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| 13. ABSTRACT (Maximum 200 Words) Ductal carcinoma in situ (DCIS) is a pre-invasive stage of breast cancer. From our allelotyping study of DCIS, chromosomal regions of 8p, 13q, 16q, 17p and 17q showed loss of heterozygosity (LOH) significantly above background (5%). We concentrated our efforts on the LOH mapping of a region on 8p (30% LOH) and identified the smallest common deletion region located at 8p22-p23 in an ~1.4 cM interval. An integrated YAC/BAC clone contig covering the deletion region was constructed using CITB and RPCI-11 BAC libraries and the publicly available YAC contig information. STSs (sequence tagged sites) developed from CITB BACs and the publicly available insert end sequences from RPCI-11 BACs on the contig were used for database search. Seven clones with full working draft sequences were identified and localized to the contig. Clone RPCI-11 184O21 contains genomic sequence for the tyrosine kinase, blk. A second predicted gene in the same clone shows 89% homology to hematopoietic cell kinase (Hck), a member of the Src family of tyrosine kinases. Clone RPCI-11589N15 contains sequences homologous to human procathepsin B, Squalene synthase and GATA-4. When the sequence of the entire deletion region becomes available, gene-finding programs can be used to identify all genes in this region and assess the likelihood of a gene being a tumor suppressor gene (TSG). Then, the putative TSGs can be evaluated on normal and tumor tissues of DCIS cases. | | | | |
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Tumor Suppressor Genes in Early Breast Cancer and its Progression

Alison M. Goate, D. Phil.

Table of Contents

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|---|-------|
| Front cover | 1 |
| SF 298 | 2 |
| Foreword | 3 |
| Table of Contents | 4 |
| Introduction | |
| A. Nature of the problem | 5 |
| B. Background | 5 |
| Body | |
| Task 1 | 6-8 |
| Task 2 | 8 |
| Task 3 | 8 |
| Task 4 | 8-11 |
| Key Research Accomplishments | 11-12 |
| Publications resulting from this research project | 12 |
| Conclusions | 12-13 |
| References | 13-16 |
| Appendices | |

Introduction

A. Nature of the problem

An increasing percentage of breast cancer is being detected at a pre-invasive stage: ductal carcinoma *in situ* (DCIS). DCIS is a form of breast cancer in which malignant cells have not penetrated the basement membrane.⁽¹⁾ The histopathological diagnosis encompasses a heterogeneous group of subtypes, including comedo, cribriform, solid, papillary and micropapillary, some of which may differ in biological behavior. The potential for associated micro-invasion and the likelihood of recurrence after breast conservation therapy are higher with the comedo subtype.^(2,3) As with invasive breast cancer, DCIS can also be stratified by nuclear grade: high, intermediate, and low. Circumstantial evidence that DCIS is a precursor lesion to invasive ductal carcinoma is based on three observations: the frequent co-existence of DCIS and invasive cancer in the same breast;⁽⁴⁾ the greatly increased risk of subsequent invasive breast cancer in women with biopsy-proven DCIS;⁽⁵⁾ and the finding that when a local recurrence is seen after breast-conserving treatment of DCIS there is a 50% chance that the recurrence will be of the invasive variety.⁽⁶⁾ DCIS is not an obligate precursor however, and other possible pathways to invasion may exist such as the *de novo* transition to malignancy of normal epithelium without an intervening non-invasive stage. For many years the standard treatment for DCIS has been total mastectomy, though lumpectomy with adjuvant radiation is being utilized currently for small, well-localized areas of DCIS.

Lobular carcinoma *in situ* (LCIS), on the other hand, is not thought to be a pre-invasive cancer but rather an indicator of increased risk of breast cancer. Interestingly, the risk is the same in both breasts regardless of the side in which the LCIS was detected. That the LCIS cells do not inevitably progress to invasive breast cancer is evidenced by the fact that, of those cancers which do develop, only half are of the invasive ductal variety.⁽⁷⁾

Atypical lobular hyperplasia (ALH) and atypical ductal hyperplasia (ADH) are considered to be high-risk lesions both associated with an increase of 4-5 fold compared to the general female population. If a strong family history of breast cancer exists, the risk is doubled to 8-9 fold.⁽⁵⁾

Our studies have concentrated on the genetic changes that occur in DCIS and the transition from DCIS to invasive breast cancer. A better understanding of the oncogenesis of breast cancer at the molecular level, and the correlation of this information with clinical data, may aid in treatment choices.

B. Background

Most solid tumors arise due to the inactivation of tumor suppressor genes and activation of oncogenes. The accumulation of genetic changes is believed to result in the invasive followed by the metastatic phenotypes. Loss of heterozygosity (LOH) of one of a pair of alleles in tumor tissue compared to matched normal control from the same individual can reveal areas of chromosomal deletion that are likely to contain putative tumor suppressor genes. A large number of articles have been published on LOH in invasive breast cancer, and virtually every human chromosome has been shown to exhibit allelic loss.^(8,9) The most frequent losses in invasive breast cancer are seen on chromosome 7q,^(8,10,11) 16q,^(8,9,12-14) 17p,^(8,9,15-18) 17q,^(8,9,19-25) and 18q.^(8,22,26,27) Less frequent losses are found on 1p,^(8,9,28) 1q,^(8,9,29,30) 3p,^(8,9,17) 6q,^(8,9) 8p,^(8,16) 11p,^(8,31) and 13q.^(9,17)

Because of the multiple putative tumor suppressor loci that exhibit LOH in invasive

breast cancer, it is not clear which loci are involved in oncogenesis and which are lost randomly due to the instability conferred by the malignant state of the genome.

Allelotyping involves the comprehensive screen of the genome for LOH in a particular cancer. Generally an initial screen will involve assaying with at least one marker from each non-acrocentric chromosomal arm, thereby the average or baseline level of LOH can be determined. This may vary from 5 to 20% depending on the type of cancer. A significant level of LOH at a single locus, indicating the site of possible tumor suppressor genes involved in oncogenesis, can be ascertained once the background level is known. Regions that show significant LOH can then be analyzed with additional markers to refine the smallest deleted region that may contain the tumor suppressor gene. The analysis of tumors with a number of markers also permits calculation of the fractional allelic loss (FAL) for each tumor. This has been defined as the total number of chromosomal arms which show LOH divided by the total number of informative arms for that tumor.^(32,33) FAL has been correlated with patient outcome in colon cancer,⁽³²⁾ and may correlate with clinical information in other tumor types.

Few reports exist on the molecular changes in DCIS that pertain to invasive breast cancer. Davidoff et al.⁽³⁴⁾ studied 6 examples of synchronous DCIS and invasive cancer for expression of p53 and found the same levels of protein expression in each tissue type. Expression of the oncogenes c-erbB-2 and c-myc is also consistent between coexisting pre-invasive and invasive breast cancer.^(35,36) Zhuang et al. studied allelic loss for two loci on 11q13 (INT2 and PYGM). They found that for every case of DCIS which showed LOH (N=15), loss of the same allele was seen in the corresponding invasive tumor.⁽³⁷⁾ O'Connell et al.⁽³⁸⁾ studied four loci TPO (2pter), D4S192 (4q25-34), D16S265 (16q21) and D17S579 (17q21) and found that 8 of 10 cases of DCIS shared LOH patterns with more advanced lesions for at least one of the 4 loci.

Body:

Task 1. The identification and characterization of the extent of chromosomal deletion in DCIS.

Materials and Methods

Study subjects

As described in previous progress reports, paraffin embedded samples of DCIS were collected from several hospitals in St. Louis (Barnes-Jewish, Deaconess Central, St. Louis University, and the Outpatient Surgery Center). Subtypes of DCIS and nuclear grade of the tumor were classified by the pathologists. Either matched archival normal lymph node DNA or leukocyte DNA was used as a control. When it was necessary to draw blood for a normal control, informed consent was obtained following Institutional Review Board approval. Samples were microdissected to enrich for tumor cells and DNA was purified as previously described.⁽³⁹⁾ After microdissection, tissue samples containing an insufficient number of tumor cells for the normal DNA extraction procedure were digested in small volumes (10-20 μ l) of lysis buffer containing proteinase K, then phenol and chloroform extracted once before sodium acetate/ethanol precipitation.

LOH analysis

We have used a panel of highly polymorphic microsatellite markers for LOH assay. DNA extracted from the tissues was resuspended in water and then aliquots were used

directly as a template (5-10 ng) for PCR amplification. Initial PCR conditions were obtained via the Genome Data Base (GDB, <http://gdbwww.gdb.org>) and then optimized in the laboratory. PCR was performed in the tumor/normal pairs and products were separated on denaturing polyacrylamide sequencing gels. LOH was determined by a combination of visual inspection and scanning densitometry of the autoradiographs. A 2.5 fold difference in the relative allele intensity ratios between an informative tumor/normal pair was scored as LOH (allele1/allele2 in tumor compared to allele1/allele2 in normal). To maintain a conservative scoring approach, marginal allele reduction by inspection was not scored as LOH. Tumor samples with equivocal results were re-dissected in an attempt to obtain a "purer" specimen and the experiments were repeated. All LOH designations were scored by two independent scientists and repeated to verify the result.

Genetic Linkage Maps

Once a region of chromosomal deletion has been identified it can be narrowed down using a panel of closely linked markers which map to that area. Since new microsatellite markers appeared in the public databases daily, they did not appear on published genetic maps. In order to determine the deletion map in the tumors, it is necessary to know the precise location of the markers being used. A fine genetic linkage map was constructed using genotypic data from a number of families generated in the lab and publicly available data (<http://www.cephb.fr/cephdb/>) of Centre d'Etude Polymorphisme Humaine (CEPH) reference families. Merged genotypic data were processed using the linkage program CRIMAP (P. Green, unpublished) with minimum odds for order of 1000:1. Having identified a small region of deletion (preferably no larger than 1cM) positional cloning techniques can be undertaken to clone the putative tumor suppressor gene contained within the region.

