_{1-2}N_0$
Stage T_2N_1
Stage T_3N_0
P = 0.0057


100
90
80
70
60
50
Percentage LRR-free survival
0+ LN
1—3+ LN
≥ 4+ LN
0
50
100
150
200
Time in Months
P = < 0.0001


100
90
80
70
60
50
Percentage LRR-free survival
0
50
100
150
200
Time in months
Age > 40 years
Age ≤ 40 years
P = 0.0001


100
90
80
70
60
50
Percentage LRR-free survival
0
50
100
150
200
Time in months
Received tamoxifen
No tamoxifen, ER–
No tamoxifen, ER +
P = 0.0067

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Controversies Regarding the Use of Radiation After Mastectomy in Breast Cancer

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Key Words. *Radiation · Mastectomy · Chemotherapy · Local-regional recurrence · Breast reconstruction*

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Explain the potential benefits of delivering radiation after mastectomy for patients with breast cancer.
2. Provide a list of appropriate indications for selecting which patients would benefit from radiation after mastectomy and chemotherapy.
3. Appreciate how radiation can potentially cause cardiovascular injuries and understand the importance of radiation technique in minimizing the risk for such injuries.
4. Appreciate how immediate breast reconstruction can affect the delivery of postmastectomy radiation.

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ABSTRACT

Despite years of clinical study, there are still many unanswered questions regarding postmastectomy radiation. It is clear that radiation therapy plays a critical role in the multidisciplinary management of patients with locally advanced or inflammatory breast cancer. It is also accepted that postmastectomy radiation is not required for most women with noninvasive disease or stage I disease. Randomized clinical trials studying radiation treatments for women with stage II or III breast cancer have shown that the addition of radiation after mastectomy can reduce local-regional recurrence rates, which then improves survival. However, other data have indicated that the risk of local-regional recurrence after mastectomy and chemotherapy is low for patients with small tumors and one to three positive lymph nodes, leading some to question whether postmastectomy radiation is useful for this group. A second controversy regards the sequencing of postmastectomy radiation and breast reconstruction. In this article we discuss these controversies, review the data that are relevant, and provide our institutional approaches to these issues. ***The Oncologist*** *2002;7:539-546*

INTRODUCTION

Approximately 25 years ago, *Gilbert Fletcher* and *Eleanor Montague*, two leading experts in breast cancer radiation treatments, wrote, "there is, perhaps, no more controversial subject in the management of cancer than the use of postoperative irradiation in conjunction with...mastectomy" [1]. It is ironic that nearly 3 decades later this statement remains true. Breast cancer is a common disease,

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Received August 27, 2002; accepted for publication September 3, 2002.

and the strategy of combining radiation with mastectomy has been investigated since the 1950s. Despite this, there is not an accepted standard of care concerning radiation use for a patient treated with a modified radical mastectomy and systemic therapy for stage II breast cancer with one to three positive lymph nodes. Some would strongly advocate that such patients receive radiation and cite randomized prospective data that support its use. Others show that the risk of local-regional recurrence (LRR) for such patients is low and argue that the potential toxicities and the costs associated with postmastectomy radiation in these patients may not be warranted.

In this article, we explore the current controversies regarding the use of radiation after mastectomy for breast cancer. Initially, we review data demonstrating that postmastectomy radiation decreases LRR after mastectomy and, correspondingly, improves breast-cancer-specific and overall survival in appropriately selected patients. We also review the potential toxicities associated with radiation after mastectomy. Given these potential benefits and risks, it is critical that appropriate selection criteria are used to determine who should receive treatment. To help clarify these selection criteria, we review data concerning patterns of failure for patients treated with mastectomy and either adjuvant or neoadjuvant chemotherapy. Finally, we discuss the controversy regarding the sequencing of postmastectomy radiation and breast reconstruction surgery.

Benefits and Risks of Postmastectomy Radiation

Meta-Analyses

Over the past 5 decades, there have been more than 25 randomized prospective clinical trials that have evaluated the benefits of radiation after mastectomy for patients with breast cancer. Given the long time period over which this topic has been studied, it is not surprising that there is considerable heterogeneity in the surgical and radiotherapy treatments among these trials. Despite the variability in trial design and treatments, a number of groups have performed meta-analyses of the data from these studies. In 1987, *Cusik et al.* published the first meta-analysis of data from postmastectomy radiation trials and reported that radiation use was associated with a poorer overall survival [2]. However, in a subsequent update of this analysis, postmastectomy radiation was found to decrease the breast cancer death rate but increase the non-breast-cancer death rate [3]. These competing results lead to an equivalent overall survival between the two groups.

A more comprehensive meta-analysis concerning postmastectomy radiation was recently updated by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) [4]. This group analyzed the actual data from over 15,000 patients treated in clinical trials investigating the use of postmastectomy radiation. The data from this analysis showed that postmastectomy radiation reduced isolated LRR rates. For patients with lymph-node-positive disease, the 10-year isolated LRR rate was 9% in the radiation group versus 24% in the no radiation group. This highly significant reduction in isolated LRR was noted both in the trials that included a standard modified radical mastectomy and the trials that allowed mastectomy with axillary sampling. Despite the reduction in isolated LRR, the 20-year overall survival rates between the postmastectomy radiation and mastectomy alone groups were nearly identical (37.1% versus 35.9%, respectively, $p = 0.06$).

The lack of a survival benefit led many to question whether postmastectomy radiation was of value. These data also led some to conclude that LRR was an unlikely source of distant metastases. However, further data provided by the EBCTCG analysis are inconsistent with this paradigm of thought. Similar to the earlier *Cusik et al.* study, the EBCTCG analysis found that postmastectomy radiation significantly improved breast-cancer-specific survival (20-year rates of 53.4% versus 48.6%, respectively, $p = 0.0001$) [4]. The most logical explanation for this finding is that when radiation substantially lowers LRR rates, the probability of being cured of breast cancer improves. Unfortunately, in the meta-analyses, the improvement in breast cancer deaths was offset by an increase in non-breast-cancer deaths ($p = 0.0003$) [4]. This has been attributed to an association with the radiation treatment techniques used in some of these trials and injury to cardiovascular structures. Indeed, the authors found that cardiovascular deaths were statistically greater in the patients treated with radiation, whereas deaths due to pulmonary toxicity or treatment-related cancers were not statistically different between the two groups [4]. Therefore, it is conceivable that postmastectomy radiation could improve overall survival if new techniques that selectively avoided treating the heart and vasculature were used.

It is important to recognize limitations of meta-analyses when considering the relevancy of these data to modern breast cancer patients. The EBCTCG meta-analyses purposely included all trials. While this has obvious benefits, there are also major shortcomings. Differing eligibility criteria (patients at low risk for LRR will have less benefit than those at high risk for LRR), radiation dose and fractionation, radiation field design, and quality assurance factors can alter the relative risk and benefits of radiation in a clinical trial. To minimize these confounding effects, *Van de Steene et al.* recently reanalyzed the EBCTCG data, excluding trials that began before 1970, trials with small sample sizes, trials with relatively poor crude survival rates, and trials that used

radiation fractionation schedules that are no longer standard practice [5]. When these less than optimal studies were excluded, postmastectomy radiation significantly improved overall survival, with an odds reduction for death of 12.4%. It should be noted that these data were predominantly powered by the Danish postmastectomy trials, which are discussed later in this paper. It is also important to note that this degree of improvement in overall survival is of the same magnitude as that achieved by the early chemotherapy trials for lymph-node-positive disease [6]. A second meta-analysis focusing on more recent randomized trials also suggested that radiation use improved the overall survival of patients with intermediate-stage breast cancer [7]. This analysis compared the outcome of breast preservation therapy (which included radiation treatment) with modified radical mastectomy and found that breast preservation therapy provided a survival advantage over mastectomy in the trials that did not include postmastectomy radiation (odds ratio favoring breast conservation therapy of 0.69). However, in the trials that compared breast conservation therapy with mastectomy plus postoperative radiation, the two treatments achieved equivalent outcomes. These data again suggest that radiation should be a component of care for women with intermediate-risk breast cancer.

Modern Postmastectomy Radiation Trials

It is generally accepted that reproduced large phase III randomized trials provide a higher level of scientific evidence than meta-analyses. A recent study found that meta-analyses frequently fail to accurately predict the results of subsequent large phase III studies [8]. One can argue that such a discrepancy exists with respect to the question of whether postmastectomy radiation improves survival in breast cancer. Subsequent to the initial meta-analyses, 10-year data from three randomized trials investigating postmastectomy radiation provided new insights into the potential benefits of radiation. These studies differed from previous trials in the radiation treatment techniques used and in their use of systemic therapy. The use of chemotherapy is relevant to the relationship between radiation use and survival in that it reduces the competing risk of distant metastatic disease development, making the prevention of LRR more important.

Perhaps the most important of the recent randomized prospective trials is the Danish Breast Cancer Cooperative Group (DBCCG) 82b trial, which randomized 1,708 premenopausal women with stage II or III breast cancer to mastectomy followed by nine cycles of chemotherapy or mastectomy, radiation, and eight cycles of chemotherapy [9]. Radiation therapy consisted of 50 Gy in 25 fractions delivered to the chest wall and draining lymphatics utilizing electron beams in the regions over the heart to minimize dose to the cardiovascular structures. The results showed that patients randomized to radiation had a lower 10-year rate of LRR (9% versus 32%, $p < 0.001$, respectively) and an improved 10-year overall survival rate (54% versus 45%, $p < 0.001$, respectively). A much smaller trial, conducted in Vancouver, Canada, was of a similar design and reported remarkably similar results [10]. In that trial, 318 premenopausal women with lymph-node-positive disease were randomized to receive mastectomy and chemotherapy plus or minus postmastectomy radiation. Patients randomized to receive radiation had a very similar reduction in their 10-year rate of LRR (13% versus 25%, $p = 0.003$, respectively) and similar improvement in 10-year overall survival (64% versus 54%, $p = 0.07$, respectively). Finally, coincident with the 82b study, the DBCCG conducted a companion trial, 82c, for postmenopausal women [11]. This trial randomized over 1,300 patients to mastectomy and tamoxifen or mastectomy, tamoxifen, and radiation. The magnitude of the benefits for the patients randomized to receive radiation was similar to the two previous studies (10-year LRR rates of 8% versus 35%, $p < 0.001$, respectively, and 10-year overall survival rates of 45% versus 36%, $p = 0.03$, respectively).

Taken together, these three studies demonstrated that by reducing postmastectomy LRR, radiation could improve overall survival. The data from these studies collectively indicated that a reduction in postmastectomy LRR from 25%-30% to 10% resulted in an absolute survival benefit of 10%, meaning that half of the patients in whom LRR was avoided survived. One important contribution to the improvement in overall survival was the lack of increase in non-breast-cancer deaths. The Danish trials treated with techniques that minimized dose to the cardiac structures. This resulted in equivalent rates of heart-disease-related hospital admissions and cardiac deaths in the patients treated with radiation compared with those in the no radiation arm [12].

Indications for Postmastectomy Radiation

Mastectomy and Adjuvant Chemotherapy

It is clear from these recent phase III clinical trials that radiation improves the overall survival of women treated with mastectomy and chemotherapy who have a 25%-30% risk of having an LRR. It is more difficult to determine from these studies which subcategories of patients have this degree of LRR risk. Prior to publication of the Danish studies, the standard indications to use postmastectomy radiation were the presence of ≥4 positive lymph nodes or T3 or T4 primary disease. In part, these indications were justified by an investigation of failure patterns in 627 patients treated with mastectomy and chemotherapy without radiation in Eastern Cooperative Oncology Group (ECOG) trials [13].

This study reported that these subgroups of patients had clinically relevant rates of LRR, whereas patients with less than four involved lymph nodes and T1 or T2 primaries had a low risk of LRR. After publication of the Danish 82b trial and the Canadian trial, it became less clear whether postmastectomy radiation should be offered to women with stage II breast cancer with one to three positive lymph nodes. In part, this controversy arose because women with one to three positive lymph nodes made up a large percentage of both study populations. Specifically, in the much larger 82b trial, 63% of the patients had one to three positive lymph nodes [9]. However, many patients in that trial did not undergo a formal level I/II axillary dissection. In the Danish 82b trial, the median number of axillary lymph nodes resected was only seven, with 76% of the patients having less than 10 lymph nodes removed, and 15% having three or fewer lymph nodes removed [9].

The potential consequences of having less than a standard axillary dissection are twofold. First, axillary sampling procedures lead to an underestimation of the true number of positive lymph nodes. It is, therefore, likely that many of the patients reported to have one to three positive lymph nodes in the Danish trial may have had four or more positive lymph nodes if a more extensive surgical procedure had been performed. Secondly, the more limited dissection also increased LRR risk by failing to remove microscopic axillary disease. Indeed, in the patients who did not receive radiation, 45% of all LRR in the Danish study occurred in the axilla [14]. This percentage is significantly higher than the contributions of axillary recurrences to total LRR reported in other series that had standard axillary dissections [15, 16].

To further investigate the risk of LRR for patients treated with mastectomy and chemotherapy, a number of groups recently have again explored failure patterns in women treated without radiation. These data are summarized in Table 1 [15-18]. The axillary surgical procedures in these series were different from the surgery performed in the Danish studies. Specifically, the median numbers of lymph nodes recovered in the ECOG and M.D. Anderson Cancer Center series were 15 and 17, respectively, over twice the median number recovered in the Danish trials [15, 16]. In addition, the patients in many of these series were treated with doxorubicin-based chemotherapy, which has been suggested to have a greater efficacy than nonanthracycline-containing regimens [6]. The average rate of LRR for patients with one to three positive lymph nodes in these series was approximately 12%, which is almost three times less than the LRR rate in the no radiation arm in the Danish trials [9, 11, 15-18]. Correspondingly, as the risk for LRR was significantly less, the expected benefit from postmastectomy radiation is unknown. Hypothetically, if postmastectomy radiation had a similar proportional reduction in LRR and improvement across all disease stages, then a patient with a 10-year risk of LRR of 12% would be expected to have an absolute rate of improvement in LRR of 8% and an absolute survival benefit of 4%. However, it is unknown whether these assumptions are accurate. It is not clear that the proportional benefit of radiation on survival remains constant as the risk of LRR decreases. One potential problem with extrapolating data from one risk group to another is that the potential toxicity of postmastectomy radiation would be expected to be roughly equivalent over all risk groups. Therefore, if radiation caused a small increase in non-breast-cancer deaths, it is likely that some threshold of LRR risk is needed for an increased overall survival.

The M.D. Anderson Cancer Center series in Table 1 provides additional information concerning LRR for patients with stage II breast cancer and one to three positive lymph nodes. An analysis of the data from this subgroup found that the presence of extracapsular extension greater than 2 mm, tumor size over 4 cm, positive or close (2 mm) surgical margins, lym-

Table 1. Ten-year local regional recurrence rates after mastectomy and systemic treatments

Investigators	*n* of patients	Systemic therapy	Local regional recurrence rate	
			Patients with 1-3 +LN	Patients with <3 +LN
ECOG [15]	2,016	CMF	13%	29%
MDACC [16]	1,031	Doxorubicin-based	10%	21%
NSABP [17]	5,758	Varied	6%-11%*	14%-25%*
Taiwan [18]	125	Varied**	16%+	not studied

*range dependent on size of primary tumor

**86 patients received tamoxifen alone

+ rate is at 4 years, no 10-year data available

Abbreviations: +LN = positive lymph nodes; CMF = cyclophosphamide, methotrexate, 5-fluorouracil; MDACC = M.D. Anderson Cancer Center; NSABP = National Surgical Adjuvant Breast and Bowel Project.

phovascular space invasion, or invasion of the skin, nipple, or pectoralis muscle all were associated with rates of isolated LRR ranging over 25% [16, 19]. The one treatment-related factor that predicted high rates of LRR in patients with one to three positive lymph nodes was resection of less than 10 lymph nodes [16]. In addition, using a recursive partition analysis, those authors found that the most important predictor of LRR was a 20% or greater lymph node involvement [20]. These data are consistent with the Danish data in that the majority of patients with one to three positive lymph nodes in the Danish trials likely had a 20% or greater lymph node involvement because of the low number of total lymph nodes resected.

Recently, both the American Society for Therapeutic Radiology and Oncology and the American Society of Clinical Oncology have published consensus statements regarding postmastectomy radiation. Both of these statements recommend radiation for women with ≥4 positive lymph nodes or advanced primary disease, and both statements highlight the need for additional prospective data concerning the use of postmastectomy radiation for women with T1 or T2 disease and one to three positive lymph nodes [21, 22].

There is currently an ongoing national Inter-Group trial designed to determine the benefits of postmastectomy radiation for patients with small tumors and one to three positive lymph nodes. The schema of that trial is shown in Figure 1. In that trial, patients with stage II breast cancer with one to three positive lymph nodes are randomized to receive postmastectomy radiation or observation after mastectomy and adjuvant chemotherapy. Patients must have 10 or more lymph nodes dissected and negative margins. Patients with gross extracapsular extension of disease or stage N2 or N3 disease are excluded. This National Cancer Institute-designated high-priority study addresses a critically important clinical question that affects thousands of breast cancer patients in the U.S.

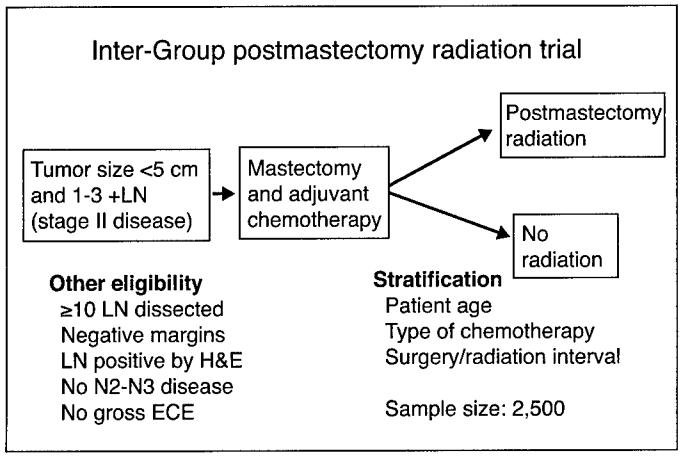

Figure 1. Schema of the current Inter-Group randomized prospective trial investigating the benefits of postmastectomy radiation for patients with stage II breast cancer with one to three positive lymph nodes. *Abbreviations: +LN = positive lymph nodes; H&E = hematoxylin and eosin staining; ECE = extracapsular extension of disease.*

Neoadjuvant Chemotherapy and Mastectomy

There are substantially less data to aid in determining which patients treated with neoadjuvant chemotherapy warrant postmastectomy radiation. In addition, determining the appropriate selection criteria is more complicated in this group of patients than in those initially treated with surgery. This is because the majority of patients treated with neoadjuvant chemotherapy have a significant change in their disease resulting from the chemotherapy. Therefore, the pathological factors that historically have been used to identify subgroups of patients with clinically relevant risk of LRR after mastectomy are less certain.

Investigators from M.D. Anderson Cancer Center Breast Cancer Group recently reported a study of LRR patterns in 150 patients treated in neoadjuvant chemotherapy trials who did not receive postmastectomy radiation [23]. This population had relatively advanced disease at diagnosis, with 59% of the patients having clinical T3 or T4 stage, and 70% having clinically suspicious lymphadenopathy. As expected, there was a significant change in disease extent with the chemotherapy treatment. After chemotherapy, the median pathological size of the primary tumor was 2 cm and the median number of positive lymph nodes was one.

In a multivariate analysis, three factors were associated with higher rates of LRR. These were clinical stage IIIB disease or greater (hazard ratio 4.5, $p < 0.001$), ≥4 positive lymph nodes (hazard ratio 2.7, $p = 0.008$), and lack of tamoxifen use (hazard ratio 3.9, $p = 0.027$) [24]. There was no clear relationship between disease response to chemotherapy and LRR. The 5-year rate of LRR for the 18 patients with a complete pathological response was 19% (95% confidence interval [CI] 6%-48%), with all of the failures in this subgroup occurring in patients with either T3 disease or clinical stage III disease at diagnosis. Another interesting subset of patients in this study was 40 patients with residual tumor sizes >5 cm and one to three positive lymph nodes. In this group, the 5-year LRR rate was 46% (95% CI 24%-76%) for patients with clinical T3 or T4 primary tumors compared with only 4% (95% CI 1%-25%) for patients with clinical T1 or T2 disease ($p = 0.002$).

Those authors also compared rates of LRR in neoadjuvant chemotherapy patients with those previously reported after mastectomy and adjuvant chemotherapy [24]. Not surprisingly, for any given pathology, the risk of LRR was higher in those treated with neoadjuvant chemotherapy.

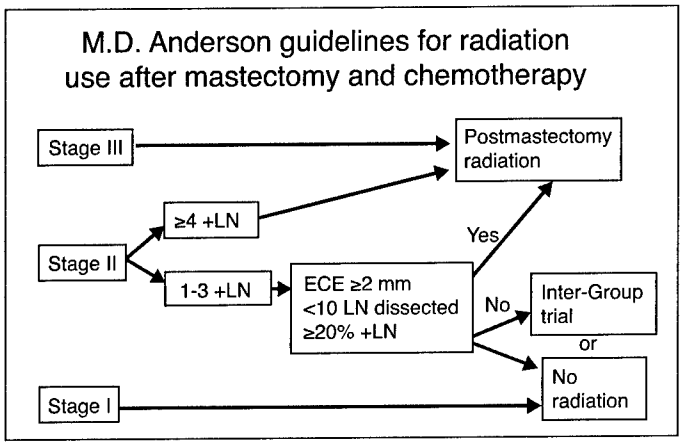

Figure 2. Guidelines used at The University of Texas M.D. Anderson Cancer Center to determine which breast cancer patients are recommended to receive postmastectomy radiation. *Abbreviations: +LN = positive lymph nodes; ECE = extracapsular extension of disease.*

These data indicate that both pretreatment clinical stage and posttreatment pathological findings should be considered when determining indications for radiation after neoadjuvant chemotherapy and mastectomy.

Figure 2 provides the postmastectomy radiation guidelines used at M.D. Anderson Cancer Center.

Sequencing of Breast Reconstruction and Postmastectomy Radiation

Many women who are treated with mastectomy for breast cancer elect to have an autologous tissue breast reconstruction or implant breast reconstruction. A number of advances in the fields of plastic surgery and surgical oncology have significantly improved the probability of achieving an excellent aesthetic outcome after breast reconstruction surgery. One of these advances has been the increased use of skin-sparing mastectomy with an immediate reconstruction. Immediate reconstruction after mastectomy not only allows patients to have one surgical procedure rather than two but also achieves a superior aesthetic result than a delayed procedure because the inframammary sulcus can be preserved. Unfortunately, the use of postmastectomy radiation also has to be considered in deciding the timing and type of postmastectomy reconstruction for breast cancer patients.

Ideally, decisions concerning the sequencing of breast reconstruction and postmastectomy radiation should be made by a closely coordinated multidisciplinary team whose focus is on avoidance of recurrence, improvement of curability, and maximization of long-term quality of life of the patient. As previously highlighted, postmastectomy radiation has been shown to improve survival for selected breast cancer patients and, for most breast cancer patients, cure of the disease is the highest priority. Therefore, the most important question concerning immediate breast reconstruction is whether the reconstruction can impair the efficacy of postmastectomy radiation. To date, this question has never been directly studied. It is clear that all types of breast reconstruction surgeries do not directly have an adverse affect on radiation (i.e., through a modification of the beam). However, breast reconstruction can substantially affect radiation field design. As discussed above, it is imperative that the entire chest wall is treated with postmastectomy radiation, while dose to the lung and heart is minimized. There are a variety of techniques available to achieve this goal. Unfortunately, some breast reconstruction surgeries significantly distort the chest wall anatomy, make the treatment of the targeted tissues more difficult, and often require an increase in the volume of lung or heart irradiated. Most problematic are the steeply sloping medial and apical contours resulting from inflated tissue expanders.

Our typical approach for chest wall radiation is to treat the medial chest wall and internal mammary lymph nodes with an anterior electron beam field that is geometrically matched to two photon fields designed to treat the lateral chest wall. An example of these field arrangements on an axial computed tomography slice is shown in Figure 3A. The medial electron field has a rapid dose fall-off after the chest wall/lung interface, which, when combined with the more lateral photon fields, treats a small volume of lung. The flat chest wall surface allows for a relatively precise junction of the fields. An example of these field arrangements in the presence of a tissue expander is shown in Figure 3B. One consequence of the expander is that the junction between the fields occurs over a steeply sloping contour. This makes the geometric match less precise, which can lead to underdosing areas of the chest wall in the area under the field junctions. A second consequence of the sloping contour is that the thickness of the chest wall across the width of the electron field becomes nonuniform. The electron beam dose falls off as a function of tissue thickness, so this nonuniformity can also lead to inhomogeneities of dose within the treatment field.

A second negative consequence of performing an immediate reconstruction when postmastectomy radiation is required concerns the impact radiation can have on the long-term aesthetics of the reconstruction. These negative effects are worse for patients with implant reconstruction than for those with autologous tissue reconstruction. Specifically, for women with implants, radiation can promote significant capsular fibrosis.

Based on these two concerns, our multidisciplinary-determined institutional philosophy is to avoid immediate

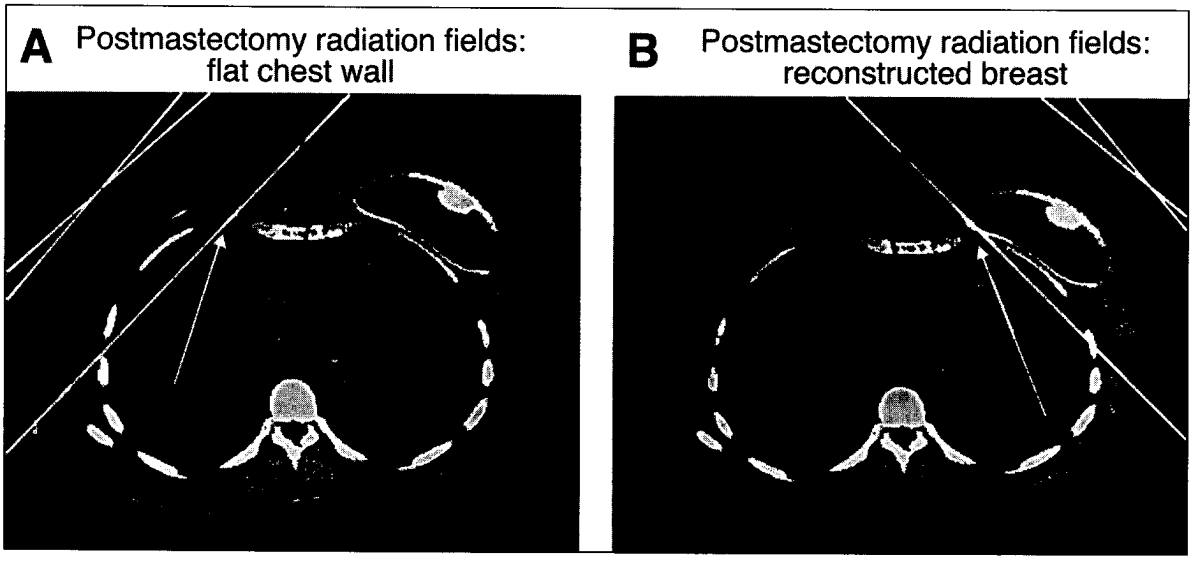

Figure 3. An axial computed tomography slice showing radiation fields used to treat the chest wall after mastectomy (A) or mastectomy with immediate reconstruction using a tissue expander (B). *The medial gray fields represent an anterior electron beam field that is geometrically matched to the white x-ray fields used to treat the lateral chest wall. The electron beam field has a rapid dose fall-off to minimize dose to the underlying lung. The gray arrows show the orientation of the radiation beams. The white arrows show the triangles of tissue in the medial chest wall that received less radiation dose at the junction of the fields. As shown, this triangle is much bigger when there is a tissue expander.*

reconstruction in all patients who have clinical features of disease that predict a high likelihood of requiring postmastectomy radiation. After radiation is completed, we then offer autologous tissue reconstruction, if the patient is a suitable candidate. Tissue expanders with implants may not be a good option for women after radiation because the tissue of the chest wall has significantly less elasticity after treatment. Unfortunately, the need for postmastectomy radiation is often determined based on the pathology of the mastectomy specimen, so in many cases, it is unclear at the time of surgery whether radiation will be indicated. It is very important that patients are aware of these issues throughout the entire decision-making process and contribute to decisions concerning the sequencing of breast reconstruction and postmastectomy radiation. How to optimally sequence postmastectomy radiation and breast reconstruction is a subject of ongoing research within our institution, and innovative approaches are still needed to further facilitate patients quality of life without compromising their treatments.

ACKNOWLEDGMENTS

Supported in part by National Cancer Institute Department of Health and Human Services grant numbers CA16672 and T32CA77050 and a grant from the Stanford and Joan Alexander Foundation, Houston, Texas. *Dr. Buchholz* is supported by grant number BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

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Molecular Biology and Genetics of Breast Cancer Development: A Clinical Perspective

Thomas A. Buchholz and David E. Wazer

Understanding the molecular and genetic events affecting breast cancer development not only helps oncologists address important questions commonly asked by their patients but also helps clinicians gain insights into the biology of the disease. Although the molecular and genetic determinants of most sporadic breast cancer remain unknown, significant advances in the understanding of events that contribute to breast cancer formation have been made. It is now recognized that mutations in some tumor suppressor genes, such as p53, BRCA1, BRCA2, PTEN, or ATM, or epigenetic functional inactivation of other tumor suppressor genes, such as SYK and NES1, appear to play important early roles in the formation of some breast cancers. In addition, alterations in proto-oncogenes, such as HER2/neu, may contribute to the development of some breast cancer. The goal of this article is to further introduce clinicians to molecular and genetic pathways that contribute to breast cancer formation. By participating in the study of breast cancer development at the molecular as well as the histopathological level, oncologists can help develop novel prevention, diagnostic, and therapeutic approaches for the future.

Recent advances in biotechnology, genetics, and molecular biology have made this the most exciting historical era in breast cancer research. During the first 980 years of the last millennium, we gained only a rudimentary understanding of the etiology of breast cancer. However, since the 1980s, there has been a consistent and exponential annual increase in the knowledge of how and why breast cancer develops. Even more exciting is the near certainty that over the next two decades, what we have learned about breast cancer development will exceed the combined sum of knowledge in the past two centuries. This rapidly increasing knowledge will affect all aspects of breast cancer care and radically reshape the practice of oncology. Our intent in this article is to introduce clinicians to the molecular and genetic processes that contribute to breast cancer development and offer some insights into the clinical implications of current and future research avenues.

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Dr. Buchholz is supported by Grant No. BC980154, a USAM Breast Cancer Research Program Career Development Award.

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1053-4296/02/1204-0002$35.00/0
doi:10.1053/srao.2002.35248

Breast Cancer Is a Genetic Disease

Breast cancer results from a series of complex genetic and epigenetic events that result in a malignant transformation of a normal epithelial cell. Genetic mutations, in which specific nucleotide base pairs of a gene are either altered or lost, are the most commonly recognized basis for these events. Genetic mutations can result in either a loss of function or an aberrant gain of function. Cells can also be altered without a change in their intrinsic genetic code, in what is known as an epigenetic phenomenon. Epigenetic changes result in an inhibition or a change in the transcription of a gene, without an alteration in its normal base-pair sequence. An example that may have relevance in breast cancer formation is hypermethylation of the promoter region of the gene. As we discuss later in this article, some genes have normal sequences that include "CpG islands." These regions are prone to methylation, which inhibits RNA binding to the promoter region and thereby prevents transcription. Correspondingly, no protein product is produced and the gene function can be lost.

For a breast cancer to develop, it must acquire the capacity to invade, recruit a vascular supply, and proliferate. Development of these phenotypes most often requires activation of oncogenes and deactivation of tumor suppressor genes, which inhibit many of these malignant traits and function to maintain the genomic integrity of cells. Some tumor suppressor genes support normal checkpoints in the cell cycle, which prevent the incorporation of genetic aberrancies into

daughter cells during mitosis, whereas others detect, process, and/or repair injuries to the DNA. Finally, still other tumor suppressor genes activate an intrinsic genetic suicide program (apoptotic pathway), so that the cell will die if damage to DNA is irreparable. Mutations in tumor suppressor genes are very common in breast cancer and likely represent early and critical events in the malignant transformation process. Examples of tumor suppressor gene mutations that have relevance to breast cancer formation include p53, BRCA1, BRCA2, PTEN, and possibly ATM.

Mutations in tumor suppressor genes can either be inherited (germline mutations) or developed during the life of a cell (somatic). Each strand of DNA (or allele) encodes genetic information, one from the original maternal and one from the paternal germ cell. In every known germline mutation that has relevance to breast cancer formation, an individual inherits a mutated allele from one parent and a normal allele from the other. For many germline tumor suppressor gene mutations, the normal allele makes sufficient protein product to allow cells to have a normal phenotype. In these instances, a "second hit" in the normal allele is needed to result in the loss of function in the tumor suppressor gene.[1] Women who inherit one abnormal allele are at much greater risk of developing this second hit.

Changes in gene sequences of tumor suppressor genes can also occur during the life of an individual (called somatic mutations). These mutations affect individual cells and their progeny. It is currently believed that most breast cancers are a consequence of somatic rather than germline mutations. Indeed, 70% of women who develop breast cancer have a negative family history of the disease and no clear familial breast cancer predisposition.

The second category of mutation that contributes to breast cancer formation is oncogene activation. Oncogenes result from mutations or alterations in proto-oncogenes and directly promote a malignant phenotype, such as uncontrolled cell growth. In contrast to tumor suppressor genes, oncogenes typically result in a gain rather than loss of function. Oncogenes are exclusively the consequence of somatic mutations (ie, they are never inherited) and may require only one abnormal allele to affect gene phenotype. Oncogenes may encode proteins that facilitate invasiveness, cell-cycle progression, or recruitment of a vascular supply for the tumor. Examples of proto-oncogenes that have relevance to breast cancer formation are HER2/neu, EGFR, Ras, Myc, and β-catenin.

Over the past decade, there have been significant discoveries of germline conditions that predispose individuals to breast cancer formation. The first discoveries occurred in families in which close to 50% of women eventually developed breast cancer (an autosomal-dominant inheritance pattern). Through these studies, breast cancer formation was found to be associated with germline tumor suppressor gene mutations in BRCA1, BRCA2, p53, and PTEN genes (these genes are discussed in further detail in the material that follows). All of these mutations are in high-penetrance genes, meaning that if the mutation is inherited, breast cancer is likely. However, together, they are associated with only 7% to 10% of all breast cancer cases. It is possible that other, yet undiscovered high-penetrance genes may account for an additional 5% of breast cancer cases. The remaining cases likely result from either sporadic mutations or germline conditions in low-penetrance genes. Low-penetrance germline conditions are much more difficult to discover, in that they lead to a subtler predisposition that affects only occasional family members.

BRCA1 and BRCA2

Each year, approximately 10,000 to 20,000 breast cancer patients in the United States have a clinical and family history suggestive of a predisposing germline mutation in a high-penetrance gene. During the 1990s, genetic linkage studies using lymphocytes from these families aided in the discovery of 2 genes, BRCA1 and BRCA2, which together accounted for the relevant germline mutation in approximately 65% of these familial breast cancer cases.[2] Management and genetic counseling issues are discussed in detail in articles by Pierce and Robson and Offit in this issue.

BRCA1 and BRCA2 are tumor suppressor genes whose protein products play important roles in the processing of DNA damage and preservation of genomic integrity. These 2 genes differ significantly in their genetic sequence and are located on different chromosomes. However, the gene products share many functional qualities. BRCA1 and BRCA2 both colocalize with Rad51 in a protein complex that is important for the

recognition, processing, and repair of double-strand DNA breaks.[3] This complex appears to play a specific, important role in a process called homologous recombination repair (covered in detail in the article by Xia and Powell), in which a sister chromatid is used as a template from which to repair the double-strand injury. Researchers have discovered that homozgyous mutations (mutations in both alleles of the gene) in either BRCA1 or BRCA2 adversely affect this interaction and consequently inhibit the repair of double-strand DNA damage.[4]

BRCA1 and BRCA2 have additional roles as tumor suppressor genes. For example, cells containing a homozygous BRCA1 mutation display diminished transcription-coupled repair and a diminished capacity for nonhomologus DNA end joining, 2 other mechanisms for repairing double-strand breaks.[5] Another recent discover is that BRCA1 joins histones H2A and H2AX at DNA break sites within minutes of damage, and that this association forms independently from Rad50 and Rad51.[6] Finally, both BRCA1 and BRCA2 proteins are preferentially expressed during G1/S and G2/M phases. In addition, wild-type BRCA1 expression has been found to be elevated in proliferating cells, and DNA damage promotes localization of BRCA1 on proliferating-cell nuclear, antigen-positive replicating structures, implying involvement in a checkpoint response.

It is possible that a cellular dysfunction in DNA double-strand break repair contributes to the high rates of breast cancer formation noted in women with a germline mutation in a BRCA gene. This dysfunction may lead to misrepaired DNA damage, which in turn can lead to other mutational events. However, normal cells are equipped with additional tumor suppressor genes, such as the p53 gene, which can prevent the propagation of misrepaired DNA. p53 acts as "a guardian of the genome" and regulates transcription of a number of genes that affect cycle, DNA damage repair, and the apoptotic pathway. Mouse embryos with a germline homozygous BRCA1 or BRCA2 mutant die early during embryonic development,[7] possibly due to p53-mediated cell-cycle arrest in response to unrepaired DNA damage. Indeed, embryos with both a homozygous BRCA1 or BRCA2 gene mutation and a homozygous mutation in p53 have later gestational lethality. These embryos presumably die due to misrepaired DNA damage rather than lack of appropriate proliferation. Interestingly, Rad51 knockout mice display embryonic lethality similar to that of BRCA knockout mice, which offers additional evidence that BRCA proteins function in Rad51-mediated DNA damage repair.[8-10] One consequence of the deficiency of double-strand break repair associated with a homozygous loss of the BRCA genes is cellular radiosensitivity. This is presented in more detail in the article by Xia and Powell in this issue. The cellular radiosensitivity associated with BRCA mutations may have a number of clinical implications. Individuals with a germline mutation in BRCA1 or BRCA2 inherit only one abnormal allele. The cancers that develop in these individuals have a loss of the other normal BRCA allele, which results in a homozygous mutation that is selectively present in the tumor cells.[9] This loss of BRCA function may offer a therapeutic advantage, in that these tumors should be selectively more sensitive to treatments such as radiation, bleomycin, and mitomycin, which achieve their therapeutic effects through double-strand DNA damage, than the normal tissue. To date, there have been no reported clinical trials investigating the efficacy of these specific chemotherapeutics for breast cancer in BRCA carriers. There are also no clear human data concerning the radioresponsiveness of tumors arising in this setting.

Understanding the function of the BRCA genes also raises concerns about the carcinogenic effects of radiation and the safety of therapeutic radiation treatment. In theory, the normal BRCA allele present in BRCA carriers should make sufficient protein to produce a normal cellular phenotype. However, in a condition known as haploinsufficiency, the phenotype of the heterozygous state is midway between the normal and the homozygous condition. If present in BRCA carriers, haploinsufficiency may increase the probability that exposure to radiation might result in additional mutational events. A recent pilot study demonstrated that fibroblasts and lymphocytes from individuals with a germline BRCA heterozygous mutation were more sensitive to radiation injury than cells from controls.[11] Foray et al earlier noted this same effect.[12] Additionally, in another recent study, lymphocytes from some individuals with germline heterozygous BRCA2 mutations evidenced genomic instability in the constitutional karyotype, as demonstrated by rearrangements at 9p23-24.[13] It is important to

recognize that other studies using genetically engineered murine cells have not found diminished damage repair in cells with heterozygous BRCA mutations. However, there are distinct differences between the murine and human BRCA genes. Most important, heterozygous mutations in BRCA1 or BRCA2 genes do not appear to predispose mice to cancer development.[7]

The question of haploinsufficiency with heterozygous BRCA mutations is also relevant with respect to the safety of radiation treatment in these patients. Thus far, retrospective studies have not found increased normal tissue sequela in breast cancer patients with germline BRCA1 or BRCA2 mutations (reviewed by Pierce in a subsequent article in this issue). Although these data offer evidence that BRCA carriers are not prone to severe complications, such as chest wall necrosis, they do not disprove the possibility that more subtle degrees of injury are possible. Given that haploinsufficiency would likely result in a modest to moderate cellular radiosensitivity, more subtle radiation complications, such as poor breast aesthetic outcomes, would be expected in BRCA carriers. Accurate analysis of this type of end point is almost impossible to achieve with a retrospective review of medical records.

A deficiency in double-strand break repair from haploinsufficiency would increase the probability of a loss of heterozygosity at the BRCA locus and increase the frequency of mutations in other tumor suppressor genes, such as p53. Indeed, breast cancers in women with BRCA1 mutations have increased rates of p53 mutation and high nuclear grade compared with sporadic breast cancers, and unlike sporadic breast cancers, the p53 mutations in BRCA1 tumors occur randomly throughout the gene.[14] However, it is not known whether this increased frequency and different locations of the p53 mutations are due to a deficiency in double-strand break repair secondary to haploinsufficiency from a heterozygous BRCA1 mutation. This is also discussed in the article by Xia and Powell in this issue.

Other Germline Tumor Suppressor Gene Mutations Relevant to Breast Cancer Formation

p53: Li-Fraumeni Syndrome

The paradigm of familial cancer syndromes was first described by Li and Fraumeni in 1969, in an epidemiologic evaluation of more than 600 medical and family history records of childhood sarcoma patients.[15] The original description consisted of kindreds with a spectrum of tumors that included soft-tissue sarcomas, osteosarcomas, breast cancer, brain tumors, leukemia, and adrenocortical carcinoma. Although it remained elusive for 20 years, the genetic link between these families was determined to be caused by the inheritance of germline alterations of the p53 tumor suppressor gene in affected family members.[16] Germline p53 mutations are primarily single-base changes in the gene (missense mutations) that yield a mutant protein that is generally more stable than the wild-type allele. The spectrum of mutations of p53 in the germline are indistinct from somatic mutations found in a wide variety of tumors, including 20% to 40% of breast cancers.[17] Patients with Li-Fraumeni syndrome are heterozygous for the mutation, and their tumors frequently have the second (wild-type) allele deleted or mutated, which is consistent with the classic modes of inactivation of this tumor suppressor gene. The risk of developing cancer in individuals with a germline p53 mutation are estimated to be 50% by age 30, and 90% by age 70. In women with Li-Fraumeni syndrome, breast cancer is the most common cancer.[18]

Germline alterations of the p53 gene have also been reported in a variable fraction of cancer patients with cancer phenotypes that resemble, but are not entirely consistent with, the classic Li-Fraumeni syndrome. In fact, only 60% to 80% of such "classic" families have detectable alterations of the gene.[19] It is unknown whether the remainder are associated with defects in other growth suppressor genes that may be functionally similar to p53, because of the presence of modifier genes, the occurrence of promoter defects yielding abnormalities of p53 expression, or simply the result of weak genotype-phenotype correlations (ie, the broad clinical definition encompasses families not actually affected by the Li-Fraumeni syndrome). Other candidate predisposition genes, such as p16, p21, BRCA1, and BRCA2 associated with multisite cancers, have not at this time been ruled out as potential targets.

Since the discovery of the p53 protein, a great deal of research effort has been expended to define the role of this gene in human cancers. Mutations in p53 are the most commonly recog-

nized genetic mutation in cancer cells.[17] The prognostic significance of p53 in distant metastasis and survival and local-regional control are discussed in articles by Esteva et al and Haffty in this issue. The structure and function of the p53 gene and its protein product are enormously complex (see reviews[20,21]). In brief, p53 protein regulates the transcription of a number of genes. Some of these genes, such as GADD45, p21, MDM2, and BAX, are transcriptionally activated by p53, whereas other genes, such as the proto-oncogenes BCL-2, c-Myc, and c-Fos, have their transcription repressed by p53. Mutations in p53 adversely affect the ability of the protein to bind to the regulatory DNA sequences of these genes and thus affect the ability of p53 to function as a transcriptional regulator.

Two cellular responses to DNA damage that are affected by p53 mutations are cell-cycle arrest and the apoptotic response. Together, these pathways are important in preventing propagation of cells with mutated genomes.[22,23] The growth arrest after wild-type p53 expression is critically dependent on p53 transcriptional activation of p21.[21] The gene product of p21 inhibits cyclin-cdk complexes and arrests cells in the transition from the G1 to S cell-cycle phases.[22] p53 affects the cellular apoptotic response to DNA injury through activation of BAX, a proapoptotic gene, and downregulation of BCL-2, a powerful antiapoptotic proto-oncogene.[24] It has been shown that preneoplastic mammary cell lines with normal p53 function undergo apoptosis after radiation injury, whereas cells with mutated p53 genes have a decreased apoptotic response.[25] It is probable that loss of p53 function produces genomic instability that allows for accumulation of additional mutations. Ultimately, this can lead to uncontrolled growth and development of a malignant phenotype. This hypothesis is supported by animal studies. Specifically, mice genetically engineered to have mutations in both alleles of p53 (knockout mice, p53 −/−) and mice with a single allele mutation (p53 +/−) have greatly increased tumor susceptibility.[26]

PTEN

Cowden's disease, also known as the multiple hamartoma syndrome, is a familial cancer syndrome with autosomal-dominant inheritance. Clinical signs and symptoms in young children include progressive macrocephaly with mild to moderate delay in psychomotor development. Characteristic lesions in adults include facial trichilemmomas, oral papillomas, lingua plicata, and hamartomas such as lipomas, fibromas, and hemangiomas. Patients are at risk of developing benign and malignant tumors at a young age. These tumors include adenoma and follicular cell carcinoma of the thyroid, polyps and adenocarcinoma of the gastrointestinal tract, fibrocystic disease, carcinoma of the breast, and cysts and carcinoma of the ovary.[27] The gene for Cowden's disease was mapped to band 10q22-23 in 1996. Within a year, PTEN (also known as MMAC-1 or TEP-1) was identified as the responsible gene.[28]

PTEN has been implicated in human mammary oncogenesis from studies that identify germline PTEN mutation as the cause of Cowden's disease. Like mutations in other tumor suppressor genes, germline mutations of PTEN are associated with loss of function of the normal allele and cancer.[29,30] The frequency of PTEN mutations in sporadic breast cancer is low, but PTEN resides at a site of frequent allelic imbalance, and PTEN protein is absent or decreased in a significant proportion of breast cancers.[31,32] Therefore, PTEN may be functionally inactivated in a higher percentage of breast cancers than can be accounted for by mutation alone. The functional significance of the PTEN gene relates to the fact that phosphatidylinositol 3-kinase (PI3K) and the PTEN protein phosphorylate and dephosphorylate the same 3′ site of the inositol ring of membrane phosphatidylinositols. The absence of the PTEN protein allows for the activation of the PI3K pathway that contributes to cell-cycle progression, decreased apoptosis, and increased metastatic capabilities. Of interest, breast cancer cells are selectively sensitive to pharmacologic and genetic manipulation of the PI3K pathway, making molecular targeting of this pathway particularly attractive as a potential therapeutic approach.

ATM

ATM is a tumor suppressor gene responsible for the autosomal recessive disorder ataxia telangiectasia. Studies of family members of children with ataxia telangiectasia reported that parents (obligate ATM heterozygotes) had a 5-fold greater risk of developing breast cancer than the general population.[33] This led to an estimate that 8% of all breast cancers occur in carriers of ATM mu-

tations.[34] After the ATM gene was cloned in 1996, a number of groups began testing breast cancer patients for the presence of germline ATM mutations. In general, these studies have found that protein-truncating mutations, such as gene deletions, are very uncommon in breast cancer patients. Instead, a fair percentage of breast cancer patients have been found to have single-base substitutions in the normal ATM gene sequence.[35,36] It is not clear whether these substitutions represent missense mutations in the gene or normal variant polymorphisms. In 1 study that sequenced the ATM cDNA in 91 patients, 33 had a least 1 single-base substitution in the gene. The frequency of the 3 most common single-base changes in the cases was compared with that in a control set with no cancer history; one of the base changes was statistically more common in the breast cancer patients (6.7% *v* 1.6%, respectively, $P = .004$). The other 2 changes were present at equal frequencies and therefore were likely normal variant polymorphisms.[36] Further studies are needed to verify these data.

The ATM gene plays an important role in maintaining genomic integrity. ATM is an upstream regulator of a number of important genes that are either involved in the cell-cycle response to DNA injury or directly participate in DNA damage repair. These genes include p53, BRCA1, DNA-PK and c-Abl.[37,38] Cells with an ATM deficiency do not display the regulatory cell-cycle arrest seen in normal cells after damage of cellular DNA, in part, because of its interaction with p53.[37] In addition, BRCA1 associates with and is phosphorylated by ATM,[38] and protein products from both genes are present in a large complex that has a role in sensing and processing DNA damage.[39] Similar to BRCA −/− cells, cells with deficient ATM function are radiosensitive and display diminished capacity for double-strand break repair. These data suggest that it is possible that mutations in ATM may increase the risk of breast cancer, in part through pathways that are also adversely affected by BRCA1 mutations.

Other Rare Inherited Syndromes

Peutz-Jeghers syndrome, an autosomal-dominant disorder occurring in approximately 1 in 20,000 live births, is characterized by hamartomatous polyps in the small bowel and pigmented macules of the buccal mucosa, lips, fingers, and toes.[40,41] It has recently been associated with an increased incidence of tumors of the breast, gastrointestinal tract, ovary, testis, and uterine cervix.[41] The gene mutated in Peutz-Jeghers syndrome is located on chromosome 19 and identified as STKII/LKB1.[42] This gene is a putative tumor suppressor that encodes a protein kinase.

Muir-Torre syndrome, a variant of hereditary nonpolyposis colon cancer (HNPCC, also called Lynch Type II syndrome), is the eponym given to the association between multiple skin tumors, and multiple benign and malignant tumors of the upper and lower gastrointestinal and genitourinary tracts.[43] Women with the syndrome reportedly have an increased risk of postmenopausal breast cancer. Multiple genes for HNPCC have been described, including MLH1 and MSH2.[44,45] Mutations in these genes are thought to lead to development of HNPCC through accumulation of DNA replication errors and associated subsequent genome instability.[46]

Inherited Low-Penetrance Genes

The majority of research concerning the inherited genetic predisposition to breast cancer has focused on germline conditions that have a strong association with breast cancer. Although these inherited conditions clearly contribute to breast cancer risk, they do so in a very small percentage of the entire breast cancer population. It is possible that low-penetrance genes contribute to breast cancer formation in a much larger percentage of breast cancer patients. By comparing the annual incidence rate of breast cancer development in twin and other relatives of women with breast cancer, the authors of a recent study concluded that the majority of breast cancers develop in a minority of genetically susceptible women.[47] However, discovering these low-penetrance genes is very difficult, in that they lead to a small or modest increase in the relative risk of cancer formation, which precludes the traditional approach of linkage analysis studies.

A different strategy for identifying the risk of breast cancer in a given individual from an inherited low-penetrance gene is to study the predisposing phenotype rather than attempt to discover the causative mutation. By evaluating a common downstream consequence of a variety of tumor suppressor gene mutations, a phenotype assay can potentially capture a much broader percentage of the breast cancer population. Furthermore, this strategy is not dependent on new gene

discovery and potentially can identify individuals who harbor relevant germline mutations in yet undiscovered genes. A number of investigators have analyzed whether cellular radiosensitivity can be used as a predictor of breast cancer risk. In one of the largest of these studies, Scott et al reported that breast cancer patients have a higher mean number of chromatid breaks in lymphocytes irradiated ex vivo than do controls with no breast cancer history.[48] Furthermore, other researchers found that the rates of chromatid breaks progressively increased in a control group, a group of women with bilateral breast cancers and a negative family history, a group of women with bilateral breast cancers and a positive family history, and a group of individuals with documented heterozygous BRCA germline mutations.[11,49] Two other studies also found that first-degree relatives of breast cancer patients had more radiation-induced chromosome breaks than did controls.[50,51] Data from one of these studies suggested that the inheritance pattern of radiosensitivity and breast cancer fits best with an inherited low-penetrance breast cancer predisposition gene.[51]

Class II Tumor Suppressors: SYK and NES1

The function of classic tumor suppressor genes (Class I tumor suppressors) such as p53 and retinoblastoma (RB) is typically lost through gene deletion or mutation.[52] However, recent studies have suggested that in a large number of genes, the functional inactivation and loss of expression is a consequence of gene silencing rather than mutation/deletion.[53] This loss of gene expression in tumor cells without evidence of gene deletion/mutation indicates a Class II tumor suppressor.[52] The number of genes categorized as Class II tumor suppressors has grown considerably. Prominent examples are RB, a cyclin-dependent kinase inhibitor (CDKI) p16, BRCA1, retinoic acid receptor-β (RARβ) 14-3-3σ, and cyclin D2.[54-58] Two recently described genes in this class that appear to be particularly relevant to breast cancer are SYK and NES1. Both are inactivated through an epigenetic pathway, referred to as hypermethylation of CpG islands. A large proportion of human genes have clusters of CpG dinucleotides (CpG islands) in their 5′-regulatory sequences. Gene silencing through methylation of these sites has been observed as part of normal cell homeostasis in embryologic development and aging.

SYK

Tyrosine kinases are proteins that play a critical role in breast cancer cell signaling pathways. Perhaps the most widely studied tyrosine kinase protein in breast cancer results from overexpression of the HER2/neu gene. Recently, another tyrosine kinase, encoded by the SYK gene, has been implicated in the inhibition of breast cancer cell growth and metastasis. This recent finding was unexpected, because SYK function has been predominantly linked to hematopoietic cell signaling. A recent study suggests that the SYK gene functions as a tumor suppressor in breast cancers.[59] SYK is expressed in normal breast ductal epithelial cells but, owing to hypermethylation of the gene, not in a subset of invasive carcinoma. Also, the loss of SYK expression seems to be associated with malignant phenotypic characteristics such as increased motility and invasion. Additionally, cells expressing transfected SYK cDNA exhibit decreased tumorigenicity.

NES-1

NES1 (also referred to as KLK10) was identified by subtractive hybridization between 76R-30, a radiation-transformed breast epithelial cell line, and its isogenic mammoplasty-derived normal parental strain, 76N.[60] The NES1 gene is expressed in normal but not in radiation-transformed mammary epithelial cells.[61] As seen with SYK, this loss of expression is not associated with gene mutation.[62] Importantly, NES1 mRNA, as well as protein expression, was dramatically downregulated or completely lost in a majority of breast cancer cell lines.[61] Transfection of NES1 cDNA into a highly aggressive NES1-negative breast cancer cell line dramatically reduced the tumorigenic phenotype.[63] These findings suggested that, in addition to providing a possible tumor marker, inactivation of the NES1 gene expression through hypermethylation of CpG islands may be linked to oncogenesis. To evaluate this further, an in situ hybridization technique with an antisense NES1 probe was used to detect NES1 mRNA in tissue sections of normal breast epithelium, atypical ductal hyperplasia, ductal carcinoma in situ, and infiltrating ductal carcinoma.[64] High levels of NES1 expression were

detected in all 30 normal breast specimens examined. Notably, 18 of 24 (75%) breast hyperplasia specimens, whether typical or atypical, showed high NES1 expression, with weak-to-moderate expression in 25%. There was a complete lack of NES1 expression in 13 of 28 (46%) ductal carcinoma in situ specimens, and the remaining 54% showed weak-to-moderate staining. Finally, 29 of 30 (97%) infiltrating ductal carcinoma specimens lacked NES1 mRNA, with weak expression in the one remaining sample. These results indicate that the analysis of NES1 expression in cellular specimens obtained from ductal lavage may prove to be useful in risk assessment and screening.

Sporadic Mutations

It is currently thought that most women with breast cancers do not have a germline mutation that specifically predisposed them to the development of the disease. Determining the etiology of cancer formation in the absence of a germline mutation is very complex.

It is highly probable that breast cancer can result from a variety of sporadic gene mutations that lead to abnormalities in multiple independent pathways. The most common sporadic gene mutations in breast cancer are mutations in p53, which have been found in up to 40% of human breast cancers. The association of Li-Fraumeni syndrome with breast cancer formation suggests an important relationship of p53 mutations with the development of some breast cancers. Furthermore, in vitro studies have shown that loss of p53 function leads to immortalization of human mammary epithelial cells.[65,66]

A significant number of mutations or alterations in the expression levels of other genes are common in breast cancer, many of which also affect the cell-cycle or apoptotic pathways. Whether these genetic events occur early or late in the transformation process remains an area of continued investigation. To date, insufficient data exist to suggest that the majority of identified gene mutations or expression abnormalities in breast cancer play a significant role in breast cancer formation. Preliminary data do indicate that amplification of the HER2/neu proto-oncogene may be important. HER2/neu is a transmembrane tyrosine kinase of the epidermal growth factor (EGF) family. Approximately 30% of breast cancers have overexpression of the HER2/neu cell surface receptor.[67] In humans, overexpression does not result from a mutation in the HER2/neu gene, but rather appears to be a consequence of abnormal gene amplification (increased number of copies of the gene), upregulation of gene transciption, and/or enhancement of protein translation. Overexpression of HER2/neu is associated with an increased proliferative capacity, enhancement of the metastatic potential, and increased rate of tumorigenesis.[68,69] Furthermore, it has been shown that downregulation of HER2/neu, via monoclonal antibodies directed to its receptor or the addition of proteins that bind to the promoter region of the HER2/neu gene, can reverse a malignant phenotype both in vitro and in vivo.[69] Dillon, Esteva et al, and Sartor, in this issue, discuss in more detail the clinical role of HER2/neu in the clinical management of breast cancer.

Future Directions

Reducing the study of breast cancer formation to a single gene product likely oversimplifies a very complex and heterogeneous genetic process for the majority of cases. As important as the discovery of BRCA1 and BRCA2 and the elucidation of other genetic cancer-related syndromes have been, they have furthered our understanding of cancer formation for only 10% of all breast cancer cases. To date, the genetic events that have relevance for the remaining 90% of cases remain uncertain. Furthermore, recent data suggest that cancer formation is not dependent solely on genomics, but rather is likely a function of the interaction of genomics of a cell and the complex interaction of the cell with its microenvironment.[70,71] For example, integrins, which function as cell-to-cell interactors, and the extracellular matrix both affect cancer formation independent of the genetic makeup of the cell.

One strategy being developed to overcome the complexity of the genetic processes and tissue microenvironment contribution is to use cDNA microarrays to produce a transcriptional profile of tumors. This strategy permits the simultaneous measurement of the expression levels of up to 30,000 genes in one patient's tissue specimens. In turn, expression levels of individual genes can be compiled to study pathways that may be important in breast cancer development. It is also

possible to study how global gene expression patterns change across the spectrum of biological processes, from atypical ductal hyperplasia to ductal carcinoma in situ, to invasive breast cancer, to breast cancer within a lymph node, to breast cancer within a systemic metastasis. This research strategy will likely uncover additional genetic and epigenetic phenomena required for the highly complex events that culminate in malignant transformation.

Conclusions

An understanding of the genetic and epigenetic conditions that contribute to breast cancer development can help clinicians inform their patients about the risks of new primary tumors and of cancer development in family members. Furthermore, increasing evidence suggests that many of the molecular contributors that affect breast cancer development also affect the biology of the cancer and its response to therapeutic interventions.

The past 2 decades has provided a wealth of new information concerning genetic and molecular contributors to breast cancer development. More exciting is the certainty that the current and future advances in this area of research will rapidly overshadow past progress. The completion of the Human Genome Project and advances in technology will undoubtedly increase the pace of discovery and make possible novel, molecular-based breast cancer risk assessment and prevention strategies within the next generation.

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ORIGINAL ARTICLE

Factors Predictive of Outcome in Patients With Breast Cancer Refractory to Neoadjuvant Chemotherapy

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PURPOSE

The purpose of this study was to determine the clinical, pathological, and treatment factors that are predictive of local-regional recurrence and overall survival for patients with breast cancer that is refractory to neoadjuvant chemotherapy.

PATIENTS AND METHODS

This study analyzed the data of the 177 breast cancer patients treated on our institutional protocols who had less than a partial response to neoadjuvant chemotherapy. The initial clinical stage of disease was II in 27%, III in 69%, and IV (supraclavicular lymph node involvement) in 4%. Surgery was performed in 94% of the patients, and 77% of these patients also received adjuvant chemotherapy.

RESULTS

After a median follow-up of 5.2 years, 106 patients experienced disease recurrence, with 98 of these having distant metastases and 45 having local-regional recurrence. The 5- and 10-year overall survivals for the entire group were 56% and 33%, respectively. The factors that were independently associated with a statistically significant poorer overall survival in a Cox regression analysis were pathologically involved lymph nodes after surgery, estrogen receptor–negative disease, and progressive disease during neoadjuvant chemotherapy. The 5-year overall survival for patients with pathologically negative lymph nodes ranged from 84% (estrogen receptor–positive disease) to 75% (estrogen receptor–negative disease), compared with rates for patients with pathologically positive lymph nodes of 66% (estrogen receptor–positive disease) and 40% (estrogen receptor–negative disease). The 5-year survival of patients with progressive disease was only 19%.

The 5- and 10-year local-regional recurrence rates for the 177 patients were 27% and 34%, respectively. Significant factors on Cox analysis that predicted for local-regional recurrence were four or more pathologically involved lymph nodes and estrogen receptor–negative disease. For the 105 patients treated with surgery and postoperative radiation therapy, the 10-year local-regional recurrence rates for the subgroups with 0, 1, or 2 of these factors were 12%, 25%, and 44%, respectively.

CONCLUSIONS

For patients with a poor response to neoadjuvant chemotherapy, conventional treatments achieve reasonable outcomes in those with lymph node–negative disease or estrogen receptor–positive disease. However, more active systemic and local therapies are needed for patients with estrogen receptor–negative disease and positive lymph nodes and for those with clinical evidence of progressive disease during neoadjuvant chemotherapy. (*Cancer J 2001;7:413–420*)

KEY WORDS

Refractory breast cancer, outcome, neoadjuvant

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Received on June 1, 2001; accepted for publication July 17, 2001.

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this article.

Supported in part by National Cancer Institute Department of Health and Human Services grant nos. CA16672 and T32CA77050. Dr. Buchholz is supported by grant no. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

This work was presented at the Radiological Society of North America, Chicago, November, 2000.

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Neoadjuvant chemotherapy has become the standard of care for patients with locally advanced nonmetastatic breast cancer, in part because it offers the earliest treatment directed against micrometastic disease. In addition, chemotherapy can convert inoperable disease to disease that is amenable to surgical resection and, in selected cases, can even permit breast conservation therapy. Finally, neoadjuvant chemotherapy permits an in vivo assessment of disease response to treatment. This

assessment has important prognostic and therapeutic implications and may help to guide the introduction of non–cross-resistant regimens for patients with a poor initial clinical response.[1]

Over the past 3 decades, we have performed prospective clinical trials to study the efficacy of neoadjuvant chemotherapy for patients with advanced breast cancer. In these studies, the clinical partial response rate after neoadjuvant chemotherapy was approximately 80%.[2,3] We have previously demonstrated that response to chemotherapy is a strong predictor both for disease-free survival (DFS) and overall survival (OS). For example, a previous analysis of our data found that patients with a complete clinical response (12% of the study population) had a 3-year DFS of 95%, compared with 36% for patients with no response or progressive disease (7% of the population).[4]

Although patients with locally advanced breast cancer who achieve an excellent response to neoadjuvant chemotherapy have outcomes similar to those of patients with early-stage disease, patients with less favorable responses to neoadjuvant chemotherapy remain a therapeutic challenge. For these patients, few data provide insight into the optimal treatment strategy and predictors for local and distant recurrences. In this report, we reviewed the outcome of patients treated in our clinical trials who, at the time of primary treatment, were prospectively classified as having had a poor response to neoadjuvant chemotherapy. Our goal in this study was to evaluate how the subsequent treatments affected local and distant disease control. In addition, to determine which cohorts of patients achieve acceptable outcomes with conventional treatments and which should be considered for more novel therapeutic approaches, we evaluated the clinical, pathological, and treatment factors predictive of local-regional recurrence (LRR) and OS.

PATIENTS AND METHODS

This study is a retrospective analysis of the data from patients treated in five prospective institutional clinical trials that investigated neoadjuvant chemotherapy for noninflammatory breast cancer. These trials were conducted at the University of Texas M. D. Anderson Cancer Center from 1974 to 1998. In these trials, 883 patients were treated with neoadjuvant doxorubicin-containing chemotherapy, and 87 were treated with neoadjuvant single-agent paclitaxel. All of the patients treated in these trials were prospectively evaluated with both physical and radiologic examinations before and after the neoadjuvant treatment. Clinical stages were assigned at study entry, after a physical examination, mammography, chest radiography, bone scan, and evaluation of the liver (liver scan, computed tomography, or ultrasound). Patients with systemic metastases or inflammatory carcinoma were treated on different protocols and were not included in this study. The clinical response to neoadjuvant chemotherapy was prospectively categorized for each patient by a multidisciplinary team on the basis of the physical examination and imaging studies (mammogram, ultrasonogram). Clinical complete response was defined as total resolution of the breast primary tumor and involved regional adenopathy. Partial response was defined as a $\geq$ 50% reduction of the product of the two longest perpendicular dimensions of the breast mass and regional adenopathy. Minimal response was defined as < 50% reduction in these measurements. Finally, clinical response could also be categorized as no change or as progressive disease ($\geq$ 25% increase of the product of the two largest perpendicular dimensions of the breast mass and regional adenopathy).

For the purpose of this analysis, we reviewed the outcome of the 177 patients who were prospectively classified as having less than partial clinical response to preoperative chemotherapy. Our study population consisted of 27 patients with progressive disease (15%), 52 patients with no change in their disease (29%), and 98 patients with a minimal response (55%).

Table 1 shows the clinical, disease, and treatment characteristics of the 177 patients included in this report. As shown, most patients (73%) had IIIA or greater disease. Ninety-four patients (53%) had estrogen receptor (ER)–positive disease, 59 (34%) had ER-negative disease, and the remaining 23 (17%) had an unknown ER status. Sixty-eight patients (38%) had progesterone receptor (PR)–positive disease, 59 (33%) had PR-negative disease, and 49 (29%) had unknown PR status.

Table 2 shows the neoadjuvant chemotherapy regimens and the number of cycles that were used for the patients in this study. The treatment regimen and its scheduling followed the specific protocol under which the patient was treated. As shown in Table 2, 90% of the 177 patients received doxorubicin-containing neoadjuvant chemotherapy, and only 10% were initially treated with paclitaxel. The full details concerning the regimens have been published in earlier reports.[2,3,5] Briefly FAC chemotherapy consisted of 500 mg/m^2 of 5-fluorouracil given on days 1 and 4, 50 mg/m^2 of doxorubicin given as a day 1 bolus or as a 72-hour continuous infusion, and 500 mg/m^2 of cyclophosphamide given on day 1. For the patients treated with dose-escalated FAC (5-fluorouracil, doxorubicin, cyclophosphamide), the doses were increased to 600, 60, and 1000 mg/m^2, respectively. The VACP (vincristine, doxorubicin, cyclophosphamide, prednisone) regimen consisted of 1.5 mg/m^2 of vincristine, 60–75 mg/m^2 of doxorubicin, 600–750 mg/m^2 of cyclophosphamide, and 40 mg of prednisone. Finally, the paclitaxel regimen consisted of a dose of 250 mg/m^2 given as a 24-hour infusion.

Ninety-four percent (166/177) of patients in our

TABLE 1 Patient, Pathological, and Treatment Characteristics

Age	<40	16% (28/177)
	40–60	70% (124/177)
	>60	14% (25/177)
Clinical stage	IIA	9% (16/177)
	IIB	18% (32/177)
	IIIA	22% (39/177)
	IIIB	47% (83/177)
	IV[a]	4% (7/177)
ER/PR status	ER+, PR−	12% (21/177)
	ER+, PR+	34% (60/177)
	ER−, PR+	5% (9/177)
	ER−, PR−	21% (37/177)
	Unknown	28% (50/177)
Surgery	None	6% (11/177)
	Mastectomy	87% (154/177)
	Breast conservation	7% (12/177)
Radiation[b]	None	16% (27/166)
	Preoperative	20% (34/166)
	Postoperative	63% (105/166)
Adjuvant chemotherapy[b]	None	23% (38/166)
	High-dose chemotherapy with autologous transplant	9% (15/166)
	MV	32% (53/166)
	CMF	5% (8/166)
	VMF	9% (15/166)
	FAC	11% (18/166)
	Paclitaxel	7% (12/166)
	Other	4% (7/166)
Adjuvant tamoxifen[b]	Yes	50% (83/166)
	No	50% (83/166)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; MV, methotrexate, vinblastine; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; VMF, vinblastine, methotrexate, 5-fluorouracil; FAC, 5-fluorouracil, doxorubicin, cyclophosphamide.

[a]Indicates ipsilateral supraclavicular lymph node involvement without systemic metastases.

[b]Percentages for the patients receiving radiation, chemotherapy, and tamoxifen include only the 166 patients treated surgically.

TABLE 2 Neoadjuvant Chemotherapy Treatment Details

Protocol	Years of the Study	Neoadjuvant Chemotherapy	Number of Cycles	Included Patients/ Total Study Population
Advanced primary	1974–1985	FAC	3	22/191
85–01	1985–1989	VACP	3	32/200
89–007	1989–1991	FAC	4	56/203
91–015	1991–1994	FAC or dose-escalated FAC	4	32/202
94–002	1994–1998	FAC or paclitaxel	4	35/174
Total	1974–1998			177/970

Abbreviations: FAC, 5-fluorouracil, doxorubicin, cyclophosphamide; VACP, vincristine, doxorubicin, cyclophosphamide, prednisone.

study population underwent surgery (Table 1). Four percent of patients did not undergo surgery because of inoperable disease, 1% because of development of early distant metastasis, and 1% because of an early treatment-related death. For the patients who underwent surgery, the median number of lymph nodes recovered was 15, with a range of three to 56.

Surgery followed by postoperative radiation therapy, the preferred treatment strategy for these patients during the study period, was used in 105 of the patients. The 34 patients who received preoperative radiation therapy did so because of local-regional disease extent. It was not possible to retrospectively determine the reasons why 27 patients were not treated with radiation therapy after surgery, although selection biases clearly played a role.

Radiation treatments were given in a consistent fash-

ion over the years of this study. Patients who received preoperative radiation therapy received a median dose of 50 Gy at 2 Gy per fraction delivered to the breast and ipsilateral axillary apex/supraclavicular fossa. Postmastectomy radiation therapy consisted of 50 Gy at 2 Gy per fraction to the chest wall, axillary apex/supraclavicular fossa, and internal mammary lymphatics, followed by a boost to the chest wall (median dose, 10 Gy). For patients treated after breast-conserving surgery, a median dose of 50 Gy at 2 Gy per fraction was delivered to the breast, followed by a tumor bed boost (median dose, 10 Gy). Treatment to the nodal basins was individualized according to the extent of nodal disease.

One hundred twenty-eight (77%) of the patients who underwent surgery were subsequently treated with adjuvant chemotherapy. Postoperative chemotherapy treatment strategies changed over the period of time included in this study. Initially, adjuvant FAC (similar to the preoperative regimen) was often used, with the hope that it would have greater efficacy after the surgical removal of bulky disease. The second approach was to use CMF (cyclophosphamide, methotrexate, 5-fluorouracil) and subsequently either VM (vinblastine, methotrexate) or VMF (vinblastine, methotrexate, 5-fluorouracil). Finally, the latest strategy used in this cohort of patients was to investigate high-dose chemotherapy with transplantation.

Table 1 provides the details of the adjuvant chemotherapy regimen used. Only 12 patients (7%) were treated with an adjuvant regimen containing a taxane. Fifteen patients (9%) received high-dose chemotherapy with stem cell transplantation after surgery on institutional protocols. The high-dose chemotherapy patients underwent mobilization of peripheral blood stem cells for collection using either granulocyte colony stimulating factor treatment alone (6 μg/kg subcutaneously every 12 hours) or after CVP chemotherapy (cyclophosphamide, 1.5 g/m²/day i.v. on days 1–3; etoposide, 250 mg/m²/day i.v. on days 1–3; and cisplatin, 40 mg/m² i.v. on days 1–3. Patients then received high-dose CBT chemotherapy (cyclophosphamide, 2.0 g/m² i.v. on days −7, −6, and −5; BCNU (carmustine), 150 mg/m² i.v. on days −7, −6, and −5; thiotepa, 240 mg/m² i.v. on days −7, −6, and −5, with mesna, 2.0 mg/m²/day by continuous i.v. infusion for 3 days). The cryopreserved blood progenitor cells were then reinfused intravenously.

We used the method of Kaplan and Meier to generate actuarial local control and survival data.[6] All event and follow-up times were measured from the date of diagnosis. Two-sided log-rank tests were used to detect differences in actuarial data. A Cox proportional hazards model was used to determine independent variables associated with OS and local control.[7] Cases with unknown factors were excluded in the initial Cox regression analysis. If a factor did not predict for the endpoint being analyzed, the cases with unknown values for that factor were again added, and the Cox regression was repeated, with that particular factor dropped.

RESULTS

Pathological response could be determined in the 166 patients who underwent surgical resection. Of these, three (2%) had a pathological complete response despite having less than a partial clinical response. The post-treatment pathological stage was complete response or stage I in 32 patients (19%), stage II in 56 patients (34%), and stage III in 78 patients (47%). The median pathologically determined diameter of residual primary disease was 5.1 cm, with a range of 0–15 cm. In 51 patients (31%), no disease was found in axillary lymph nodes, 48 (29%) had one to three positive lymph nodes, 43 (26%) had four to nine positive lymph nodes, and 24 (14%) had 10 or more positive lymph nodes.

The clinical stage after neoadjuvant chemotherapy correlated with the pathological stage in only 42% (69/166) of the patients treated surgically. Seventeen percent of these patients (28/166) had higher-stage disease on pathological examination than was clinically evident, whereas 42% (69/166) had a lower pathological stage. All surgically treated patients underwent complete resection of their known disease.

After a median follow-up of 5.2 years, disease recurred in 60% of patients (106/177). In 98 (55% of the total population), distant metastases developed, and in 45 (25% of the total population), LRR, either alone or in combination with distant metastases, developed. In the 106 patients with disease recurrence, LRR was the first site of recurrence in 24%, simultaneous LRR and distant metastases occurred in 10%, distant metastases followed by LRR occurred in 25%, and distant metastases occurred alone in 41%.

Figure 1 shows the actuarial OS and DFS for the 177 patients. The actuarial OS rates at 5 and 10 years were 56% and 33%, respectively. The 5- and 10-year actuarial DFSs were 41% and 25%, respectively. The following factors predicted for a decreased OS on univariate analysis: progressive disease ($P < 0.0001$), ER-negative disease ($P = 0.003$), pathologically positive lymph nodes after surgery ($P = 0.03$), PR-negative disease ($P = 0.006$), and initial clinical stage III or IV disease ($P = 0.003$). On multivariate analysis, progressive disease (hazards ratio, 4.15; $P < 0.0001$), ER-negative disease (hazards ratio, 2.17; $P = 0.002$), and pathologically positive axillary lymph nodes after surgery (hazards ratio, 2.83; $P = 0.001$) independently predicted for poorer OS. PR-negative disease and stage III/IV disease were no longer significant in the multivariate analysis.

The 5- and 10-year OS rates for the significant factors found on multivariate analysis are shown in Table 3.

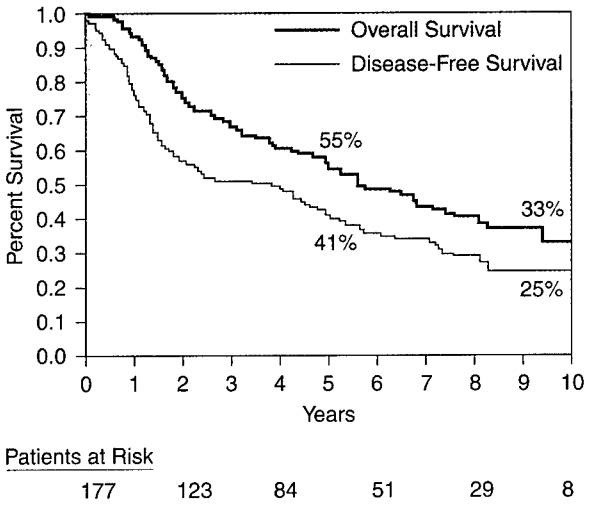


FIGURE 1 Actuarial overall survival and disease-free survival for the study population.

Table 4 defines subgroups of patients with favorable and unfavorable survival rates based on stratification according to these factors. As shown, patients with negative lymph nodes and patients with ER-positive disease achieved reasonably good survival rates with the given therapies. Conversely, all patients with progressive disease faired poorly, and patients with both ER-negative disease and positive lymph nodes had a 5-year survival rate of only 40%.

Figure 2 shows the relationship between OS and use of surgery and adjuvant chemotherapy. By univariate analysis, both the ability to perform surgery and the use of adjuvant chemotherapy were associated with a significantly improved survival, whereas radiation (P = 0.91), hormonal therapy (P = 0.46), and high-dose chemotherapy (P = 0.80) were not (data not shown). Multivariate analysis, including treatment and clinical-pathological factors, found that none of the treatments were independent predictors of improved OS.

The overall rates of LRR at 5 years and 10 years were 28% and 34%, respectively. Factors that predicted for LRR on univariate analysis were clinical stage III/IV disease (P = 0.017), progressive disease during neoadjuvant chemotherapy (P < 0.0001), four or more pathologically involved lymph nodes (P = 0.040), ER-negative disease (P = 0.0003), and PR-negative disease (P = 0.019). Only ER-negative disease and four or more pathologically involved lymph nodes remained significant in a Cox model analysis.

TABLE 3 Overall Survival According to the Significant Factors Found on a Cox Regression Analysis

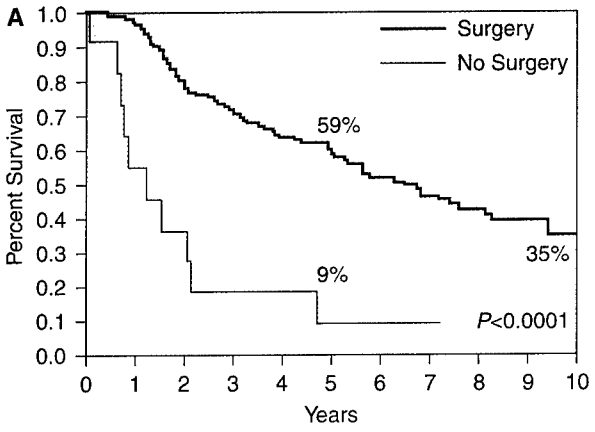
Category	Factor	5-Year Survival[a]	10-Year Survival[a]
Clinical response	Progressive disease	19% (6%–37%)	No data
	No change or response	62% (53%–70%)	37% (25%–49%)
Involved lymph nodes	0	64% (50%–75%)	48% (33%–61%)
	1–3	59% (42%–73%)	39% (22%–56%)
	4–9	49% (32%–64%)	0%
	10+	45% (24%–64%)	No data
ER status	ER− disease	43% (29%–55%)	29% (17%–43%)
	ER+ disease	70% (58%–79%)	47% (34%–60%)

Abbreviation: ER, estrogen receptor.
[a]Numbers in parentheses represent 95% confidence intervals.

TABLE 4 Prognostic Factors that Predict for Favorable and Unfavorable Survival Rates

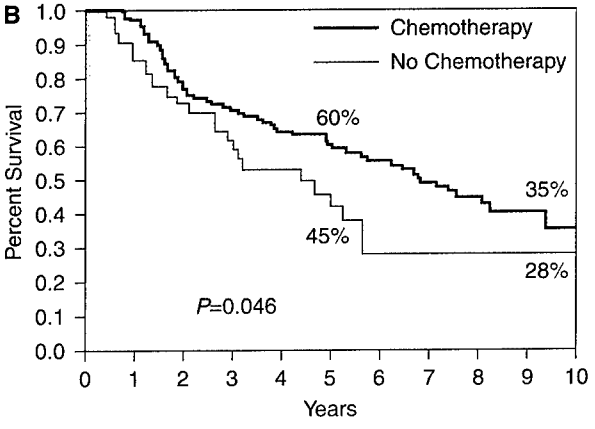
Group	Clinical Response	Pathological Lymph Node Status	ER Status	5-Year Survival[a]	10-Year Survival[a]
1	No change or response	Negative	Positive	84% (62%–94%)	70% (42%–86%)
2	No change or response	Negative	Negative	75% (46%–90%)	52% (24%–74%)
3	No change or response	Positive	Positive	66% (51%–78%)	41% (24%–57%)
4	No change or response	Positive	Negative	40% (21%–58%)	No data
5	Progressive disease	Either	Either	19% (6%–37%)	No data

Abbreviation: ER, estrogen receptor.
[a]Numbers in parentheses represent 95% confidence intervals.



Patients at Risk

166	118	67	50	29	8
11	4	2	1	0	0

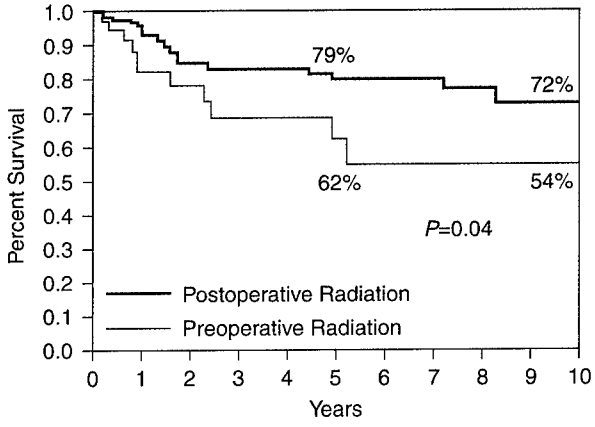


Patients at Risk*

134	93	68	44	25	7
40	27	15	6	3	1

*3 patients excluded because it was uncertain whether additional chemotherapy was given

FIGURE 2 Actuarial overall survival for patients divided according to treatment with surgery (A) and divided according to the use of adjuvant chemotherapy (B).



Patients at Risk*

105	75	57	38	21	6
34	18	12	5	3	1

FIGURE 3 Actuarial local-regional control curves for patients treated with surgery and radiation therapy divided according to the sequencing of these therapies.

Patients who underwent surgery had a significantly decreased LRR rate ($P < 0.0001$) compared with those not treated with surgery. In the group of patients treated surgically, the use of radiation therapy ($P = 0.19$) did not correlate with local control, likely reflecting the fact that patients with low-risk disease did not receive radiation therapy, whereas those with high-risk features did. Neither adjuvant chemotherapy ($P = 0.49$) nor high-dose chemotherapy with transplantation ($P = 0.60$) affected local control. Figure 3 shows the comparative local control data between patients treated with postoperative radiation therapy versus those treated with preoperative radiation therapy. The 5- and 10-year freedom from local regional recurrence was 79% and 72%, respectively, for the postoperative radiation group, compared with 5- and 10-year rates of 62% and 54%, respectively, for the preoperative radiation therapy group ($P = 0.04$). However, the local-regional disease extent after neoadjuvant chemotherapy was the primary factor in determining the sequencing of surgery and radiation therapy.

For the 30 patients with ER-positive disease and less than four involved lymph nodes who were treated with surgery and postoperative radiation therapy, the 5- and 10-year rates of freedom from LRR were 95% (CI of 71%–99%) and 88% (CI of 58%–97%), respectively. The 10-year freedom from LRR for the 105 patients treated with surgery and postoperative radiation therapy who had either ER-negative disease or four or more involved lymph nodes was 75% and decreased to only 56% for the patients with both of these factors.

DISCUSSION

Many studies have indicated that the outcome of patients with locally advanced breast cancer can be equivalent to those with early-stage disease if a favorable response to neoadjuvant chemotherapy is achieved.[4,5,8,9] For example, previous data from our institution reported that women with advanced nonmetastatic breast cancer who have negative axillary lymph nodes after neoadjuvant doxorubicin-based chemotherapy have an OS rate exceeding 80% at 5 years.[10] At the other spectrum of disease response, however, the prognosis of the 20% of patients with a disease that is refractory to neoadjuvant chemotherapy remains poor. To date, few studies have

provided insights into how to optimally manage such patients. In this report, we provide one of the first clinical data sets specifically evaluating the treatment and pathological factors that affect outcome in breast cancer patients who have a poor clinical response to neoadjuvant chemotherapy. We demonstrate that this subset of patients is a heterogeneous population, in part reflecting the imprecision of physical examination and radiographic imaging in assessing disease response. Despite the fact that every patient in this study was prospectively determined to have less than a partial response to treatment, 19% of those who subsequently underwent surgery were found to have either a complete pathological response or a primary tumor < 2 cm and negative axillary lymph nodes. Other authors have also noted this discordance between clinical and pathological response to neoadjuvant chemotherapy treatment.[11,12]

One of our objectives in performing this study was to identify treatment and pathological factors that would help distinguish patients who have acceptable outcomes after conventional treatments from those for whom more novel therapeutic strategies should be considered. We found that poor clinical response to neoadjuvant therapy by itself does not always carry a dismal prognosis after conventional treatments. The actuarial 5-year survival in this series was greater than 50%, and 25% of the patients were estimated to be alive and free of recurrence at 10 years. These outcomes were achieved despite the fact that more than half of the study population had stage IIIB or IV disease at presentation. We found that the most powerful independent prognosticators for a poor OS were progressive disease during neoadjuvant treatment, ER-negative disease, and pathologically positive axillary lymph nodes found after surgery.

The negative prognostic effect of lymph node disease after neoadjuvant chemotherapy has been reported by other authors, although these previous studies focused on entire populations of patients treated with neoadjuvant chemotherapy rather than just those with clinically refractory disease. For example, in an earlier report from our institution, Kuerer et al[13] reported that the 5-year DFS rate was 87% for patients with negative lymph nodes after neoadjuvant chemotherapy, compared with 51% for patients with residual nodal disease. ER-negative disease has been noted by numerous authors to correlate with improved response rates to neoadjuvant chemotherapy,[7,14,15] although other authors have not found a correlation between response and ER status.[16] Despite a possible association between ER-negative disease and chemotherapy response, the presence of ER-negative disease has never been demonstrated to correlate with an improved survival after neoadjuvant chemotherapy. In fact, in our study of patients who failed to achieve a favorable response to neoadjuvant chemotherapy, we found that ER-negative disease strongly correlated with a poorer survival. The significance of ER disease on OS is unlikely to be attributable to tamoxifen use because we found no relationship between use of hormonal therapy and survival ($P = 0.46$).

Our data suggested that in addition to the relatively high rates of distant metastases, patients with clinically refractory disease have high rates of LRR despite surgery and radiation therapy. The local recurrence rate in the subgroup of patients who were treated with both of these modalities exceeded 25% at 10 years. Furthermore, one third of the patients in this series had a LRR as an isolated event or as a component of the first site of failure. These data suggest that improvements in local-regional treatment may be one way to improve OS. The independent factors that predicted for high rates of LRR after neoadjuvant chemotherapy and surgery were ER-negative disease and four or more pathologically involved lymph nodes. For patients treated with mastectomy and postoperative radiation therapy, the local control rate for patients with both of these factors was only 56%.

Numerous therapeutic approaches appear to be reasonable for patients with clinical refractory breast cancer. We continue to advocate surgery for patients with operable disease. On univariate analysis, surgery highly correlated with improved OS and local control, although selection biases played a role in determining which patients underwent surgery. Surgery is also important for determining the number of positive lymph nodes after neoadjuvant treatment, a factor we found to highly correlate with outcome. After surgery, we currently advocate the use of additional chemotherapy and post-mastectomy radiation therapy for all patients with chemotherapy-refractory disease. On univariate analysis, the use of adjuvant chemotherapy appeared to improve. In addition, the most patients in this study were treated before the availability of taxanes. Taxanes have been reported to achieve clinically significant response rates for patients with anthracycline-resistant metastatic disease.[17,18] Thus, the use of taxanes may further improve the outcome data we report. For subsets of patients who are also at high risk for LRR (ER-negative disease or four or more positive lymph nodes), protocols are needed to investigate concurrent taxanes and post-mastectomy radiation therapy. Both paclitaxel and docetaxel have radiosensitizing properties for tumor cells in vivo,[19,20] and their concurrent use with radiation therapy after mastectomy may simultaneously minimize the risks of local and distant failure. In addition, the use of 5-fluorouracil derivatives or cisplatin can also be investigated as radiosensitizers and non–cross-reactive chemotherapeutics.

In conclusion, patients with a poor clinical response to neoadjuvant chemotherapy represent a heterogeneous population, and therefore, decisions regarding

prognosis and further treatment should be based on pathological determinants as well as clinical response. In addition, improvements in imaging (e.g., magnetic resonance, positron emission tomography) are needed to more accurately predict pathological response. In this study, we have shown that patients with ER-positive disease or pathologically negative lymph nodes have reasonable outcomes with conventional treatment approaches. In contrast, patients with progressive disease, ER-negative disease, and/or a large number of pathologically involved lymph nodes develop high rates of both distant and local recurrences. Protocols investigating new therapeutic strategies should be directed toward these patients.

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APPENDIX 10

Coronary Artery Dosimetry in Intact Left Breast Irradiation

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PURPOSE

The purpose of this article is to report dose-volume histograms of coronary vessels from irradiation of the intact left breast.

PATIENTS AND METHODS

Fifteen women with cancer of the left breast underwent computed tomographic treatment planning for radiation treatments of an intact left breast. Images through the heart were reconstructed at 1-mm increments to permit contouring of the coronary vessels. Five treatment plans were created for each patient; one plan from the simulated treatment fields and four additional plans that were generated from virtual treatment fields created by shifting the isocenter 5 mm and 10 mm both superficially and deep. The radiation dose was calculated using a three-dimensional treatment planning system that incorporated heterogeneity correction factors.

RESULTS

With no adjustment to the perpendicular lung distance, a mean volume of 12% of the left anterior descending coronary artery received 20 Gy, 6% received 30 Gy, and 3% received 40 Gy. The dose to the left anterior descending coronary artery varied significantly with changes in the perpendicular lung distance. From the mean perpendicular lung distance of 1.87 for the simulated fields, a 5-mm increase in the perpendicular lung distance resulted in an increase of 20%, 15%, and 12% in the percentage of the left anterior descending coronary artery treated to 20 Gy, 30 Gy, and 40 Gy, respectively. With a 10-mm increase, the respective volumes were increased to 49%, 41%, and 34%, respectively. A 5-mm reduction of lung distance in the original plan resulted in a decrease of 10%, 5%, and 3% in the percentage treated to 20 Gy, 30 Gy, and 40 Gy, respectively. The dose to the left main coronary artery, the right main coronary artery, and the left circumflex coronary artery was limited to scatter and was less than 7 Gy. Changes in the perpendicular lung distance did not significantly affect the dose administered to these vessels.

DISCUSSION

The left anterior descending coronary artery is anatomically located at the edge of the cardiac silhouette on traditional treatment films. Small changes in the perpendicular lung distance can significantly change the dose delivered to this vessel. A fundamental change in the shape of the dose-volume histogram occurs at a perpendicular lung distance of 2.3 cm, whereas the dose is very low when the perpendicular lung distance is less than 1.3 cm. These points may serve as clinically important values in the treatment planning for cancer of an intact breast. *(Cancer J 2001;7:492–497)*

KEY WORDS

Coronary artery, breast cancer irradiation, dose-volume histogram

Acute and late cardiovascular injury after radiation therapy for cancer of the left breast remains a clinical concern for patients and clinicians. It is clear that radiation treatment of breast cancer can increase mortality rates from cardiovascular injuries if care is not taken to avoid radiation exposure to the cardiac structures. With the advent of modern treatment planning tools, left-breast cancers can be treated by use of more accurate dose localization. It is hoped that this will prevent the life-threatening injuries to the coronary vessels and the epicardial surface that have occurred after chest wall or mediastinal irradiation.[1,2]

Using computed tomography–based treatment planning tools, many authors have begun to report the dose-volume relationships of the whole heart when various radiation treatment techniques are employed. Not surprisingly, most techniques allow a very low volume of the entire heart to receive significant doses. However, the entire heart may not be the most appropriate target

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Received on June 28, 2001; accepted for publication July 26, 2001.

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this article. Supported in part by National Cancer Institute Department of Health and Human Services grant nos. CA16672 and T32CA77050. Dr. Buchholz is supported by Grant No. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

This work was presented at the American Radium Society Annual Meeting, April 2001.

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for the assessment of the risk of acute and late injury. An initial step in defining a relationship between radiation therapy and late complications is accurately measuring the dose to the individual coronary structures that may be responsible for causing late effects. The exact target or targets responsible for the cardiovascular injury from radiation are not known with certainty. Because myocardial infarction is the most common cardiovascular injury, our hypothesis is that injury to the major epicardial vessels is the cause of many later complications. It is possible for portions of the coronary vessels to receive high doses of radiation, even when only a small volume of the entire heart is irradiated. We undertook this study because there are no dosimetric studies describing the relationship of dose to the epicardial vessels for fields commonly used to treat left-breast cancers.

PATIENTS AND MATERIALS

Patient Selection

This study was approved by the institutional review board of The University of Texas M.D. Anderson Cancer Center, and all participants provided written informed consent. We studied 15 patients who were undergoing intact breast irradiation for cancer of the left breast. The study lasted 3 to 4 months, with approximately one patient enrolled per week. Breast size and location, size, and features of the tumor were not used for patient selection.

Computed Tomography Protocol

A computed tomography protocol was designed specifically for this study with the help of the Section of Thoracic Imaging in the Department of Diagnostic Radiology. Spiral computed tomographic scans of the treatment field using 3-mm-thick cuts were obtained using a flat table inset, a breast board, and a vacuum-sealed mold to duplicate the patient's treatment position. Scans were obtained with the patients breathing normally. Intravenous contrast material was not used for this study. The field of view for the scans was as small as possible for each patient in order to maximize the resolution. From the initial scan, images were reconstructed every 1 mm through the region of the heart to allow visualization of the coronary vessels. Approximately 150 images were obtained for each patient. These images were electronically transferred to the treatment planning computer for the dose calculations.

Treatment Field Arrangements and Prescription Point

The technique for breast irradiation used at our institution has recently been described in detail.[3] Using a fluoroscopic simulator, medial and lateral tangential treatment fields were designed to include the entire left breast while minimizing the volume of lung and heart in the fields. No cardiac blocks were used in this study, but fields were collimated to match the chest wall slope and to exclude most of the cardiac silhouette as seen under fluoroscopy. The isocenter of the field was placed in the breast at the interface of the chest wall and the breast tissue at a point that approximated the mid-separation distance. The dose was then normalized so that the 100% isodose line covered the breast tissue as seen on the treatment-planning computed tomographic images.

Contouring

The heart was contoured beginning at the origin of the superior coronary vessel to create a consistent, objective superior border that otherwise might vary by 5–10 mm. The ventricles were contoured together and as four separate units. The left anterior descending (LAD), left main, right main, and left circumflex coronary arteries were contoured as individual regions of interest. Each vessel was contoured for the entire length of the vessel that was seen on the scan. In almost all cases, vessels were visible on approximately 75% of the slices from the origin of the vessel to the final contour, with consistent imaging of the proximal vessel and approximately two-thirds visualization of the middle and the distal vessel.

Treatment Planning

For this study, a commercially available three-dimensional treatment planning system was used to calculate dose (ADAC Pinnacle[3]; ADAC Laboratories, Milpitas, CA). Heterogeneity corrections for differences in tissue density were used for the dosages reported in this study, as they are for routine treatment planning. The plans were optimized using wedges and differential field weighting to minimize the dose inhomogeneity throughout the three-dimensional treatment volume. The treatment fields designed for the patient treatments were used for the baseline treatment plans. From this baseline, four additional deep field edges were created for each patient with isocenter shifts of –5 mm, –10 mm, 5 mm, and 10 mm. The isocenters were shifted on a line perpendicular to the nondivergent posterior field edges of the original tangent field, thereby increasing or decreasing by 5 or 10 mm the amount of lung seen on a traditional simulation field. Wedges, weighting, and monitor units were not changed for the additional four plans, because none of the shifts resulted in significant changes in the shape of the isodose curves in the first three patients.

RESULTS

The coronary vessels were adequately visualized in all 15 patients, with the contouring of the LAD and left main coronary arteries judged to be accurate within 1–2 mm. Visualization of the right main and left circumflex arteries was significantly more difficult and was on occasion defined by the anatomic region of the heart in which the vessel is most commonly found. Consequently, we believe that the dose calculations for the LAD and left main arteries were very accurate, whereas the information for the right main and left circumflex arteries was much less reliable. However, in each case, the right main and left circumflex arteries were outside the tangential radiotherapy fields and therefore received only a scatter dose. Table 1 shows the length and volume of each contoured vessel and other regions of interest.

Figure 1 shows the mean composite dose-volume histogram (DVH) for the LAD coronary artery obtained from the plans from the simulated treatment fields and the previously described four additional plans. The mean perpendicular lung distance in the simulated treatment fields was 1.87 (range, 0.7–2.7 cm) (curve 3 of Fig. 1). In the plans from these fields, the mean volumes of the LAD artery that received 20 Gy, 30 Gy, and 40 Gy were 12%, 6%, and 3%, respectively. With a 5-mm increase in the perpendicular lung distance (curve 2; mean, 2.37 cm), the mean LAD artery volumes receiving 20 Gy, 30 Gy, and 40 Gy increased to 20%, 15%, and 12%, respectively. With a 10-mm increase, the respective volumes were 49%, 41%, and 34% (curve 1; mean, 2.87 cm). In contrast, after a 5-mm reduction in the perpendicular lung distance of the original plan, the percentage treated to 20, 30, and 40 Gy decreased to 10%, 5%, and 3%, respectively (curve 4; mean, 1.37 cm). With a 10-mm reduction in lung volume, the mean LAD artery volumes receiving these dosages approached 0% (curve 5; mean, 0.87 cm).

Figure 2 again presents the DVH data for the LAD artery, this time stratified by the perpendicular lung distance. Arbitrary cut-off points of 1.5 cm, 2.0 cm, 2.5 cm, and 3.0 of perpendicular lung distance were used to create the five curves. These curves represent the average values of 12, 12, 15, 14, 12, and 10 plans, respectively. Similar to Figure 1, Figure 2 illustrates that a significant change in the DVH occurs when the perpendicular lung distance exceeds 2.2 cm. Beyond this distance, a large volume of the vessel began to receive moderate-to-high doses of radiation. Figure 3 illustrates the most dramatic results in the current 15-patient series. This patient was treated for a lateral breast

TABLE 1 Length and Volume of Contoured Vessels and Other Areas of Interest

Structure	Mean Volume (Range) cm³	Mean Length (Range) cm
Heart	512 (355–672)	—
Ventricles	316 (243–426)	—
LAD	1.52 (0.72–2.81)	5.58 (4.2–6.9)
LM	1.36 (0.41–3.10)	3.96 (2.1–6.3)
LC	0.93 (0.31–1.55)	3.33 (1.4–4.8)
RM	2.46 (0.50–3.29)	1.04 (0.4–1.7)

Abbreviations: LAD, left anterior descending coronary artery; LM, left main coronary artery; LC, left circumflex coronary artery; RM, right main coronary artery.

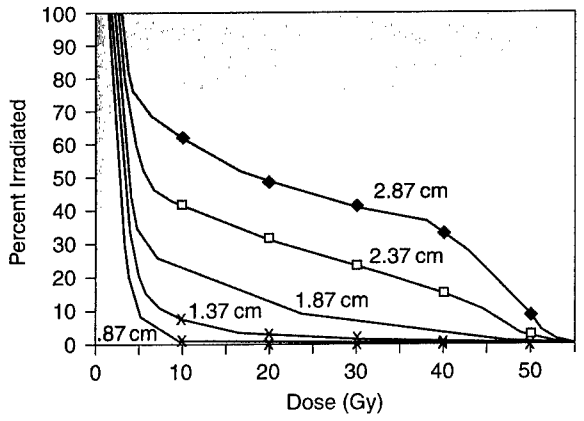

FIGURE 1 Dose to the left anterior descending (LAD) coronary artery stratified by offsets in the perpendicular lung distance. The mean dose-volume histograms for the LAD artery using plans with offsets of –10 mm, –5 mm, 0, +5 mm, and +10 mm from the mean perpendicular lung distance. The central curve (1.87 cm) represents an offset of 0.

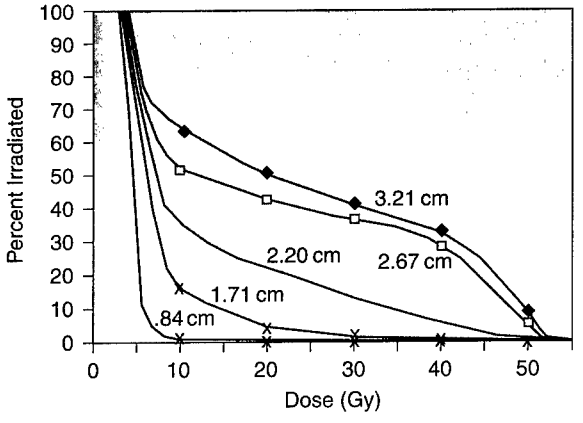

FIGURE 2 Dose to the left anterior descending (LAD) coronary artery stratified by the perpendicular lung distance. The mean dose-volume histograms for the LAD artery stratified by perpendicular lung distances of 0–1.5 cm, 1.5–2.0 cm, 2.0–2.5 cm, 2.5–3.0 cm, and > 3.0 cm. Values in the figure represent the mean perpendicular lung distance for that subset of plans.

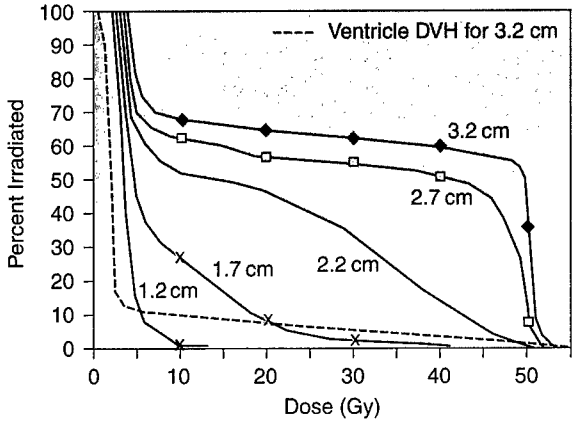

FIGURE 3 An example of a left anterior descending (LAD) coronary artery dose-volume histogram from a patient with a perpendicular lung distance of 2.2 that resulted in 32% and 14% of the LAD irradiated to 30 Gy and 40 Gy, respectively. In addition, the dose-volume histograms for offsets in perpendicular lung distance by –10 mm, –5 mm, +5 mm, and +10 mm are shown. Finally, the dose-volume histogram for ventricle for the perpendicular offset of +10 mm is shown for comparison.

tumor with a perpendicular lung distance of 2.2 cm, and as shown, a 1.0-cm shift in the perpendicular lung distance placed in LAD artery almost entirely within the prescription isodose region.

Figures 4 and 5 display the median and the upper and lower extreme DVH values for the heart and ventricles, respectively. The dose to the heart and the ventricles at different perpendicular lung distances varied to a much smaller degree than did the dose to the LAD artery. Composite DVHs for the right main and left circumflex arteries were also calculated, but doses were limited to scatter effects at all five perpendicular lung distances. These vessels received radiation doses ranging from 2 to 7 Gy.

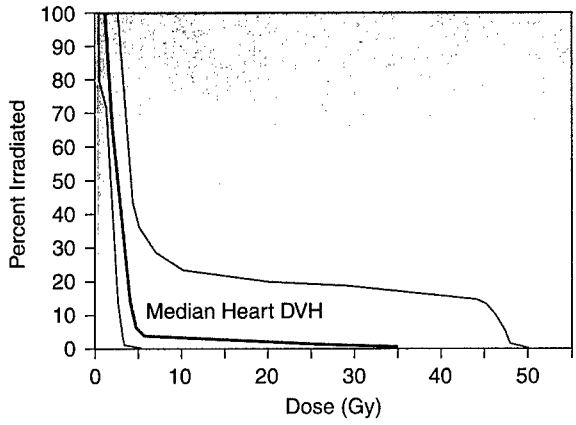

FIGURE 4 Median and range of the heart dose-volume histogram curves for the 15 patients in this study.

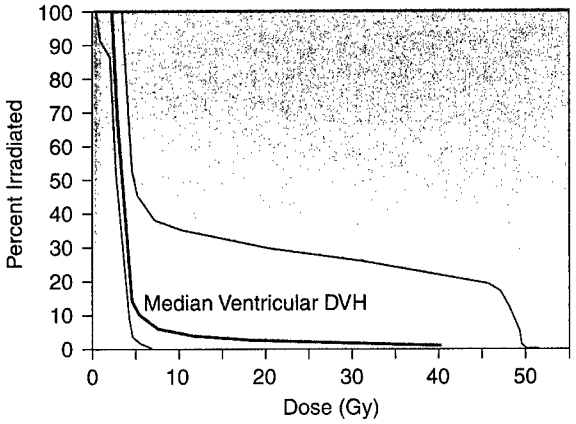

FIGURE 5 Median and range of the ventricles dose-volume histogram curves for the 15 patients in this study.

DISCUSSION

We undertook this study to accurately describe the dose delivered to the coronary vessels during a standard course of radiation therapy to the intact left breast. It is our belief that the doses delivered to these individual vessels may be better predictors of long-term cardiac morbidity from breast irradiation than the dose delivered to the entire cardiac volume. Although we studied a small number of patients, we believe that our results represent typical doses delivered to the individual coronary arteries. In this study, we demonstrated that the LAD artery lies at the edge of the cardiac silhouette as seen on a traditional simulation film, an example of which is shown in Figure 6.

As shown in Figures 1 and 2, the shape of the DVH for the LAD artery changes significantly when the perpendicular lung distance exceeds 2.2 cm. Beyond 2.2 cm, the volume of the artery that receives doses greater than 30 Gy or even 45 Gy increases rapidly. In the current trial, four patients were treated with perpendicular lung distances $\geq$ 2.2 cm, even though particular attention was paid to limiting the volume of lung in the treatment fields. As illustrated in Figures 1 to 5, the DVH for the heart or the ventricles would appear to be perfectly acceptable, whereas the DVH of the LAD artery may cause clinical concern. For such cases, further attempts to minimize the perpendicular lung distance by slightly adjusting the medial and lateral entry points appear warranted. Additionally, these patients may represent the subset of patients that ultimately benefit from the use of more conformal radiotherapy techniques. In contrast, for patients in whom the target volume is adequately covered by a field with a perpendicular lung distance of 1.5 or less, further adjustments are unlikely to affect the radiation dose to the heart, ventricles, or LAD artery.

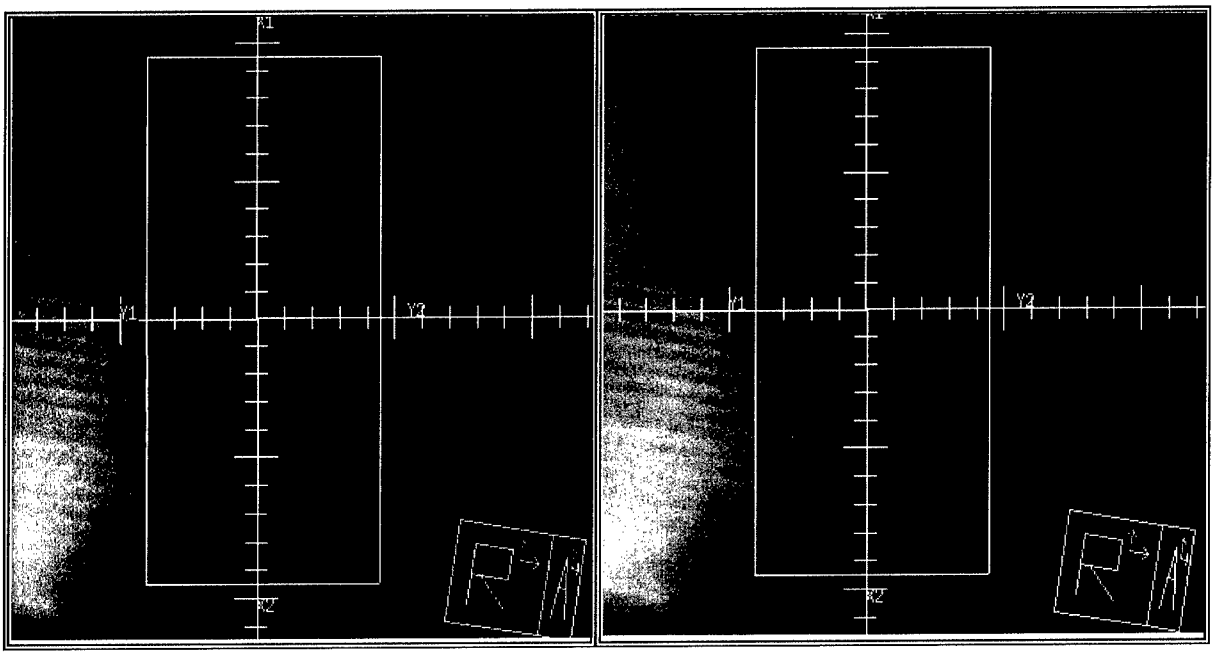

FIGURE 6 Beam's eye view of a medial left breast tangent with and without the contoured left anterior descending (LAD) coronary artery highlighted.

To date, studies evaluating the cardiac dosimetry of breast cancer radiation treatments have generally reported the dose to the entire heart. We believe that the individual coronary vessels may serve as much more accurate predictors of late coronary morbidity. After radiation therapy to the chest, patients are more likely to experience a myocardial infarction than a cardiomyopathy or a restrictive carditis.[2,4,5] We believe that this may be related to damage to small vessels in the epicardial region of the heart or to coronary vessels. Published series have documented significant coronary disease that corresponds to the region of high-dose radiation therapy after radiation therapy to the chest.[6] In addition, recent data derived from positron emission tomographic imaging of cardiac perfusion have shown that the blood flow to the myocardium may be compromised during a course of radiation treatments for left-breast cancer.[7]

To our knowledge, this is the only study documenting radiation therapy doses to individual coronary vessels in a series of breast cancer patients. Our DVH data for the heart is comparable to other data evaluating tangential irradiation for left intact breast cancer, which show relatively small volumes of the heart irradiated to high doses. In 1995, Mallik et al[8] reported very similar DVH information using computed tomographic planning for left-sided breast cancer. In that study, 11.9% of the total heart volume was irradiated, with a median perpendicular lung distance of 25.4 mm.

As newer techniques become available for the treatment of breast cancer, it is imperative that we have a solid understanding of the doses delivered to vital structures during tangential breast irradiation. We believe that this study identifies a subset of patients who are at high risk of having a significant portion of their LAD arteries treated to doses in excess of 40 Gy. One mechanism for avoiding this degree of irradiation to the LAD artery for patients in whom the targeted volume to be treated must include the LAD is gated therapy. A recent publication reported that deep inspiration favorably displaces the cardiac structures relative to the treatment fields used in breast cancer.[9] For patients in whom the LAD artery dosage is high, our data predict that this degree of displacement would lead to a significant improvement in the DVH of the LAD artery. Gated delivery of radiation during inspiration may therefore be a method of improving the DVH of the LAD artery without compromising the coverage of the target volume.

Although this protocol was designed to maximize the visualization of all of the coronary vessels, we found that the technique presented in the current article was adequate for visualizing only the LAD and left main coronary vessels. We attempted to use a contrast medium in an initial subset of patients but abandoned this protocol after noting that the contrast in the ventricles decreased the level of detail visible in the regions of the coronary vessels. On the basis of our experience, we believe that high-resolution computed tomographic scanning and three-dimen-

sional treatment planning should permit radiation oncologists to visualize and contour the LAD artery during treatment planning. This information appears to be particularly important when the perpendicular lung distance approaches 2.2 cm.

In this study, the treatment fields were arbitrarily moved by changing the perpendicular lung distance to determine the effects of these shifts. Certainly, other methods for adjusting treatment fields can also significantly affect the dose to the heart or the LAD artery. Das et al[10] recently published data showing that in a series of 52 patients, the beam angle affected the dose to the heart, whereas perpendicular lung distance was a much less important factor. We elected to look at shifts in the perpendicular lung distance rather than changes in gantry angle because we believed that this was a more accurate depiction of the way treatment fields were optimized at the treatment simulation by breast radiation oncologists.

In conclusion, small changes in the perpendicular lung distance can significantly change the dose delivered to the LAD artery. This vessel could play a major role in the development of long-term cardiac morbidity after intact breast irradiation. Fundamentally, the first step in understanding the development of long-term toxic effects is to accurately describe the dose to the structures involved. We hope that as more information becomes available, a better understanding can be gained of the risks that a particular patient may incur as a result of left-sided intact breast irradiation.

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Advances in Radiation Treatments of Breast Cancer

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Supported in part by grants CA16672 and T32CA77050 from the National Cancer Institute. Dr. Buchholz is supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154.

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Running head: Radiation Advances in Breast Cancer

Abstract

Significant advances have occurred in the radiation treatment of breast cancer. Over the past decade, improvements in treatment planning tools, computer and imaging technologies and new therapeutic modalities allow radiation to be delivered in a conformal fashion while minimizing treatment toxicity. It is critical that physicians involved in breast cancer care recognize the numerous advances that have occurred in the delivery of radiation therapy. Three specific changes in the treatment planning and delivery have revolutionized the way we approach breast cancer treatment: the design of radiation fields using computed tomography (CT) data sets, the development of three-dimensional dose calculation algorithms, and the development of new methods to modulate the delivery of radiation dose. With the advent of CT simulators, individual patient anatomy and pathology can be readily visualized and reconstructed in an axial, coronal, and sagittal view. With an improved anatomic delineation between the target volumes and critical organ structures, the treatment fields are better able to be designed that are more congruous to the areas at highest risk. Within the last few years, new three-dimensional dose calculation algorithms have been generated that more accurately calculate dose distributions throughout the treatment planning volume. Finally, modern linear accelerators allow for modulation of the dose intensity of the radiation beam, which leads to improved aesthetics and decreased side effects while ensuring that the volumes at high risk receive the prescribed dose. Radiation therapy may be delivered safely and effectively to breast cancer patients.

Introduction

Radiation therapy is a critically important component of treatment for the majority of patients diagnosed with breast cancer. For patients with ductal carcinoma *in-situ* (DCIS) and patients with early stage breast cancer treated with breast-preserving surgery, phase III randomized trials have conclusively demonstrated that radiation use reduces the probability of breast cancer recurrence. Radiation use after mastectomy is also considered to be the standard of care for patients with advanced disease and patients with stage II breast cancer with 4 or more positive lymph nodes. Despite clinical trials and studies clearly showing the benefits of radiation therapy, its use remains underutilized both in the United States and Europe. This underutilization of radiation therapy may in part reflect a decision by the referring physician and/or the patient to forgo appropriate care due to concern over the toxicity of radiation treatment. Therefore, it is critical that physicians involved in breast cancer care recognize the numerous advances that have occurred in the delivery of radiation therapy. Particularly over the past decade, improvements in treatment planning tools, computer technologies, imaging technologies, and new therapeutic modalities allow radiation to be delivered in a much more conformal fashion. It is predicted that these technological improvements will further minimize the risk of long-term treatment-associated morbidity. These changes have been revolutionary and have made the past decade one of the most intellectually and clinically exciting in the century-long history of radiation oncology. Unfortunately, radiation oncology is a relatively small field within the medical profession and these advances have been under-appreciated by most physicians and breast cancer patients.

For radiation to kill breast tumor cells and avoid normal tissue injury there must be a therapeutic ratio, which allows for a selective killing of residual disease while preserving normal tissue structure. For radiation treatments, both biology and physics determine this therapeutic ratio. For example, normal breast tissue and tumors have different abilities to repair sub-lethal radiation damage. With fractionated therapy, this repair difference can be exploited and lead to accumulation of damage selectively within tumors to the threshold of lethality. The biological considerations of radiation treatment delivery have not significantly changed over the past decade. A number of the very early historical breast clinical trials used fractionation schemes for treatment delivery that are no longer considered standard, while within the United States, the delivery of 5 – 6 weeks of a daily dose of 1.8- 2.0 Gy has been standard for some time. Correspondingly, most of the exciting recent advances in radiation treatments for breast cancer have been physics-based. Whereas, the biology of fractionated therapy attempts to spare normal tissue within the treatment field, a primary goal of radiation physics is to determine the optimal delivery of radiation dose. Meticulous treatment planning is required to deliver the appropriate radiation dose selectively to the areas recognized to be at risk for cancer recurrence. The planning of radiation therapy requires an in-depth knowledge of clinical and radiographic anatomy, and must be individualized by taking into account patient anatomy, volumes of tissue at risk, and critical organ structures.

In this brief review, we will review important recent advances in radiation treatments for breast cancer. Three specific changes in the treatment planning and delivery have revolutionized the way we approach breast cancer treatment: the design of radiation fields using computed tomography (CT) data sets, the development of three-

dimensional dose calculation algorithms, and the development of new methods to modulate the delivery of radiation dose. The purpose of this article is to characterize these advances and to provide an overview of the consequences of these changes.

Designing Radiation Fields Using Computed Tomography Data Sets

One of the most critical recent advances in the field of Radiation Oncology has been in how treatment fields are designed. The goal of all radiation treatments is to include the region at risk of recurrence completely within the irradiated volume while minimizing the volume of normal tissue that receives coincidental treatment. Historically, the delineation of radiation fields for breast cancer was done based on an empiric understanding of anatomy and visualization of anatomical structures using fluoroscopy. Currently, at our institution, the construction of radiation fields is performed using a three-dimensional rendering of the virtual anatomy obtained with treatment planning computed tomography (CT). Many Radiation Oncology departments currently have dedicated CT scanners that are specifically designed for the purpose of radiation treatment planning. These CT scanners differ from diagnostic units in that they are equipped with lasers to provide orientation to three-dimensional points in space, which are used to verify the day to day patient alignment. They precisely correspond with similar laser localized points associated with the linear accelerators that are used to deliver the radiation treatments. In addition, large bore treatment planning CT scanners are available to allow for accommodation of various bodies sizes, treatment positions and

immobilization devices. These devices are important to assure that the patient is scanned in the exact position in which they will receive their radiation therapy.

Each treatment field is individualized according to the patient's anatomy, sites felt to be at risk of recurrence, and areas felt necessary to avoid complications. The use of CT in designing these fields is an exciting development for a number of reasons. First, the goal of radiation therapy in most breast cancer patients is to kill the residual microscopic tumor cells present after surgery. Based on data documenting the recurrence patterns in early and advanced stage breast cancer, radiation therapy is used most often in the adjuvant setting to facilitate local control of disease in the breast, chest wall, and selective regional lymphatics. The breast and chest wall can be readily visualized with either fluoroscopy or CT simulation. However, historical simulations using fluoroscopy often had a more difficult time precisely localizing radiation fields to regional lymphatics at risk. Specifically, attempts to include the axilla and/or internal mammary lymph nodes were historically done based on an empiric understanding of lymphatic anatomy referenced to bony landmarks. Two-dimensional fluoroscopic simulation did not allow direct visualization of lymph nodes in the axilla, infraclavicular and supraclavicular fossa, or internal mammary chain. Conversely, with three-dimensional CT simulation the individual patient anatomy including the lymphatics, muscles, vasculature and nerves can be readily visualized and reconstructed in an axial, coronal, and sagittal view. Multiple iterations of treatment fields can be virtually generated on CT data sets in order to optimize the radiation fields.

To assist in the demarcation of targeted regions such as lymph node basins, the anatomical areas at risk can be digitized on sequential axial CT slices. Subsequently, a

three-dimensional volume of each structure can be generated and the relationship of this volume to the radiation field easily visualized. Therefore, the probability of an area at risk falling outside of the treatment field should be dramatically reduced with three-dimensional CT simulations. An example of the type of reconstructed anatomical rendering is shown in Figure 1. In this example, the extent of an axillary dissection is easily visualized and included in a radiation field. Additionally, a digitized reconstruction of the upper internal mammary lymph nodes was obtained by outlining the anatomical region on sequential axial CT slices. These images significantly aid in the design of an optimal treatment plan. Ultimately, by minimizing the risk of a marginal miss, CT treatment planning may both improve the efficacy of treatment and decrease dose to normal tissues.

In addition to providing important information regarding the anatomic location of high-risk regions, another important result of CT simulation planning is the reduction in the dose to normal tissues. In the past, it has been clearly demonstrated that improper design of radiation fields used for breast cancer treatment can increase the risk of cardiovascular deaths.[1] Presumably, these deaths were secondary to treatment effects on the heart and coronary vasculature. When treating the left breast or left chest wall, care must be taken to avoid irradiation of the heart and coronary vessels. The apex of the heart is often difficult to visualize and precisely localize on tangential fluoroscopic images. Unlike fluoroscopic imaging, CT imaging provides better visualization of these critical organ structures and consequently can help to design fields that minimize dose to these critical organ structures. For example, the left anterior descending artery (LAD) typically lies in close proximity to the border of the deep border of tangential fields used

to treat the breast or chest wall. CT planning may permit the visualization of the LAD, and may provide the Radiation Oncologist the opportunity for modification of the design of the treatment fields to better optimize for the individual patient (Figure 2). Using a fluoroscopic simulator, such precision could not be accomplished and there would be a risk for both missing part of the targeted volume and including more normal tissue than is actually necessary.

Three-Dimensional Treatment Planning

Once the geometric design of the treatment fields are optimal to assure inclusion of important therapeutic targets with the minimization of normal tissues with the volume to be treated, the process of dosimetric treatment planning occurs. The dose varies according to physical properties of the radiation beam and tissue being treated. For example, in general, the dose of radiation decreases as the beam travels deeper into tissue. To accommodate for this, often two opposed fields are used and the dose-fall-off from each beam is attempted to be matched in order to provide a relatively uniform dose throughout the treatment volume. The technique of opposing fields is the standard for treatment of the breast, which is typically treated in a matched pair of medial and lateral tangent beams. However, the separation distance at the apex of the breast along the plane of the beam is much less than that at the base, so the corresponding dose in the apex is higher than that at the base (Figure 3). To correct for these differences in dose across the volume dosimetry planning is performed. Dosimetry planning optimizes dose

uniformity, ensures that the target volume receives the prescribed dose, and verifies that the critical organ structures are spared unnecessary amounts of radiation.

Historically, treatment planning consisted of optimizing the dose distribution in a single two-dimensional axial plane in the center of the field. An external contour of the breast or chest wall in the center of the field was obtained during the simulation by using a plaster cast or a wire and transposed. This external cast was transferred onto treatment planning graph paper. Dosimetrists then calculated the resulting dose-distributions on this single axial slice. The dose distribution could then be optimized through preferential weighting of the two fields and through introduction of beam modification devices called wedges, which could decrease the dose at the apex relative to the base. Within the last few years, new three-dimensional dose calculation algorithms have been generated that more accurately calculate dose throughout the entire 3-D volume included in the field. In addition to permitting dose visualization in areas outside of the central plane, these new treatment planning systems more accurately calculate dose because they account for differences in the density of the tissue within the fields (determined according to the CT Hounsfield unit). This improvement is clinically important in many ways. For example, we have demonstrated that two-dimensional plans can fail to recognize a significant underdosage in the axillary lymph nodes (e.g. 77% of nominal dose). With 3-D treatment planning, the dose in this region, which is superior to the central plane, is now calculated and can be corrected to assure adequate coverage.[3] Furthermore, other areas in the upper or lower portion of the field, which would not be recognized and corrected with traditional 2-D planning would commonly receive in excess of 110% of prescribe dose.

Modifying Radiation Dose

One of the more recent and exciting advances in the radiation treatment of breast cancer lies in the ability to selectively modify the intensity of the radiation dose in the treatment volume. With the advent of the dynamic multileaf collimator, which sits inside the treatment head of modern linear accelerators, static intensity-modulated radiation beams can reduce the hot spots in the treatment volume and optimize the homogeneity of dose delivered in the treatment volume. This dose modulation is achieved by selectively designing additional fields which block out the hot spots of radiation dose in the treatment volume while bringing up the dose in the cool regions, a technique commonly known as field-in-field (FIF). A dosimetrist prior to the start of treatment performs forward planning intensity-modulation. Often times, 6 to 8 fields are used to create a more uniform dose distribution, as opposed to the 2 field arrangements that were conventionally used. The multileaf collimators allow computer-controlled segments to be move into or out of the treatment field. The resulting planned is then reviewed and either approved or modified by the Radiation Oncologist.

Modification of the dose within the treatment volume to achieve a homogeneous dose distribution traditionally used external fixed wedges. These fixed wedges provided the physician with a limited number of solutions for excessive "hot spots" (regions where dose is excessively higher than prescribed dose) and "cold spots" (excessively lower than prescribed dose) that arose within the treatment volume. One clinical consequence of "hot spots" within the normal tissue and treatment volume is damage to breast tissue that can contribute to a poor aesthetic outcome. The consequences of "cold spots" within the

target volume include an increased risk of local recurrence. With new generation treatment planning software and multi-leaf collimators (MLC), an infinite number of solutions may be created to optimize dose distribution within the treatment volume. Therefore, no matter how different one patient's anatomy is from another, "hot spots" and "cold spots" can be minimized, thereby proving each individual patient an optimal solution for the specifics of their disease and anatomy.

At our institution, we use the FIF technique for dose modulation. The FIF technique is a form of intensity modulated radiation therapy (IMRT), but varies from the classic "IMRT" in that fields are created in a step-wise fashion to generate the optimal solution. In the classic "IMRT" treatment the physician defines planning goals and restrictions and the treatment planning software defines the optimal solution.[4] The inverse planning usually results in the use of many beams and often delivers a low dose to a larger volume of normal tissues. Because of the potential carcinogenic risk of low dose delivery to the lungs and contralateral breast we have chosen not to use the inverse planning method.

After CT simulation of the patient, the Radiation Oncologist defines target volumes and designs treatment fields as discussed above, and then selects the dose and fractionation schedule that best achieves the therapeutic ratio. A medical dosimetrist then enters the data sets into a treatment planning software system and generates an initial optimized open-beam treatment solution. The dose distribution in the target volume is defined with isodose curves, which display the volume of breast tissue treated to various doses (Figure 4). Isodose line increments of 5% above the prescribed dose are sequentially evaluated and eliminated. For example, after an initial optimized open beam

treatment solution is generated, there is often a 110% isodose "hot spot" in the apex of the breast. An isodose "cloud", which represents in three dimensions the portion of the treatment volume that has 10% higher dose than was prescribed by the physician, is then created. The medical dosimetrist creates a new field with the original treatment field which specifically blocks out the "dose cloud" (Figures 5a-c). The dose and weighting of each treatment field is then modified so that the 110% "dose cloud" disappears. In practical terms, adjusting the MLC in the linear accelerator while treating the patient will generate this new field. Sequential "dose clouds" are then generated and additional field reductions are performed. The dose is modified as stated above and this process undergoes multiple iterations until the ideal treatment planning solution is achieved. The treatment planning system not only increases dose uniformity by permitting selective reduction in high dose areas, but facilitates increased planning efficiency and faster treatment delivery time.

Summary/Conclusion

In conclusion, exciting new recent advances in the radiation treatment planning of breast cancer has occurred over the last decade. With the advent of CT simulators, there is an improved anatomic delineation between the target volumes and critical organ structures and treatment fields can be designed that are more congruous to the areas at highest risk. Through the development of the 3-D treatment planning computer systems and improved dosimetric calculation algorithms, we are better able to calculate and visualize dose distributions throughout the treatment planning volume. Finally, and

perhaps most critically, modern accelerators allow for modulation of the dose intensity of the radiation beam, which leads to improved cosmesis and decreased side effects without compromising the prescribed dose to the target volume.

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Legends

Figure 1: CT treatment planning permits the visualization of the high-risk areas of recurrence and regional lymphatics, which are identified in this supraclavicular treatment field. The visualization of the axillary lymph nodes and the internal mammary chain nodes permit optimal radiation treatment planning and delivery.

Figure 2: The left anterior descending artery (LAD) is contoured in a breast-conservation therapy patient, and its proximity to the radiation treatment field is better visualized than with traditional flouroscopic treatment planning. (Reproduced with permission from *The Cancer Journal*)[2]

Figure 3: The standard medial and lateral tangents for a breast conservation patient illustrates the dosimetric challenge that include increasing hot-spots at the apex of the breast, i.e. increased hot spots at the apex of the breast. The shorter separation distance at the apex along the plane of the beam may increase normal breast tissue toxicity because of the higher dose of delivered radiation.

Figure 4: This sagittal view of a breast conservation patient illustrates the isodose distribution (as % of prescribed dose) that is generated by medial and lateral tangential radiation treatment fields.

Figures 5a-c: (a) A beams eye view of a 110% hot spot "dose cloud" in the apex of the right breast. (b) Static intensity-modulated radiation beams i.e. "Field-in-Field" (FIF) can reduce the hot spots in the treatment volume and optimize the homogeneity of dose delivered in the treatment volume. (c) FIF selectively blocks out the hot spots of radiation dose in the treatment volume while maintaining the prescribed dose to the target volume.

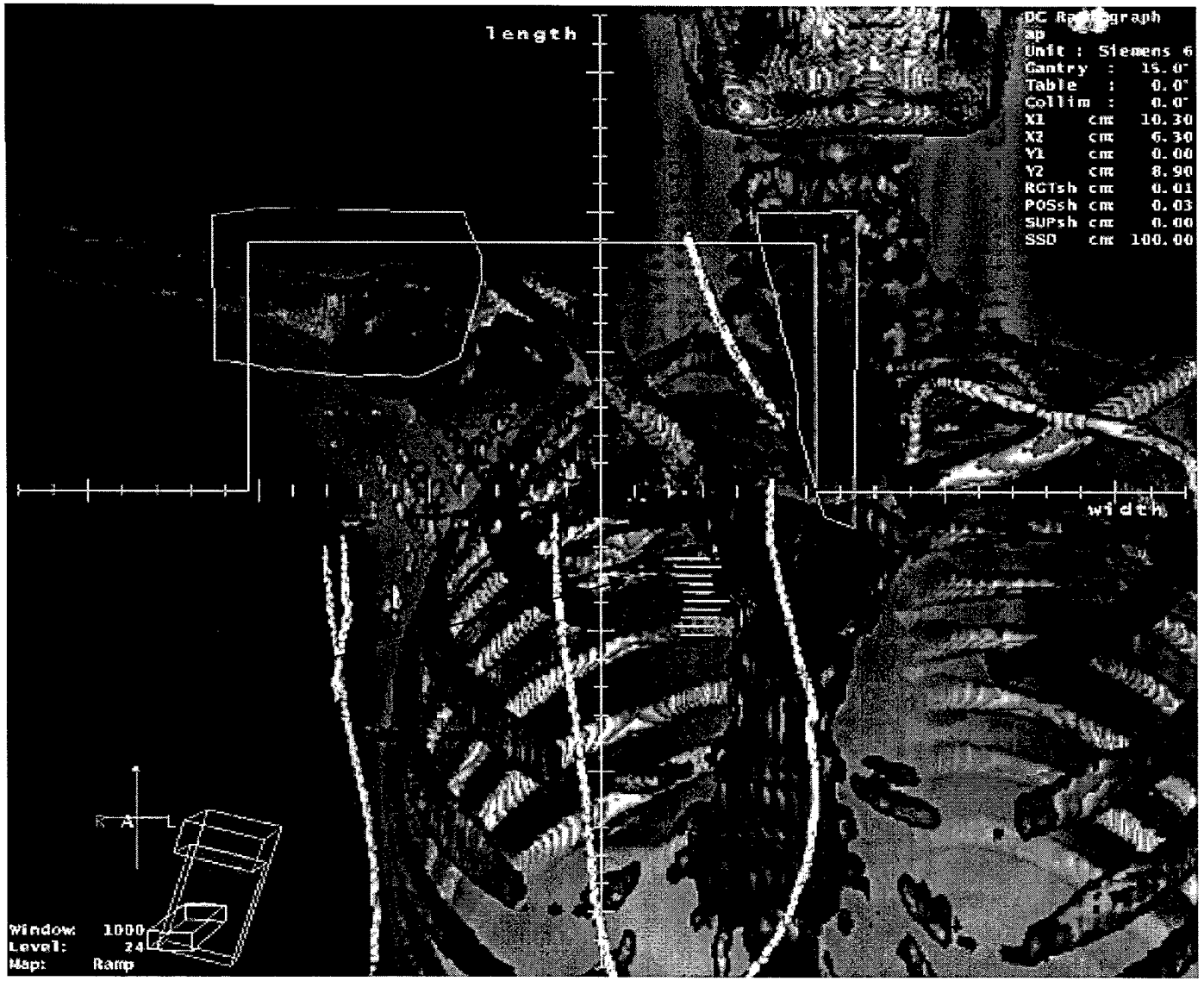
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Figure 1

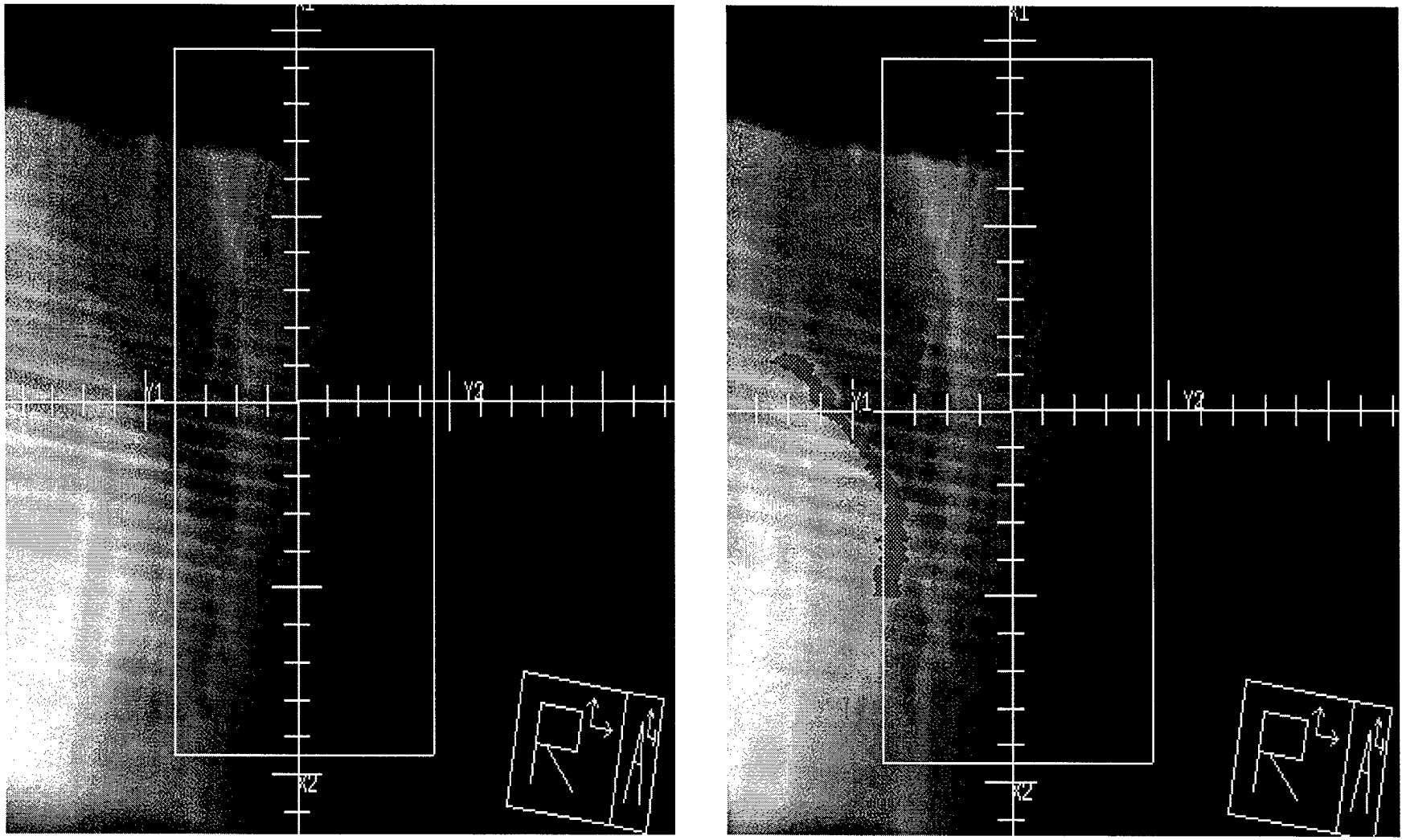


Figure 2

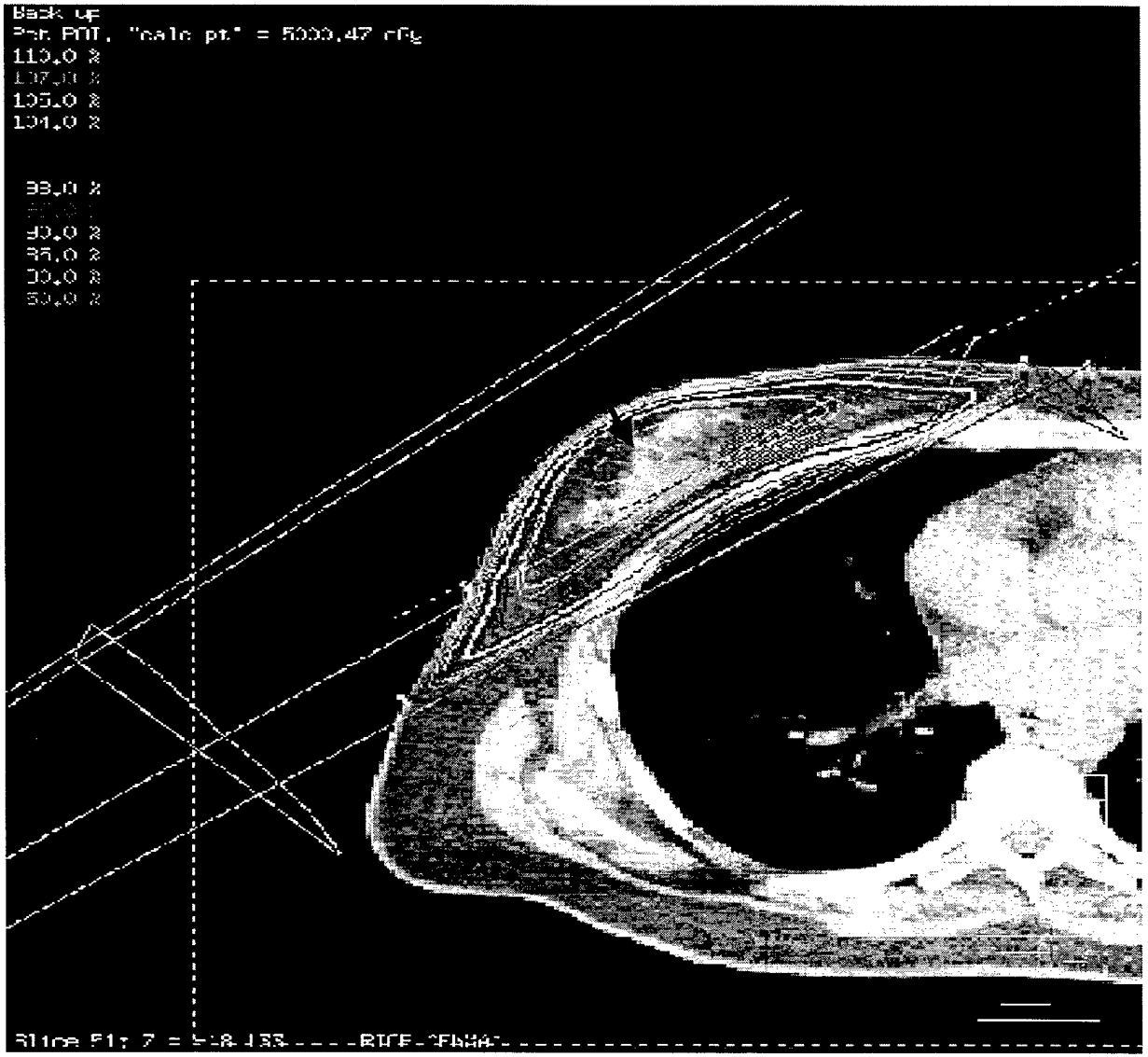
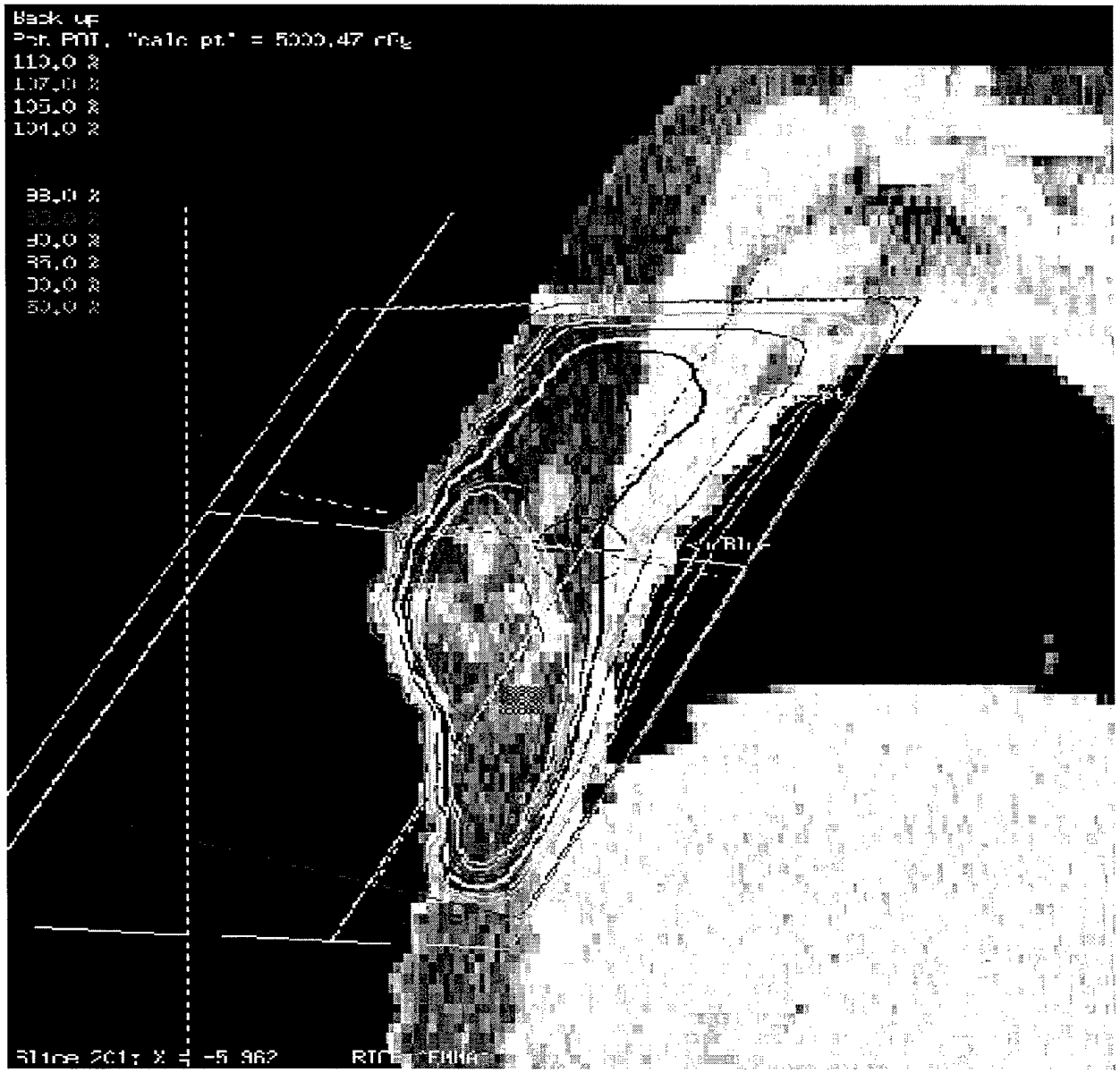


Figure 3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RTD: FMMA

Figure 4

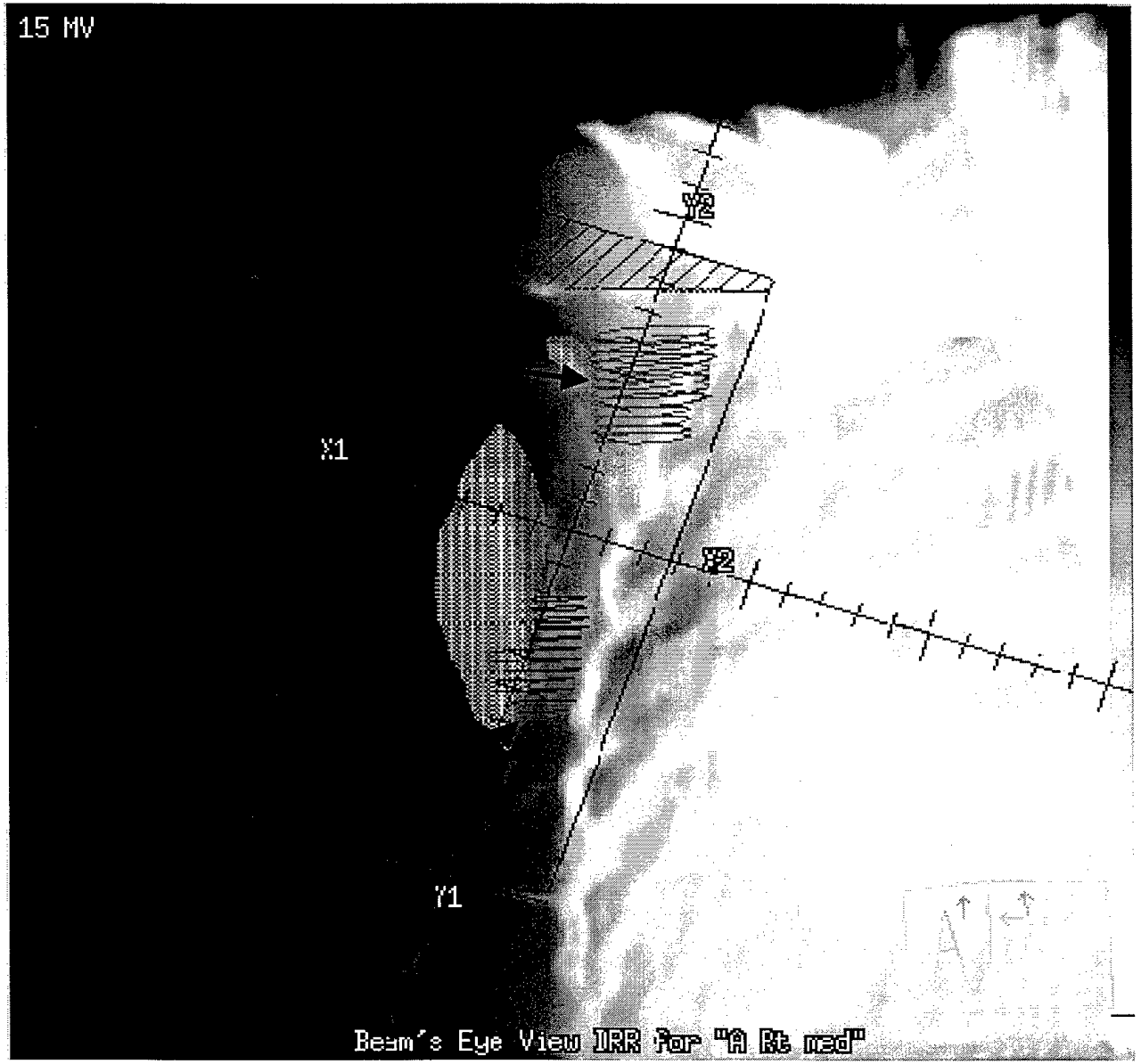

Figure 5a

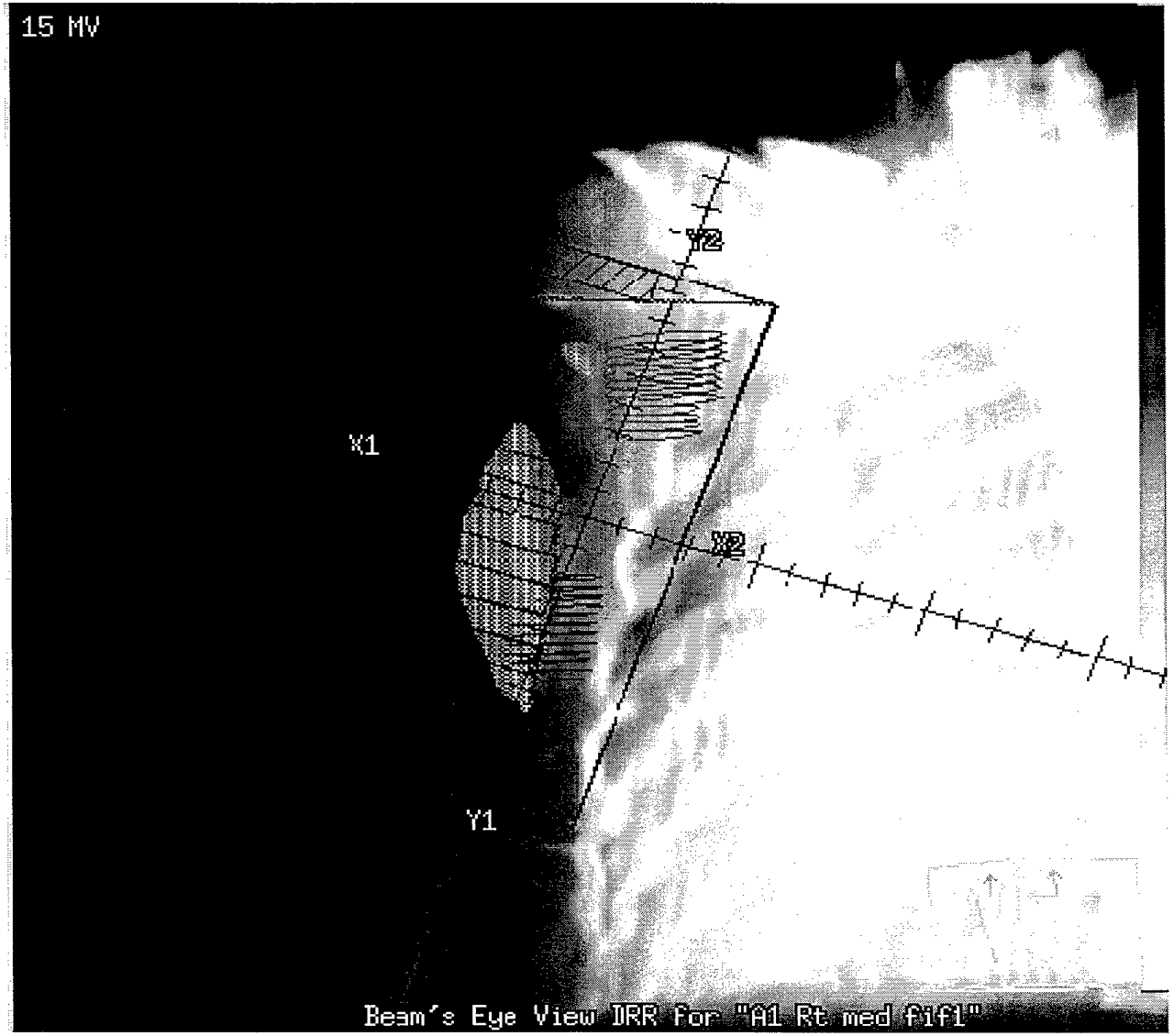

Figure 5b

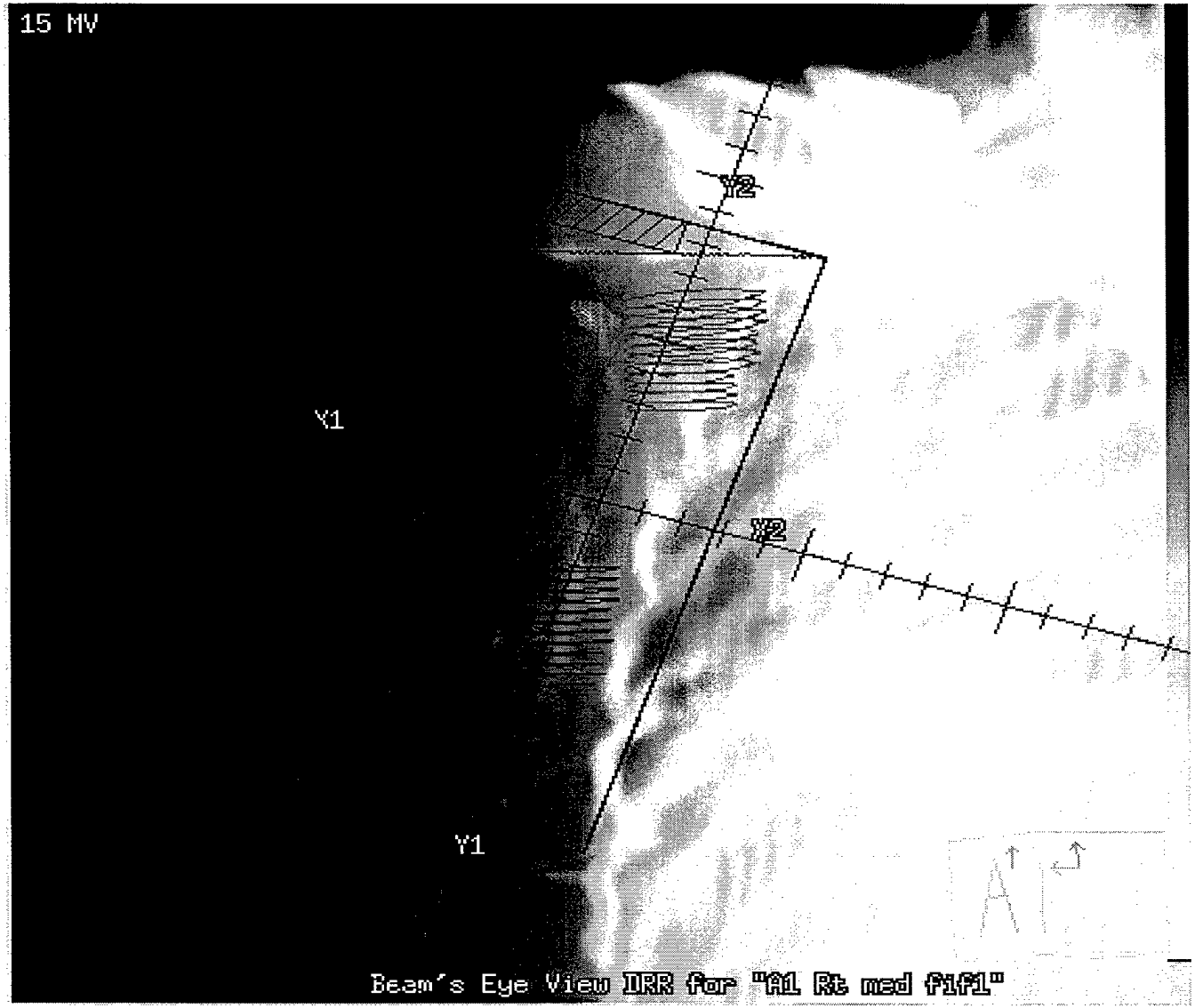

Figure 5c

Global Gene Expression Changes During Neoadjuvant Chemotherapy for Human Breast Cancer

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PURPOSE

The purpose of this study was to analyze global gene expression changes in serial tumor core biopsy specimens taken during neoadjuvant chemotherapy for primary breast cancer.

PATIENTS AND METHODS

Core biopsy specimens from tumors were obtained before treatment and 24 and/or 48 hours after treatment from 21 women who were beginning chemotherapy for breast cancer. RNA was extracted, and radiolabeled complementary DNA was synthesized. The complementary DNA probes were hybridized to high-density microarray membranes that contained more than 25,000 human sequence clones. Hierarchical cluster analysis was used to compare the degree of similarity between expression profiles.

RESULTS

Twenty-five (45%) of the 56 available core specimens yielded sufficient quantity and quality RNA for microarray analysis. Microarray profiles were performed only on samples from patients with pretreatment and posttreatment specimens, resulting in serial data sets for five patients (14 specimens). The serial samples from individual patients clustered more closely than the samples taken from different patients. Analyses of the variance of individual gene expression showed that there were significantly fewer genes with fivefold differences in expression in an individual tumor at different times (average, 359 genes) versus pretreatment samples of different tumors (average, 732 genes). Patients with a good pathological response to treatment had gene patterns that clustered distinctly from those of poor responders. Significant transcriptional response occurred in all patients during therapy. Surprisingly, all patients had different genes change after chemotherapy, with no single gene having a significant expression change in all five patients.

DISCUSSION

This is the first report to show global gene expression changes during chemotherapy in a human solid tumor. Comprehensive gene expression profiles of more than 25,000 genes can be obtained from core biopsy specimens. A remarkable diversity in transcriptional response was observed for individual cases. Further data are needed to determine whether gene profiling can predict response to chemotherapy. *(Cancer J 2002;8:461–468)*

KEY WORDS

DNA microarray, breast cancer, gene expression, chemotherapy response

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Received on June 28, 2002; accepted for publication August 1, 2002.

The author or one or more of the authors have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article. In addition, benefits have been or will be directed to a research fund, foundation, educational institution, or other nonprofit organization with which one or more of the authors is associated. This work was supported in part by National Cancer Institute Department of Health and Human Services grant nos. CA16672 and T32CA77050. Dr. Buchholz is supported by grant no. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award. Dr Pusztai is supported by a research grant from Millennium Pharmaceuticals.

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Historically, human studies aimed at investigating the molecular predictors of breast cancer response to therapy have correlated treatment outcome with presence or absence of expression of a particular gene product. This line of investigation has yielded clinically important findings, such as the relationship between estrogen and progesterone receptors and response to hormone therapy and the relationship between protein overexpression of the HER2-neu oncogene to response to trastuzumab therapy.[1,2] However, there currently is no clinically useful molecular marker that predicts response to chemotherapy. Considering the complex molecular biology of apoptosis and cellular response to a toxic insult, as well

as the genetic heterogeneity of breast cancer, it is unlikely that a single gene product will prove to be a dominant determinant of treatment outcome.

Microarray technology enables the simultaneous assessment of several thousand gene products. Although this technology is still in development, many believe that it will soon revolutionize the practice of medicine. One area of cancer medicine in which microarray analysis of gene expression may be valuable is the identification of molecular predictors of chemotherapy response. It is hoped that the study of the simultaneous expression pattern of thousands of genes will be more predictive of the response of cancer to a particular chemotherapy regimen than the presence or absence of a single gene product. One approach for microarray studies is to correlate gene expression profiles of samples taken at the time of diagnosis with treatment response. A second approach is to study transcriptional response to chemotherapy by assessing expression changes over time. Expression changes that occur during treatment are in part due to the cellular response to injury, including molecular pathways that initiate or block apoptosis. Studying gene expression profiles changes over time may better predict treatment outcome than only evaluating expression profiles of baseline specimens. In addition, this strategy may provide new targets to modulate the cytotoxicity of chemotherapy drugs.

To investigate this, we began this pilot trial to assess global gene expression change in human breast cancers during chemotherapy. Our objectives were to test the feasibility of obtaining comprehensive gene expression profiles from serial core biopsies of the primary tumor during chemotherapy and to assess the magnitude and the pattern of transcriptional changes resulting from therapy at 24 and 48 hours after treatment.

PATIENTS AND METHODS

Patients

This study was prospectively performed at The University of Texas M.D. Anderson Cancer Center. All study subjects voluntarily participated in a protocol that was approved by the human subjects institutional review board and provided written informed consent. Study eligibility rules required that participants have an untreated primary breast cancer that was amenable to serial core biopsies and that was going to be treated with chemotherapy as the first therapeutic intervention. This was a pilot study to assess feasibility and accrual, so eligibility was not restricted to patients who were given any particular chemotherapy regimen. Serial core biopsies of the primary tumor were performed before treatment and 24 and 48 hours after the initiation of the first course of their neoadjuvant chemotherapy. The biopsies were performed under local anesthesia using a spring-loaded 18-gauge needle (Bard Co, Inc., Covington, GA). The repeat biopsies were all performed in the same area of the tumor using the same skin entry site as was used in the initial pretreatment biopsy. The study was closed after it met the predetermined accrual goal of 30 participants.

Tissue Handling, RNA Extraction, and Transcriptional Gene Expression Profiling

All core biopsy specimens were snap-frozen in liquid nitrogen within a minute of the biopsy procedure and stored in liquid nitrogen until further processing. Tissues were used to quantify apoptosis and apoptosis-related markers by immunohistochemistry and also for RNA extraction by RNAeasy kit (Qiagen, Valencia, CA). The amount and the quality of RNA were evaluated with Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA). This system allows a concentration estimate on as little as 5 ng of total RNA and also assesses the quality of RNA. First-strand complementary DNA (cDNA) synthesis was performed with Superscript II in the presence of [^{33}P] dCTP (100 mCi/mL, Amersham, Piscataway, NJ) from 1–2 μg of total RNA. The generated cDNA probes were hybridized without further amplification to a panel of five high-density cDNA microarray membranes. The membranes in this panel were developed by, and are proprietary to, Millennium Pharmaceuticals, Inc. (Cambridge, MA). The panel contained 25,855 human sequence clones. Duplicate, parallel, independent transcriptional profiling experiments were performed for all specimens to learn about the reproducibility of the results. For data acquisition, the filters were placed on a Fuji phosphorimager screen for 48 hours and then analyzed on a BAS 2500 phosphorimager (Fuji Medical Systems, Stanford, CT). The radioactivity of the cDNA probes bound to target cDNA were quantified using Array Vision software (Imaging Research, St. Catherine, ON, Canada).

Statistical Methods

For each sample, the expression values were normalized to the mean expression value within each of the five membranes. After normalization, expression levels were log-transformed. An initial assessment of reproducibility was performed with hierarchical clustering using

$$(1 - \rho_{A,B})$$

as a correlation based distance between A and B. This was performed for each of the five arrays in the set (not shown). Sample TX.0.A branched extremely far away from the other 27 samples on array #2, and so the measurements for TX.0.A, array #2 were discarded.

For each gene, the replicate experiments were used

to estimate the measurement error by use of the median absolute deviation as a robust estimate of scale. Replicate pairs whose error exceeded expected quantiles $p/2$ and $1-p/2$ were discarded, where p is the Bernoulli multiple testing corrected value corresponding to 0.05. Remaining replicate pairs were then averaged to produce up to 14 expression profiles for each gene (corresponding to each of the samples) in the subsequent analysis. For array 2, TX.0, the values from sample TX.0.B only were used.

The replicate pairs allowed estimation of error; if the observed expression level is given by

$$X = \mu_{g,j} + \varepsilon_g$$

where $\mu_{g,j}$ is the mean for gene g and sample j (and hence may vary from sample to sample) and ε_g is the error for replicates $A_{g,j}$ and $B_{g,j}$,

$$D_{g,j} = A_{g,j} - B_{g,j}.$$

Assuming that the error ε_g is approximately normally distributed, $D_{g,j}$ is also normal, with mean 0. Because for each gene there were 14 replicate pairs, we then estimated the variance of $D_{g,j}$ and used this estimate to discard replicate pairs for which $D_{g,j}$ was too far into the tail; i.e., we simply performed a t-test on the null hypothesis that the mean value of the difference was 0. For a given P value (here, 0.05), we used the Bernoulli multiple testing adjustment $1 - (1 - p)^{n-1}$ to set the confidence threshold.

Measurement error increases with signal level in microarray gene expression experiments; therefore, picking a fixed fold-difference threshold level to detect significant changes increases the false-positive rate for genes expressed at low levels and decreases the power for genes expressed at a higher level. We adjusted for the variation in measurement error by use of the following: location and scale for each gene were estimated using the median (M) and median absolute deviation (MAD). A LOWESS regression model was used to fit MAD as a function of M. The fitted values of the MAD were used for calculating the T statistic of the differences in expression levels between, e.g., 0 and 24 hours.

RESULTS

Patient Characteristics

The principal investigator discussed the protocol with approximately 60 eligible patients, from whom 30 volunteered to participate. During the study, five (17%) patients elected not to undergo any further biopsy after the first or had false-negative biopsy results that yielded no tumor. Sixteen of the remaining 25 patients underwent biopsies at all three time points, and nine patients underwent biopsies at two time points. Of these 25 patients, 76% had clinical stage III disease, and 72% had either a T3 or a T4 primary tumor. The initial chemotherapy for the 25 patients was AT (doxorubicin/docetaxel) for 16 patients, TX (single-agent paclitaxel) for eight, and FAC (5-fluorouracil, doxorubicin, cyclophosphamide) for one.