Results & Discussion

During the first year of this project we completed the allelotyping of DCIS. A total of 61 samples of DCIS were assayed. The average number of informative tumors examined for each marker was 19 (range 8 to 48). The median fractional allelic loss (FAL) was 0.037. The highest % of LOH was shown for loci on 8p (18.7%), 13q (18%), 16q (28.6%), 17p (37.5%) and 17q (15.9%). LOH on 18q was found in 10.7% of informative tumors. FAL was associated with LOH on 17p with high nuclear grade and with the comedo subtype of DCIS. LOH on 17p correlated with LOH on 17q and on 13q. Additional markers were employed for 16q and 17p to determine the smallest common region of deletion and maps of 17p and 16q were generated.⁽⁴⁰⁾ Aldaz et al. also studied allelic loss in a total of 23 examples of DCIS. They found the most frequent sites of loss to be on chromosomes 7p, 16q, 17p and 17q.⁽⁴¹⁾

During the second year of the project we concentrated our efforts on the refinement of the area of loss on 8p. In a more detailed analysis of LOH on 8p we genotyped 18 polymorphic markers in 65 sporadic DCIS cases and observed LOH in 29% of informative samples.⁽⁴²⁾ Several investigators have reported two distinct regions of loss on 8p in breast cancer, located at 8p21 and 8p22. Yaremko et al. studied 20 examples of invasive ductal cancer and found the overall rate of LOH on 8p to be 55% with loss at 8p22 observed more frequently than at 8p21.⁽⁴³⁾ Recently, Yokota et al. reported allelic loss in two regions (8p12-21 and 8p22-23.1 encompassing D8S549 and D8S1992) in advanced tumors, implying that allelic loss on 8p may contribute to the progression of breast carcinoma.⁽⁴⁴⁾

Simultaneously with LOH analysis, we have generated a meiotic linkage map for 8p to resolve previously reported conflicting map orders from the literature. The map spanned from the telomere of 8p to just beyond the centromere with a distance of 80.8 cM. The average distance between markers is 3.5 cM. Our genetic mapping study refined the deleted region to a 1.4 cM interval between markers D8S520 and D8S265.⁽⁴⁵⁾ Anbazhagan et al. found the most common region of loss in 60 invasive ductal breast tumors to be at 8p21.3-p23.3 between D8S560 and D8S518.⁽⁴⁵⁾ The 1.4 cM region of loss we have identified lies within this region.

Task 2. The study of chromosomal deletions in hyperproliferative breast conditions.

We assayed 3 pairs of LCIS/normal samples using several markers on 8p. No LOH was observed.

Task 3. The study of chromosomal deletions in the progression of DCIS to invasive and metastatic phenotypes.

To study genetic changes and the evolution of breast cancer we have assayed for loss of heterozygosity (LOH) in twelve sets of synchronous carcinoma *in situ* (CIS) and invasive cancer, compared to normal control DNA. Microsatellite markers were used which map to each non-acrocentric autosomal arm. Eight tumor sets demonstrated LOH of the same allele in both concurrent invasive cancer and DCIS, for a total of eighteen chromosomal loci. Three of nine tumor sets showed LOH on 11p. In two of these sets LOH was seen on 11p only in the invasive tumor, not the corresponding CIS. One of these tumors also exhibited allelic loss in the invasive tumor for 4 loci, all of which were retained in the non-invasive tumor. For two tumor sets LOH was mirrored in matched DCIS, invasive tumor and lymph node metastases. The maintenance of LOH for certain loci throughout the stages of breast cancer suggests clonality of the cancer cells. Tumor suppressor loci on 11p may be involved in the invasive phenotype.⁽⁴⁷⁾

Task 4. Cloning a tumor suppressor gene involved in breast cancer.

Materials and Methods

Radiation hybrid maps

As described in previous progress reports, G3 and GB4 are the two radiation hybrid mapping panels (Research Genetics, Inc. Huntsville, AL) we used to construct the RH placement map. The G3 panel, comprised of 83 RH clones from the whole human genome, was created at the Stanford Human Genome Center and is considered a medium resolution panel (i.e. 500 Kb resolution). The GB4 panel, comprised of 93 RH clones from the whole human genome, has lower resolution (i.e. 1000 Kb). STS markers were assayed by PCR amplification and sized on 3% agarose gels stained with ethidium bromide. Each assay was performed twice, i.e. PCR products at the expected size were observed on each of the duplicate gels in order to be scored as positive. Data generated from the GB4 panel were submitted to the Whitehead Institute Center for Genome Research (WICGR) Mapping Service Center. Data generated using the G3 panel were submitted to the Stanford RH server. A list of the most tightly-linked mapped markers, the LOD score between the linked markers, and the distance in cR between the submitted marker and the linked marker on the map reported from the servers were used as a reference for the framework. We then used the program RHMAP, version 3.0 (<http://www.sph.umich.edu/group/statgen/software>)⁽⁴⁸⁾ to

integrate our RH data from the six markers covering the deletion region into the framework map.⁽⁴⁵⁾

YAC and BAC clone contig construction

The CEPH YAC contig WC-1195 from WICGR was used for the initial contig construction. YAC clones used in the mapping were propagated on YPD medium plates. Ten isolated colonies from each YAC clone were tested using a "whole cell PCR" assay to identify colonies positive for marker(s) from the region. For "whole cell PCR", a small amount of an isolated colony was suspended in 5 μ l of deionized water and the suspension was used directly as the template in a standard PCR reaction. The standard ligation-mediated PCR method was used to develop new STSs from YACs.

For BAC contig construction, we screened the human BAC library CITB-978SK-B & CITB-HSP-C, constructed by the California Institute of Technology (obtained from Research Genetics, Inc., Huntsville, AL). The library screen involved a total of 120 PCR reactions for each STS assay. As with YAC clone isolation, each identified BAC clone was then verified by a "whole cell PCR" assay, using 10 randomly selected colonies as candidates. After the positive BAC clones were verified, single BAC colonies were propagated in liquid medium, cells harvested, and insert DNA prepared using the Plasmid Midi-Kit from Qiagen Inc. (Chatsworth, CA). Each BAC clone was partially sequenced on an ABI 373 DNA sequencer (PE/ABI), from the insert ends using modified T7 and Sp6 primers. The sequence generated was analyzed for candidate PCR primer sequences using the program PRIMER 0.5 (Lincoln and Lander, MIT Center for Genome Research). STSs developed from BACs were tested using the NIGMS (#2) rodent/human hybrid panel, in which each hybrid cell contains a single human chromosome, and only chromosome 8 specific STSs were used as entry points for chromosome walking.

The insert sizes of BAC clones were determined by digesting clones with Not I and restriction fragments were separated on 1% agarose gels in 0.5X TBE buffer using MidRange II PFG markers (NE BioLabs) as size standards. Gels were electrophoresed at 6 V/cm with an initial pulse of 5 seconds and a final pulse of 15 seconds for 18 hrs at 14 C in a BioRad CHEF-DR II apparatus.

Colony Hybridization

To effectively sequence the deletion region with the high-throughput technique at the Genome Sequencing Center of Washington University, a second BAC library (RPCI-11) was screened using hybridization and fingerprinting. We used end sequences derived from CITB-978SK-B and CITB-HSP-C BAC clones to generate 23 overgos using the techniques described in Current Protocols in Human Genetics. Briefly, two 24-mer oligonucleotides with an overlap of 8 bp are designed from the target sequence and then annealed to create two 16-bp overhangs. Klenow fragment with incorporated radionucleotides is used to fill in the 16-bp overhangs to generate a double-stranded 40-mer probe with high specific activity. These overgo probes were pooled and hybridized to nylon filters containing immobilized DNA from the RPCI-11 BAC library (Research Genetics, Inc., Huntsville, AL). After hybridization, the filter was washed to remove non-specifically bound probe and visualized by autoradiography.

Fingerprinting

Two colonies for each positive BAC clone from the hybridization screening were isolated and propagated in liquid YT medium containing chloramphenicol. A modified alkaline lysis procedure^(49,50) was followed for DNA preparation. Samples were digested with restriction endonuclease Hind III and electrophoresed on 1% SeaKem LE (FMC BioProducts) agarose gels. After electrophoresis, gels were stained with 1:10,000 dilution of SYBR Green (FMC BioProducts) and then imaged using a Molecular Dynamics FluorImager SI. Fingerprinting data were collected as the distance that restriction fragments migrate on an agarose gel relative to fragments in the standard marker (Boehringer-Mannheim marker V in our study) lane using the program FPC version 3.2^(49,51) (<http://www.sanger.ac.uk>). The relative mobilities of restriction fragments from newly isolated RPCI clones were compared with the fingerprinting data of CITB clones to assemble the comprehensive sequence-ready contig.

Identification of tumor suppressor gene candidates

First, the TIGR BAC database was searched to identify insert end sequences of incorporated RPCI-11 BAC clones identified from the fingerprinting. Then, the end sequences of RPCI-11 BACs identified from the database and those we developed from the CITB BACs were used to screen publicly available nucleotide databases of fully sequenced clones using the program BLAST (<http://www.ncbi.nlm.nih.gov>).

Identified clones with full sequences at the working draft stage were examined with the dbEST, UniGene databases (<http://www.ncbi.nlm.nih.gov>) and the program GENSCAN (<http://ccr-081.mit.edu/GENSCAN.html>) to detect known genes and possible exons. Finally, publicly available protein databases (<http://www.ncbi.nlm.nih.gov>) were used to search for homologies between predicted peptides and known genes.

Results & Discussion

We have constructed a radiation hybrid map for the 1.4 cM deletion interval between markers D8S520 and D8S265 within chromosome 8p22-p23.⁽⁴⁵⁾ This map provides an independent means of ordering the markers in this region and helped to verify the linkage map marker order, a necessary step prior to the construction of a clone contig and gene identification. The distance of 42.3 cR, or approximately 1,565 kb (assuming 37 kb per cR)⁽⁵²⁾ between the markers D8S265 and D8S520 confirmed the approximate size of 1.4 cM deletion region from our genetic mapping result.

After we confirmed the genetic marker orders with RH mapping, an integrated YAC/BAC clone contig covering the ~1.4 cM LOH interval near D8S550 was constructed.⁽⁴⁴⁾ This contig spanning ~1730 kb consists of 13 YACs and 27 CITB BACs. Eleven ESTs localized to the region near D8S550 were mapped to our contig. Two of these 11 ESTs, N29512 and AA018590, identified by the marker SGC30677 in UniGene, were developed from a cDNA clone with sequence homology to human Farnesyl Diphosphate Farnesyltransferase. EST H16027, which is one of the ESTs detected by the marker WI-8953, is derived from a cDNA clone with homology to human procathepsin B.

Since only 11 ESTs were mapped to the contig, most of which gave no clue to their function, we decided to use a sequencing approach for the TSG candidate search. As described in previous progress reports, our collaboration with the Genome Sequencing Center of Washington University has increased the depth of the contig. A total of 57

clones from the second BAC library (RPCI-11) screening were isolated and incorporated into a refined sequence-ready contig by hybridization and fingerprinting. The BACs are now being sequenced.

During the last project year, we have focused on DNA sequence analyses. The STSs we developed from CITB BACs and the publicly available insert end sequences of RPCI-11 BACs were used to search the public databases with the program BLAST. Seven clones with full working draft sequences were identified and localized to the contig by electronic PCR using STS assays (Figure. 1). A total of 13 STSs were localized to clone RPCI-11 177H2 and the overlapping clone RRPCI-11 184L12. Although many exon fragments were detected in these two clones and the other overlapping clone RPCI-11 44D1 using GENSCAN, there are currently no corresponding full length cDNAs in the public databases.

Six STSs and one EST (WI-6800) were localized to clone RPCI-11 110L10 (Figure 1) by homology search to the htg database. One gene predicted by the GENSCAN program was also identified and localized to this clone by homology to a Unigene cluster (gi=7020537). The function of this gene remains unknown. Two additional genes are predicted by GENSCAN. Full length cDNAs for these genes do not currently exist in the public databases, however, ESTs corresponding to exon fragments are detected (Figures 1 & 2).

Fourteen STSs, one EST (AA504989) and two microsatellite markers (D8S1695 and D8S1759) were placed on clone RPCI-11 148O21 (Figure 1). Two genes were detected in this clone by homology to the Unigene database and by the GENSCAN program. One of these two predicted genes shows 89% homology with amino acids 369-531 of hematopoietic cell kinase (Hck), a member of the Src family of tyrosine kinases. The other putative gene shows >97% homology to a protein tyrosine kinase (blk) (Figure 3).

Three STSs and 5 ESTs (R01183, R01769, H16027, Z45810 and N29512) were placed on clone RPCI-11 589N15 (Figures 1). Three known genes, squalene synthase, human procathepsin B and GATA-4 map within this clone. However, this clone currently exists as 33 discontinuous fragments. It is clear from figure 4 that they are not yet in the correct order since exons for squalene synthase are randomly distributed throughout the clone. The large number of fragments makes the GENSCAN predictions less meaningful since we cannot predict which exons belong to which gene except for those with clear homology to known genes.

Key research accomplishments

- Chromosomal deletions on 8p, 13q, 16q, 17p, and 17q were observed from our initial alleotyping study of DCIS.
- Our LOH assay of LCIS suggested that tumor suppressor loci on 11p may be involved in the invasive phenotype.
- Our data identified the smallest common LOH region of DCIS to be located at 8p22-p23.
- The smallest deletion region of ~1.4 cM has been determined and confirmed by meiotic linkage and radiation hybrid mapping.

- We constructed a ~1.7-Mb sequence-ready YAC/BAC contig covering the deletion region that may contain a tumor suppressor gene.
- Based on the currently available databases, 7 clones with full working draft sequences were identified using the information and clones on the physical map.
- Clone RP11-148O21 contains two putative tyrosine kinases. The *blk* gene, which has previously been mapped to 8p22-p23, is within this clone. A second polypeptide of 601 amino acids that is predicted by GENSCAN shows 89% homology to hematopoietic cell kinase (*Hck*) between aa369 and aa531 of the hypothetical gene sequence. *Hck* is a member of the *Src* family of tyrosine kinases.
- Clone RP11-589N15 that maps to the proximal end of the contig contains sequences homology to human procathepsin, squalene synthase and GATA-4.

Publications resulting from this research project

1. Wang, J.C., Radford, D., Wylie C., McPherson, J., Donis-Keller, H., Goate, A.. Physical mapping and identification of tumor suppressor gene candidates involved in ductal carcinoma *in situ* on chromosome 8p22-p23. The 200Era of Hope Proceedings, Volume 1, p151.
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Conclusion

From our initial allelotyping study of DCIS we determined that the background rate of loss of heterozygosity (LOH) is low (5%). The chromosomal regions showing LOH significantly above background were 8p, 13q, 16q, 17p and 17q. In our study of 65 cases of DCIS, using 18 markers on 8p and one on 8q, LOH was seen on 8p in 29% of informative cases. Our genetic linkage analysis and RH mapping effort localized the smallest deletion region to 8p22-p23 in an approximately 1.4 cM interval between D8S265 and D8S520. We also constructed an integrated YAC/BAC clone contig

covering this deletion region. The STS information and the overlapping BAC clones on the contig provided the starting material for large scale sequencing of this genomic region. To date, 7 BAC clones with full sequence at the working draft stage have been identified in the databases. A total of ~175 exons were predicted from these clones using the program GENSCAN. In addition, five genes were detected in two clones, RP11-148O21 and RP11-589N15.

When the sequencing for the entire deletion region becomes publicly available, gene-finding programs can be used to identify all genes in this region and assess the likelihood of a gene being a tumor suppressor gene. Then, the putative TSGs can be evaluated on normal and tumor tissues of DCIS cases.

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Appendix I.

Figure 1. An integrated YAC/BAC clone contig spanning the 1.4cM deletion region

Figure 2. Predicted putative genes in clone RPCI-11 110L10.

Figure 3. Predicted putative genes in clone RPCI-11 148O21.

Figure 4. Predicted putative genes in clone RPCI-11 589N15.

Figure 2. Predicted putative genes in clone RPCI-11 110L10.

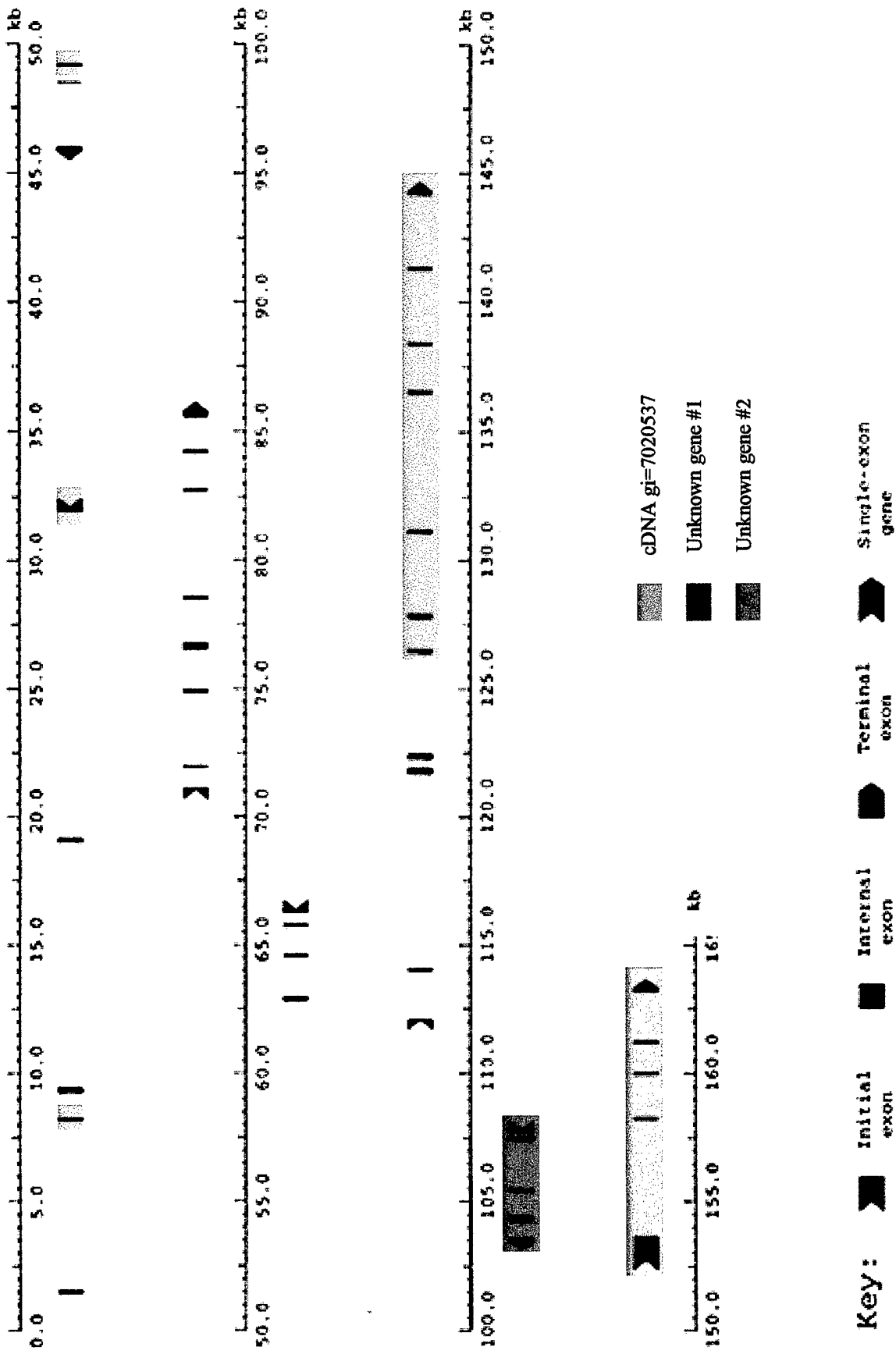


Figure 2. Clone RPCI-11 110L10

I. Genes predicted by GENSCAN and by homology to the Unigene database.

| FEATURES | Location/Qualifiers |
|-----------------|--|
| exon | complement(31913..32180) /note="putative exon 1 of cDNA gi=7020537" |
| exon | complement(49076..49226) /note="putative exon 2 of cDNA gi=7020537" |
| exon | complement(8146..8282) /note="putative exon 3 of cDNA gi=7020537" |
| exon | 126355..126546 /note="putative exon 4 of cDNA gi=7020537" |
| exon | 127715..127980 /note="putative exon 5 of cDNA gi=7020537" |
| exon | 131046..131225 /note="putative exon 6 of cDNA gi=7020537" |
| exon | 136445..136590 /note="putative exon 7 of cDNA gi=7020537" |
| exon | 138199..138456 /note="putative exon 8 of cDNA gi=7020537" |
| exon | 141214..141375 /note="putative exon 9 of cDNA gi=7020537" |
| exon | 144075..145232 /note="putative exon 10 of cDNA gi=7020537" |
| CDS | complement(145740..146043) /note="EST marker WI-6800" |
| misc_feature | 79763..92911 /note="assembly_fragment clone_end:SP6 vector_side:left" |