RNA Yield and Reproducibility of Profiles

Six of the 25 patients did not have tissue frozen and processed for RNA extraction. RNA was extracted from a total of 56 specimens obtained from the remaining 19 patients. Of the 56 specimens, 26 (46%) yielded some RNA. Profiling studies were performed only on samples from patients with pretreatment and posttreatment specimens that yielded RNA of sufficient quality and quantity. Given this restriction, we profiled 14 specimens from five patients. Four of these patients underwent a complete series of biopsies, and one underwent a baseline and a 24-hour biopsy only. The average RNA yield from a single-core biopsy, when sufficient quality RNA was obtained, was 3.7 μg (0.1–15 μg). Table 1 shows

TABLE 1. Treatment and Outcome Characteristics

Patient	Clinical Stage	Nuclear Grade	Estrogen Receptor	Progesterone Receptor	Her2–neu	Planned Neoadjuvant Chemotherapy	Additional Neoadjuvant Chemotherapy	Primary Size Pretreatment	Extent of Residual Disease
AT1	T4N2M1	3	Negative	Negative	Negative	AT (4 cycles)	None	5 cm[a]	0.8 cm
AT2	T3N1M0	2	Positive	Positive	Negative	AT (4 cycles)	None	10 cm[a]	5.3 cm
AT3	T4N2M0	3	Negative	Negative	Negative	AT (4 cycles)	AT (2 cycles) CMF (3 cycles)	13 cm[a]	3.0 cm
TX	T2N0M0	3	Negative	Negative	Negative	Paclitaxel (12 weekly cycles)	FAC (4 cycles)	2.5 cm[a]	1.4 cm
FAC	T4N1M0	2	Positive	Negative	Negative	FAC (3 cycles)	Taxanes + HDC with transplant	8 cm[a]	3.5 cm+

Abbreviations: AT, doxorubicin/docetaxel (dose: 60/60 mg/m² given as an i.v. bolus); FAC, 5-fluorouracil, doxorubicin, cyclophosphamide (dose: 500 mg/m² of 5-fluorouracil given days 1 and 4, 50 mg/m² of doxorubicin given as a 72-hour continuous infusion, and 500 mg/m² cyclophosphamide given on day 1); TX, paclitaxel (dose: 80 mg/m² as an i.v. bolus); HDC, high-dose chemotherapy.
[a]As determined by physical examination, breast ultrasound, and mammogram.

the patient characteristics and pathological response to therapy of the five cases.

Table 2 shows the correlation coefficients between the replicate pairs of 70 individual hybridization experiments (five membranes/profile × 14 specimens). Eighty-one percent (57/70) had correlation coefficients of ≥ 90. With one notable exception, experiment TX 0 (pretreatment) on array #2, all correlations were 83 or greater. In unsupervised hierarchical cluster plots, one of the two replicates of TX0 on array #2 joined the main tree only at the root node, suggesting that this single replicate was suboptimal. Consequently, the data from this particular array were omitted from further analysis. For the remaining samples, an average of the two replicate experiments was used in further hierarchical cluster analyses.

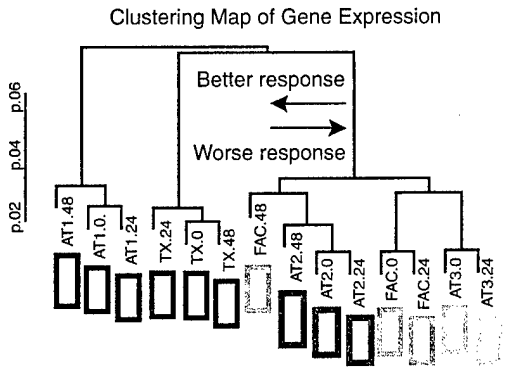

FIGURE 1 Hierarchical clustering map of genetic profiles from the samples of the 5 cases available for the study, with repeated data averaged to one value (exception: TX 0). The most similar data sets clustered in the terminal regions of the hierarchical cluster map. The degree of similarity/dissimilarity is represented by the proximity of each sample to one another along the cluster tree and the length of each branch of the cluster.

Transcriptional Profiling Results: Serial Samples From the Same Patient Cluster Together

Figure 1 displays a hierarchical cluster map that includes the profiling data for all cases and time points. As shown in this figure, samples from an individual patient obtained at different times had much more similar expression patterns than samples taken from different patients. Figure 2 further illustrates that the heterogeneity of gene expression was much greater across individuals than it was in serial samples from the same individual. Although this figure represents data from only two patients, all five patients had the same characteristics. In accordance, an analysis of variance-adjusted gene/expressed sequence tag expression levels revealed that there were fewer genes with fivefold differences in expression in an individual tumor at different times (average, 359 genes/expressed sequence tag) than in pretreatment samples of different tumors (average, 732 genes/expressed sequence tags).

As shown in Figure 1, the samples also clustered according to the pathological response of disease to treatment and according to the baseline estrogen receptor status, although the limited number of cases precludes a statistical analysis of the correlation of gene expression and disease response.

Transcriptional Changes During Therapy

Table 3 illustrates the number of genes from individual tumors with significant transcriptional changes during chemotherapy treatment. For this table, we defined sig-

TABLE 2 Correlation Coefficients of Replicated Experiments for Each Array Membrane

Sample	Time of Biopsy	Array 1	Array 2	Array 3	Array 4	Array 5
AT1	0 h	91.6%	83.3%	89.9%	92.1%	88.2%
	24 h	93.7%	91.8%	91.5%	88.2%	86.8%
	48 h	96.0%	94.1%	95.5%	90.0%	94.5%
AT2	0 h	92.5%	96.6%	97.0%	96.2%	93.2%
	24 h	94.8%	95.3%	89.1%	96.5%	93.8%
	48 h	96.0%	95.2%	96.0%	94.8%	92.8%
AT3	0 h	91.4%	92.0%	94.0%	92.1%	92.7%
	24 h	96.4%	96.0%	96.1%	96.1%	96.4%
FAC	0 h	96.8%	97.0%	97.0%	96.0%	96.4%
	24 h	98.4%	97.9%	97.9%	97.0%	97.4%
	48 h	97.1%	96.2%	97.0%	95.7%	96.4%
TX	0 h	82.9%	76.6%	92.9%	88.8%	87.9%
	24 h	96.5%	89.3%	93.3%	96.4%	95.4%
	48 h	93.3%	85.1%	91.7%	94.5%	87.1%

Abbreviations: AT, doxorubicin/docetaxel (dose: 60/60 mg/m² given as an i.v. bolus); FAC, 5-fluorouracil, doxorubicin, cyclophosphamide (dose: 500 mg/m² of 5-fluorouracil given days 1 and 4, 50 mg/m² of doxorubicin given as a 72-hour continuous infusion, and 500 mg/m² cyclophosphamide given on day 1); TX, paclitaxel (dose: 80 mg/m² as an i.v. bolus).

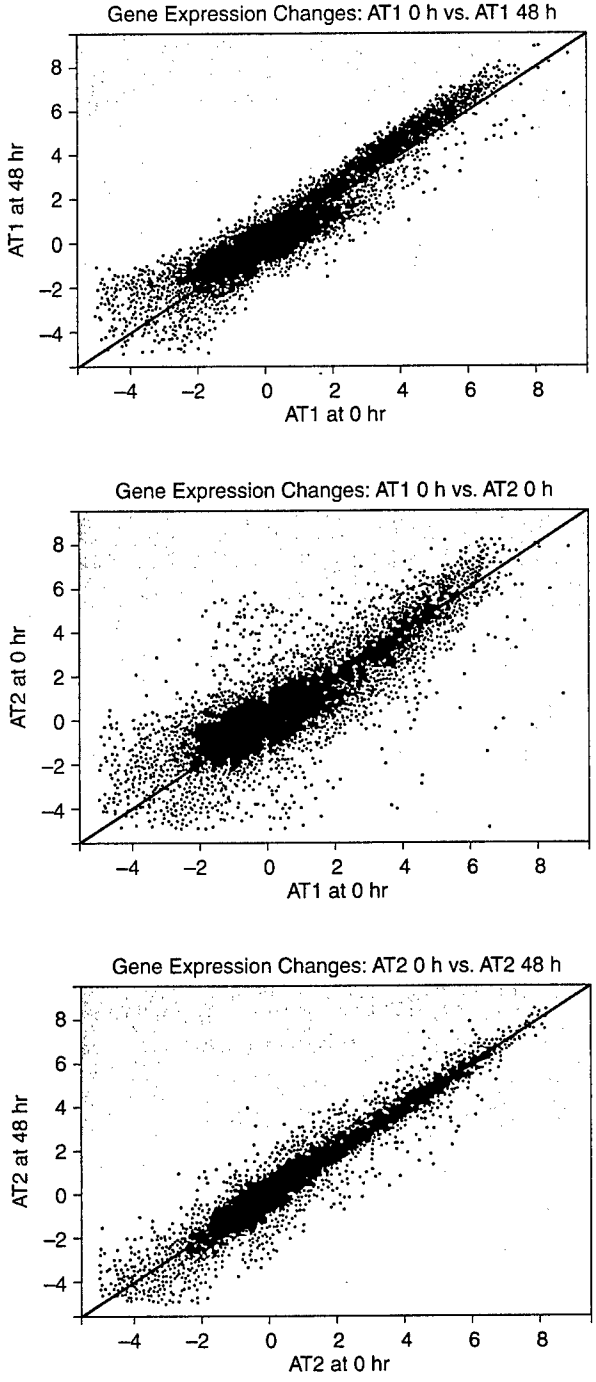

FIGURE 2 Comparison plot of expression profile for samples AT1–0 vs. AT1–48, AT1–0 vs. AT2–0, AT2–0 vs AT2–48, respectively. The axis and ordinate are the expression reading of a particular gene for the two comparative samples. If the samples were identically expressed in both samples, they would align on the line x = y. The degree of distance from this line represents the degree of dissimilarity in expression between the two samples. The greater degree of dissimilarity seen at low expression levels (at the bottom right corner of each graph) is typical for microarray data and represents a greater uncertainty of the data at low expression values. These data show an example of how there is greater genetic variation across the baseline of different individuals (B) than in samples taken serially from the same patient (A,C).

nificant changes as those differences that had a 1% or lower probability of being due to chance alone. On average, 125 genes were significantly up-regulated (range, 65–267) at 24 hours when compared with pretreatment, and 116 genes were significantly down-regulated (range, 53–190) at that time. These averages were higher at 48 hours (193 and 238, respectively). No individual genes were up-regulated or down-regulated across every tumor or even across the three tumors treated with AT chemotherapy.

Table 3 also shows data from a second strategy that can be used to assess the changes in individual genes over time. Specifically, the last row of Table 3 shows averages of the individual gene expression levels obtained from the five tumors for each time (only four tumors at 48 hours). Only three of the 162 genes up-regulated at 24 hours were also up-regulated at 48 hours, and only one gene that was down-regulated at 24 hours was also significantly down-regulated at 48 hours. We also classified the individual genes that were significantly up- or down-regulated in this combined set into functional categories (Table 4). The greatest effects were noted in the genes involved in nucleoside/nucleotide metabolism, protein metabolism, signal transduction, and the cell cycle.

DISCUSSION

In this paper, we demonstrated that it is possible to assess the expression of 25,000 human sequence clones in serial core biopsies of human breast cancer, and we report that significant transcriptional changes occur during chemotherapy.

A growing number of reports indicate that studying global gene expression patterns in human breast cancer with DNA microarray technology may lead to new insights into breast cancer biology and may result in improved classification schemes of the disease. For example, in a study of 65 breast cancers, Perou et al[3] discovered that estrogen receptor-negative breast cancers have a gene expression profile that is distinct from estrogen receptor-positive breast cancers, and this finding has also been noted by others.[4,5] Microarray analyses have also been used to identify a series of genes that distinguishes sporadic breast cancers from *BRCA1*-associated and/or *BRCA2*-associated breast cancer.[6] Finally, van't Veer et al[7] reported that cDNA profiling of 97 lymph node–negative breast cancers helped identify approximately 5100 genes that strongly predicted for distant relapse and then validated these data in a second set of patients. However, there are no published data concerning the use of cDNA arrays to predict response to chemotherapy in breast cancer. Lonning et al[8] studied breast cancer specimens obtained both before and after neoadjuvant chemotherapy from 20 breast cancer

TABLE 3 Numbers of Individual Genes With Significant Transcriptional Changes During Chemotherapy[a]

	24 hours		48 hours	
Sample	**Up-Regulated**	**Down-Regulated**	**Up-Regulated**	**Down-Regulated**
AT1	82	120	125	218
AT2	132	53	160	207
AT3	82	140	N/A[b]	N/A[b]
TX	267	190	129	210
FAC	65	79	356	320
Overall combined[c]	166	62	72	53

Abbreviations: AT, doxorubicin/docetaxel (dose: 60/60 mg/m² given as an i.v. bolus); FAC, 5-fluorouracil, doxorubicin, cyclophosphamide (dose: 500 mg/m² of 5-fluorouracil given days 1 and 4, 50 mg/m² of doxorubicin given as a 72-hour continuous infusion, and 500 mg/m² cyclophosphamide given on day 1); TX, paclitaxel (dose: 80 mg/m² as an i.v. bolus); N/A, not applicable.

[a]Nominal *P* value, 0.005.

[b]AT3 was not successfully profiled at 48 hours.

[c]Data represent the averages of the genes from all the tumors.

patients. These authors found that the genetic profile of tumors clustered according to various biologic markers, such as HER2–neu overexpression, keratin-related genes, or estrogen receptor genes. Their report did not address gene expression changes in response to therapy.

We undertook this study to assess global gene expression changes in the same tumor over the course of treatment. We elected to study expression patterns shortly after the initiation of treatment because important molecular pathways, such as those involved in apoptosis, are often seen within 48 hours after chemotherapy exposure.[9–11] We observed a remarkably diverse transcriptional response to therapy; specifically, no individual genes were significantly changed in a consistent manner across every specimen. These data suggest that the acute molecular effects of chemotherapy on tumors are likely to be complex.

An observation from our study is that serial biopsy specimens from the same tumor show gene expression profiles that are much more similar to each other during therapy than to the expression profiles of samples taken from tumors of different individuals. By analyzing the same tumor serially through time, we found that on average, there were half as many genes with fivefold expression difference in serial samples from the same individual, despite chemotherapy, than differentially expressed genes between tumors of different individuals. These data are similar to those in two previous reports. Both Perou et al[3] and Loning et al[8] found that gene expression profiles of tumor specimens from the same individual were more closely related than expression profiles of tumors from different individuals. These data suggest that intratumor transcriptional heterogeneity due to sampling of distinct subpopulations of cells, with or without external disturbance (i.e., chemotherapy), is much less than the heterogeneity among individual tumors.

One goal of studying transcriptional profile expression patterns of breast cancers is to use these patterns to differentiate chemotherapy-sensitive from chemotherapy-resistant tumors. A second important goal of such studies is to aid in the discovery of relevant genes involved in resistance to chemotherapy. One methodology used to achieve both of these goals is to study differentially expressed genes. For example, using this approach, Hedenfalk et al[6] defined a set of 75 genes in the 2000 they evaluated that were distinctly different in *BRCA1* versus *BRCA2* versus sporadic tumors. This process, called "supervised clustering," is one strategy to eliminate the background genetic "noise" that is associated with the interindividual variation in gene expression. However, the process requires a validation study to prove that the identified gene sets were not affected by selection biases. In this report, we have shown that studying tumors serially may be an alternative strategy for eliminating some of this interindividual genetic noise. In addition, studying tumors serially over time can give investigators many distinct sets of differential genes that can be used to categorize tumors. Our finding that very few of the genes are similar at 24 and 48 hours suggests that the differentiating gene sets at each time may be distinct. Finally, serial samples also allow the identification of genes that have the greatest expression changes over time. It is possible that transcriptional responses to treatment affects chemosensitivity to a greater extent than the genetic makeup of the untreated tumor.

In conclusion, we showed that is it feasible to study the changes in global gene expression patterns of more than 25,000 human sequence clones during a course of chemotherapy. This approach will likely be useful to learn about the complex cellular and tissue response to therapy. A larger set of patients with uniform treatment will be needed to identify clusters of genes whose altered

TABLE 4 Categories of Genes With Significant Up- or Down-Regulation During Chemotherapy

Category	No. of Genes Down-Regulated 0 vs. 24 hours	No. of Genes Up-Regulated 0 vs. 24 hours	No. of Genes Down-Regulated 0 vs. 48 hours	No. of Genes Up-Regulated 0 vs. 48 hours
Amino acid metabolism		1		
Carbohydrate metabolism		1		1
Cell adhesion	2	3	3	
Cell cycle	4	5	2	2
Cell growth and maintenance				1
Cell motility	2	2		1
Cell organization and biogenesis	1		1	
Cell proliferation	2	5	1	
Cell shape and cell size control		1		
Cell surface – signal transduction	4	1	1	
Cell–cell signaling			1	2
Co-translational membrane targeting		1		
Death (apoptosis)		4		
Developmental processes		2		1
Drug resistance		1		
Ectoderm development		5		
Embryogenesis and morphogenesis	1	1	1	2
Energy pathways				2
Induction of apoptosis by p53		1		
Intracellular protein traffic	2	2		2
Intracellular signaling cascade	2			
Lipid metabolism			1	1
Membrane fusion		1		
Mesoderm development		2		1
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	8	2	6	7
Oncogenesis	1	2		2
Perception of external stimulus	3	1	1	
Phosphate metabolism	1			
Physiological processes	1	2	1	
Protein metabolism and modification		7	5	3
Radiation response	3	1	1	
Sex differentiation		1		
Signal transduction	6	3	3	
Small molecule transport		3	1	
Spermatogenesis		1		1
Stress response	4	2		
Transport	1	1		2
Vitamin A metabolism	1			

expression in response to therapy could predict clinical outcome.

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Factors Predictive for Local-Regional Recurrence After Neoadjuvant Chemotherapy and Mastectomy: Implications for Radiation Treatment

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In a recent article published in the *Journal of Clinical Oncology*, we investigated factors predictive of local-regional recurrence (LRR) for a group of breast cancer patients treated with neoadjuvant chemotherapy and mastectomy without radiation.[1] The purpose of this study was to ascertain clinical and pathological factors that could be used to identify subgroups of patients for whom postmastectomy radiation should be considered. On multivariate analysis, we found that advanced clinical stage at presentation and the presence of ≥4 residual positive lymph nodes (+LN) were independent predictors of LRR. Pathological complete response to chemotherapy did not correlate with local control. Patients with advanced clinical stage independent of response to chemotherapy and patients with ≥4 +LN after chemotherapy had clinically relevant rates of LRR. Data involving patients with clinical stage I or II disease who had 1 to 3 +LN after the neoadjuvant chemotherapy were insufficient to ascertain whether radiation treatments should be considered in this subset.

Postmastectomy Radiation: Past and Present

Our incentive in pursuing this clinical research project was the recognition of the importance of defining which subsets of breast cancer patients may benefit from radiation. For more than two decades, it has been recognized that postmastectomy radiation reduces breast cancer death rates.[2,3] However, many of the early postmastectomy trials found that non–breast cancer death rates (mostly cardiovascular) were equally increased in patients treated with radiation.[2,3] Fortunately, more modern studies that have used improved radiation treatment techniques, which minimized radiation dose to the heart, have found no increase in cardiovascular morbidity and mortality after postmastectomy radiation.[4] Correspondingly, the three most recently published prospective randomized clinical trials investigating the use of postmastectomy radiation have demonstrated an improved overall survival (OS) for the patients randomized to receive this treatment.[5-7] In general, these three trials found that radiation led to an overall 20% absolute reduction in the 10-year LRR rate (30% versus 10%). More importantly, each trial also found an absolute improvement of 9% in the 10-year OS.[5-7] This improvement in OS is in the same range as the overall 10-year survival benefit seen for first-generation polychemotherapy regimens for patients with +LN (12%—Early Breast Cancer Trialists' Collaborative Group Meta-Analysis[8]).

While together the data from these three trials strongly suggest that reducing rates of LRR after mastectomy can improve OS in breast cancer, the trials have not resulted in a consensus as to which patients should be treated. We

For a more detailed discussion, please see the following: Buchholz TA, Tucker SL, Masullo L, et al. Predictors of local-regional recurrence after neoadjuvant chemotherapy and mastectomy without radiation. *J Clin Oncol.* 2002;20:17-23.

Table 1

Rates of LRR as a Function of Clinical and Pathological Extent of Disease After Neoadjuvant Chemotherapy

Factor	Category	5-Year LRR Rate (n = number of patients)
Clinical stage	I	0% (n=1)
	IIA	5% (n=21)
	IIB	16% (n=44)
	IIIA	17% (n=35)
	IIIB	50% (n=38)
	IV	79% (n=11)
Extent of residual primary disease	<2 cm	18% (n=74)
	2-5 cm	36% (n=56)
	>5 cm	46% (n=14)
Extent of residual nodal disease	0 +LN	12% (n=62)
	1-3 +LN	18% (n=42)
	≥4 +LN	53% (n=41)

began investigating this issue by reviewing the LRR rates in more than 1000 patients treated with a mastectomy followed by adjuvant 5-fluorouracil, doxorubicin, and cyclophosphamide (FAC) chemotherapy without radiation. We reported that patients with primary tumors >5 cm and/or patients with ≥4 +LN had 10-year LRR rates that exceeded 20%.[9] For patients with smaller tumors and 1 to 3 +LN, the 10-year LRR rates were low, with notable exceptions: patients with ≥2 mm extracapsular extension of disease, ≤10 dissected lymph nodes or ≥20% involvement of axillary lymph nodes, close (<2 mm) or positive margins, or pectoralis muscle invasion.[9-11]

Neoadjuvant Chemotherapy and Mastectomy

Historically, pathological factors have been used to identify which subgroups of patients have a clinically relevant risk of LRR after mastectomy. In part, this is due to the fact that clinical stage often underestimates the extent of disease, particularly with respect to axillary lymph nodes.[12,13] This fact makes determining the selection criteria for postmastectomy radiation for patients treated with neoadjuvant chemotherapy more complicated. In these patients, the pathological extent of disease is available only after 4 to 6 courses of treatment. Furthermore, 80% to 90% of breast cancers obtain a partial or complete response to neoadjuvant chemotherapy,[14,15] which suggests that the pathological extent of disease after chemotherapy is likely to be much different than the pathological extent of disease at diagnosis. Thus, pathological factors associated with LRR after mastectomy may be different for patients treated with neoadjuvant chemotherapy compared to patients treated adjuvantly.

There have been few published data concerning risk factors for LRR after neoadjuvant chemotherapy and mastectomy. The University of Texas M.D. Anderson Cancer Center Breast Cancer Group began investigating neoadjuvant chemotherapy in a series of prospective clinical trials dating back to 1974. We conducted a retrospective analysis of the data from these trials and identified 150 patients treated in these studies who did not receive postmastectomy radiation.[1] This population had relatively advanced disease at diagnosis, particularly in the trials with the longest follow-up. The percentage of each clinical stage in our study population was: stage I, 1%; stage II, 43%; stage III, 48%; and regional stage IV, 7%. Of these patients,

81% were initially treated with 3 to 4 cycles of FAC, and 19% received 4 cycles of single-agent paclitaxel in the preoperative setting.

Prior to chemotherapy, 59% of the patients had clinical stage T3 or T4 disease, and 70% had clinically suspicious lymphadenopathy. After chemotherapy, the median pathological extent of residual primary tumor disease was 2 cm, with 49% having disease less than 2 cm, 37% having disease measuring 2 to 5 cm, and 9% having disease greater than 5 cm (the disease was not quantified in 3%). The median number of +LN was 1, with 41% having no axillary disease, 28% having 1 to 3 +LN, and 27% having 4 or more +LN.

Predictors of LRR

On a Cox regression analysis, the three significant factors associated with higher rates of LRR were clinical stage IIIB disease or greater (hazard ratio 4.5, $P<0.001$), ≥4 +LN (hazard ratio 2.7, $P=0.008$), and lack of tamoxifen use (hazard ratio 3.9, $P=0.027$). Table 1 shows the 5-year rates of LRR according to clinical stage, extent of residual primary disease, and extent of residual nodal disease. We also analyzed LRR according to disease response and did not find a relationship. The 5-year rate of LRR for the 18 patients with a complete pathological response was 19% (95% CI, 6%-48%). The 4 patients who had a complete pathological response and a subsequent LRR had either T3 disease or clinical stage III disease at diagnosis.

Another interesting subset was the 40 patients with residual tumor sizes >5 cm and 1 to 3 +LN.[16] We analyzed this group separately and found that the 5-year LRR rate was 46% (95% CI, 24%-76%) for the patients with clinical T3 or T4 primary tumors compared to only 4% (95% CI, 1%-25%) for the patients with clinical T1 or T2 disease ($P=0.002$).

Discussion

These data indicate that both pretreatment clinical stage and posttreatment pathological findings should be considered when determining indications for radiation after neoadjuvant chemotherapy and mastectomy. Our data suggest that patients with advanced disease at presentation maintain clinically relevant risks of LRR even if they achieve a favorable histologic response to chemotherapy. Not surprisingly, patients with residual primary disease that remains ≥5 cm and those with residual metastatic disease in ≥4 axillary nodes also have rates of LRR that are clinically significant.

Each trial found an absolute improvement of 9% in the 10-year overall survival.

There are important limitations of our analysis that should be recognized. The median follow-up of surviving patients treated with neoadjuvant chemotherapy was only 4.1 years, and the rates of LRR we report will likely increase with further follow-up time. For example, in our earlier analysis of LRR failure patterns in the patients treated with mastectomy and adjuvant chemotherapy, 21% of the LRR developed after 5 years.[9] Second, the sample size of the patients treated with neoadjuvant chemotherapy was much smaller than the number of patients we analyzed who were treated with mastectomy and adjuvant chemotherapy. Correspondingly, the rates of LRR are less certain. This is particularly true with respect to the subgroup analyses we performed. It is clear that more data are needed to determine whether radiation is indicated for women with early stage breast cancer who have small residual tumor sizes and 1 to 3 +LN after chemotherapy. There have been two published randomized prospective clinical trials investigating neoadjuvant versus adjuvant chemotherapy for early breast cancer, and data from these trials may provide additional insights into this important question.[17,18]

In conclusion, based on our clinical studies, our institutional policies for using radiation after mastectomy are:

Adjuvant Chemotherapy
Stage III breast cancer (T3-T4 or N2-N3), or
≥4 +LN, or
≤5 cm primary tumors and 1 to 3 +LN with one of the following:
- Extracapsular extension ≥2 mm
- ≤10 axillary lymph nodes dissected
- ≥20% of axillary lymph nodes positive
- Close or positive margins
- Pectoralis fascia invasion

Neoadjuvant Chemotherapy
Clinical stage III breast cancer, or
≥4 +LN after chemotherapy, or
Residual tumor size >5 cm, or
pathological T4 primary disease, or
Residual tumor size ≤5 cm and 1 to 3 +LN with one of the following:

Extracapsular extension ≥2 mm
<10 axillary lymph nodes dissected
≥20% of axillary lymph nodes positive
Close or positive margins
Pectoralis fascia invasion

We feel strongly that additional research is needed to define the role of radiation in patients with early stage breast cancer and 1 to 3 positive lymph nodes who are treated with mastectomy and either neoadjuvant or adjuvant chemotherapy. ■

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Supported in part by National Cancer Institute Department of Health and Human Services grant Nos. CA16672 and T32CA77050 and a grant from the Stanford and Joan Alexander Foundation, Houston, Texas. Dr. Buchholz is supported by grant No. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

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Am. J. Hum. Genet. 67:1437–1451, 2000

Haplotypes at *ATM* Identify Coding-Sequence Variation and Indicate a Region of Extensive Linkage Disequilibrium

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Genetic variation in the human population may lead to functional variants of genes that contribute to risk for common chronic diseases such as cancer. In an effort to detect such possible predisposing variants, we constructed haplotypes for a candidate gene and tested their efficacy in association studies. We developed haplotypes consisting of 14 biallelic neutral-sequence variants that span 142 kb of the *ATM* locus. *ATM* is the gene responsible for the autosomal recessive disease ataxia-telangiectasia (AT). These *ATM* noncoding single-nucleotide polymorphisms (SNPs) were genotyped in nine CEPH families (89 individuals) and in 260 DNA samples from four different ethnic origins. Analysis of these data with an expectation-maximization algorithm revealed 22 haplotypes at this locus, with three major haplotypes having frequencies ≥.10. Tests for recombination and linkage disequilibrium (LD) show reduced recombination and extensive LD at the *ATM* locus, in all four ethnic groups studied. The most striking example was found in the study population of European ancestry, in which no evidence for recombination could be discerned. The potential of *ATM* haplotypes for detection of genetic variants through association studies was tested by analysis of 84 individuals carrying one of three *ATM* coding SNPs. Each coding SNP was detected by association with an *ATM* haplotype. We demonstrate that association studies with haplotypes for candidate genes have significant potential for the detection of genetic backgrounds that contribute to disease.

Introduction

Qualifying and quantifying the genetic contribution to the etiology of common complex disease remains one of the great quests of modern medical genetics. The complexity of multifactorial diseases challenges the paradigms and tools of conventional genetic research. Traditional methods of genetic analysis do not have the statistical power or sensitivity for the task of teasing out a genetic contribution when it is subtle or when several genes may be working together (Risch and Merikangas 1996). Genomewide association studies, as well as population studies with candidate genes, have been touted as possible alternatives to linkage analysis (Risch and Merikangas 1996; Collins et al. 1997; Kruglyak 1999; Risch 2000). These approaches focus on finding either a causative variant or a genetic variant closely linked with the disease phenotype. Some studies utilizing single-nucleotide polymorphisms (SNPs) have succeeded in detecting the risk for disease, notably in the case of the apolipoprotein type E (apoE) gene and both coronary artery disease (Boerwinkle et al. 1996) and Alzheimer disease (Strittmatter and Roses 1995). These studies were able to directly assess the risk conferred by known apoE functional variants. In some other cases, however, the attempt to correlate single-locus alleles with phenotypes have produced mixed results (Josefsson et al. 1998; Kraft et al. 1998; Storey et al. 1998).

Haplotype association with disease by the linkage disequilibrium (LD) approach has been used successfully for the identification of genomic regions containing loci responsible for disease phenotypes (MacDonald et al. 1992; Yu et al. 1996). The same principle can be applied by use of haplotypes of biallelic markers to detect disease association. Using several SNPs distributed across 100–200 kb should result in statistical sensitivity that is greater than that in studies using fewer loci. Another strength of such an approach is the ability to use purely epidemiological populations for detection of chromosomal backgrounds lending risk for disease.

All of these approaches are, to one extent or another, dependent on LD. An understanding of LD relationships between markers will inform the efficacy and design of future LD-based strategies for detection of genetic contributions to common disease. Simulation studies have estimated the length of useful LD to be as low as 3 kb (Kruglyak 1999). Recent investigations support the no-

Received August 22, 2000; accepted for publication September 21, 2000; electronically published November 14, 2000.

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0002-9297/2000/6706-0010$02.00

tion that LD varies throughout the genome (Collins et al. 1999; Taillon-Miller et al. 2000) and that it can extend to considerable lengths, such as several hundred kilobases (Collins et al. 1999; Eaves et al. 2000; Moffatt et al. 2000; Taillon-Miller et al. 2000). Reports of such extreme differences indicate the need for further study of the extent and nature of LD.

Allelic variation leading to functional variants of genes may predispose to risk for seemingly sporadic cases of common disease (Lander 1996; Collins et al. 1997). Here we describe a strategy for exploring the possible effects of functional variants of genes involved in familial cancers. We use a resequencing approach to detect SNPs across a large (184 kb) genomic region containing the *ATM* gene. *ATM* is responsible for the autosomal recessive disease ataxia-telangiectasia (A-T) (Savitsky et al. 1995). A-T is characterized by cerebellar ataxia, oculocutaneous telangiectasia, immune deficiency, sensitivity to ionizing radiation, increased incidence of tumors, and chromosomal instability (Gatti et al. 1991). A-T heterozygotes may be at increased risk for development of cancers, most prominently—and controversially—breast cancer (Swift et al. 1987, 1991; Morrell et al. 1990; Stankovic et al. 1998; Gatti et al. 1999). With carrier frequencies estimated to be from 0.5% to >1% (Swift et al. 1986; Gatti et al. 1999), assessment of cancer risk for this population is a compelling endeavor. In addition, the *ATM*-gene product is centrally involved in cellular responses to DNA damage, including DNA double-strand break repair and signaling leading to cell-cycle arrest and apoptosis (reviewed in Rotman and Shiloh 1999). We genotyped 295 individuals from four ethnic groups, for 14 SNP markers that spanned 142 kb. An expectation-maximization algorithm estimated 22 *ATM* haplotypes from these data. Tests for recombination and LD revealed (*a*) no evidence for recombination in the white European American study population and (*b*) perfect disequilibrium extending the full length marked by these SNPs. We then conducted a model association study with these haplotypes and a population of samples that possessed one of three different coding SNPs (cSNPs) in the *ATM* gene. The results of this study provide strong support for the utility of complex SNP haplotypes as a means to detect polymorphisms in a population-based sample.

Subjects and Methods

Human Subjects

For SNP discovery, genomic DNA from five unrelated white European Americans was sequenced. This DNA was extracted from lymphoblast and fibroblast cell lines. For SNP genotyping, individuals from four ethnic groups were sampled: African American ($n = 71$), Asian American ($n = 39$), white European American ($n = 77$), and Hispanic ($n = 73$). All ethnic samples (self-described ethnicity) were part of a collection of 941 DNA purified samples from anonymous blood donors in community-based blood drives in southeastern and central Texas. Samples analyzed in the model association study were also from this DNA collection. Members of nine CEPH families were also analyzed. In all families, four grandparents, two parents, and four children were examined; since two of these families share a grandparent, 89 individuals were genotyped, and the number of segregating chromosomes is 70.

Samples from Great Apes

Six great-ape samples were genotyped in this study: two from common chimpanzees (*Pan troglodyte*), one from a bonobo (*P. paniscus*), two from western lowland gorillas (*Gorilla gorilla*), and one from an eastern lowland gorilla (*G. g. graueri*).

PCR and Sequencing Primers

Primers for DNA amplification and sequencing were designed by MacVector, version 6.0.1. The 184-kb genomic sequence of *ATM* was masked for repetitive sequence, by Repeat Masker. Thirty-six primer sets were designed to amplify regions containing little or no repeat sequence, distributed evenly throughout the sequence. Primers were selected that met strict criteria for melting temperature and that amplified regions containing very little or no repeat sequence. The same primers were used for PCR and sequencing reactions and are listed in Appendix A.

PCR Amplification of Genomic DNA

Genomic DNA from five unrelated individuals was amplified by means of 29 of the 36 primer sets mentioned above. The 50-μl reactions included DNA (200 ng), standard PCR buffer, dNTPs (0.1 mM each), *Taq* (0.5 μl; Perkin-Elmer), and primers (1 μM each). PCR was performed in a Perkin Elmer 9700 analyzer, with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 7 min. For all amplicons, 6 μl of PCR product was run on a 1.5% agarose gel.

DNA Sequencing

PCR products were purified and sequenced. Preparation of DNA for sequencing included incubation of ~60 ng of PCR product with shrimp alkaline phospha-

tase (2 U; Amersham) and exonuclease I (10 U; Amersham) at 37°C for 15 min, followed by enzymatic inactivation at 80°C for 15 min. Sequencing of each PCR product was performed with the Thermo Sequenase [^{33}P]-radiolabeled terminator-cycle sequencing kit (Amersham Pharmacia), according to the manufacturer's instructions. Sequencing reactions were performed in a Perkin Elmer 9700 analyzer, with an initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Samples were run on 6% polyacrylamide gels, fixed for 15 min in 5% acetic acid/20% methanol, and dried.

Multiplex PCR

Sequencing revealed 17 SNPs in 15 different regions of the gene. These 15 PCR amplicons were multiplexed into two PCR reactions. Multiplex group 8 amplifies eight fragments, and Multiplex group 7 amplifies seven fragments. The 50-μl reactions for group 7 included DNA (400 ng), standard PCR buffer (2 ×), dNTPs (0.2 mM each), and *Taq* (0.5 μl; Perkin-Elmer). The 50-μl reactions for group 8 included DNA (400 ng), standard PCR buffer (1.8 ×), dNTPs (0.2 mM each), and *Taq* (0.5 μl; Perkin-Elmer). Primers include some of those originally designed for sequencing and some of those newly designed to alter the size of the amplicons. Products were separated by ≥20 bp, so that they could be resolved from one another on a 2.5% agarose gel. Multiplex PCRs were checked to have amplified all products, by running 6 μl of product on a 2.5% agarose gel. The concentrations and primer sequences used for PCR are listed in Appendix B.

Allele-Specific Oligonucleotide (ASO) Hybridizations

Genotypes for each SNP were determined in all sample populations, by ASO hybridizations. ASO hybridizations were performed as described by DeMarchi et al. (1994). We performed ASO hybridization for 14 SNPs for each individual typed. These 14 SNPs were chosen from the original 17 because they perform consistently well under standard ASO-hybridization conditions. Hybridizations were performed under conditions that allowed for annealing of only the probe that is an exact match for the substrate DNA. Genotypes for SNPs were read on at least two independent occasions. The sequences of the ASO-hybridization probes are listed in Appendix C.

Estimation of Haplotypes and Frequencies

Haplotypes and their frequencies were estimated on the basis of unphased genotype data, by the computer program EMHAPFRE. Described in the work of Excoffier and Slatkin (1995), EMHAPFRE uses an expectation-maximization algorithm that determines the maximum-likelihood frequencies of multilocus haplotypes in diploid populations. Only individuals who were scored for all 14 SNPs were included in the data analysis.

Haplotype Assignment to Genotype Data

A short script written in Microsoft Excel Visual Basic and named "Assign" was used to assign genotypes to individual samples. The script was given, as input, the list of haplotypes produced by EMHAPFRE and the raw unphased genotype data. It produces a list of samples input, with a pair(s) of haplotypes that satisfies the genotype data assigned to each; in cases in which multiple pairs of haplotypes were listed, one pair is chosen, by use of a haplotype frequency–based method. A probability is calculated for each haplotype pair, by multiplication of the haplotypes' frequencies in the control population. The haplotype pair with the highest probability is assigned to the individual.

Statistical Analysis for Recombination and LD

To test for recombination, we used the four-gamete test and the Hudson and Kaplan (1985) recombination statistic, R. For a given haplotype AB, mutation may result in either Ab or aB. Haplotype ab arises only in the case of either recombination or repeat mutation. The four-gamete test was executed on unphased genotype data, in a pairwise fashion, across all SNP loci. On the basis of the resulting matrix of the four-gamete test, R estimates the location and number of recombination events that have occurred in the sample.

Initial LD analysis was computed by performance of pairwise comparisons for all SNP loci. Fisher's exact test was used to determine significance levels. SNPs having a minor-allele frequency of .05 were excluded from LD analyses. LD statistic D is a pairwise comparison of gametic frequencies such that D = p11p22–p12p21. D', the relative disequilibrium, is $D' = D/|D|\max$, where $|D|\max = \max(p1p2,q1q2)$ if $D<0$ and $|D|\max = \min(q1p2,p1q2)$ if $D>0$. D' ranges from 1 to −1, and this range is not influenced by allele frequency.

All recombination and LD statistics were generated by the software program DnaSP 3.00 (written by J. Rozas and R. Rozas, University of Barcelona).

Statistical Analysis for Association Study

Testing for significance in the model association study was done by use of contingency tables for independence. P values for significance of association at the haplotype level were determined by use of 2 × 2 tables and 3 × 3 tables for the genotype level. Significance values refer to a one-sided test.

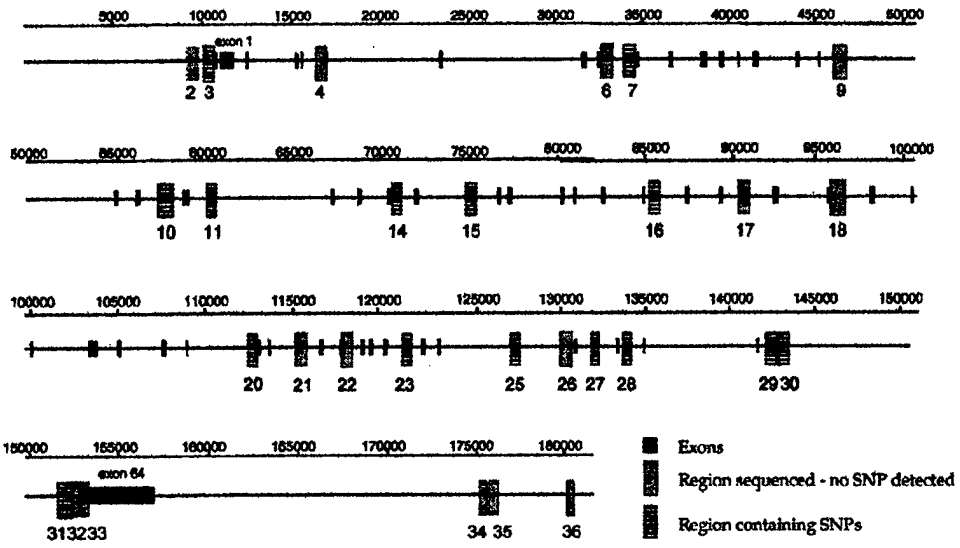

Figure 1 Schematic of *ATM*. The 184 kb of the *ATM* locus is illustrated, with the 64 exons represented by black boxes. Twenty-nine ~500-bp regions were amplified by PCR in five unrelated individuals. These regions were sequenced and found to contain 17 SNPs.

Results

SNP Discovery

Our initial objective was to discover common neutral sequence variants spanning the length of the *ATM* gene. A gel-based resequencing strategy was employed to detect SNPs at the *ATM* locus. Genomic DNA of five unrelated individuals was amplified, by PCR, for [^{33}P]-radiolabeled sequencing. For detection of markers spanning the entire locus, PCR primers were designed for amplicons dispersed approximately evenly throughout the 184-kb genomic region containing the gene (fig. 1). Approximately 13.5 kb of the 184-kb total sequence was read in each individual. The nucleotide diversity, π, calculated for this sequence data was .00057. Seventeen SNPs were found, which span 142 kb and all of which are located in introns (table 1). This yielded an average of 1 SNP/794 nucleotides sequenced.

Genotyping and Haplotype Development

To begin construction of haplotypes from these SNPs, we genotyped nine three-generation CEPH families (Dausset et al. 1990). By using three-generation families, we could determine haplotypes from genotype data, through inference. This allowed us both to determine the efficacy of the computer algorithm used to predict haplotypes (see below) and to optimize our genotyping assay. We performed ASO hybridization on nine CEPH families (89 individuals; 70 chromosomes), for 14 of the original 17 SNPs. These 14 SNPs were chosen from the original 17 because they performed consistently well under standard ASO-hybridization conditions.

We then used two different methods for deciphering the haplotypes derived from the genotype data, in a side-by-side comparison. First, haplotypes were inferred by

Table 1

Seventeen *ATM* Noncoding SNPs Detected by Resequencing

SNP[a]	Location in Genomic Sequence with GenBank Accession Number U82828
Prior to 5′UTR t→a[b]	10182
IVS8−356t→c	34293
IVS19−1276a→g	57469
IVS21−77t→c	60136
IVS26+491c→g[c]	71049
IVS27−193c→t[c]	75083
IVS34+754g→a	85811
IVS46−257a→c	112721
IVS55+186c→t	121819
IVS57+3570t→c	127195
IVS58+997g→a	132032
IVS59+414g→t[c]	133986
IVS61−55t→c	142611
IVS62+60g→a	142789
IVS62+424g→a	143153
IVS62−973a→c	151964
IVS62−694c→a	152243

[a] Nomenclature is according to the guidelines recorded by the Ad Hoc Committee on Mutation Nomenclature (1996).

[b] This SNP is named in reference to the genomic sequence having GenBank accession number U82828 because of the highly variable nature of the 5′UTR.

[c] Not used in genotyping or haplotype analysis.

Table 2

***ATM* Haplotypes of 295 Humans from Five Ethnic Groups and of Three Species of Great Apes**

		Frequency in Humans[a]					
Haplotype	Sequence	Overall (n = 295)	African American (n = 71)	Asian American (n = 39)	White European American (n = 77)	Hispanic American (n = 73)	CEPH (n = 35)
1	ACTCTACTTCCCTC	.002				.007	
2[b]	ACTCTACTTCTTTC	.313	.190	.500	.292	.315	.394
3	ACTCTCCTTCTTTC	.037			.065	.048	.061
4	ACTTCACTCCTCTC	.002	.007				
5	ACTTTACTCTCCTC[c]	.002					
6[b]	ACTTTACTTCCCTC	.066	.218	.013	.013	.027	.015
7	ACTTTACTTCTTTC	.019	.077				
8	ATTCTACTTCTTTC	.012	.007	.051		.007	
9	ATTCTCCTTCTTTC	.000		.013			
10	ATTTCACTCCCCTC	.002	.007				
11	ATTTCATCCTCCCC	.002		.013			
12	TCTCTACTTCTTTC	.007				.021	.015
13	TCTTCACTCTCCTC	.010	.035			.007	
14	TCTTCATCCTCCCC	.002				.007	
15[b]	TTCTCACTCTCCTA	.090	.028	.013	.175	.041	.227
16	TTTCTATCCTCCCC	.005		.017		.007	
17[b]	TTTTCACCCTCCTC	.100	.141	.068	.097	.110	.015
18	TTTTCACCCTCTTC	.002				.007	
19	TTTTCACTCCTTTC	.002	.007				
20	TTTTCACTCTCCTA	.002				.007	
21[b]	TTTTCACTCTCCTC	.048	.162	.013	.006	.027	
22[b]	TTTTCATCCTCCCC	.277	.113	.291	.351	.363	.273
	TTTCTACCCTCCTC[c]	...		.009			
	ACTTTACCCTCCTC[c]	...	.007				
Total		1.000	1.000	1.000	1.000	1.000	1.000

		Frequency in Great Apes[d]		
		Chimpanzee (n = 2)	Bonobo (n = 1)	Gorilla (n = 3)
1	TCTTTACTCTCCTC	.750	1.000	.000
2	TCTTTACTCTCTTC	.250	.000	.000
3	TATTTACTCTCCTC	.000	.000	1.000

[a] Samples were genotyped by ASO hybridization, then haplotypes and their frequencies were estimated from unphased genotype data, by the EM algorithm EMHAPFRE.

[b] Haplotype present in all four ethnic groups studied.

[c] Low-frequency haplotypes in which some differences were seen in the combined data set and in individual ethnic populations.

[d] Samples were genotyped by ASO hybridization and fluorescent sequencing.

hand. We began with homozygotes and predicted other haplotypes on the basis of transmission and by establishing the phase through the pedigrees. Seven haplotypes were identified in the sample of CEPH families. Subsequently, we subjected the same data set to an expectation-maximization algorithm, to estimate haplotypes and their frequencies. The computer program EMHAPFRE is a maximum-likelihood program developed to predict multilocus haplotypes from unphased genotype data (Excoffier and Slatkin 1995). It produces both a list of haplotypes and their estimated frequencies in the input sample population. The haplotype predictions from EMHAPFRE were in complete accordance with those that had been inferred manually, giving us confidence that this program was suitable for data of this nature.

Haplotype and Allele Frequencies

To determine frequencies of haplotypes and of individual SNPs in different ethnic populations, we performed ASO hybridization on anonymous African American (n = 71), Asian American (n = 39), white European American (n = 77), and Hispanic (n = 73) DNA samples collected in central and southeastern Texas. Genotype data were analyzed by the EMHAPFRE program. For the total population, 22 haplotypes and their frequencies were predicted by EMHAPFRE (table

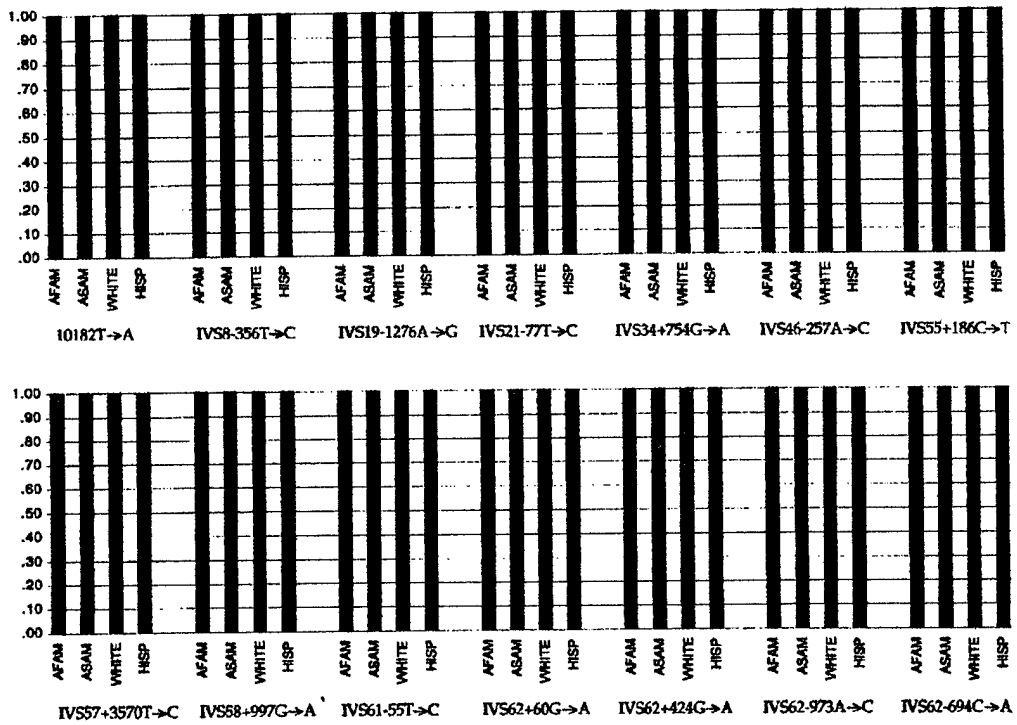

Figure 2 ATM SNP allele frequencies for 14 ATM SNPs in each of four ethnic groups. A total of 260 individuals (71 African American, 39 Asian American, 77 white European American, and 73 Hispanic) were genotyped by ASO hybridization.

2). Three predominant haplotypes were found at frequencies ≥10%. An independent study that examined neutral sequence variants at the *ATM* locus also found three major haplotypes (Li et al. 1999).

The majority of SNPs identified in this study have a frequency, in all ethnic groups, of ≥25% (fig. 2). Of the 14 SNPs, 3 (IVS19−1276a→g, IVS46−257a→c, and IVS62−694c→a) have a minor-allele frequency of <10% in most ethnic groups. SNP frequencies vary across ethnic groups. Three SNPs (IVS55+186c→t, IVS62+424g→a, and IVS62−973a→c) have a frequency of 11% in African Americans while being present at a frequency of >30% in all other ethnic groups. SNP IVS46−257a→c was not found in the samples from African Americans. Of the three low-frequency SNPs, two (IVS19−1276a→g and IVS62−694c→a) have a frequency of >18% in the white European American population and of <6% in the others. This is not surprising, given that the original five samples used for SNP detection were white European Americans.

To begin to describe the haplotype phylogeny at the *ATM* locus, we wanted to determine what haplotypes were present in each ethnic population. The genotype data were analyzed, by EMHAPFRE, as four separate data sets segregated by ethnic group. However, this analysis led to small discrepancies from what was predicted from the complete data set. In each case, changes were found in the lowest-frequency haplotypes (table 2). The efficacy of EMHAPFRE is known to decay as data sets decrease in size (Excoffier and Slatkin 1995). Thus, a second approach to ascription of haplotypes and their frequencies to each ethnic group was taken. To this end, a simple script was written in Microsoft Excel Visual Basic. This script, named "Assign," takes a list of haplotypes and a data set of unresolved genotypes and then assigns to each individual sample one or more pairs of haplotypes that can resolve its genotype data; Assign lists every pair of haplotypes that can resolve an individual's genotype data. We input each ethnic group's data set individually with the 22 haplotypes. In this way we were able to determine which of the haplotypes suggested by EMHAPFRE were necessary for resolution of our genotype data, thus further refining the results. The genotype of every sample in this study could be accounted for by at least one pair of the 22 haplotypes predicted by EMHAPFRE from the complete data set. Six of the 22 haplotypes exist in all ethnic populations, and 11 of them are unique to a single population and hereafter are referred to as "private" haplotypes (table 2); each of these 11 haplotypes has a frequency of <1%.

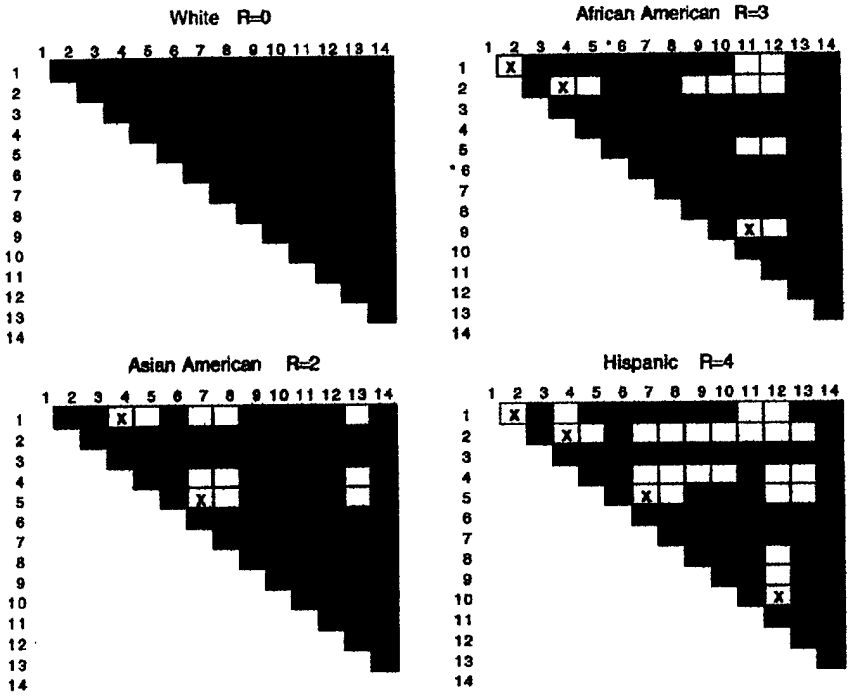

Figure 3 Four-gamete test for recombination in *ATM*. White boxes denote site pairs having four gametic types, which implies that recombination has occurred between these two sites. Also shown is the Hudson and Kaplan recombination statistic R, which is an estimate of the number and sites of recombination events needed to explain the results of the four-gamete matrix. A white box containing an " × " denotes a potential site of recombination. The asterisk (*) denotes an SNP that is not polymorphic in the sample population.

We analyzed primate DNA in order to approximate an ancestral *ATM* haplotype. Three haplotypes were found in 12 chromosomes (table 2). Two common chimpanzees, one bonobo, and three gorillas were genotyped by ASO hybridization and fluorescent sequencing; in cases in which ASO hybridization gave ambiguous results, fluorescent sequencing was used to confirm the genotype. None of the ape haplotypes was found among the 22 human haplotypes. One ape haplotype differs from a human haplotype by a single-base variant. This human haplotype is one of the least common (frequency .007) and occurs only in our African American study group. Only one of the human SNPs showed variation in the apes; the remainder were monomorphic. One common chimpanzee was heterozygous for IVS62+424g→a. The gorillas shared all but one allele with the chimpanzees. At IVS8−356t→c, gorillas are homozygous for a third allele (A), which is not found in either humans or chimpanzees.

Intragenic Recombination and LD

The small number of haplotypes seen in our study population suggests the possibility that recombination is reduced at the *ATM* locus. This is further evidenced by the results of the four-gamete test (fig. 3) (Hudson and Kaplan 1985). For a given haplotype AB, mutation may result in either Ab or aB. Haplotype ab arises only in the case of either recombination or repeat mutation. For the purpose of this analysis, we will consider repeat mutation to be rare and will use the four-gamete test as a measure of recombination. The four-gamete test was executed on unphased genotype data, in a pairwise fashion across SNP loci. This was done for each ethnic group separately. Interestingly, the four-gamete test found no site pairs with four gametes in the samples from white European Americans, implying a complete lack of recombination in that population. Low recombination was indicated for the other groups, as shown in figure 3.

Another test for recombination is that of Hudson and Kaplan (1985). Based on the resulting matrix of the four-gamete test, the Hudson and Kaplan parameter *R* is an estimate of the minimum number of recombination events in the history of the sample. For the white European American population, this estimate is 0 (fig. 3). For the other ethnic groups, *R* ranges from 4, in His-

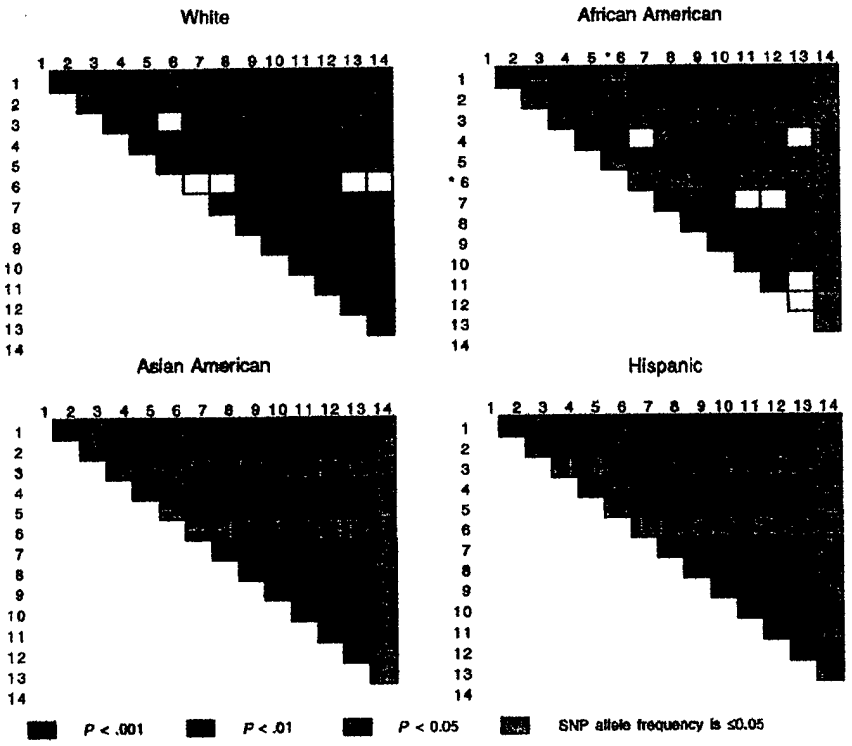

Figure 4 Fisher's exact test for LD in *ATM*. White boxes denote site pairs that do not have a significant value by Fisher's exact test, indicating linkage equilibrium. Gray columns and rows denote SNPs that have a minor-allele frequency ≤.05. The asterisk (*) denotes an SNP that is not polymorphic in the sample population.

panics, to 2, in Asian Americans. The predicted sites of recombination are similar among ethnic groups. African Americans and Hispanics share two possible recombination sites in the 5′ end of the gene, and a third, in the 3′ end, could also be in the same location. The Asian American population shares one of the 5′ end sites and has, in the middle of the gene, another potential site of recombination, which is also present in Hispanics.

Further support for the hypothesis that there is minimal recombination at the *ATM* locus is provided by the results of Fisher's exact test (Weir 1996). We computed all possible pairwise comparisons between sites, to determine the degree of nonrandom association between sites. The majority of site pairs across all data sets show significance ($P < .001$), indicating that there is extensive disequilibrium at this locus (fig. 4). It has been demonstrated that alleles with frequencies ≤.05 do not have the power for detection of disequilibrium (Lewontin 1995; Goddard et al. 2000). In this analysis, we included only SNPs having an allele frequency >.05. The Hispanic and Asian American populations were in complete disequilibrium. In the white European American population, the pattern of equilibrium followed the SNP with the lowest-frequency (.06) allele.

Disequilibrium was next measured by use of the statistic D', in a pairwise fashion across the 14 SNP loci (fig. 5). $D' = D/|D|\text{max}$, where $D = \text{p11} - \text{p1p2}$ and $|D|\text{max} = \text{max}(\text{p1p2},\text{q1q2})$ if $D < 0$ and $|D|\text{max} = \text{min}(\text{q1p2},\text{p1q2})$ if $D > 0$. D' ranges from 1 to −1, and this range is not influenced by allele frequency. A score of either 1 or −1 is considered to represent perfect disequilibrium. Interestingly, the results of this test are virtually superimposable on the results of the four-gamete test. The majority of site pairs are in perfect disequilibrium. The white European American population is in perfect disequilibrium across all sites. For the other groups, the sites with $|D'| < 1$ are exactly the same sites that have four gametes. We conclude that the *ATM* locus exhibits reduced recombination and extensive disequilibrium in all four ethnic groups, with the white European American population being the most extreme case.

Association Study

Ultimately, we aim to use these *ATM* haplotypes for association studies in populations with cancer. To evaluate the potential that these haplotypes have for identification of a particular mutation or polymorphism, we

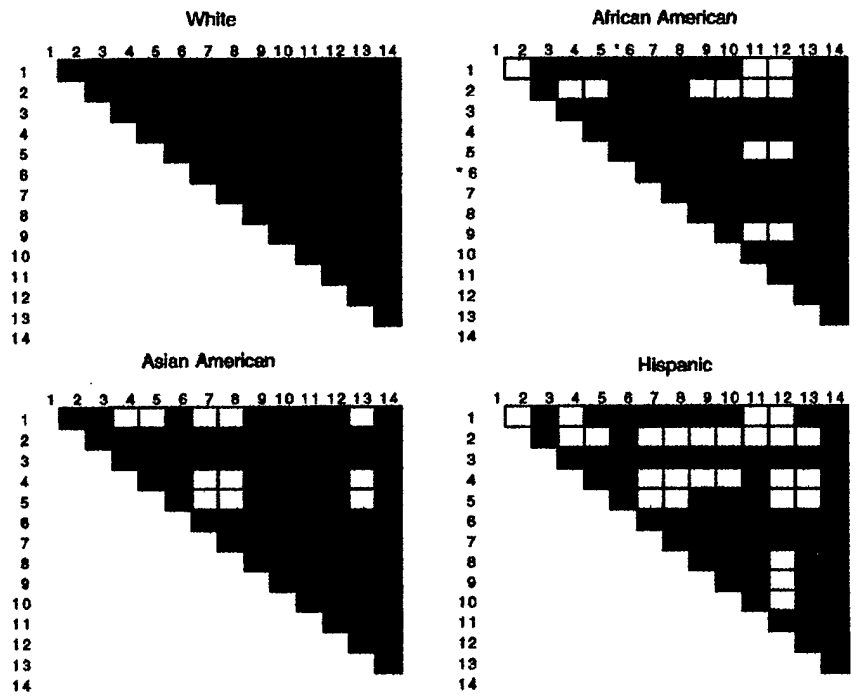

Figure 5 *D'*, measured as $D' = D/|D|\max$ in a pairwise fashion across 14 SNP loci. A score of either 1 or −1 is considered perfect disequilibrium. Black boxes denote site pairs with perfect disequilibrium; white boxes denote site pairs with $|D'| < 1$. The asterisk (*) pair denotes an SNP that is not polymorphic in the sample population.

performed a model association study. We tested the ability of these haplotypes to detect, by association, three different cSNPs in the *ATM* gene. These cSNPs were found by sequencing the reverse transcriptase–PCR products from *ATM* mRNA isolated from peripheral blood lymphocytes from cancer patients. cSNP1 is located in exon 4 and results in Ser49Cys. Positioned in exon 38, cSNP2 results in Asp1853Asn; and cSNP3, which results in Pro1054Arg, is located in exon 23. A population of 941 individuals was screened for these three cSNPs, by ASO hybridization. The resulting frequencies of the cSNPs in this population are shown in table 3.

In the model association study, samples from white European Americans in the 941-individual collection that were found to possess one of the three cSNPs were considered to be the "case" population. The "control" population consisted of samples from white European Americans from the same collection that were randomly chosen and negative for the cSNPs; because of the low frequency of these cSNPs in other ethnic groups, only samples from white European Americans were used in this association study. All case and control samples were genotyped for the 14 *ATM* neutral sequence variants, via ASO hybridization. To assign haplotypes to individual samples, we used Assign and the initial 22 *ATM* haplotypes.

Each cSNP showed a significant association with a different, specific *ATM* haplotype (table 4). cSNP1 showed an association with haplotype 2, cSNP2 with haplotype 15, and cSNP3 with haplotype 17. Haplotype 2 was present at a frequency of .29 in the control population (no. of chromosomes [*c*] = 152) and at a frequency of .64 in the cSNP1 population ($c = 14$); haplotype 15 was present at a frequency of .07 in the control population ($c = 112$) and at a frequency of .57 in the cSNP2 population ($c = 56$); and haplotype 17 was pre-

Table 3

Frequencies for Three *ATM* cSNPs in the Control Population

	Frequency in 941 Individuals			
	White European Americans	African Americans	Asian Americans	Hispanic Americans
cSNP1	.005	.000	.001	.001
cSNP2	.066	.001	.002	.017
cSNP3	.015	.001	.000	.005

Table 4

Association of *ATM* Haplotypes and *ATM* cSNPs in Individual "Case" and Control Populations

		Frequency in[a]		*P* for	
	Haplotype	Control Population	"Case" Population	Genotype Association[b]	Haplotype Association[c]
cSNP1	2	.29 (c = 152)	.64 (c = 14)	.0478	.0166
cSNP2	15	.07 (c = 112)	.57 (c = 56)	.0000	.0000
cSNP3	17	.08 (c = 146)	.52 (c = 54)	.0000	.0000

Note.—Samples carrying one of three *ATM* cSNPs were genotyped, by ASO hybridization, for the 14 *ATM* noncoding SNPs.

[a] Each cSNP was found to occur on separate *ATM* haplotypes. c = total number of white European American chromosomes genotyped.

[b] By 2 × 2 contingency table.

[c] By 3 × 3 contingency table.

sent at a frequency of .08 in the control population (c = 146) and at a frequency of .52 in the cSNP3 population (c = 54). These are 2-fold, 8-fold, and 6.5-fold increases in the frequencies of haplotypes 2, 15, and 17, respectively; and the *P* values for these associations are .0166, .0000, and .0000, respectively. Genotype correlations were also present, with *P* values of .0478, .0000, and .0000, respectively (table 4).

One of the great challenges in studying the genetics of a complex disease such as cancer is its multifactorial etiology. As presented in table 4, the data from our simulated association study model a scenario in which all "cases" are caused by a single mutation. To more accurately simulate an association study with a complex disease, we reanalyzed our data. We considered the three groups of samples carrying the variant cSNPs as one "case" population. In this analysis, two of the three haplotypes that originally had shown an association demonstrated a significant increase in frequency (table 5). No increase in frequency was apparent for haplotype 2, which had previously shown a twofold increase in the cSNP1 population; haplotype 15 showed a fourfold increase (P = .0002); and haplotype 17 showed a threefold increase (P = .0002). Thus, we successfully demonstrated the ability of these *ATM* haplotypes to discern members of our case population who carry a particular SNP. The results of these studies indicate a significant potential for the use of haplotypes extending over a large genomic region, to detect disease associations through a case-control–study design in a general population.

Discussion

In this study, we have presented a strategy for uncovering the genetic contribution to complex disease. Specifically, we have demonstrated the utility of a complex SNP-based–haplotype approach to association studies and have detected significant LD at the *ATM* locus, extending ~142 kb. The results of this study provide proof of principle for the use of SNP-haplotype data in the detection of genetic factors contributing to complex disease.

We sequenced 13.5 kb of the *ATM* gene in five unrelated individuals and detected 17 SNPs in noncoding regions. We then utilized these neutral sequence variants spanning 142 kb of the *ATM* gene to construct haplotypes for this genomic locus. The expectation-maximization algorithm EMHAPFRE (Excoffier and Slatkin 1995) was used to predict haplotypes from genotype data on 295 individuals from four ethnic groups. Twenty-two haplotypes and their frequencies were predicted by EMHAPFRE, for the total population. Three of these 22 haplotypes have a frequency of ≥10%. This concurs with the findings of Li et al. (1999), who also used neutral sequence variants to detect three major haplotypes at the *ATM* locus. Six of the 22 haplotypes exist in all four ethnic populations in our study and are also the most commonly occurring haplotypes. There are 11 private haplotypes, each of which has a frequency of <1%.

We verified the reliability of the haplotype-prediction algorithm by using several tests. First, we genotyped individuals from nine three-generation CEPH families (n = 87). This allowed us to determine haplotypes by inspection of allele segregation. The CEPH genotype data were also analyzed by EMHAPFRE, and the resulting haplotypes agreed completely with those in-

Table 5

Association of *ATM* Haplotypes and *ATM* cSNPs in Combined Case Population

	Frequency in Combined Case Population	*P* for	
Haplotype	(c = 124)	Genotype Association	Haplotype Association
2	.19	.9644	.7606
15	.27	.0000	.0002
17	.24	.0000	.0002

Note.—See footnotes to table 4.

ferred on the basis of transmission data. Next, we used Assign, a script written in Microsoft Excel Visual Basic, to assign pairs of haplotypes to individual genotypes. Given the 22 haplotypes predicted by EMHAPFRE, Assign successfully resolved the genotype data for all 295 individuals in this study. The results of EMHAPFRE were tested against another haplotype-prediction program, one that does not use the expectation-maximization algorithm and that does not assume that Hardy-Weinberg is in effect. This program, termed "Data Mining," uses the resulting matrix of the four-gamete test to inform the process of haplotype prediction so that recombination may influence outcome (N. Wang, R. Chakraborty, M. Kimmel, and L. Jin, personal communication). There were minor differences in the results of this comparison. For the population of white European Americans, the outcome of each program was identical. This is not surprising, since the four-gamete test reveals no evidence for recombination in this population. The results of these trials confirm that EMHAPFRE was successful in estimating the correct haplotypes necessary to sufficiently resolve our data set. We feel confident that the size and diversity of our data set has allowed us to describe in relative depth the haplotype architecture of *ATM*. Consequently, we have chosen to use, as the foundation for further studies, the 22 haplotypes predicted from the complete data set.

With a minimal amount of sequencing (13.5 kb in five individuals), we were able to detect highly informative neutral sequence variants spanning a large genomic region. In sequencing 10 chromosomes form white European Americans, we found SNPs that have a common occurrence in four different ethnic groups. In all ethnic groups, the majority (11 of 14) of SNPs identified in this study have a minor-allele frequency of ≥25%. SNPs with frequencies in the range of .2–.5 have the highest information content for association and LD studies (Kruglyak 1997). Although most SNPs had a high minor-allele frequency in all ethnic groups, allele frequencies varied across ethnic groups. This is in accordance with several other studies that have found population differences in SNP-allele frequencies (Lai et al. 1998; Nickerson et al. 1998; Cargill et al. 1999; Halushka et al. 1999; Goddard et al. 2000). Variations in allele frequencies are most pronounced in the African American population. Four SNPs (IVS21−77t→c, IVS55+186c→t, IVS62+424g→a, and IVS62−973a→c) have a minor-allele frequency that is reduced by 40%–75% in African Americans, compared with that in other ethnic groups. A fifth SNP, IVS46−257a→c, was not found in the African American samples. These differences illustrate that there is population structure in SNP-allele frequencies that is an important factor to consider when SNP-based association and LD studies are designed.

Comparison of genotype data from six great apes was instructive for approximating ancestral haplotypes and SNP alleles. Genotyping revealed three haplotypes in this population, none of which is identical to the human *ATM* haplotypes. Of the 14 SNPs, 2 showed variation in the ape population. One common chimpanzee was heterozygous for IVS62+424g→a, and all three gorillas were homozygous for a third allele (A) at IVS8−356t→c. The extent of homozygosity in this sample indicates that most of the SNPs found varying in the human population have arisen since the divergence of the human lineage from the last common ancestor shared with the chimpanzee. This agrees with the assertion by Hacia et al. (1999)—that is, that most current neutral human polymorphisms are not shared with the chimpanzee (Hacia et al. 1999). It may also imply that these SNPs are not hypermutable sites, since more variation might be expected in the 12 primate chromosomes analyzed. Although these SNPs are common in man, they are not due to hypermutability; rather, they are old enough to be found throughout diverse ethnic groups.

The results of this study show a remarkable lack of recombination at the *ATM* locus. This effect is most profound in the white European American population, in which no evidence for recombination is detected by the four-gamete test and in which D' shows perfect disequilibrium across all SNPs. Low recombination is implicated for the African American, Asian American, and Hispanic groups as well. The possibility of low recombination was suspected on the basis of the seemingly small number of haplotypes found at this locus. Twenty-two haplotypes with 14 loci is not considerably greater than the $n + 1$ (i.e., 15) that would be expected if there is no recombination. Another study, performed in parallel with this one, used the same approach as that described here and serves as a direct comparison: D. Trikka, Z. Fang, A. Renwick, S. Jones, R. Chakraborty, M. Kimmel, and D. L. Nelson (unpublished data) used neutral sequence variants dispersed across the BLM, WRN, and RECQL loci, to derive haplotypes for these regions; their study used the same sample population, with fewer SNPs (8, 13, and 11 respectively) for haplotype construction, and found considerably larger numbers of haplotypes (50, 56, and 47, respectively) at each locus. The key difference between these loci and *ATM* is the amount of recombination and LD reported. Trikka et al. found more evidence for recombination and linkage equilibrium when the four-gamete test and Fisher's exact test were used. For *ATM*, the four-gamete test revealed few site pairs with four gametes. The Hudson-Kaplan recombination statistic R ranged from 0, in white European Americans, to 4, in Hispanics. Analysis by both Fisher's exact test and D' indicated extensive LD for *ATM*, in all ethnic groups studied. Figure 5

shows extensive disequilibrium, with >72% of site pairs having perfect disequilibrium in all ethnic groups.

Using a model association study, we have successfully demonstrated the ability of *ATM* haplotypes to identify chromosomes carrying specific coding polymorphisms. The three cSNPs that we used as candidates for detection had varying frequencies in our control population of white European Americans (cSNP1, .005; cSNP2, .066; and cSNP3, .015). When each of the three cSNP populations was analyzed individually, each cSNP showed a significant association with a different *ATM* haplotype. cSNP1 showed an association with haplotype 2, cSNP2 with haplotype 15, and cSNP3 with haplotype 17 (P = .0166, .0000, and .0000, respectively); the increase in haplotype frequency in cases versus controls was 2-fold, 8-fold, and 6.5-fold, respectively. To model the potential for multiallelic etiology of a complex disease, we combined the three populations of samples carrying the cSNPs into one "case" population. In this analysis, two haplotypes demonstrated a readily detectable increase in frequency: haplotype 15 showed a fourfold increase, and haplotype 17 showed a threefold increase in frequency; no frequency increase was apparent for haplotype 2, which had previously shown a twofold increase in the cSNP1 population.

The association that becomes undetectable (i.e., haplotype 2 with cSNP1) involves the haplotype occurring most commonly (frequency .29) in the general population. Haplotype 15 shows the greatest increase in frequency and is the least common of the three haplotypes, with a control frequency of .05. This leads us to an important point for future association studies. Haplotypes with lower frequencies in control populations may be more effective for detection of associations. However, it is important to note that haplotype 17, which is the third most frequent haplotype (frequency .10), nevertheless showed a 2.6-fold increase in frequency in the combined cSNP population. An additional factor contributing to detection in this study is frequency of the mutation. In the case of cSNP1 and haplotype 2, in which the association becomes undetectable, the most frequent haplotype was associated with the least common SNP (cSNP1, .006). The difference in frequency between cSNP1 and the other cSNPs is a factor of 10. Both the haplotype frequency and the cSNP frequency contribute to detection. This underscores the idea that several factors, including frequency of haplotype, frequency of mutation, and age of mutation, contribute to limits of detectability.

This model association study demonstrates proof of principle for the use of complex SNP haplotypes covering candidate genes, in the detection of genetic factors contributing to complex disease. We have successfully demonstrated the ability of these *ATM* haplotypes to discern members of our "case" population that carry a particular coding SNP. The results of these studies indicate that haplotypes extending over a large genomic region have a significant potential for detection of disease associations.

There is much interest in the use of SNPs in genomewide association studies and other LD-based strategies. Our approach and analyses bear on those strategies, in several regards. First, LD estimates from simulation studies have been as low as 3 kb of meaningful LD (Kruglyak 1999). This calculation suggests that a very-high-density map with as many as 0.5–3 million SNPs would be necessary for effective association studies (Kruglyak 1999). Our results and those of other studies (Collins et al. 1999; Eaves et al. 2000; Moffatt et al. 2000; Taillon-Miller et al. 2000) indicate, to the contrary, that significant LD can be found extending as far as several hundred kilobases. This should reduce the number of SNPs necessary for genomewide linkage studies. Comparison of LD at *ATM* versus the results of the *LPL* study (Clark et al. 1998) in which LD patterns were complex over just 9.7 kb supports the idea that LD varies widely throughout the genome, indicating that some regions will require SNPs that are more densely spaced. Second, higher-frequency (.2–.5) SNPs are more robust, whereas rare SNPs may be less useful and, in some analyses, may confound results. More than half of the SNPs used to construct haplotypes in the *LPL* study had a relative allele frequency of <.2. This resulted in 67 of 71 individuals having a unique haplotype. By using fewer markers (14) with higher frequency (.20), we were able to effectively elucidate the haplotype architecture and the LD and recombination profiles for the *ATM* genomic locus (142 kb). These haplotypes were used successfully in association studies, to detect coding polymorphisms in the *ATM* gene. We conclude that reasonably spaced, highly informative SNPs have the ability to define a larger number of ancestral chromosomes and have increased power for population-based association studies.

Acknowledgments

The authors thank Ranajit Chakraborty of the University of Texas Health Science Center (Houston) and Marek Kimmel of Rice University (Houston), for discussion and review of the manuscript; Jason Deats, for his valued contribution to the design and code for Assign; and Melissa Bondy, Alice Sigurdson, and Scott Manatt, of M.D. Anderson Cancer Center, for collection of normal DNAs. P.E.B was supported by a fellowship from the W.M. Keck Center for Computational Biology (Houston) (funded by U.S. National Library of Medicine Training Grant 1T15LM07093). T.A.B. was supported by Department of Defense Breast Cancer Research Program Career Development Award CBC980154. This work was supported by the Kleburg Fund for New and Innovative Research and by U.S. National Cancer Institute grant CA75432.