Figure 2. Clone RPCI-11 110L10

II. Genes predicted by GENSCAN only.

| FEATURES | Location/Qualifiers |
|-----------------|--|
| promoter | complement(109688..109727) /note="putative promoter of unknown gene #1" |
| exon | complement(107656..107818) /note="putative exon 1 of unknown gene #1" |
| exon | complement(107384..107556) /note="putative exon 2 of unknown gene #1" |
| exon | complement(105399..105504) /note="putative exon 3 of unknown gene #1" |
| exon | complement(104204..104473) /note="putative exon 4 of unknown gene #1" |
| exon | complement(103471..103610) /note="putative exon 5 of unknown gene #1" |
| polyA_signal | complement(88650..88655) /note="polyA site of unknown gene #1" |
| promoter | 146854..146893 /note="putative promoter of unknown gene #2" |
| exon | 152752..153653 /note="putative exon 1 of unknown gene #2" |
| exon | 158221..158313 /note="putative exon 2 of unknown gene #2" |
| exon | 159955..160055 /note="putative exon 3 of unknown gene #2" |
| exon | 161173..161277 /note="putative exon 4 of unknown gene #2" |
| exon | 163185..163321 /note="putative exon 5 of unknown gene #2" |
| polyA_signal | 163891..163896 /note="polyA site of unknown gene #2" |

Figure 3. Predicted putative genes in clone RPCI-11 148O21

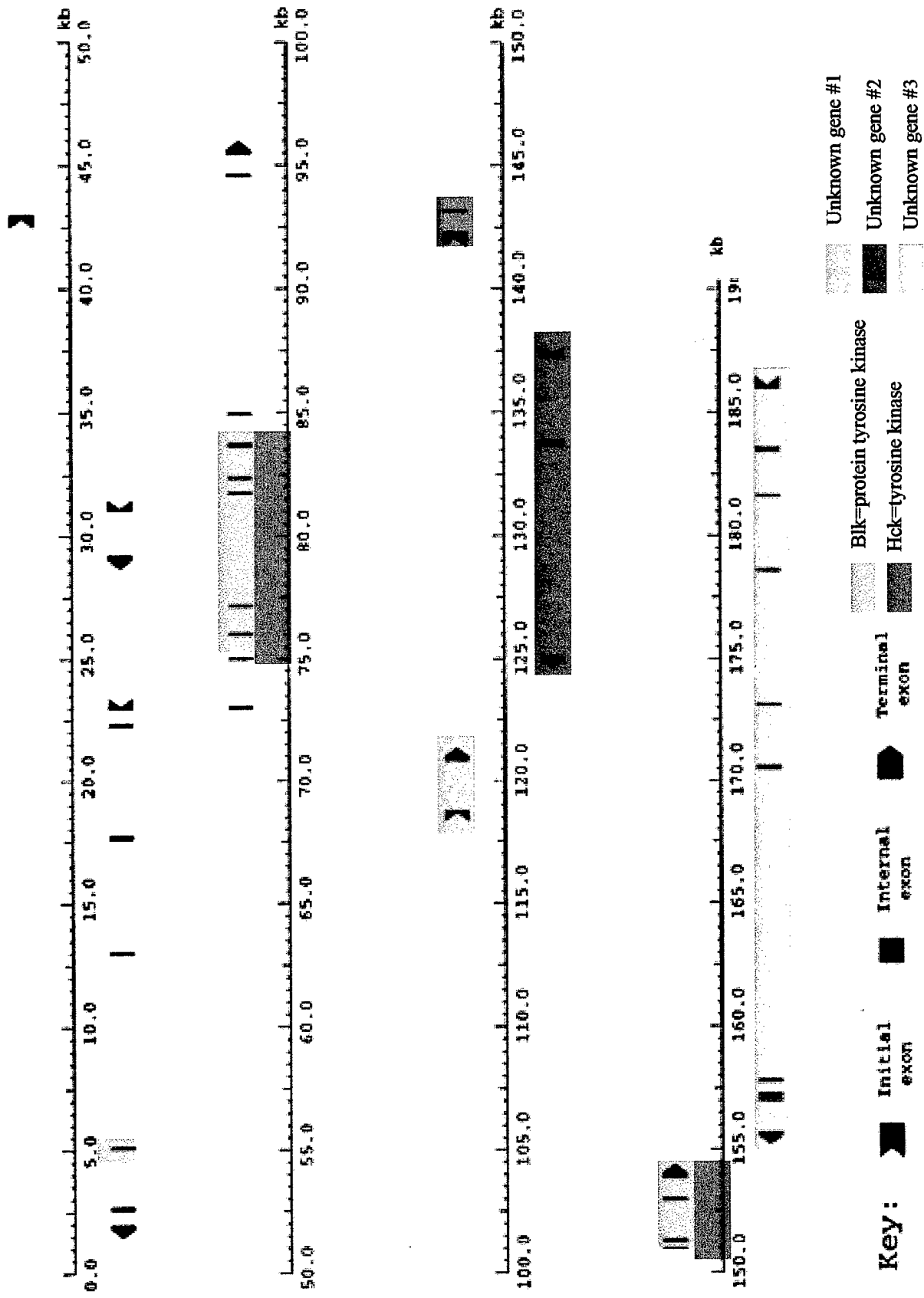


Figure 3. Putative genes in clone RPCI-11 148021

I. Genes predicted by GENSCAN and by homology to the Unigene database.

| FEATURES | Location/Qualifiers |
|--------------|--|
| exon | complement(5000..5152) /note="blk=protein tyrosine kinase exon 1" |
| exon | 74962..75057 /note="blk=protein tyrosine kinase exon 2" |
| exon | 75955..76060 /note="blk=protein tyrosine kinase exon 3" |
| exon | 77089..77196 /note="blk=protein tyrosine kinase exon 4" |
| exon | 82253..82422 /note="blk=protein tyrosine kinase exon 5" |
| exon | 83589..83776 /note="blk=protein tyrosine kinase exon 6" |
| exon | 84892..84980 /note="blk=protein tyrosine kinase exon 7" |
| exon | 151219..151383 /note="blk=protein tyrosine kinase exon 8" |
| exon | 152902..153074 /note="blk=protein tyrosine kinase exon 9" |
| exon | 153814..154216 /note="blk=protein tyrosine kinase exon 10" |
| exon | 154303..154529 /note="blk=protein tyrosine kinase exon 11" |
| polyA_signal | 154509..154514 /note="polyA site of blk=protein tyrosine kinase" |
| exon | 75786..75887 /note="tyrosine kinase Hck exon 1" |
| exon | 76920..77027 /note="tyrosine kinase Hck exon 2" |
| exon | 81504..81636 /note="tyrosine kinase Hck exon 3" |
| exon | 82153..82249 /note="tyrosine kinase Hck exon 4" |
| exon | 83420..83601 /note="tyrosine kinase Hck exon 5" |
| exon | 142091..142310 /note="tyrosine kinase Hck exon 6" |
| exon | 143133..143204 /note="tyrosine kinase Hck exon 7" |
| exon | 151084..151138 /note="tyrosine kinase Hck exon 8" |
| exon | 151354..151508 /note="tyrosine kinase Hck exon 9" |
| exon | 153033..153166 /note="tyrosine kinase Hck exon 10" |
| exon | 153955..154163 /note="tyrosine kinase Hck exon 11" |

CDS 114740..115158
 /note="EST marker AA504989"
 marker complement (67759..68150)
 /note="microsatellite marker D8S1695"
 marker 130656..131033
 /note="microsatellite marker D8S1759"

II. Genes predicted by GENSCAN only.

| FEATURES | Location/Qualifiers |
|--------------|---|
| promoter | 107727..107766 /note="putative promoter of unknown gene #1" |
| exon | 118691..118763 /note="putative exon 1 of unknown gene #1" |
| exon | 120794..120993 /note="putative exon 2 of unknown gene #1" |
| polyA_signal | 121734..121739 /note="polyA site of unknown gene #1" |
| promoter | complement (140238..140277) /note="putative promoter of unknown gene #2" |
| exon | complement (137281..137287) /note="putative exon 1 of unknown gene #2" |
| exon | complement (133691..133885) /note="putative exon 2 of unknown gene #2" |
| exon | complement (124967..125115) /note="putative exon 3 of unknown gene #2" |
| polyA_signal | complement (122570..122575) /note="polyA site of unknown gene #2" |
| exon | complement (185949..186048) /note="putative exon 1 of unknown gene #3" |
| exon | complement (183404..183567) /note="putative exon 2 of unknown gene #3" |
| exon | complement (181565..181643) /note="putative exon 3 of unknown gene #3" |
| exon | complement (178527..178625) /note="putative exon 4 of unknown gene #3" |
| exon | complement (173083..173148) /note="putative exon 5 of unknown gene #3" |
| exon | complement (170443..170610) /note="putative exon 6 of unknown gene #3" |
| exon | complement (157719..157842) /note="putative exon 7 of unknown gene #3" |
| exon | complement (157131..157280) /note="putative exon 8 of unknown gene #3" |
| exon | complement (156970..157057) /note="putative exon 9 of unknown gene #3" |
| exon | complement (155555..155662) /note="putative exon 10 of unknown gene #3" |
| polyA_signal | complement (154916..154921) /note="polyA site of unknown gene #3" |

Figure 4. Predicted putative genes in clone RPCI-11 589N15.

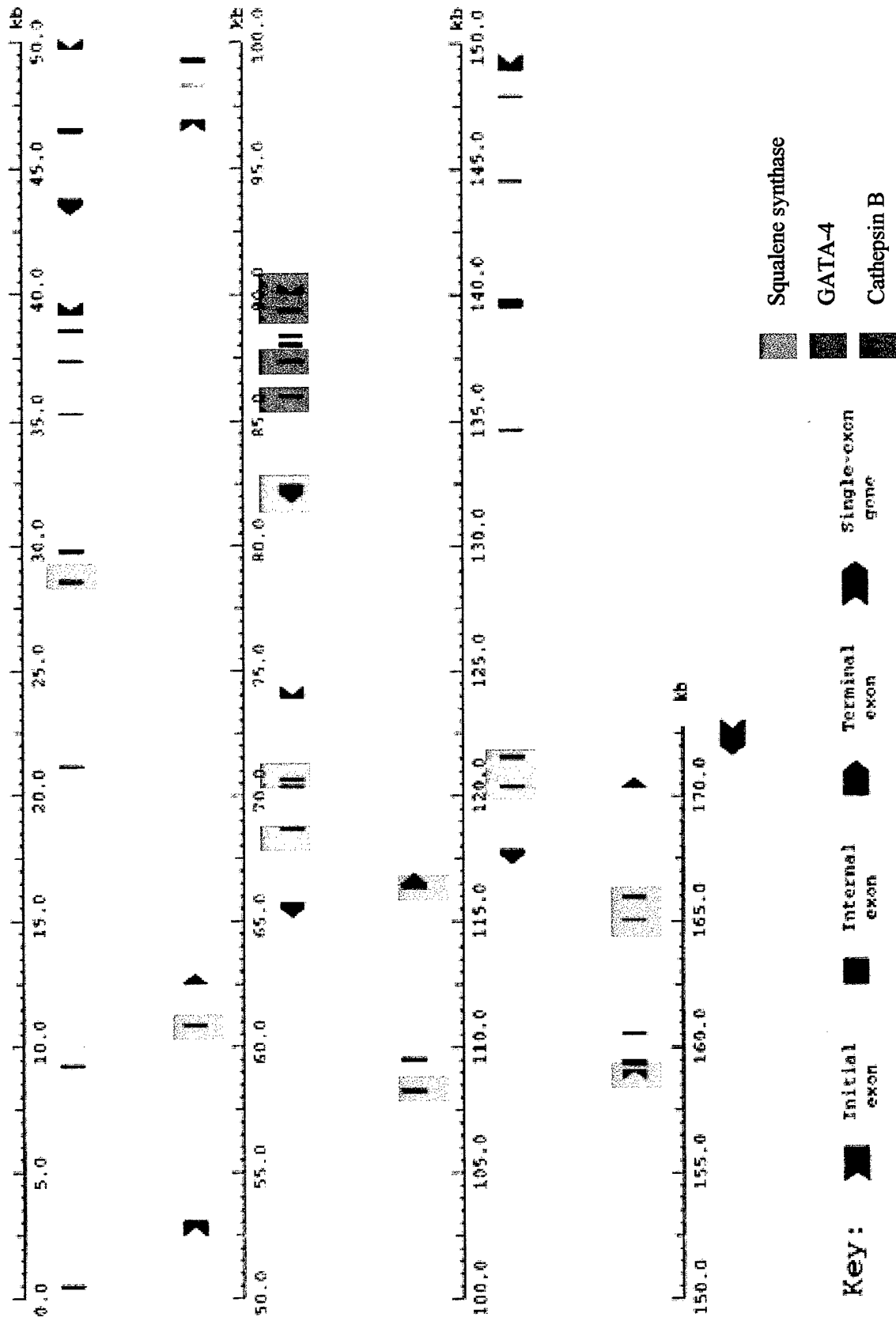


Figure 4. Putative genes in clone RPCI-11 589N15

Genes predicted by GENSCAN and by homology to the Unigene database.

| FEATURES | Location/Qualifiers |
|----------|--|
| exon | complement (65516..65899) /note="exon of transcriptional coactivator pc4mRNA" |
| exon | 158970..159156 /note="exon 1 of squalene synthase gi=435676" |
| exon | 165015..165115 /note="exon 2 of squalene synthase gi=435676" |
| exon | 165890..166076 /note="exon 3 of squalene synthase gi=435676" |
| exon | 60811..60940 /note="exon 4 of squalene synthase gi=435676" |
| exon | 28685..28471 /note="exon 5 of squalene synthase gi=435676" |
| exon | 108165..108362 /note="exon 6 of squalene synthase gi=435676" |
| exon | 109437..109608 /note="exon 7 of squalene synthase gi=435676" |
| exon | 116298..117234 /note="exon 8 of squalene synthase gi=435676" |
| exon | complement (87938..88058) /note="exon 1 of cathepsin B gi=348706" |
| exon | complement (87310..87429) /note="exon 2 of cathepsin B gi=348706" |
| exon | complement (85921..86054) /note="exon 4 of cathepsin B gi=348706" |
| exon | complement (84590..85484) /note="exon 5 of cathepsin B gi=348706" |
| exon | complement (121493..121675) /note="exon N1 of GATA-4 gi=508483" |
| exon | complement (120340..120471) /note="exon N2 of GATA-4 gi=508483" |
| exon | complement (70582..70670) /note="exon N3 of GATA-4 gi=508483" |
| exon | complement (68632..68783) /note="exon N4 of GATA-4 gi=508483" |
| exon | complement (82806..83647) /note="exon N5 of GATA-4 gi=508483" |
| CDS | 42481..42864 /note="EST marker R01183" |
| CDS | 81946..82373 /note="EST marker R01769" |
| CDS | complement (84714..85098) /note="EST marker H16027" |
| CDS | 108843..109120 /note="EST marker Z45810" |
| CDS | complement (116770..117222) /note="EST marker N29512" |

Appendix II.

Reprints

1. Wang, J.C., Radford, D., Wylie C., McPherson, J., Donis-Keller, H., Goate, A.. Physical mapping and identification of tumor suppressor gene candidates involved in ductal carcinoma *in situ* on chromosome 8p22-p23. The 200Era of Hope Proceedings, Volume 1, p151.
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6. Radford, D.M., Phillips, N.J., Fair, K.L., Ritter, J.H., Holt, M., and Donis-Keller, H. (1995). Allelic loss and the progression of breast cancer. *Cancer Res.* 55:5180- 5183
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PHYSICAL MAPPING AND THE IDENTIFICATION OF TUMOR SUPPRESSOR GENE CANDIDATES INVOLVED IN DUCTAL CARCINOMA *IN SITU* ON CHROMOSOME 8P22-P23.

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From our allelotyping study of ductal carcinoma *in situ* (DCIS), we identified the smallest common region of loss of heterozygosity (LOH) to be located at chromosome 8p22-23 in an approximately 1.4-cM interval between D8S265 and D8S520. An integrated YAC/BAC clone contig covering this DCIS deletion region was constructed using the CITB BAC library. This contig spanning ~1730 kb consists of 13 YACs and 27 BACs. Eleven ESTs localized to the region near D8S550 from the UniGene map were verified by PCR assay and mapped to our contig. Eight of these 11 ESTs appeared to be unique since they did not identify homologous sequences in the publicly available databases. Two ESTs, N29512 and AA018590 identified by the marker SGC30677 in UniGene, were developed from a cDNA clone with sequence homology to human Farnesyl Diphosphate Farnesyltransferase. EST H16027 which is one of the ESTs detected by the marker WI-8953 is derived from a cDNA clone with homology to human Cathepsin B precursor. To effectively sequence the deletion region with the high through-put technique at the Genome Sequencing Center of Washington University, a second BAC library (RPCI-11) was screened using the insert end sequences of BAC clones on the YAC/BAC contig. Fifty seven RPCI BACs were isolated and incorporated into a refined sequence-ready contig by hybridization and fingerprinting. Twenty four of these clones were contained entirely within other clones. Clones forming a minimal tiling path have been subcloned into M13 and shotgun sequencing is in progress.

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Sequence-Ready Contig for the 1.4-cM Ductal Carcinoma *in Situ* Loss of Heterozygosity Region on Chromosome 8p22–p23

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We report the construction of an ~1.7-Mb sequence-ready YAC/BAC clone contig of 8p22–p23. This chromosomal region has been associated with frequent loss of heterozygosity (LOH) in breast, ovarian, prostate, head and neck, and liver cancer. We first constructed a meiotic linkage map for 8p to resolve previously reported conflicting map orders from the literature. The target region containing a putative tumor suppressor gene was defined by allelotyping 65 cases of sporadic ductal carcinoma *in situ* with 18 polymorphic markers from 8p. The minimal region of loss encompassed the interval between D8S520 and D8S261, and one tumor had loss of D8S550 only. We chose to begin physical mapping of this minimal LOH region by concentrating on the distal end, which includes D8S550. A fine-structure radiation hybrid map for the region that extends from D8S520 (distal) to D8S1759 (proximal) was prepared, followed by construction of a single, integrated YAC/BAC contig for the interval. The ~1730-kb contig consists of 13 YACs and 27 BACs. Fifty-four sequence-tagged sites (STSs) developed from BAC insert end-sequences and 11 expressed sequence tags were localized within the contig by STS content mapping. In addition, four unique cDNA clones from the region were isolated and fully se-

quenced. This integrated YAC/BAC resource provides the starting point for transcription mapping, genomic sequencing, and positional cloning of this region. © 1999 Academic Press

INTRODUCTION

Chromosome loss reveals constitutional recessive mutations that can result in tumor development (Sager, 1989; Marshall, 1991). Individuals heterozygous for a mutated tumor suppressor allele express the mutant phenotype (cancer) when the normal allele is lost. Thus, loss of heterozygosity (LOH) analysis may be used to map putative tumor suppressor genes when the DNA from normal and tumor-derived cells for the same patient are compared, using polymorphic markers flanking the locus. Frequent LOH of chromosome 8p has been reported in several types of human cancer, e.g., tumors of the prostate (Vocke *et al.*, 1996), lung (Emi *et al.*, 1992; Wood *et al.*, 1994), colon (Emi *et al.*, 1992; Wood *et al.*, 1994), larynx (Sunwoo *et al.*, 1996), liver (Emi *et al.*, 1992), and breast (Chuaqui *et al.*, 1995; Kerangueven *et al.*, 1995; Adelaide *et al.*, 1998; Dahiya *et al.*, 1998).