Appendix A

Primers Used for PCR and Sequencing

f1.atm, ATGGTCATCTCGTTACAGGCAATGC
r1.atm, CCCCAAGTGACTGAAGGCATCTAGG
f2.atm, TGGTGGAACCATTTCCGTTTAACG
r2.atm, GCGCCCTTCTAATAACCCGCC
f3.atm, GCCCAGAACCTCCGAATGACG
r3.atm, CGACTTAGCGTTTGCGGCTCG
f4.atm, TGGCTGGCAACATTACCAACTGC
r4.atm, TGCATCTTTTTCTGCCTGGAGGC
f5.atm, TGTGTGCTAGGGAGGAATCTGGTGG
r5.atm, GGCTGTCTCTAGGCTTGTTGAGGGC
f6.atm, CCATCATCCGAAAGGAGCCAAAAC
r6.atm, GCAGCAATTTCCCTGTTTCTGCC
f7.atm, AAATTGGCAGGATGATGAGGATGC
r7.atm, GCTGTCAAGCTGCATCAGCGTTAG
f8.atm, CCAAAGCGTGCCAGAATGGTATG
r8.atm, CCAAAGCGTGCCAGAATGGTATG
f9.atm, GGTATGCGTAGCGGGGCTAGTGAG
r9.atm, CGCAGGAAAAAGCCAGATGCAATC
f10.atm, GCCCTAGCCCCAGTGTATGTGGAG
r10.atm, GGCAGCCAGTTTCCGAGAACTACC
f11.atm, TTTTTGGCAAGGTGAGTATGTTGGC
r11.atm, TGCGAACTTGGTGATGATTGTCAGC
f12.atm, AGATTGTTCCAGGACACGAAGGGAG
r12.atm, TTTCTTCCCATTGTCACCTGTTCCC
f13.atm, TGCGAAAAACAGGCTTTGTTTGC
r13.atm, GGTGATGGAAAAGAGACGGGGC
f14.atm, GCAAGTCCCCTCACCAGCAACAC
r14.atm, GATGCCTTCCCATCATCCTGATACC
f15.atm, TCTGGGAAGAAGTTACGCAGGGAAC
r15.atm, CTGACTGGCACTAGAATTTGCTGGC
f16.atm, GGCGGAATGAATGTGAGTTATGCG
r16.atm, CCAGGTGATTTCTCCATCCCGTG
f17.atm, CTGCCTAAAGCAGCAGTTTTTGCC
r17.atm, TGTTGCTATCCCGAAGCTGAAACC
f18.atm, GGTGTGTAAGCAAGAATGCCTGGG
r18.atm, GCCACAGATTTTGAGACCACTGCAC
f19.atm, TAGTTTGTATGGCTGTGGTGGAGGG
r19.atm, CATCCCTCTGCTTCAGGAGTATCCC
f20.atm, CCAGTAGGGGGTCCCTCATTTCC
r20.atm, TGAGAAGCTGGGAGTGTTTCTGCC
f21.atm, CCCCGTACATGAAGGGCAGTTG
r21.atm, TGGGTGGCTGGGCTAATGAAGAG
f22.atm, GGTTCAGCGAGAGCTGGAGTTGG
r22.atm, GCAGCAGGGGGAAAACCCAC
f23.atm, CCACAGATTAGCAACAAGTTGGGGC
r23.atm, TGGCATAAGCACACGGAAACTCTCC
f24.atm, AGGTTCCGATGGCAAGGAGAGG
r24.atm, CTGTGTCTTTCCACCACTCCCCAG
f25.atm, CAGTCATGGTTCTGGGGAGAGAAGC
r25.atm, GCCTTTCTGATTTCCCTTCCTGCC
f26.atm, CTTGATGGTGGGAGGGACTTAGGG
r26.atm, TGCCTAGATGTTTGAGAGCCTGCC
f27.atm, CAGGGCACACAGGGTACAGTGTAGG
r27.atm, TCAGTTCAGACCATCTCATGCCTCC
f28.atm, CAGGGGGATGATAGTGATGATGTGG
r28.atm, TTCAAAACATACATGCCCTGCCTTC
f29.atm, CAAAGACTGAGAGCTGAGCCCAGTG
r29.atm, GCACAATCTCCTCCTTTCTGCTGC
f30.atm, TGGTTTAGAAATGCCTTCAGCCCC
r30.atm, TGCACTCTACCTGCCATGCTTCC
f31.atm, GCCATGTCAGTGCCCAACTTGAAG
r31.atm, TTGGTGCTGCGTTTGGAATCTTG
f32.atm, GATTCCAAACGCAGCACCAAACC
r32.atm, GGTAGTTGATGGGGGAGGGGAAC
f33.atm, GTTCCCCTCCCCCATCAACTACC
r33.atm, GAGCACAGTGCCTTCTTCCACTCC
f34.atm, CCCTGACAATCTGGGGCACAAAC
r34.atm, CCGTGGCTTTTGCTGGCATTC
f35.atm, GTCCTGTGGCATTGTGCATAACTCC
r35.atm, GCAGACATTAGGCATAAGCCCCTTC
f36.atm, GATGACTGCCCTTGTTCCCCAAG
r36.atm, TGGTTAAGTTGCTTTTCCCCCCAG

Appendix B

Primer Sequences and Concentrations Used for Multiplex PCR

Group 8:
3F *ATM*, 5′-GCCCAGAACCTCCGAATGACG-3′; and 3R-2 *ATM*, 5′-GCCGTGAAGCGAAAGAGGCG-3′ (0.25 μM)
11F *ATM*,5′-TTTTTGGCAAGGTGAGTATGTTGGC-3′; and 11R *ATM*, 5′-TGCGAACTTGGTGATGATTGTCAGC-3′ (0.25 μM)
14F *ATM*, 5′-GCAAGTCCCCTCACCAGCAACAC-3′; and 14R *ATM*, 5′-GATGCCTTCCCATCATCCTGATACC-3′ (0.25 μM)
23F-2 *ATM*, 5′-GGTGGAATCTGGTCTAGTTACCC-3′; and 23R *ATM*, 5′-TGGCATAAGCACACGGAAACTCTCC-3′ (0.25 μM)
27F *ATM*, 5′-CAGGGCACACAGGGTACAGTGTAGG-3′; and 27R *ATM*, 5′-TCAGTTCAGACCATCTCATGCCTCC-3′ (0.188 μM)
29R *ATM*, 5′-CAAAGACTGAGAGCTGAGCCCAGTG-3′; and 29R *ATM*, 5′-GCACAATCTCCTCCTTTCTGCTGC-3′ (0.125 μM)
30F *ATM*, 5′-TGGTTTAGAAATGCCTTCAGCCCC-3′; and 30R-2 *ATM*, 5′-CAGCCAGTCCAACATAAATCAG-3′ (0.25 μM)
31F *ATM*, 5′-GCCATGTCAGTGCCCAACTTGAAG-3′; and 31R *ATM*, 5′-TTGGTGCTGCGTTTGGAATCTTG-3′ (0.25 μM)
Group 7:
7R *ATM*, 5′-GCTGTCAAGCTGCATCAGCGTTAG-3′; and 7F-2 *ATM*, 5′-GTTGGATTACCATGTTCACCAG-3′ (.188 μM)
10F *ATM*, 5′-GCCCTAGCCCCAGTGTATGTGGAG-3′and 10R-2 *ATM*, 5′-GCAGAGATAATCATGGGCAGG-3′ (0.25 μM)
15F *ATM*, 5′-TCTGGGAAGAAGTTACGCAGGGAAC-3′; and 15R-2 *ATM*, 5′-TGGGGAGACTATGGTAAAAGAGG-3′ (0.31 μM)
16F *ATM*, 5′-GGCGGAATGAATGTGAGTTATGCG-3′; and 16R *ATM*, 5′-CCAGGTGATTTCTCCATCCCGTG-3′ (0.25 μM)
20F *ATM*, 5′-CCAGTAGGGGGTCCCTCATTTCC-3′; and 20R *ATM*, 5′-TGAGAAGCTGGGAGTGTTTCTGCC-3′ (0.25 μM)

25F *ATM*, 5′-CAGTCATGGTTCTGGGGAGAGAAGC-3′; and 25R-2 *ATM*, 5′-CTATCAATATCTAGCTCTGGGGC-3′ (0.15 μM)
28F *ATM*, 5′-CAGGGGGATGATAGTGATGATGTGG-3′; and 28R *ATM*, 5′-TTCAAAACATACATGCCCTGCCTTC-3′ (0.5 μM)

Appendix C

Probes Used for ASO Hybridization

*ATM*Aso 3T, 5′-TAACCCTCCTTCCCGC-3′
*ATM*Aso 3a, 5′-TAACCCTCCATCCCGC-3′
*ATM*Aso 7T, 5′-AAGGAACTTGTAATATTTTTC-3′
*ATM*Aso 7c, 5′-AGGAACTCGTAATATTTTTC-3′
*ATM*Aso 10T, 5′-TGGGAAACATGACCAGGG-3′
*ATM*Aso 10c, 5′-GGGAAACACGACCAGGG-3′
*ATM*Aso 11T, 5′-GTAACTTATAATAACCTTTC-3′
*ATM*Aso 11c, 5′-GAAGTAACTTACAATAACC-3′
*ATM*Aso 14C, 5′-TCTGTACAAGAAAAATTTG -3′
*ATM*Aso 14g, 5′-TCTGTAGAAGAAAAATTTG-3′
*ATM*Aso 15C, 5′-TTTCCTCTCAGTCTACAGG-3′
*ATM*Aso 15t, 5′-TTTTTCCTCTTAGTCTACAGG-3′
*ATM*Aso 16C, 5′-TAGAGATGATGTCGGCTTC-3′
*ATM*Aso 16t, 5′-CTAGAGATGATGTTGGCTTC-3′
*ATM*Aso 20A, 5′-GTAATGTCAGAGTATTAAA-3′
*ATM*Aso 20c, 5′-TAATGTCAGCGTATTAAA-3′
*ATM*Aso 23T, 5′-CAAAAGCTTCTCTTGCTTT-3′
*ATM*Aso 23c, 5′-AAAAGCTTCTCCTGCTTTC-3′
*ATM*Aso 25C, 5′-TTTTTTGTGGCATTCACAC-3′
*ATM*Aso 25t, 5′-TTTTTTGTGGTATTCACAC-3′
*ATM*Aso 27C, 5′-CTGCTCATGCCTCCTCTC-3′
*ATM*Aso 27t, 5′-CTGCTCATGTCTCCTCTCC-3′
*ATM*Aso 28C, 5′-TTCTATTAAACAGTATTA-3′
*ATM*Aso 28a, 5′-TTCTATTAAAAAGTATTA-3′
*ATM*Aso 29.1T, 5′-GATAAAGATATGTTGACAA-3′
*ATM*Aso 29.1c, 5′-GATAAAGATACGTTGACAA-3′
*ATM*Aso 29.2C, 5′-ACTTCCTGACGAGATACAC-3′
*ATM*Aso 29.2t, 5′-ACTTCCTGATGAGATACAC-3′
*ATM*Aso 30c, 5′-CCTAAGCCACGTTCCTCTA-3′
*ATM*Aso 30t, 5′-CCTAAGCCATGTTCCTCTA-3′
*ATM*Aso 31.1C, 5′-AAATAGAGCGATTTTGGTT-3′
*ATM*Aso 31.1t, 5′-AAATAGAGAGATTTTGGTTC-3′
*ATM*Aso 31.2C, 5′-AGAAATTCCTCATGAACTC-3′
*ATM*Aso 31.2a, 5′-AGAAATTCATCATGAACTC-3′

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank Overview, http://www.ncbi.nlm.nih.gov/Genbank Overview.html (for genomic sequence [accession number U82828])

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Women's Oncol Rev 2001;1:349–354

APPENDIX 15

Sequencing of surgery, systemic therapy and radiation for patients with invasive breast cancer

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INTRODUCTION

The majority of patients with a newly diagnosed invasive breast cancer will require treatment with surgery, radiation and systemic therapy. While in the past, chemotherapy and hormonal therapy were reserved for women with lymph node-positive breast cancer, randomized trials have now conclusively demonstrated that systemic therapy can also improve survival of most women with early-stage disease[1]. In addition, radiation therapy has been established to be an integral component of therapy for all women treated with a breast conserving surgery and for selected women treated with mastectomy and chemotherapy[2,3]. As the positive benefits of chemotherapy and radiation have been elucidated, how to optimally sequence and integrate these treatments has become an important clinical question.

Despite breast cancer being the most common malignancy in women, it is interesting that patients with early-stage breast cancer receive a variety of different treatment sequencing recommendations that are dependent on the biases of their physicians. Some physicians advocate neoadjuvant chemotherapy, surgery, additional chemotherapy, and subsequent radiation with either concurrent or sequential tamoxifen for the majority of women with lymph node-positive disease. Many others feel that the initial treatment for women with operable disease should always be surgery, with the sequencing of adjuvant radiation and chemotherapy determined by the surgical margin and lymph node status of the individual case.

This article will review the important topic of treatment sequencing in breast cancer and offer suggestions as to how oncologists can consider the wide variety of sequencing options that are inherent to nearly every case of breast cancer. The most important consideration that the article will suggest is that each case should be handled in a multidisciplinary fashion, and that optimal management of breast cancer truly requires a concerted effort on the part of surgeons, medical oncologists, radiation oncologists, pathologists and diagnostic radiologists.

THE NEOADJUVANT CHEMOTHERAPY DEBATE

SURVIVAL CONSIDERATIONS

Breast cancer most commonly becomes life-threatening only after the development of systemic metastases. The outcome data from breast cancer patients treated with local-regional therapy alone suggest that a proportion of patients have pre-existing micrometastatic disease at the time of treatment. The survival benefit associated with adjuvant chemotherapy almost certainly results from the eradication of disease in these pre-existing distant sites. It is also highly probable that the success of systemic therapy in eradicating systemic micrometastases is dependent, in part, on the tumor burden at the time of treatment. Neoadjuvant chemotherapy has the theoretical advantage of offering therapy against micrometastatic disease at the time of lowest disease burden. An initial delay in chemotherapy to first allow for local-regional therapy runs the risk of increasing disease in pre-existing micrometastatic sites to the point where chemotherapy can no longer be curative.

Conversely, there are also potential downsides to initial systemic treatment. The majority of patients currently cured of breast cancer are cured as a result of local-regional therapy. The reason for this fact is that the majority of patients currently cured of breast cancer do not have micrometastases at the time of their diagnosis. For these patients, an initial surgery removes the source of metastatic events (i.e. the primary tumor and any involved lymph nodes) at the earliest possible time interval. If these patients were treated with neoadjuvant chemotherapy, this source of metastatic disease would be left in place an additional 4–6 months thereby permitting micrometastases during neoadjuvant chemotherapy.

To investigate the optimal sequencing of chemotherapy and surgery, the National Surgical Bowel and Breast Project (NSABP) conducted a large prospective

randomized trial (designated B-18) to evaluate the efficacy of neoadjuvant versus adjuvant chemotherapy[4]. In this study, 1523 patients with early-stage breast cancer were randomized to receive four cycles of doxorubicin/cyclophosphamide (AC) either prior to or after a definitive surgical procedure. After 5 years, the overall survival and risk of distant metastases were identical between the two groups. These data can be interpreted as either demonstrating that there was no benefit for early treatment with systemic therapy against pre-existing micrometastases or that this benefit was exactly offset by the corresponding risk of continued seeding from the primary tumor.

To further explore this issue, we modeled the benefits and risks of neoadjuvant chemotherapy as a function of the stage of initial disease[5]. We estimated the rates of new systemic metastases developing per month as a function of the primary tumor size and lymph node status. These estimations were derived from the outcome of over 1722 breast cancer patients that were treated with local-regional therapy alone. The median follow-up of this population was 15 years. Our model predicted that for the women with large tumors or lymph node-positive disease, the benefit of early treatment of pre-existing micrometastases outweighed the risk of continued primary seeding. In contrast, for those women with a low risk of pre-existing micrometastases, the risk from continued primary seeding exceeded the benefits of early systemic treatment. Specifically, our model suggested that women with T1N0 disease would be more optimally treated with adjuvant chemotherapy whereas stage IIB or greater disease would likely benefit from neoadjuvant chemotherapy. Unfortunately, the NSABP trial has not reported their B-18 data stratified according to the initial stage of disease. However, a smaller trial from France that compared chemotherapy prior to or after local therapy (predominantly radiation alone) for women with stage II and III breast cancer in part supported our modeling study. In this study, there was an initial statistically improved survival rate among the women treated with neoadjuvant treatment compared to adjuvant[6]. With longer follow-up, a decreased rate of distant metastasis favoring patients treated with neoadjuvant chemotherapy was found on multivariate analysis[7].

Breast conservation

A major conclusion of the NSABP B-18 trial was that breast conservation rates were increased in women treated with neoadjuvant chemotherapy compared to those treated with adjuvant chemotherapy (67% versus 60%, respectively; $p = 0.002$)[4]. This advantage of neoadjuvant chemotherapy obviously only pertains to women who are not optimal breast conservative candidates at the time of diagnosis. In the B-18 study, the higher breast conservative rates in the neoadjuvant arm was predominantly attributed to an increased rate of breast conservation in the subgroup of women with T3 disease (22% versus 8% for the neoadjuvant versus adjuvant arms, respectively).

Importantly, the B-18 trial reported that overall ipsilateral breast recurrences as first events were equivalent in the neoadjuvant chemotherapy arm compared to the adjuvant arm, with respective (NSABP) B-18 rates of 7.9% and 5.8% ($p = 0.23$). However, the ipsilateral breast recurrence rate for women treated with neoadjuvant chemotherapy who were not optimally suited for breast conservation at the time of diagnosis were higher than the patients who were candidates for breast preservation at diagnosis (14.5% versus 6.9%, respectively; $p = 0.04$). These crude rates of recurrence are also likely to increase with longer follow-up. Therefore, whether large primary tumors can safely be treated with neoadjuvant chemotherapy and breast conservative therapy in community settings is still in question. In contrast to these data, selective experiences from single institutions have reported excellent local control rates for women with large T2 or T3 primary tumors treated with breast conservation therapy after neoadjuvant chemotherapy[8,9]. In our own institutional experience, the rates of local recurrence after neoadjuvant chemotherapy followed by breast conservation therapy for women with large primary tumor mirror the rates achieved in early-stage primary disease[9]. For women treated with neoadjuvant chemotherapy, it is critical that breast conservation treatment be carefully coordinated amongst treatment specialists. Patients need to be re-imaged during chemotherapy and for patients with excellent responses, metallic markers are required to demarcate the tumor bed. It is also important that careful attention to margin status is given after neoadjuvant chemotherapy. Breast cancer is an infiltrative disease and disease response to neoadjuvant chemotherapy is often not concentric. In our own institution, a multidisciplinary team sees the majority of potential breast conservation patients before neoadjuvant chemotherapy and again before surgery. Furthermore, the breast surgeons work closely with pathologists and radiologists to assess specimen radiographs and margin status at the time of breast conserving surgery.

Other considerations

Another advantage of neoadjuvant chemotherapy over adjuvant chemotherapy is that neoadjuvant treatment of gross disease permits a measurement of disease response. This advantage allows the chemotherapy to be changed for patients with suboptimal response. However, the relevancy of this advantage is limited to a small minority of patients because approximately 80% of patients achieve a partial or complete clinical response to neoadjuvant chemotherapy[4]. Furthermore, if the treating oncologist is predisposed towards treating with an anthracycline regimen sequentially followed by a taxane, the decision to use both agents is often independent of clinical response. Specifically, many feel that the group that is most likely to benefit from the addition of taxanes are those patients with anthracycline-responsive disease. In addition, the majority of oncologists feel that a taxane is warranted as a secondary agent for the patients with anthracycline-resistant disease.

An under-appreciated negative aspect of neoadjuvant chemotherapy concerns the change in value and potential utilization of prognostic information. A number of studies have indicated that pathological disease status after neoadjuvant chemotherapy continues to provide important prognostic information with respect to the endpoint of survival[4,10,11]. What is less clear, however, is how the pathological information should be used to determine indications for post-mastectomy radiation. Currently, post-mastectomy radiation is offered selectively to women felt to be at moderate or high-risk for local-regional recurrence. These risk determinations have been based on pathological parameters following an initial surgery. We recently reviewed our experience comparing pathological risk factors for post-mastectomy recurrence for patients treated with neoadjuvant chemotherapy compared to those treated with adjuvant chemotherapy. We found that the relative risk of having a local-regional recurrence was 1.5–2.5-fold higher for any given pathological tumor size or category of lymph node status in the neoadjuvant group compared to those receiving post-mastectomy adjuvant chemotherapy[12]. It should be recognized that the difficulty of determining indications for post-mastectomy radiation is not an inherent problem with neoadjuvant chemotherapy. Instead, more data concerning predictors of local-regional failure after neoadjuvant chemotherapy and mastectomy are needed in order to determine the proper indications for radiation use.

Neoadjuvant chemotherapy: conclusions

The advantages and disadvantages of sequencing chemotherapy prior to surgery will continue to be a controversial subject for many years to come. Based on the B-18 trial, NSABP has adopted neoadjuvant chemotherapy as a standard treatment. In its subsequent randomized B-27 trial, all three study arms received neoadjuvant chemotherapy:

AC × 4 + surgery
AC × 4 + surgery + docetaxel × 4
AC × 4 + docetaxel × 4 + surgery

With respect to treatment sequencing, this trial will provide important information concerning the efficacy of eight cycles versus four cycles of neoadjuvant chemotherapy and whether it is safe to defer local regional therapy for approximately 6 months in order to first deliver systemic therapy.

Based on the available clinical data, we feel that neoadjuvant chemotherapy is an appropriate sequencing strategy. The most relevant group of patients for which this approach should be considered are those at high-risk for pre-existing micrometastases and those interested in breast conservation therapy whose primary tumor extent precludes this approach at treatment onset. Treatment with neoadjuvant chemotherapy requires a closely coordinated effort on the part of surgeons, medical oncologists, radiation oncologists, pathologists and diagnostic radiologists. We recommend that patients be examined by medical, radiation and surgical oncologists at baseline for optimal timing and design of local/regional therapy. There is no practical advantage for using neoadjuvant chemotherapy for women who are excellent candidates for breast conservation therapy at the time of diagnosis, especially if the type and duration of systemic therapy has been predetermined. While for locally advanced and most stage III patients neoadjuvant chemotherapy is preferable, its use should be evaluated further in earlier stages to define its role and rule out the theoretical disadvantages.

SEQUENCING OF ADJUVANT CHEMOTHERAPY AND RADIATION

Breast conservation patients

The majority of breast cancer patients treated in the United States continues to have surgery as the initial component of their therapy. The most common treatment-sequencing question concerning these patients is the timing of adjuvant chemotherapy and radiation. One of the first studies addressing this issue was reported by the Joint Center of Radiation Therapy (JCRT) in

1991[13]. In this retrospective analysis of patients with lymph node-positive breast cancer treated with breast conservation therapy, the authors found that a delay in radiation of greater than 16 weeks from the time of surgery led to an increase in local recurrence, with a 5-year actuarial rate of 35%. Subsequent to this report, a number of other institutions have also reported similar analyses, with some confirming that radiation delay increases failure risk and others refuting this[14,15].

Based on their retrospective work, the JCRT conducted a small prospective trial that randomized patients treated with breast conservation therapy to receive either radiation followed by four cycles of chemotherapy or the same chemotherapy followed by radiation[16]. The initial results of this trial indicated that patients who received chemotherapy prior to radiation had a significantly lower rate of distant metastasis than the group that had radiation immediately following surgery (36% versus 25% at 5 years; $p = 0.05$). A higher percentage of patients treated with chemotherapy first experienced local recurrence within the breast, but the difference did not reach statistical significance (14% versus 5% at 5 years; $p = 0.07$). In an analysis of patient subsets, the higher rate of distant metastasis with chemotherapy delay was exclusively seen in the patients with four or more positive lymph nodes. Furthermore, the increase risk of local recurrence with radiation delay was seen exclusively in the patients with close or positive surgical margins. In general the results of this trial have significantly influenced the sequencing of chemotherapy and radiation in the United States and have led most institutions to sequence adjuvant chemotherapy prior to radiation.

There are some interesting aspects of the JCRT trial that warrant consideration. First, over 85% of patients in this study had lymph node-positive disease. It is not appropriate to extrapolate these data to patients with lymph node-negative disease because the risk of pre-existing micrometastatic disease is much less in this cohort. We recently reviewed our institutional experience of chemotherapy and radiation sequencing in 124 patients treated with breast conservation therapy for lymph node-negative disease and found that the rates of local and distant failures were not significantly influenced by the chemotherapy and radiation treatment sequencing strategy[17]. As we have emphasized obtaining negative margins for decades, only two patients in this study had positive surgical margins. Furthermore, the breast recurrence rates overall were very low, suggesting that women with negative surgical margins treated with both adjuvant radiation and chemotherapy have excellent local control rates with either sequencing strategy. It is our current practice to deliver adjuvant chemotherapy first for most women with early-stage disease. For the patients with positive margins in whom radiation delay may increase breast recurrence risk, we often advocate for a re-excision.

Another important aspect of the JCRT trial was the fact that it preceded the use of taxanes and included only four cycles of chemotherapy. Currently, it is not uncommon for patients with breast conservation therapy to receive eight cycles of chemotherapy. There are no current published data to assess the risk and benefits of sequencing radiation and eight cycles of chemotherapy. The results of B-27 should provide comparative information regarding local control after four or eight preoperative cycles of chemotherapy. In addition, the data from CALGB 9844 (AC × 4 versus AC × 4 followed by paclitaxel × 4) should provide important information regarding local control after 4 versus 8 cycles of neoadjuvant chemotherapy for patients treated with breast conservation.

Finally, it should be recognized that the JCRT trial was a relatively small study and the degree of reduction in metastatic disease development found in the trial is somewhat surprising. As mentioned, the difference in distant metastases was 11% lower with early chemotherapy at 5 years, decreasing from 36% to 25%[16]. This represents a 31% reduction in the risk of metastatic disease development. This degree of reduction is of the same magnitude reported in the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis of trials comparing adjuvant chemotherapy to no chemotherapy[1]. This suggests that for the JCRT findings to be true, the delay in chemotherapy to first give radiation completely ablated any possible benefit to post-radiation chemotherapy. The B-18 trial and other studies suggest that this degree of delay in administration in chemotherapy would be unlikely to completely negate its positive benefits[4]. Unfortunately, it is very unlikely that the JCRT sequencing trial will ever be repeated to confirm these findings. As such, it remains the only source of randomized data pertaining to adjuvant chemotherapy and radiation sequencing, and it is difficult to not respect the positive finding of the study. The results of this trial are likely to be updated soon and it will be interesting to see whether the data showing an increased rate of metastatic disease with chemotherapy delay continues to be present with longer follow-up.

There are alternative strategies for sequencing chemotherapy and radiation other than sequential administration of therapies. Examples of such alternatives

are concurrent chemoradiation and sandwich therapy. However, the EBCTCG meta-analysis indicated that an adjuvant anthracycline-containing regimen achieve had an improved outcome compared to a non-anthracycline regimen[1]. The high toxicity associated with concurrent anthracycline and radiation precludes a chemoradiation treatment approach using the most effective agents. A second alternative to sequential sequencing is to deliver radiation halfway through the adjuvant chemotherapy course. This 'split course' strategy has been tried in the past with radiation and in general has been unsuccessful, presumably due to repopulaton of tumor clonogens during the break from treatment. For these reasons, our preference has been to complete all adjuvant chemotherapy prior to use of radiation.

POST-MASTECTOMY SEQUENCING

Even fewer data are available concerning the sequencing of chemotherapy and radiation for women treated with initial mastectomy[14]. The recent randomized trials showing a benefit for radiation incorporated radiation after one to three cycles of chemotherapy[3,18]. However, many of the same considerations that were reviewed above for breast conservative treatment apply to the post-mastectomy setting. The mechanism underlying the increase in the distant metastasis rate seen in the group randomized to chemotherapy delay in the JCRT trial presumably reflects growth of pre-existing micrometastatic disease to the point where the chemotherapy is less effective. This phenomenon should be independent of the type of surgery performed.

TAMOXIFEN AND RADIATION SEQUENCING

The final area of clinical controversy surrounding treatment sequencing is whether to administer tamoxifen concurrently or sequentially with radiation. Historically, sequential treatment was recommended based on preclinical data suggesting that tamoxifen may arrest breast cancer cells in radioresistant cell cycle phases, which theoretically would decrease the efficacy of radiation treatment. However, this similar concern was frequently raised for the concurrent use of hormonal therapy and radiation for prostate cancer. Ironically, subsequent randomized clinical trials in prostate cancer suggested an improvement in outcome with concurrent hormone and radiation use compared to radiation alone[19].

Another possible drawback of concurrent tamoxifen and radiation use concerns the potential risk of pulmonary toxicity. A study of radiation-related lung changes using clinical data from a small randomized, prospective trial comparing post-mastectomy radiation versus post-mastectomy radiation with concurrent tamoxifen reported increased lung fibrosis in the tamoxifen-treated patients[20]. However, no symptom data was reported in this study and the clinical significance of this finding is unknown to date. It is also unclear from these data whether concurrent use would increase lung fibrosis compared to sequential use.

There are few data that specifically compare sequential versus concurrent tamoxifen use with respect to the endpoint of efficacy. One retrospective series from Yale University found that local recurrence rates were not affected by tamoxifen/radiation sequencing, with respective breast recurrence rates of 9% for concurrent tamoxifen, 4% for sequential tamoxifen, and 15% for those not treated with tamoxifen[21].

ADJUVANT TREATMENT: CONCLUSIONS

It is reasonable to consider the risk and benefits of any adjuvant sequencing approach according to the particulars of each case. While the JCRT randomized sequencing study was a relatively small trial that has never been repeated or reproduced, it suggests that chemotherapy delay can increase the risk of distant metastasis. These data were obtained from a patient cohort that predominantly had lymph node-positive disease. This study and others also suggest that the breast recurrence risk may be increased with radiation delay if negative margins are unable to be achieved. Therefore, a reasonable sequencing strategy would attempt to achieve negative surgical margins and administer chemotherapy prior to radiation. It is equally reasonable to consider using radiation prior to chemotherapy for lymph node-negative patients in whom margin status is close or positive. Our bias is to avoid split course therapy. In the future, data from the NSABP B-27 trial and others will provide greater insight as to whether the detrimental effect of tumor cell repopulation previously described with split course radiotherapy is also seen with split course chemotherapy.

ACKNOWLEDGEMENTS

Dr. Buchholz is supported by grant number BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

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APPENDIX 16

Neoadjuvant Chemotherapy For Breast Carcinoma

Multidisciplinary Considerations of Benefits and Risks

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Supported by Grant BC980154, a U. S. Army Medical Research and Materials Command (USAMRMC) Breast Cancer Research Program Career Development Award (Thomas A. Buchholz).

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Received May 5, 2003; accepted June 3, 2003.

DOI 10.1002/cncr.11603

BACKGROUND. The majority of patients with breast carcinoma receive chemotherapy as a component of multimodality treatment. Over the past decade, it has become increasingly more common to deliver chemotherapy first, but this has raised new questions within all disciplines of cancer management.

METHODS. The authors reviewed published studies on the effect of neoadjuvant chemotherapy for breast carcinoma on the practice of medical oncology, surgical oncology, radiation oncology, pathology, and radiology.

RESULTS. Treating breast carcinoma with neoadjuvant chemotherapy has several advantages, such as providing the earliest possible treatment against preexisting micrometastases, offering selected patients breast conservation therapy, and allowing for measurement of disease response, which can then be used to customize subsequent chemotherapy. However, neoadjuvant chemotherapy affects the practice not only of medical oncology, but also has important implications for the specialties of surgery, radiology, pathology, and radiation oncology. The current review addressed the new opportunities and challenges within the multidisciplinary care of breast carcinoma provided by neoadjuvant chemotherapy.

CONCLUSIONS. The complexity of the issues led the authors to conclude that patients who receive neoadjuvant chemotherapy are likely to benefit from a coordinated multidisciplinary approach to their care. ***Cancer* 2003;98:1150–60.**

KEYWORDS: breast carcinoma, neoadjuvant chemotherapy, surgery, radiation, pathology, diagnostic imaging.

Neoadjuvant chemotherapy, also called preoperative chemotherapy, is becoming an increasingly popular sequencing strategy in the multimodality treatment of breast carcinoma. It has several potential advantages compared with the traditional strategy of surgery followed by adjuvant chemotherapy. Neoadjuvant chemotherapy substantially reduces the size of the primary tumor and lymph node metastases in greater than 80% of cases, increasing the probability that breast-conserving surgery can be performed.[1–4] A second advantage of this sequencing schedule is that it permits the assessment of response of the primary tumor to a particular chemotherapy regimen. This assessment allows the opportunity to "cross over" to a different regimen for an individual patient if there is minimal or no response to the first regimen.

These and other theoretic advantages for neoadjuvant chemotherapy must be balanced carefully with other aspects of individual patient management. Neoadjuvant chemotherapy affects not only medical oncology decisions, but also those of all disciplines that participate in the multidisciplinary management of breast carcinoma, including surgical oncology, breast imaging, breast pathology, and

radiation oncology. Accordingly, it is imperative that clinicians from all of these disciplines participate in the decisions regarding treatment sequencing and work together to create a multidisciplinary infrastructure that is able to address new clinical questions raised by the increasing use of neoadjuvant chemotherapy.

A number of previously published articles, including some from The University of Texas M. D. Anderson Cancer Center (MDACC), have reviewed the use of neoadjuvant chemotherapy for breast carcinoma.[5,6] In general, these articles have focused on important medical oncology considerations concerning this form of treatment. The purpose of the current review is to provide a more comprehensive discussion of how neoadjuvant chemotherapy affects the multidisciplinary management of patients with breast carcinoma.

Benefits of Neoadjuvant Chemotherapy: Randomized Prospective Clinical Trials

One of the first considerations for studying neoadjuvant chemotherapy for breast carcinoma was to investigate whether earlier delivery of chemotherapy offered the possibility of improved survival in patients with locally advanced breast carcinoma. Breast carcinoma deaths most often result from progression of metastatic disease that was present at a microscopic level at the time of diagnosis. Therefore, it was rational to question whether initiating chemotherapy at diagnosis (when the micrometastatic tumor burden is lowest) would improve outcome compared with delaying chemotherapy until after surgical resection. Preclinical animal studies have indicated that the removal of the primary tumor could increase the growth rate of existing micrometastases.[7] It was reported that treating animals with either chemotherapy or tamoxifen before resection of the primary tumor abrogated this adverse effect.[7]

To test these concepts, the National Surgical Adjuvant Breast and Bowel Project (NSABP) began the B-18 trial to test whether sequencing chemotherapy before surgery would improve outcomes.[1,3] This trial enrolled 1523 patients with early-stage, operable breast carcinoma and randomized them to receive four cycles of doxorubicin/cyclophosphamide (AC) either before or after surgical treatment. The primary end points of this trial were disease-free and overall survival. With respect to these end points, the trial was a negative study. After 9 years, the overall survival and disease-free survival were nearly identical between the two groups ($P = 0.80$, $P = 0.50$, respectively). A second large, randomized prospective trial that directly compared the sequencing of chemotherapy and surgery was performed by the European Organization for Research and Treatment of Cancer (EORTC).[4] The trial randomized 698 patients to preoperative or postoperative chemotherapy comprised of four cycles of 5-fluorouracil (5-FU), epirubicin, and cyclophosphamide. Like the NSABP B-18 trial, the EORTC study demonstrated equivalent survival and rates of distant metastases between the two treatment arms.

The question of whether chemotherapy delay adversely affects outcome has also been addressed in earlier retrospective and prospective studies that investigated the sequencing of chemotherapy with radiation. The majority of these studies also found that delaying chemotherapy to first administer local treatments did not lead to a higher rate of distant metastasis.[8–10] The one notable exception was an early report of a randomized prospective clinical trial of adjuvant chemotherapy and radiation sequencing in early-stage breast carcinoma. The initial publication of this trial reported that chemotherapy given before radiotherapy decreased the risk of distant metastasis compared with radiotherapy given before chemotherapy.[11] However, with longer follow-up, this benefit was no longer present.[12] Therefore, in aggregate, the preponderance of the data suggests that delaying chemotherapy for short periods to first deliver local therapy does not compromise distant disease-free and overall survival.

Neoadjuvant Chemotherapy and Breast Preservation: the Costs and Benefits

Despite the finding that survival after neoadjuvant chemotherapy is not improved compared with survival achieved with adjuvant chemotherapy, there are other potential advantages associated with treatment with chemotherapy first. For example, a significant response to chemotherapy in the primary tumor may increase the potential to offer breast-conserving therapy (BCT). In the B-18 trial, the rates of BCT were 68% in the neoadjuvant arm and 60% in the adjuvant chemotherapy arm.[1,3] Importantly, 27% of the patients in the B-18 trial for whom mastectomy was originally planned were able to have a lumpectomy after the tumor responded to neoadjuvant chemotherapy. Similarly, the EORTC study found that 23% of patients requiring a mastectomy at presentation were able to undergo BCT after treatment with preoperative chemotherapy.[4]

Although increasing the rates of BCT is worthwhile, many details must be carefully considered. First, this benefit is limited to patients whose tumor size at diagnosis precludes BCT. In addition, the tumor-to-breast size ratio is only one of several possible reasons why a patient might not be a candidate for

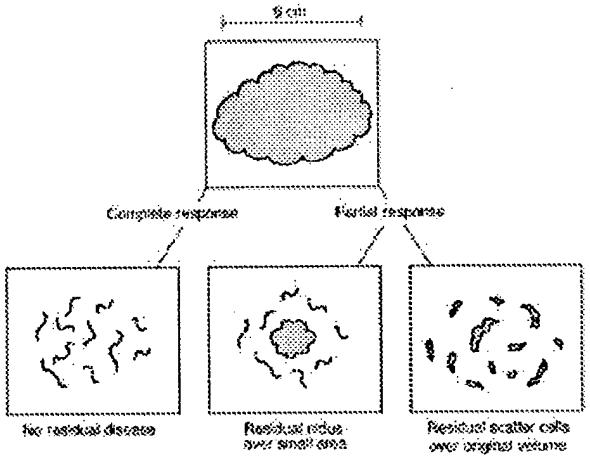


FIGURE 1. Examples of the various pathologic responses observed after neoadjuvant chemotherapy. In some instances, malignant cells are clustered around a residual nidus after disease response. In other cases, residual tumor cells are scattered over the residual volume of disease. A breast-conserving surgical procedure directed toward a central nidus may leave different volumes of residual disease in these two clinical scenarios.

BCT. Other reasons, which cannot be overcome by administering neoadjuvant chemotherapy, include multicentric disease, extensive microcalcifications throughout the breast, and coexisting medical conditions that predispose to radiotherapy injuries.

A second important issue concerning BCT is whether treatment with neoadjuvant chemotherapy increases the rate of ipsilateral breast carcinoma recurrence. Primary tumors respond to neoadjuvant chemotherapy in a variety of ways (Fig. 1). In some cases, a partial response to chemotherapy may leave nests of residual microscopic disease throughout the original volume of the primary tumor. A breast-conserving surgical procedure directed at the identifiable nidus of residual cancer may leave residual microscopic disease, increasing the risk of disease recurrence in the breast. In the B-18 trial, the crude ipsilateral breast carcinoma recurrence rate (first events only) was 10.7% in the patients treated with neoadjuvant chemotherapy versus 7.6% in those treated with adjuvant chemotherapy ($P = 0.12$).[3] The slight increase in the breast carcinoma recurrences for patients treated with neoadjuvant chemotherapy was, in part, attributable to a higher rate in the subset of 69 patients who were considered to be candidates for mastectomy at diagnosis but became BCT candidates after the tumor responded to chemotherapy. The breast carcinoma recurrence rate in these 69 patients was 15.9% versus a 9.9% rate in the patients treated with neoadjuvant chemotherapy who were candidates for BCT at diagnosis ($P = 0.04$).[3] In the EORTC trial, after a median follow-up of 56 months, there were no differences in the rates of breast carcinoma recurrence between patients treated with neoadjuvant chemotherapy and those treated with adjuvant chemotherapy.[4]

Other multicenter studies have reported higher rates of breast carcinoma recurrence after neoadjuvant chemotherapy and BCT. Rouzier et al.[13] retrospectively analyzed the breast carcinoma recurrence rate in 257 patients treated in various French hospitals with neoadjuvant chemotherapy followed by BCT. The 5-year and 10-year ipsilateral breast carcinoma recurrence rates were 16% and 21%, respectively. The tumor recurrence rates were highest in women younger than 40 years old (40% at 10 years) and in patients with residual primary disease of 2 cm or greater (30% at 10 years). A second French multiinstitutional trial also reported high rates of breast carcinoma recurrence after neoadjuvant chemotherapy and BCT. In that trial, patients with primary tumors greater than 3 cm in dimension were randomized to receive mastectomy and adjuvant chemotherapy or neoadjuvant chemotherapy with the goal of breast preservation.[14] Of the 40 patients treated with neoadjuvant chemotherapy and BCT, the crude total breast carcinoma recurrence rate was 22.5% and the crude isolated breast carcinoma recurrence rate was 15% (the median follow-up period was 124 months). In contrast to these data from multicenter trials, selected studies from single institutions have not reported increased rates of breast carcinoma recurrences in patients treated with neoadjuvant chemotherapy. Bonnadona et al.[15] noted a breast carcinoma recurrence rate of 6.8% (the median follow-up period was 65 months) among 456 women with initially large primary tumors treated with BCT after they responded to neoadjuvant chemotherapy. Similarly, in a series of 109 patients with a median primary tumor size of 4 cm who were treated with neoadjuvant chemotherapy at MDACC, the 5-year locoregional disease recurrence (LRR) rate was 5%.[16]

The differences in outcome between studies may be due to a number of reasons. First, single institutions are likely to have stricter selection criteria. Table 1 lists the MDACC criteria for selecting patients for BCT after neoadjuvant chemotherapy. In the MDACC series, all patients treated with BCT after neoadjuvant chemotherapy had pathologically negative margins.[16] In contrast, in the Rouzier et al. study,[13] which showed a relatively high rate of breast tumor recurrence, 11% of the patients had positive margins and 18% had close margins. The differences in outcome may also be attributable to better coordination of treatment across various disciplines at some institutions. Neoadjuvant

TABLE 1
Selection Criteria and Contraindications for Breast-Conserving Therapy after Neoadjuvant Chemotherapy

Selection criteria
Ability to completely resect residual disease and maintain an acceptable aesthetic outcome
Contraindications for BCT
Diffuse microcalcifications throughout breast
Multicentric disease
Inability to achieve negative margins
Inability to localize primary tumor secondary to a complete clinical response
Abnormal postoperative mammogram
Inability or unwillingness to be treated with radiation
Medical contraindications to radiation

BCT: breast-conservation therapy.

chemotherapy presents special challenges for the breast surgeon, the radiologist, and the pathologist. To obtain optimal results, responses to these challenges need to be coordinated among the treating team and addressed prospectively. In the sections that follow, we have outlined specific considerations concerning the use of neoadjuvant chemotherapy in breast carcinoma according to the various specialties involved in the management of breast carcinoma.

Medical Oncology Considerations concerning Neoadjuvant Chemotherapy

Optimizing response rates

Most of the initial experience with neoadjuvant chemotherapy was obtained in patients with locally advanced breast carcinoma treated with three or four cycles of anthracycline and alkylating agent-containing regimens (cyclophosphamide, doxorubicin/epirubicin, with or without 5-FU). Under these conditions, objective responses, defined as greater than 50% reductions in the product of the two longest perpendicular diameters of the primary tumor, ranged from 80% to 90% and complete clinical remission rates ranged from 5% to 13%.[1–4,17,18]

Pretreatment tumor size, duration of chemotherapy, and use of alternating chemotherapy regimens may alter these response rates. For example, one report indicated a correlation between tumor size and clinical complete remission rate and found that 60% of patients with T1 tumors achieved a complete clinical response.[2] Longer treatment duration may also improve maximal tumor response for some patients. The initial practice of administering three or four cycles of neoadjuvant chemotherapy was arbitrary. However, patients for whom the major objective of neoadjuvant chemotherapy is to maximize the possibility of BCT, careful monitoring of changes in tumor dimensions might justify the administration of more treatment cycles before surgery. The feasibility of administering up to eight cycles of neoadjuvant chemotherapy has been demonstrated in several recent prospective clinical trials.[19,20]

Using sequential, non–cross-resistant chemotherapy regimens in the neoadjuvant setting also may increase response rates. In addition, the delivery schedule for chemotherapy may be an important variable. In a prospective clinical trial conducted at MDACC, 29% of the patients treated with 12 cycles of weekly paclitaxel followed by four cycles of 5-FU, doxorubicin, and cyclophosphamide (FAC) achieved a pathologic complete response.[21] This pathologic response rate was more than twice that of patients randomized to receive four cycles of neoadjuvant single-agent paclitaxel given every 3 weeks, followed by four cycles of FAC. In addition, a Scottish trial found that combining four cycles of neoadjuvant cyclophosphamide, vincristine, doxorubicin, and prednisolone (CVAP) with four cycles of docetaxel achieved a better overall response rate (94% vs. 66%), clinical complete remission rate (62% vs. 34%), pathologic complete remission (pCR) rate (34% vs. 16%), and disease recurrence-free survival rate (92% vs. 73% at 3 years) compared with treatment with eight cycles of CVAP.[22]

Finally, combining non–cross-resistant chemotherapy regimens in the neoadjuvant setting has also been adopted in the most recent NSABP trial, protocol B-27. This three-arm study randomized patients to receive neoadjuvant AC for four cycles and then surgery, AC for four cycles followed by surgery then docetaxel for four cycles, or four cycles of AC and four cycles of docetaxel followed by surgery. The arm in which both AC and docetaxel were given preoperatively produced a better overall response rate (91% vs. 85%), clinical complete response rate (65% vs. 40%), and complete pathologic response rate (26% vs. 14%) than the other two arms.[20] The long-term data from this trial will indicate whether this improved response rate translates into higher disease recurrence-free and overall survival rates.

Benefits of measuring response

One of the important practical benefits of neoadjuvant chemotherapy is the ability to take serial measurements of the primary tumor and any involved lymph nodes. When chemotherapy is given after surgical resection of all macroscopic disease, the effectiveness of adjuvant chemotherapy for an individual case cannot be determined until long-term outcome data are available. The ability to quantify response of disease to neoadjuvant chemotherapy offers two potential advantages. Data from Phase II and III clinical trials

indicated that 3–5% of patients will have total drug resistance and progressive disease during neoadjuvant FAC/5-FU, epirubicin, and cyclophosphamide (FEC) chemotherapy.[1–4,17,18] For these unusual cases with no response or progression, the treatment can be stopped, thus preventing further toxicity, inconvenience, and cost. The chemotherapy regimen can be changed to a non–cross-resistant regimen or a new strategy (surgical resection/radiation/high-dose chemotherapy with stem cell transplant) can be instituted without further delay.

Prognostic factors are different with neoadjuvant versus adjuvant chemotherapy

Most patients with breast carcinoma have a significant decrease in the volume of disease after treatment with neoadjuvant chemotherapy. Therefore, prognostic factors, such as tumor size and number of involved lymph nodes, often change during the course of treatment. Data suggest that the prognosis of patients treated with neoadjuvant chemotherapy is dependent on both the initial extent of disease and the response of the disease to treatment. Although initial clinical stage and tumor size are important, the most powerful predictor of long-term outcome is the extent of residual disease present after completion of neoadjuvant chemotherapy. Data from multiple clinical trials have indicated that pCR, i.e., no residual cancer in the breast or lymph nodes after neoadjuvant chemotherapy, is associated with an excellent long-term prognosis.[2,17,18] For example, data from MDACC indicated that the 5-year survival rate was 89% in patients in whom a pCR was achieved versus a rate of 64% in those in whom it was not ($P < 0.01$).[18]

Neoadjuvant chemotherapy as a clinical and translational research tool

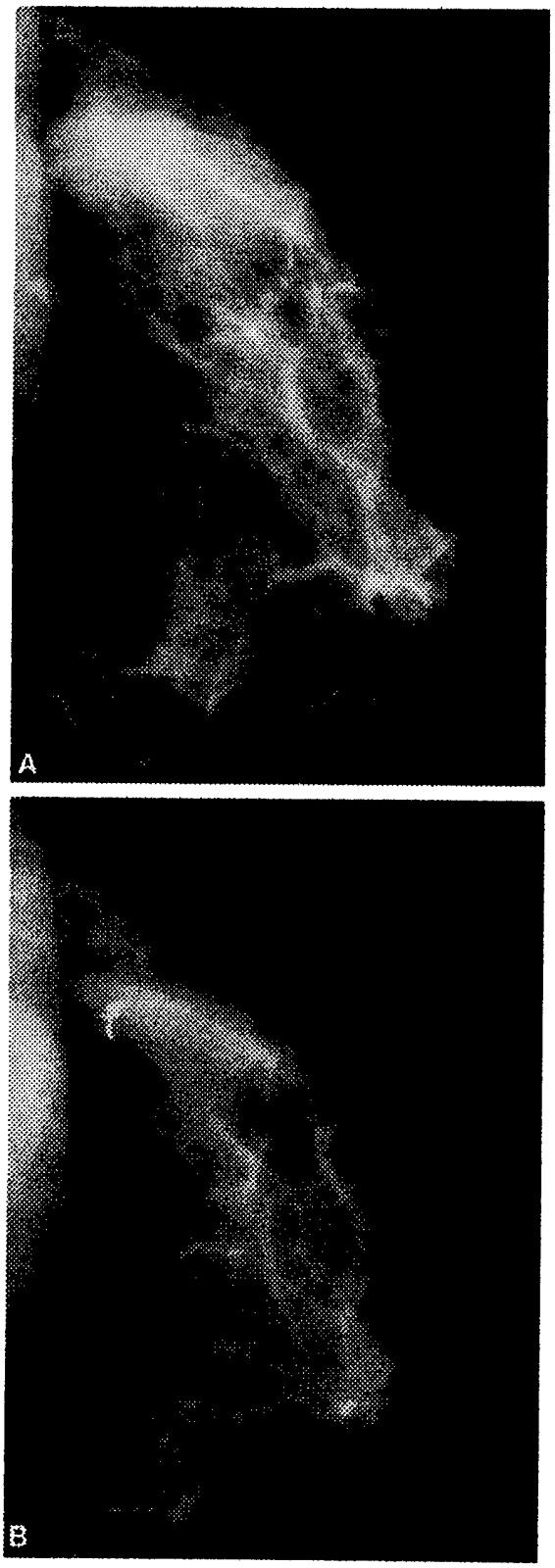
Neoadjuvant chemotherapy is also a valuable research tool. Because pCR rates strongly correlate with excellent long-term survival, they can be used as a short-term surrogate of the success of treatment. For example, the Phase III randomized trial that indicated that 12 cycles of weekly paclitaxel followed by four cycles of FAC achieved nearly a doubling of the pCR rate compared with four cycles of paclitaxel every 3 weeks followed by the same FAC schedule provided immediate data, indicating that the weekly delivery of paclitaxel had greater activity.[21] For a similar adjuvant chemotherapy trial, this finding could only be ascertained after 5–10 years of follow-up. The greatest value of using pCR as a surrogate end point is found for studies that directly compare two regimens in which all chemotherapy is given preoperatively.

In addition to being an important tool for assessing the success of clinical treatment strategies, neoadjuvant chemotherapy can also facilitate translational research investigating mechanisms of chemotherapy-induced cell death and mechanisms of chemotherapy resistance. For example, Buchholz et al.[23] demonstrated recently that it is feasible to simultaneously measure expression of more than 50,000 genes using microarrays and to determine how the expression of these genes changes during a course of neoadjuvant treatment.[23] In addition, Pusztai et al.[24] recently identified an expression profile of a set of genes that correlated with the probability of achieving a pCR after paclitaxel/FAC chemotherapy. Thesestudies are likely to provide significant insights into the heterogeneity of tumor response and identify new targets for therapies.

Breast Imaging Considerations concerning Neoadjuvant Chemotherapy

Imaging plays a critical role both in the initial assessment of disease in patients who are to be treated with neoadjuvant chemotherapy and in the assessment of disease response. During the initial assessment of the disease, imaging can be used to determine tumor volumes and identify multifocal and multicentric disease, features that will likely affect decisions concerning subsequent locoregional treatments. In addition, imaging can be utilized at the time of initial staging and during neoadjuvant chemotherapy to evaluate the regional (axillary, infraclavicular, supraclavicular, and internal mammary) lymph nodes for evidence of metastases. For example, we routinely use sonography to assess the regional lymph nodes and perform ultrasound-guided fine-needle aspiration biopsies to confirm the presence of regional disease. This documentation of initial disease extent also frequently affects subsequent surgery and radiation oncology treatment decisions.

In patients with a favorable response to neoadjuvant chemotherapy, metal clips should be placed at the tumor site to assure that the tumor bed can be clearly localized for a subsequent breast-conserving procedure. At MDACC, 3-mm stainless steel MicroMark II clips (Ethicon Endo-Surgery Inc., Cincinnati, OH) are placed at the tumor site with stereotactic guidance and 0.89-mm platinum embolization coils (Cook, Inc., Bloomington, IN) or metal markers (UltraClip, Inrad, Inc., Kenturrid, MI) are placed with sonographic guidance. If the tumor disappears during imaging and a marker was not placed, techniques to perform needle localizations with mammographic guidance are now available. Placing metal markers is preferred, however, as mammographic needle localization of disappearing tumors is labor intensive and



somewhat complicated, requiring careful planning and appropriate original mammograms (including straight lateral views). Figure 2 shows an example of pretreatment and posttreatment mammograms for a patient treated with neoadjuvant chemotherapy for an upper-outer quadrant breast carcinoma. Metal coils were placed in the tumor bed after an initial response to treatment and the curls can be localized easily on the postchemotherapy mammogram. In addition to being of value for surgical localization of the tumor bed, these metal markers aid in the histopathologic evaluation of the tumor site.

Calcifications associated with a primary tumor mass can often act as an intrinsic marker of tumor location. Although a decrease in the size of the primary tumor mass is achieved in 80% of cases, malignant-appearing calcifications very rarely regress during a course of treatment.[25] Therefore, patients who present with calcifications over a significant volume are very unlikely to become candidates for breast conservation after treatment with neoadjuvant chemotherapy.

Although imaging has a significant value in directing therapy, newer modalities are needed to improve the accuracy of disease response monitoring. Currently, imaging tools are unable to define patients who have achieved a pCR. In addition, further work is needed to define pretreatment characteristics that may predict the tumors that will have a favorable response to treatment. A number of groups are currently investigating the value of magnetic resonance imaging scans, positron emission tomography, and sestamibi imaging to monitor response to neoadjuvant chemotherapy. Imaging of expression of particular gene products is now possible and may eventually prove to be a noninvasive method to direct biologically targeted therapies.

Surgical Oncology Considerations concerning Neoadjuvant Chemotherapy

Ideally, the breast surgeon should assess patients for their surgical options before chemotherapy is initiated or early in the course of therapy to help patients understand the feasibility of BCT. Although the final determination of the feasibility of BCT is often based on the patient's degree of response, in some cases the

FIGURE 2. An example of (A) prechemotherapy and (B) postchemotherapy mammograms in a woman with an upper-outer quadrant breast tumor. Mediallateral oblique images are shown. The initial mass decreased in size with chemotherapy. To ensure that the tumor bed can be localized for surgery, metal clips were inserted into the tumor bed using ultrasound guidance.

surgeon can determine at diagnosis that the probability of successful BCT is low. Examples include patients who present with diffuse pleomorphic microcalicifications throughout the breast and patients with known multicentric disease.

For patients who are candidates for BCT at diagnosis, it is very unusual for them to become ineligible for BCT because of disease progression during treatment. For patients whose disease extent at diagnosis makes them suboptimal candidates for BCT, the major determinant regarding the feasibility of BCT after neoadjuvant chemotherapy is the disease response. The MDACC institutional selection criteria and contraindications for BCT after neoadjuvant chemotherapy are shown in Table 1. In addition, Newman et al.[26] recently demonstrated that patients with lobular carcinomas are less likely to become candidates for BCT after neoadjuvant chemotherapy. Based on these criteria, the probability that a patient who is not a candidate for BCT will become a BCT candidate after neoadjuvant chemotherapy is approximately 20–30%.[16,26] The aim of a segmental mastectomy after neoadjuvant chemotherapy is to remove all residual foci of clinically evident or radiographically visible disease and achieve negative histologic margins. The target volume of resection is the postchemotherapy abnormality, and attempts are not made to remove the prechemotherapy volume of disease.

A second important surgical consideration for patients treated with neoadjuvant chemotherapy is the feasibility and efficacy of sentinel lymph node surgery. It is conceivable that sentinel lymph node identification rates would be lower secondary to the fibrosis of lymphatic channels caused by the chemotherapy. In addition, false-negative rates may be higher due to a fibrotic reaction to disease response within a sentinel lymph node that prevents radiocolloid or dye uptake or to selective eradication of disease within the sentinel lymph node but not within nonsentinel axillary lymph nodes. These concerns have led a number of groups to investigate sentinel lymph node dissections for patients treated with neoadjuvant chemotherapy. Similar to the technique performed before chemotherapy, there is a learning curve to successfully performing sentinel lymph node surgery after neoadjuvant chemotherapy. For example, Breslin et al.'s initial identification rate of sentinel lymph nodes was 65% after neoadjuvant chemotherapy, but this has subsequently improved with experience to a rate of 94%.[27] In their first reported experience, Breslin et al.[27] reported three false-negative events out of a total of 25 patients with positive lymph nodes. Table 2 lists the identification rates and false-negative rates for various studies.[27–36] The integration of lymphatic mapping into the surgical management of patients receiving neoadjuvant chemotherapy has been reviewed in further detail by Kuerer and Hunt.[37]

TABLE 2
Success of Sentinel Lymph Node Surgery after Neoadjuvant Chemotherapy

Reference	No. of patients	Identification rate (%)	False-negative rate (%)
Mamounas et al.[28]	325	83	11
Breslin et al.[27]	51	84	12
Fernandez et al.[33]	40	85	25
Miller et al.[32]	35	86	0
Stearns et al.[30]	34	85	14
Haid et al.[34]	33	88	0
Julian et al.[35]	31	94	0
Tafra et al.[29]	29	93	0
Balch et al.[36]	26	96	7
Nason et al.[31]	13	87	33

For patients who require or elect mastectomy after preoperative chemotherapy, careful consideration must be given to the use of reconstructive surgery. Postmastectomy radiation should be recommended for patients with locally advanced disease at presentation but may not be required for patients with limited, early-stage disease based on prechemotherapy imaging and clinical examination. However, some patients believed to have early-stage disease before chemotherapy may have a significant volume of microscopic disease identified in the breast and regional lymph nodes after surgery. Postmastectomy radiotherapy would be indicated in these patients. If immediate breast reconstruction has been performed with either implants or autologous tissue, this may complicate the design of the radiation treatment fields. We discuss the possibility of postmastectomy radiotherapy with all patients treated with preoperative chemotherapy for whom mastectomy with immediate breast reconstruction is planned. This allows the plastic surgeon to plan appropriately for the possibility of radiotherapy and discuss with the patient the potential impact of radiotherapy on the overall cosmetic outcome.

Pathology Considerations concerning Neoadjuvant Chemotherapy

Biologic predictive factors of chemotherapy response

Table 3 summarizes the relationship between the biologic features of breast carcinomas and the response to chemotherapy. Histologic or nuclear grade has the strongest correlation with response.[2,38] Well differentiated tumors seldom, if ever, achieve a pCR, whereas nearly all of the pCR occur in patients with poorly

TABLE 3
Biologic and Pathologic Factors and Their Association with Response to Neoadjuvant Chemotherapy

Factors that are consistently associated with a favorable response
High nuclear grade
Factors that may be associated with a favorable response
ER-negative disease
Mitotic index
Mitosin staining
High apoptosis
Decrease in Bcl-2
Factors that may be associated with an unfavorable Response
ER-positive disease
HER-2/*neu* overexpression
P53 mutation
Low Bax
Factors that are associated consistently with an unfavorable response
Well differentiated tumors

ER: estrogen receptor.

differentiated tumors. In addition to high nuclear grade, high tumor proliferative rate assessed by mitotic index or immunohistochemical evaluation of proliferation-related proteins such as mitosin and Ki-67 has been reported to correlate with pCR.[38] Some reports have also found that patients with estrogen receptor (ER)-negative tumors respond more often and more completely to neoadjuvant chemotherapy than patients with ER-positive tumors, although this may, in part, be due to the greater likelihood that ER-negative tumors are high grade.[18,39,40] HER-2/*neu* amplification/overexpression has not been shown consistently to affect response to FAC chemotherapy, but retrospective analyses of several trials have suggested that patients with HER-2/*neu*–positive tumors benefit more from treatment with anthracycline-containing regimens than from other treatments.[41]

Finally, in a small pilot trial, Buchholz et al.[42] demonstrated that the degree of treatment-induced apoptosis, determined 48–72 hours after the initiation of treatment, may also predict response to chemotherapy. Similarly, a decrease in the tumor expression of *bcl*-2, a negative regulator of apoptosis, was found to be correlated with chemotherapy response.

Pathologic responses of breast carcinoma to neoadjuvant chemotherapy

Primary breast carcinomas display a varied response pattern to neoadjuvant chemotherapy. Figure 1 illustrates various examples. Often, tumors decrease concentrically, resulting in a residual nidus of tumor. The breast parenchyma around such tumors frequently shows therapy effects, such as increased fibrosis. Other tumors that also have a clinically apparent response to neoadjuvant chemotherapy have scattered microscopic foci throughout the original volume of disease. It is more difficult to achieve negative surgical margins with these tumors and there is a greater risk that residual disease will remain if limited surgery is performed.

Careful pathologic processing and assessment of specimens is essential to optimizing the outcome of BCT after neoadjuvant chemotherapy. Within MDACC, the handling of specimens is a closely coordinated effort between surgeons, radiologists, and pathologists. Before surgery, patients are screened carefully to assure that the clinical features listed in Table 1 are met. For BCT candidates, the surgeon carefully orients the segmental mastectomy specimen. After the specimen is inked, the tissue is sectioned and a specimen radiograph of the serial slices is obtained (Fig. 3). This allows for re-resection of regions in which the margin status is close or positive during the same operative procedure.

On final processing, measurements of tumor size and final margin assessment are provided, including comments regarding evidence of treatment-induced fibrosis. For example, scattered foci of disease over a 5-cm region should be distinguished from a 5-cm residual bulky primary tumor. In addition, during the evaluation of axillary lymph nodes, fibrosis in regions of previous involvement can be seen. This information may help the radiation oncologist to select the appropriate target of radiation fields.

Radiation Oncology Considerations concerning Neoadjuvant Chemotherapy

The use of neoadjuvant chemotherapy has posed new questions concerning radiotherapy treatments for breast carcinoma. In general, the radiation dose and the treatment fields used to treat the breast are not affected by the sequencing of chemotherapy and surgery. However, the use of neoadjuvant chemotherapy may affect decisions regarding regional lymph node irradiation. Specifically, the number of positive lymph nodes is often the primary determinant on whether the axillary apex and the supraclavicular fossa are included in the radiation fields. There are too few data available to ascertain whether patients treated with neoadjuvant chemotherapy should have a different threshold of axillary disease extent, which justifies the additional morbidity of adding treatment to the axillary apex and the supraclavicular region compared with patients who are treated with surgery first.

An analogous and more common question regarding the appropriate thresholds for adding radiation treatments concerns patients who undergo a mastectomy after neoadjuvant chemotherapy. Historically, indications for postmastectomy radiation have

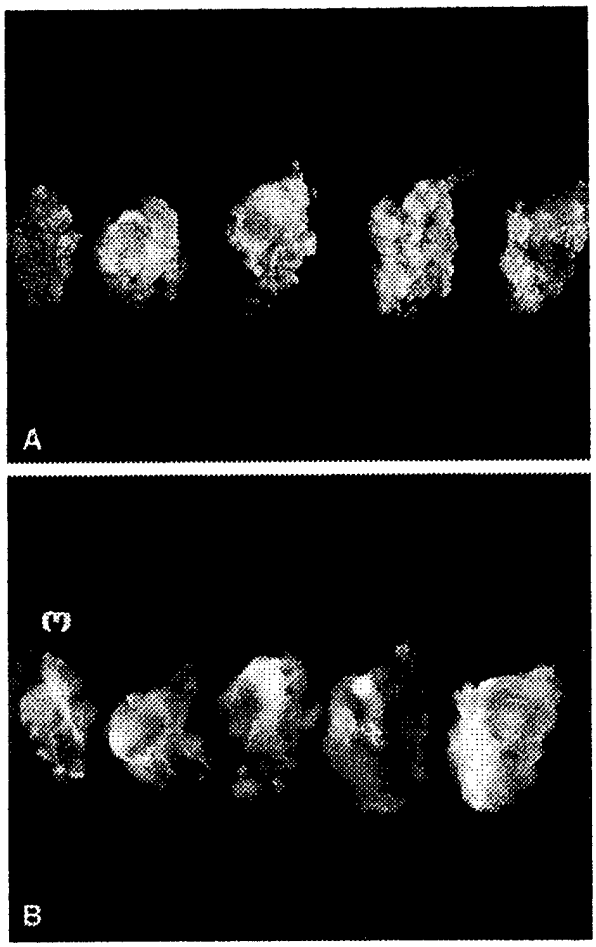


FIGURE 3. (A) Serial sections of a macroscopic specimen resected as a component of breast-conserving surgery. Before sectioning, the specimen is inked with multiple colors for orientation of the margins. (B) Specimen radiographs then are taken to assess the proximity of disease to the surgical margin. This allows for re-excision during the same operative procedure. Metal coils were localized in the center of the specimen, but an area of increased density approached a margin. Additional tissue was resected in this region and negative final margins were achieved.

been based on the pathologic extent of disease. The American Society of Therapeutic Radiation Oncology and the American Society of Clinical Oncology have published consensus statements that women with four or more involved axillary lymph nodes and patients with locally advanced disease should receive postmastectomy radiation.[43,44] For women with Stage II breast carcinoma with one to three involved lymph nodes, the use of radiation after mastectomy and chemotherapy is controversial.

As neoadjuvant chemotherapy changes the extent of pathologic disease in the majority of patients, the appropriate selection criteria for postmastectomy radiation remain largely unknown. Recently, Buccholz et al.[45] studied this issue by investigating LRR patterns in a group of patients treated with neoadjuvant chemotherapy followed by mastectomy without radiation. They demonstrated that both the initial clinical stage and the final pathologic extent of disease were important in assessing LRR risk. Specifically, they found that the LRR risks associated with any postchemotherapy pathologic primary tumor size and any category of positive lymph nodes (none, one to three, four, or more) were higher than those associated with the same volume of untreated disease.[46] These data indicate that the risk for LRR is a function of both the extent of pathologic residual disease and the initial clinical stage. Most noteworthy, in a small subset of patients with locally advanced breast carcinoma who achieved a pCR, the LRR rate remained relatively high (19%; 95% confidence interval, 6–48%). A high risk of LRR (46%) was also observed in a group of patients who initially had clinically T3 or T4 primary tumors but after chemotherapy had tumors less than 5 cm in dimension and 1–3 positive lymph nodes.[47]

We currently recommend postmastectomy radiation for all patients with clinical T3 tumors or clinical Stage III disease, regardless of their response to the chemotherapy regimen. For patients with clinical Stage I or II breast carcinoma, we offer postmastectomy radiation for patients with four or more positive lymph nodes after chemotherapy or the unusual case in which the primary tumor exceeds 5 cm in diameter.

Conclusions

Neoadjuvant chemotherapy has a number of advantages for patients with operable breast carcinoma. It affords selected patients their only option for BCT, it permits an in vivo assessment of chemotherapy response, and it provides a model system for the study of new systemic treatment strategies. It is clear that neoadjuvant chemotherapy can be administered safely without compromising clinical management. However, optimal treatment requires close coordination among the various oncology disciplines including medical oncology, surgical oncology, radiation oncology, diagnostic radiology, and pathology.

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ORIGINAL ARTICLE

Chemotherapy-Induced Apoptosis and Bcl-2 Levels Correlate with Breast Cancer Response to Chemotherapy

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PURPOSE

The relevance of apoptosis to breast cancer response to chemotherapy is unclear. We investigated whether changes in tumor cell apoptosis and Bcl-2 expression immediately after chemotherapy correlated with response to breast cancer treatment.

PATIENTS AND METHODS

Serial core biopsies of 25 breast cancer primary tumors were performed at either two or three time points: before treatment ($N = 24$) and approximately 24 hours ($N = 22$) and/or 48 hours ($N = 19$) after the initiation of the first cycle of chemotherapy. Apoptosis levels were quantified by use of a fluorescent terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) stain, and Bcl-2 and Bax were measured by semiquantitative immunohistochemical assays. All calculated *P* values were two sided.

RESULTS

The apoptosis level at 48 hours was significantly higher in the tumors with pathological complete response or < 1 cm of residual disease (median, 22%; range, 6%–51%) than in the tumors with > 1 cm residual disease (median, 7%; range, 1%–36%); Mann-Whitney test. This difference was also present in the subgroup of 16 tumors treated with docetaxel/doxorubicin chemotherapy (25% vs 4%, respectively). A decrease in Bcl-2 expression after chemotherapy relative to the expression from the pretreatment sample also correlated with disease response. Specifically, three of the nine tumors with a decrease in Bcl-2 had a pathological complete response, compared with 0 of the 15 tumors with stable levels of Bcl-2 (Fisher's exact test). There was no relationship between serial measurements of Bax and response.

DISCUSSION

These data suggest that apoptosis may play an important role in determining breast cancer response to chemotherapy and that the level of treatment-induced apoptosis may have some value as a predictive marker. *(Cancer J 2003;9:33–41)*

KEY WORDS

Apoptosis, breast cancer, Bcl-2, doxorubicin, docetaxel

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Received on July 16, 2002; accepted for publication September 25, 2002.

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this article. This study was funded by a clinical and translational research grant from The University of Texas M. D. Anderson Cancer Center Physician Referral Service and supported in part by The University of Texas M. D. Anderson Breast Cancer Tumor Bank and the National Cancer Institute Department of Health and Human Services grant nos. CA16672 and T32CA77050. Dr. Buchholz is supported by grant no. BC980154, a USAMRMC Breast Cancer Research Program Career Development Award. Dr. Esteva is supported by a National Cancer Institute Career Development Award (grant no. K23-CA82119). This work was presented in part at the San Antonio Breast Cancer Symposium, December, 2001.

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Gaining further insight into the mechanisms involved in chemotherapy-induced breast cancer cell death is a goal that has significant clinical relevance. If early molecular determinants of chemotherapy response are established, greater individualization of systemic treatments should become possible. In addition, an improved understanding of the factors influencing tumor response may identify relevant targets against which new therapies can be designed.

Numerous studies have investigated the role that the apoptotic pathway may play in determining the response of solid tumors to chemotherapy. For example, Meyn et al[1] reported that cyclophosphamide treatment of mice bearing the murine mammary adenocarcinoma MCa-4 increased the apoptotic index from 2.5% at baseline to more than 20% immediately after treatment. This degree of apoptosis was also found after treatment with cisplatin, doxorubicin, and ionizing radiation.[2,3] Other in-

vestigators subsequently reported that taxanes also initiate early cell death after treatment. In an in vivo study, three of four murine mammary adenocarcinomas showed a significant increase in apoptosis over baseline (e.g., from 1.2% to 23.7%) shortly after treatment with paclitaxel.[4] Furthermore, the baseline and paclitaxel-induced levels of apoptosis statistically correlated with tumor growth delay, whereas the peak percentage of cells displaying mitotic arrest did not.[4] A similar study investigating docetaxel found no correlation between apoptosis and chemotherapy response in vivo.[5] However, this study did demonstrate a significant degree of cell lysis within 72 hours of chemotherapy, and the degree of cell lysis was greater in tumors with a significant growth delay.[5] Only two preliminary reports have been published that investigated the correlation between apoptosis levels and chemotherapy response in human breast cancer.[6,7] Both of these studies suggested that early cell death might be an important predictor of the success of chemotherapy.

The available human data from studies correlating pretreatment expression of biomarkers involved in the apoptotic pathway with breast cancer outcome have been inconsistent. Specifically, there have been contradictory results concerning the predictive and prognostic importance of *p53* mutations and Bcl-2 expression in human breast cancer.[8-14] We hypothesized that expression of Bcl-2 and other markers important in the apoptotic pathway would be more predictive of chemotherapy response if they were measured shortly after exposure to chemotherapy rather than before. The purpose of this study was to investigate changes in apoptosis and expression of Bcl-2 and Bax 24 and 48 hours after treatment, and determine whether these changes correlate with breast cancer response to preoperative chemotherapy.

PATIENTS AND METHODS

This study was prospectively performed after receiving approval from the human subjects institutional review board at The University of Texas M.D. Anderson Cancer Center and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services. All participants provided written informed consent for this study. Thirty patients with breast cancer who were scheduled to begin definitive treatment with neoadjuvant chemotherapy consented to undergo serial core biopsies. One patient was treated for synchronous bilateral breast cancers and underwent biopsies of both cancers. Six patients elected not to undergo the postchemotherapy biopsies or had false-negative posttreatment biopsy results (no tumor material obtained from the biopsy) and therefore were not further considered in this report, leaving a total of 25 tumors in 24 patients for this analysis.

Biopsy Methods

Core needle biopsies were performed under local anesthetic using a spring-loaded 18-gauge core needle (Bard Co., Inc., Covington, GA). Generally, four to six cores were obtained during each biopsy session. Core samples were fixed in formalin and embedded in paraffin. Serial cores were performed using the same skin entry site as that used in the pretreatment biopsy.

An attempt was made to perform one pretreatment and two posttreatment biopsies on every study participant. The second biopsy was scheduled for approximately 24 hours after the first course of chemotherapy was initiated, and the third biopsy was scheduled for approximately 48 hours after the first course of chemotherapy. One patient underwent the second posttreatment biopsy 66 hours after treatment. Because this protocol was done for research purposes only, patients could elect not to undergo the second or third biopsy at their discretion. From the 30 participants, useful biopsy material was available from a posttreatment specimen in 25. Of these cases, 24 had a pretreatment specimen, 22 had a specimen from 24 hours, and 19 had a specimen from 48 hours.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded sections of the core biopsy using the avidin-biotin peroxidase complex method. The staining was performed in batch after the study was complete. Briefly, 5 μm sections were deparaffinized with xylene and ethanol. For antigen retrieval, the slides were heated with antigen retrieval solution in a steamer heater for 10 minutes. Intrinsic peroxidase activity in tissue was blocked by treatment for 5 minutes with 3% hydrogen peroxide in methanol. Immunolocalization was carried out with the avidin-biotin peroxidase enzyme complex Elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions.

Rabbit polyclonal antibodies for Bcl-2 (Santa Cruz cat # sc783) and Bax (Santa Cruz cat # sc493) were used in 1:250 and 1:200 dilutions, respectively. Mouse monoclonal antibody for p53 (oncogene cat # OP43) was used in 1:200 dilution. Peroxidase activity was developed by 3-3 diaminobenzidine tetrahydrochloride (Sigma Chemicals, St. Louis, MO). Appropriate positive and negative controls were used throughout. Harris's hematoxylin was used to counterstain the slides.

The result of staining in a section of tumor was considered positive if unequivocal staining of cytoplasm for Bcl-2 and Bax and nuclear staining for p53 was seen. The level of staining was categorized as 0, none or

< 5% positive cells; +, 5%–25% positive cells; ++, 26%–50% positive cells; +++, > 50% positive cells. Staining for Bcl-2 and Bax were performed on both pretreatment and posttreatment specimens, whereas p53 staining was performed only on the pretreatment specimen. For the Bcl-2 or Bax samples, the semiquantitative value had to change by one category in order for consecutive biopsies from an individual tumor to be classified as having an increase or a decrease. Scoring of the immunohistochemical staining was performed by a breast pathologist (A.S.) without the knowledge of clinical data.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) staining[15] was performed using a commercial kit according to the manufacturer's protocol (Promega, Madison, WS). Briefly, the tissue sections were deparaffinized and fixed in 4% paraformaldehyde at room temperature for 5 minutes. The nuclei of tissue sections were stripped of proteins by incubation with 20 μg/mL of proteinase K for 10 minutes. The tissue sections were permeabilized by incubating with 0.5% Triton X-100 in phosphate-buffered saline for 5 minutes at room temperature. After they were rinsed two times with phosphate-buffered saline for 5 minutes, the slides were incubated with terminal-deoxynucleotidal-transferase buffer for 10 minutes. Terminal deoxynucleotidal transferase, reaction buffer, and deoxyuridine triphosphate conjugated with fluorescein isothiocyanate were then added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 hour. The slides were stained with 10 μg/mL of propidium iodide for 10 minutes and washed three times with phosphate-buffered saline for 5 minutes. Cover slips were mounted using Prolong solution (Molecular Probes, Eugene, OR). Immunofluorescence microscopy was performed using a 20× objective (Zeiss Plan-Neofluar, Thornwood, NY) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel to select for green fluorescence in three to five random fields in each biopsy. The calculated apoptotic index represented the percentage of cells that stained positive with TUNEL that were quantified from images processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Statistical Methods

The primary endpoint used to determine the success of chemotherapy was the pathological response of the primary tumor. This was evaluated by the extent of residual disease present after the surgical procedure. We selected a pathological rather than a clinical endpoint because measurements of clinical response are often discordant with the true pathological extent of disease.[16-18] Before data analysis, we elected to study the response of only the primary tumor (and not lymph nodes) because all core specimens were taken from the primary tumor. This pathological endpoint was divided into four categories: (1) pathological complete response [pCR] (category 1), (2) partial response with residual breast disease < 1 cm (category 2), (3) response with residual breast disease > 1 cm (category 3), and (4) clinical evidence of progressive disease during chemotherapy (category 4). The extent of residual disease of < 1 cm or > 1 cm was selected a priori as a cut point in an effort to divide the subjects into two populations that were more equal in number than would have been possible using only a pCR cut point. Clinical progression of disease was defined as disease becoming more extensive during the course of chemotherapy. This was considered as a separate category because patients with progressive disease may not have been eligible for surgical resection.

We first analyzed the relationship of apoptosis levels to disease response with a Spearman correlation test; the response categories of 1 versus 2 versus 3 versus 4 were used. In addition, we used a Mann-Whitney test to analyze the relationship of the median value of apoptosis and chemotherapy response. We compared the apoptosis and biomarker levels in categories 1 and 2 with those in categories 3 and 4, and we compared category 1 with others. We selected these two definitions of a favorable response versus an unfavorable response because they were objective and represented either a comparison of complete eradication of primary disease or a response of disease to a microscopic level. We used a Fisher's exact test to analyze how changes in Bcl-2 levels (defined as decrease versus stable/increase) and Bax (defined as increase versus stable/decrease) correlated with a favorable versus an unfavorable response. All *P* values were two sided.

RESULTS

Table 1 shows the clinical, disease, and treatment characteristics of the 25 cases. Seventy-six percent of the tumors were stage IIIA or greater disease, and 72% of the primary tumors were clinical stage T3 or T4. For all but one tumor, neoadjuvant chemotherapy was given as part of a prospective clinical trial. Sixteen tumors were treated with bolus neoadjuvant AT (doxorubicin, docetaxel) given at a dose of 60/60 mg/m^2 every 3 weeks, with granulocyte macrophage colony-stimulating factor 250 mg/m^2 daily for 10–14 days. Ten of these tumors were treated with four cycles of AT before surgery, three were treated with six cycles of AT before surgery, one was treated with six cycles of AT and three cycles of cyclophosphamide/methotrexate/5-fluorouracil before surgery, and two tumors progressed during treatment and were not able to be resected. The next most common

Patient, Pathological, and Treatment Characteristics

Characteristic	Result
Age	
Median	51
Range	34-71
Clinical T stage	
T1	0% (0/25)
T2	28% (7/25)
T3	32% (8/25)
T4	40% (10/25)
Clinical N stage	
N0	12% (3/25)
N1	40% (10/25)
N2	44% (11/25)
N3	4% (1/25)
Clinical stage	
II	24% (6/25)
IIIA	28% (7/25)
IIIB	32% (8/25)
IV (supraclavicular)	16% (4/25)
ER/PR status	
ER+, PR+	24% (6/25)
ER+, PR-	24% (6/25)
ER−, PR+	12% (3/25)
ER−, PR−	40% (10/25)
Her2-neu	
Positive	32% (8/25)
Negative	68% (17/25)

ER, estrogen receptor; PR, progesterone receptor.

chemotherapy regimen was single-agent paclitaxel, which was given to seven patients (eight tumors) in a randomized study comparing a 3-weekly versus a weekly schedule. The dose per cycle for the every-3-week schedule (given to five tumors) was 175–225 mg/m^2, and the dose per cycle of weekly schedule (given to three tumors) was 80 mg/m^2. After receiving four cycles of the every-3-week schedule or 12 doses of weekly schedule, these eight cases were also treated with four cycles of neoadjuvant FAC (5-fluorouracil/doxorubicin/cyclophosphamide) before surgery. The FAC chemotherapy consisted of 500 mg/m^2 of 5-fluorouracil given on days 1 and 4, 50 mg/m^2 of doxorubicin given as a 72-hour continuous i.v. infusion, and 500 mg/m^2 of cyclophosphamide given intravenously on day 1. One of these eight tumors progressed during chemotherapy but was still able to be resected. The final patient was treated outside of a clinical trial and received three cycles of neoadjuvant FAC. This patient had progressive inoperable disease after FAC and subsequently received one cycle of paclitaxel and three cycles of docetaxel and then went on to high-dose chemotherapy with peripheral autologous hematopoietic stem cell transplantation before surgery.

In summary, surgery was performed in 23 of the 25 cases after the neoadjuvant chemotherapy. Resection of the primary tumor only was performed in two of these cases, whereas resection of both the primary tumor and the axillary lymph nodes was performed in the remaining 21. Two of the four tumors with clinical evidence of progressive disease during chemotherapy did not have surgery.

After neoadjuvant chemotherapy, 12% (3/25) of the tumors had a pCR, 32% (8/25) of the tumors had residual disease measuring < 1 cm, 40% (10/25) of the tumors had > 1 cm of residual disease, and the remaining 16% (4/25) had clinical progression of disease during chemotherapy. Of the tumors that clinically progressed, two were unresectable, one had 9 cm of residual disease after paclitaxel and FAC, and one had 3.5 cm of residual disease after FAC, taxane therapy, and high-dose chemotherapy with transplantation. In the subset of tumors treated with AT chemotherapy, 13% (2/16) tumors had a pCR of the primary tumor, 44% (7/16) had residual disease < 1 cm, 31% (5/16) had > 1 cm of residual disease, and the remaining 13% (2/16) progressed during chemotherapy.

There was no correlation between pathological response to chemotherapy and pretreatment measurements of apoptosis levels, histologic grade, p53, Bcl-2, or Bax. The median baseline apoptosis level in the tumors with a pCR or < 1 cm of residual disease was 3 (range, 0–10), compared with 6 (range, 1–17) in the group with > 1 cm residual cancer and the clinically progressing disease group ($P = 0.177$). Overexpression of p53 (defined nuclear staining of at least 5% of the cells) was found in 45% (5/11) of tumors with a favorable response (pCR or < 1 cm), versus 31% (4/13) of the tumors with an unfavorable response ($P = 0.675$). Baseline + + + expression of Bcl-2 or Bax occurred in similar percentages in the two subgroups of tumors. Finally, there was no correlation between pretreatment estrogen receptor status, progesterone receptor status, or Her2-neu overexpression and disease response, although the small sample size limits the certainty of any of these negative findings.

The median apoptosis level in the tumors significantly increased after chemotherapy (0 hours: 2%, range, 0%–17%, vs 24 hours: 8%, range, 0%–29% vs 48 hours: 10%, range, 1%–51%). Figure 1 shows the apoptotic indices for the 25 tumors in this report, with the 16 tumors treated with AT chemotherapy displayed by the numbers not included in a box. The 24- and 48-hour posttreatment levels were both significantly higher than the pretreatment level, as displayed by the Mann-Whitney test ($P = 0.0163$ and $P = 0.0003$, respectively). The use of a Mann-Whitney test was justified because there was no correlation between pretreatment levels and either of the posttreatment levels in individual

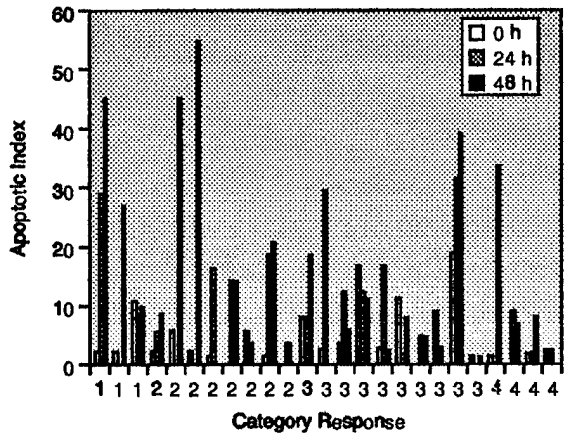

FIGURE 1 Apoptotic index for the all tumors and the tumors treated with AT chemotherapy. Bar graphs of the pre- and post-treatment apoptotic levels of the 25 tumors in the study and the subgroup of 16 tumors treated with AT (doxorubicin, docetaxel) chemotherapy (identified by having the response number on the x-axis in black). The response categories represent: pCR (1.00), < 1 cm of residual disease (2.00), < 1 cm of residual disease (3.00), and progressive disease (4.00). Overall, the median apoptosis levels 48 hours after treatment was significantly higher in the patients with pCR or < 1 cm of residual disease versus those with > 1 cm of residual disease (22% vs 7% $P=0.018$). This difference was also significant for the subgroup of patients treated with AT chemotherapy, (25% vs 4% $P=0.015$). pCR, pathological complete response.

tumors ($P = 0.558$ and $P = 0.917$, respectively, Spearman correlation test). The level of apoptosis at 48 hours correlated significantly higher with tumor response ($P = 0.0497$, Spearman correlation test). Furthermore, the median apoptosis levels of tumors with a response of < 1 cm or better was higher than that of tumors with a less favorable response (22%, range 8%–51% vs 7%, range 1%–36%, respectively; $P = 0.018$, Mann-Whitney test). Figures 2A and 2B show two examples of pretreatment and posttreatment specimens stained to measure apoptosis levels. These specimens were from a tumor with a pCR of the primary tumor and from a tumor with > 1 cm of residual disease, respectively. The apoptosis level difference in 48 hours between those with a good response and those with an unfavorable response was also observed in the 16 tumors treated with AT chemotherapy (25%, range, 8%– 51% vs 4%, range 1%–19%, respectively for the tumors with pCR or < 1 cm of residual disease vs those with an unfavorable response, $P = 0.015$). However, for both the entire group of tumors and the subset treated with AT, there was no significant difference between the median apoptosis level at 24 hours in the patients with a pCR or < 1 cm of residual disease and those with an unfavorable response (entire set of cases: 11% vs 9%, respectively, $P = 0.293$).

In addition to apoptosis levels at 48 hours, a decrease in tumor Bcl-2 expression at either the 24- or 48-hour time point compared with the expression from the pretreatment sample correlated with having a pCR. Specifically, three of the nine tumors with a decrease in Bcl-2 achieved a pCR, compared with zero of 15 of the tumors with a stable Bcl-2 level ($P = 0.042$) (Table 2). In the 16 tumors treated with AT chemotherapy, there was no statistical correlation between decrease in Bcl-2 expression and disease response (pCR vs others, $P = 0.175$).

Measurement of Bax expression in the serial specimens showed no relationship to pathological response (Table 2).

DISCUSSION

The preliminary data we present in this article suggest that cellular and molecular changes in tumor cells 24 to 48 hours after treatment correlate with response to neoadjuvant chemotherapy. These data add to the very small number of studies that have demonstrated that chemotherapy induces apoptosis in a human solid tumor. In addition, our finding that apoptosis levels at 48 hours correlated with chemotherapy response suggests that the apoptotic cell death pathway is a relevant determinant of the outcome of breast cancer treatment. These data are consistent with those from preclinical mice studies that first noted that the degree of treatment-induced apoptosis correlated with the response to chemotherapy. Our data also indicated that prolonged apoptotic response as measured at 48 hours is more powerfully associated with response than measurements at 24 hours. The preclinical studies that served as the basis of this protocol found that the apoptosis levels peaked in the 48- to 72-hour range.[2,19] It is possible that a later time point (72 or 96 hours) would have uncovered an even stronger correlation.