Studies in colorectal cancer have indicated the presence of at least two tumor suppressor genes (TSG) on 8p, at 8p11–p21.3 and 8p21–p22 (Farrington *et al.*, 1996). Similarly, three distinct regions, at 8p23, 8p22, and 8p12–p21, were defined by LOH studies for prostate cancer (Bova *et al.*, 1996; Vocke *et al.*, 1996; Perinchery *et al.*, 1999) and tumors of the head and neck (Sunwoo *et al.*, 1996; Wu *et al.*, 1997). Wright *et al.* (1998) identified three regions of loss on 8p (one in 8p22 and two in 8p23) for ovarian adenocarcinomas.

Markers in the region at 8p12–p22 have been shown by linkage analysis to have significant lod scores in several German breast cancer families, suggesting that BRCA3 may reside within that region (Seitz *et al.*,

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1997a,b). Recently Yokota *et al.* (1999) reported allelic loss in two regions (8p12–p21 and 8p22–p23.1, encompassing D8S549 and D8S1992) in advanced tumors, indicating that allelic loss on 8p may contribute to the progression of breast carcinoma.

In 1932 Broders proposed the term “*in situ*” to describe a condition in which malignant cells are present but have not yet penetrated the basement membrane. Ductal carcinoma *in situ* (DCIS) of the breast is a preinvasive form of breast cancer and can be a precursor of invasive ductal breast cancer. Several studies have described allelic losses in DCIS at multiple regions of chromosome 8p (Radford *et al.*, 1995; Yaremko *et al.*, 1995, 1996; Anbazhagan *et al.*, 1998), suggesting that these regions may be involved in early development of breast cancer.

The objective of our study was to map physically a genomic region defined by LOH assays of 65 DCIS tumor/normal pairs. We report DCIS allelic losses in 8p22–p23 and the construction of a 1.7-Mb sequence-ready contig from 8p22–p23 that may contain a tumor suppressor gene. Fifteen expressed sequence tags (ESTs) and cDNA clones from the region were also mapped to the contig, and nearly 25 kb of sequence was generated during the course of the study. This integrated YAC/BAC contig map provides a very useful resource for additional transcriptional mapping, positional cloning, and sequencing of this important region.

MATERIALS AND METHODS

LOH analysis. Sixty-five archival paraffin-embedded samples of DCIS were collected from several hospitals in St. Louis (Barnes-Jewish, Deaconess Central, St. Louis University, and the Outpatient Surgery Center). Subtypes of DCIS and nuclear grade were classified by the pathologists (N.J.P., J.H.R., K.DeS.). Either matched archival normal lymph node DNA or leukocyte DNA was used as a control. When it was necessary to draw blood for a normal control, informed consent was obtained following Institutional Review Board approval. Samples were microdissected to enrich for tumor cells, and DNA was purified as previously described (Radford *et al.*, 1993). After microdissection, tissue samples containing an insufficient number of tumor cells for the normal DNA extraction procedure were digested in small volumes (10–20 μ l) of lysis buffer containing proteinase K and then phenol- and chloroform-extracted once before sodium acetate/ethanol precipitation. This material was resuspended in water, and then aliquots were used directly as a template (5–10 ng) for PCR amplification. Initial PCR conditions were obtained via the Genome Data Base (GDB, <http://gdbwww.gdb.org>) and then optimized in the laboratory. PCR products were separated on 7 M urea denaturing polyacrylamide sequencing gels and dried before exposure to Kodak XAR film. LOH was determined by a combination of visual inspection and scanning densitometry of the autoradiographs (Radford *et al.*, 1995). A 2.5-fold difference in the relative allele intensity ratios between tumor and normal DNA in an informative tumor/normal pair was scored as LOH (allele1/allele2 in tumor compared to allele1/allele2 in normal). To maintain a conservative scoring approach, marginal allele reduction by inspection was not scored as LOH. Tumor samples with equivocal results were redissected in an attempt to obtain a “purer” specimen, and the experiments were repeated. All LOH designations were scored by two independent scientists and repeated to verify the result.

Genetic linkage maps. Publicly available data (<http://www.cephb.fr/cephdb/>) of 8 Centre d’Etude Polymorphisme Humaine (CEPH)

reference families from CEPH version 7 were used for loci D8S264, D8S258, and D8S133. An additional 8 CEPH families (66, 1333, 1334, 1340, 1341, 1345, 1375, and 1377) were typed for markers D8S262, D8S277, D8S439, D8S351, D8S503, D8S516, D8S552, D8S261, LPL, SFTP2, NEFL, D8S137, D8S259, PLAT, and D8S166 and then merged with other family genotypes from CEPH. For loci D8S520, D8S550, D8S265, D8S511, and D8S549, an additional 12 families were genotyped (the extra 4 families being 104, 1344, 1346, and 1408). Merged data were then processed using the linkage program CRIMAP (P. Green, unpublished data) with minimum odds for order of 1000:1 during the “build.” The program permutation option “flips” was used to determine the most likely marker order from the “build” results.

Radiation hybrid (RH) maps. We used the G3 radiation hybrid mapping panel (Research Genetics, Inc., Huntsville, AL) to construct the RH placement map. This 8 Krad panel, comprising 83 RH clones representing the whole human genome, was created by the Stanford Human Genome Center and has 500-kb resolution (Stewart *et al.*, 1997). Sequence-tagged site (STS) markers were assayed by PCR amplification and sized on 3% agarose gels stained with ethidium bromide. Each assay was performed twice; i.e., PCR products at the expected size were observed on each of the duplicate gels to be scored as positive. Marker data generated using the G3 panel were submitted to the Stanford RH server (<http://www-shgc.stanford.edu/RH/rhserverformnew.html>), which subsequently returned the results of analysis with a list of the highest-linked mapped markers, the lod score of the linkage, and the distance in centirays between the submitted marker and the linked marker on the map. However, the server compares only one submitted marker and the highest-linked marker at a time. To construct a RH placement map containing all of our markers, we also used the program RHMAP, version 3.0 (Lunetta *et al.*, 1996; <http://www.sph.umich.edu/group/statgen/software>) to analyze our G3 panel data together with the RH data from two flanking markers, SHGC-1955 (AFM287we5) and SHGC-13122 (D8S2061), from Stanford’s RH map (<http://www-shgc.stanford.edu/mapping/rh/>).

YAC and BAC clone contig construction. The CEPH YAC contig WC-1195 from WICGR was used for the initial contig construction. A copy of the CEPH YAC library was maintained in our laboratory, and clones used in the mapping were propagated on YPD medium plates. Ten isolated colonies from each YAC clone were tested using a “whole-cell PCR” assay to identify colonies positive for a marker(s) from the region. For whole-cell PCR, a small amount of an isolated colony was suspended in 5 μ l of deionized water, and the suspension was used directly as the template in a standard PCR. A standard ligation-mediated PCR method (Mueller and Wold, 1989; Vocero-Akbani *et al.*, 1996) was used in the development of the STS sJCW from YAC 770E9. PCR primers developed for the marker sJCW are 5’-TCAACAGCAGATTAGACACAGC-3’ and 5’-GYGGCTCTTATGC-CATGTAC-3’.

For BAC contig construction, we screened the human BAC library CITB-978SK-B and CITB-HSP-C, constructed by the California Institute of Technology (obtained from Research Genetics, Inc.). The library screen involved a total of 120 PCR for each STS assay. As with YAC clone isolation, each identified BAC clone was then verified by a whole-cell PCR assay using 10 randomly selected colonies as candidates. After the positive BAC clones were verified, single BAC clones were propagated in liquid medium, cells were harvested, and insert DNA was prepared using the Plasmid Midi-Kit from Qiagen Inc. (Chatsworth, CA). Each BAC clone was partially sequenced on an ABI 373 DNA sequencer (PE/ABI) from the insert ends using modified T7 (5’-GTAATACGACTCACTATAGGG-3’) and Sp6 (5’-CGCCAAGCTATTTAGGTGACAC-3’) primers. The sequence generated was analyzed for candidate PCR primer sequences using the program PRIMER 0.5 (Lincoln and Lander, MIT Center for Genome Research). STSs developed from BACs were tested using the NIGMS (No. 2) rodent/human hybrid panel, in which each hybrid cell contains a single human chromosome, and only chromosome 8-specific STSs were used as entry points for chromosome walking.

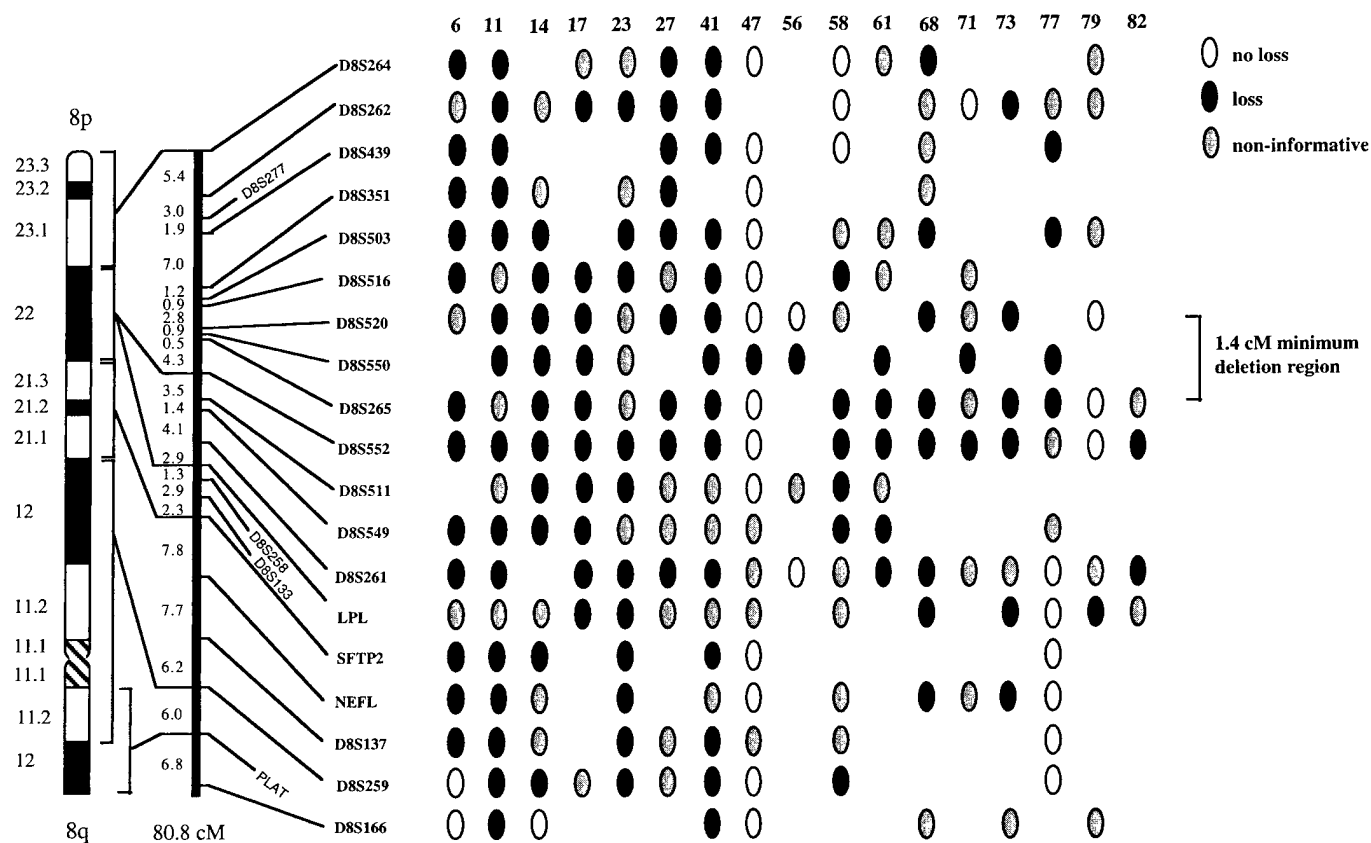


FIG. 1. LOH analysis using 18 mapped markers on 8p and one on 8q. An 80.8-cM sex-average multipoint linkage map is presented as a vertical bar to the right of the cytogenetic idiogram. Map distances are given to the left of the vertical map in cM. Genetic loci uniquely placed with odds for order of at least 1000:1 are given to the right of the map.

To estimate insert sizes, BAC clones were digested with *NotI*, and restriction fragments were separated on 1% agarose gels in 0.5× TBE buffer using MidRange II PFG markers (NE BioLabs) as size standards. Gels were electrophoresed at 6 V/cm with an initial pulse of 5 s and a final pulse of 15 s for 18 h at 14°C in a Bio-Rad CHEF DR II apparatus.

RESULTS

LOH Observed for DCIS on 8p

Sixty-five cases of DCIS were assayed for LOH using 18 mapped microsatellite markers on 8p and 1 on 8q. Of 61 samples informative for at least 1 marker on 8p, LOH was found in 17 tumor DNA samples (27.8%). Of the informative tumors, LOH was observed in 11/35 comedo, 3/14 cribriform, 1/4 solid, and 2/3 micropapillary samples. None of the 3 informative mixed and 2 informative papillary samples showed LOH. Sixteen of 44 informative samples with high nuclear grade showed LOH, 1/13 showed low nuclear grade, and 0/4 showed intermediate nuclear grade.

Ten tumor samples demonstrate loss of most of the short arm of chromosome 8 (Fig. 1). For example, tumor 6 had apparently lost an allele from all loci tested telomeric to D8S259. Tumors 56, 58, 71, and 77 contain deletions that localize the putative tumor suppressor gene between D8S520 and D8S261. Tumor 47 showed LOH for D8S550 but retained loci D8S265 and

D8S520, confining the deleted region to a minimum interval of 1.4 cM at 8p22-p23, between the markers D8S520 and D8S265 (Figs. 1 and 2). Tumor 79 showed LOH for LPL at 8p22 but retained three loci that were more telomeric, suggesting a second, more centromeric deletion region at 8p22-p23. The frequency of LOH for each marker tested is listed in Table 1. The highest rates of loss were found with markers D8S550, D8S552, and D8S503. No statistical correlation was found between LOH on 8p and histologic parameters such as subtype, nuclear grade, or the presence or absence of associated invasion. Tumor 47 showed LOH for D8S550 only, suggesting that the tumor suppressor gene would be found near this locus. Thus we concentrated our physical mapping efforts on this region.

RH Mapping of the Region near D8S550

We constructed a refined radiation hybrid map (Table 2) for the deletion interval using the Stanford G3 panel. A total of 10 markers including 6 microsatellite markers (D8S265, D8S520, D8S550, D8S1695, D8S1755, and D8S1759), one novel STS (sJCW), and 3 ESTs (A005E28, T96924, and WI-6800) that were identified through database searches were ordered on the map using our typing data and the program RHMAP, version 3.0. The marker D8S520 was mapped distal to the centromere and D8S1759 proximal to the centromere.

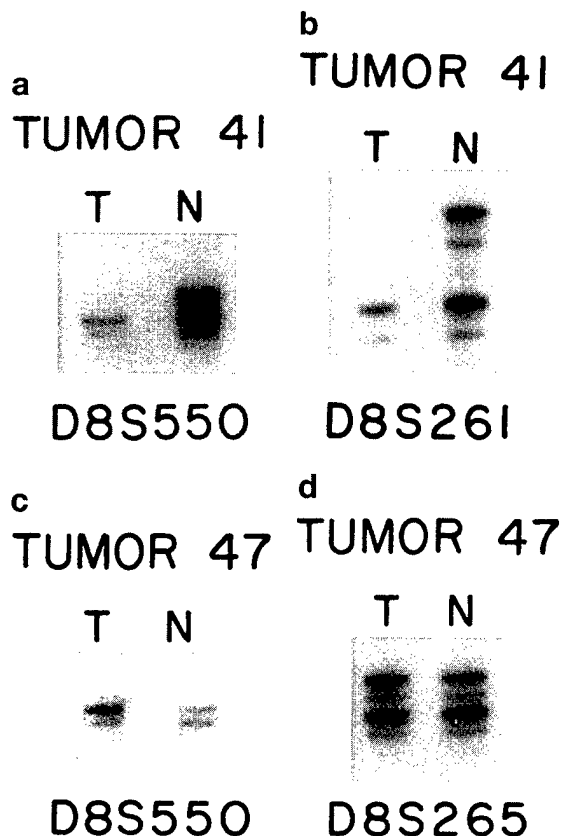


FIG. 2. Examples of LOH in DCIS samples. T indicates tumor DNA and N matched normal control. (a) Tumor 41 marker D8S550. The fold difference in integrated allele ratios between tumor and normal is 6.13. (b) Tumor 41 marker D8S261. The fold difference in integrated allele ratios between tumor and normal is 10.9. The upper allele is lost in the tumor in both cases. (c) Tumor 47 marker D8S550. (d) Tumor 47 marker D8S265. The lower allele is lost in the tumor for marker D8S550, but both alleles are retained for marker D8S265.

mere. The order of markers D8S265, D8S520, and D8S550 confirmed our genetic linkage map order. The order of other markers on the RH map also matched the position of those markers on our contig map except that of D8S1755 (Fig. 3, Table 2). When only 6 microsatellite markers were typed, D8S1755 was placed between markers D8S265 and D8S550, which corresponded to the contig map. However, when the RH data from 3 EST markers were integrated, D8S1755 mapped between D8S265 and D8S1759. Data errors such as false-positives or false-negatives or the limited resolution of the RH panel could explain this discrepancy. The distance of 42.3 cR, or approximately 1565 kb (assuming 37 kb per centiray; Stewart *et al.*, 1997), between the markers D8S265 and D8S520 demonstrated ~1:1 correspondence between genetic and physical distances for this interval.

Construction of an Integrated YAC/BAC Contig from D8S520 to D8S1759

We constructed a single, continuous, integrated YAC/BAC clone contig spanning a total distance of

TABLE 1
Eighteen Loci on 8p and One Locus on 8q Assayed for LOH

| Locus | No. LOH ^a | No. inf. ^b | % LOH |
|--------|----------------------|-----------------------|-------|
| D8S264 | 5 | 26 | 19.2 |
| D8S262 | 7 | 32 | 21.9 |
| D8S439 | 5 | 22 | 22.7 |
| D8S351 | 3 | 18 | 16.6 |
| D8S503 | 8 | 21 | 38 |
| D8S516 | 6 | 26 | 23 |
| D8S520 | 7 | 25 | 28 |
| D8S550 | 8 | 22 | 36.4 |
| D8S265 | 10 | 44 | 22.7 |
| D8S552 | 13 | 32 | 40.6 |
| D8S511 | 4 | 19 | 21 |
| D8S549 | 6 | 19 | 31.6 |
| D8S261 | 9 | 31 | 29 |
| LPL | 5 | 23 | 21.7 |
| SFTP2 | 5 | 30 | 16.6 |
| NEFL | 5 | 22 | 22.7 |
| D8S137 | 4 | 22 | 18.2 |
| D8S259 | 5 | 26 | 19.2 |
| D8S166 | 2 | 21 | 9.5 |

^a No. LOH, number of LOH cases detected from informative tumor samples tested for the marker.

^b No. inf., number of informative samples observed from 65 tumor cases tested.