Other small series have also attempted to evaluate the significance of chemotherapy-induced apoptosis levels in breast cancer patients. Symmans et al[6] measured apoptosis levels from serial fine needle aspirate samples obtained from 11 breast cancer patients treated with single-agent paclitaxel. This study also demonstrated that the cumulative apoptotic response over the first 4 days after chemotherapy correlated with a good pathological response. In addition, Chang et al[7] reported the results of apoptosis levels in 28 breast cancer patients treated with mitoxantrone/methotrexate chemotherapy who underwent serial fine needle aspirations of the primary at time 0 and after treatment at 24 or 72 hours. They also noted an increased median rate of apoptosis after chemotherapy and demonstrated a correlation between the degree of treatment-induced apoptosis and tumor response.

Although this report and these other two small series

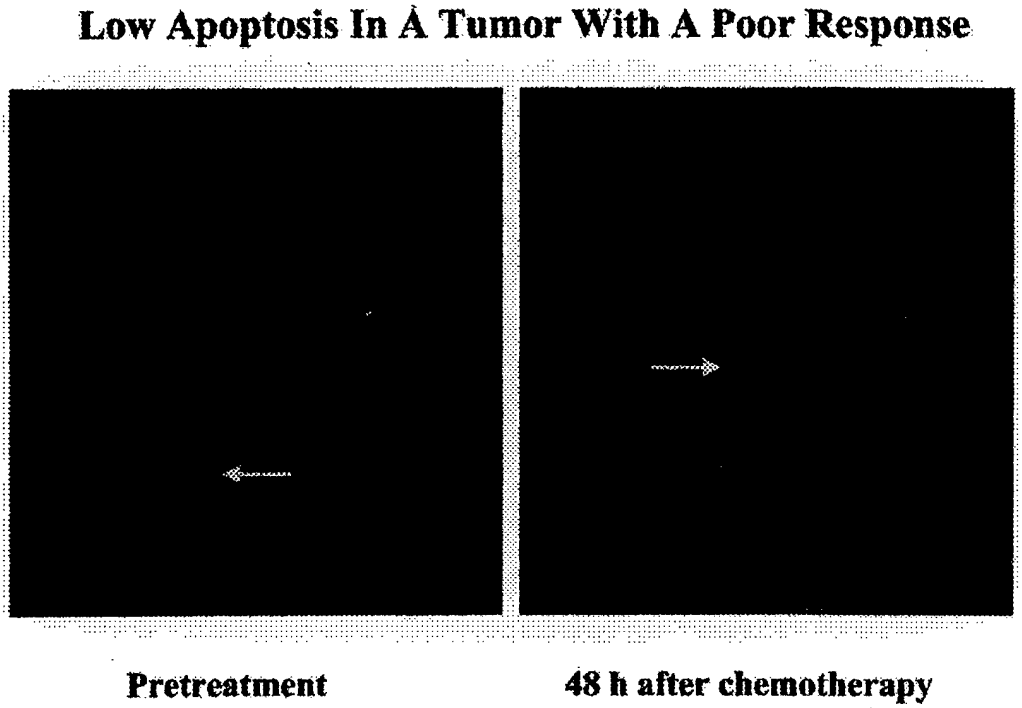

FIGURE 2 A, High apoptosis in a patient with a pCR. B, Low apoptosis in a patient with a poor response. Examples of pre- and posttreatment fluorescent TUNEL stain used to quantify the percentage of apoptotic cells in the tumor specimens. Fig. 2A is an example from a patient with a complete pathological response of the primary tumor to chemotherapy and Fig. 2B is an example from a patient whose tumor had an unfavorable pathological response to chemotherapy. The fluorescent-tagged cells indicate cells with DNA fragmentation, whereas the red cells indicate intact tumor cells that were counterstained with propidium iodide.

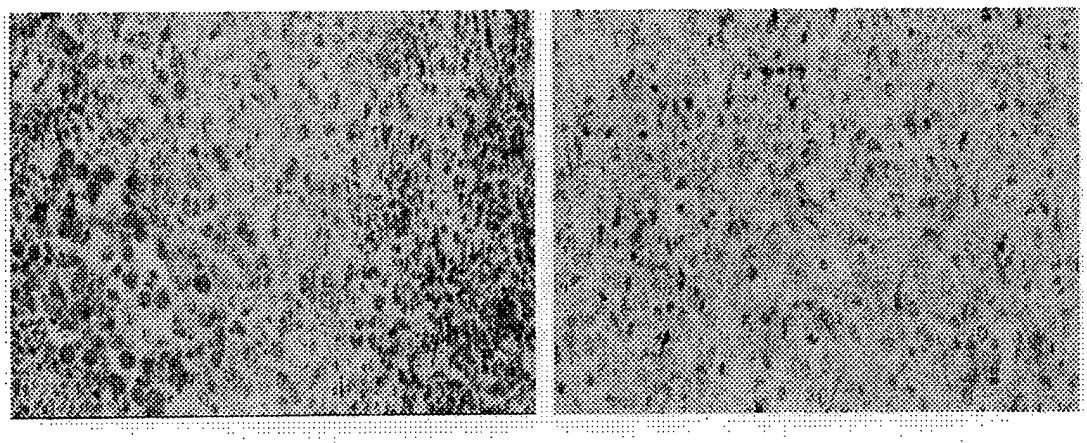

FIGURE 3 Decrease in Bcl-2 expression after 48 hours in a patient with a pCR Immunohistochemistry stains of Bcl-2 expression from a tumor sampled at prior to (left panel) and 48 hours (right panel) after neoadjuvant chemotherapy. The percentage of positive cells and the expression intensity decreased, as shown by the brown cytoplasmic staining, decreased in the 48-hour sample. The pathology of the tumor in this patient after chemotherapy indicated a complete pathological response of the primary.

Bcl-2 and Bax Changes After Neoadjuvant Chemotherapy

Response	Bcl-2 Change	Bax Change
pCR	Decreased (100%)	Increased (33%)
(N = 3)	No Change (0%)	No change (67%)
	Increased (0%)	Decreased (0%)
< 1 cm	Decreased (38%)	Increased (25%)
(N = 8)	No Change (62%)	No change (62%)
	Increased (0%)	Decreased (13%)
> 1 cm	Decreased (20%)	Increased (40%)
(N = 11)	No Change (80%)	No change (50%)
	Increased (0%)	Decreased (10%)
Clinical progression	Decreased (25%)	Increased (75%)
(N = 4)	No Change (75%)	No change (25%)
	Increased (0%)	Decreased (0%)

Abbreviation: pCR, pathological complete response.

are the first to study the importance of the apoptotic pathway in human breast cancer, many previous studies have examined how the expression of apoptosis-related molecular markers in human breast cancer specimens correlate with chemotherapy response. The majority of these studies have analyzed only baseline untreated tumor samples for such markers as p53, Bcl-2, Bax, and bag-1.[8-14,20,21] Most of these studies have tested whether molecular inhibitors of apoptosis, such as presence of a *p53* mutation, high Bcl-2 expression, or low Bax expression, inhibit response to chemotherapy. Unfortunately, the data concerning *p53* mutations and chemo-

therapy response have been mixed, and p53 assessment is not currently recommended as a factor for determining therapeutic management.[22] *Bcl-2* is a proto-oncogene that inhibits apoptosis and is in the same family of genes as *Bax*, which has proapoptotic effects.[23,24] Similar to the p53 data, the literature concerning baseline Bcl-2 expression as a prognostic or predictive factor in breast cancer have been mixed.[10-14]

Although the study of mutations in apoptosis-related genes in pretreatment samples seems relevant, we believe that it is intuitively more interesting to measure expression levels of proteins involved in the apoptotic response to cellular injury after chemotherapy treatment rather than before. Similar to several previous reports, we did not find a correlation between pretreatment p53, Bcl-2, or Bax expression and outcome. However, by measuring the response of Bcl-2 after treatment, we demonstrated a correlation between a decrease in the level of Bcl-2 expression and achievement of a pCR. These data are consistent with a study that found that low Bcl-2 expression in cervical cancer after administration of 10.8 Gy of radiation positively correlated with response.[25] However, Chang et al[7] also studied Bcl-2 expression in serial samples taken from breast cancer and paradoxically found that an increased Bcl-2 expression correlated with a good response to chemotherapy. The authors suggested that this may represent a selection of apoptotic resistant cells in the tumor specimen. The reason for the differences in the findings of the Chang et al study and our own is unclear, but it may be related to differences in chemotherapy regimens, definition of response (pathological versus clinical), timing of Bcl-2 measurements, and Bcl-2 antibodies used to evaluate expression.

Despite the finding of a statistical correlation between apoptosis and Bcl-2 and response, it is important to recognize that some patients in our report had a marked apoptotic response at 24 and 48 hours and yet had minimal response to therapy. Furthermore, one of our patients who had a pCR had only a minimal change in the apoptosis rate during the three sampling times. In addition, some of the patients with a poor response to treatment had a decrease in Bcl-2 expression. It is therefore likely that the apoptosis pathway of cell death is only one determinant of chemotherapy response. Other possible contributors include degree of tumor cell repopulation and other mechanisms of cell death that would not be histologically evident at these early time points.

A drawback of this study is the patients did not all receive the same chemotherapy regimen. In a murine tumor system, Meyn et al[3] found that different chemotherapeutics induce different levels of apoptosis. However, we were able to demonstrate that in the largest patient subgroup (the patients treated with AT chemotherapy), there was a statistically significant correlation between apoptosis and tumor response. The data for the patients treated with paclitaxel or FAC was not sufficient to evaluate whether apoptosis correlated with response in these patients. A second potential drawback is that our data may have been subject to a sampling bias. We attempted to obtain biopsy samples from the same region of the tumor at each time point, but it is possible that we sampled different microenvironments within the tumor, which could have influenced biomarker expression. However, this sampling bias would likely affect all time points equally and would therefore be unlikely to have a significant impact on the comparison of the various time point measurements.

Together, our study and two previous reports indicate that the apoptotic pathways of cell death may have relevance in the determination of the response of breast cancer to chemotherapy. These data suggest that cellular and molecular changes within a tumor occurring early after chemotherapy may provide more useful insights into molecular mechanisms that determine response than simple assessment of markers at baseline. If confirmed in a larger set of patients, these data may have clinical relevance by providing clinicians with an early assay that is predictive of chemotherapy response. A validated predictive assay available within 2 days of initiation of treatment would allow for an immediate cross-over to alternative chemotherapy regimens or earlier intervention with local-regional therapy. However, it is clear that significantly more data are needed before early or molecular markers of chemotherapy response can be used clinically.

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APPENDIX 18

Changes in the 2003 American Joint Committee on Cancer Staging for Breast Cancer Dramatically Affect Stage-Specific Survival

By Wendy A. Woodward, Eric A. Strom, Susan L. Tucker, Marsha D. McNeese, George H. Perkins, Naomi R. Schechter, S. Eva Singletary, Richard L. Theriault, Gabriel N. Hortobagyi, Kelly K. Hunt, and Thomas A. Buchholz

Purpose: **To evaluate how implementation of the 2003 American Joint Committee on Cancer (AJCC) staging system will affect stage-specific survival of breast cancer patients.**

Patients and Methods: **Records of 1,350 patients treated on sequential institutional protocols with mastectomy and adjuvant doxorubicin-based chemotherapy were reviewed. Pathologic stage was assigned retrospectively according to the 1988 and the 2003 AJCC staging criteria. Overall stage-specific survival (OS) was calculated using the Kaplan-Meier method, and hypothetical differences were compared by the log-rank test.**

Results: **Six hundred five of 1,087 patients with stage II disease according to the 1988 classification system had stage II disease according to the 2003 system. The 10-year OS for patients with stage II disease was significantly improved using the 2003 system (76% [2003] v 65% [1988]; $P < .0001$). Two hundred eighty-nine of 633 patients with stage IIb disease using the 1988 system were stage IIb with the 2003 system, and 10-year OS was 58% (1988) versus 70% (2003; $P = .003$). The number of patients with stage III disease increased from 207 (1988) to 443 (2003), and the 10-year OS changed from 45% (1988) to 50% (2003; $P = .077$). Most of this difference resulted from changes within stage IIIa: OS, 45% (1988) versus 59% (2003; $P < .0001$).**

Conclusion: **Stage reclassification using the new AJCC staging system for breast cancer will result in significant changes in reported outcome by stage. It is imperative that careful attention is devoted to this effect so that accurate conclusions regarding the efficacy of new treatment strategies can be drawn.**

J Clin Oncol 21:3244-3248.

TUMOR STAGING systems provide information about extent of disease that can be used to guide treatment recommendations and provide estimates of patient prognosis. In addition, the staging system provides a framework for reporting treatment outcomes and thereby permits the efficacy of new treatments to be assessed. Changes in the staging system are periodically required to incorporate new diagnostic and therapeutic advances that affect risks of disease recurrence and patient survival. The American Joint Committee for Cancer (AJCC) has recently published new staging criteria for breast cancer, which were implemented in January 2003.[1] In this study, we examined the impact that changing the staging criteria will have on reporting stage-specific outcomes for breast cancer. We compared the stage-specific overall survival of 1,350 patients treated on prospective adjuvant chemotherapy breast cancer protocols at the University of Texas M.D. Anderson Cancer Center with mastectomy and doxorubicin-based chemotherapy using both the 2003 and 1988 AJCC staging criteria. We found that the change in the staging system had a dramatic effect on the reporting of stage-specific survival outcomes for breast cancer.

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Submitted March 10, 2003; accepted June 18, 2003.

Supported in part by grant Nos. CA16672 and T32CA77050 from the National Cancer Institute, the Nellie B. Connally Breast Cancer Research Fund, and a grant from the Stanford and Joan Alexander Foundation, Houston, TX. T.A.B. is supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154.

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0732-183X/03/2117-3244/$20.00

PATIENTS AND METHODS

Patient, Tumor, and Treatment Characteristics

We retrospectively reviewed the records of 1,501 patients with breast cancer who were treated with mastectomy and doxorubicin-based adjuvant systemic therapy with or without tamoxifen or radiation in five prospective clinical trials at the University of Texas M.D. Anderson Cancer Center between 1975 and 1994.[2-8] Each protocol was reviewed and approved by the institutional review board, and participants gave written informed consent. One hundred fifty-one patients treated on these protocols were excluded from this analysis because insufficient information was available to assign a pathologic stage in both the 1988 and 2003 AJCC staging classifications, leaving a total of 1,350 patients who were assessable for this study. Each patient was retrospectively assigned a 1988 and a 2003 disease stage based on pathologic data from the chart.

End Points and Statistical Analysis

Ten- and 15-year actuarial overall stage-specific survival was calculated by Kaplan-Meier method with comparison among the groups performed using two-sided log-rank test.[9] All P values were two-tailed, with a value of less than .05 considered to be significant. The P values were strictly hypothetical for the purpose of this exercise as no change in outcome truly occurred.

RESULTS

Staging Subgroups

The AJCC pathologic staging criteria for breast cancer from 1988 and 2003 are listed in Table 1. Table 2 demonstrates the distribution

Table 1. American Joint Committee on Cancer Staging Systems (pathologic)

1988		2003	
Primary tumor (T)		Primary tumor (T) unchanged from 1988	
TX	Primary tumor cannot be assessed	TX	Primary tumor cannot be assessed
TO	No evidence of primary tumor	TO	No evidence of primary tumor
Tis	Carcinoma in situ: intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumor	Tis	Carcinoma in situ: intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumor
T1	Tumor 2 cm or less in greatest dimension*	T1	Tumor 2 cm or less in greatest dimension*
T2	Tumor more than 2 cm but not more than 5 cm in greatest dimension	T2	Tumor more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumor more than 5 cm in greatest dimension	T3	Tumor more than 5 cm in greatest dimension
T4	Tumor of any size with direct extension (a) to the chest wall (b) skin, only as described below	T4	Tumor of any size with direct extension (a) to the chest wall (b) skin, only as described below
	T4a extension to chest wall		T4a extension to chest wall
	T4b edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast		T4b edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
	T4c (a) and (b)		T4c (a) and (b)
	T4d inflammatory carcinoma		T4d inflammatory carcinoma
Regional lymph nodes (N)		Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed	NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis	N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph node(s)	N1	Metastasis in one to three axillary lymph nodes, and/or in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent*
N1a	Only micrometastasis	N1mi	Micrometastasis (> 0.2 mm, ≤ 2.0 mm)
N1bi	Metastasis in one to three lymph nodes (> 0.2 cm, < 2 cm)	N2	Metastasis in four to nine axillary lymph nodes, or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
N1bii	Metastasis in four or more lymph nodes (> 0.2 cm, < 2 cm)	N3	Metastasis in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more axillary lymph nodes; or in more than three axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
N1biii	Extension beyond the capsule (involved node > 0.2 cm, < 2 cm)		
N1biv	Metastasis to lymph node greater than 2 cm		
N2	Metastasis to ipsilateral axillary lymph nodes fixed to one another or to other structures		
N3	Metastasis to ipsilateral internal mammary lymph node(s)		
Stage groupings		Stage groupings	
Stage 0	TisN0M0	Stage 0	TisN0M0
Stage I	T1N0M0	Stage I	T1N0M0
Stage IIa	T0N1M0, T1N1M0, T2N0M0	Stage IIa	T0N1M0, T1N1M0, T2N0M0
Stage IIb	T2N1M0, T3N0M0	Stage IIb	T2N1M0, T3N0M0
Stage IIIa	T0N2M0, T1N2M0, T2N2M0, T3N1M0, T3N2M0	Stage IIIa	T0N2M0, T1N2M0, T2N2M0, T3N1M0, T3N2M0
Stage IIIb	T4 any N, any T N3	Stage IIIb	T4 N0M0, T4N1M0, T4N2M0
		Stage IIIc	Any T N3
Stage IV	Any T any N M1	Stage IV	Any T any N M1

*Groups are further subcategorized in the American Joint Committee on Cancer staging guidelines.[1]

of patients in each stage subgroup using the 1988 and the 2003 staging systems. In general, the 2003 staging system shifted higher-risk patients from the stage II group into the stage III group. Using the 1988 staging system, 1,087 patients had stage II disease, whereas only 605 of these patients remained in stage II using 2003 system. The number of patients with stage III disease increased from 207 (1988) to 701 (2003). The number of patients in stage IIa decreased from 454 (1988) to 316 (2003), and the number of patients in stage IIb decreased from 633 (1988) to 289 (2003). Conversely, the number of patients in stage IIIa increased from 196

Table 2. Patient Distribution by Stage

1988 Stage	2003 Stage						Total
	I	IIa	IIb	IIIa	IIIb	IIIc	
I	**44**	0	0	0	0	0	44
IIa	0	**315**	0	95	0	44	454
IIb	0	0	**288**	219	0	126	633
IIIa	0	1	1	**121**	0	73	196
IIIb	0	0	0	1	**7**	3	11
IV	0	0	0	0	0	**12**	12
Total	44	316	289	436	7	258	

(1988) to 436 (2003). The number of patients with stage IIIb was relatively unchanged, and 258 patients were classified as stage IIIc (2003), a stage that did not exist in the 1988 system. Because most stage changes manifested in this cohort of patients were based on the number of pathologically involved axillary nodes, the 2003 staging criteria did not affect the number of patients in stage I (node-negative disease).

Overall Survival

Figures 1A through 1C show overall survival curves for the patients with stage IIa, IIb, and IIIa disease defined according to the 1988 staging system. For each of these figures, the overall survival curves were then reclassified into stages IIa, IIb, and IIIa according to the 2003 system. Within each of the 1988 stage groupings, there was a highly significant difference in overall survival between the new stage groupings ($P < .001$, $P = .0002$, and $P = .0013$ for stage IIa, IIb, and IIIa, respectively).

Figures 2A through 2D superimpose the Kaplan-Meier stage-specific overall survival curves in the two staging systems. For each stage of disease, the patients whose stage was assigned according to the 2003 system had a better overall survival than those staged according to the 1988 standard. The changes in the 10-year and 15-year actuarial overall survival by stage are listed in Table 2.

DISCUSSION

Regarding the topic of geographic migration during the Great Depression of the 1930s, humorist-philosopher Will Rogers once said, "When the Okies left Oklahoma and moved to California, they

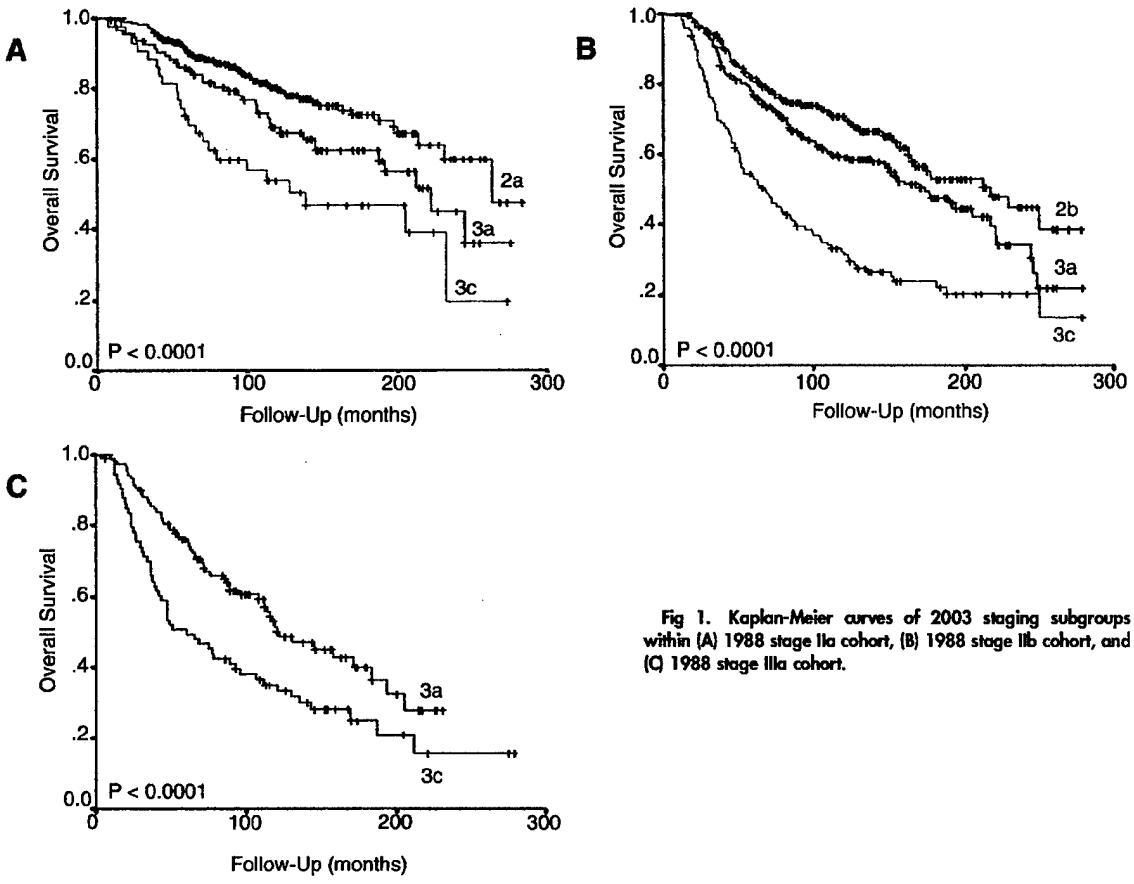

Fig 1. Kaplan-Meier curves of 2003 staging subgroups within (A) 1988 stage IIa cohort, (B) 1988 stage IIb cohort, and (C) 1988 stage IIIa cohort.

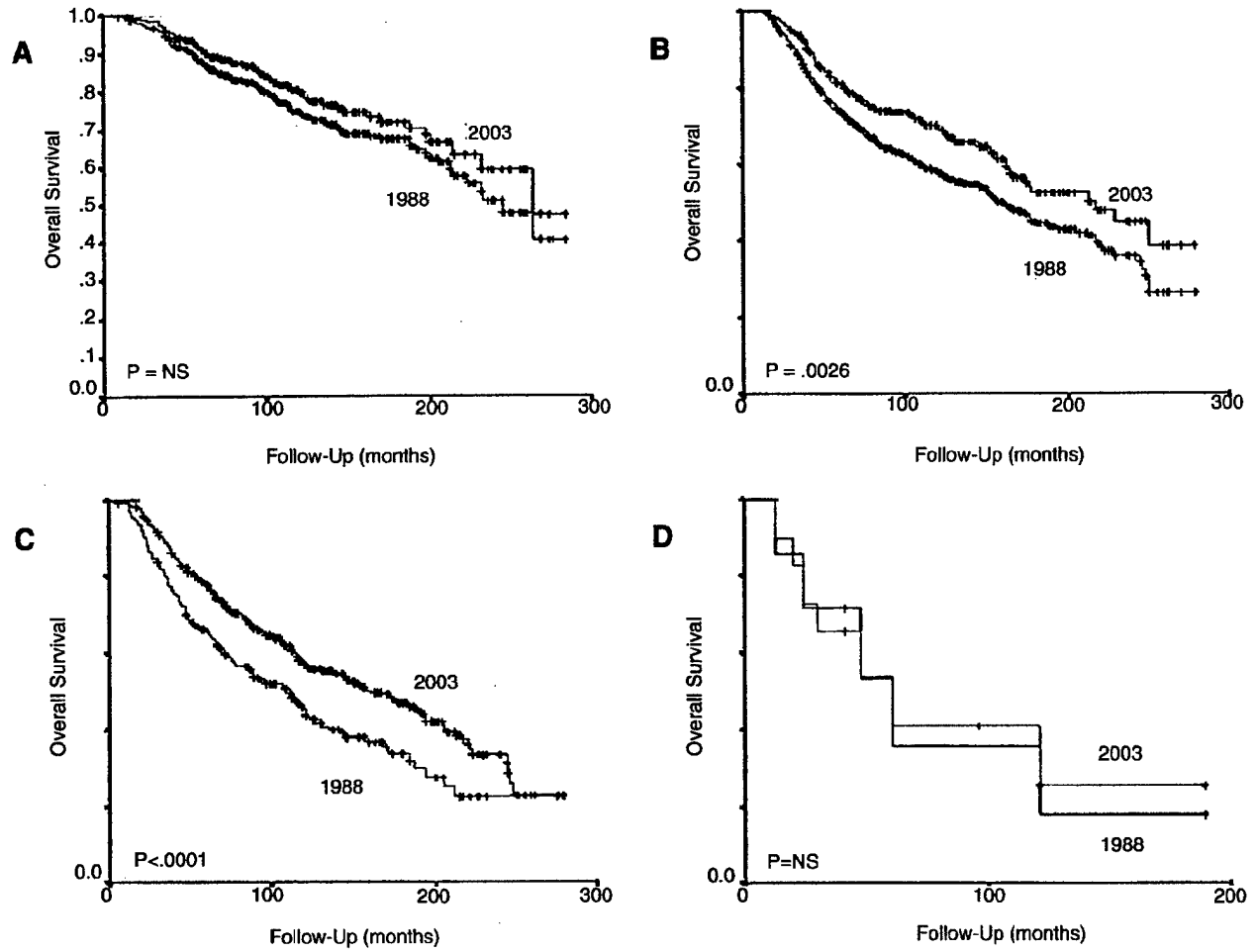

Fig 2. Kaplan-Meier curves comparing stage subgroups classified using both the 1988 and 2003 staging systems. (A) stage IIa, *P* = not significant; (B) stage IIb, *P* = .0026; (C) stage IIIa, *P* < .0001; (D) stage IIIb, *P* = not significant.

raised the intellectual level in both states."[10] This effect has come to be known as the Will Rogers phenomenon and describes an important source of bias in clinical research. Feinsten et al[10] first suggested that this phenomenon could cause improvement in survival rates between two different groups without there actually being a change in individual outcome. In this report, we have shown that the recent change in the breast cancer staging system has this effect. In our data, implementing the 2003 AJCC staging for breast cancer improved stage-specific overall survival by as much as 15%. Furthermore, the improvement in stage-specific overall survival for patients staged according to the 2003 staging system applied across nearly every disease stage. The data we report used the same group of patients for comparison, so our data obviously do not reflect a change in the inherent survival of the group. Rather, the result reflects a shifting of the population in each stage towards more advanced stages. As the poorer prognostic cohort of each stage was shifted toward a higher stage, the overall survival for any one stage was improved.

The data presented in Figure 1 support the recent revision of the 1988 staging system. These curves demonstrated that various subgroups within each stage had significantly different prognoses. For example, using the 1988 staging system, despite a markedly different prognosis, a woman with a 2.1-cm primary tumor and one of 25 positive lymph nodes and a patient with a 4.6-cm primary and 15 of 17 positive lymph nodes were both considered to have stage IIb disease. The 2003 staging system has incorporated three new areas of prognostic value regarding the evaluation of the axilla: the number of positive lymph nodes, the relevance of micrometastatic disease, and the significance of the method of detection. Of these, we found that the most significant shifting of stage resulted from the number of positive lymph nodes. This may in part reflect the era in which our sample population was treated. The recategorization of patients according to the number of involved lymph nodes leads to a change in stage for a large percentage of patients with stage II and III breast cancer (as shown in Table 2), and this change translates into a significant Will Rogers Phenomenon (as shown in Table 3).

The data we present do not imply that the new staging system is not an important improvement. Indeed, Bunnell and Winer[11] conclude their editorial on the 2003 system by saying, "The stage is set for a time when we better understand the heterogeneity of breast

Table 3. Overall Survival, 1988 and 2003 Staging Systems

Stage	1988 Staging System		2003 Staging System		P
	10-Year OS	15-Year OS	10-Year OS	15-Year OS	
II	53	44	76	62	< .0001
IIa	75	67	81	72	NS
IIb	58	45	70	52	.0026
III	45	33	50	40	.077
IIIa	45	34	59	49	< .0001
IIIb	42	28	36	18	NS
IIIc			36	28	
IV	18	18			

Abbreviations: OS, overall survival; NS, not significant.

cancer and use this information in making treatment decisions." Our data indicate, rather, that comparisons among patients staged with the different staging systems will be inaccurate and may be inappropriately interpreted as reflecting improvements in treatment efficacy when none exists. In addition, ongoing randomized trials that span the transition from the old to the new staging system need to consider these findings and consistently stage patients according to one rather than two systems. Finally, it is recommended that during this time of transition, investigational protocols and treatment reports should also include a clear statement about which staging system is used.

Our review was limited by the diagnostic tests available at the time these patients were diagnosed, and so we do not have adequate information on whether the method of histologic or diagnostic detection of lymph nodes affects outcome. The patients whose outcome we analyzed were staged before the use of immunohistochemistry to detect micrometastatic disease in lymph nodes, and the patients in this report did not undergo routine computed tomography, ultrasound examination, or lymphoscintigraphy to detect abnormal internal mammary or infraclavicular nodes. As a result, the stage of very few patients were reclassified on the basis of clinically positive internal mammary nodes or infraclavicular nodes, and no patients were restaged on the basis of micrometastatic nodal disease or the method of detecting lymph node disease. In addition, because patients with stage IV disease were excluded from participation in these protocols, few patients with positive supraclavicular lymph nodes are included in our analysis. For these reasons, these data reflect restaging primarily as a result of incorporating the number of positive lymph nodes into the staging system and may therefore be an underestimate of the changes in overall survival using the 2003 staging system. Lastly, it should be noted that relatively few stage I patients were enrolled in these protocols. This impact is not likely to impact population-based outcomes because this reporting is not stage-specific.

In conclusion, a significant percentage of patients whose 1988 stage of disease was IIa and IIb will be reclassified into higher disease stages using the 2003 staging system. Higher-risk patients are removed from the stage II groups, whereas the stage III groups seem to have an increased proportion of favorable tumors. This results in patients with an assigned 2003 stage of IIa/IIIa having significantly improved stage-specific overall survival compared with cohorts with similar stage disease defined by the 1988 staging system. It is imperative that careful attention is devoted to this effect so that accurate conclusions regarding the efficacy of new treatments can be drawn.

ACKNOWLEDGMENT

We thank Jessica Erwin and Ramani Krishnan for their contributions to the database.

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Predictors of Local-Regional Recurrence After Neoadjuvant Chemotherapy and Mastectomy Without Radiation

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Purpose: **To define clinical and pathologic predictors of local-regional recurrence (LRR) for patients treated with neoadjuvant chemotherapy and mastectomy without radiation.**

Patients and Methods: **We analyzed the outcome of the 150 breast cancer cases treated on prospective institutional trials with neoadjuvant chemotherapy and mastectomy without postmastectomy radiation. Clinical stage at diagnosis was I in 1%, II in 43%, IIIA in 23%, IIIB in 25%, and IV in 7%. No patient had inflammatory breast cancer.**

Results: **The median follow-up period of surviving patients was 4.1 years. The 5- and 10-year actuarial rates of LRR were both 27%. Pretreatment factors that positively correlated with LRR were increasing T stage ($P < .0001$) and increasing combined clinical stage ($P < .0001$). Pathologic and treatment factors that positively correlated with LRR were size of the residual primary tumor ($P = .0048$), increasing number of involved lymph nodes ($P < .0001$), and no use of tamoxifen ($P = .0013$). The LRR rate for the 18 patients with a pathologic complete response of both the primary tumor and lymph nodes (pCR) was 19% (95% confidence interval, 6% to 48%). In a forward stepwise Cox logistic regression analysis, clinical stage IIIB or greater (hazard ratio of 4.5, $P < .001$), pathologic involvement of four or more lymph nodes (hazard ratio of 2.7, $P = .008$), and no use of tamoxifen (hazard ratio of 3.9, $P = .027$) independently predicted for LRR.**

Conclusion: **Advanced disease at presentation and positive lymph nodes after chemotherapy predict for clinically significant rates of LRR. Achievement of pCR does not preclude the need for postmastectomy radiation if warranted by the pretreatment stage of the disease.**

J Clin Oncol 20:17-23.

NEOADJUVANT CHEMOTHERAPY is an increasingly popular treatment strategy for patients with breast cancer. This treatment sequencing offers the earliest treatment of micrometastatic disease and allows for an assessment of whether there is resistance to the chemotherapy regimen being administered. In addition, neoadjuvant chemotherapy has been shown to allow selected patients with advanced primary disease the option of being treated with breast-conserving local therapies.[1,2]

A number of reports have indicated that the pathologic response after neoadjuvant chemotherapy strongly correlates with disease-free and overall survival (OS).[3-7] What is less clear is how the posttreatment pathology should affect treatment recommendations. This is particularly confusing with respect to determining the appropriate indications for postmastectomy radiation. Recent randomized trials have indicated that postmastectomy radiation can improve OS for patients with pathologic features predictive for local-regional recurrence (LRR).[8-10] The currently available data that correlate pathologic factors with LRR after mastectomy are from patients who had not received chemotherapy before surgical resection. For patients treated with neoadjuvant chemotherapy, the pathologic factors predictive of LRR are likely to be different than those for patients treated with surgery first.

In this report, we reviewed the LRR patterns for a subset of patients treated on consecutive institutional clinical trials investigating the role of neoadjuvant chemotherapy for breast cancer. From these patients, we studied those who underwent mastectomy and subsequently did not receive adjuvant radiation.

PATIENTS AND METHODS

In this study, we retrospectively analyzed data from five consecutive prospective institutional clinical trials of neoadjuvant chemotherapy for noninflammatory breast cancer. These trials were conducted at the University of Texas M.D. Anderson Cancer Center from 1974 to 1998. In these trials, 883 patients were treated with neoadjuvant doxorubicin-

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Submitted March 29, 2001; accepted July 20, 2001.

Supported in part by United States Department of Health and Human Services National Cancer Institute grant nos. CA16672 and T32CA77050. T.A.B. is supported by grant no. BC980154, a U.S. Army Medical Research and Material Command Breast Cancer Research Program Career Development Award.

This work was presented at the Annual Meeting of the American Radium Society, Coronado, CA, April 25-29, 2001.

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Table 1. Patient and Clinical Characteristics

Characteristic	No. of Patients	%
Age		
< 40 years	30/150	20
40-60 years	88/150	59
> 60 years	32/150	21
Clinical stage		
I	1/150	1
IIA	21/150	14
IIB	44/150	29
IIIA	35/150	23
IIIB	38/150	25
IV*	11/150	7
ER status		
ER+	72/150	48
ER-	56/150	37
Unknown	22/150	15
PR status		
PR+	46/150	31
PR-	42/150	28
Unknown	62/150	41
Adjuvant chemotherapy		
None	12/150	8
FAC	79/150	53
VACP	19/150	13
FAC + CMF	10/150	7
FAC + MV	10/150	7
CMF	4/150	3
Other	16/150	11
Adjuvant tamoxifen		
Yes	47/150	31
No	99/150	66
Uncertain	4/150	3

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; FAC, fluorouracil, doxorubicin, and cyclophosphamide; VACP, vinblastine, doxorubicin, cyclophosphamide, and prednisone; VMF, vinblastine, methotrexate, and fluorouracil; MV, methotrexate and vinblastine; CMF, cyclophosphamide, methotrexate, and fluorouracil.

*Indicates ipsilateral supraclavicular lymph node involvement without systemic metastases.

containing chemotherapy and 87 were treated with neoadjuvant single-agent paclitaxel.

A multidisciplinary team prospectively assigned the clinical stages of patients treated in these trials after a review of both physical and radiologic examination findings. Patients with systemic metastases or inflammatory carcinoma were treated on different protocols and were not included in this study.

For the purpose of this analysis, we reviewed the outcome of the 150 patients who underwent a mastectomy after neoadjuvant chemotherapy and did not subsequently receive adjuvant postmastectomy radiation. The choice of surgical procedure and the use or omission of postmastectomy radiation was determined by patient and physician selection biases, and these treatment decisions were not specified according to the protocols.

Table 1 lists the clinical, disease, and treatment characteristics of the 150 patients included in this report. As shown, most patients (56%) had IIIA or greater disease, and only one patient had stage I disease. Table 2 lists the neoadjuvant chemotherapy regimens and the number of cycles that were used for the patients in this study. The treatment regimen and its scheduling followed the specific protocol under which the patient was treated. One hundred twenty-one of the 150 patients received doxorubicin-containing neoadjuvant chemotherapy; the remaining 29 were treated with single-agent paclitaxel. The full details concerning the regimens have been published in earlier reports.[4,11,12] Briefly, chemotherapy consisted of 500 mg/m^2 of fluorouracil administered on days 1 and 4, 50 mg/m^2 of doxorubicin given on day 1 as a bolus or as a 72-hour continuous infusion, and 500 mg/m^2 of cyclophosphamide given on day 1 (FAC). For the patients treated with dose-escalated FAC, the doses of these drugs were increased to 600, 60, and 1,000 mg/m^2, respectively. The VACP regimen consisted of 1.5 mg/m^2 of vincristine, 60 to 75 mg/m^2 of doxorubicin, 600 to 750 mg/m^2 of cyclophosphamide, and 40 mg of prednisone. Finally, the paclitaxel regimen consisted of a dose of 250 mg/m^2 given as a 24-hour infusion.

All patients in this report underwent mastectomy after neoadjuvant chemotherapy. The median number of lymph nodes recovered was 15. One hundred thirty eight (92%) of the patients were subsequently treated with adjuvant chemotherapy after surgery. Postoperative chemotherapy strategies changed over the period of time included in this study. Initially, adjuvant FAC (similar to the preoperative regimen) was recommended. The second approach was to use cyclophosphamide, methotrexate, and fluorouracil and subsequently either vinblastine and methotrexate or vinblastine, methotrexate, and fluorouracil. Finally, the last strategy used in this cohort of patients was implemented to investigate taxanes. Adjuvant tamoxifen was used in 32%.

The method of Kaplan and Meier was used to generate local control and survival curves.[13] All event and follow-up times were measured from the date of diagnosis. Two-sided log-rank tests were used to detect differences in time to local recurrence or death. A Cox proportional hazards model was used to determine independent variables associated with OS and local control.[14] Cases with unknown factors were excluded in the initial Cox regression analysis. If a factor did not predict for the end point being analyzed, the cases with unknown values

Table 2. Neoadjuvant Chemotherapy Treatment Details

Protocol	Years of Study	Neoadjuvant Chemotherapy	No. of Cycles	Included Patients/Total Study Population
Advanced Primary	1974-1985	FAC	3	40/191
85-01	1985-1989	VACP	3	23/200
89-007	1989-1991	FAC	4	15/203
91-015	1991-1994	FAC or dose-escalated FAC	4	60/202
94-002	1994-1998	FAC or paclitaxel	4	60/174
Total	1974-1998			150/970

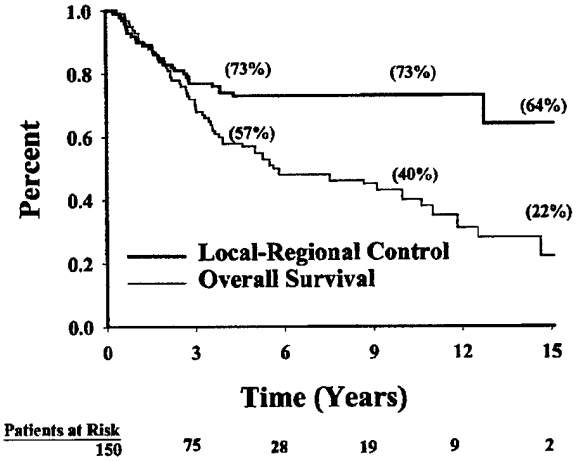


Fig 1. OS and LRR-free survival for the study population.

for that factor were again added into the analysis, and the Cox regression was repeated with that particular factor dropped.

RESULTS

The median residual tumor size after neoadjuvant chemotherapy was 2 cm, with a range of 0 to 16 cm. Seventy-four patients (49%) had residual primary disease ≤ 2 cm, 56 patients (37%) had tumors measuring 2.1 to 5.0 cm, and 14 patients (9%) had tumor sizes in excess of 5.0 cm. In the remaining six patients, the residual primary tumor could not be accurately measured. The median number of involved lymph nodes was one, with a range of 0 to 23. Sixty-two patients (41%) had negative axillary lymph nodes, 42 patients (28%) had one to three positive lymph nodes, 30 patients (20%) had four to nine positive lymph nodes, and 11 patients (7%) had 10 or more positive lymph nodes. In the remaining five patients, an accurate number of positive lymph nodes could not be determined from the records. Fifteen patients in this series (10%) had a complete pathologic response. The pretreatment clinical stage in these patients was IIA in two patients, IIB in two patients, IIIA in four patients, and IIIB in seven patients.

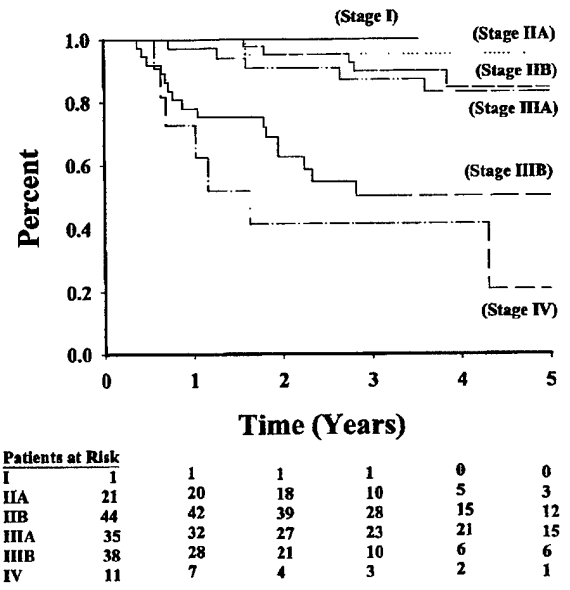
After a median follow-up period of 4.1 years among surviving patients (range, 1.5 to 17.7 years), disease recurred in 70 patients (47%). Distant metastases (DM) developed in 63 (42%), and LRR developed in 35 patients (23%). Of the 35 patients who experienced LRR, LRR was an isolated first event in 23 (66%), simultaneous with DM in five (14%), and subsequent to DM in seven (20%). Figure 1 shows the actuarial OS and local recurrence-free survival for the 150 patients. The actuarial OS rate at 5 and 10 years was 57% (95% confidence interval [CI], 47% to 65%) and 40% (95% CI, 29% to 51%), respectively. The LRR rate was 27% (95% CI, 20% to 37%) for both 5 and 10 years.

Table 3 lists 5-year LRR rates for patients divided according to clinical and pathologic characteristics of the primary tumor and the lymph nodes. Patients with increasing clinical T stage were associated with higher rates of LRR. The LRR rate for patients with clinically positive nodes was not significantly higher than that for patients with clinically negative lymph nodes. However, the patients with

Table 3. Five-Year Rates of LRR According to Single Variables Describing the Extent of Disease

Factor	5-Year Rate (%)	P	Crude Rate		Sites of Failure
			No.	%	
Clinical T stage					
T1	0	< .0001	0/5	0	
T2	12		5/56	9	CW-3, SCF-3, AX-1, ICF-2, IMC-0
T3	25		9/44	20	CW-6, SCF-4, AX-2, ICF-1, IMC-1
T4	51		20/45	44	CW-18, SCF-4, AX-3, ICF-0, IMC-0
Clinical LN status					
Negative	23	.307	7/42	17	CW-5, SCF-4, AX-1, ICF-0, IMC-0
Positive	27		25/106	24	CW-20, SCF-8, AX-5, ICF-3, IMC-1
Pathologic primary size					
≤ 2.0 cm	18	.005	12/74	16	CW-11, SCF-3, AX-1, ICF-0, IMC-0
2.1-5.0 cm	36		15/56	27	CW-10, SCF-7, AX-4, ICF-3, IMC-1
> 5.0 cm	46		6/14	43	CW-5, SCF-2, AX-1, ICF-0, IMC-0
Pathologic LN status					
0 + LN	12	< .0001	6/62	10	CW-5, SCF-3, AX-0, ICF-0, IMC-0
1-3 + LN	18		7/42	17	CW-5, SCF-2, AX-2, ICF-1, IMC-1
4-9 + LN	57		14/30	47	CW-12, SCF-5, AX-1, ICF-2, IMC-0
> 10 + LN	30*		3/11	27	CW-3, SCF-0, AX-0, ICF-0, IMC-0

Abbreviations: LN, lymph node; CW, chest wall; SCF, supraclavicular fossa; AX, axilla; ICF, infraclavicular fossa; IMC, internal mammary chain.
*Rate at 3 years; no patients were at risk at 5 years.



Patients at Risk						
I	1	1	1	1	0	0
IIA	21	20	18	10	5	3
IIB	44	42	39	28	15	12
IIIA	35	32	27	23	21	15
IIIB	38	28	21	10	6	6
IV	11	7	4	3	2	1

Fig 2. LRR-free survival for patients divided according to clinical stage of the disease.

more advanced clinical nodal disease stage (N2, N3, or M1 *v* N0 or N1) had significantly higher rates of LRR ($P < .0001$; data not shown). LRR did not correlate with pretreatment patient age, estrogen receptor status, or progesterone receptor status.

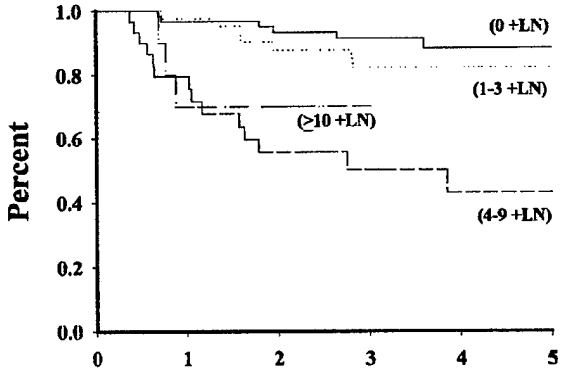
Pathologic disease extent after neoadjuvant chemotherapy also positively correlated with LRR. Figure 2 shows the actuarial LRR curves for the patients divided according to the number of pathologically involved lymph nodes after neoadjuvant chemotherapy. On univariate analysis, LRR was also associated with higher pathologic size of the primary (Table 3).

Table 4 provides LRR data according to clinical stage, clinical T stage combined with pathologic nodal status, and combined pathologic extent of primary disease and nodal status. The local recurrence-free survival curves for the patients divided according to the clinical stage of disease are shown in Fig 3.

A pathologic complete response (pCR) of both the primary tumor and lymph nodes was achieved in 18 patients. The 5-year LRR rate for patients with a pCR was 19%, which was not significantly different from the LRR rate of 28% in those who did not achieve a pCR ($P = .413$). However, the small sample size of the pCR group led to a large 95% CI (6% to 48%). The pretreatment clinical stages of the four patients with pCR who experienced an LRR were T3N0, T2N2, T4NX, and T4N2. Three of the 18 patients with pCR had residual noninvasive disease in the breast. If one considered these patients as not having a pCR, the LRR rate for the pCR group was 15% and continued to not be significantly different from that of the remaining patients ($P = .29$).

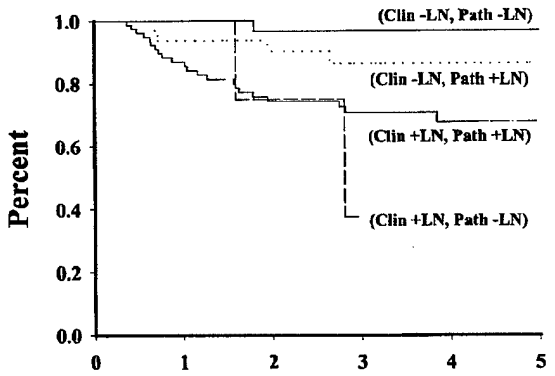
Table 4. Five-Year Rates of LRR According to Multiple Variables Describing the Extent of Disease

Factor	5-Year Rate (%)	P	Crude Rate		Sites of Failure
			No.	%	
Clinical stage					
I	0*	< .0001	0/1	0	
IIA	5		1/21	5	CW-1, SCF-0, AX-0, ICF-0, IMC-0
IIB	16		5/44	11	CW-5, SCF-2, AX-1, ICF-1, IMC-0
IIIA	17		5/35	14	CW-3, SCF-2, AX-1, ICF-1, IMC-1
IIIB	50		16/38	42	CW-14, SCF-5, AX-3, ICF-0, IMC-0
IV	79		7/11	64	CW-4, SCF-3, AX-1, ICF-1, IMC-0
Clinical T stage, pathologic LN status					
T1-2, negative LN	5	.004	1/19	5	CW-1, SCF-0, AX-0, ICF-0, IMC-0
T3-4, negative LN	34		6/23	26	CW-4, SCF-4, AX-1, ICF-0, IMC-0
T1-2, positive LN	13		4/42	10	CW-2, SCF-2, AX-1, ICF-1, IMC-0
T3-4, positive LN	36		21/64	33	CW-18, SCF-5, AX-4, ICF-1, IMC-1
Pathologic T size, pathologic LN status					
≤ 2.0 cm, negative LN	10	.002	2/21	10	CW-2, SCF-1, AX-0, ICF-0, IMC-0
2.1-5.0 cm, negative LN	49		3/14	21	CW-1, SCF-2, AX-1, ICF-0, IMC-0
> 5.0 cm, negative LN	20		1/5	20	CW-1, SCF-1, AX-0, ICF-0, IMC-0
≤ 2.0 cm, positive LN	20		9/52	17	CW-8, SCF-2, AX-1, ICF-0, IMC-0
2.1-5.0 cm, positive LN	30		11/41	27	CW-8, SCF-5, AX-3, ICF-3, IMC-1
> 5.0 cm, positive LN	63		5/9	55	CW-4, SCF-1, AX-1, ICF-0, IMC-0



Patients at Risk						
0 +LN	62	60	53	37	27	20
1-3 +LN	42	40	34	26	15	11
4-9 +LN	30	20	14	9	6	6
≥10 +LN	11	7	5	1	0	0

Fig 3. LRR-free survival for patients divided according to the pathologic status of axillary lymph nodes. Abbreviation: +LN, positive lymph nodes.



Patients at Risk						
-LN, -LN	29	28	27	18	12	9
-LN, +LN	32	30	25	18	15	11
+LN, -LN	4	3	2	0	0	0
+LN, +LN	79	63	51	36	21	17

Fig 4. LRR-free survival for patients divided according to both the clinical and pathologic stage of the axillary lymph nodes. Abbreviations: −LN, negative lymph nodes; +LN, positive lymph nodes; Clin, clinical stage; Path, pathologic stage.

Figure 4 shows LRR rates for patents divided according to their clinical and pathologic nodal disease status. The 5-year LRR rate for patients with clinically and pathologically negative lymph nodes was 3% (95% CI, 0.5% to 22%), compared with rates of 14% (95% CI, 5% to 33%) for clinically negative and pathologically positive lymph nodes, 63% (95% CI, 19% to 99%) for clinically positive but pathologically negative lymph nodes, and 32% (95% CI, 22% to 46%) for clinically positive and pathologically positive lymph nodes.

The only treatment-related factor associated with LRR was the use of tamoxifen. The 5-year rate of LRR was 7% in the patients treated with tamoxifen compared with 36% for those not treated with tamoxifen ($P = .0013$).

In a forward stepwise multivariate analysis by Cox logistic regression, the three factors that were independently associated with increased risk for LRR were initial clinical stage (IIIB or greater; hazard ratio of 4.5; $P < .001$), pathologic involvement of four or more lymph nodes (hazard ratio of 2.7; $P = .008$), and failure to use tamoxifen (hazard ratio of 3.9; $P = .027$).

DISCUSSION

In this article, we provide the first data concerning postmastectomy LRR after neoadjuvant chemotherapy and mastectomy. We demonstrated that both the initial clinical extent of disease and the pathologic findings have to be considered when determining LRR risk. It is clear from our data that patients with stage IIIB or greater disease and patients with four or more positive lymph nodes were at high risk of LRR. The only cohort of patients we identified to be at low risk for LRR were those patients with stage I/II disease who had clinically and pathologically negative lymph nodes. A number of other categories of patients, such as patients with one to three positive lymph nodes, carried intermediate risk for LRR.

The data from this study are important because postmastectomy radiation can reduce LRR and improve OS for women with a high-risk for LRR. The Danish 82b and 82c trials and a randomized prospective trial from Vancouver, British Columbia, all demonstrated that the addition of radiation to mastectomy and chemotherapy resulted in an approximately 20% proportional reduction in the risk of death.[8-10] It is clear, however, that radiation is not needed in every breast cancer case treated with mastectomy and chemotherapy. For example, many women treated initially with surgery for early-stage disease have LRR rates of less than 10%.[15] Therefore, the cost and potential toxicities of adjuvant radiation for these patients clearly outweigh any small potential benefit. However, our data suggest that patients with more advanced disease who are downstaged after neoadjuvant chemotherapy still have clinically relevant rates of LRR and may ultimately benefit from postmastectomy radiation.

A number of studies have been conducted to define selection criteria for the use of postmastectomy radiation. We recently conducted a retrospective study of more than

1,000 women treated with mastectomy and adjuvant chemotherapy without radiation.[15] Patients with tumor size greater than 4 cm, patients with four or more positive lymph nodes, and patients with gross extracapsular extension of disease, positive surgical margins, or skin invasion all had rates of LRR exceeding 15%. These data support a similar study of patients treated on prospective trials by the Eastern Cooperative Oncology Group.[16] Both of these studies indicated that the most powerful predictors of LRR were pathologic factors.

Neoadjuvant chemotherapy is becoming increasingly popular in the United States. This treatment sequencing was originally formulated for women with advanced, inoperable disease. However, as clinical trials demonstrated a benefit for adjuvant chemotherapy independent of a patient's lymph node status, many groups began using neoadjuvant chemotherapy in early-stage disease. To test the efficacy of neoadjuvant chemotherapy, the National Surgical Adjuvant Breast and Bowel Project conducted a prospective clinical trial in which patients who had stage I to III breast cancers were randomized between neoadjuvant chemotherapy followed by surgery versus surgery followed by adjuvant chemotherapy.[1] This trial demonstrated an equivalent disease-free survival and OS between the two groups. However, the rates of breast conservation were higher in the neoadjuvant chemotherapy patients. Given these data, the National Surgical Adjuvant Breast and Bowel Project has adopted neoadjuvant chemotherapy as standard treatment in their current early-stage breast cancer trials. The currently ongoing B-27 trial is a three-arm study in which all patients receive neoadjuvant chemotherapy.

A significant difference between neoadjuvant and adjuvant chemotherapy is that the pathologic information gained after the surgical procedure has different meanings. A number of groups, including our own, have shown that the pathologic extent of disease after neoadjuvant chemotherapy is the most powerful determinant of survival.[2-7] For example, Kuerer et al[7] reported a 5-year survival rate of 89% in patients who achieved a pCR compared with a 64% rate in those who did not have a pCR ($P < .01$). Although disease extent continues to be a powerful predictor for survival, it is unclear how disease extent affects LRR risks after mastectomy. Although it is generally accepted that patients with extensive lymph node or primary disease after neoadjuvant chemotherapy are likely to be at high risk for LRR, it is unclear whether patients with clinically advanced disease who achieve an excellent response to neoadjuvant chemotherapy warrant postmastectomy radiation. Our data indicate that indeed this group remains at high risk for LRR.

The relatively small number of LRR events in this series does not allow us to make conclusive statements regarding the appropriate treatment volume that should be included in postmastectomy radiation fields. It has been our philosophy to treat with comprehensive fields that include the chest wall and draining lymphatics.

There are two limitations of this study that should be considered when interpreting the rates of LRR for these intermediate-risk patients. The limited sample size of patient subgroups and the relatively short median follow-up period of the population increase the uncertainty of our findings. For example, in our previous report of failure patterns after mastectomy and adjuvant chemotherapy, 21% of the total number of LRRs developed after 5 years of follow-up.[15] Therefore, the estimates of LRR in this report are likely underestimates of lifetime risk of LRR.

In conclusion, we have demonstrated that both the clinical and pathologic extent of disease must be considered when deciding whether to administer postmastectomy radiation to a patient treated with neoadjuvant chemotherapy. Patients with locally advanced disease (independent of the pathologic response) and patients with positive lymph nodes may benefit from postmastectomy radiation.

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Impact of Systemic Treatment on Local Control for Patients With Lymph Node–Negative Breast Cancer Treated With Breast-Conservation Therapy

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Purpose: **To determine the impact of tamoxifen and chemotherapy on local control for breast cancer patients treated with breast-conservation therapy.**

Patients and Methods: **The data from 484 breast cancer patients who were treated with breast-conserving surgery and radiation were analyzed. Only patients with lymph node–negative disease were studied to provide comparative groups with a similar stage of disease and a similar competing risk for distant metastases. Actuarial local control rates of the 277 patients treated with systemic therapy (128, chemotherapy with or without tamoxifen; 149, tamoxifen alone) were compared with the rates for the 207 patients who received no systemic treatment. Only 10% of the patients had positive (2%), close (3%), or unknown margin status (5%).**

Results: **Patients treated with systemic therapy had improved 5-year (97.5% v 89.8%) and 8-year (95.6% v 85.2%) local control rates compared with those that did not receive systemic treatment ($P = .004$, log-rank test). There was no statistical difference in local control between patients treated with chemotherapy and patients treated with tamoxifen alone ($P = .219$). Systemic treatment, margin status, young patient age, estrogen and progesterone receptor status, and primary tumor size were analyzed in a Cox regression analysis. The use of systemic treatment was the most powerful predictor of local control: patients who did not receive systemic treatment had a relative risk of local recurrence of 3.3 (95% confidence interval, 1.5 to 7.5; $P = .004$).**

Conclusion: **In this retrospective analysis, systemic therapy appears to contribute to long-term local control in patients with lymph node–negative breast cancer treated with breast-conservation therapy.**

J Clin Oncol 19:2240-2246.

THE PERCENTAGE of breast cancer patients diagnosed with early stage disease has significantly increased over the past two decades.[1] Simultaneously, there has been a dramatic increase in the use of breast-conserving local treatments for these women. This change in practice resulted from randomized studies showing that breast conservation therapy provided an outcome equivalent to a modified radical mastectomy for patients with early stage breast cancer.[2,3] The ipsilateral breast recurrence rates in these randomized trials were approximately 1% per year, which permitted 90% of the women treated with breast-conservation therapy to avoid mastectomy during a 10-year period of follow-up.[2,3] Also during this era, clinical trials proved that systemic treatment with chemotherapy and/or tamoxifen improved the probability of survival for both women with lymph node–positive disease and the majority of women with lymph node–negative disease.[4,5]

The randomized trials investigating systemic therapy in early stage breast cancer had recurrence-free survival and overall survival as their endpoints. Less information is available concerning the impact of systemic treatment on local recurrence rates after breast-conserving local treatment. This issue is becoming more important as the percentage of breast cancer patients with stage I disease increases. In these patients, local recurrences account for one third to one half of the total number of treatment failures.

In this article, we report the results of a retrospective analysis investigating the impact of systemic therapy on local recurrence rates after breast-conserving surgery and radiation. An inherent difficulty and potential shortcoming of such a retrospective analysis is that a number of clinical and pathologic factors likely influenced the decision to use or not use systemic treatment. To minimize some of the major confounding factors, we elected to study only women with lymph node–negative breast cancer that were treated with breast-conserving surgery and radiation. Since nearly

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Submitted May 3, 2000; accepted January 11, 2001.

Supported in part by National Cancer Institute Department of Health and Human Services grant nos. CA16672 and T32CA77050. T.A.B. is supported by grant no. BC980154, an USAMRMC Breast Cancer Research Program Career Development Award.

Presented at the Eighty-Second Annual Meeting of the American Radium Society, London, England, April 1-5, 2000.

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all of the women with lymph node–positive disease treated during the years of this study received systemic treatment, we could not evaluate the impact of systemic treatment on local control in patients with this stage of disease. Focusing our study on patients with lymph node–negative disease also selected a population with a low competing risk for the development of metastatic disease.

PATIENTS AND METHODS

Between 1987 and 1995, 484 breast cancer patients with pathologically negative lymph nodes were treated with breast-conserving surgery and radiation at our institution. The records from these patients were retrospectively analyzed. We chose the years included in this study, before data abstraction, because they encompassed an era when an attempt to achieve negative surgical margins was the routine.

All patients underwent a segmental mastectomy with axillary lymph node dissection. Final surgical margins were negative (≥ 2 mm) in 90% of the cases. In the remaining 10% of the cases, 2% had positive margins (tumor cells present at inked margin), 3% had close margins (< 2 mm), and in 5% the margin status was unknown. All patients were treated with radiation involving only the ipsilateral breast. The median dose to the breast was 50 Gy delivered in 25 fractions over a 5-week treatment course with photons from a linear accelerator. A tumor bed boost (median dose, 10 Gy) was delivered in 57%, and only two patients were treated with a brachytherapy boost.

Two hundred seven patients (43%) were treated with surgery and radiation alone, whereas 277 patients received systemic therapy in addition to the surgery and radiation. The decision to use systemic treatment was made by the treating medical oncologist and the patient and was likely influenced by prognostic variables of the particular case. Of those treated systemically, 149 patients were treated with tamoxifen alone and 128 patients were treated with systemic chemotherapy with or without tamoxifen. Doxorubicin-based combination chemotherapy was used in 107 of these patients, with 77 receiving six cycles of 5-fluorouracil, doxorubicin, and cyclophosphamide. An additional 18 patients were treated with cyclophosphamide, methotrexate, and 5-fluorouracil, and three patients were treated with other non–doxorubicin-containing regimens. Of the patients treated with chemotherapy, only 13 were subsequently treated with tamoxifen. Fifty-nine patients received radiation followed by chemotherapy (46%), and 69 patients received chemotherapy followed by radiation (54%). The median time interval from surgery to radiation in patients treated with chemotherapy first was 6.7 months. The median time interval from surgery to chemotherapy in patients treated with radiation first was 3.0 months. We have previously reported the effect of the sequencing of chemotherapy and radiation on clinical outcome in the patients treated with chemotherapy.[6]

The method of Kaplan and Meier[7] was used to generate actuarial local control curves for various subgroups of patients. All event and follow-up times were measured from the date of diagnosis. Two-sided log-rank tests were used to detect differences in actuarial data. The data were also analyzed using the cumulative incidence methodology.[8] Because these analyses essentially provided the same results, only the results using the Kaplan-Meier data were reported. A Cox proportional hazards model was used to determine independent variables associated with local control.[9] Cases with unknown factors were excluded in the initial Cox regression analysis. If a factor did not predict for local control, the cases with unknown values for that factor were again added, and the Cox regression was repeated with that particular factor dropped. A Fisher's exact test was used to compare clinical and tumor characteristics of populations. Unknown values were not included in the Fisher's exact tests.

Table 1. Patient Characteristics

	No Systemic Treatment (n = 207)		Systemic Treatment (n = 277)		
	No.	%	No.	%	P
Tumor size					< .001
0.1-1 cm	81	39	26	9	
1-2 cm	98	47	171	62	
2.1-5.0 cm	24	12	76	27	
Unknown	4	2	4	1	
Age					
< 40 years	29	14	46	17*	.435†
40-60 years	116	56	135	49	
> 60 years	62	30	96	35	
Negative margins	190	92	242	87	.014‡
Close	0	0	16	6	
Positive	6	3	6	2	
Unknown	11	5	13	5	
ER-positive	102	49	181	65§	.017
ER-negative	63	30	67	24	
Unknown	42	20	29	10	
PR-positive	90	43	153	55§	.222
PR-negative	62	30	82	30	
Unknown	55	27	42	15	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

*The distribution of age according to the type of systemic treatment was chemotherapy: under 40, 34%; 40 to 60, 61%; over 60, 5%; tamoxifen: under 40, 2%; 40 to 60, 38%; over 60, 60%.

†The *P* value represents a comparison of the percentage of the population under 40 years old.

‡The *P* value represents a comparison of positive plus close versus negative. A comparison of positive versus close plus negative has a *P* = .405 value. If all four values are used, *P* = .001.

§The distribution of ER and PR according to the type of systemic treatment was chemotherapy; ER-positive, 34%; ER-negative, 47%; ER unknown, 19%; tamoxifen: ER-positive, 93%; ER-negative, 4%; ER unknown, 3%.

RESULTS

Table 1 lists the patient and tumor characteristics for the patients divided according to the use of systemic treatment. As shown, the two groups (systemic treatment *v* no systemic treatment) were comparable with respect to the percentage of patients under the age of 40 and the progesterone receptor status. However, within those receiving systemic treatment, the patients treated with chemotherapy had a higher percentage of patients under 40 compared to those treated with tamoxifen alone. As expected, several other prognostic features differed in the two populations. Specifically, the patients treated with systemic therapy more often had primary tumor sizes exceeding 2 cm and more often had close or positive surgical margins. In contrast, the patients

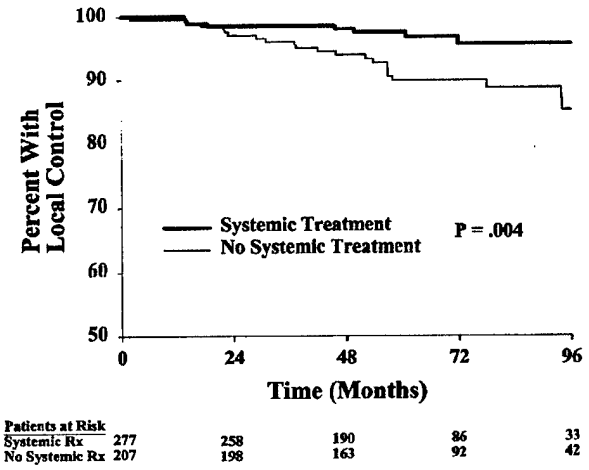

Fig 1. Actuarial local control curves according to the use of systemic treatment.

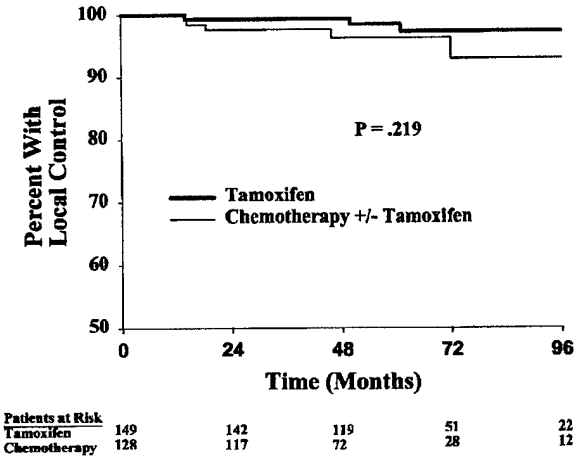

Fig 2. Actuarial local control curves for the cohort of patients treated with systemic therapy according to use of tamoxifen alone or chemotherapy.

who did not receive systemic treatment more often had estrogen receptor–negative disease.

The median follow-up for surviving patients in this study was 66 months. The 5-year and 8-year overall survival for the study population was 92% and 83%, respectively. There were eight local recurrences in the 277 systemically treated patients compared with 21 local recurrences in the 207 patients who did not receive systemic treatment. There were no patients with regional recurrences. Figure 1 displays the actuarial local control curves for the patients divided according to the use of systemic therapy. Local control was higher in patients treated with systemic therapy compared with those not receiving systemic treatment ($P = .004$): the 5-year rates were 97.5% versus 89.8% and the 8-year rates were 95.6% versus 85.2%, respectively. There were no local recurrences in the patients followed for over 8 years, so the 10-year rates were identical to the 8-year rates (patients still at risk at 10 years, n = 27). There was no difference in local control according to whether patients were treated with chemotherapy or whether they received tamoxifen alone ($P = .219$) (Fig 2): the 5-year and 8-year local control rates for those receiving chemotherapy versus those receiving tamoxifen alone were 96% versus 98% and 93% versus 97%, respectively. Local control was improved in the patients treated with chemotherapy versus those with no systemic treatment, but this difference was not statistically significant ($P = .162$). As previously reported, there was not a statistically significant difference in local regional control in chemotherapy patients divided according to their sequencing of chemotherapy and radiation (local control rates at 8-years: sequenced chemotherapy-radiation *v* sequenced radiation-chemotherapy, 91% *v* 94%, respectively; $P = .351$).[6] The improvement in local control seen between the patients treated with tamoxifen alone and those not receiving systemic treatment was significant ($P = .004$). The median interval to local recurrence was 44 months for the entire group of patients treated with systemic therapy, 32 months for those treated with chemotherapy, 55 months for those treated with tamoxifen alone, and 46 months for those not receiving systemic treatment.

The effect that estrogen and progesterone receptor status, primary tumor size, surgical margins, and young patient age had on local control was also analyzed. Of these variables, only patient age influenced local control. However, it is important to note that only 2% of the patients in this report had positive surgical margins, so evaluation of the significance of positive margins on local control could not be adequately studied. Figure 3 displays the actuarial local control curves for patients less than 40 years old versus those 40 years or older. The 5-year and 8-year local control rates for the patients under 40 years old compared with those 40 or older were 87% versus 95% and 84% versus 92%, respectively ($P = .017$).

A Cox proportional hazards model revealed that young patient age and no use of systemic therapy were independent risk factors for local recurrence. The hazard ratio for local recurrence for patients under 40 compared with those 40 or over was 2.8 (95% confidence interval [CI], 1.3 to 6.2; $P = .011$). The hazard ratio for local recurrence for patients not treated with systemic therapy compared with those treated with systemic therapy was 3.3 (95% CI, 1.5 to 7.5; $P = .004$). A Cox regression analysis was also performed to independently compare the hazards of local recurrence both for the chemotherapy treated group and the tamoxifen alone

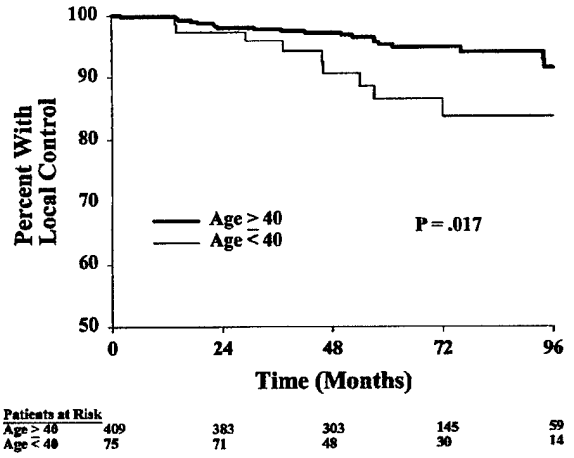

Fig 3. Actuarial local control curves according to patient age < 40 or ≥ 40 at the time of diagnosis.

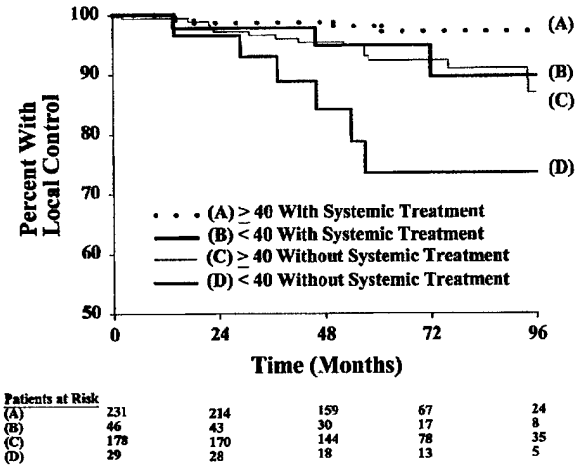

Fig 4. Actuarial local control curves according to patient age < 40 or ≥ 40 and the use of systemic treatment.

treated patients against those not treated with systemic therapy. The hazard ratio of local recurrence for patients not treated with systemic therapy compared with those treated with chemotherapy was 2.7 (95% CI, 1.0 to 7.5; $P = .057$). The hazard ratio of local recurrence for patients not treated with systemic therapy compared with those treated with tamoxifen alone was 4.2 (95% CI, 1.2 to 14.3; $P = .022$).

To further account for the different age distributions between the control, chemotherapy, and tamoxifen groups, we also compared the local control rates of the women older than 50 treated with tamoxifen (n = 127) to the women older than 50 in the control group (n = 112). The 8-year local control rates for these two groups were 99% in the tamoxifen group versus 88% in the control group ($P = .013$). In addition, we compared the local control rates of the women less than or equal to 50 years old treated with chemotherapy (n = 106) to the women less than or equal to 50 in the control group (n = 95). The 8-year local control rates for these two groups were 93% in the chemotherapy group versus 82% in the control group ($P = .057$).

Figure 4 displays actuarial local control curves for the entire population of patients divided according to both age (< 40 v ≥ 40) and the use of systemic treatment. The 5-year local control rates were 95% in the 46 patients less than 40 treated with systemic treatment and 74% in the 29 patients less than 40 who were not treated with systemic therapy ($P = .077$). The median intervals to local recurrence for the two systemically treated subgroups were 59 months for the younger patients and 34.5 for the older patients. The median intervals to local recurrence for the two subgroups not treated with systemic therapy were 46 months for the younger patients and 39.5 for the older patients.

DISCUSSION

Screening mammography and public education have led to a shift in the stage at which breast cancer is diagnosed to much earlier disease stages. Therefore, optimizing the management of early stage breast cancer has become increasingly important. The goal of combined-modality treatment of early stage breast cancer is to achieve the highest possible rates of breast preservation and overall cure by minimizing both the risk of local and distant recurrence. Randomized trials have clearly demonstrated that radiation plays a critical role in minimizing the risk of local recurrence after breast-conserving surgery.[2,3] Randomized data have also demonstrated that systemic therapy can reduce distant metastases for all stages of disease.[4,5] As such, systemic therapy has now become a standard component of therapy for breast cancer patients with a clinically relevant lifetime risk of distant metastases. This includes all women with lymph node–positive disease and the majority of women with lymph node–negative disease.[10]

The randomized data concerning the efficacy of systemic treatment in early stage breast cancer have focused on its role in minimizing the development of distant metastases. Fewer data are available on how the use of systemic therapy affects local control rates after breast-conserving surgery. It is clear that systemic therapy cannot be safely used as a substitute for breast radiation in the treatment of early stage breast cancer. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-06 trial, the 12-year rate of local recurrence in patients treated with lumpectomy and chemotherapy (no radiation) was 40% compared with a 10% rate in patients treated with lumpectomy and radiation.[2] In the

Scottish randomized trial, favorable breast cancers treated with either lumpectomy and tamoxifen or lumpectomy and chemotherapy had higher 6-year local recurrence rates compared with patients receiving breast radiation.[11]

Although systemic therapy does an inadequate job of preventing local recurrence after treatment with breast-conserving surgery alone, it has a positive impact on local control when it is used in conjunction with surgery and breast radiation. In this report, we found that the use of systemic therapy was the most powerful independent prognostic indicator for achieving local control after treatment with breast-conserving surgery and radiation. This effect was achieved even in a population considered to be at relatively low risk for local recurrence. Specifically, we focused our study on patients treated during the era in which surgical margins were routinely assessed, and we evaluated only patients with lymph node–negative disease.

The patients in this study had a median follow-up of only 66 months, so we cannot rule out the possibility that systemic treatment may delay rather than prevent local recurrences. It should also be noted that the small number of local recurrences that developed in our study population limits the certainty of our results. Another aspect of this study that warrants consideration is our decision to present the majority of our data with all patients treated with systemic therapy combined into one group. We felt this was justified in that there are published data suggesting both types of systemic treatments decrease local recurrence. Indeed, in our Cox regression analyses, both chemotherapy and tamoxifen use were associated with a reduced hazard ratio for local recurrence compared with the patients who did not receive systemic treatment.

The data from this series support the results of a number of randomized trials that have also suggested that systemic therapy has a positive benefit on local control after breast-conserving surgery and radiation. In the NSABP B-06 trial, patients treated with lumpectomy and breast radiation received systemic chemotherapy only if they had lymph node–positive disease. The 12-year local failure rate was less than 5% in lymph node–positive patients treated with lumpectomy, radiation, and chemotherapy compared with 10% in the lymph node–negative patients treated with surgery and radiation alone.[2] The NSABP B-13 and B-14 trials were randomized studies designed to test the systemic efficacy of chemotherapy (B-13) or tamoxifen (B-14) for women with lymph node–negative breast cancer. Both of these studies showed that systemic treatment decreased ipsilateral breast recurrences. In the patients treated with breast-conservation therapy in the B-13 trial, the use of chemotherapy decreased the 8-year rate of breast recurrences from 13% to 2.6% ($P = .001$).[12] The B-14 trial showed that tamoxifen use also decreased ipsilateral breast recurrences (10-year recurrence rates of 3.4% for those treated with tamoxifen *v* 10.3% for those not treated with tamoxifen; $P < .001$).[13] In an abstract publication of NSABP B-21, tamoxifen similarly improved local control rates in patients with lymph node–negative breast cancers measuring less than 1 cm. There was only a 0.36% annual breast tumor recurrence rate in women randomized to lumpectomy, radiation, and tamoxifen compared with a 1.2% annual rate in women treated with lumpectomy and radiation alone.[14] Finally, data from a Swedish randomized trial investigating tamoxifen use in early stage disease showed that tamoxifen reduced ipsilateral (hazard ratio, 0.4) and contralateral (hazard ratio, 0.4) breast tumor recurrences in the 432 patients with lymph node–negative disease treated with breast-conserving surgery and radiation.[15]

In contrast to these data, Fowble et al[16] did not find a statistically significant decrease in local recurrence according to tamoxifen use in 491 women with estrogen receptor–positive tumors treated with breast-conserving surgery and radiation. The 5-year actuarial local recurrence rate in the 337 patients who did not receive tamoxifen was 7% compared with a 4% rate in the 154 patients treated with tamoxifen ($P = .21$). In the subgroups of patients with lymph node–negative disease (319, no tamoxifen; 87, tamoxifen) there was also no difference in local recurrence ($P = .29$). This series differed from ours in that the control group included only patients with estrogen receptor–positive tumors, and therefore the median age of the patients was higher (62 years old). In another retrospective analysis, Wazer et al[17] also reported that tamoxifen was not an independent predictor of local control, although the 10-year rate of local recurrence was only 1.9% for those treated with tamoxifen versus 8.4% in those not treated with tamoxifen. In contrast to these studies and similar to our current study, Haffty et al[18] reported that the use of systemic therapy and age over 35 were independent predictors of local control. The Haffty et al study included both patients with lymph node–negative and lymph node–positive disease, so there was a significant difference in the stage of the two comparison groups.

Our series also differs from the above retrospective studies because only 2% of the study population had positive surgical margins. For example, 36% of the patients in the Fowble et al[16] report had positive, close, or unknown margin status compared with a rate of only 10% in our study. There have been a number of recent articles evaluating whether systemic therapy overcomes the negative prognostic aspect of margin status. A report of 184 patients treated at the Royal Marsden Hospital with lumpectomy, radiotherapy, and chemoendocrine therapy noted a local

recurrence rate of only 1.9% (median follow-up, 57 months), even though 38% of their population had unexcised, microscopically involved margins.[19] In a report of 533 patients treated with conservative surgery and radiation at the Joint Center of Radiation Therapy, a multivariate analysis revealed that systemic treatment and margin status were the only two independent predictors of local control.[20] Moreover, the crude local recurrence rate at 8 years was 7% in the 45 patients with focally positive margins treated with systemic therapy versus 18% in the 77 patients with focally positive margins who did not receive systemic therapy. For the patients with more extensive margin involvement, the use of systemic therapy did not reduce local recurrence (26% *v* 29%). Data from Fox Chase Cancer Center suggested that systemic therapy may delay but does not prevent local recurrences in patients with close or positive margins. In this cohort of patients, systemic therapy appeared to reduce the local recurrence at 5 years (11% *v* 5%), but at 10 years this effect was much less (16% *v* 12%).[21] In the Fox Chase study, the use of systemic therapy did not appear to affect local recurrence in their subset of patients with negative margins.[21]

In addition to margin status, young age has been recognized by many authors to be an independent risk factor for breast tumor recurrence.[18,22-25] Our own results corroborate these data and suggest that the negative effect of young age may be minimized by the use of systemic therapy. Specifically, the 5-year local recurrence rate in the patients under 40 who received systemic treatment in addition to surgery and radiation was only 5%. In contrast, the 5-year local recurrence rate in the patients under 40 who did not receive systemic treatment was 26%. Given the small size of the young age groups in our comparison, there is a need for additional data investigating this question.

We did not demonstrate a difference in local control according to the type of systemic treatment (tamoxifen alone *v* chemotherapy; $P = .219$). Furthermore, in our Cox regression analyses, both the use of tamoxifen ($P = .022$) and the use of chemotherapy ($P = .057$) decreased the hazard ratios for a local recurrence compared to that of the patients not receiving systemic treatment. We did not have sufficient data to compare whether the use of both types of systemic therapy further reduces the risk for local recurrence. However, in the NSABP B-20 trial, which compared the efficacy of chemotherapy and tamoxifen versus tamoxifen alone in lymph node–negative patients treated with breast-conservation therapy, there was a lower local recurrence rate in patients treated with both chemotherapy and tamoxifen (annual rate, 0.22% to 0.48%) versus tamoxifen alone (annual rate, 0.88%; $P < .025$).[26]

In conclusion, our data indicate that the 8-year breast local recurrence rate for patients treated with breast-conserving surgery, radiation, and systemic treatment is less than 5%. Tamoxifen is currently advocated for patients with noninvasive breast cancer because of its benefit in reducing local and contralateral breast recurrences.[27] Our data add to the accumulating literature suggesting that systemic treatment may have an equally beneficial role in reducing local recurrences in invasive disease. Therefore, clinicians should consider both the degree of reduction in distant metastases and the degree of improvement in local control when making decisions regarding systemic treatment, particularly in young patients.

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Int. J. Radiation Oncology Biol. Phys., Vol. 57, No. 2, pp. 327–335, 2003

0360-3016/03/$–see front matter

ELSEVIER

doi:10.1016/S0360-3016(03)00594-7

CLINICAL INVESTIGATION **Breast**

CARDIOVASCULAR DEATH AND SECOND NON–BREAST CANCER MALIGNANCY AFTER POSTMASTECTOMY RADIATION AND DOXORUBICIN-BASED CHEMOTHERAPY

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Purpose: To assess the incidence of long-term toxicity after postmastectomy radiation and doxorubicin-based adjuvant chemotherapy.

Methods: Records of 470 patients treated with mastectomy, doxorubicin-based chemotherapy, and postmastectomy radiation in five institutional prospective trials were retrospectively reviewed. Actuarial toxicity rates were compared with those of 1031 patients treated with mastectomy and doxorubicin-based chemotherapy who did not receive postmastectomy radiation. For those treated with radiation, the chest wall received a median dose of 55 Gy with Co-60 (42%) or electrons (51%). Adjuvant chemotherapy consisted of a doxorubicin-based regimen, often followed by 2 years of cyclophosphamide, methotrexate, and fluorouracil.

Results: Median follow-up was 10 years. The overall 10-year actuarial rates of RTOG toxicity Grade >1 and ≥3 after radiation were 4% and 2%, respectively. The overall 10- and 15-year actuarial rates of second non–breast cancer malignancy were 3.8% and 7%, respectively. There was no statistical difference between the rates of non–breast cancer second malignancy in the radiated and unirradiated cohorts (3.4% vs. 4.7% 10-year actuarial rates). Increasing age and treatment with >10 cycles of chemotherapy were associated with higher rates of second malignancy ($p = 0.025$, $p = 0.016$). The 10-year actuarial rate of death from myocardial infarction (MI) was 2.4% (eight events) and 0.5% (five events) in the radiated and unirradiated groups, respectively ($p = 0.058$). Of the 8 irradiated patients who died of MI, 2 patients had left-sided breast cancer.

Conclusions: We found very low rates of serious sequelae after postmastectomy radiation, including death from myocardial infarction and non–breast cancer second malignancy. The rate of second non–breast cancer malignancy was increased among patients treated with >10 cycles of cyclophosphamide-containing chemotherapy. © 2003 Elsevier Inc.

Myocardial infarction, Second malignancy, Radiation, Postmastectomy breast.

INTRODUCTION

Postmastectomy radiotherapy for breast cancer has been demonstrated in multiple randomized trials to improve rates of freedom from locoregional recurrence (1–11). In addition, three more recent trials have demonstrated a benefit in overall survival (8, 10, 11). However, long-term follow-up has shown that radiation use can have long-term toxicity that is, in part, dependent on radiation technique. A meta-analysis of the randomized trials investigating radiation use in breast cancer suggested that radiation use improved breast cancer–specific mortality, but this improvement was offset by an increase in deaths from cardiovascular disease (1). In addition, some data have suggested that radiation use for breast cancer is associated with increased rates of second cancers (12). Data from retrospective analyses have also shown an increased risk of acute leukemia and myelodysplastic syndrome after alkylating agent-containing chemotherapy, which is related to age and cumulative dose of the alkylating agent (13). Similarly, the National Surgical Adjuvant Breast and Bowel Project (NSABP) has reported that the incidences of acute leukemia and myelodysplastic syn-

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Supported in part by grants CA16672 and T32CA77050 from the National Cancer Institute, the Nellie B. Connally Breast Cancer Research Fund, and a grant from the Stanford and Joan Alexander Foundation, Houston, Texas. Dr. Buchholz is supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154.

Acknowledgments—The authors also wish to thank Jessica Erwin and Ramani Krishnan for their contributions to the database.

Received Jan 29, 2003, and in revised form Apr 28, 2003. Accepted for publication May 1, 2003.

drome were greater after surgery and chemotherapy than after surgery alone, and were further increased after surgery, chemotherapy, and radiotherapy (14). Finally, numerous retrospective studies have reported increased risks of skin and soft-tissue toxicity, pulmonary toxicity, rib fractures, and diminished range of motion after postmastectomy radiotherapy (15–22). Many of these reports, however, include patients treated with older radiation techniques; very few of these studies included patients who were treated with doxorubicin-based chemotherapy.

The goal of this study was to report the long-term rates of toxicity from our multidisciplinary approach to treatment, including radiation-related events, second malignancy, and death resulting from cardiovascular events after postmastectomy radiation and doxorubicin-based chemotherapy. We have compared the rates of second malignancy and deaths resulting from cardiovascular events among patients treated with postmastectomy radiation and doxorubicin-based chemotherapy with those from a similar cohort, treated on the same clinical trials, that did not receive postmastectomy radiation.

PATIENTS AND METHODS

Patient, tumor, and treatment characteristics

We retrospectively reviewed the records of the 462 patients with breast cancer (including 8 cases of bilateral breast cancer for a total of 470 cases) who received postmastectomy radiation therapy between 1975 and 1994, and 1031 patients with breast cancer who did not receive postmastectomy radiation. This population included all the patients who were treated with mastectomy and doxorubicin-based adjuvant systemic therapy with or without tamoxifen in five prospective clinical trials at The University of Texas M. D. Anderson Cancer Center (23–29). Each protocol was reviewed and approved by the institutional review board, and participants gave written informed consent. In four of the five prospective trials, referral for postoperative irradiation was at the discretion of the treating oncologists. The remaining trial intended to include a randomization for radiation treatment. The 184 patients who received radiation therapy in this trial (141radiation per physician preference, 43 randomized to radiation) were included in this analysis. Only patients with Stage II or IIIA disease were eligible for these five trials. Patients older than age 75 years, those with evidence of distant dissemination at diagnosis, and those with a prior or concurrent malignancy were excluded.

All patients underwent mastectomy before adjuvant systemic therapy. Patients were then randomly assigned to one of several chemotherapy regimens. Each protocol stipulated a minimum of six cycles of a doxorubicin/cyclophosphamide–containing regimen and a minimum doxorubicin dose of 40–50 mg/m^2 per cycle. Adjuvant chemotherapy generally consisted of fluorouracil (400–500 mg/m^2 on Days 1 and 8), doxorubicin (40–50 mg/m^2 intravenously [IV] on Day 1 or by 72-h continuous infusion), and cyclophosphamide (400–500 mg/m^2 IV on Day 1 (FAC) administered every 21 days. No patients received preoperative chemotherapy in these trials. Patients who completed six cycles of FAC on the first two protocols received cyclophosphamide, methotrexate, and fluorouracil (CMF) every 3–4 weeks until completing 2 years of chemotherapy.

End points and statistical analysis

The distributions of patient and tumor characteristics in the two groups were compared using the chi-square test, and are reported in full in the accompanying article on locoregional recurrence in these cohorts also published in this issue (30). Five- and 10-year actuarial rates of freedom from complication were calculated according to the Kaplan-Meier method (31), with comparisons among groups performed using two-sided log–rank tests. The end point "any complication" included any radiation-related toxicity regardless of grade. These included brachial plexopathy, rib fracture, decreased range of motion, pulmonary fibrosis, pneumonitis, pigment changes, telangiectasia, soft-tissue fibrosis, and soft-tissue necrosis. Arm edema could not be assessed because of insufficient documentation in the medical record. Heart injuries and second non–breast cancers were addressed separately. Major complications were defined as any Grade 3 toxicity or higher (among the radiation-related toxicities listed previously). Second malignancy was defined as cancer of any site other than the breast, including hematologic malignancy, diagnosed after the breast cancer.

RESULTS

Patient and treatment characteristics

Patient and treatment characteristics of the irradiated and unirradiated cohorts are listed in Table 1. Additional data have been reported in a related article published in this journal. The median age was 49 years (interquartile range 42 to 57 years) in both the radiation group and the no-radiation group. A total of 303 patients received radiation within our institution; 167 patients received radiation treatment elsewhere. Complete records concerning radiation treatments were available for review for 350 patients. In these patients, the median dose to the chest wall (including a chest wall boost, where applicable), to the internal mammary chain (IMC), and to the supraclavicular fossa (SCV) was 55 Gy (interquartile range 50–60 Gy), 50 Gy (interquartile range 50–50 Gy), and 50 Gy (interquartile range 50–50 Gy), respectively. The median dose per fraction to the chest wall, IMC, and SCV was 2 Gy (interquartile range 2 Gy–2 Gy). The chest wall boost was a scar boost with generous margins to include much of the postmastectomy flaps. The chest wall was treated with Co-60 in 42.4% of patients and with electrons in 51% (4–17 MeV). Twenty-four patients were treated with 6-MV photons or with mixed-energy beams. A separate field was used to target the IMC in 326 patients. This field was treated with electrons and generally targeted the first three intercostal spaces. The median number of chemotherapy cycles given was 8 (inter-

Table 1. Patient, tumor, and treatment characteristics

Characteristic	No Radiation		Radiation		p*
	No.	%	No.	%	
Age					NS
Median	49 years		49 years		
Interquartile range	42–57 years		42–57 years		
≤40	226	22	98	21	
41–50	382	37	165	35	
51–60	262	25	126	27	
>60	161	16	81	17	
Tumor size					<0.001
Median	2.5 cm		3.0 cm		
Interquartile range	1.9–3.9 cm		2.1–5.0 cm		
No. of positive nodes					<0.001
Median	3 nodes		6 nodes		
Interquartile range	1–6 nodes		3–10 nodes		
No. of nodes examined					NS
Median	17		17		
Interquartile range	13–22		12–23		
Hormonal treatment					<.001
Yes	318	31	58	12	
No	645	62	403	86	
Unknown	68	7	9	2	
Chemotherapy cycles					<.001
Median	8		10		
Interquartile range	6–14		7–24		

Abbreviations: No. = number; NS = not significant.
* Chi-squared test for equality of distributions in the radiation and no radiation groups.
Due to small differences in rounding numbers, percentages do not always equal 100%.

quartile range 6–14) overall, 10 (interquartile range 7–24) in the irradiated cohort, and 7 (interquartile range 6–10) in the unirradiated cohort ($p < 0.01$). Three hundred seventy-nine patients received more than 10 cycles of chemotherapy, and 994 received fewer than 10 cycles. The number of cycles was unknown in 128 cases. For the patients who received more than 10 cycles, the most common regimen was 6 cycles of FAC followed by a year or two of maintenance CMF ($n = 198$). In addition to cytotoxic chemotherapy, 58 patients (12%) in the irradiated cohort with estrogen receptor (ER)- or progesterone receptor (PR)-positive tumors also received tamoxifen, compared with 318 patients (31%) in the unirradiated cohort ($p < 0.001$). Median follow-up from the date of initial histologic diagnosis for all patients alive at the time of analysis was 120 months (range 6–262 months).

Radiation-related complications

In the cohort of 470 patients treated with postmastectomy radiation, the 5-, 10-, and 15-year actuarial rates of freedom from any complication were 90%, 89%, and 89%, respectively. The corresponding rates for freedom from major complication were 99%, 98.5%, and 98%, respectively (Fig. 1). In total, 46 patients experienced a complication of any grade, and 7 patients experienced a major complication. The crude incidence of specific toxicities and grade are listed in Table 2. Neither total dose to the chest wall (≤50 Gy vs. >50 Gy), beam type (electron or cobalt), fraction size, treatment of the IMC, or treatment of the SCV predicted for increased rates of complications. Univariate analysis of pulmonary complications alone, including pneumonitis and pulmonary fibrosis, also failed to identify factors that predict for significantly increased pulmonary complications. Factors examined included beam type, tamoxifen use, age, treatment of the IMC, and treatment of the SCV. In this

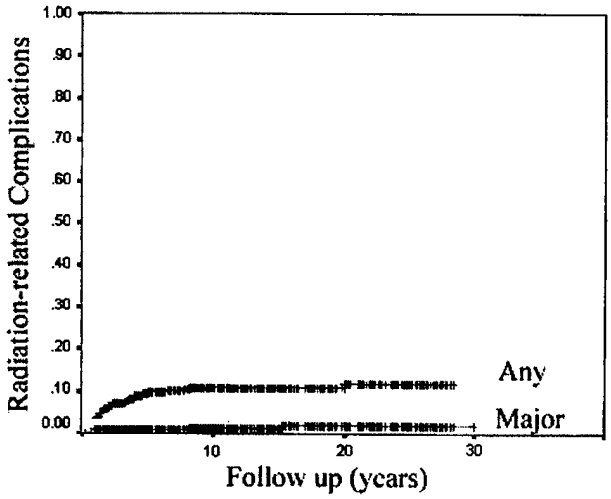

Fig. 1. Kaplan-Meier curves of any complication (RTOG ≥1) and major complication (RTOG ≥3) after mastectomy, radiotherapy, and doxorubicin-based chemotherapy.

Table 2. Radiation-related toxicity and grade

Toxicity	No	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5 (Fatal)
Any complication	64*	36	16	6	5	1
Brachial plexopathy	0	0 Subjective symptoms	0 Objective symptoms	0 Impaired function	0 Paralysis	0
Rib fracture	12	4 1 fracture	6 2–3 ribs	2 >5 ribs	NA	0
ROM	2	0 Requiring medication	1 Moderate stiffness/ constant pain	1 Severe functional limitation	NA	0
Pneumonitis	4	0 Mild	4 Moderate	0 Severe: hospitalization	0 Life-threatening	0
Pulmonary fibrosis	22	18 Asymptomatic	1 Exertional dyspnea	2 Normal activity dyspnea	1 Dyspnea at rest	0
Pigment change	3	1 Mild, present > 1 year	2 Severe	NA	NA	0
Soft tissue fibrosis/ necrosis	18	11 Mild	1 Moderate	1 Severe: impaired ROM	4 Necrosis	1
Telangiectasia	3	2 Scattered	1 Patchy: <1/2 treated area	0 Confluent: >1/2 treated area	0 Ulceration	0

Abbreviation: ROM = range of motion.

* Represents total number of radiation-related complications in the 470 cases. Some patients experienced multiple complications so this number is higher than the total number of patients experiencing a complication, 46.

Grading criteria represent an institutional adaptation of the National Cancer Institute Common Toxicity Criteria: *http://ctep.cancer.gov/forms/CTCManual_v4_10-4-99.pdf*

cohort, 1 patient died of radiation-related complications. This patient developed a radiation-induced necrosis of the glenohumeral joint, underwent attempted surgical repair with a complicated postoperative course, and ultimately died during the hospitalization for this repair. Part of the glenohumeral joint was in the treatment field.

Cardiac-related deaths

In total, eight deaths were attributed to myocardial infarctions (MI) among patients who were treated with postmastectomy radiation. The rate of MI-related death was higher in the irradiated cohort than in the unirradiated group (10-year rate of 2.4% vs. 0.5%, respectively, $p = 0.057$). Laterality of the breast cancer and dose delivered to patients who received radiation and subsequently died of a MI are listed in Table 3. As indicated, 2 patients had MIs during adjuvant systemic therapy. Excluding these 2 cases, the median time to MI was 109 months. Only 2 of the 8 patients who died of MI had left breast cancer. None of the irradiated patients treated with tamoxifen died of MI, and there was no

Table 3. Cardiovascular toxicity

	Dose (energy)		
Event	Chest wall (Gy)	IMC (Gy)	Laterality
MI*	50/25 (7 MeV)	50/25 (17 MeV)	Left
MI	47.75 (Co60)	50 (MeV)	Left
MI*	NA	NA	Right
MI	50/25 (4 MeV)	0	Right
MI	55 (13 MeV)	50 (9 MeV)	Right
MI	NA	NA	Right
MI	NA	NA	Right
MI	62.5	57.5	Right
CHF	NA	46 (Co60)	Left
CHF	60/29 (Co60)	56.25 (MeV)	Right
Pericarditis	55/20 (MeV)	56.25 (MeV)	Left
Pericarditis	NA	NA	Left

Abbreviations: MI = myocardial infarction; CHF = congestive heart failure; NA = not available.

* MI temporally related to chemotherapy administration.

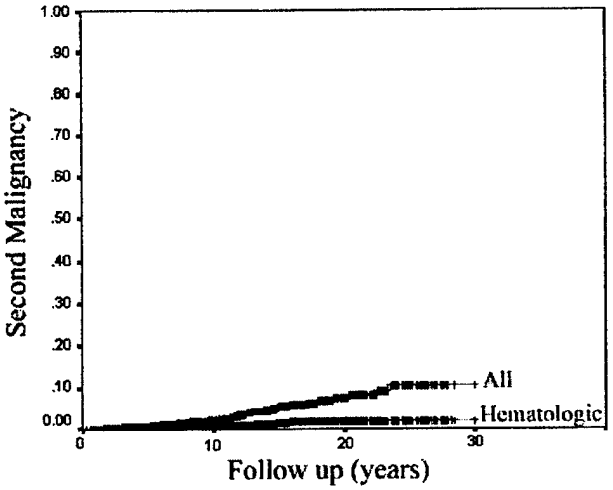

Fig. 2. Kaplan-Meier curves of second non–breast cancer malignancy (including hematologic malignancies) and hematologic malignancies for patients treated with mastectomy and doxorubicin-based chemotherapy with and without adjuvant radiation treatment.

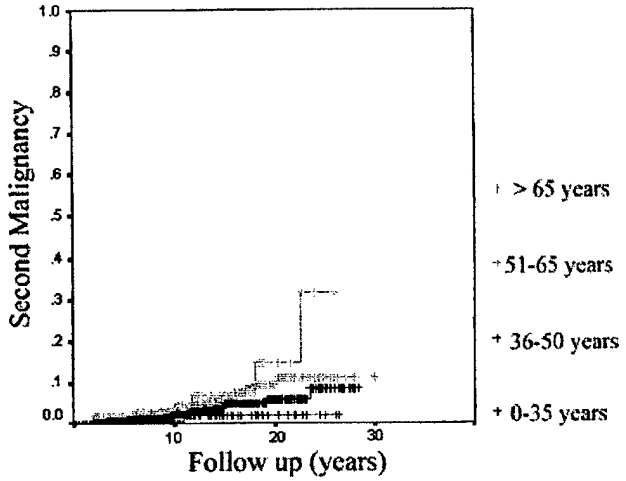

Fig. 3. Kaplan-Meier curves of second non–breast cancer malignancy (including hematologic malignancies) for all patients stratified by age ($p < 0.044$).

significant difference in the rate of death from MI when analyzed by tamoxifen use. In addition to the deaths from MI, there were two documented cases of pericarditis and two cases of congestive heart failure. No deaths were attributed to stroke after postmastectomy radiation. No nonfatal MIs were identified.

Second malignancy after treatment

The overall 15-year actuarial rate ($n = 1501$) of non–breast second cancer development was 7%. The overall 10-year actuarial rate of hematologic malignancy was 2% (Fig. 2). The rates of second cancers increased with increasing age at the time of breast cancer diagnosis (Fig. 3; $p = 0.04$). There was no significant difference in second cancer development in the radiation vs. no radiation group (Fig. 4a). Of the 25 cancers in patients treated with radiation, only 2 cases developed near the radiation treatment fields (two lung cancers). Site of second malignancy is listed in Table 4. Among irradiated patients, the hematologic system was the most common site of second malignancy ($n = 8$), followed by the gynecologic system ($n = 7$). Among unirradiated patients the gynecologic system was the most common site of second malignancy ($n = 9$), followed by the hematologic system ($n = 5$). Treatment with tamoxifen did not significantly increase the risk of second cancers (Fig. 4b). Patients treated with more than 10 cycles of chemotherapy had increased rates of second cancers as well as increased rates of hematologic malignancy compared with those who received fewer than 10 cycles of chemotherapy (Fig. 4c, $p = 0.016$). The most common chemotherapy regimen among patients treated with >10 cycles of chemotherapy was FAC followed by maintenance CMF. Analysis of patients treated with CMF-containing chemotherapy regimens compared to patients treated with non–CMF-containing regimens demonstrated a trend toward increased 10-year actuarial rates of second malignancy, 6.1% vs. 3.3% ($p = 0.08$).

DISCUSSION

The long-term toxicity associated with radiotherapy and chemotherapy for breast cancer has been of considerable interest for many decades. In this series, we found low rates of major complications attributed to radiation including non–breast cancer second malignancy and death attributed to MI after mastectomy and adjuvant doxorubicin-based chemotherapy. This article is important because it has a large sample size and a median follow-up of 10 years. In addition, unlike the majority of data assessing the toxicity of postmastectomy radiation therapy, all of the patients in our series received doxorubicin-based chemotherapy. Doxorubicin is currently considered to be a standard component of therapy for the vast majority of patients treated with postmastectomy radiation (32). Another advantage of this series is that the majority of patients in this series were treated in a single institution, with standardized techniques and dosages.

Four meta-analyses (Surveillance, Epidemiology and End Results [SEER], Cuzick *et al.*, Early Breast Cancer Trialists' Collaborative Group [EBCTCG], Swedish Cancer Registry) have suggested a significant association between postmastectomy radiation therapy for breast cancer and cardiovascular toxicity observed after 10 years of follow-up (1,33–35). In the meta-analysis by Cuzick *et al.*, the authors reported an increased rate of death in patients randomized to receive radiation vs. those not receiving radiation treatment (34). The cause of death was not available in that study. In a meta-analysis performed by the EBCTCG (1), radiation treatment reduced the risk of death from breast cancer, but increased the risk of death from cardiovascular disease.

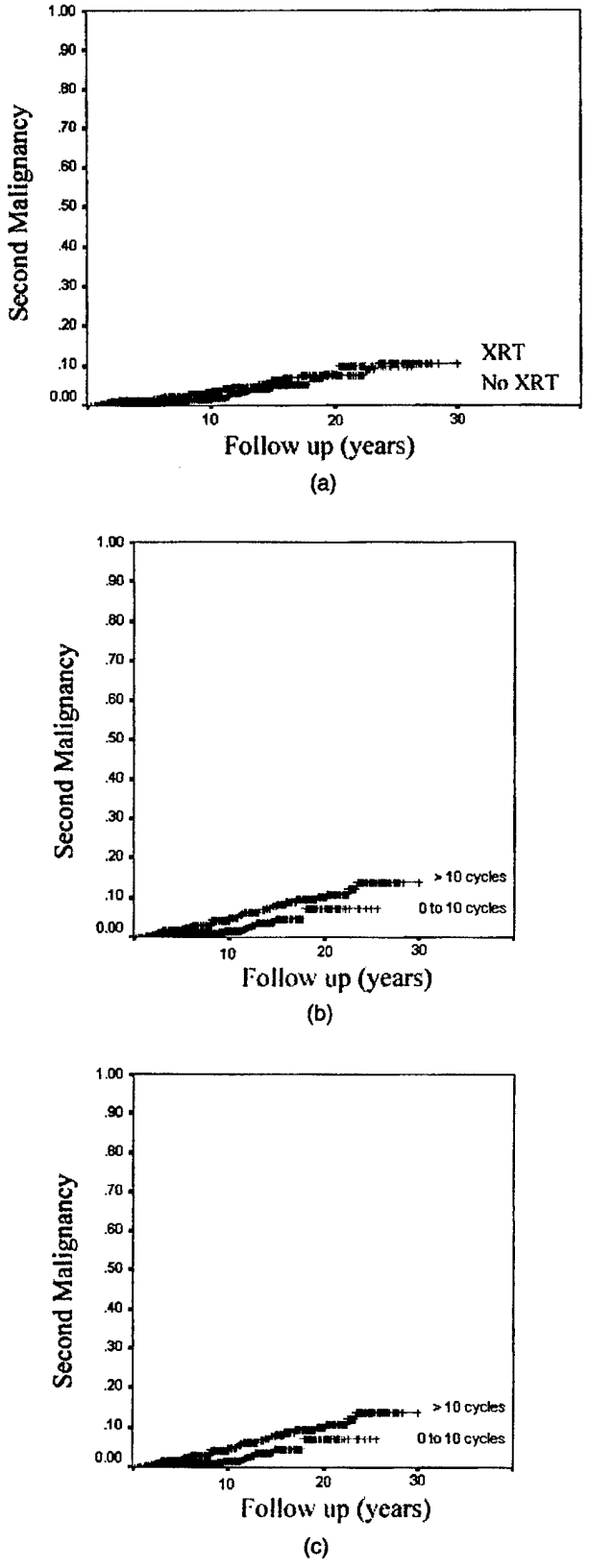

Fig. 4. Kaplan-Meier curves of second non–breast cancer malignancy stratified by initial adjuvant treatment: (a) radiation (p = NS), (b) tamoxifen (p = NS), or (c) chemotherapy, greater than 10 cycles, $p = 0.017$).

Table 4. Site of second malignancy

System	XRT*	No XRT
Hematologic	8	5
AML	5	2
PML	1	0
Large cell lymphoma	2	2
CLL		1
Gynecologic	7	9
Ovary	4	3
Endometrium	3	3
Cervix	1	1
Uterine sarcoma	0	1
Vulva	0	1
Gastrointestinal	3	5
Colon	1	3
Esophagus	1	1
Pancreas	1	0
Gastric	0	1
Head and neck	2	2
Tonsil	1	0
Thyroid	1	1
Tongue	0	1
Skin	2	2
Squamous	2	0
Melanoma	0	2
Genitourinary	1	1
Bladder	1	0
Kidney	0	1
Lung	2	5

Abbreviations: XRT = radiation therapy; AML = acute myelocytic leukemia; PML = promyelocytic leukemia; CLL = chronic lymphocytic leukemia.

* Three patients had two second malignancies; therefore, total equals 25.

Importantly, the difference in cardiovascular injuries was not apparent in the first 9 years after treatment, but became significantly increased between posttreatment years 10 through 20. Clearly, long-term follow-up is required to assess the risk of cardiovascular toxicity after postmastectomy radiotherapy.

The published meta-analyses that have addressed cardiovascular toxicity after radiotherapy have been criticized for including trials using radiation techniques and equipment considered suboptimal by today's standards.

Consequently, the dose delivered to the cardiac structures, the volume of the cardiac structures irradiated, and the daily fraction sizes were likely much higher than is currently acceptable. It is clear that radiation dose and technique affect the risk of radiation-related injury. A trial conducted in Norway and Sweden noted increased risk of cardiac-related mortality in the initial phase of the trial, but after the treatment techniques were changed to include more cardiac sparing, no difference in cardiac-related mortality was observed (6, 7). Therefore, it is hoped that with increasing use of more modern radiation therapy techniques, cardiac-related morbidity will become less significant.

In this series, we found a trend toward increased deaths from MI in the irradiated patients vs. those not receiving radiation ($p = 0.057$). However, only 8 of the 470 patients

treated with radiation died of cardiovascular disease, and multiple confounding factors make it is impossible to determine whether radiation treatments actually contributed to the deaths from MI. For example, 6 of the 8 patients who died of MI after radiation were receiving treatments for a right breast cancer. Although some dose to the right heart is possible with postmastectomy radiation for a right breast cancer (particularly if the IMC is also treated), the volume of heart potentially receiving some dose is substantially lower for right and left breast cancer treatment. In fact, a report that used SEER data for breast cancer patients treated between 1970 and 1985 suggested that the risk of MI was greater after postmastectomy radiation for left-sided breast cancer than for right-sided breast cancer (33). Indeed, with modern techniques, it is often possible to entirely avoid irradiating the heart, particularly for right-sided breast cancers. Therefore, the relative absence of fatal MI among women with left-sided breast cancer argues against the conclusion that radiation contributed to death from MI. Last, 2 patients who received radiation died of a MI during adjuvant chemotherapy treatment. This very short interval between radiation treatment and death may suggest that the deaths were unrelated to radiotherapy. We cannot conclude that the higher incidence of MI deaths we report is clearly attributed to radiation. However, as previously noted, the EBCTCG meta-analyses suggested that the majority of increased cardiovascular deaths associated with radiation occurred after 10 years. Therefore, further follow-up on these patients will be necessary.

The low rate of mortality from myocardial infarction we report is consistent with other series that have used modern radiation techniques to deliver postmastectomy radiation. The Danish Breast Cancer Cooperative Group conducted two concurrent trials (one in premenopausal breast cancer patients, the other in postmenopausal breast cancer patients) that investigated the value of postmastectomy radiation and found that radiation improved overall survival (8, 10). In these studies, electrons, which have a sharp fall-off of dose and thereby a limited dose to the cardiac structure, were used to treat the anterior chest wall. An important secondary component of these studies included surveillance for cardiac injury. After a median follow-up of 10 years, there was no increased risk of cardiac-related morbidity or mortality (36). The use of doxorubicin-based chemotherapy in our series is an important difference between this report and the Danish studies, because the recognized cardiac toxicity of this chemotherapy may affect rates of radiation-related cardiovascular injury.

In addition to demonstrating a low absolute risk of death resulting from cardiovascular injury, our series is important in demonstrating no increased risk of second cancers after postmastectomy radiation treatments. Although it is clear that radiotherapy for breast cancer increases the risk of second solid tumors, the absolute risk is very small. The most commonly accepted cancer thought to be a consequence of radiation is soft-tissue sarcoma, which did not occur in our series. These findings are consistent with the EBCTCG meta-analysis of radiotherapy trials, which did not demonstrate an increased risk of death from second non–breast cancer malignancy in patients treated with radiation for breast cancer compared with patients who did not receive radiation (1).

This is one of the first reports to demonstrate an association between non–breast cancer second malignancy and increasing number of chemotherapy cycles. In this study, the patients at greatest risk for second cancers were patients treated with six cycles followed by a year or more of maintenance therapy with CMF. This duration of adjuvant treatment, with prolonged administration of alkylating agents is not commonly practiced today. Our data support those from a Swiss randomized trial that also demonstrated an increased risk of second malignancies after breast cancer for patients treated with increased doses of CMF chemotherapy (37). This trial randomized 491 patients with breast cancer and one to three positive axillary lymph nodes to CMF chemotherapy or CMF chemotherapy with prednisone. They reported that the use of prednisone allowed for an increased dose of CMF, which was associated with a small increase in second malignancies. In our study, analysis of second malignancies stratified by treatment regimens with or without CMF demonstrated an increased rate of second malignancies for patients treated with any CMF containing regimens regardless of number of cycles. In our series, all patients treated with CMF had a high total cumulative dose of cyclophosphamide. The NSABP cooperative group has also published data suggesting that high total dosages of alkylating agents may increase the rate of non–breast cancer second malignancy (14). In the most recent update of the NSABP experience spanning six complete trials, Smith *et al.* report that the incidence of AML was sharply increased among patients receiving intensified regimens of cyclophosphamide at 5 years, 1.01% vs. 0.21% (37). The impact of increased dose of cyclophosphamide on second cancers other than AML was not reported in this study. We found that greater than 10 cycles of chemotherapy increases not only hematologic malignancies, but other solid tumors as well. These authors report that breast radiotherapy appeared to be associated with an increased risk of AML (38). In contrast, we have not found this to be the case in the current study.

With respect to other treatment-related toxicities, we found that the 15-year actuarial rate of major complication was only 2%. Furthermore, all major complications of Grade 3 or higher occurred in the first 5 years of follow-up. The most frequent complication observed was pulmonary fibrosis, which occurred in 11% of the patients. This rate included asymptomatic pulmonary fibrosis detected only on chest radiography, generally limited to the lung apex corresponding to the supraclavicular field. Pulmonary fibrosis has been reported to be increased in patients treated with IMC fields and in patients receiving tamoxifen simultaneously with radiation (15). We did not find these factors significant. In general, our philosophy has been to limit the maximum depth of lung included in tangent fields to 2 cm,

and only 12% of the patients in the irradiated cohort received tamoxifen. These factors may have contributed to the small number of events. It is important to note that no cases of brachial plexopathy were observed in our cohort. One patient did develop radiation-induced necrosis of the glenohumeral joint and died after attempted surgical repair. This patient had 24 lymph nodes resected (17 were positive) and was described as having severe and chronic arm edema. She was treated to the SCV and axilla with Co-60, 50 Gy in 25 fractions. The degree of bone necrosis observed in this case is extremely unusual, particularly with this standard fraction size. This unusual complication highlights the unpredictability of individual patient variation in sensitivity to radiation. More work is needed to identify patients who are genetically predisposed to radiation injury and for whom altered fractionation schemes or treatment regimens should be considered. Nevertheless, the low rate of complications for the cohort in general suggests that the standard dose of 50 Gy to the chest wall followed by a 10-Gy boost to the chest wall is well-tolerated.

We acknowledge that this is a retrospective study and limited by documentation biases. In addition, it is important to note that this cohort represents 1501 patients treated on prospective clinical trials that had inclusion criteria. As such, these results may not necessarily reflect those of patients with supraclavicular disease at presentation or patients older than 75 years of age who were ineligible for the study. Also, it might be helpful to compare these results with those of patients treated with surgery alone to better assess the impact of chemotherapy on rates of second malignancy. Finally, patients received radiation at the discretion of the treating physician. As reported in the accompanying article on locoregional recurrence in these cohorts also published in this issue (30), patients received radiation at the discretion of the treating physician, and patients in the radiated cohort had increased rates of factors that predict for decreased overall survival and locoregional control.

Postmastectomy radiation has been shown to improve survival and reduce locoregional recurrence in women with breast cancer. In this report, we affirm that this benefit can be provided with a low risk of significant treatment-related morbidity or mortality after 10 years. Although all radiation-related complications occurred within 5 years of treatment, strong evidence suggests that the risk of second malignancy and MI continue to increase with time, and long-term follow-up is necessary to evaluate these risks. Nevertheless, the low rates of complications with postmastectomy radiation and anthracycline-based chemotherapy are reassuring: we report a very low incidence of long-term complications despite the very long (and currently no longer used) duration of chemotherapy with alkylating agents.

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ELSEVIER

APPENDIX 22

Int. J. Radiation Oncology Biol. Phys., Vol. 57, No. 2, pp. 336–344, 2003

0360-3016/03/$–see front matter

doi:10.1016/S0360-3016(03)00593-5

CLINICAL INVESTIGATION **Breast**

LOCOREGIONAL RECURRENCE AFTER DOXORUBICIN-BASED CHEMOTHERAPY AND POSTMASTECTOMY: IMPLICATIONS FOR BREAST CANCER PATIENTS WITH EARLY-STAGE DISEASE AND PREDICTORS FOR RECURRENCE AFTER POSTMASTECTOMY RADIATION

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Purpose: To compare rates of locoregional recurrence (LRR) after mastectomy, doxorubicin-based chemotherapy, and radiation with those of patients receiving mastectomy and doxorubicin-based chemotherapy without radiation and to determine predictors of LRR after postmastectomy radiation.

Methods: Kaplan-Meier freedom-from-LRR rates were calculated for 470 patients treated with mastectomy, doxorubicin-based chemotherapy, and postmastectomy radiation in five single-institution clinical trials. The LRR rates in these patients were compared to previously reported rates in 1031 patients treated without radiation in the same trials.

Results: Median follow-up was 14 years. Irradiated patients had significantly less favorable prognostic factors for LRR than did unirradiated patients. Despite this, in all subsets of node-positive patients, postmastectomy radiation led to lower rates of LRR. This included patients with T1 or T2 tumors and one to three positive nodes (10-year LRR rates of 3% vs. 13%, $p = 0.003$). Multivariate analysis of LRR for patients with this stage of disease revealed that no radiation, close/positive margins, gross extracapsular extension, and dissection of <10 nodes predicted for increased LRR (hazard ratios 6.25, 4.61, 3.27, and 2.66, respectively). Significant predictors of LRR for patients treated with postmastectomy radiation were higher number and ≥20% positive nodes, larger tumor size, lymphovascular space invasion, and estrogen receptor (ER)-negative disease. Recursive partitioning analysis revealed ER-negative status to be the most powerful discriminator of LRR in irradiated patients.

Conclusions: Postmastectomy radiation decreases LRR for patients with breast cancer, including those with Stage II breast cancer and one to three positive lymph nodes. © **2003 Elsevier Inc.**

Postmastectomy, Radiation, Breast, Locoregional recurrence.

INTRODUCTION

Despite an abundance of data from studies spanning more than 30 years, significant controversy remains over which subsets of patients with breast cancer should receive postmastectomy radiation therapy (1–11). The three most recent prospective randomized trials investigating this question demonstrated that improvements in locoregional control can lead to improved overall survival for some patients who receive postmastectomy radiation (8, 10, 11). These data led both the American Society of Clinical Oncology (ASCO) and the American Society for Therapeutic Radiology and Oncology (ASTRO) to publish consensus statements regarding the value of postmastectomy radiation therapy (12, 13). Both statements independently concluded that radiation therapy is clearly beneficial for patients with four or more positive lymph nodes or Stage III disease. However, the value of postmastectomy radiation for patients with less extensive disease is still unclear.

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Presented at the American Society for Therapeutic Radiology and Oncology annual meeting, New Orleans, LA, Oct. 6–10, 2002.

Supported in part by grants CA16672 and T32CA77050 from the National Cancer Institute and a grant from the Stanford and Joan Alexander Foundation, Houston, Texas. Dr. Buchholz is supported by Department of Defense Breast Cancer Research Program Career Development Award, BC980154.

Acknowledgments—The authors also wish to thank Jessica Erwin and Ramani Krishnan for their contribution to this database.

Received Jan 29, 2003, and in revised form Apr 28, 2003. Accepted for publication May 1, 2003.

Recently, several groups, including our own, have studied locoregional recurrences (LRR) after mastectomy and chemotherapy in an attempt to better define which patients may benefit from postmastectomy radiation (14, 15). The data from these series have shown that the long-term rate of locoregional recurrence after mastectomy and chemotherapy is greater than 20% in patients with four or more lymph nodes or T3 disease. In contrast, for the majority of patients with Stage II breast cancer and one to three positive lymph nodes, the long-term risk of LRR after mastectomy and chemotherapy is less than 15%. Within this subset, only patients with extracapsular extension (ECE) of disease measuring ≥2 mm or patients with less than 10 lymph nodes recovered at axillary dissection have been demonstrated to have substantially higher rates of LRR (15).

Although these data have helped define the risk of LRR without radiation, few data are available that define the risk of LRR with radiation, particularly for patients with Stage II breast cancer and one to three positive lymph nodes (8, 10, 11). In addition, few data are available to demonstrate factors predictive of LRR for patients who receive postmastectomy radiation. Such data might be useful for determining subsets of patients for whom different locoregional treatment strategies should be considered.

Our study had two main goals. The first was to determine the magnitude of benefit from postmastectomy irradiation for patients with various subsets of disease—especially patients with Stage II breast cancer and one to three positive lymph nodes. The second goal was to identify clinicopathologic predictors of LRR after postmastectomy radiation therapy. Herein we report the LRR rates for 470 patients treated with doxorubicin-based chemotherapy, mastectomy with consistent technique and full level I and II axillary dissection, and radiation. We compared these rates with those of the 1031 patients who did not receive postmastectomy radiation that were previously reported by Katz *et al.* (15). All of these patients were treated during the same era on the same institutional prospective clinical trials. The incidence of radiation-related complications, deaths resulting from myocardial infarction, and second malignancies in this cohort is reported in a related manuscript published elsewhere in this journal.

PATIENTS AND METHODS

Patient, tumor, and treatment characteristics

Between 1975 and 1994, 1805 patients were treated with doxorubicin-based adjuvant systemic therapy with or without tamoxifen in five prospective clinical trials at The University of Texas M. D. Anderson Cancer Center (16–22). Each protocol was reviewed and approved by the Institutional Review Board, and participants gave written informed consent. For this study, we reviewed the records of the 462 patients (including 8 cases of bilateral breast cancer for a total of 470 cases) who received postmastectomy radiation therapy and compared these data to those previously reported from the 1031 patients who did not receive radiation. The remaining 312 patients treated in these trials were treated with breast-conserving surgery and were not analyzed in this report.

In four of the five prospective trials, referral for postoperative irradiation was at the discretion of the treating oncologists. The remaining trial intended to include a randomization for radiation treatment (17). The 184 patients who received radiation therapy in this trial (141 radiation per physician preference, 43 randomized to radiation) were included in this analysis. Only patients with Stage II or IIIA disease were eligible for these five trials. Patients older than age 75 years, those with evidence of distant dissemination at diagnosis, and those with a prior or concurrent malignancy were excluded. Pathology information for this study was obtained from report from the review at our institution, which was done before treatment for all patients. Pathology materials were not specifically reexamined for the purpose of this study. Pathologic tumor size was determined from the report of the surgical specimen.

All patients underwent mastectomy before adjuvant systemic therapy and no patient received neoadjuvant chemotherapy. Patients were then randomly assigned to one of several chemotherapy regimens. Each protocol stipulated a minimum of 6 cycles of a doxorubicin-containing regimen and a minimum doxorubicin dose of 40–50 mg/m^2 per cycle. In the radiation cohort, the median number of chemotherapy cycles was 10 (interquartile range 7 to 24). In addition to cytotoxic chemotherapy, 58 patients (12%) with estrogen receptor (ER) or progesterone receptor (PR)-positive tumors also received tamoxifen.

The median radiation dose to the chest wall was 50 Gy, delivered with Co60 or electrons except 13 patients who were treated with 6-MV photons. Routine chest wall irradiation at this institution targets the chest wall, internal mammary lymph nodes. and supraclavicular lymph nodes. Techniques used to target the chest wall often provide coverage of the level I and level II axilla, although this is not a specified target. Additionally, patients routinely receive chest wall boost, which includes the mastectomy scar with generous margins. In the radiated cohort, 93 patients received a chest wall boost. The median dose to the chest wall including the chest wall boost was 55 Gy (interquartile range 50–60 Gy). The median dose to both the internal mammary lymph nodes and supraclavicular lymph nodes was 50 Gy (interquartile range 50–50 Gy). Dose information was available for 351 cases. Radiation treatment was delivered at an outside institution in 167 cases.

Follow-up

Patient follow-up consisted of physical examination, routine laboratory studies, chest radiographs, mammogram of the remaining breast, and bone scans, according to protocol guidelines. Ultrasound and computerized tomography (CT) were not routinely ordered during the follow-up period but were performed when indicated to further evaluate clinical findings consistent with possible recurrence. It should be noted that CT technology was not available for routine

Table 1. Patient, tumor, and treatment characteristics

Characteristic	No radiation No.	No radiation (n = 1031)	No radiation %	Radiation No.	Radiation (n = 470)	Radiation %	p*
Age, years							0.735
Median		49 years			49 years		
Interquartile range		42–57 years			42–57 years		
≤40	226		22	98		21	
41–50	382		37	165		35	
51–60	262		25	126		27	
>60	161		16	81		17	
T stage							<0.001
T1	332		30	89		19	
T2	509		50	214		46	
T3	103		10	74		16	
T4	0		0	9		2	
TX	87		10	84		18	
Tumor size							<0.001
Median		2.5 cm			3.0 cm		
Interquartile range		1.9–3.9 cm			2.1–5.0 cm		
<1.0 cm	62		6	16		3	
1.1–2.0 cm	270		26	75		16	
2.1–3.0 cm	280		27	106		22	
3.1–4.0 cm	160		15	70		15	
4.1–5.0 cm	69		7	42		9	
>5.0 cm	103		10	76		16	
Unknown	87		9	85		18	
T & N groupings							<0.001
T1/2 N0	122		12	4		1	
T1/2 N1-3	404		39	98		21	
T1/2 N4+	312		30	195		41	
T3 N0	7		1	4		1	
T3 N1-3	33		3	21		5	
T3 N4+	61		6	47		10	
Unknown	92		9	101		21	
No. of positive nodes							<0.001
Median		3 nodes			6 nodes		
Interquartile range		1–6 nodes			3–10 nodes		
0	141		14	12		3	
1–3	466		45	140		30	
4–9	263		26	175		38	
≥10	156		15	131		29	
Unknown	5		<1	12		<1	
No. of nodes examined							0.834
Median		17			17		
Interquartile range		13–22			12–23		
<10	100		10	46		10	
≥10	918		89	406		86	
Unknown	13		1	18		4	
LVSI							0.629
Positive	364		35	166		35	
Negative	643		62	277		59	
Unknown	24		2	27		6	
Margin status							0.148
Positive	12		1	3		1	
Negative	965		94	427		91	
Close	17		2	14		3	
Unknown	37		3	26		5	
Grade							<0.001
Well-differentiated	29		3	64		14	
Mod differentiated	401		39	92		20	
Poorly differentiated	366		35	48		10	
Unknown	235		23	266		56	

continued

Table 1. Patient, tumor, and treatment characteristics (*cont'd*)

Characteristic	No radiation No.	(n = 1031) %	Radiation No.	(n = 470) %	p
Extracapsular extension					0.006
None	714	64	291	62	
<2 mm	83	9	34	7	
≥2 mm	141	16	57	12	
Present, NOS	68	8	54	11	
Unknown	25	3	34	7	
Percent positive nodes†					<0.001
<20%	435	50	127	29	
≥20%	442	50	315	71	
Menopausal status					0.096
Premenopausal	493	48	204	43	
Postmenopausal	525	51	262	56	
Unknown	13	1	4	1	
ER status					<0.001
Positive	466	45	102	22	
Negative	391	38	101	22	
Unknown	174	17	267	56	
Hormonal treatment					<0.001
Yes	318	31	58	12	
No	645	62	403	86	
Unknown	68	7	9	2	

Abbreviations: XRT = radiation therapy; No. = number; NS = not significant; LVSI = lymph-vascular space invasion; NOS = not otherwise specified; ER = estrogen receptor.

* Chi-square test for equality of distributions in the radiation and no radiation groups.

† Excluding node-negative patients.

Because of small differences in rounding numbers, percentages do not always equal 100%.

clinical use at M. D. Anderson Cancer Center before the early 1980s and was not used systematically for some time after this.

End points and statistical analysis

The distributions of patient and tumor factors in the two groups were compared using the chi-square test. LRR was defined as recurrence on the ipsilateral chest wall, or in axillary, supraclavicular, infraclavicular, or internal mammary nodes. Recurrence at any other site was considered distant metastasis. Five- and 10-year actuarial rates of overall survival, disease-free survival, and total LRR were calculated according to the Kaplan-Meier method, with comparisons among groups performed using two-sided log–rank tests (23). Total LRR was defined as any LRR with or without prior or simultaneous distant metastasis. We elected to study total LRR rather than isolated LRR as an endpoint because analyses based on time to first event underestimate the true LRR rate (24). In addition, the purpose of our study was to evaluate pathologic factors predictive of LRR. We assumed that prior distant metastases would be unlikely to reseed the locoregional area and therefore believed the source of locoregional events was independent from the development of distant disease. Multivariate analysis was performed using the Cox proportional hazards model. All p values were two-tailed, with values of ≤0.05 considered to be significant (23).

To determine factors predictive of LRR in the patients treated with radiation, a recursive partitioning analysis was performed in addition to univariate analyses (25). For the recursive partitioning analysis, all covariates were initially entered into the model. For ER and PR status, an unknown value was considered as an independent category from positive and negative. We analyzed age both as a continuous variable and dichotomized as young vs. old (young defined as ≤35 years then repeated as ≤40 years). For positive lymph node status, a separate category was created for each number of positive lymph nodes with the exception of patients with 10 or more positive lymph nodes, whom we elected to evaluate as a single group.

RESULTS

Patient, tumor, and treatment characteristics

Patient, tumor, and treatment characteristics for the irradiated and unirradiated cohort are listed in Table 1. Neither age nor menopausal status was significantly different between the radiation and no radiation groups. The median age for patients treated with radiation was 49 years (interquartile range 42–57 years). The median tumor size in the radiation group was 3.0 cm with an interquartile range of 2.1–5.0 cm. The median number of nodes examined in this group was 17 (interquartile range 12–23), and the median number of positive nodes was 6 (interquartile range 3–10). Of the pathologic features examined, T stage, tumor size, number of positive nodes, grade, extracapsular extensions (ECE), per-

Table 2. Sites of locoregional recurrence (LRR)*

Site	Total LRR, No Radiation		Total LRR, Radiation	
	No.	(%)	No.	(%)
Chest wall	122	68	31	76
Supraclavicular	71	40	16	39
Axilla	25	14	1	2
Infraclavicular	12	7	0	0
Internal mammary	15	8	1	2
Any site	179	100	41	100

* Percentages represent the fraction of LRRs that include the specific site as a component of recurrence. Because some patients experienced more than one site of recurrence, percentages do not total 100%.

centage of positive nodes, ER status, and hormonal treatment were all statistically imbalanced between the groups, with the radiation group demonstrating a significant selection bias toward poor prognostic features for LRR (all $p <$ 0.007). Median follow-up from the date of initial histologic diagnosis for all patients alive at the time of analysis was 120 months (range 6–262 months).

Survival and recurrence rates for the entire cohort

Overall and disease-free survival rates for all patients at 10 years were 54% and 51%, respectively. The 5-, 10-, and 15-year actuarial rates of freedom from total LRR for the entire cohort were 92%, 90%, and 90%, respectively, with 95% confidence intervals of 89–94%, 87–93%, and 86–92%, respectively. Of the 41 total LRRs in the patients treated with radiation, 19 occurred as an isolated first event, 14 occurred simultaneously with a distant metastasis, and 8 occurred after a distant metastasis. The 10-year freedom from LRR rates for the radiation and no radiation cohorts were 90% vs. 81%, respectively ($p < 0.0001$). Table 2 lists the sites of LRR in this cohort and the previously reported cohort of patients not treated with radiation. The chest wall and the supraclavicular fossa were the most common sites of locoregional failure in both cohorts. Axillary, infraclavicular, and internal mammary nodal chain recurrences were much less common in the cohort without radiation, and very uncommon in the patients receiving adjuvant radiation. The median interval to any LRR was 29 months in the unirradiated group and 32 months in irradiated patients.

Locoregional recurrence rates according to use of radiation and disease stage

In all categories of patients with positive lymph nodes, 10-year actuarial freedom from LRR rates were improved with radiation. Ten-year freedom from LRR rates for patients with 1–3, 4–9, and 10 or more positive lymph nodes, respectively, were 86%, 80%, and 66% for patients not treated with radiation, compared with 95%, 92%, and 83% for patients in the radiation group ($p <$ 0.003, $p < 0.0001$, $p < 0.001$, respectively). Figure 1 shows the improvement in actuarial freedom from LRR for the 502 patients with T1 or T2 tumors and one to three positive lymph nodes according to the use of radiation. The 10-year actuarial freedom from LRR rates were 97% and 87% in the irradiated and unirradiated groups, respectively ($p = 0.003$). Of these 502 patients, 151 had either positive or close margins, ≥2 mm ECE, or fewer than 10 lymph nodes recovered at the axillary dissection. When these patients were excluded, radiation continued to show a trend for improving the freedom from LRR (10-year actuarial rates of 98% vs. 91%, $p = 0.072$, $n =$ 351). Multivariate analysis all 502 patients with T1 or T2 tumors and one to three positive lymph nodes (treated with and without radiation) revealed four prognostic factors for increased rate of LRR: no radiation, close or positive margins, ≥2 mm ECE, and dissection of fewer than 10 axillary lymph nodes (Table 3). Of these four factors, the greatest hazard ratio was associated with no radiation. These same factors were identified using both forward and backward step-wise analysis. Of note, close or positive margins were included in this multivariate analysis, although it was not a significant predictor of LRR on univariate analysis—likely because there were only 9 patients with close or positive margins. Excluding

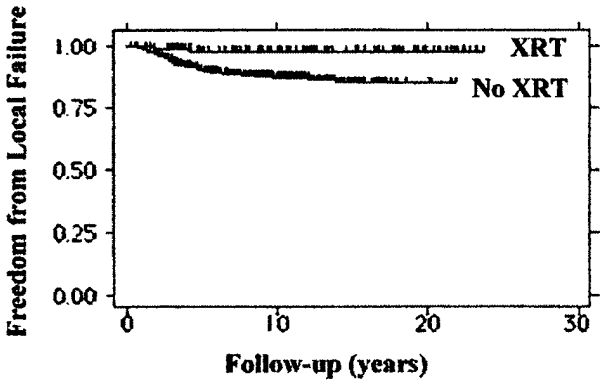

Fig. 1. Locoregional control in patients with T1 or T2 tumors with one to three positive lymph nodes treated with and without radiation therapy. The rates of freedom from LRR at 10 years were 97% (radiation) and 87% (no radiation), $p = 0.003$.

Table 3. Multivariate analysis of predictors for LRR in 502 patients with T1 or T2 tumors and one to three positive nodes treated with and without adjuvant radiation therapy

Factor	Hazard ratio	95% Confidence interval	*p*
No radiation therapy	6.25	1.58–27.3	0.009
Close or positive margins	4.61	1.10–19.3	0.036
Gross ECE	3.27	1.66–6.42	0.0001
<10 nodes recovered	2.66	1.35–5.34	0.0005

Abbreviations: LRR = locoregional recurrence; ECE = extracapsular extension.

close or positive margins from the analysis yields similar risk factors for increased LRR: no radiation, ≥2 mm ECE, dissection of fewer than 10 axillary lymph nodes, and T2 tumors (hazard ratios 6.45, 3.16, 2.56, and 1.91, respectively).

Figure 2 shows the improvement in the actuarial freedom from LRR among the subgroup of patients for whom postmastectomy radiation therapy is routinely offered: those with T3 or T4 tumors or four or more positive lymph nodes. In this subset, the 10-year actuarial freedom from LRR rates were 93% in the irradiated cohort and 60% in the unirradiated cohort ($p < 0.001$). Table 4 shows freedom from LRR rates for all patients in the study stratified according to primary tumor category and number of positive lymph nodes. Radiation reduced LRR in nearly all subsets of patients.

Predictors of locoregional recurrence after postmastectomy radiation therapy

Table 5 shows the correlation between patient, disease, and treatment characteristics and LRR after postmastectomy radiation. In these univariate analyses, increasing involvement of lymph nodes was a significant predictor of LRR. Pathologic identification of lymph-vascular-space invasion was also predictive of higher rates of LRR. Patients with ER-negative disease had higher rates of LRR compared with patients with ER-positive disease, and accordingly, patients who received tamoxifen had lower LRR rates. Thirty-eight of the 101 patients with known ER-positive

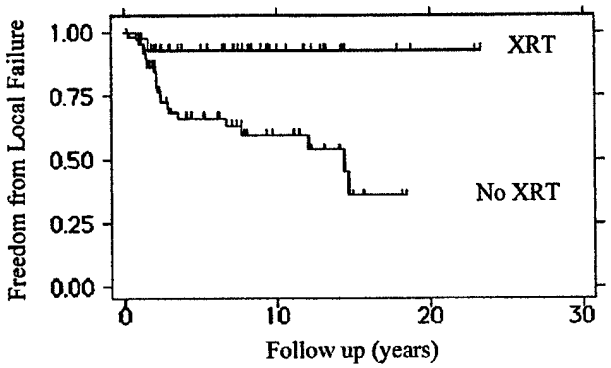

Fig. 2. Locoregional control in patients with T3 or greater disease or four or more positive nodes treated with and without radiation therapy. The rates of freedom from LRR at 10 years were 93% (radiation) and 60% (no radiation), $p = 0.001$.

disease received tamoxifen vs. only 9 of the 102 patients with ER-negative disease.

A recursive partitioning analysis of 13 variables affecting LRR was performed to explore which subgroups of patients had excellent rates of locoregional control and which patients had less favorable rates. Figure 3 shows the results of this analysis. In this model, the presence of ER-negative disease was the most powerful predictor of LRR. The 10-year rate of LRR among the patients with ER-negative disease was 17%. The model did not further split this subgroup. For patients with either ER-positive disease or unknown ER status, the model further divided patients according to whether they had <10 or ≥10 positive lymph nodes. As shown, this break defined a favorable subgroup with a very low LRR rate at 10 years and a subgroup with a less favorable outcome, similar to that of the patients with ER-negative disease.

DISCUSSION

Our findings suggest that the addition of radiation therapy after mastectomy and doxorubicin-based chemotherapy reduces the rate of LRR in all patients with

Table 4. Ten-year actuarial rates of locoregional recurrence (LRR) with regard to T-stage and nodal status*

	1–3 Positive nodes		4–9 Positive nodes		≥10 Positive nodes		Total	
T stage	No radiation	Radiation	No radiation	Radiation	No radiation	Radiation	No radiation	Radiation
T1	9% (15/190)	3% (1/38)	18% (14/72)	0% (0/29)	17% (4/29)	19% (3/17)	12% (33/291)	5% (4/84)
	$p = 0.246$		$p = 0.012$		$p = 0.904$		$p = 0.055$	
T2	16% (33/214)	2% (1/60)	27% (31/132)	10% (8/89)	34% (22/79)	17% (8/60)	23% (86/425)	9% (17/209)
	$p = 0.004$		$p = 0.008$		$p = 0.070$		$p = 0.0002$	
T3	23% (8/33)	10% (2/21)	40% (14/33)	10% (2/22)	39% (9/28)	5% (1/25)	33% (31/94)	8% (5/68)
	$p = 0.140$		$p = 0.019$		$p = 0.009$		$p = 0.0002$	
Total	13% (56/437)	3% (4/119)	26% (59/237)	8% (10/140)	31% (35/136)		15% (12/102)	
	$p = 0.003$		$p < 0.0001$		$p = 0.007$			

* Patients with unknown T stage or unknown number of positive nodes are excluded. The fractions in parentheses are the total number of patients with LRR over the total number at risk in each cohort.

Table 5. Univariate analysis of factors associated with locoregional recurrence (LRR) in patients receiving radiation therapy

Factor	Total LRR 10-year actuarial (%)	Crude	p
Age, years			
≤40	15	14/98	0.060
41–50	6	10/165	
51–60	12	13/126	
>60	6	4/81	
Tumor size			
≤1.0 cm	0	0/16	0.012
1.1–2 cm	6	4/75	
2.1–3 cm	5	5/106	
3.1–4 cm	8	5/70	
4.1–5 cm	25	8/42	
>5.0 cm	12	7/76	
Number of positive nodes			
0	20	2/12	0.023
1–3	5	6/140	
4–9	8	14/175	
≥10	16	17/131	
Percentage of positive nodes			
<20%	6	7/137	0.046
≥20%	11	32/315	
Lymph-vascular space invasion			
Negative	7	17/277	0.023
Positive	14	20/166	
Hormonal treatment			
None	10	38/403	0.039
Tamoxifen	2	1/58	
Estrogen receptor status			
Positive	7	7/101	0.023
Negative	17	15/102	
Unknown	8	19/267	

Pathologic tumor size, extracapsular extension, pathologic node size, and margin status were not predictive of LRR in this analysis ($p \geq 0.25$).

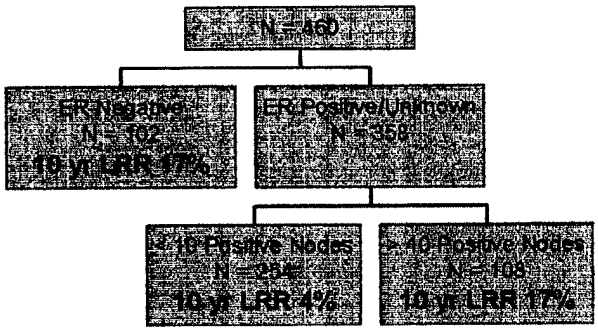

Fig. 3. Results of recursive partitioning analysis for 460 patients treated with postmastectomy radiation.

node-positive breast cancer. This finding is especially compelling in light of the fact that the cohort treated with radiation had a higher percentage of patients with advanced disease and other pathologic features associated with higher rates of LRR. Among patients with Stage II tumors and one to three positive lymph nodes, the greatest predictor for higher LRR was not receiving radiation therapy. Higher tumor stage, increased number of positive nodes, increased percentage of positive nodes, lymph-vascular space invasion, no hormonal therapy, and ER-negative status were significant predictors of LRR among patients treated with radiation. Recursive partitioning analysis revealed that ER-negative status was the most powerful predictor of LRR after radiation therapy.

Our finding that postmastectomy radiation significantly reduced the rate of LRR further supports the findings of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis of the clinical trials investigating the value of radiation therapy for treatment of breast cancer (1). However, most of the trials of postoperative radiotherapy included in the EBCTCG meta-analysis were conducted before the routine use of systemic therapy. Our report is one of the first large series to demonstrate the LRR benefit for patients treated with doxorubicin-based chemotherapy, which is now considered as a standard component of therapy for all patients with lymph node–positive disease (26). This reduction in LRR was achieved despite the fact that the radiation group contained a higher percentage of patients with advanced disease and other adverse pathologic features.

The three most recently published randomized trials investigating postmastectomy radiation all demonstrated that when large reductions of LRR rates were achieved, radiation could improve disease-specific survival (8, 10, 11). In addition, a meta-analysis of 18 trials including more than 6000 women concluded that radiation reduced mortality for lymph node–positive patients treated with surgery and systemic therapy (27). Furthermore, in the Danish and British Columbia trials, the reduction in LRR from approximately 30% to 10% associated with postmastectomy radiation corresponded to a 9% overall survival advantage. On the basis of these data, it is clear that postmastectomy radiation should be offered to all patients with a 20% to 30% LRR risk. However, from these trials, it is difficult to determine what categories of patients have this degree of LRR risk. In part, this difficulty has arisen because the axillary dissection used in the Danish studies differed from the standard level I/II axillary dissection routinely used in the United States. For example, the median number of lymph nodes recovered in the Danish trials was 7 compared with a median number of 17 in this current report. Correspondingly, the rate of axillary recurrence in the unirradiated group in the Danish trial was substantially higher than the rate in our report.

For these reasons, a number of groups (14, 15) have recently investigated rates of LRR after mastectomy and chemotherapy to determine the patient and pathologic features predictive of clinically relevant rates of LRR. In aggregate, these studies demonstrated that patients with four or more positive lymph nodes or T3 disease treated without radiation have rates of LRR similar to those found in the Danish trial. However, the LRR rates for

patients with Stage II breast cancer with one to three positive lymph nodes are much lower. Specifically, in an analysis of 2016 patients treated with mastectomy and chemotherapy on Eastern Cooperative Group Trials, Recht *et al.* reported 10-year total LRR of 28.7% for patients with four or more positive lymph nodes, and 12.9% for patients with one to three positive lymph nodes (14). Similarly, our data suggested a 10-year total LRR rate of 25–34% for patients with four or more positive nodes, and 13% 10-year total LRR for patients with one to three positive nodes (15).

Based on the results of the randomized prospective clinical trials and the studies of LRR patterns after mastectomy and chemotherapy, both ASCO and ASTRO published consensus statements regarding postmastectomy radiation (12, 13). These statements both suggested that postmastectomy radiation should be considered as a standard component of therapy for patients with four or more positive lymph nodes. For patients with Stage II breast cancer and one to three positive lymph nodes, both statements concluded that there is insufficient evidence to make recommendations or suggestions for the routine use of postmastectomy radiation.

Perhaps the most interesting finding of this analysis is the demonstration that radiation reduced the risk of LRR for all categories of patients with lymph node–positive disease, including those with Stage II breast cancer and one to three positive lymph nodes. This is one of the first studies to evaluate the benefit of postmastectomy irradiation for patients with this stage of disease who were treated with a standard modified radical mastectomy and adjuvant doxorubicin-based chemotherapy. In these patients, we found a 10% absolute improvement in the 10-year rate of LRR with the addition of radiation (3% vs. 13%, $p = 0.001$). Furthermore, on multivariate analysis, no radiation was the strongest predictor for LRR of the 13 variables examined for this group. Whether this degree of improvement in LRR can affect overall survival is unknown. Because of selection biases that affected radiation use, we could not study whether radiation use improved overall or disease-free survival.

In light of the abundance of data on postmastectomy irradiation, it is surprising that there are few published data concerning factors that predict for increased risk of LRR after postmastectomy radiation. Using a recursive partitioning analysis, we found ER status to be the most powerful discriminator of LRR. For those with ER-positive disease or unknown status, the presence of 10 or more involved lymph nodes also predicted for a higher rate of LRR. If these findings are validated in an independent set of patients, they could be used to identify patients for new trials aimed at improving LRR by escalating radiation dosages or using concurrent radiosensitizing agents.

It is important to recognize the limitations of this study. Foremost, this was a retrospective review of two separate cohorts with significant differences in the distribution of variables that confound LRR, the primary endpoint of our study. However, these biases would be predicted to disproportionately increase the rates of LRR in the cohort treated with radiation. These imbalances may also mean that the reported differences in various subgroups underestimate the true effects of radiation. It is also important to note that all patients met eligibility for clinical trials and therefore there were no patients older than age 75 and no patients with M1 disease.

In conclusion, our data suggest that the addition of radiation therapy after mastectomy and doxorubicin-based chemotherapy improves LRR rates in all patients with lymph node-positive breast cancer. However, whether the magnitude of benefit we demonstrated for patients with Stage II breast cancer with one to three positive lymph nodes warrants postmastectomy radiation remains controversial.

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APPENDIX 23

Int. J. Radiation Oncology Biol. Phys., Vol. 53, No. 5, pp. 1225–1233, 2002

0360-3016/02/$–see front matter

ELSEVIER

PII S0360-3016(02)02878-X

CLINICAL INVESTIGATION **Breast**

LOCOREGIONAL TREATMENT OUTCOMES FOR INOPERABLE ANTHRACYCLINE-RESISTANT BREAST CANCER

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Purpose: To assess the therapeutic outcomes and treatment-related morbidity of patients treated with radiation for inoperable breast cancer resistant to anthracycline-containing primary chemotherapy.

Methods and Materials: We analyzed the medical records of breast cancer patients treated on five consecutive institutional trials who had been designated as having inoperable locoregional disease after completion of primary chemotherapy, without evidence of distant metastases at diagnosis. The cohort for this analysis was 38 (4.4%) of 867 patients enrolled in these trials. Kaplan–Meier statistics were used for survival analysis, and prognostic factors were compared using log–rank tests. The median follow-up of surviving patients was 6.1 years.

Results: Thirty-two (84%) of the 38 patients were able to undergo mastectomy after radiotherapy. For the whole group, the overall survival rate at 5 years was 46%, with a distant disease-free survival rate of 32%. The 5-year survival rate for patients who were inoperable because of primary disease extent was 64% compared with 30% for those who were inoperable because of nodal disease extent ($p = 0.0266$). The 5-year rate of locoregional control was 73% for the surgically treated patients and 64% for the overall group. Of the 32 who underwent mastectomy, the 5-year rate of significant postoperative complications was 53%, with 4 (13%) requiring subsequent hospitalization and additional surgical revision. Preoperative radiation doses of ≥54 Gy were significantly associated with the development of complications requiring surgical treatment (70% vs. 9% for doses <54 Gy, $p = 0.0257$).

Conclusion: Despite the poorer prognosis of patients with inoperable disease after primary chemotherapy, almost one-half remained alive at 5 years and one-third were free of distant disease after multidisciplinary locoregional management. These patients have high rates of locoregional recurrence after preoperative radiotherapy and mastectomy, and the morbidity associated with this approach may limit dose-escalation strategies. Alternative therapeutic strategies such as novel systemic agents, use of planned myocutaneous repair for closure, or radiation combined with radiosensitizing agents, should be considered in this class of patients. © **2002 Elsevier Science Inc.**

Preoperative radiotherapy, Breast cancer, Inoperable disease.

INTRODUCTION

Primary (neoadjuvant) systemic chemotherapy is a vital component of the management of locoregionally advanced breast cancer. Prospective and retrospective analyses have reported that approximately 80% of patients treated with primary chemotherapy achieve a partial or complete response (1–8). Correspondingly, for patients who present with disease that is initially inoperable, most are able to undergo surgical resection after primary chemotherapy.

Many series, including our own, have indicated that the tumors that fail to respond to primary chemotherapy have higher metastatic rates compared with those that respond (1, 7, 9–18). We recently reported our experience treating 177 patients with disease refractory to primary chemotherapy and found that these patients had high rates of both locoregional and distant recurrence. Most of those who did not achieve a partial response to chemotherapy continued to have operable disease, and we found that surgery was critically important for both achieving locoregional control and minimizing the risk of death from breast cancer (9).

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Supported in part by National Cancer Institute Department of Health and Human Services Grants CA16672 and T32CA77050. Dr. Buchholz is supported by Grant BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

Received Jan 3, 2002, and in revised form Apr 3, 2002. Accepted for publication Apr 8, 2002.

For the patients whose tumors remain inoperable after chemotherapy, the optimal management strategy is less clear. Historically, we have considered inoperable disease as either gross residual disease in the axilla or supraclavicular fossa that could not be completely resected without excessive morbidity or significant residual disease in the breast that could not be completely resected using primary skin closure. Our management approach for these patients has been to use preoperative radiotherapy (RT) in the hope that a modified radical mastectomy will become possible. Currently, little or no published data are available regarding the success and toxicity of preoperative RT for patients with inoperable breast cancer after primary chemotherapy. These data are needed to provide information about the selection of the radiation dose and the determination of factors that are predictive of outcome.

In this paper, we reviewed the data from patients treated on consecutive institutional trials involving the use of primary chemotherapy for breast cancer. We analyzed the clinical outcome and postoperative morbidity for the patients who had inoperable disease after primary chemotherapy and subsequently received RT.

METHODS AND MATERIALS

We retrospectively analyzed the data from 5 consecutive prospective clinical trials conducted at The University of Texas M. D. Anderson Cancer Center that investigated the role of primary chemotherapy for patients with nonmetastatic breast cancer. Between 1985 and 1998, 867 patients were enrolled into these trials. The eligibility criteria for these trials changed over the course of time. However, all trials required that patients have T3 primary disease or Stage III–IV disease. Patients with Stage IV disease were eligible only if they had ipsilateral involvement of supraclavicular lymph nodes without additional evidence of metastatic disease. A total of 186 patients (21%) were prospectively judged to have less than a partial response to the primary chemotherapy. Of these, only 38 patients (4.4% of the total population of the 5 studies) make up the population of this current report because they had disease characteristics that required RT for inoperable disease after failure of anthracycline-containing primary chemotherapy. The other 148 patients underwent surgery followed by RT or palliative care if distant disease developed during primary chemotherapy. These patients were assessed jointly by a medical oncologist, surgeon, radiologist, and radiation oncologist after completion of primary chemotherapy and determined to be inoperable. Twenty patients were thought to have inoperable disease because of unresectable adenopathy (fixed axillary disease and/or supraclavicular disease), and 18 patients were thought to have inoperable disease because the primary disease extent precluded a primary skin closure.

Table 1 shows the clinical, disease, and treatment characteristics of the 38 patients in this study. The multidisciplinary team prospectively assigned the clinical stages according to the American Joint Committee on Cancer Staging and End Results Reporting guidelines (19) after physical examination, mammography, chest radiography, bone scan, and liver evaluation (liver scan, ultrasonography, or CT). Patients who had systemic metastases or inflammatory carcinoma were treated on different protocols and were not included in this study. Twenty-nine of the patients (76%) in this series had Stage IIIB or greater disease at diagnosis. The 2 patients with Stage IIB disease had primary tumor sizes >5 cm without nodal involvement. The 9 patients with Stage IV disease had ipsilateral supraclavicular node involvement without other systemic metastases (regional Stage IV).

Table 2 describes the primary chemotherapy regimens the patients received. All patients were treated with doxorubicin-containing combinations; 6 patients also received taxane-based chemotherapy. The details regarding these regimens have been published in earlier reports (1, 20, 21). In

Table 1. Patient characteristics

Median follow-up* (y)	6.1
Age (y)	
Mean	47.3 ± 8.9
≤40	7 (18)
Clinical stage	
IIB	2 (5)
IIIA	7 (18)
IIIB	20 (53)
IV†	9 (24)
T stage	
T0	1 (3)
T1	0
T2	3 (8)
T3	8 (21)
T4	26 (68)
N stage	
N0	6 (16)
N1	10 (26)
N2	19 (50)
N3	3 (8)
Adjuvant chemotherapy	
None	16 (42)
VM	14 (37)
VMF	7 (18)
FAC + VM	1 (3)
Adjuvant tamoxifen	
Yes	12 (32)
No	26 (68)
Estrogen receptor status	
Positive	10 (26)
Negative	21 (55)
Unknown	7 (19)
Progesterone receptor status	
Positive	11 (29)
Negative	18 (47)
Unknown	9 (24)

Data in parentheses are percentages.

* Of surviving patients.

† Indicates ipsilateral supraclavicular lymph node involvement without systemic metastases.

Abbreviations: VM = vinblastine, methotrexate; VMF = vinblastine, methotrexate, 5-fluorouracil; FAC = 5-fluorouracil, doxorubicin, cyclophosphamide.

Table 2. Primary chemotherapy treatment

Protocol	Years of study	Primary chemotherapy	Cycles (*n*)	Patients/total population (*n*)
85-01	1985–1989	VACP	3	11/200
89-007	1989–1991	FAC	4	11/203
91-015	1991–1994	FAC or dose-escalated FAC	4	9/202
94-002	1994–1998	FAC	4	1/174
97-099	1998–2000	AT	6	6/88

Abbreviations: FAC = 5-fluorouracil, doxorubicin, cyclophosphamide; VACP = vincristine, doxorubicin, cyclophosphamide, and prednisone; AT = doxorubicin, docetaxel.

summary, FAC chemotherapy consisted of 500 mg/m^2 5-fluorouracil given on Days 1 and 4 or 8, 50 mg/m^2 doxorubicin given as a Day 1 bolus or as a 72-h continuous infusion, and 500 mg/m^2 cyclophosphamide given on Day 1. For those patients receiving dose-escalated FAC, the doses of these drugs were increased to 600, 60, and 1000 mg/m^2, respectively. The VACP regimen consisted of 1.5 mg/m^2 vincristine, 60–75 mg/m^2 doxorubicin, 600–750 mg/m^2 cyclophosphamide, and 40 mg prednisone. Finally, the AT regimen consisted of 60 mg/m^2 doxorubicin and 60 mg/m^2 docetaxel given as i.v. boluses.

After chemotherapy, the medical team prospectively determined the clinical response of the primary tumor and regional lymph nodes according to standard response categories: (*1*) complete response (CR)—total resolution as assessed by physical or radiologic examination; (*2*) partial response (PR)—≥50% reduction of the product of the 2 largest perpendicular dimensions of the mass; (*3*) minor response—<50% reduction; (*4*) no change; and (*5*) progressive disease. Response was evaluated by a combination of physical examination, serial mammograms, and more recently, serial sonograms.

All 38 patients received RT (Table 3) to the breast and surrounding lymphatic regions immediately after primary chemotherapy. The involved breast was treated with conventional tangential fields to a median dose of 50 Gy (range 30–65) using a beam energy of 6 MV in 21 patients and ^{60}Co γ rays in the remaining 17 patients. An anterior field treating the supraclavicular fossa and axillary apex to a median dose of 50 Gy (range 30–64) was prescribed for all patients. Additionally, the midplane axilla was boosted to a median dose of 45 Gy (range 26–50) using a posterior axillary field. The internal mammary chain was treated to a median dose of 50 Gy (range 30–66) in 25 patients, with 22 receiving electron beam treatments to minimize the dose to the underlying thoracic structures. Six patients received a boost to the primary tumor bed using external beam RT (range 4–14 Gy), and 2 received interstitial brachytherapy boosts of 15 Gy. Five patients received 5-fluorouracil concurrently with RT. One patient received palliative RT consisting of 30 Gy to both breasts because locally progressive disease had extended to the contralateral breast during primary chemotherapy.

Table 3. Radiotherapy

Site	Patients (*n*)	Median dose (Gy)
Breast	38	50 (30–65)
SCV	38	50 (30–64)
Axilla (midplane)	38	45 (26–50)
IMC	25	50 (30–66)
Tumor bed boost	8	10 (4–15)

Data in parentheses are the range.

Abbreviations: SCV = supraclavicular fossa/axillary apex; IMC = internal mammary chain.

After completion of RT, 32 patients (84%) underwent mastectomy. Surgery was generally performed 4–6 weeks after RT completion. Postoperatively, 22 patients (58%) received additional chemotherapy. These regimens changed during the period of the clinical trials and included the use of vinblastine and methotrexate, vinblastine, methotrexate, and 5-fluorouracil, and FAC (similar to the preoperative regimen). Twelve patients (32%) received tamoxifen postoperatively.

The Kaplan–Meier method (22) was used to calculate the actuarial statistics for overall survival (OS), distant disease-free survival (DDFS), locoregional control, locoregional recurrence (LRR), and postoperative morbidity. OS and DDFS were measured from the date of diagnosis. Locoregional control, LRR, and postoperative morbidity were measured from the date of mastectomy. Two-sided log–rank tests (23) were used to detect differences in OS, DDFS, LRR, and postoperative morbidity associated with independent clinical or pathologic variables. Cases with unknown values were excluded from the univariate analyses.

Locoregional control was defined as clinically free of disease after completion of surgery and/or RT. LRR was defined as having a recurrence (only after achieving locoregional control) in the ipsilateral chest wall, skin, or regional nodes, with or without prior, simultaneous, or subsequent distant metastases. Distant disease was defined as visceral metastatic disease, not including the ipsilateral supraclavicular nodes. For DDFS calculations, distant disease recurrence was scored as an event, and nonbreast cancer deaths were censored. The postoperative complications analyzed included wound infection, wound dehiscence, wound/flap

Table 4. Inoperable breast cancer after primary chemotherapy: clinical response assessed after chemotherapy and RT

	Response to CT (%)	Response to RT (%)
Primary		
CR	0 (0)	5 (13)
PR	7 (18)	5 (13)
MR	11 (29)	18 (47)
NC	12 (32)	4 (11)
PD	7 (18)	3 (8)
No primary at diagnosis	1 (3)	1 (3)
Unclear	—	2 (5)
Nodes		
CR	2 (5)	14 (37)
PR	7 (18)	8 (21)
MR	4 (11)	3 (8)
NC	13 (34)	4 (11)
PD	9 (24)	5 (13)
No nodes at diagnosis	3 (8)	3 (8)
Unclear	—	1 (3)

Abbreviations: CT = chemotherapy; RT = radiotherapy; CR = complete response; PR = partial response; MR = minor response; NC = no change; PD = progressive disease.

necrosis, lymphedema, brachial plexopathy, rib fracture, and chronic pain requiring long-term pain management.

RESULTS

From a total population of 867 breast cancer patients treated with primary anthracycline-containing chemotherapy, 38 patients (4.4%) had inoperable residual disease after chemotherapy and subsequently received RT in attempt to make mastectomy possible. These patients were considered to be inoperable because they had either gross residual disease in the axilla or supraclavicular fossa that could not be completely resected without excessive morbidity or residual disease in the breast that could not be completely resected using primary skin closure.

The clinical response rates to primary chemotherapy and RT are shown in Table 4. In these patients, primary chemotherapy resulted in an overall clinical tumor response of 18% (0% CR, 18% PR) and an overall nodal response of 23% (5% CR, 18% PR). RT resulted in an overall tumor response of an additional 26% (13% CR, 13% PR) and an overall nodal response of an additional 58% (37% CR, 21% PR).

Thirty-two patients (84%) underwent surgery consisting of a modified radical mastectomy, radical mastectomy, or a simple mastectomy. Thirty patients (79%) underwent axillary dissection. Ten patients (31%) required myocutaneous reconstruction: 3 had trans-rectus abdominis myocutaneous flaps, 6 had latissimus dorsi flaps, and 1 had a gluteal flap. Two of the patients underwent mastectomy for palliative reasons after the development of distant disease during and after RT. All 5 patients who were treated with concurrent 5-fluorouracil and RT were able to undergo mastectomy. Of the 6 patients who did not undergo surgery, 1 patient no longer had any detectable disease and 5 patients experienced progressive disease during RT (1 had locally progressive disease in the axilla and 4 developed distant metastases).

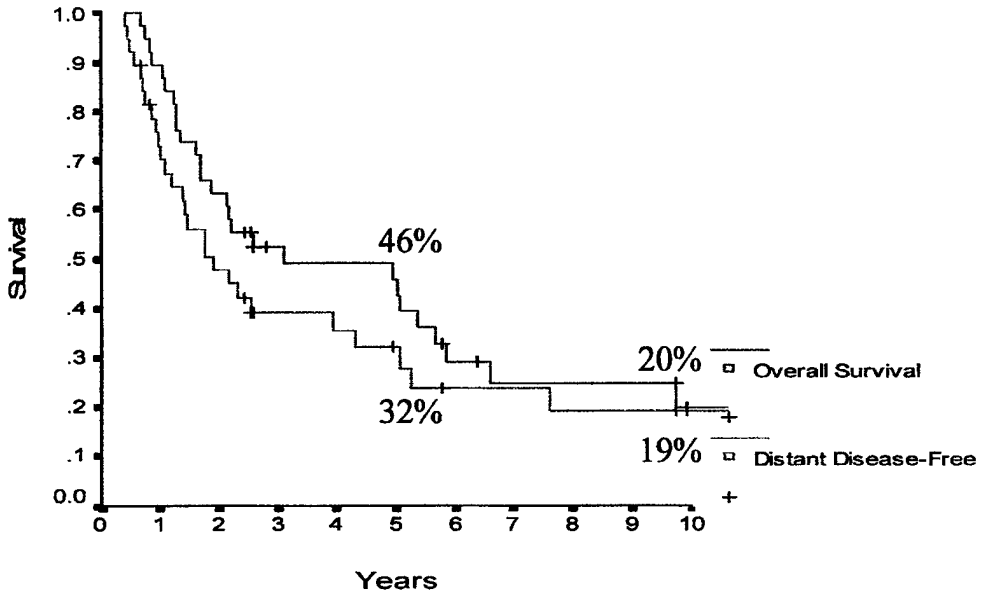
The median clinical tumor size at diagnosis was 8 cm (range 0–17). On completion of chemotherapy before RT, the median clinical tumor size was 7 cm (range 0–15). Of those who had mastectomy after RT, the median pathologic tumor size was 3.4 cm (range 0–13.0). Eight patients (25%) had residual primary tumors of $\leq$2 cm, 14 patients (44%) had tumors $>$2 cm but $\leq$5 cm, and 7 patients (22%) had tumors $>$5 cm. No residual primary disease could be identified in 3 patients (9%). The median number of positive lymph nodes was 2 (range 0–17). Of those who underwent axillary dissection, 13 patients (43%) had 1–3 positive nodes, 6 (20%) had 4–9 positive nodes, and 3 (10%) had $\geq$10 positive nodes. No positive nodes were identified in 8 patients (27%). In this series of patients, only 2 (5%) had a complete pathologic response; their clinical stage at diagnosis was IIIB (T4N2M0) and IV (T4N1M1). The surgical margins were $>$2 mm in 24 (75%), $\leq$2 mm in 4 (13%), and positive in 4 (13%) patients. Pathologic skin involvement was present in 8 patients (25%), and lymphvascular invasion was present in 15 patients (47%).

Thirty-one patients (82%) were initially rendered disease free after RT and mastectomy. Of the 7 patients with residual disease, 5 did not undergo surgery because of progressive disease, and 2 underwent palliative mastectomy after distant disease developed during and after RT.

Clinical outcomes and prognostic factors

After a median follow-up of 6.1 years among surviving patients, 29 patients (76%) experienced progressive disease after completion of all therapies. As a component of their first failure, 5 (13%) had LRR alone, 21 (55%) developed distant metastatic disease alone, and 3 (8%) developed both. Of the 9 patients (24%) who remained disease free, 3 died of other causes (motor vehicle accident, pneumonia, and congestive heart failure).

The OS and DDFS rates for all patients were 46% and 32% at 5 years and 20% and 19% at 10 years, respectively (Fig. 1). Table 5 lists the 10-year rates of OS and DDFS categorized according to the clinical and pathologic characteristics. When clinically assessed after primary chemotherapy, patients who were inoperable because of nodal disease extent had significantly worse OS and DDFS than did those who were inoperable only because of primary breast disease extent (Fig. 2). Also, having advanced nodal stage (N2 or N3) or poor nodal response (minor response, no change, or progressive disease) after chemotherapy was associated with significantly worse OS and DDFS (data in Table 5). Although not statistically significant, patients with $\geq$4 pathologically positive nodes had a lower rate of DDFS (0% vs. 33%, $p = 0.0576$). A tumor size $>$5 cm correlated with significantly worse DDFS and showed a trend toward worse OS (data in Table 5). OS and DDFS were not associated



Patients at Risk:			
Overall Survival	38	14	3
Distant Disease-Free	38	8	2

Fig. 1. OS and DDFS for all patients measured from the date of diagnosis.

with clinical stage, T stage or N stage at diagnosis, primary response to chemotherapy, primary or nodal response to RT, or radiation dose to the breast ($p > 0.2$ for all comparisons).

Locoregional control was initially achieved in 33 patients (87%), with 5- and 10-year rates of 64%. For those who achieved locoregional control, the 5- and 10-year rate of LRR was 27%. Of the 7 patients who had LRR, recurrence was an isolated first event in 3, an event simultaneous with distant disease in 3, and an event subsequent to distant disease in 1. The sites of locoregional failure were as follows: 4 patients had recurrences in the chest wall, 1 had recurrence in the axilla, and 2 had recurrences at both sites. At last follow-up, 6 patients had died of distant disease, and 1 was alive with locoregional disease.

Although not statistically significant, 2 factors were found to be associated with LRR. Patients with nodal disease that did not respond to RT (minor response, no change, or progressive disease) had a higher rate of LRR (82% vs. 29%, $p = 0.0526$). In addition, a trend was noted for a higher rate of LRR in the patients who received radiation doses to the breast of ≤50 Gy (80% vs. 49%, $p = 0.0726$), although in this analysis, we included the 1 patient treated palliatively to 30 Gy. LRR was not associated with clinical stage, T stage or N stage at diagnosis, primary or nodal response to chemotherapy, primary or nodal response to RT, pathologic tumor size, or the number of pathologically positive nodes ($p > 0.2$ for all comparisons). All 7 patients with LRR had negative margins.

Postoperative morbidity

For the 32 patients who underwent mastectomy, the 5-year rate of significant postoperative morbidity was 53% (Fig. 3). The complications were wound infection in 4 patients, wound dehiscence in 2, flap necrosis in 2, significant lymphedema in 3, brachial plexopathy in 1, rib fracture in 1, and chronic pain requiring pain medications in 7. Four of these patients (13%) required hospital admission and additional surgery: 2 for wound dehiscence, 1 for flap necrosis, and 1 for rib fracture.

The rate of postoperative complications requiring surgical revision was significantly associated with radiation doses of ≥54 Gy to the involved breast (70% vs. 9%, $p = 0.0257$). Although not statistically significant, patients receiving doses >50 Gy also had a higher overall rate of postoperative complications (85% vs. 43%, $p = 0.0983$). Factors that were not significant included radiation dose to the midplane axilla, use of photon beams vs. ^{60}Co γ rays, use of 5-fluorouracil concurrently with RT, use of myocutaneous flap closure vs. primary closure, clinical T stage, tumor size by physical examination, pathologic tumor size, clinical N stage, and the number of pathologically positive lymph nodes ($p > 0.1$ for all comparisons). Of the 2 patients who had brachytherapy boosts, 1 had a rib fracture and the other remained complication free.

DISCUSSION

We present data regarding the clinical outcomes and toxicity of RT for patients with inoperable disease after primary chemotherapy. It is generally expected that these patients have very poor prognoses. Numerous studies investigating the role of neoadjuvant chemotherapy have established that patients who do not achieve at least a PR have

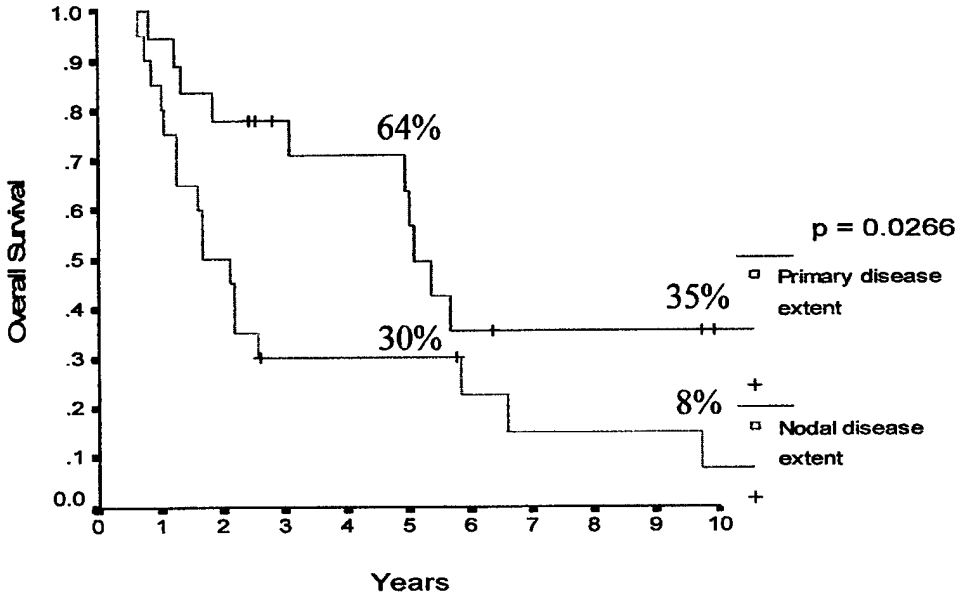
Table 5. Inoperable breast cancer after primary chemotherapy: 10-year rates of survival according to single prognostic variables

		Distant disease-free survival		Overall survival	
Factor	Patients (*n*)	10-y rate	*p*	10-y rate	*p*
Clinical stage at diagnosis					
IIB-IIIA	9	13	0.0585	28	0.4699
IIIB	20	29		17	
IV	9	22		33	
T stage at diagnosis					
≤T2	4	25	0.6215	0	0.8637
T3	8	19		31	
T4	26	20		20	
N stage at diagnosis					
N0-1	16	15	0.8097	31	0.3693
N2-3	22	27		14	
T stage after CHT					
≤T2	6	33	0.1562	0	0.4624
T3	5	33		40	
T4	27	18		18	
N stage after CHT					
N0-1	19	27	0.0130	37	0.0276
N2-3	19	11		7	
Inoperable after CHT					
Primary disease extent only	18	26	0.0174	35	0.0266
Nodal disease extent	20	14		8	
Pathologic primary size (cm)					
≤2	11	56	0.0172	16	0.0687
>2–5	14	17		21	
>5	7	0		0	
Pathologic node status					
0-3 +LN	21	33	0.0576	22	0.5962
>4 +LN	9	0		21	
Primary response to CHT					
Yes	7	57	0.1829	48	0.2403
No	30	13		16	
Nodal response to CHT					
Yes	9	65	0.0041	40	0.0178
No	26	4		6	
Primary response to RT					
Yes	10	15	0.8124	0	0.8344
No	25	17		21	
Nodal response to RT					
Yes	22	13	0.3883	15	0.9893
No	12	8		10	
RT Dose to breast (Gy)					
≤50	27	16	0.6367	14	0.9361
>50	11	44		44	

Abbreviations: CHT = chemotherapy; RT = radiotherapy; LN = lymph node; Response = clinically assessed as complete or partial response.

significantly higher metastatic rates than do those who do respond (1, 7, 10–18), with 5-year survival rates of 0–24% (1, 10). Because of their guarded outcome, the patients who remain inoperable after chemotherapy are often considered for Phase I studies exploring new chemotherapy regimens as a last resort.

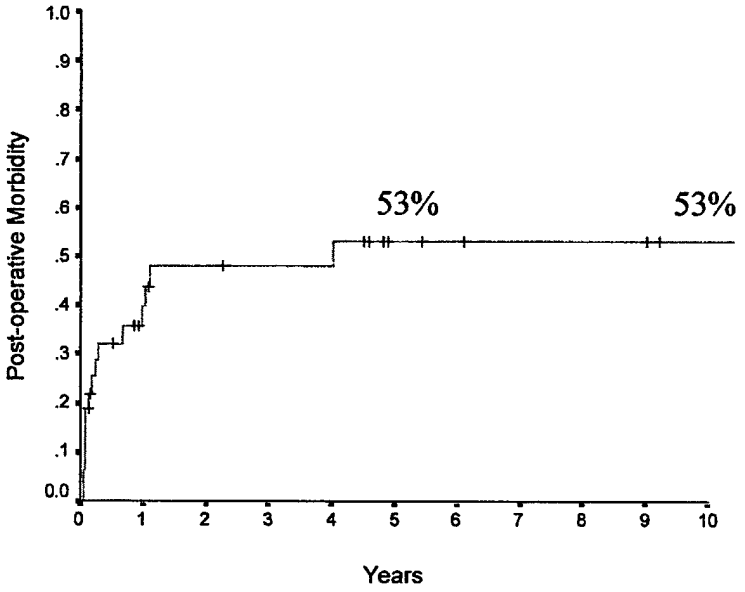
Our approach for these patients has been to use aggressive locoregional management, initiating preoperative RT in the hope of proceeding with mastectomy. This strategy is considered superior because the combination of both RT and surgery after primary chemotherapy has been shown to decrease locoregional failure and increase survival compared with RT alone after chemotherapy (16, 24–28). Using this approach, almost one-half of the patients in this series remained alive at 5 years, and one-third were free of distant disease. These outcomes (5-year OS rate 48%) are not significantly worse than those (5-year OS rate 36–65%) for the overall population of women treated for locally advanced breast cancer reported by a number of investigators (1, 10, 12, 29–31). Our retrospective data therefore suggest that having inoperable disease after primary chemotherapy, by itself, is not predictive of significantly worse survival, and multidisciplinary locoregional treatment may be able to achieve a chance of prolonged survival.



Patients at Risk:			
Primary disease extent	18	9	2
Nodal disease extent	20	5	1

Fig. 2. OS for patients who were inoperable because of nodal disease extent compared with those who were inoperable only because of primary disease extent.

Unfortunately, but not totally unexpectedly, our series of patients had a high rate of LRR after RT and mastectomy (5-year rate 27%). Furthermore, the high probability of treatment-related morbidity precluded investigating whether radiation dose escalation could improve locoregional control. Of those who underwent mastectomy, more than one-



Patients at Risk:	32	6	2

Fig. 3. Postoperative morbidity of the 32 patients who underwent mastectomy measured from the time of surgery.

half had a significant postoperative complication, and several patients required additional surgical revision. The complication rates were highest in those who received a dose of ≥54 Gy. Similar complication rates of 40–65% have been published by other institutions investigating preoperative RT and mastectomy for locally advanced breast cancer (32–35). These data collectively support the need to develop novel treatment strategies such as RT combined with radiosensitizing agents. Alternatively, we are also investigating whether patients with extensive inoperable primary disease after chemotherapy could be better treated with surgical procedures using myocutaneous repair for closure followed by postmastectomy RT.

The possibility of long-term survival, combined with the high risk of postoperative morbidity, has important implications regarding treatment recommendations for this class of patients. Because they are inoperable after primary chemotherapy, the crucial therapeutic decision is whether to proceed with locoregional treatment despite the poor response to initial therapy. In our analysis, patients who were inoperable after chemotherapy only because of primary disease extent (tumor size precluding a primary skin closure), rather than nodal disease extent (N2–3 or M1 disease), had significantly more favorable OS and DDFS. Similarly, having a less advanced nodal stage (N0 or N1) or a clinical nodal response (CR or PR) after chemotherapy was associated with better outcomes. Our data indicate that these patients should proceed with definitive locoregional treatments. In contrast, for those patients who are inoperable because of advanced nodal disease extent, quality-of-life issues regarding the high risk of treatment-related morbidity should be weighed very carefully given their poor prognosis, and it may be appropriate to consider these patients for Phase I clinical trials.

The sample size of this series was relatively small because primary chemotherapy is effective at achieving disease response. More than 95% of patients who were treated with chemotherapy in our institutional protocols were able to proceed with surgery as the initial form of local therapy. Our limited sample size may not have had enough power to detect other prognostic factors that could be incorporated into treatment recommendations.

CONCLUSION

Despite the poor prognosis of having inoperable disease that persists after primary chemotherapy, aggressive locoregional management using preoperative RT and mastectomy offers these patients long-term survival that is surprisingly better than expected. Using this approach, almost one-half of the patients remained alive at 5 years. Our data indicate that patients who are inoperable only because of primary disease extent have significantly better outcomes than those who are inoperable because of nodal disease extent. These clinical prognostic factors, combined with the high risk of LRR and postoperative morbidity, should be carefully considered when making therapeutic decisions after primary chemotherapy. These concerns emphasize the need to develop novel treatment strategies such as RT combined with radiosensitizing agents, more extensive surgical procedures combined with myocutaneous repair for closure, or new effective systemic agents.

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Int. J. Radiation Oncology Biol. Phys., Vol. 53, No. 4, pp. 880–888, 2002

0360-3016/02/$–see front matter

ELSEVIER

PII S0360-3016(02)02850-X

CLINICAL INVESTIGATION **Breast**

PATHOLOGIC TUMOR SIZE AND LYMPH NODE STATUS PREDICT FOR DIFFERENT RATES OF LOCOREGIONAL RECURRENCE AFTER MASTECTOMY FOR BREAST CANCER PATIENTS TREATED WITH NEOADJUVANT VERSUS ADJUVANT CHEMOTHERAPY

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Purpose: To compare the pathologic factors associated with postmastectomy locoregional recurrence (LRR) in breast cancer patients not receiving radiation who were treated with neoadjuvant chemotherapy (NEO) vs. adjuvant chemotherapy (ADJ).

Methods and Materials: We retrospectively analyzed the rates of LRR of subsets of women treated in prospective trials who underwent mastectomy and received chemotherapy but not radiation. These trials were designed to answer chemotherapy questions. There were 150 patients in the NEO group and 1031 patients in the ADJ group. In the NEO group, 55% had clinical Stage IIIA or higher vs. 9% in the ADJ group ($p < 0.001$, chi-square test).

Results: Despite the more advanced clinical stage in the NEO group, the pathologic size of the primary tumor and the number of positive lymph nodes (+LNs) were significantly less in the NEO group than in the ADJ group ($p < 0.001$ for both comparisons). However, the 5-year actuarial LRR rate was 27% for the NEO group vs. 15% for the ADJ group ($p = 0.001$, log–rank). The 5-year risk for LRR was higher in the NEO patients for all pathologic tumor sizes: 0–2 cm (18% vs. 8%, $p = 0.011$), 2.1–5 cm (36% vs. 15%, $p < 0.001$), and >5 cm (46% vs. 28%, $p = 0.028$). The risk of LRR by the number of +LNs was similar in the NEO and ADJ groups, except for the subset of patients with ≥4 +LNs (53% vs. 23%, $p < 0.001$). The rates of LRR in the patients with primary tumors measuring ≤2.0 cm and 1–3 +LNs were similar in both groups. However, for the patients with a pathologic tumor size of 2.1–5.0 cm and 1–3 +LNs, the LRR was higher in the NEO group than in the ADJ group (30% vs. 15%, $p = 0.016$). Most failures in this NEO subgroup had clinical Stage III disease. In a subset of NEO and ADJ patients matched for clinical stage, no significant differences were found in the rates of LRR according to primary tumor size and number of +LNs when these variables were analyzed independently. Again, however, differences were found in the subgroup of patients with tumors pathologically measuring 2.1–5.0 cm with 1–3 +LNs (32% NEO vs. 8% ADJ, $p = 0.030$).

Conclusion: The rates of postmastectomy LRR for any pathologic tumor size are higher for patients treated with initial chemotherapy than for patients treated with initial surgery. Radiotherapy should be offered to all patients with ≥4 +LNs, tumor size >5 cm, or clinical Stage IIIA or greater disease, regardless of whether they receive neoadjuvant or postoperative chemotherapy. The information assessing LRR rates in patients with clinical Stage II disease who receive neoadjuvant chemotherapy, particularly if 1–3 lymph nodes remain pathologically involved, is insufficient to determine whether these patients should receive radiotherapy. © 2002 Elsevier Science Inc.

Neoadjuvant chemotherapy, Adjuvant chemotherapy, Mastectomy, Locoregional recurrence.

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Supported in part by National Cancer Institute Department of Health and Human Services Grant CA16672 and T32CA77050 and a grant from the Stanford and Joan Alexander Foundation, Houston, TX. Dr. Buchholz is supported by Grant BC980154, a USAMRMC Breast Cancer Research Program Career Development Award.

Presented at the American Society for Therapeutic Radiology and Oncology 2001 Meeting, San Francisco, CA.

Received Nov 21, 2001, and in revised form Mar 13, 2002. Accepted for publication Mar 19, 2002.

INTRODUCTION

Postmastectomy radiotherapy (RT) is indicated for selected breast cancer patients whose locoregional extent of disease places them at increased risk of locoregional recurrence (LRR). A number of randomized trials have demonstrated that RT reduces the LRR rate after mastectomy by approximately two-thirds (1). Furthermore, recent trials have shown that the addition of RT to mastectomy and chemotherapy can improve the overall survival of selected populations of breast cancer patients who have a 25–30% probability of LRR without RT (2–4). The improvement in survival noted in these randomized trials was thought to be a consequence of sterilization of residual tumor in the chest wall and regional lymph nodes by RT. This hypothesis is consistent with a study that compared metastasis curves in patients with and without local failure, which also suggested a secondary dissemination in patients with LRR (5). Therefore, a survival benefit from postmastectomy RT would be unlikely in a population of breast cancer patients who have a low probability of LRR after mastectomy.

This consideration is important, because although modern techniques have significantly improved the safety of treatment, postmastectomy RT still carries the risk of normal tissue injury. Such injuries can include radiation pneumonitis, chronic arm edema, and, possibly, cardiovascular injuries (1, 6). In an effort to define the patient and pathologic characteristics that predict for elevated rates of LRR, we recently reviewed a series of patients treated in prospective institutional clinical trials to determine the clinical and pathologic features that predict for LRR after mastectomy and chemotherapy (7). We demonstrated that the relevant risk factors that increased LRR were related to the locoregional disease extent (7). This finding is consistent with the reports of others, who have also demonstrated that the extent of nodal disease involvement and size of the primary were significant independent factors predictive of LRR (8, 9). In addition, two independent consensus statements regarding the indications for postmastectomy RT concluded that it is recommended for patients with ≥4 positive lymph nodes (+LNs) (10, 11).

The use of neoadjuvant chemotherapy has, however, complicated the selection criteria for postmastectomy RT. The initial surgery is both therapeutic and diagnostic in that it provides an assessment of the pathologic size of the primary and extent of nodal involvement. More than 80% of patients treated with neoadjuvant chemotherapy achieve a partial or complete response to treatment (12–13), meaning that for most patients the pathologic extent of disease after neoadjuvant chemotherapy is different than the extent at diagnosis. It is not clear how these treatment-induced changes in the pathologic extent of nodal and primary disease influence their value as predictive factors for LRR. To address these questions, we recently investigated the clinical and pathologic predictors for LRR after neoadjuvant chemotherapy and mastectomy (14). In our initial study, we found that both the clinical extent of disease and the residual pathologic extent of disease predicted for LRR (14). Although this study provided one of the first sets of data defining the risk factors for LRR for patients treated with neoadjuvant chemotherapy, we recognized that the quantification of the clinical extent of disease was very imprecise. For example, previous data from our institution and others have shown that the discordance between the clinical and pathologic assessment of disease extent is significant (15, 16). For this reason, pathologic criteria have been considered a more objective and reproducible basis for treatment selection. The purpose of the present study was to compare how pathologic tumor size and lymph node status correlated with LRR in patients treated with neoadjuvant chemotherapy followed by mastectomy vs. those treated with mastectomy and adjuvant chemotherapy. It is our hope that these data will help clinicians in defining which patients treated with neoadjuvant chemotherapy and mastectomy should receive postmastectomy RT.

METHODS AND MATERIALS

We retrospectively reviewed the data from the records of 1181 of the 2638 patients treated in consecutive prospective clinical trials performed at The University of M. D. Anderson Cancer Center between 1974 and 1998. These trials were designed primarily to answer chemotherapy-related questions. Because the purpose of this study was to assess the pathologic risk factors predictive of LRR after mastectomy and chemotherapy, we only analyzed the data from patients who did not receive RT. In this analysis, we compared the pathologic predictors of outcome of 1031 patients treated with mastectomy and adjuvant chemotherapy (ADJ) to that of 150 patients treated with neoadjuvant chemotherapy (NEO) followed by mastectomy. The ADJ and NEO groups were treated in different protocols. These patients represented 57% (1031 of 1805 ADJ) and 17% (150 of 833 NEO) of the total number of patients enrolled in these studies.

All patients underwent staging evaluations to rule out the presence of metastatic disease before enrollment in the study. Patients with inflammatory breast cancer were treated in different protocols and were not included in this analysis. The treatment details concerning chemotherapy were dictated by the protocol guidelines and have been previously reported (7, 14). All patients in the ADJ group received a doxorubicin-containing chemotherapy regimen after mastectomy. Additional details concerning the chemotherapy regimens used in these patients have been previously published (7). For the NEO group, 121 patients were treated with a doxorubicin-containing chemotherapy regimen before mastectomy and the remaining 29 were treated with single-agent paclitaxel. The median number of cycles before surgery was 4. Postoperatively, 92% (138 of 150) of the NEO group received additional chemotherapy treatments. Additional details concerning the chemotherapy for this group have been previously published (14). The rate of

Table 1. Patient and disease characteristics

	Overall (%)		Matched subgroup (%)	
Characteristic	ADJ	NEO	ADJ	NEO
Age				
<40	20 (208/1031)	20 (30/150)	28 (55/198)	20 (20/99)
40–60	64 (663/1031)	59 (88/150)	57 (113/198)	64 (63/99)
>60	16 (160/1031)	21 (32/150)	15 (30/198)	16 (16/99)
Clinical stage				
I	4 (41/1031)	1 (1/150)	0	0
IIA	36 (369/1031)	14 (21/150)	21 (42/198)	21 (21/99)
IIB	42 (430/1031)	29 (44/150)	44 (88/198)	44 (44/99)
IIIA	10 (104/1031)	23 (34/150)	34 (68/198)	34 (34/99)
IIIB	0	25 (37/150)	0	0
IV*	0	7 (11/150)	0	0
Unknown	8 (87/1031)	0 (2/150)	0	0
ER status				
ER+	45 (466/1031)	48 (72/150)	45 (89/198)	56 (55/99)
ER−	38 (391/1031)	37 (56/150)	39 (77/198)	31 (31/99)
Unknown	17 (174/1031)	15 (22/150)	16 (32/198)	13 (13/99)
PR status				
PR+	30 (312/1031)	31 (46/150)	32 (64/198)	39 (39/99)
PT−	35 (358/1031)	28 (42/150)	67 (66/198)	33 (33/99)
Unknown	35 (361/1031)	41 (62/150)	34 (68/198)	27 (27/99)

* Indicates ipsilateral supraclavicular lymph node involvement without systemic metastases.
Abbreviations: ADJ = adjuvant chemotherapy; NEO = neoadjuvant chemotherapy; ER = estrogen receptor; PR = progesterone receptor.

tamoxifen use for the ADJ and NEO groups was 31% (318 of 1031) and 31% (47 of 150), respectively.

The median follow-up of the surviving patients was 4.1 years for the NEO group and 9.6 years for the ADJ group, the difference in part reflecting a change from adjuvant to neoadjuvant chemotherapy that occurred during the years included in this study. The percentage of patients whose breast cancer was diagnosed before 1990 was 78% for the ADJ group and 45% for the NEO group.

Freedom from LRR was calculated using the Kaplan–Meier method (17), with all event and follow-up times measured from the date of diagnosis. We considered all LRRs, whether they developed before, simultaneous with, or after distant metastases. This approach was preferred to methods based on the time to first event, which underestimate the LRR rate (18). Our rationale for considering overall LRRs rather than isolated LRRs was that the purpose of our study was to evaluate the pathologic factors predictive of locoregional events. We assumed it unlikely that prior distant metastases would lead to a reseeding of the locoregional area and thus believed the source of locoregional events was independent of the development of distant disease. Log–rank tests were used to compare the LRR data. Chi-square tests were used to compare the categorical variables between the groups. All reported p values were 2-sided.

We also analyzed LRR in a matched subgroup that included only patients with clinical Stage IIA, IIB, or IIIA disease and that was weighted 2:1 (ADJ/NEO). A total of 6 NEO and ADJ subgroups that were matched for clinical stage were randomly generated. From these matched subgroups, we selected the one matched pair for our analysis that had no significant differences in age or estrogen and progesterone receptor status and that had the most similar actuarial freedom from LRR curves. The rationale for selecting the matched pair with the most equivalent LRR curves was that we wanted to minimize the confounding variables that could influence our assessment of the relationship of the pathologic features and LRR. We also assumed that the sequencing of chemotherapy and surgery would be unlikely to affect rates of postmastectomy LRR.

RESULTS

Table 1 provides the patient and disease characteristics for the 2 groups of patients overall and the 2 matched control subgroups. No statistically significant differences were found between the 2 matched subgroups with respect to the characteristics shown in Table 1.

Table 2 shows the pathologic characteristics of the populations. The pathologic characteristics of the NEO group represent the extent of disease after chemotherapy, and the characteristics of the ADJ group represent disease that was not previously treated. In general, both the NEO group overall and the matched subgroup had a less advanced pathologic primary size than did the ADJ group ($p < 0.001$ for both comparisons). In addition, both the NEO group overall and the matched subgroup had fewer pathologically involved lymph nodes than did the ADJ group ($p < 0.001$ for both comparisons). In the NEO group, 56% of patients had residual primary disease measuring <2.0 cm, even though only 1 patient in this cohort had clinical Stage T1

Table 2. Pathologic characteristics of primary and nodal disease

Characteristic	Overall (%)		Matched subgroup (%)	
	ADJ	NEO	ADJ	NEO
Pathologic primary size (cm)				
≤2.0	31 (314/1031)	49 (74/150)	15 (29/198)	58 (57/99)
2.1–5.0	49 (509/1031)	37 (56/150)	51 (100/198)	34 (34/99)
>5.0	10 (103/1031)	9 (14/150)	33 (66/198)	3 (3/99)
Unknown	10 (105/1031)	4 (6/150)	2 (3/198)	1 (1/99)
Pathologic LN status				
0 + LNs	14 (141/1031)	41 (62/150)	6 (11/198)	45 (45/99)
1–3 +LNs	45 (466/1031)	28 (42/150)	43 (85/198)	38 (38/99)
≥4 +LNs	41 (419/1031)	27 (41/150)	50 (99/198)	16 (16/99)
Unknown	<1 (5/1031)	3 (5/150)	2 (3/198)	2 (2/99)

Abbreviations: ADJ = adjuvant chemotherapy; NEO = neoadjuvant chemotherapy; LN = lymph node.

disease at diagnosis; 46% had negative lymph nodes after chemotherapy and only 28% had clinical Stage N0 disease at the time of diagnosis.

The rate of LRR in the NEO group was higher than that in the ADJ group (5-year rate 27% vs. 15%, respectively, $p = 0.001$). Of the patients with LRR in the NEO group, 66% had LRR as isolated first events and 34% had LRR either simultaneously with or after distant metastasis. For the ADJ group, these percentages were 69% and 31%. For the 2 matched subgroups, the overall LRR rates were similar (5-year rate 12% for NEO vs. 16% for ADJ, $p = 0.404$).

Figure 1 displays the 5-year rates of LRR according to pathologic measurement of primary tumor size for the ADJ and NEO groups (Fig. 1a) and the matched subgroup of patients with clinical Stage II-IIIA disease (Fig. 1b). As shown, the LRR rate was significantly higher for any given pathologic tumor size in the NEO group than in the overall ADJ group (p values represent comparison of the Kaplan–Meier data and not the 5-year rates). However, these differences were no longer significant when the groups were matched for clinical stage.

Figure 2 displays the 5-year rates of LRR according to the number of +LNs for the ADJ and NEO groups (Fig. 2a) and the ADJ and NEO matched subgroups (Fig. 2b). Unlike the comparison of primary tumor size, in which differences were seen across all tumor sizes, the LRR was significantly higher only for the subgroup of patients with ≥4 +LNs in the NEO patients compared with the ADJ patients. No statistically significant differences in LRR were noted as a function of the number of +LNs in the matched subgroups.

We analyzed separately the data in patients with tumor sizes ≤5 cm who had 1–3 +LNs. Figure 3 displays the freedom from LRR curves for the patients with tumor sizes 0–2 cm and 1–3 +LNs. The differences were not statistically significant, and at 5 years, both curves had LRR rates of <20%. Figure 4 displays the freedom from LRR curves for the patients with tumor sizes 2–5 cm and 1–3 +LNs. In contrast to the smaller tumor sizes shown in Fig. 3, the differences in the curves for both Fig. 4a,b were statistically significant.

To determine how the clinical stage affected LRR in the NEO patients with tumor sizes ≤5 cm who had 1–3 +LNs, we compared the LRR rates in those patients with clinical Stage T3 or T4 primary tumors with those with clinical Stage T1 or T2 tumor. The LRR rate for these 2 groups was 46% vs. 4% ($p = 0.0019$), with all but 1 of these patients who experienced LRR having T3 or T4 disease at diagnosis.

DISCUSSION

In this study, we demonstrated that the risks of LRR according to pathologic criteria alone are different for patients treated with chemotherapy before mastectomy than for patients receiving chemotherapy after mastectomy. For patients treated with neoadjuvant chemotherapy, both the initial clinical extent of disease and the extent of residual disease at surgery are useful when assessing LRR risk.

The use of neoadjuvant chemotherapy is becoming increasingly common in the United States. Once reserved for patients with locally advanced disease, this sequencing approach has been studied in all stages of the disease. Neoadjuvant chemotherapy has the advantage of offering clinicians the opportunity to evaluate the response of the disease to a particular chemotherapy regimen. In theory, patients with resistant disease can be identified, and the treatment changed to a potentially more effective regimen. Neoadjuvant chemotherapy also avoids any delay in systemic treatment. To test whether the avoidance of delay improved outcome, the National Surgical Adjuvant Breast Project (NSABP) conducted a randomized clinical trial that compared the efficacy of neoadjuvant chemotherapy with that of adjuvant chemotherapy (12). Survival and distant disease-free survival were equivalent (12). The NSABP adopted neoadjuvant chemotherapy as its new standard for current clinical studies, because the breast preservation rates were higher in the patients randomized to neoadjuvant chemotherapy.

One consequence of the increasing use of neoadjuvant chemotherapy is that the indications for delivering postmastectomy RT are less clear. The American Society for Clinical Oncology (ASCO) consensus conference regarding postmastectomy RT after neoadjuvant chemotherapy spe-

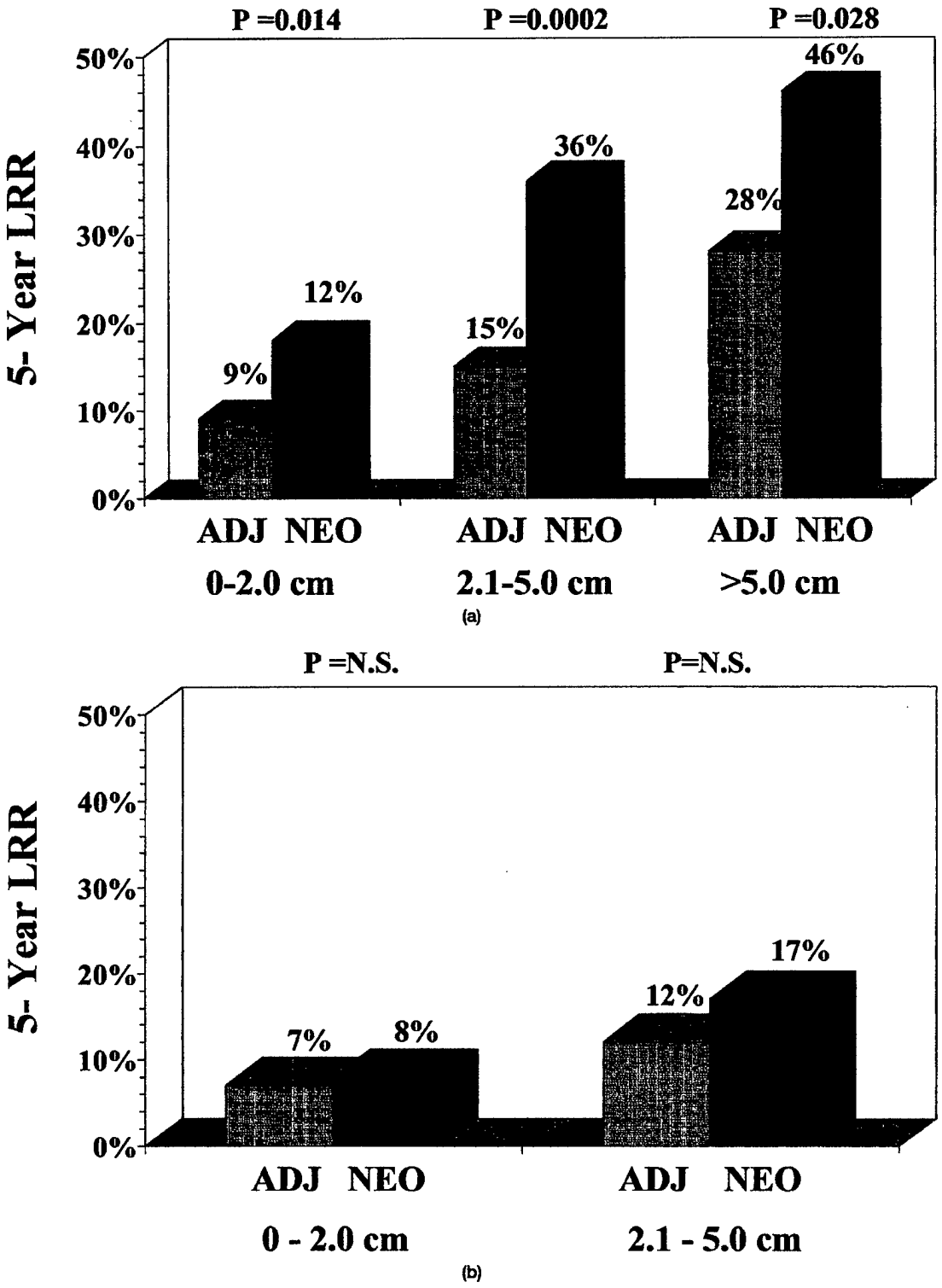


Fig. 1. Five-year rates of LRR as a function of primary tumor size measured pathologically. (a) Overall groups. (b) Matched subgroups.

cifically cited that no recommendations could be made because of the lack of data concerning failure patterns after neoadjuvant chemotherapy and mastectomy compared with mastectomy followed by adjuvant chemotherapy (11). In addition, neoadjuvant chemotherapy has been used during more recent years and so long-term recurrence patterns cannot yet be assessed. A second potential problem introduced by neoadjuvant chemotherapy with respect to defin-

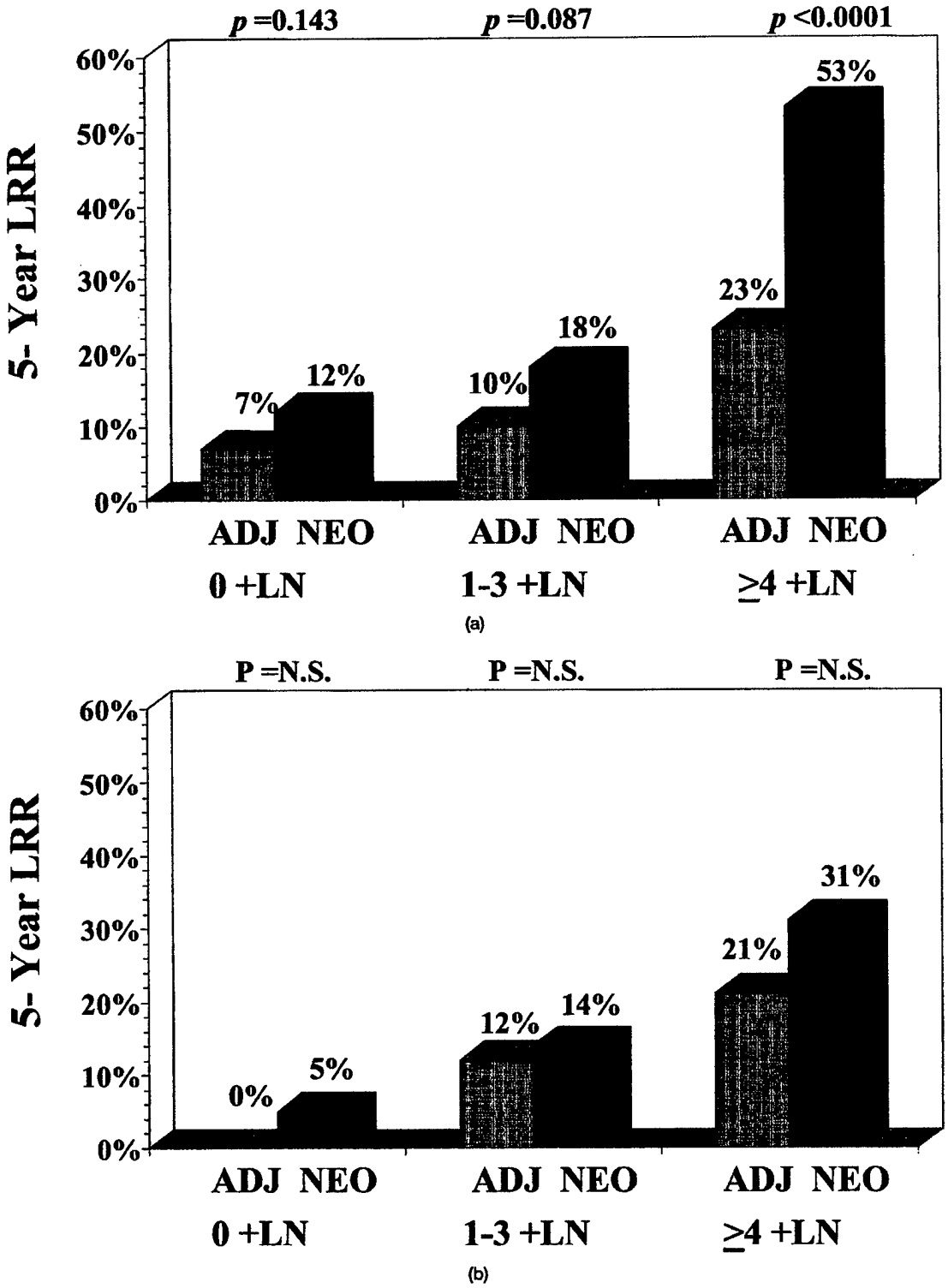
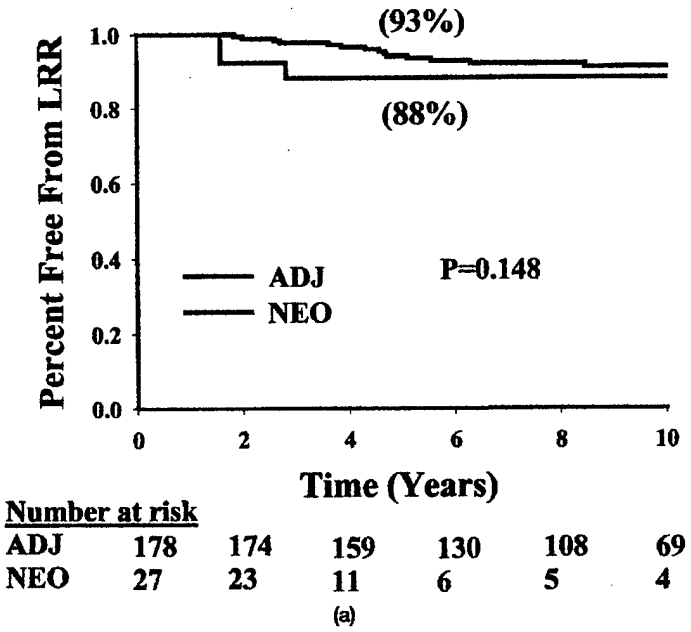


Fig. 2. Five-year rates of LRR as a function of number of pathologically involved lymph nodes. (a) Overall groups. (b) Matched subgroups.

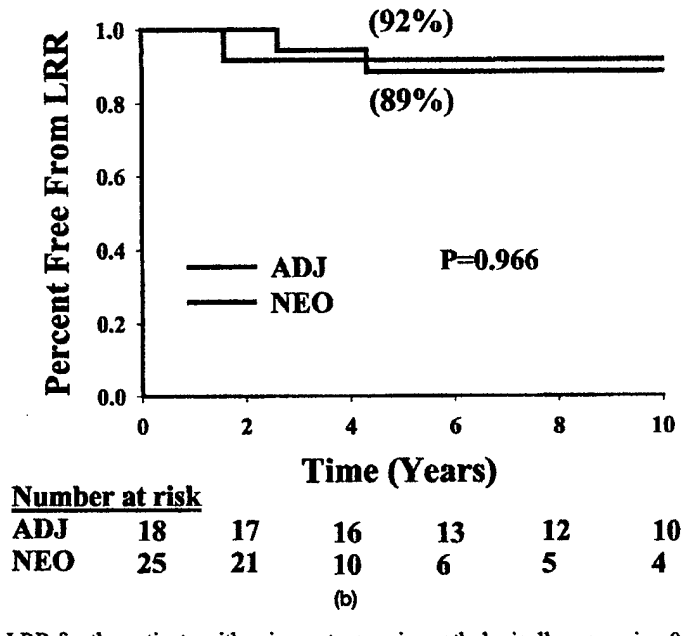
ing the selection criteria for postmastectomy RT is that the clinical assessment of disease is less reliable than the pathologic determination of disease extent. A number of studies have shown that 20–30% of patients with clinically negative lymph nodes will have positive disease on pathologic examination (19, 20). Furthermore, using the available clinical tools, it is rarely possible to identify the subgroup of patients with ≥4 +LNs at diagnosis, which is the threshold



Number at risk

ADJ	178	174	159	130	108	69
NEO	27	23	11	6	5	4

(a)



Number at risk

ADJ	18	17	16	13	12	10
NEO	25	21	10	6	5	4

(b)

Fig. 3. Freedom from LRR for the patients with primary tumor size pathologically measuring 0–2 cm and 1–3 +LNs. (a) Overall groups. (b) Matched subgroups.

that the American Society for Therapeutic Radiology and Oncology and ASCO consensus statements use to definitely recommend postmastectomy RT (10, 11).

In this study, we demonstrated that for any pathologic tumor size, the rates of LRR are higher in patients first treated with neoadjuvant chemotherapy than in those treated with surgery first and then chemotherapy. These data imply that the disease response to chemotherapy does not reduce the risk of LRR to a level associated with untreated pathologic features with the same extent of disease. From these data, it can be concluded that the risk of LRR after neoadjuvant chemotherapy and mastectomy is a function of both the original disease extent and the extent of disease after treatment.

The most important reason to investigate the rates of LRR after mastectomy is to help understand which patients should receive postmastectomy RT. Currently, it is controversial as to what predicted threshold of LRR warrants postmastectomy RT. As previously mentioned, the randomized clinical trials that demonstrated a survival advantage with the addition of postmastectomy RT to chemotherapy had LRR rates of 25–30% in the patients randomized to not receive RT (2–4). A subgroup of patients for whom the use of postmastectomy RT has been controversial is the group

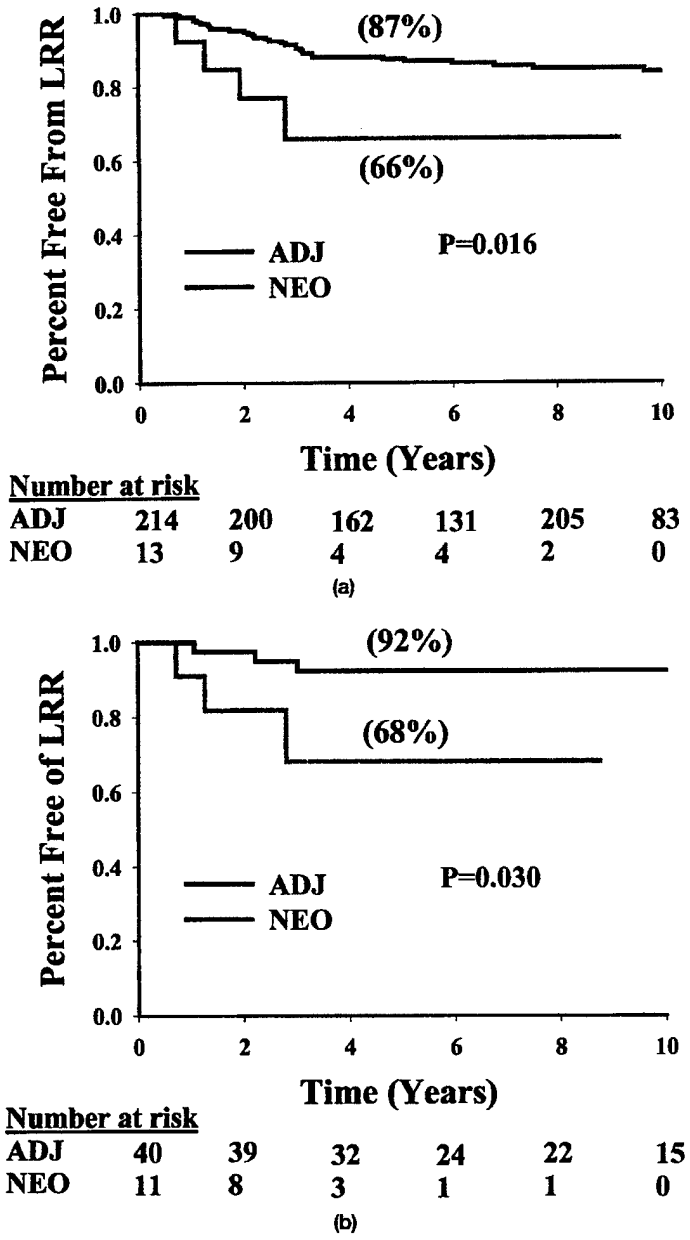


Fig. 4. Freedom from LRR for the patients with primary tumor size pathologically measuring 2.1–5.0 cm and 1–3 +LNs. (a) Overall groups. (b) Matched subgroups.

of patients with Stage II disease and 1–3 +LNs. For patients treated with adjuvant chemotherapy in this series, the 5-year risk of LRR ranged from 7% to 13%, depending on tumor size. We have demonstrated that, after neoadjuvant chemotherapy, for patients with clinical Stage II disease, the risk associated with tumor sizes of ≤2 cm and 1–3 +LNs was similar to that in the ADJ group. However, in the NEO patients with residual tumor sizes between 2 and 5 cm and 1–3 +LNs, the 5-year LRR rate was 32%, significantly higher than in the comparable group of ADJ patients. This higher rate was a direct consequence of LRRs in the patients with initial clinical Stage III disease.

When interpreting these data, it is important to recognize some limitations of this study. Our sample size of patients treated with neoadjuvant chemotherapy was limited, so the data concerning LRR in the patient subgroups have significant uncertainties. In addition, despite matching a subgroup of NEO and ADJ patients for stage, estrogen receptor status, and age, it is possible that other confounding variables important for LRR were unequally distributed. In addition, the NEO group had shorter follow-up and therefore the 5-year LRR rates were less certain than those of the ADJ cohort. It is also likely that the overall rates of LRR will increase within between 5 and 10 years in the NEO group. For example, we previously reported that 21% of the total number of LRR in the ADJ group occurred after 5 years.

It is important to recognize that this study does not suggest that the use of neoadjuvant chemotherapy is associated with higher rates of LRR compared with adjuvant chemotherapy. We believe that the higher rates of LRR in the overall NEO group compared with the ADJ group were a function of the imbalance in disease stage rather than the differences in chemotherapy and surgery sequencing. The important finding of our study is that the LRR as a function of a given pathologic variable is different for the two groups.

We found in our study that neoadjuvant chemotherapy was more likely to affect the prognostic significance of the primary tumor size than that of lymph node status. This is because changes in primary tumor size after chemotherapy are more common than changes in the number of affected lymph nodes. In this series, 56% of patients had a pathologic size of the primary disease measuring <2.0 cm after neoadjuvant chemotherapy, despite only 1 patient in this cohort having clinical Stage T1 disease at diagnosis. Changes in tumor size after neoadjuvant chemotherapy may be more common than changes in the number of +LNs because the number of logs of cell kill required to sterilize a +LN with 1 cm of disease is 3–5-fold greater than the number of logs of cell kill required to reduce a 4.0-cm tumor to 1.0 cm (depending on assumed tumor-clonogen density and effectiveness of treatment).

CONCLUSION

Our study is the first to compare how pathologic factors associated with LRR are different between those treated with neoadjuvant chemotherapy and those treated with adjuvant chemotherapy. We have demonstrated in this retrospective study that the LRR rate for any pathologic tumor size is higher for patients treated with neoadjuvant chemotherapy than for those treated with adjuvant chemotherapy when the initial clinical stage is ignored. Patients with clinical Stage III disease at presentation should receive postmastectomy RT regardless of the degree of response that occurs in the primary tumor and the regional lymphatics. Additional data or a clinical trial are needed to determine the value of RT for patients with Stage II breast cancer treated with neoadjuvant chemotherapy and mastectomy.

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APPENDIX 25

Int. J. Radiation Oncology Biol. Phys., Vol. 51, No. 3, pp. 671–678, 2001

0360-3016/01/$–see front matter

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PII S0360-3016(01)01684-4

CLINICAL INVESTIGATION **Breast**

RELATIONSHIP OF SENTINEL AND AXILLARY LEVEL I–II LYMPH NODES TO TANGENTIAL FIELDS USED IN BREAST IRRADIATION

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Purpose: To evaluate the volume of nodal irradiation associated with breast-conserving therapy, we defined the anatomic relationship of sentinel lymph nodes and axillary level I and II lymph nodes in patients receiving tangential breast irradiation.

Methods and Materials: A retrospective analysis of 65 simulation fields in women with breast cancer treated with sentinel lymph node surgery and 39 women in whom radiopaque clips demarcated the extent of axillary lymph node dissection was performed. We measured the relationship of the surgical clips to the anatomic landmarks and calculated the percentage of prescribed dose delivered to the sentinel lymph node region.

Results: A cranial field edge 2.0 cm below the humeral head the sentinel lymph node region was included or at the field edge in 95% of the cases and the entire extent of axillary I and II dissection in 43% of the axillary dissection cases. In the remaining 57%, this field border encompassed an average of 80% of cranial/caudal extent of axillary level I and II dissection. In 98.5% of the cases, all sentinel lymph nodes were anterior to the deep field edge and 71% were anterior to the chest wall–interface, whereas 61% of the axillary dissection cohort had extension deep to the chest wall–lung interface. If the deep field edge had been set 2 cm below the chest wall–lung interface, the entire axillary dissection would have been included in 82% of the cases, and the entire sentinel lymph node would have been covered with a 0.5-cm margin. The median dose to the sentinel lymph node region was 98% of the prescribed dose.

Conclusions: By extending the cranial border to 2 cm below the humeral head and 2 cm deep to the chest wall–lung interface, the radiotherapy fields used to treat the breast can include the sentinel lymph node region and most of axillary levels I and II. © 2001 Elsevier Science Inc.

Breast cancer, Sentinel lymph nodes, Axillary lymph nodes.

INTRODUCTION

For women with early-stage breast cancer, breast conservation therapy (BCT) consisting of segmental mastectomy, axillary lymph node dissection, and breast radiotherapy (RT) has proved to be highly effective, producing local recurrence rates of only 1% annually (1). Given this high success rate, more recent research efforts concerning BCT have focused on reducing the treatment-related morbidity. Although serious toxicity after BCT is very unusual, nearly all patients experience a number of iatrogenic injuries that impair their quality of life. All patients who undergo axillary dissection experience loss of sensation in the axillary region, temporary range of motion limitations of the shoulder, and postoperative arm and axillary pain. Furthermore, axillary dissection increases the risk of edema and cellulitis of either the breast or arm (2). For these reasons, primary RT and sentinel lymph node dissection with or without adjuvant RT have been proposed as alternatives to standard axillary dissection.

For these 2 alternatives, to ensure that the axilla receives appropriate treatment, an understanding of the anatomic relationship of the axillary lymph nodes to the RT fields used in BCT is of critical importance. For women receiving RT as a component of BCT, standard RT fields tangentially traverse the anterior thorax to include the entire ipsilateral breast while minimizing the dose to the underlying intrathoracic contents. In selected cases, a matched field or fields are added above the tangential fields to treat the high axilla and supraclavicular fossa. For women who have undergone a standard level I–II dissection, these additional fields are needed when treatment of the undissected regional lymph nodes is desired. However, for women who have undergone only sentinel lymph node surgery or no axillary surgery, most lymph nodes at highest risk of

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Oral presentation, American Radium Society 2001 Meeting, San Diego, CA.

This work was supported in part by Grant T32CA77050 awarded by the National Cancer Institute, U.S. Department of Health and Human Services. A USAMRMC Career Development Award from the Breast Cancer Research Program (BC980154) supports T.A.B.

Received Feb 2, 2001, and in revised form May 11, 2001. Accepted for publication May 19, 2001.

containing disease will be within or near the fields used to treat the breast. For these women, effective treatment of the regional lymphatics is more dependent on the proper design of the tangential "breast" fields than any field or fields added above the cranial edge of the tangents.

A consideration of the axillary anatomy in the design of the tangential fields used to treat the breast is not standard practice. However, an understanding of the relationship of the axillary anatomy to the breast tangential fields intuitively has significant clinical relevance. For women in whom primary RT is used to treat the axilla, the knowledge of the axillary anatomy is necessary for the proper design of the tangential field borders. This understanding is also important for patients who undergo sentinel lymph node surgery. The inclusion of undissected axillary lymph nodes in the tangent RT fields may minimize the risk of regional failure in the event of false-negative sentinel lymph node surgery. Finally, consideration of the axillary anatomy is relevant to an ongoing American College of Surgeons multi-institutional Phase III trial. A portion of this trial was designed to investigate whether axillary dissection can be safely omitted in patients with ≥ 1 positive sentinel lymph nodes. Patients with positive sentinel lymph nodes are randomized to axillary dissection or no additional axillary surgery, with the further stipulation that adjuvant RT be directed to the "breast only," without attempting to irradiate the undissected axilla. To understand the impact of that stipulation, it will be necessary to understand the anatomic relationship of the axillary lymph nodes to the tangential RT fields used for treatment of the breast.

In this study, we attempted to define the relationship of the sentinel lymph nodes and axillary level I–II anatomy with respect to the tangential breast fields routinely used as a component of breast irradiation. The specific questions to be addressed by the study were as follows: How often are sentinel lymph nodes included in breast tangential RT fields? What percentage of the anatomic region of axillary levels I and II is included in the breast tangential fields? What anatomic landmarks can RT oncologists use to design tangential fields that adequately cover the axillary lymph nodes? What percentage of prescribed RT dose is delivered to the axilla during a course of breast RT?

METHODS AND MATERIALS

Data from 2 patient sets were retrospectively analyzed to determine the relationship of the sentinel lymph nodes and axillary anatomy to the breast tangential fields. The first data set included patients who underwent sentinel lymph node surgery and breast irradiation without axillary dissection at the University of Texas M. D. Anderson Cancer Center. Between September 1997 and June 2000, 165 patients underwent sentinel lymph node surgery at our institution. To be included in the study, we required that RT was given at our facility, radiopaque clips were placed in the region of the sentinel lymph node surgery, sentinel lymph node clips were distinguishable from tumor bed clips, and the patients were receiving BCT. Of 165 patients, we identified 65 patients who met our criteria and were treated with sentinel lymph node surgery and breast RT. The second patient cohort consisted of 39 patients consecutively treated by a single surgeon (M.I.R.) with a level I–II axillary dissection. This surgeon's cases were selected because he used vascular clips throughout the entire dissection; therefore, the clips could be used to demarcate the entire extent of the axillary surgery.

As part of our case selection criteria, all patients had to have conventional simulation films from breast tangential RT fields available for review. In general, these fields were designed to encompass the breast without a specific attempt to include the lymph node regions. For this reason, we measured the relationship of the surgical clips to several objective anatomic structures on the simulation film, as well as the borders of the planned treatment field. The relationship of the clips to the treatment field was studied in both a cranial–caudal plane and a superficial–deep plane. The cranial–caudal landmarks used in this study included the distance of the proximal and distal clips to the humeral head, the ipsilateral abducted and externally rotated arm, and the cranial field edge. The landmark used to identify the superficial–deep plane was the perpendicular distance from the chest wall–lung to the deep field edge. The distances cranial or deep to the specified border were recorded as negative measurements, and the distances caudal or superficial to the border were recorded as positive measurements. Figure 1 shows examples of these relationships for 2 patients in the sentinel lymph node cohort. Figure 2 shows examples of these measurements for 2 patients who underwent axillary lymph node dissection. An estimate of the percentage of axillary levels I and II relative to the anatomic and field landmarks was determined by calculating the proportion of the cranial–caudal extent of the clips above and below the particular landmark being analyzed. Furthermore, the effects that the gantry angle of the medial field, body index of the patient, and field separation distance had on these relationships were analyzed.

For 22 patients in the sentinel lymph node cohort in whom CT-based three-dimensional treatment planning was available, the dose to the region of the sentinel lymph node clips was calculated. For this component of the study, a commercially available three-dimensional treatment planning system was used (ADAC Pinnacle Treatment Planning System). The dose calculations were performed using the CC Convolve dose calculation engine, which incorporates off-axis factors and scatter contribution and uses tissue inhomogeneity corrections based on CT Haunsfield units. The RT dose was normalized in the central plane of the treatment field at mid-separation. The depth of the prescription point was one third the distance from the posterior field edge to the apex of the breast. For patients with sentinel lymph node clips within the treatment fields, the dose to these clips relative to this normalization point was calculated. For patients with

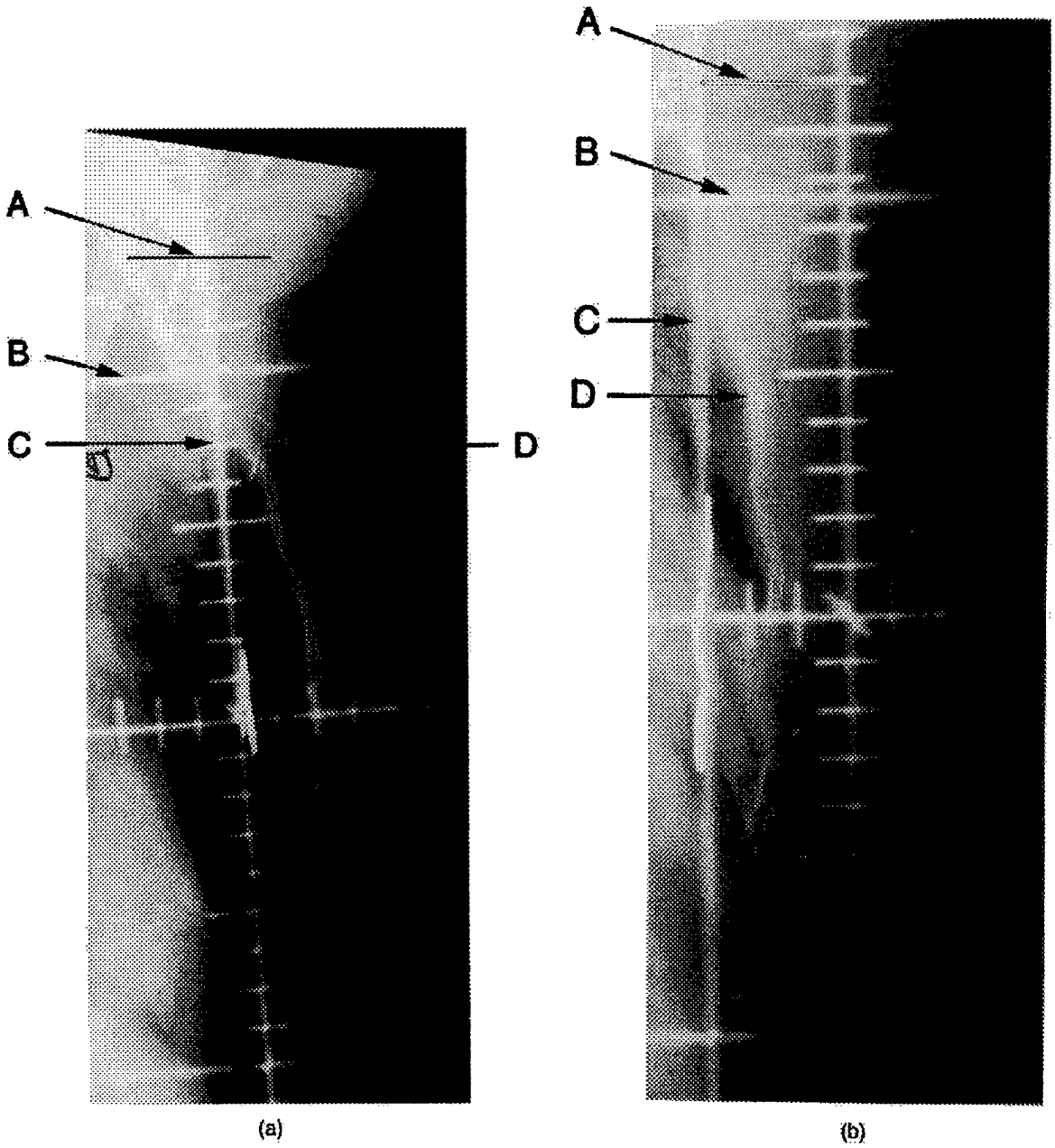

Fig. 1. Relationship of sentinel lymph node clips to a medial tangent RT field used to treat the breast. The two representative cases were selected because of their variability with respect to the cranial field edge. (A) Clips well below the cranial border and humeral head. (B) Clips close to the humeral head and above and below the cranial field edge. Anatomic and treatment field landmarks: A = humeral head; B = cranial edge; C = deep edge; and D = chest wall edge.

more than one sentinel lymph node, the minimal dose was recorded for each plan.

RESULTS

Patient and treatment characteristics

Table 1 shows the patient and treatment characteristics of the 2 study cohorts. The height and weight of each patient at the time of RT planning was used to calculate the quetlet (in kilograms per meters squared). The mean quetlet value was 26.83. The mean separation of the deep field edge was 19.27 cm. Two thirds of patients had their ipsilateral arm abducted approximately 120°, the remaining patients had their arm abducted approximately 90°. The mean distance of the cranial field edge to the humeral head was 3.1 cm.

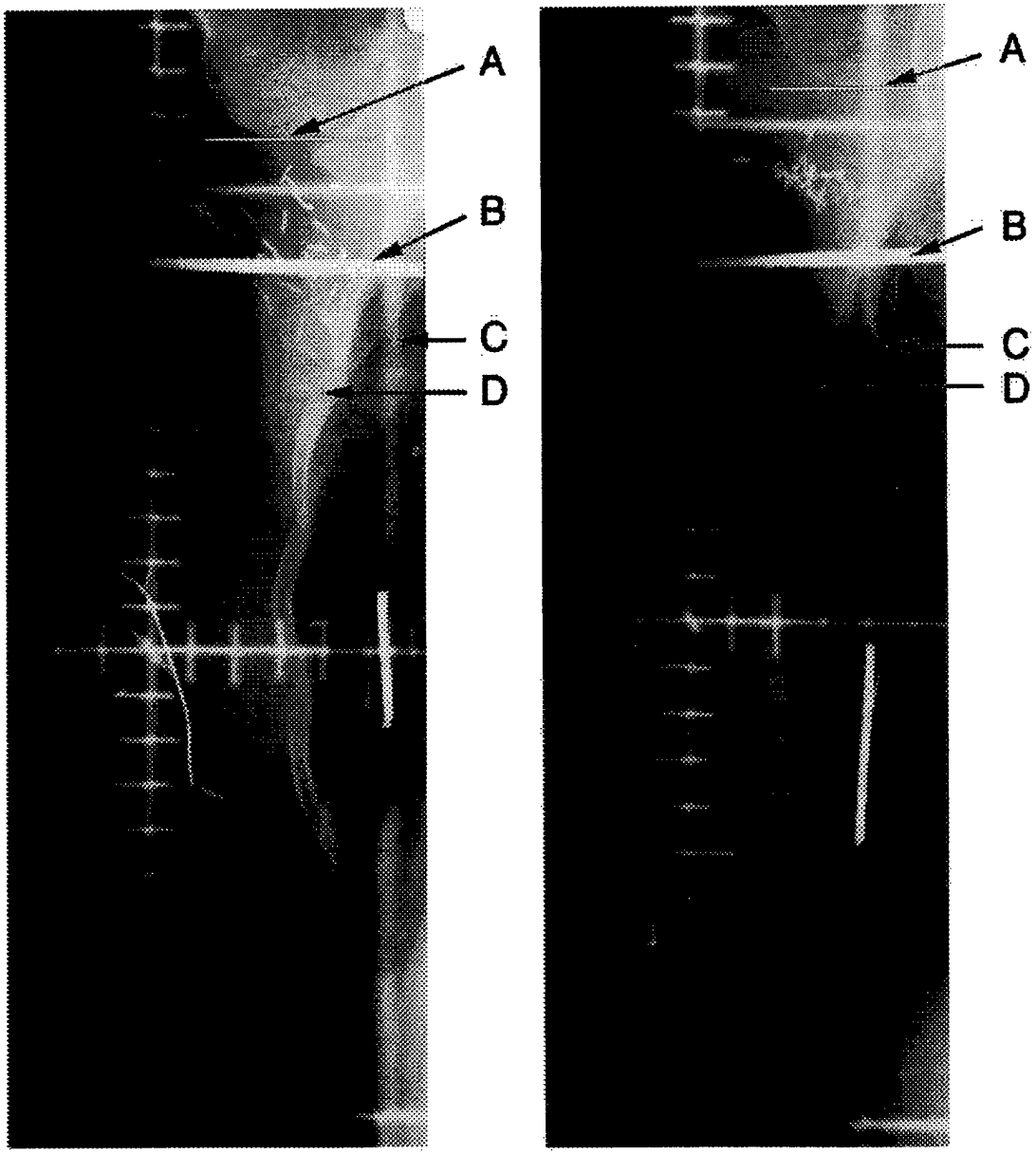

Fig. 2. Relationship of axillary dissection clips to a medial tangential RT field used to treat the breast. The two representative cases were selected because of their variability with respect to the deep field edge. (A) Clips superficial to the chest wall and the humeral head. (B) Clips extend beyond the deep field border. Anatomic and treatment field landmarks: A = humeral head; B = cranial edge; C = deep edge; and D = chest wall edge.

Sentinel lymph node surgery cohort

Table 2 provides data concerning the anatomic relationship of the region of sentinel lymph nodes to the medial tangential RT beams used to treat the breast. On average, the sentinel lymph node area was 4.75 cm (range 1.0–10.0) caudal to the humeral head. In 95% of the cases, the sentinel lymph nodes would have been treated with nondivergent, high tangential fields that were <2 cm from the humeral head. Furthermore, in 95% of the cases, the sentinel lymph nodes would have been treated with nondivergent, high tangential fields that exited below the ipsilateral arm. The actual treatment fields used in the patients studied were not designed to specifically include the sentinel lymph nodes. Despite this, the clips were included within the standard breast tangential fields in 85% of the cases. In the 15% of patients with sentinel lymph node clips outside the field edge, 60% were within 1 cm of the cranial edge and all were within 3 cm. The range in distance from the clips to cranial field edge was 3.0 cm above the field to 7.5 cm within the field. We did not find a correlation between patient size, separation distance, or gantry angle and the probability of having a sentinel lymph node cranial to the field edge or ipsilateral arm.

Table 1. Patient and treatment characteristics

Characteristic	Sentinel lymph node cohort	Axillary dissection cohort
Patients (*n*)	65	39
Mean no. of nodes removed	2.5 (1–8)	16 (8–28)
Mean quetlet	26.83 (17.54–38.52)	26.02 (20.8–39.3)
Mean separation (cm)	19.27 (9.5–28)	20.03 (14.2–26.5)
Gantry angle right (degrees)	307.25 (286–349.3)	304.4 (274.5–314.0)
Gantry angle left (degrees)	56.78 (42.5–66)	52.13 (36.5–63.0)

Numbers in parentheses are the range.

With respect to the deep–superficial location of the sentinel lymph node clips, on average, the clips were located 1.8 cm superficial to the chest wall–lung interface. The range of measurements was 0.5 cm deep to the interface to 8.0 cm superficial to the interface. In 98.5% of the cases, all sentinel lymph nodes were included within the deep border of the tangential RT field used to treat the patient. Only 1 of these patients had the sentinel lymph node clip directly within the deep field border. The average distance from the deep field edge to the more superficial sentinel lymph nodes was 2.5 cm.

The calculated mean dose to the sentinel lymph node region as a percentage of the prescribed dose to the isocenter of the field was 98% (standard deviation 6%; range 77%–107%). Only 1 case (4.5%) had a dose <90%, and only 9% had dosages <95% of the prescribed isocenter dose.

Axillary dissection cohort

Table 3 provides data concerning the anatomic relationship of the axillary level I–II lymph nodes in relation to the medial tangential RT beams used to treat the breast. In 43% of the cases, the entire extent of the axillary dissection would have been treated with nondivergent, high tangential fields that were <2 cm from the humeral head (4 of these had their most proximal clip at exactly 2 cm). In the remaining 57% of patients, an average of 80% of the cranial–caudal extent of the clips would have been included in a field, with the cranial border set at 2 cm below the humeral head. The remaining 20% of the region would have been above the 2-cm mark. In evaluating the relationship of the axillary dissection extent to the actual breast tangential fields used (which were not designed to include the axillary lymph nodes), we found that the clips extended an average of 1.44 cm above and 3.7 below the cranial field edge.

In 39% of the cases, the entire extent of the axillary dissection was anterior to the chest wall–lung interface. In the remaining 61% of patients, the deepest axillary dissection clip averaged 1.6 cm below the chest wall–lung interface, with the range extending to 4.5 cm deep to the chest wall–lung interface. If 2 cm of lung were included in the cranial aspect of the RT fields, 82% of the cases would have had the deepest component of the axillary dissection within the RT field. We did not find a correlation between any of the patient (i.e., quetlet) or treatment characteristics (i.e., separation or gantry position) and the probability of extension of clips deep to the chest wall–lung interface.

Table 4 shows the percentage of included sentinel and axillary lymph node regions with respect to the anatomic landmark distances measured in 1-cm increments.

Table 2. Distance of surgical clips in relation to anatomic landmarks in sentinel lymph node cohort

Site	Range (cm)	Mean (cm)	Within field (%)
Humeral head	1.0–10.0	4.75	NA
Arm*	−2.5–10.0	4.5	NA
Cranial edge	−3.0–7.5	4.5	85
Deep edge	0.0–8.0	2.5	100
Chest wall	−1.5–6.5	1.8	NA

* Edge of visible skin surface of the proximal medial arm on the simulation film.

DISCUSSION

When surgical and RT oncologists discuss RT management of the axilla, they most often consider whether to include a third treatment field matched to the cranial or superior border of 2 tangential fields primarily designed to irradiate the breast. This consideration is indeed relevant for individuals who have undergone level I–II axillary dissection. For these patients, the predominant risk of nodal recurrence is within the infraclavicular–supraclavicular area rather than within the dissected portion of the axilla. For example, in a recent report from our institution that provided data on failure patterns in 172 women with locoregional recurrence after treatment with a standard level I–II dissection, the dissected axilla was a component of failure in only 14%. In contrast, 47% had an infraclavicular or supraclavicular failure as a component of recurrence (3).

Newer trends in the management of breast cancer are changing the risk of level I or II recurrence. Specifically, alternative therapeutic strategies to standard level I–II lymph node dissection are being investigated in an effort to minimize treatment-related morbidity. These include axillary lymph node sampling, primary RT management of the axilla, and sentinel lymph node dissection. When less than a standard level I–II dissection is performed, axillary recur-

Table 3. Distance of surgical clips in relation to anatomic landmarks in axillary dissection

Site	Range (cm)	Mean (cm)	Within field (%)
Humeral head	0.0–9.5	4.1	NA
Arm*	−2.5–9.5	3.75	NA
Cranial field edge	−5.5–8.0	2.3	23
Deep field edge	−2.5–7.5	2.3	82
Chest wall	−4.5–6.0	1.75	NA

Abbreviation: NA = not applicable.
* Edge of visible skin surface of the proximal medial arm on the simulation film.

rences are much more common. For example, in a recent randomized trial in which mastectomy was accompanied by lymph node sampling, axillary failures occurred in almost 50% of the patients experiencing a locoregional recurrence (4, 5). For these reasons, proper RT techniques for treating level I–II lymph nodes are needed to minimize the risk of an axillary recurrence.

For patients treated with less than a formal level I–II dissection, the axillary lymph nodes at greatest risk of recurrence are likely to be located below (caudal to) the third field that is sometimes added to the high axilla and supraclavicular fossa. Therefore, it is critically important to design the breast tangential RT fields to cover the region of the low axilla. An abstract by Kiel *et al.* (6) also described the relationship of the surgical axillary clips to the bony landmarks, including the humeral head and the most medial chest wall. They found that the tangential fields did not always include the entirety of the axillary surgical bed. They recommended that the cranial field edge be 1.2 cm below the humeral head and that 2.5 cm of the lung be in the breast tangential field for adequate coverage of the axillary contents. Our study provides one of the first data sets describing the anatomic relationship of both axillary lymph node dissection and sentinel lymph node dissection to breast tangential fields. As each tangential RT field is individually designed according to patient anatomy and the particulars of the case, it is expected that the relationship of the lymph nodes to RT fields will vary from patient to patient. Indeed, we found a range in axillary anatomy in the patients evaluated in this study. However, using objective relationships between the lymph node anatomy and the RT fields, we defined relationships that can be helpful in the design of the treatment fields.

For patients in whom RT was used as the primary management of the axilla, our data demonstrated that the lymph nodes at greatest risk (i.e., the sentinel lymph nodes) were ≥2 cm below (caudal to) the humeral head on a medial breast tangential field in 95% of cases. In addition, high tangential RT fields with the cranial border set at 2 cm below the humeral head would have covered the entire axillary dissection content in 43% of the cases and covered 80% of levels I and II in the remaining cases.

In addition to the cranial border of the tangential fields, effective coverage of the axilla also necessitates adequate deep coverage. We found that the sentinel lymph nodes were anterior to the chest wall–lung interface in 71% of cases and anterior to the deep field edge set at the routine breast simulation in 98.5% of cases. However, the dissected axilla extended ≤4.5 cm deep to the chest wall–lung interface. Typical fields designed to treat the breast only do not include this degree of lung in the cranial-most portion of the field. On the basis of our data, for patients with an undissected axilla, we advocate opening the deep border to include 2.0 cm of lung in the cranial third of the tangential field. To minimize the total lung treated, a lung–heart block can be added to the lower two thirds of the tangential field, which is below level I of the axilla.

It may also be important to design RT fields to include the axilla for patients who have undergone sentinel lymph node surgery. The risk of axillary recurrence after falsely negative sentinel lymph node surgery could be minimized with carefully designed RT fields. Currently, data from large institutions with experienced surgeons in sentinel lymph node surgery report very low rates of false-negative results (7–9). However, false-negative results are likely to be much more common as this procedure is adopted throughout the community. For example, for a series of community sur-

Table 4. Distance of surgical clips to anatomic landmarks in sentinel lymph node dissection and axillary lymph node dissection cohorts

	Sentinel lymph node cohort (%)	Axillary lymph node cohort (%)
Distance below humeral head*† (cm)		
0	100 (62/62)	100 (37/37)
1	98 (61/62)	76 (28/37)
2	87 (54/62)‡	43 (16/37)
Distance deep to chest wall (cm)		
Anterior or at the chest wall	80 (52/65)	39 (15/39)
−1	94 (61/65)	67 (26/39)
−2	100 (65/65)§	82 (32/39)

* In the sentinel lymph node cohort, 62 of 65 patients had an assessable humeral head.
† In the axillary lymph node cohort, 37 of 39 patients had an assessable humeral head.
‡ Five patients had sentinel lymph node clips exactly at 2 cm; including these patients would give a value of 95%.
§ All clips were ≤1.5 cm.

geons in Vermont, Krag *et al.* (9) reported false-negative rates ≤29%. From the data presented here, it is likely that most positive lymph nodes remaining after falsely negative sentinel lymph node surgery can be effectively treated with properly designed tangential RT fields used for the treatment of the breast.

Our data have relevance to 2 ongoing national trials. The National Surgical Adjuvant Breast and Bowel Project B-32 trial compares sentinel node resection with conventional axillary dissection for patients with pathologically negative sentinel lymph nodes. This study includes both patients treated with BCT and patients treated with mastectomy. Correspondingly, it will be interesting to see whether patients treated with mastectomy and sentinel lymph node surgery (who do not receive RT) will have higher axillary recurrence rates compared with patients treated with RT as a component of BCT. In addition to the B-32 trial, an American College of Surgeons Oncology Group Trial (ZOO10 and ZOO11) randomizes patients with positive sentinel lymph nodes to standard axillary dissection or no additional surgery. All patients included in this trial are treated with BCT, and the RT is specified to include the "breast only." Previous studies have found that 55%–65% of patients with positive sentinel lymph nodes who undergo axillary dissection have additional axillary disease (10, 11). The anatomic location of these additional lymph nodes is not well described. However, our data indicate that in the vast majority of patients, RT designed to treat the breast will coincidentally treat a significant portion of the lymph node region at risk. It is likely that this will affect the outcome of the American College of Surgeons Trial. Selected patients with positive lymph nodes after sentinel lymph node surgery will also be at risk of recurrence in the level III and supraclavicular regions, which anatomically are above the tangential fields. It is an accepted practice in radiation oncology to include a matched third field above the tangential fields for all patients with ≥4 positive lymph nodes. In the series of Krag *et al.* (9), the rates of ≥4 positive lymph nodes after axillary dissection were 16% for patients with a single involved sentinel lymph node, 30% for patients with 2 involved sentinel lymph nodes, and 60% for patients with 3 involved sentinel lymph nodes. Therefore, even if tangentially designed breast fields effectively treated the low axilla, a fair percentage of patients with ≥2 involved sentinel lymph nodes would be at risk of disease in areas beyond the tangential field.

A number of studies have indicated that primary RT is an effective modality for the treatment of clinically negative axilla, because the rates of axillary recurrence in these series were all ≤3% (12–14). On the basis of these data, it was assumed that the dose to the axillary region during breast irradiation was adequate to control microscopic disease, although no previous data have specifically addressed this important issue. The final significance of our study was to confirm that the dose delivered to the sentinel lymph node regions included in the tangential RT fields was ≥90% of the prescribed dose in 90% of the patients. We recommend that patients who receive RT for primary management of the axilla, should have dosages calculated in this anatomic area with three-dimensional treatment planning to ensure adequate dose delivery.

One limitation of this study was our decision to include only data from one surgeon for our axillary dissection cohort. This decision was made because he was the only available surgeon who used surgical clips to demarcate the entire extent of the axillary level I–II dissection. It is well known that the anatomic volume resected during axillary dissections may vary according to the surgeon. Therefore, a data set that includes many surgeons may be needed to verify the results of this study. In addition, because the surgical clips were predominately used for homeostasis, it is uncertain whether they correctly defined the anatomic region of the lymph nodes.

CONCLUSIONS

We advocate careful design of the tangential RT fields to include the low axilla for all patients believed to be at risk of having microscopic disease within the level I–II axilla. On the basis of our data, we recommend extending a nondivergent cranial field edge to within 2 cm of the humeral head. The deep field border in this cranial portion of the field should include 2 cm of underlying lung, with appropriate collimation or custom blocking to minimize the volume of lung and heart within the caudal aspect of the tangential field.

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Int. J. Radiation Oncology Biol. Phys., Vol. 49, No. 2, pp. 533–537, 2001

0360-3016/01/$–see front matter

ELSEVIER

PII S0360-3016(00)01502-9

RADIATION-INDUCED CHROMATID BREAKS AS A PREDICTOR OF BREAST CANCER RISK

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Purpose: In *in vivo* models, radiation-induced genomic instability correlates with the risk of breast cancer development. In addition, homozygous mutations in tumor suppressor genes associated with breast cancer development adversely affects the processing and repair of radiation-induced DNA damage. We performed a case-control study to determine whether an assay measuring radiation-induced chromatid breaks correlated with the risk of having bilateral breast cancer.

Methods and Materials: Patients were prospectively studied on an institutional review board-approved protocol. We included only women with bilateral breast cancer as cases to obtain patients with a presumed genetic susceptibility for breast cancer. Controls were healthy women without a previous cancer history. A mutagen sensitivity assay using γ-radiation was performed on lymphocytes obtained from 26 cases and 18 controls. One milliliter of whole blood was cultured with 9 mL of blood medium for 91 h and then treated with 125 cGy using a Cs-137 irradiator. Following an additional 4 h in culture, cells were treated with Colcemid for 1 h to arrest cells in metaphase. The number of chromatid breaks per cell was counted using a minimum of 50 metaphase spreads for each sample.

Results: Cases had a statistically higher number of γ-radiation-induced chromatid breaks per cell than controls, with mean values of 0.61 ± 0.24 vs. 0.45 ± 0.14, respectively ($p = 0.034$, Wilcoxon rank sum test). Using the 75th percentile value in the control group as a definition of radiation sensitivity, the radiation-sensitive individuals had a 2.83-fold increased odds ratio for breast cancer development compared with individuals who were not radiation sensitive (95% confidence intervals of 0.83 and 9.67).

Conclusions: These preliminary data suggest that sensitivity to radiation-induced chromatid breaks in lymphocytes correlates with the risk of bilateral breast cancer. Although the differences between cases and controls were statistically significant, the small sample size necessitates that this finding be validated in a larger study. More data are also needed to determine whether this sensitivity is limited to breast cancer patients with a genetic susceptibility for the disease or also applies to the general breast cancer population. © 2001 Elsevier Science Inc.

Breast cancer, Radiosensitivity, Chromatid breaks, Radiation.

INTRODUCTION

A biologic predictive assay of breast cancer development risk would have significant relevance to a large cohort of women. Breast cancer is the most common nondermatologic cancer in women, with an estimated 182,800 new cases diagnosed annually in the United States (1). Furthermore, breast cancer remains the second leading cause of cancer deaths in women, with 40,800 predicted to die of the disease in the year 2000 (1). A biologic assay quantifying an individual's risk of breast cancer development could help identify candidates for judicious clinical and radiographic screening, for trials evaluating chemoprevention strategies, and for consideration of prophylactic surgical interventions.

There are two types of biologic predictors of breast cancer risk: genotype sequencing and phenotype screening. The discovery and cloning of BRCA1 and BRCA2 have permitted the development of a commercially available sequencing test to identify germline mutations in these two genes. In addition, the relationships of mutations in other candidate genes, such as ATM (ataxia telangiectasia, mutated), to breast cancer development are being investigated by a number of groups. While genotype sequencing has had a dramatic impact on understanding breast cancer risk in selected cases, only a small percentage of breast cancer patients develop the disease in the setting of a known predisposing germline mutation (2, 3).

In this preliminary report, we investigate a phenotype-screening assay for breast cancer. Phenotype screening has a number of advantages and disadvantages compared with

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Presented at ICTR 2000, Lugano, Switzerland, March 5–8, 2000.

This work was supported by T32CA77050 awarded by the National Cancer Institute, U.S. Department of Health and Human Services and T.A.B. is supported by a USAMRMC Career Development Award from the Breast Cancer Research Program (BC980154).

Accepted for publication 31 August 2000.

genotype sequencing. By evaluating a common downstream consequence of a variety of tumor suppressor gene mutations, a phenotype assay can potentially capture a much broader percentage of the breast cancer population. Furthermore, this strategy is not dependent on new gene discovery and potentially can identify individuals who harbor relevant germline mutations in yet undiscovered genes. A phenotype-screening assay also affords the possibility of quantifying the importance of an individual's genotype. For example, a phenotype-screening assay may be able to quantitatively distinguish between different mutations in a tumor suppressor gene that entail different risks of breast cancer development.

The assay we investigated in this study was cellular radiosensitivity, as defined by the number of chromatid breaks per cell following *in vitro* treatment of lymphocytes with γ-radiation.

METHODS AND MATERIALS

Approval for this prospective study was obtained through The University of Texas M. D. Anderson Institutional Review Board. Informed consent was obtained from all cases and controls in this study.

Cases were 26 females with a history of bilateral breast cancer. All patients had at least one breast cancer treated in our institution. No samples were obtained from the cases during chemotherapy or radiation treatment because these treatments could potentially affect the number of chromatid breaks. Controls were 18 females with no personal cancer history who were recruited for a simultaneous study investigating lung cancer.

All participants donated 10–20 mL of blood for the mutagen sensitivity assay. The details of the mutagen sensitivity assay have been previously described (4), although in this study γ-radiation rather than bleomycin was used as the mutagenic agent. All cultures were set up within 24 h of the blood draw. One milliliter of blood was added to 9 mL of RPMI-1640 medium supplemented with 20% fetal calf serum and phytohemagglutinin. The cultures were then incubated at 37°C for 91 h, after which the cultures were treated with 125 cGy of γ-radiation delivered from a Cs-137 irradiator. The cultures were then incubated for 4 h to allow time for DNA repair. Subsequently, the cultures were treated for 1 h with Colcemid (0.04 μg/mL) to arrest cells in metaphase. Cells were then harvested, fixed, washed, and stained with Giemsa as previously reported (4). For each case and control, the number of chromatid breaks per cell were counted. A minimum of 50 metaphase spreads per sample were examined.

The mean values, standard deviations, and standard errors were calculated. A Wilcoxon rank sum test for non-normal distribution was used to compare cases and controls. This test analyzed the data as categorical variables to minimize the impact that a single high mutagen sensitivity score could have on the mean value. Odds ratios for bilateral breast cancer were determined by comparing the incidence of bilateral breast cancer in mutagen-sensitive individuals and mutagen-resistant individuals. Consistent with previous reports, the value for being categorized as mutagen sensitive was 75% value of the control population. This value was determined before the analysis of the data and represents an accepted quartile cutoff point.

RESULTS

Characteristics of cases

The median age of the cases at the time of first breast cancer diagnosis was 49 years with a range of 25–79. Sixty-five percent of the cases had a history of breast cancer in either a primary relative (38%) or a secondary relative (27%), 31% of the cases denied a breast cancer family history, and in 1 case the family history was unknown. The majority of cases (88%) were Caucasian. Only two of the cases were of Ashkenazi Jewish descent, and 1 of these was known to have a germline mutation in BRCA1. From a published monogram for predicting the probability of having a BRCA1 mutation based on personal cancer history, family cancer history, age of diagnosis, and whether the individual is of Ashkenazi descent (5), the approximate average probability of having a BRCA1 mutation for our cases was 15%.

Mutagen sensitivity assay results

The number of chromatid breaks per cell was significantly higher in our cases vs. controls, with respective values of 0.61 ± 0.24 (standard deviation) and 0.45 ± 0.14 ($p = 0.034$). Figure 1 shows the distribution of cases and controls according to the number of chromatid breaks per cell. As shown, the distribution of the cases is skewed to the radiosensitive end of the graph.

The data were also analyzed to determine the odds ratio for breast cancer development for mutagen-sensitive and mutagen-resistant individuals. Consistent with previous studies using the mutagen sensitivity assay, we dichotomized cases and controls as being sensitive or resistant at the 75% level of the controls (0.56 chromatid breaks per cell). This analysis revealed that the mutagen-sensitive individuals had an odds ratio for breast cancer development of 2.83 (95% confidence interval of 0.83–9.67).

A comparison was also performed between the cases with a positive ($n = 17$) or negative ($n = 8$) family history. These results revealed that the cases with a positive family history had a higher number of chromatid breaks per cell than those with a negative family history, although the difference between the two groups was not statistically significant (0.67 ± 0.14 vs. 0.49 ± 0.25, $p = 0.07$). The distribution of these results is shown in Fig. 2.

DISCUSSION

In this article, we present evidence that the phenotype of cellular radiosensitivity, as defined by a chromatid-break assay, correlates with the risk of having bilateral breast

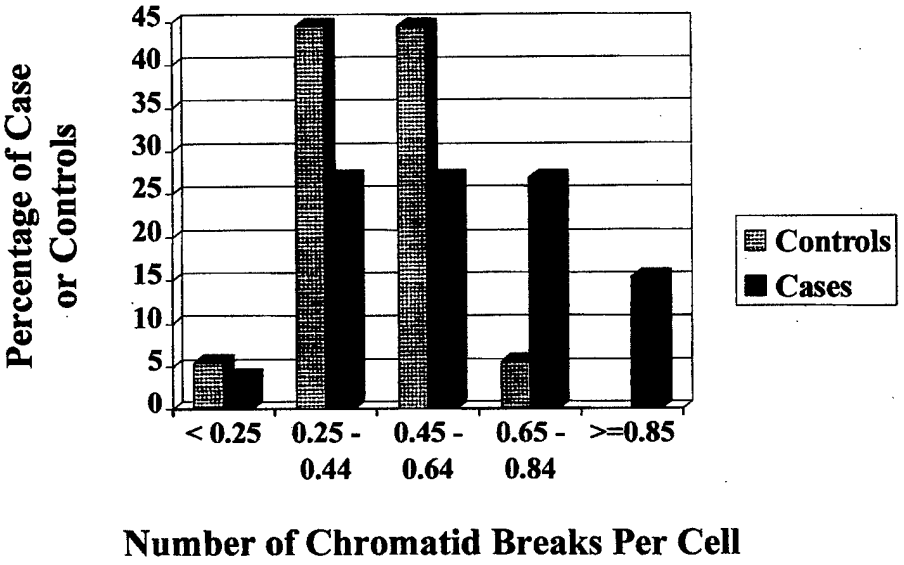

Fig. 1. Distribution of the case and control populations as a function of the mutagen sensitivity assay results.

cancer. Specifically, we found that radiation induced a greater number of chromatid breaks in lymphocytes from patients with a history of bilateral breast cancer compared to female controls without a cancer history.

This study followed an earlier negative report from our institution investigating the value of the mutagen sensitivity assay in predicting breast cancer risk. In 1989, Hsu *et al.* (4) reported no increase in the number of chromatid breaks per cell in 82 breast cancer cases compared with 335 controls (0.64 ± 0.36 vs. 0.60 ± 0.35, respectively). We designed this current protocol with important differences from the earlier Hsu *et al.* study (4). First, by evaluating only patients with a personal history of bilateral breast cancer (2/3 of whom also had a positive family history of breast cancer) we selected cases that had a greater probability of having a predisposing genotype. In the original Hsu study, patients with a history of a single breast cancer were selected without regard to age at diagnosis or family history status. A second important difference between the two studies was our use of γ-radiation as a mutagen compared with the bleomycin that was used in the earlier study.

The rationale for reinvestigating the mutagen sensitivity assay in our breast cancer study population is as follows. While the majority of breast cancers are believed to develop

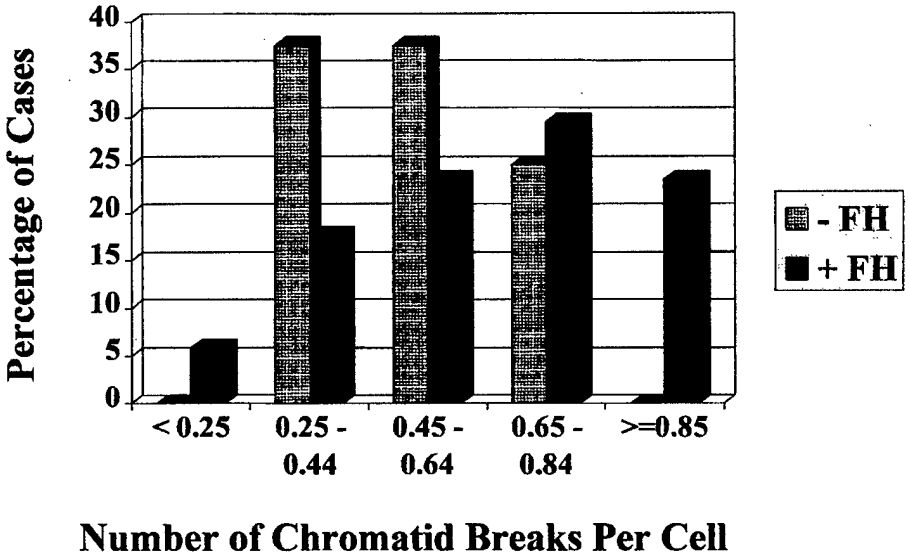

Fig. 2. Distribution of the bilateral breast cancer patients with a positive (+ FH) or negative (− FH) breast cancer family history as a function of the mutagen sensitivity assay results.

independently of an individual's genotype, it is clear that family history of breast cancer, particularly in a premenopausal first-degree relative, is an important risk factor for the development of this disease. This increased risk is likely due to inheritance of a predisposing genotype. The specific genes contributing to this predisposition are unknown in most women with breast cancer and a positive family history. Less than 7% of all breast cancers are thought to occur in the setting of a germline mutation in BRCA1 or BRCA2 (2, 3). A phenotype assay, such as the one described in this report, is not dependent on the discovery of these unknown genetic conditions. An assay that can capture a common downstream functional effect of a variety of predisposing mutations would be relevant to a much broader population of women than a genotype sequencing approach.

A possible shortcoming of using radiation-induced chromatid-breaks as a predictor for breast cancer development is that this phenotype may not be a consistent consequence of all predisposing genetic conditions. For example, there is no evidence that individuals with Li-Fraumeni syndrome (a germline mutation in p53) have increased susceptibility to chromatid-breaks. However, mutations in BRCA1, BRCA2, and ATM all affect cellular radiosensitivity and the success of double-strand break repair following ionizing radiation. Specifically, both BRCA1 and BRCA2 colocalize with Rad51 following radiation-induced double-strand injuries (6, 7). In addition, normal function of BRCA1 is required for transcription-coupled repair following damage from ionizing radiation (8). Finally, BRCA1 has also been shown to associate with hRad50-hMre11-p95 in directing a cellular DNA damage response following ionizing radiation (9). A third tumor suppressor gene that may have relevance to breast cancer formation, ATM, also plays a critical role in the successful repair of DNA strand breaks following radiation (10). This role may in part be explained by the finding that BRCA1 protein function is dependent on phosphorylation by the ATM protein (11). It is clear that homozygous mutations in any of these three genes (BRCA1, BRCA2, or ATM) result in a radiosensitive phenotype (7–12).

The second rationale for using radiation as a mutagen for our experiment is that ionizing radiation is the most clearly recognized environmental carcinogen for breast cancer. The first evidence of the carcinogenic effect of radiation came from longitudinal studies of Japanese atomic bomb survivors (13). The importance of radiation as a breast carcinogen was further confirmed by the findings of increased breast cancer rates in women treated with radiation for nonmalignant conditions such as tuberculosis and enlargement of the thymus (14, 15). The use of radiation as a cancer treatment also has been shown to carry carcinogenic risks. In a large study of girls treated with mantle irradiation for Hodgkin's disease, the 30-year actuarial risk of developing breast cancer approached 35% (16). Furthermore, there appeared to be a dose–response relationship between radiation exposure and breast cancer development.

Our finding that radiation-induced chromatid breaks correlated with the risk of having bilateral breast cancer is in part supported by animal studies. Ponnaiya *et al.* (17) noted that the significantly higher rates in radiation-induced mammary carcinoma in BALB/c mice compared to C57BL/6 mice correlated with differences in radiation-induced genomic instability in mammary epithelial tissue. After 16 population doublings, irradiated mammary cells from BALB/c mice had significantly more chromatid breaks than C57BL/6 mice. These data suggested that a genotype that increases breast cancer susceptibility correlated with a phenotype of sensitivity to radiation-induced chromatid breaks.

Following the initial investigation of chromatid breaks in breast cancer patients by Hsu *et al.* (4), a number of other investigators have also evaluated whether a chromatid break assay could predict the risk of breast cancer development. Three series with relatively small numbers of breast cancer cases all showed an increase in the median number of induced lymphocyte chromatid breaks in cases vs. controls (18–20). In the largest series to date, Scott *et al.* (21) found a statistically significant increase in chromatid breaks per cell in 135 women with a single breast cancer compared to 105 controls with no breast cancer history. Together these data, along with our current study, suggest the phenotype of sensitivity to radiation-induced chromatid breaks correlates with the risk of breast cancer development. However, as shown in Fig. 1, there is a considerable degree of overlap in the assay results between cases and controls. This suggests that the assay is unlikely to develop into a test with high-sensitivity and high-specificity. Nonetheless, the test may be of clinical value for an individual found to have a high number of chromatid breaks. In our study, a value of 0.65 or greater captured 40% of the cases compared with only 5% of the controls.

Two studies that have investigated radiation-induced chromatid breaks in first-degree relatives have provided further evidence that the radiosensitivity noted in breast cancer cases is genetically based. Patel *et al.* (20) reported that first-degree relatives of breast cancer patients had more radiation-induced chromosome breaks compared with controls. Additionally, Roberts *et al.* (22) recently reported that 62% of first-degree relatives of 16 radiosensitive breast cancer patients from the Scott *et al.* study (21) were also radiosensitive. This compared with a rate of only 7% in first-degree relatives of four breast cancer patients with a low number of chromatid breaks per cell (22). Furthermore, Roberts *et al.* (22) modeled the inheritance pattern of radiosensitivity and breast cancer and suggested that the data fit with a marker of an inherited low-penetrance breast cancer predisposition gene(s).

A potential shortcoming of the lymphocyte assay that we used in this study is that lymphocyte response to radiation is likely dependent on a number of factors. For example, it is possible that cytokines, released either from cancer cells or in response to having cancer, can affect lymphocyte response. To more precisely distinguish the genetic and epigenetic influences on lymphocyte chromatid breaks, more data comparing the rates of

the mutagen sensitivity assay in individuals with known predisposing genotypes, individuals with single breast cancers and no family history, and individuals without a cancer history will be needed.

In conclusion, increasing data suggest that screening for the phenotype of radiation-induced chromatid breaks may prove useful as a biologic predictor for breast cancer risk. We believe that the preliminary data in this report needs additional confirmatory data, as the aggregate data of our study and those reported in the literature is relatively small and is subject to publication bias (negative studies of this type are unlikely to be reported).

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APPENDIX 27

Int. J. Radiation Oncology Biol. Phys., Vol. 48, No. 4, pp. 951–957, 2000

0360-3016/00/$–see front matter

ELSEVIER

PII S0360-3016(00)00761-6

CLINICAL INVESTIGATION **Breast**

LOCAL-REGIONAL CONTROL IN BREAST CANCER PATIENTS WITH A POSSIBLE GENETIC PREDISPOSITION

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Purpose: Local control rates for breast cancer in genetically predisposed women are poorly defined. Because such a small percentage of breast cancer patients have proven germline mutations, surrogates, such as a family history for breast cancer, have been used to examine this issue. The purpose of this study was to evaluate local-regional control following breast conservation therapy (BCT) in patients with bilateral breast cancer and a breast cancer family history.

Methods and Materials: We retrospectively reviewed records of all 58 patients with bilateral breast cancer and a breast cancer family history treated in our institution between 1959 and 1998. The primary surgical treatment was a breast-conserving procedure in 55 of the 116 breast cancer cases and a mastectomy in 61. The median follow-up was 68 months for the BCT patients and 57 months for the mastectomy-treated patients.

Results: Eight local-regional recurrences occurred in the 55 cases treated with BCT, resulting in 5- and 10-year actuarial local-regional control rates of 86% and 76%, respectively. In the nine cases that did not receive radiation as a component of their BCT, four developed local-regional recurrences (5- and 10-year local-regional control rates of BCT without radiation: 49% and 49%). The 5- and 10-year actuarial local-regional control rates for the 46 cases treated with BCT and radiation were 94% and 83%, respectively. In these cases, there were two late local recurrences, developing at 8 years and 9 years, respectively. A log rank comparison of radiation versus no radiation actuarial data was significant at $p = 0.009$. In the cases treated with BCT, a multivariate analysis of radiation use, patient age, degree of family history, margin status, and stage revealed that only the use of radiation was associated with improved local control (Cox regression analysis $p = 0.021$). The 10-year actuarial rates of local-regional control following mastectomy with and without radiation were 91% and 89%, respectively.

Conclusions: Patients with a possible genetic predisposition to breast cancer had low 5-year rates of local recurrence when treated with breast conserving surgery and radiation, but the local failure rate exceeded 50% when radiation was omitted. Our data are consistent with the hypothesis that patients with an underlying genetic predisposition develop cancers with radiosensitive phenotypes. © **2000 Elsevier Science Inc.**

Breast cancer, Breast conservation therapy, Genetic predisposition, Radiation.

INTRODUCTION

The discovery of tumor suppressor genes including BRCA1 and BRCA2 has resulted in an increased interest in whether germline mutations affect tumor biology. The majority of individuals with a predisposing germline mutation in a tumor suppressor gene inherit one normal gene allele and one abnormal allele (a heterozygous mutation). Breast cancers that arise in these individuals have a loss of heterozygosity in the normal locus, with a resulting loss in the normal function of the tumor suppressor gene (1, 2). It is conceivable that this loss of function may influence the treatment outcome of cancers arising in genetically predisposed individuals. Unfortunately, there currently are insufficient clinical data to determine the optimal treatment strategy for breast cancers occurring in patients with a genetic predisposition. This deficiency in data has led to a controversy surrounding whether these patients can be safely treated with breast conserving therapy (BCT) or rather require mastectomy.

It is presumed that a variety of germline mutations in

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Presented at the 1999 ASTRO meeting, San Antonio, Texas, October 1999.

Acknowledgments—This work was supported by Grant T32CA77050, awarded by the National Cancer Institute, U.S. Department of Health and Human Services. T.A.B. is supported by a Career Development Award (BC980154), Department of Defense Breast Cancer Research Program.

Accepted for publication 21 June 2000.

Table 1. Patient and tumor characteristics

	BCT + XRT	BCT no XRT	Mastectomy + XRT	Mastectomy no XRT
Number of breasts	46	9	37	24
Average age at diagnosis	49	56	44.7	48.7
Histology:				
IDC	37 (80%)	7 (78%)	32 (86%)	17 (71%)
ILC	2 (4%)	1 (11%)	4 (11%)	3 (13%)
Other	7 (15%)	1 (11%)	1 (3%)	4 (17%)
Family history:				
At least one first degree relative	30 (65%)	7 (78%)	27 (73%)	17 (71%)
One or more second degree relatives	13 (35%)	2 (22%)	10 (27%)	7 (29%)
Stage at diagnosis:				
0	1 (2%)	1 (11%)	0	2 (8%)
I	27 (54%)	4 (44%)	4 (11%)	11 (46%)
II	18 (39%)	2 (22%)	18 (49%)	5 (21%)
III	2 (4%)	2 (22%)	13 (35%)	3 (13%)
Unknown	0	0	2 (5%)	3 (13%)

tumor suppressor genes contribute to the fivefold increased risk of breast cancer development noted in individuals with a family history of cancer. Germline mutations in either BRCA1 or BRCA2 contribute to 7–10% of all newly diagnosed breast cancer cases. The 15–20% of breast cancer patients with a positive family history for breast cancer who do not have a germline mutation in a BRCA gene may have other yet undiscovered predisposing mutations. How these mutations affect the natural history and treatment response of a tumor is unknown.

In this paper, we studied the outcome of women with a possible genetic predisposition using clinical parameters as a surrogate for genetically predisposed disease. This strategy captures a broader population than studying only individuals with a BRCA1 or BRCA2 mutation. While other authors have used positive family history as a surrogate for genetically predisposed disease, we elected to study the outcome of women with a history of bilateral breast cancer as well as a positive family history. The rationale for selecting this surrogate group was that these patients had a higher likelihood of having disease influenced by a germline mutation in a tumor suppressor gene. The clinically relevant finding we hoped to demonstrate was that BCT is appropriate for breast cancer patients who may be genetically predisposed to the development of the disease.

METHODS AND MATERIALS

Between 1959 and 1998, 58 women with bilateral breast cancer and a family history of breast cancer were treated at The University of Texas M.D. Anderson Cancer Center. The medical records of these patients were reviewed for clinical and pathologic characteristics of each breast cancer. Parameters studied included family history, age and stage at diagnosis, pathologic information, type of treatment, and outcome.

For the 58 women, the mean age at diagnosis of first and second breast cancer was 48 (range 24–94) and 53 (range 29–94), respectively. Sixty-seven percent of the patients were Caucasian, 19% were African American, and the remaining 14% were members of other ethnic populations. The majority (72%) of patients presented with cancer in only one breast, with only 16 women (28%) being diagnosed with synchronous disease. More than half of the tumors (56%) were self-detected, 26% were mammographically detected, and 18% were detected following a clinical examination.

Each case was staged individually according to 1997 American Joint Committee on Cancer (AJCC) staging guidelines (3). The stage at presentation was defined as the pathological stage except in the 17 cases treated with neoadjuvant chemotherapy. Due to possible downstaging, the clinical stage at diagnosis was used for analysis of these tumors. As for location within the breast, 45% of the cancers were in the upper outer quadrant, 14% in the upper inner quadrant, 9% in the lower outer quadrant, 8% in the lower inner quadrant, and 5% in the central breast. Fifteen breasts (13%) had disease involving multiple quadrants. The exact location of 8 tumors (7%) could not be verified. Patient and tumor characteristics as well as stage at presentation are seen in Table 1.

Family history was considered positive if at least one first- or second-degree relative had a diagnosis of breast cancer. The number of relatives with a family history of breast cancer as well as the relationship to the patient is shown in Table 2. Sixty-nine percent of the patients had at least one first-degree relative with a history of breast cancer. The remaining 31% had one or more second-degree relatives with a history of breast cancer. Twenty-two percent of

Table 2. Family history

One first degree relative	18
Multiple first degree relatives	6
One second degree relative	9
Multiple second degree relatives	9
One first degree and one second degree relative	7
Multiple first and second degree relatives	6
One first degree and multiple second degree relatives	3

Table 3. Tumor characteristics

Characteristic	Number BCT	Number mastectomy
Degree of differentiation		
Well	3	4
Moderate	20	15
Poor	15	16
Unknown	17	26
Hormone status		
ER−	17	17
ER+	18	19
ER Unknown	20	25
PR−	15	11
PR+	17	13
PR Unknown	23	37
Margins		
Positive	4*	3
<2 mm	4	5
Negative	40	36
Unknown	7	17
DCIS component		
Yes	13	10
Lymph vascular invasion		
Yes	8	14
No	26	16
Unknown	21	31

* Two of four positive for in situ disease at margin.

the women had a total of 3 or more relatives with breast cancer. Five women had a female relative with a history of bilateral breast cancer, and one patient had a male relative with breast cancer.

In addition to breast cancer family history, 27 patients had at least one other family member with a history of cancer other than breast. Two of these patients had family histories of ovarian cancer.

Eight of the women in this study were diagnosed with another type of cancer prior to or following their breast cancer diagnoses. Specifically, three had ovarian cancer, two had thyroid cancer, one had lung cancer, one had stomach cancer, and one patient developed a malignant fibrous histiocytoma of the chest wall after treatment in an irradiated field.

Pathological analysis

Details of pathology were obtained from review of pathology reports. Breast cancer histology was infiltrating ductal carcinoma in 78%, invasive lobular carcinoma in 9%, and other or not specified in 13%. Table 3 shows other pathological features of the 116 breast cancers studied in this report. Information regarding nuclear grade was available in 73 cases.

Treatment

BCT was performed in at least one breast in 36 of the 58 patients. In terms of the 116 breast tumors treated, the primary surgical management was a breast conserving procedure in 55 cases and a mastectomy in 61 cases. For patients undergoing BCT, a segmental mastectomy was performed. Axillary lymph node dissection was performed in 38/55 (70%) of the conservatively treated breasts. Reasons for lack of dissection were noninvasive disease, advanced age, and patient preference. The majority of the patients without axillary lymph node dissections had Stage I disease.

Radiation therapy was routinely recommended following breast conserving operative procedures. Average dose to the intact breast with initial fields was 4888 cGy (4500 cGy–5040 cGy). During the early years of the study, 30 breasts were irradiated with cobalt-60 gamma rays, but in the mid-1980s, photons (median energy 6 MV) were used. Thirty-two breasts received an additional 5–20 Gy (average dose 1193 cGy) to the tumor bed as a boost. Four patients underwent iridium implant as boost therapy. Treatment fields were designed to cover areas at risk for each case. All breasts were treated with medial and lateral tangent fields. Additionally, 28 breasts received at least one other field, with 10 patients receiving treatment using five fields. Five-field therapy included the breast tangents, an internal mammary chain treatment, supraclavicular and axillary apex coverage, and a posterior axillary boost field.

For the 61 cases treated with mastectomy, the surgical procedure was a simple mastectomy in 5 cases, a radical mastectomy in 6 cases, and a modified radical mastectomy in 50 cases. Sixty-one percent (37/61) of these cases also received radiation as a component of care.

Various chemotherapy regimens were used in 36 patients, 12 of whom were treated with BCT. Three of the 12 women received their chemotherapy as neoadjuvant therapy. Twenty-eight of the 36 patients given chemotherapy received it as treatment of their first cancer; and an additional 8 women were treated with chemotherapy at the diagnosis of the second cancer. Twenty-three of the patients receiving chemotherapy had lymph node-positive disease. Numerous regimens were utilized over the time course of this study, but doxorubicin was a component of the regimen in 33 of the 36 women, including all but one woman treated with chemotherapy and BCT. Hormonal medications were a component of care in 19 women, 13 of whom had received prior chemotherapy. Only six women treated with BCT underwent hormonal therapy as a component of care.

Statistical analysis

Actuarial statistics using methods of Kaplan-Meier were used to estimate local control (4). Patients were censored at the time of last follow-up or at the time of death. Time zero was the date of pathologic diagnosis for each breast cancer. Local-regional recurrences were defined as an ipsilateral breast, chest wall, or lymph node recurrence occurring as the first evidence of recurrent disease without simultaneous distant metastases. Local-regional recurrences following the development of metastatic disease were often not reliably recorded in medical records and therefore were not felt to be appropriate for this retrospective analysis. Actuarial data were compared with two-sided log rank tests. Multivariate

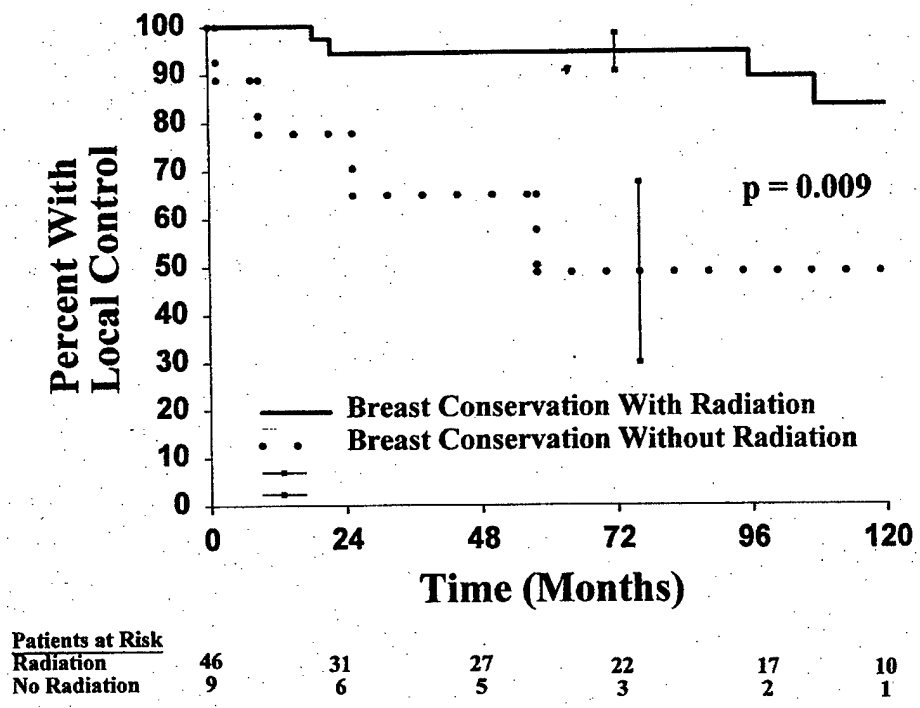

Fig. 1. Figure 1 displays actuarial local control for the BCT patients divided according to the use of radiation. Log rank comparison of the data noted a statistically significant difference for the subgroups (p = 0.009).

analysis was performed using the Cox proportional hazard model (5).

RESULTS

Treatment results

The median follow-up for the index breast cancers treated with BCT was 68 months (3–245). The median follow-up of patients from the date of the pathological diagnosis of their first breast cancer was 98 months (range 9–461 months). There were 8 local-regional recurrences in the 55 breasts treated with BCT, including those patients who did not receive radiation as a component of care. The 5- and 10-year actuarial local-regional control rates for the BCT cases were 86% and 76%. Nine of the 55 BCT cases did not have radiation therapy as part of treatment, and 4 of these 9 cases developed local recurrences (crude recurrence rate of 44.4%). The 5- and 10-year actuarial local regional control rates for the BCT cases treated without radiation were 49% and 49% (±18.73% standard error (S.E.)). Only 4 local recurrences occurred in the 46 BCT cases treated with breast conservation surgery followed by radiation therapy (crude recurrence rate of 8.7%). The 5- and 10-year actuarial local regional control rates for these cases were 94% (±4.0% S.E.) and 83% (±8.2% S.E.), respectively. Figure 1 displays the actuarial local-regional control curves for the BCT cases divided according to the use of radiation. The difference in loco-regional control for these two groups was statistically significant at $p = 0.009$. This difference remained significant ($p = 0.021$) in a multivariate analysis that incorporated radiation use, age (under or over 40 at first diagnosis), degree of family history (primary vs. secondary), margin status (negative vs. positive or close), and stage.

All four of the recurrences that occurred in the patients who did not receive radiation as a component of care were detected within five years of diagnosis. However, in the patients treated with BCT and radiation, two of the four recurrences occurred at a time greater than eight years from the original diagnosis. Table 4 shows treatment and outcome details for the eight cases of local recurrence that followed BCT. Two recurrences involved both the breast and lymph nodes while the other six involved only the breast. The two late recurrences were both only within the irradiated breast, and one of these recurred in a separate breast quadrant.

Complications possibly related to radiation were noted in 16 cases, eight in cases treated with BCT and eight in cases treated with mastectomy. The most common complication, seen in eight patients, was arm edema. Each of these patients received treatment in the early era with cobalt-60. Other complications included seven cases of Grade III skin toxicity and two cases of radiation pneumonitis. Only one patient treated with photons developed a Grade III toxicity. In this case, treatment was stopped at 46 Gy because of an acute skin reaction. One patient developed a malignant fibrous histiocytoma of the chest wall 20 years following cobalt-60 radiation of an intact breast. This patient also suffered a Grade III skin complication and radiation pneumonitis (noted above).

Table 4. Information on patients with recurrences

Patient	Family history	Time to recurrence	Status	Stage	XRT*	Chemo*	Margins
1	Multiple secondary	18 months	DOD	T2N0	Yes	No	Negative
2	Single primary	8 years	Alive after salvage	T2N0	Yes	No	Negative
3	Single primary	25 months	DOD	T2N0	No†	No	Unknown
4	Multiple primary	22 months	DOD	T1N1	Yes	No	Negative
5	Single secondary	1 month	DOD	T1N0	No‡	No	Negative
6	Single primary	9 months	Dead, other cause	T1N0	No§	No	Negative
7	Multiple primary and multiple secondary	58 months	Alive after salvage	T2N0	No‖	Yes	Negative
8	Multiple secondary	9 years	Alive after salvage	T2N0	Yes	No	Unknown

Abbreviations: Chemo = chemotherapy; DOD = dead of disease; XRT = radiation.
*Radiation and chemotherapy specified at the time of initial treatment.
†Refused radiation.
‡Developed recurrence at the start of radiation, 7.4 Gy delivered.
§Radiation not offered (patients 94 years old).
‖Refused radiation.

In the 61 breasts treated with mastectomy, there were five local recurrences, three occurring in patients who did not receive radiation. The 10-year actuarial rates of local-regional control following mastectomy with and without radiation were 91% and 89%, respectively. All local recurrences involved the chest wall with three of these cases also failing in a draining lymph node region. Of the patients with stage I–II disease, local recurrences developed in 3/16 cases treated with mastectomy alone and 1/22 of the cases treated with mastectomy and radiation. Median follow-up was 57 months for the mastectomy-treated cases.

DISCUSSION

Data from this study support the use of breast conservation surgery and radiation for women with personal and family histories suggestive of an underlying genetic predisposition to breast cancer. In this paper, we reported a 5-year 94% local control rate for women with a personal history of bilateral breast cancer and a family history of breast cancer following treatment with breast-conserving surgery and radiation. This local control rate is identical to our overall institutional experience for BCT, which is predominantly determined by treatment of sporadic breast cancer. For the 1,406 cases treated with BCT in our institution between 1955–1995, the local-regional recurrence rate following BCT in 1,406 cases was also 94% (median follow-up of 6.5 years) (6).

The majority of clinical data concerning how germline mutations may affect tumor biology have come from studies of BRCA1. Our strategy of studying women with bilateral breast cancer and a positive family history captured a much broader population than studies specifically focused on individuals with BRCA1 or BRCA2 mutations. Indeed, the patient population analyzed in this study may have a variety of predisposing genetic mutations and would be predicted to have a low overall rate of having a germline BRCA1 mutation. Shattuck-Eidens et al. published data on the probability of having a germline mutation of BRCA1 given an individual's personal cancer history, age at diagnosis, family history, and whether the individual is of Ashkenazi Jewish descent (7). From these data, we estimated the probability of having a BRCA1 mutation for each patient in our population. The average of these probabilities for the patients in our series was 15%. This estimation does not account for the impact of having multiple relatives with breast cancer or the impact of having family members with bilateral breast cancer history. As 60% of our study population had at least one of these factors, 15% is likely an underestimation. Additionally, these graphs account only for possibility of BRCA1 mutations and do not predict for other genetic mutations.

It is possible that a number of patients in this study developed bilateral breast cancers independent of an underlying genetic condition. Unfortunately, the percentage of women in our study population for whom a genetic factor contributed to their disease development cannot be determined.

A number of other institutions have also investigated local recurrence rates following BCT using clinical surrogates for genetic predisposition. Chabner *et al.* studied local control rates following BCT in young breast cancer patients with a positive family history treated at the Joint Center for Radiation Therapy (JCRT) (8). These authors compared 29 women age 36 or less who had a first-degree relative with a history of breast cancer before age 50 or a family history of ovarian cancer to 172 women age 36 or less who did not meet these family history criteria. This study found no statistically significant differences in the rates of local recurrence, distant failure, or second non-breast cancer in the two groups. The 5-year crude local recurrence rate was 3% in those with positive family history versus 14% who did not have a family history.

Haas *et al.* reported the University of Pennsylvania experience with BCT for young age patients with positive

family histories (9). In 1,021 patients, there was no difference in local control when the patients were divided according to family history of breast cancer. In patients less than or equal to 40 years old, the 5-year local failure rate was 8% for those with a first-degree relative with breast cancer, 2% for those with a non-first-degree relative with breast cancer, and 12% with a negative family history ($p \geq 0.18$). Of note, the previously mentioned Shattuck-Eidens graphs estimate that the rate of a BRCA1 mutation in young women with one breast cancer and a positive family history is only about 8–10%.

Finally, a study published by Harrold *et al.* from Yale University investigated the relationships between young age, family history, and local relapse in 984 women treated with BCT (10). The 10-year actuarial local recurrence rate for the entire group was 15%. In the 52 women with recurrent disease and in a matched control set of 52 cases with local control, there was no difference in the family history pedigrees.

Taken together, these three series and our data suggest that contributing genetic factors for breast cancer development do not increase the risk of developing a local recurrence with BCT. However, because the number of patients with BRCA mutations in these series was likely small, these data do not adequately address the risk of recurrence for individuals with known mutations. Recently, three series have evaluated the relationship of local control rate following BCT for individuals with known BRCA1 or BRCA2 mutations. In 52 patients with ipsilateral breast recurrences following BCT, Turner *et al.* reported a 15% rate of BRCA1 germline mutations (11). However, this study did not provide data specifically addressing whether the local recurrence rate following BCT is increased for women with a BRCA1 or a BRCA2 mutation. In a multi-institutional study comparing 73 women with proven BRCA1 or BRCA2 mutations to 219 matched controls with presumed sporadic breast cancer, Pierce *et al.* reported local failure-free survival rates of 99% in the genetic cohort and 96% in the sporadic cohort (median follow-up of 5.3 and 4.6 years, respectively) (12). Finally, Robson *et al.* reported that Ashkenazi women with a BRCA founder mutation had a rate of ipsilateral breast recurrence of 22% ($n = 28$) versus 6.9% ($n = 277$) who did not have a BRCA mutation ($p = 0.25$) (13). Interestingly, in the patients with a mutation, the rate of contralateral breast cancers was 27%, which raises the question as to whether the ipsilateral breast events were true recurrences or new primary tumors.

Despite developing cancers with aggressive biological phenotypes (more common to have high nuclear grade, ER −, PR − disease, and p53 mutations (14–16)), breast cancer patients with a BRCA1 or BRCA2 germline mutation do not have a worse prognosis (15, 17, 18). This disparity may be attributable to an increased sensitivity to DNA-damaging therapies in BRCA-related cancers. As previously indicated, loss of BRCA1, BRCA2, or ATM function results in a cellular deficiency in double-strand DNA break repair (19–23). Therefore, tumor cells with a homozygous mutation in any of these genes would be predicted to have a marked sensitivity to DNA-damaging therapies, such as ionizing radiation. Our clinical data support the hypothesis that breast cancers developing in genetically predisposed individuals have biologically aggressive phenotypes that are sensitive to ionizing radiation. In the small number of women who were treated with BCT without radiation, the crude local recurrence rate was 44%, and all of these recurrences developed within five years. When radiation was used as a component of breast conservation therapy, the 5-year local control rate was 94%.

The exquisite radiosensitivity associated with homozygous tumor suppressor gene mutations has not been demonstrated with heterozygous BRCA mutations (19, 20). This suggests that women with BRCA germline mutations can be safely treated with radiation therapy. Gaffney *et al.* reported no increase in radiation complications in 21 breast cancer patients with a known BRCA1 or BRCA2 mutation (24). Similarly, Pierce *et al.* found no deleterious effect of radiotherapy in women with a heterozygote mutation in BRCA1 or BRCA2 (12). In our study, only one minor complication occurred in the patients treated with photon radiation. The complication rate for our population was similar to the overall BCT experience at our institution (10% complication rate, most of which were mild and self-limited).

Patients with a genetic predisposition for breast cancer are likely to have an increased risk for developing new breast cancer primaries. In the JCRT study, the young patients with a positive family history had a 5.7-fold increased risk of developing contralateral breast cancer compared with those with a negative family history (6). In our study, 2 of the 4 patients who developed a breast recurrence following BCT with radiation treatment had a recurrence after eight years. It is quite possible that these recurrences represented new primaries. In the Yale case-control study relating local-regional control to BRCA mutation status, the median interval of ipsilateral tumor recurrence for individuals with a germline BRCA1 or BRCA2 mutation was 7.8 years, compared to a median interval of 4.7 years for women without one of these mutations (11). These authors also speculate whether a number of these late local-regional recurrences are new primary tumors within the ipsilateral breast.

In conclusion, our results further support that BCT, including both surgery and radiation therapy, is appropriate treatment for women with a possible genetic predisposition to breast cancer. We hypothesize that these tumors are sensitive to DNA-damaging therapies. These patients need to be carefully followed for the development of new primary breast cancers.

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APPENDIX 28

RADIATION RESEARCH

Volume 2: Proceedings

Edited by

M. Moriarty C. Mothersill C. Seymour

M. Edington J. F. Ward R. J. M. Fry

Eleventh International Congress
of Radiation Research
Dublin, Ireland
July 18–23, 1999

ATM Heterozygosity in the Normal Tissue Response to Radiotherapy

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Introduction

In simple terms, the likelihood of tumour control can be increased by increasing the dose of radiation. And while this notion is very straightforward, normal tissue toxicity of the surrounding tissue does not allow implementation. Radiation doses are routinely prescribed such that there is a low probability of causing a significant injury. Nonetheless, a small percentage of patients will have an exaggerated normal tissue response. It is often impossible to find clear contributing reasons as to why these patients were predisposed to injury. Overall, the clinical picture is consistent with a genetic etiology for their radiosensitivity.

Elucidating a genetic predisposition to radiation sensitivity would have important clinical consequences for radiation oncology. In addition to modifying radiation treatments to minimise the risk for injury in genetically predisposed patients, dose escalation may be possible in patients without a radiosensitive genotype. Modest escalations of dose have the potential to lead to clinically significant changes in tumour control probability. In addition to these dose modification possibilities, new strategies for normal tissue radioprotection might also be developed.

The genetic determinants of radiosensitivity are likely complex, and multiple genetic pathways may be involved in determining one's risk of radiation injury. In addition, inherited genes may have variable penetrance amongst individuals, and genetic predisposition almost certainly is influenced by environmental factors. For human genetic studies, there are two approaches to relate a genotype with a phenotypic trait. The first of these is through the careful studies of families, but for the phenotypic end point of radiosensitivity this method would be impractical. The second method is through association studies. For this type of study, expression of a mutated candidate gene would be associated with radiation sensitivity. We have begun to study just such a gene, the *ATM* gene, as a prototype for such a genotype association study. *ATM* was selected because of the well-described radiosensitivity syndrome, ataxia telangiectasia (AT), which is caused by homozygous *ATM* mutations as well as the important role *ATM* plays in the DNA damage repair pathway (1–3).

The association of *ATM* with radiation sensitivity was first described from clinical studies involving patients with homozygous *ATM* mutations. Homozygous *ATM* mutations cause the rare neurodegenerative disorder of ataxia telangiectasia. Two important characteristics of this disease are a cancer predisposition, particularly lymphomas and leukaemia, as well as extreme sensitivity to radiation injury. This radiosensitivity was first described after ionising radiation treatments of lymphoma in three AT patients. All three of these patients suffered fatal normal tissue complications after doses of 20–30 Gy, approximately half the dose routinely used in the treatment of carcinomas (1). Subsequent to this clinical finding, the radiation sensitivity of fibroblasts from individuals with AT has been studied in vitro. The

average D_0 value for AT fibroblasts is approximately half of that required to kill normal fibroblasts (4).

Patients with ataxia telangiectasia are not a major concern for the radiation oncologist, because individuals with AT are easily identifiable, they display classical phenotypic traits of the disease, and AT is rare. Our research efforts have been directed at studying the relationship of *ATM* heterozygosity and radiation injury. Unlike homozygous mutations, *ATM* heterozygotes are phenotypically normal with no identifiable traits that could be used to predict their genetic status. Heterozygous *ATM* individuals are much more common than homozygous *ATM* individuals. It is estimated that 1% of the U.S. population and 4% of the U.S. cancer population are *ATM* heterozygotes (5, 6). In vitro evidence suggests that these individuals may have a cellular susceptibility to radiation injury because some *ATM* heterozygous cell lines display an intermediate radiosensitivity compared to normal controls and homozygous *ATM* cell lines (4). An association between *ATM* heterozygosity and radiation injury would have significant clinical relevance. Classically, significant radiation toxicity was thought to be stochastic, meaning that a certain percentage of patients develop an injury with any given treatment dose. Clinically useful predictive assays to determine which categories of patients are at an increased risk of developing a radiation-related injury are not currently available. Having such technology would allow for adjustment of technique and dose to minimise the normal tissue complications for patients where radiation treatment is necessary.

Molecular techniques are now available to test the aforementioned epidemiological predictions. Thus far, the preliminary studies using molecular screening for *ATM* mutations in breast cancer patients have produced conflicting results (8–11). The discrepancy in the results of these four reports is difficult to interpret, in that each studied a different subset of breast cancer patients, most had very small patient numbers, and each used different assays to detect *ATM* mutations. Some studies, including some of the studies alluded to above, have used a protein truncation assay for *ATM* heterozygosity studies, because 80% of the mutations found in AT patients lead to protein truncations. But it is unclear whether the mutations that are relevant to cancer or enhanced radiosensitivity are the same as those necessary for the development of AT. For example, a recent study of T-cell leukaemia demonstrated that relevant mutations can exist as missense mutations, which do not truncate the protein product (7). This study directly sequenced tumour DNA and found *ATM* abnormalities in 46% of the studied specimens. Moreover, 15 of the 17 *ATM* mutations were subtle missense or frameshift mutations that had never previously been demonstrated in the AT patients.

The same uncertainty exists for enhanced radiosensitivity. A genetic link to clinical complications after irradiation could not be established in *ATM* heterozygotes in small studies of obligate heterozygotes or breast cancer patients (12, 13). However, in heterozygous *Atm* mice, sublethal radiation exposures resulted in statistically significant life shortening (14). Again, the sample size, methodology and model used limit useful interpretation. These studies highlight the importance of having a large cohort of clinical samples, a robust methodology by which to screen patients, and comparative data from a large general population sample to properly address this question.

Methods

Mutations of the *ATM* gene are not contained within hot-spot regions and can be present as single base-pair changes, small insertions, or small deletions. Therefore, cDNA sequencing of the 13 kb mRNA was chosen as a rapid and cost-effective methodology by which to map mutations and polymorphisms within *ATM*. Total RNA was extracted from peripheral blood lymphocytes donated by patients with RTOG grade 3–4 adverse reactions to radiotherapy, from unselected breast cancer patients, from breast cancer patients with multiple primary tumours, and from patients with so-called radiation-induced cancers. cDNA was synthesised from the total RNA by RT-PCR using eight primer pairs that resulted in eight cDNA sequences from *ATM* that sequentially overlap each other. These cDNA sequences were then sequenced by Terminator Cycle Sequencing. Alterations in coding sequence were identified by comparison to the *ATM* sequence listed in GenBank (HSU33841).

Blood samples from 941 individuals were obtained from the general populace during institutional blood drives in the Houston, TX community. A sample of this size would provide sufficient statistical power to determine whether polymorphisms repeatedly found in patient groups are unique to these populations, or equally common in the general population. The allele-specific oligonucleotide (ASO) assay was used to detect specific polymorphisms found in the patient population. Briefly, radiolabeled oligonucleotides specific to either the wild-type or the polymorphic sequence were used to probe genomic DNA from the control population. High stringency conditions such that even a single base mismatch inhibits binding were used during the hybridisation steps. The result is a simple plus/minus determination of a single base change at that location. Blot signal intensity was quantified by storage phosphor technology.

Results

At present 146 patients have volunteered. From these the *ATM* cDNA has been partially sequenced in 128 individuals and completely sequenced in 63. No protein truncation mutations have been found as yet. However, 21 single base-pair substitutions have been identified. Seventeen of these substitutions result in amino acid alterations. Thirteen patients have 2 or more base substitutions, while 3 patients are homozygous for their polymorphisms. Overall, *ATM* polymorphisms have been identified in 34% of patient samples. Stratifying patients by selection criteria results in polymorphism rates of: 36% (8/22), radiation injury; 62% (8/13), radiation-induced cancers; 27% (10/37), unselected breast cancer; and 29% (11/38), breast/multiple primaries. One specific single nucleotide base substitution, which results in an amino acid change at amino acid 1853, was seen in 25 patient samples. In two cases the patients were homozygous for this substitution. This amino acid lies in the RAD3 domain. Two other substitutions of note were found at amino acid 1054, where arginine is substituted for proline, and at amino acid 49, where cysteine is substituted for serine. Both alterations were seen four times each and could cause significant changes in protein structure. One final substitution of note was found at amino acid 1380, where tyrosine was substituted for histidine. This substitution is within the ABL binding motif; however, it was only seen once.

ASO analysis of the first three polymorphisms above identified these single

nucleotide substitutions at 0.71, 7.47 and 2.34%, respectively. Within the patient samples these polymorphisms were identified 5.0, 32.1 and 4.3%, respectively. Given the sample size, statistical analysis suggests that only the first two polymorphisms appear within the control and patient cohorts at different rates.

Discussion

At present some 21 polymorphisms within the cDNA of the *ATM* gene have been identified from a selected patient population. Seventeen of these polymorphisms result in single amino acid changes at the protein level. No mutations that would result in protein truncation were identified. The frequencies of two of these polymorphisms were elevated in the cancer population compared to the control population. However, whether there is a genetic link between these polymorphisms and cancer or enhanced radiosensitivity cannot be determined until the patient sample size is increased. Functional assays of the ATM protein generated from constructs that contain these single nucleotide changes may also help to elucidate any cause-effect relationships at the molecular level. This study of association between enhanced radiosensitivity of normal tissue and/or breast cancer with single nucleotide alterations within the *ATM* gene serves as a model for examining relative risks for other genes as well. Large-scale sequencing projects are rapidly identifying polymorphisms within the coding sequences of a number of genes associated with DNA repair, cell cycle regulation, signal transduction and apoptosis. DNA-based assays such as the ASO assay allow for the rapid screening of large numbers of samples from patients to determine if such polymorphisms are associated with increased risk for normal tissue complications. These types of assays are the first steps in developing rapid, clinically useful prognostic information on patient susceptibility to unacceptable radiotherapeutic complications.

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Classifying Local Disease Recurrences after Breast Conservation Therapy Based on Location and Histology

New Primary Tumors Have More Favorable Outcomes than True Local Disease Recurrences

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Supported in part by the Department of Health and Human Services National Cancer Institute (Grants CA16672 and T32CA77050 to Dr. Buchholz) and by USAMRMC Breast Cancer Research Program Career Development Awards (Grants BC980154 and BC961478 to Dr. Hunt).

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Received February 28, 2002; revision received May 14, 2002; accepted June 20, 2002.

BACKGROUND. To distinguish true local recurrences (TR) from new primary tumors (NP) and to assess whether this distinction has prognostic value in patients who develop ipsilateral breast tumor recurrences (IBTR) after breast-conserving surgery and radiotherapy.

METHODS. Between 1970 and 1994, 1339 patients underwent breast-conserving surgery at The University of Texas M. D. Anderson Cancer Center for ductal carcinoma in situ or invasive carcinoma. Of these patients, 139 (10.4%) had an IBTR as the first site of failure. For the 126 patients with clinical data available for retrospective review, we classified the IBTR as a TR if it was located within 3 cm of the primary tumor bed and was of the same histologic subtype. All other IBTRs were designated NP.

RESULTS. Of the 126 patients, 48 (38%) patients were classified as NP and 78 (62%) as TR. Mean time to disease recurrence was 7.3 years for NP versus 5.6 years for TR ($P = 0.0669$). The patients with NP had improved 10-year rates of overall survival (NP 77% vs. TR 46%, $P = 0.0002$), cause-specific survival (NP 83% vs. TR 49%, $P = 0.0001$), and distant disease-free survival (NP 77% vs. TR 26%, $P < 0.0001$). Patients with NP more often developed contralateral breast carcinoma (10-year rate: NP 29% vs. TR 8%, $P = 0.0043$), but were less likely to develop a second local recurrence after salvage treatment of the first IBTR (NP 2% vs. TR 18%, $P = 0.008$).

CONCLUSIONS. Patients with NP had significantly better survival rates than those with TR, but were more likely to develop contralateral breast carcinoma. Distinguishing new breast carcinomas from local disease recurrences may have importance in therapeutic decisions and chemoprevention strategies. This is because patients with new carcinomas had significantly lower rates of metastasis than those with local disease recurrence, but were more likely to develop contralateral breast carcinomas. *Cancer* **2002;95:2059–67.** *© 2002 American Cancer Society.*
DOI 10.1002/cncr.10952

KEYWORDS: breast conservation therapy, local disease recurrence, new primary tumor, histology.

The optimal management of patients with ipsilateral breast tumor recurrences (IBTR) after breast-conserving surgery and radiation therapy (BCT) is not well defined. Specifically, should all subsets of these patients receive systemic therapy? Numerous reports indicate that IBTR after BCT is an independent predictor of the risk of developing distant metastatic disease. An analysis of the results from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-06

trial found that the risk of distant failure for patients with IBTR after BCT is at least threefold greater compared with those without IBTR.[1] Other studies have also shown a poor prognosis for these patients, with 5-year overall and distant disease-free survival rates of approximately 60–70% and 45–65%, respectively.[2–4]

Despite information from these studies, it is not clear that all IBTR are equal in terms of predicting a poor prognosis. Other studies have suggested that there are subgroups of patients who have a relatively favorable prognosis after an IBTR. Older age, small tumors, noninvasive or focally invasive histology, negative axillary lymph nodes, low histologic grade, and location remote from the primary tumor site have all been identified as factors of an IBTR that indicate a more favorable distant disease-free survival period.[2–7] The most important prognostic indicator that has been identified so far, however, is the time interval to IBTR. Studies have demonstrated repeatedly that patients with an IBTR less than 1–5 years after the primary tumor have reduced overall and distant disease-free survival periods compared with those with IBTR occurring more than 5 years after the primary tumor.[2,4–11]

One hypothesis is that some subgroups of patients have a favorable prognosis because IBTR consists of two distinct types of disease: true local recurrences (TR) and new ipsilateral primary tumors (NP). This distinction was first articulated by Veronesi et al.[5] who described TR as "cases consistent with the regrowth of malignant cells not removed by surgery or not killed by radiotherapy," (page 20) whereas NP were described as "de novo cases of malignancies arising from mammary epithelial cells of the residual breast tissue" (ibid). Theoretically, an NP IBTR is independent of the primary breast carcinoma and the prognosis of these patients may be more favorable than those with a TR. Another hypothesis that may distinguish NP from TR is that the development of an NP may indicate an underlying genetic predisposition for breast carcinoma and thereby be associated with higher rates of carcinoma in the contralateral breast. If these hypotheses are true, the clinical management and chemoprevention strategies for patients with IBTR should reflect this distinction.

In this study, we classified IBTR as either NP or TR based on tumor location and histology and assessed whether this distinction has prognostic value for patients with IBTR after BCT. We recognized that using only clinical and pathologic features to distinguish NP from TR is likely to be less precise than molecular methods, but chose this methodology because these criteria are readily available to every clinician.

MATERIALS AND METHODS

Between 1970 and 1994, 1339 breast carcinoma patients were treated at the University of Texas M. D. Anderson Cancer Center by breast-conserving surgery, 139 (10.4%) of whom had an IBTR as the first site of failure. An IBTR was defined as a histologically confirmed recurrence of disease within the previously treated breast. We excluded 13 of the 139 patients because their records lacked information regarding their IBTR. The remaining 126 patients formed the study population.

For their primary therapy, all patients underwent breast-conserving surgery for primary breast neoplasms and 86 (68%) patients also underwent axillary lymph node dissection. All patients were treated with postoperative radiotherapy delivered to the entire ipsilateral breast with medial and lateral tangential fields using photon beams (median dose, 50 Gy), with or without regional lymph node irradiation as clinically indicated. Seventy-eight (62%) patients also received a boost to the primary tumor bed delivered by electron beams (median dose, 10 Gy) and 16 (13%) patients received a brachytherapy boost. Of the primary tumors, 112 (89%) were invasive carcinomas and 14 (11%) were ductal carcinoma in situ (DCIS). The decision to use systemic therapy was made by the patient and the treating medical oncologist according to the prognostic variables of each case. For treatment of the primary tumor, 25 (20%) patients were treated with chemotherapy, 3 (2%) patients received tamoxifen, in addition to chemotherapy, and 1 (1%) received tamoxifen alone.

After evaluating hospital records, operative reports, pathology reports, mammography reports, and radiotherapy records of the 126 patients, we classified each IBTR as either an NP or TR based on its location and histology. For the purposes of this study, an IBTR was designated as a TR if it was located within 3 cm of the primary tumor bed and if the histologic subtype was consistent with the primary tumor (i.e., infiltrating ductal carcinoma [IDC], lobular carcinoma, medullary carcainoma, tubular carcinoma). If the IBTR failed to meet either of these two criteria, it was designated as an NP. In most cases, the hospital records documented the specific location of the tumors and whether the IBTR recurred at or near the vicinity of the primary tumor site. When the location or histology of the tumors was unclear, mammograms and pathology slides were obtained and reevaluated.

Two patients in which there was a change in histology from DCIS to IDC were considered histologically characteristic of TR because this change is consistent with a natural progression of breast carcinoma.

TABLE 1
Treatment of Patients with IBTR

Treatment	All Patients (n = 126) (%)	NP (n = 48) (%)	TR (n = 78) (%)	P value
Surgery				
Local reexcision	8	6	10	0.439
Salvage mastectomy	82	94	75	0.006
None	10	0	15	0.004
Systemic therapy				
Chemotherapy	26	20	31	0.222
Hormonal therapy	14	10	17	0.330
Both	17	13	19	0.325

IBTR: ipsilateral breast tumor recurrence; NP: new primary tumor; TR: true local disease recurrence.

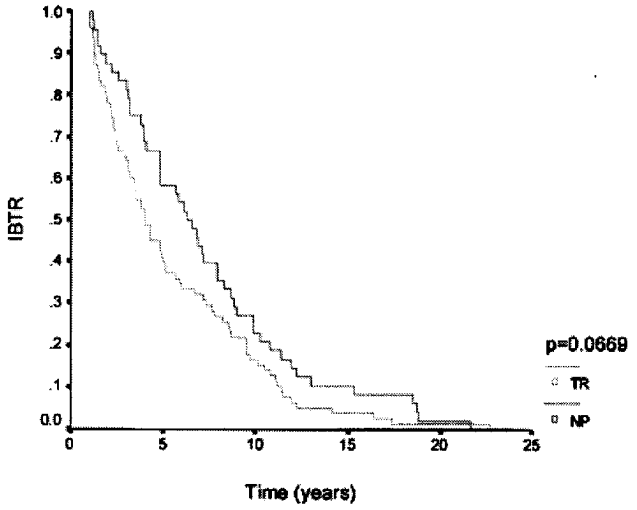

FIGURE 1. Actuarial curves showing the time interval from the primary tumor to development of ipsilateral breast tumor recurrences (IBTR). In patients with true local disease recurrnce, IBTR developed earlier than in patients with new primary tumors.

However, three patients in which there was a change from an IDC to DCIS were considered histologically characteristic of NP. Only one of these three patients had DCIS as a component of her primary tumor. The time to disease recurrence for the three patients were 4.1, 9.9, and 10.8 years, respectively. In three patients, the location of the IBTR could not be delineated because the tumor mass encompassed the entire breast at the time of disease recurrence. Because the histology was consistent with the original primary tumor, we classified these three patients as TR.

The therapeutic management of patients with IBTR depended on the clinical circumstances of each patient. The decision to treat with completion mastectomy and/or systemic therapy was made by the patient and her treating physician. Table 1 shows the treatment of the IBTR according to classification of NP versus TR. There were no significant differences between the two groups with respect to systemic therapy. However, a greater percentage of patients with NP were treated with completion mastectomy. Of the 12 (15%) patients with TR who did not receive surgery for their IBTR, 10 had tumor masses larger than 3 cm (3 of whom had carcinomas encompassing the entire residual breast tissue), 1 had lymph node involvement at the time of IBTR diagnosis, and 1 developed distant disease within 1 month after diagnosis. Eleven of these patients received chemotherapy with or without tamoxifen and one patient refused any treatment because of the development of distant disease.

All patients were classified as having either an NP or TR before any analysis of the outcome data. The Kaplan–Meier method[12] was used to calculate actuarial statistics for the time interval to IBTR and the rates of overall survival, cause-specific survival, distant disease-free survival, and contralateral breast carcinoma-free survival. For survival statistics, all event and follow-up times were measured from the date of IBTR diagnosis. Comparisons of survival between patients with NP versus TR were made using the log rank test.[13] To reduce any bias introduced by the more favorable survival of patients without invasive disease, we also calculated survival statistics for the 114 patients with invasive IBTR, excluding 12 patients whose IBTR consisted entirely of DCIS. Of these 12 patients, 7 had disease classified as NP and 5 as TR. Univariate analyses comparing various clinical and pathologic characteristics between patients with NP versus TR were performed. Proportions and means were compared using the chi-square two-sided test and the Student t test, respectively. Cases with unknown values were excluded from the univariate analysis.

After this analysis was completed, we further divided the NP patients into three subgroups on the basis of their IBTR classification criteria: different location, different histology, or both. Outcomes and time to disease recurrence for each subgroup were calculated using Kaplan–Meier survival curves and compared with one another using the log rank test. This additional analysis ensured that these subgroups were similar to one another, allowing their collective grouping into the category "NP."

RESULTS

For the 126 patients studied, the median follow-up period for the surviving patients was 12.4 and 7.0 years after diagnosis of the primary tumor and IBTR, respectively. The length of follow-up was similar between the patients with NP versus TR (12.3 and 7.2 years vs. 12.8 and 7.0 years, respectively). Figure 1 shows that the

TABLE 2
Comparison of Location and Histology between Patients with NP and TR

	All patients (n = 126) (%)		NP (n = 48) (%)		TR (n = 78) (%)		P value	
	Primary	IBTR	Primary	IBTR	Primary	IBTR	Primary NP vs. TR	IBTR NP vs. TR
Location								
Central	10	12	10	13	10	10	.977	0.697
UOQ	42	41	36	36	48	45	.186	0.295
UIQ	28	24	31	19	26	27	.495	0.296
LOQ	10	15	8	19	10	13	.721	0.366
LIQ	10	8	15	13	6	5	.129	0.137
Histology								
Invasive ductal	72	77	68	69	76	81	.397	0.124
Invasive lobular	5	6	4	7	5	5	.806	0.790
Both invasive ductal and lobular	6	4	4	4	6	4	.593	0.806
DCIS only	11	10	11	16	12	7	.846	0.066
Other	6	3	13	4	1	3	.008	0.618

NP: new primary tumor; TR: true local disease recurrence; IBTR: ipsilateral breast tumor recurrence; DCIS: ductal carcinoma in situ.

patients with TR developed their IBTR after a shorter interval from their initial treatment than patients with NP (mean time interval: TR 5.6 vs. NP 7.3 years; log rank comparison of curves, $P = 0.0669$).

Forty-eight (38%) had their disease recurrence classified as an NP and 78 (62%) as a TR. Table 2 shows the characteristics of the patients according to the classification criteria used to distinguish NP from TR. Thirty-three percent of the IBTR were located at a site different from the primary and 17% were composed of a different histologic subtype. Of the patients classified as having NP disease, 88% had different location, 44% had different histology, and 48% differed in both respects. It is noteworthy that 10% of the tumors designated as NP were classified solely on the basis of histology because the IBTR occurred at or near the primary tumor bed.

Actuarial survival rates showed that patients with IBTR classified as NP had more favorable outcomes, regardless of whether the analysis included the 12 patients whose IBTR consisted entirely of DCIS. Figure 2A shows that the 10-year overall survival rate of all patients classified as having NP was 77%, which was better than the 46% rate of patients with TR ($P = 0.0002$). In addition, patients with NP also had better 10-year rates of cause-specific survival (NP 83% vs. TR 49%, $P = 0.0001$) and distant disease-free survival (NP 77% vs. TR 26%, $P < 0.0001$; Fig. 3A). When the 12 patients with DCIS were excluded from the analysis, patients with NP still had significantly better 10-year overall survival (NP 71% vs. TR 44%, $P = 0.0012$), cause-specific survival (NP 80% vs. TR 46%, $P = 0.0005$), and distant disease-free survival rates (NP 77% vs. TR 23%, $P < 0.0001$; Figs. 2B, 3B). The overall survival ($P = 0.0029$), cause-specific survival ($P = 0.0016$), and distant disease-free survival rates ($P < 0.0001$) also remained significant when the 12 patients who did not have surgery for their IBTR were excluded from the analysis. In addition to poor survival rates, patients with TR showed a higher rate of developing a second or third local recurrence after salvage treatment of the first IBTR (TR 18% vs. NP 2%, $P = 0.008$).

Patients with NP had a significantly higher rate of contralateral breast carcinoma. Figure 4 displays the contralateral breast carcinoma-free 10-year survival rate for all patients studied (NP 71% vs. TR 92%, $P = 0.0043$; Fig. 4A) and for patients with invasive disease only (NP 68% vs. TR 92%, $P = 0.0035$; Fig.4b).

Table 3 displays a comparison of clinical characteristics for patients with NP versus TR. No significant differences were found between the two groups with respect to patient age, history of primary carcinomas other than breast carcinoma, and treatment with tamoxifen, chemotherapy, or radiation boost to the primary tumor bed ($P > 0.1$ for all comparisons). However, only four patients in this study were treated with tamoxifen, so its value in preventing NP IBTR could not be assessed. Patients with NP had a higher rate of having a first-degree relative with breast carcinoma, but this difference was not statistically significant (NP 19% vs. TR 13%, $P = 0.366$).

Table 4 summarizes the pathologic characteristics that were compared between patients with NP versus TR. No significant differences were found between the NP and TR patients with respect to primary tumor stage, primary tumor size, axillary lymph node in-

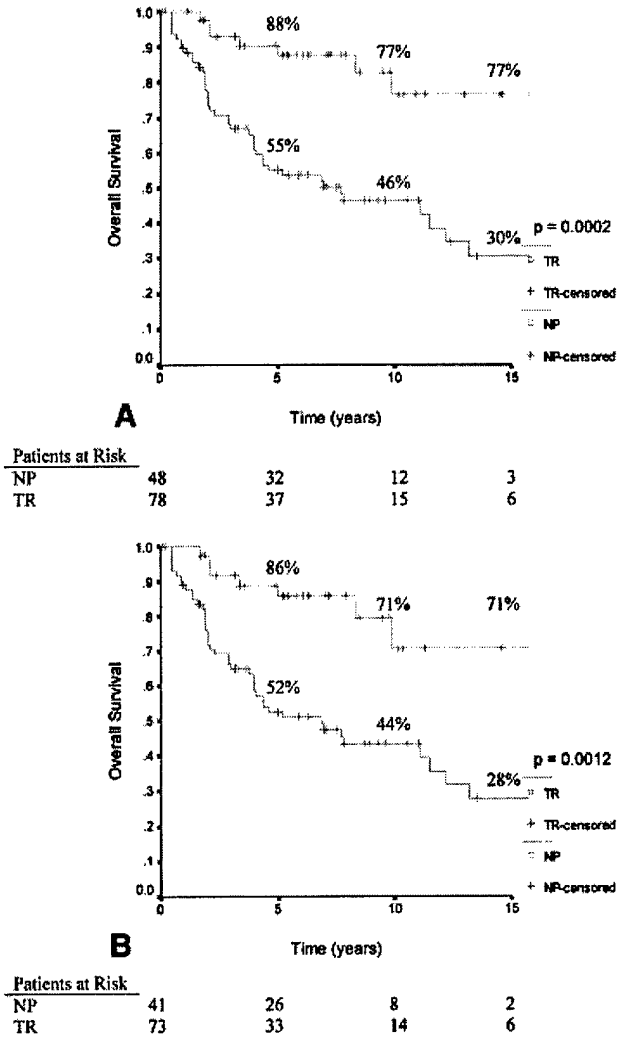

FIGURE 2. Actuarial curves showing improved overall survival for patients classified as having a new primary tumor compared with patients with true local disease recurrence in (A) all 126 patients studied and (B) the 114 patients with invasive carcinoma only.

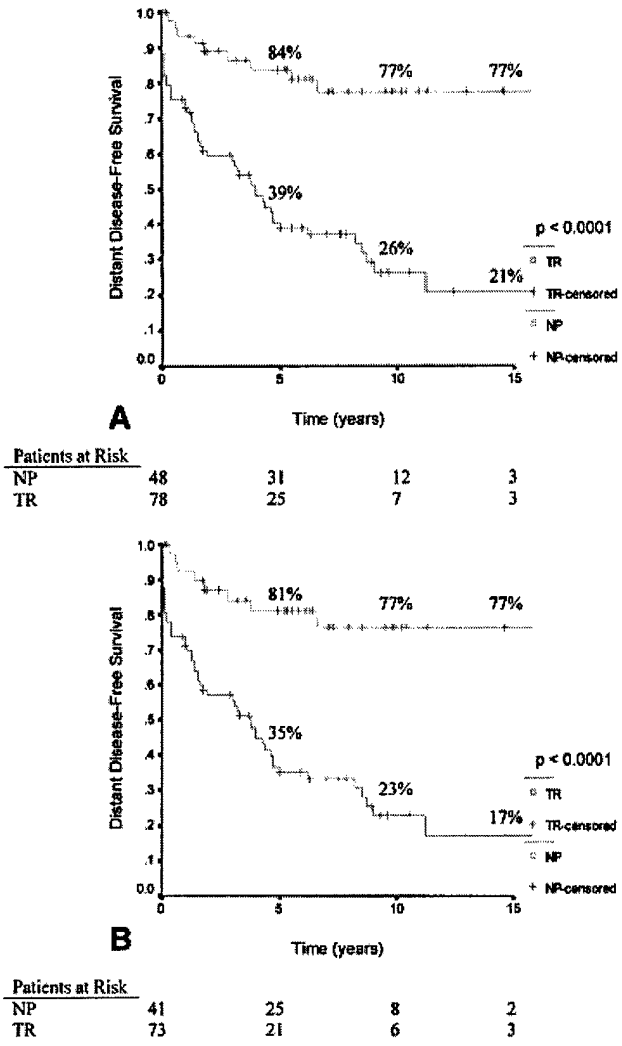

FIGURE 3. Actuarial curves showing improved distant disease-free survival for patients classified as having a new primary tumor compared with patients with true local disease recurrence in (A) all 126 patients studied and (B) the 114 patients with invasive carcinoma only.

volvement, positive margins, extensive intraductal component, nuclear grade, lymph node involvement at IBTR, and estrogen/progesterone receptor status of the IBTR ($P > 0.1$ for all comparisons). Patients designated as having NP disease had a higher rate of primary tumors with positive estrogen receptor status (NP 77% vs. TR 53%, $P = 0.049$) and positive progesterone receptor status (NP 75% vs. TR 42%, $P = 0.014$). Patients with TR had a higher rate of skin involvement by the IBTR (TR 28% vs. NP 2%, $P = 0.003$).

Among the three subgroups of NP patients according to classification criteria (different location, different histology, or both), no differences were found in rates of overall survival, cause-specific survival, and distant disease-free survival, and time interval to disease recurrence ($P = 0.5672$, 0.3490, 0.7487, and 0.6385, respectively).

DISCUSSION

In this study, we used location and histology to classify IBTR as either NP or TR. Using these criteria, 38% of patients with IBTR after long follow-up had clinical findings compatible with NP. Despite the relatively imprecise method used to distinguish NP and TR, our classification had significant prognostic value. Patients classified as having NP had more favorable overall, cause-specific, and distant disease-free survival rates than those with TR. Our findings support data from other studies that have attempted to define indicators of prognosis following IBTR. Specifically, the

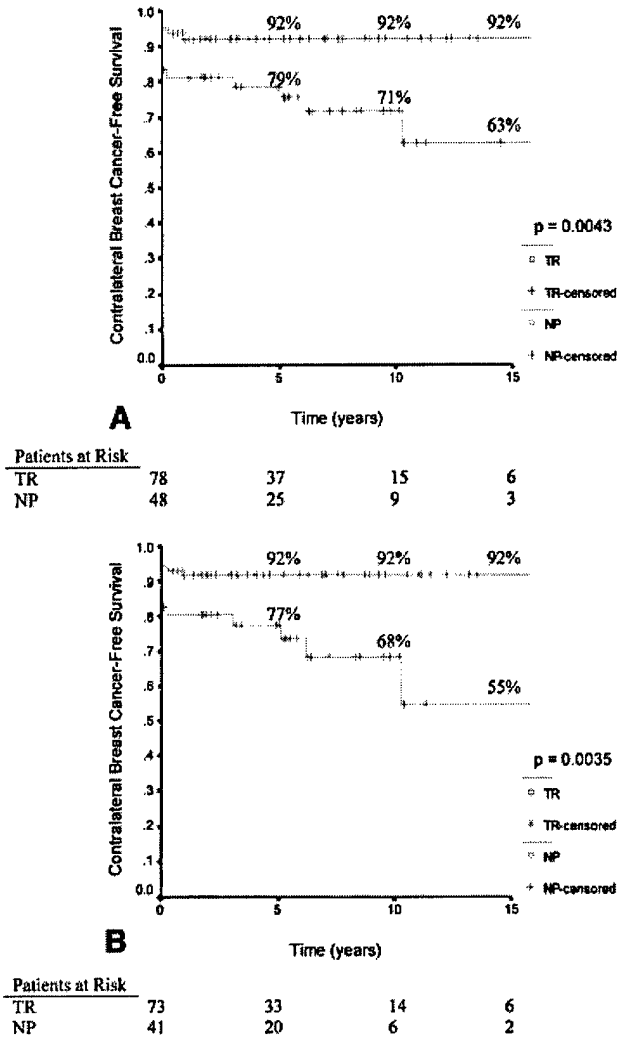

FIGURE 4. Actuarial curves showing a higher rate of contralateral breast carcinoma in patients classified as having a new primary tumor compared with patients with true local disease recurrence in (A) all 126 patients studied and (B) the 114 patients with invasive carcinoma only.

features of NP tumors have been correlated with better outcomes, including longer time interval to IBTR and location remote from the primary tumor site.[2–4,10,11] Conversely, the TR tumors shared traits that have been correlated with poor outcomes such as early onset of IBTR, location near the primary tumor site, and pathologic evidence of skin involvement.[2,4–7,14]

Our data also support the hypothesis that an NP tumor is a disease entity independent from the primary breast carcinoma. The subgroup of patients with NP had a much better outcome than patients with IBTR.[1–4] Specifically, the 5-year overall and distant disease-free survival rates for our patients with NP were 88% and 84%, respectively, compared with previously reported 5-year rates of 60–70% and 45–65%, respectively.[2–4] The overall and distant disease-free survival rates for our patients with NP (10-year rate: 77% and 77%) are more comparable to the survival rates reported for women treated with BCT for a primary carcinoma who did not experience an IBTR (10-year rate: 70–80% and 60–70%).[1,15,16] This observation makes intuitive sense because NP patients should have a prognosis similar to patients with de novo early-stage primary breast carcinomas. In addition, our findings suggest that previous studies reporting a poor prognosis for patients with IBTR following BCT[1–4,7] may actually underestimate the mortality rate of a TR, which was approximately 50–60% at 10 years in our study. Those studies may have overestimated the rates of survival because they included a subgroup of patients with NP in the overall statistical analysis.

In addition to assessing outcomes, we attempted to identify clinical and pathologic risk factors that may be predictive for developing NP versus TR. Theoretically, TR develop from residual surviving tumor clonogens. Therefore, the risk factors for developing TR should be related to issues regarding the local treatment of the primary tumor (e.g., surgical margin status, radiotherapy technique). However, we did not find positive margins or the use of a radiation tumor bed boost to be associated significantly with the development of either TR or NP disease. Conversely, because NP are believed to be de novo occurrences of breast carcinoma, the risk factors for developing NP should not be related to issues surrounding the surgical and radiation treatment of the primary tumor. Rather, they are more likely related to issues reflecting genetic predisposition and susceptibility to breast carcinoma such as family history and young age at diagnosis.[17–19] Our finding that patients with NP have significantly higher rates of carcinoma in the contralateral breast adds some support to this hypothesis because previous studies have shown a correlation between family history and the development of contralateral breast carcinoma.[20,21] However, we did not find family history or patient age to be associated significantly with IBTR classified as NP.

This distinction between NP and TR has important implications in the clinical management of IBTR. Currently, the decision to use systemic therapy for the treatment of IBTR is controversial. We have shown that patients with NP generally have a favorable long-term prognosis. Therapeutic decisions concerning systemic therapy for these patients should be similar to those used for patients with equivalent stage first primary breast carcinomas. However, the risk of developing contralateral breast carcinomas, coupled with the possibility of a genetic predisposition, highlights the need for better chemoprevention strategies

TABLE 3
Comparison of Clinical Characteristics between Patients with NP and TR

Characteristic	All patients (n = 126) (%)	NP (n = 48) (%)	TR (n = 78) (%)	P value
Age				
At primary (mean ± SE)	44.7 ± 1.4	43.9 ± 1.6	45.3 ± 1.3	0.497
Younger than 40 yrs at primary (%)	36	42	32	0.274
At IBTR (mean ± SE)	51.0 ± 1.5	51.2 ± 1.7	50.8 ± 1.4	0.852
Younger than 40 yrs at IBTR (%)	22	21	22	0.898
Time to IBTR in yrs (mean ± SE)	6.2 ± 0.6	7.3 ± 0.7	5.6 ± 0.5	0.0669
Two or more local disease recurrences	12	2	18	0.008
Family history (First-degree relative)	15	19	13	0.366
Carcinomas other than breast	14	13	15	0.654
Radiation boost for primary	74	79	71	0.283
Hormonal therapy for primary	3	0	5	0.111
Chemotherapy for primary	20	19	21	0.810

NP: new primary tumor; TR: true local disease recurrence; SE: standard error; IBTR: ipsilateral breast tumor recurrence.

TABLE 4
Comparison of Pathologic Characteristics between Patients with NP and TR

Characteristics	All patients (n = 126) (%)	NP (n = 48) (%)	TR (n = 78) (%)	P value
Stage of primary tumor				
0	11	10	12	0.846
1	46	46	46	0.972
2	43	44	42	0.874
Tumor size of primary (n = 111)				
Tumor size in cm (mean ± SE)	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	0.804
Larger than 2 cm (%)	41	45	39	0.369
Positive axillary lymph nodes at primary (n = 86)	30	27	32	0.598
Positive margins versus close/negative margins (n = 81)	14	10	16	0.471
Extensive intraductal component in primary (n = 124)	26	22	28	0.427
Modified Black's nuclear Grade 3				
Primary (n = 69)	33	29	35	0.579
IBTR (n = 87)	40	33	43	0.307
Lymph node involvement at IBTR	9	4	12	0.155
Skin involvement at IBTR (n = 113)	19	2	28	0.003
Positive estrogen receptor status				
Primary (n = 64)	63	77	53	0.049
IBTR (n = 53)	68	75	65	0.468
Positive progesterone receptor status				
Primary (n = 55)	56	75	42	0.014
IBTR (n = 45)	47	46	47	0.965

NP: new primary tumor; TR: true local disease recurrence; SE: standard error; IBTR: ipsilateral breast tumor recurrence.

in these patients. One strategy would be to recommend tamoxifen for patients with NP, as randomized trials have demonstrated its benefit in reducing contralateral and ipsilateral disease recurrences with minimal side effects.[22–24] The NSABP P-1 trial showed that 5 years of tamoxifen reduced the 5-year risk of developing breast carcinoma by as much as 50% in all age groups.[24] The beneficial effects of tamoxifen in decreasing rates of NP could not be studied adequately in this population due to its infrequent use. However, Buchholz et al.[25] reported that tamoxifen use significantly decreased the rate of IBTR after BCT. In that study, the 8-year rate of IBTR was only 3% for lymph node-negative breast carcinoma patients treated with BCT and tamoxifen. It is noteworthy to speculate to what degree the reduction in IBTR with tamoxifen use is reflective of the therapeutic versus chemopreventive effects of this agent.

In contrast to patients with NP, patients with IBTR classified as TR have a poor prognosis in terms of both

survival rates and the development of a second or third local disease recurrence. These data highlight the need for adjuvant systemic therapy for this category of patients. In addition, the 18% rate of second local disease recurrences suggests that aggressive surgery is warranted.

The main limitation of this study is that the IBTR were classified using clinical and pathologic criteria without molecular confirmation. In the future, more precise molecular studies will likely be able to identify the clonal relatedness of the IBTR and the primary tumor. However, our methodology has greater clinical applicability than molecular techniques that require sophisticated analyses. Our criteria are based on readily available information and our results demonstrate that these criteria can identify subgroups of patients with significantly different outcomes after IBTR.

Smith et al.[26] also classified IBTR as NP or TR based on clinical and pathologic criteria and investigated the outcomes of these patients in light of this distinction. Similar to our findings, they reported that NP patients had a longer time to disease recurrence and significantly more favorable overall, cause-specific, and distant disease-free survival rates. In addition, they found that patients whose tumors were classified as NP were younger than those with TR (mean age: 49 vs. 55 years). They noted that all eight patients who tested positive for BRCA 1/2 mutations developed NP. This finding adds support to the hypothesis that patients who are genetically predisposed to developing breast carcinoma are more likely to have NP recurrences.

In conclusion, based on differences in location and/or histology between the primary tumor and the IBTR, we classified more than one-third of patients as having NP rather than TR. These patients have outcomes that are similar to those for patients treated for early-stage primary breast carcinoma and significantly better than those for patients with TR. Accordingly, this distinction between NP and TR should be incorporated into the therapeutic management of IBTR. Our data support the use of systemic therapy and aggressive local management for patients with TR and the need to investigate chemoprevention strategies for patients with NP.

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