~1730 kb covering the putative TSG region of ~1100 kb based on sizes of BAC clones. This sequence-ready contig comprises 13 YACs and 27 BACs including 54 STSs from BAC end sequences (Table 3).

YAC Clone Contig

Based on the publicly available contig maps from WICGR (<http://carbon.wi.mit.edu>), we isolated 13 YACs using the six microsatellite markers listed in RH

TABLE 2
RH Map Spanning the Deletion Interval

| Marker | Distance (cR) |
|----------------|---------------|
| Telomere | |
| SHGC-1955 | |
| (AFM287we5) | 4.7 |
| A005E28 | 4.8 |
| sJCW | 4.9 |
| D8S520 | 20.8 |
| D8S550 | 16.2 |
| WI-6800 | 0.0 |
| T96924 | 5.3 |
| D8S265 | 20.6 |
| D8S1755 | 17.6 |
| D8S1759 | 0.0 |
| D8S1695 | 11.6 |
| SHGC-13122 | |
| (D8S2061) | |
| Centromere | |

Note. 1 cR \approx 37 kb (Stewart *et al.*, 1997). Boldface type indicates markers we assayed using the Stanford G3 panel.

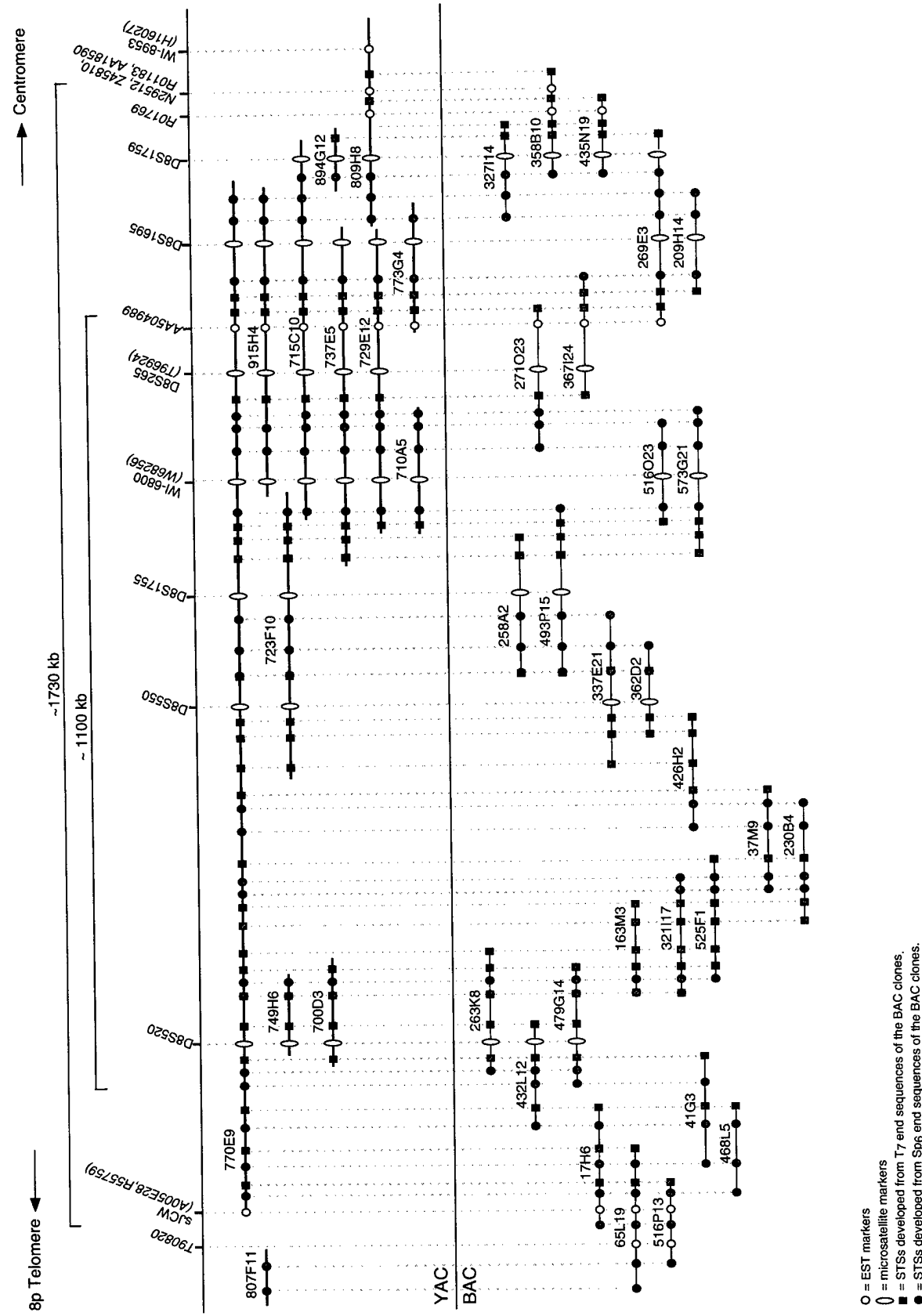


FIG. 3. An integrated YAC/BAC clone contig spanning the 1.1-cM deletion region.

○ = EST markers
 ○ = microsatellite markers
 ■ = STSs developed from T7 end sequences of the BAC clones.
 ● = STSs developed from Sp6 end sequences of the BAC clones.

TABLE 3

Primer Sequences and Annealing Temperatures for 54 Novel STS Markers on BAC Contig

| STS | PCR primer | GenBank Accession No. | Product size (bp) | Annealing temperature (°C) |
|------------------------|--|--------------------------|----------------------|----------------------------------|
| 17H6-Sp ₆ | 5'-CAGCCCTCAACAAAGGTAGG-3'/5'- CTCTTTCAGGTGTGGCCATT-3' | AF122927 | 117 | 56 |
| 17H6-T ₇ | 5'-TCTTCACCTCGTGGCACTG-3'/5'- GGACAATCCATCCATCCATC-3' | AF122928 | 198 | 55 |
| 37M9-Sp ₆ | 5'-GGCTTGTGTTGTGGGCTTATA-3'/5'- GGTGAGTTTCAAGAGGGGTT-3' | AF122943 | 148 | 56 |
| 37M9-T ₇ | 5'-TAACACTCTCCTGCACTCTGCCTC-3'/5'- AATGTGCTCTTTCCCTGCCCAAAGG-3' | AF122944 | 198 | 62 |
| 41G3-Sp ₆ | 5'-ACAAGCTAAGGGCCATCCTT-3'/5'- GATGCCACCAGCATGTGG-3' | AF122933 | 126 | 56 |
| 41G3-T ₇ | 5'-GGTATTTGTGAGCCTTCCCA-3'/5'- GAAACACAGCACAGATAGCA-3' | AF122934 | 228 | 59 |
| 65L19-Sp ₆ | 5'-CCCAAAACAGTCATTGAAACG-3'/5'- CGATAAATGCTTGCTTCATCC-3' | AF122931 | 143 | 54 |
| 65L19-T ₇ | 5'-GGGAAACAGGCACTTCACAT-3'/5'- TTTGATCATGGGGTGGATTT-3' | AF122932 | 163 | 56 |
| 163M3-Sp ₆ | 5'-ACTCAACCTCCTGGGTTCCCT-3'/5'- GTTGACAGCCTCACCTGTT-3' | AF122937 | 133 | 58 |
| 163M3-T ₇ | 5'-CTCCTCACTTGGGAAAATGTG-3'/5'- CTCCAAGAAAATGATGACTGC-3' | AF122938 | 127 | 58 |
| 209H14-Sp ₆ | 5'-CCAGCTTCCCTTTGAGACAG-3'/5'- AGGCAGGGCTTACTTCACCT-3' | AF124288 | 136 | 58 |
| 209H14-T ₇ | 5'-GACTCAGTTGCTTCCAAGGC-3'/5'- TCAGCCATCAAGCTTCACAG-3' | AF124289 | 108 | 58 |
| 230B4-Sp ₆ | 5'-TGATCCAGGGACAGAGCAAGAAG-3'/5'- GAGCCAGGAGCATGAGAATATGG-3' | AF122939 | 121 | 62 |
| 230B4-T ₇ | 5'-CCATCTTTATTCCACAGGAAGAGG-3'/5'- GCAACAAGAGTGAAACTCCGTCTC-3' | AF122940 | 180 | 62 |
| 258A2-Sp ₆ | 5'-CCAACCTAGCCGTGGTTAGT-3'/5'- TGTGTTCAAGTACCATGGGT-3' | AF124276 | 162 | 57 |
| 258A2-T ₇ | 5'-CATCAGGCATTGAAGTCACA-3'/5'- TAAGCACGCACACAGCC-3' | AF124277 | 146 | 55 |
| 263K8-Sp ₆ | 5'-TGAGCACACAGGATTTTACG-3'/5'- CGTCATCATACCCAGAGTC-3' | AF124266 | 150 | 55 |
| 263K8-T ₇ | 5'-TCTCGTTTGCTTATGACCTG-3'/5'- CTAACAACTAGATTGGAAGGAGC-3' | AF124267 | 138 | 57 |
| 269E3-Sp ₆ | 5'-AGTGCTGGGGGGGATTTTGTG-3'/5'- ACGTGGCAGTGGGTTAGAGTTG-3' | AF124290 | 148 | 61 |
| 269E3-T ₇ | 5'-GCAGCTGATCTCTCTCCACC-3'/5'- CCTTTCCTCCATGTTGGCT-3' | AF124291 | 202 | 58 |
| 271O23-Sp ₆ | 5'-GTCCTGGGGGTCAAAAATCT-3'/5'- GCTTGTGTATCCACTCCGTT-3' | AF124284 | 131 | 56 |
| 271O23-T ₇ | 5'-TGCCCACTTTCCCTCATGG-3'/5'- TTCTAAGCCAGTGAGTTGATGC-3' | AF124285 | 155 | 57 |
| 321I17-Sp ₆ | 5'-TGTGCGCATAGTAGGAATGA-3'/5'- CGCAAAAAGGTGAGAGAGAG-3' | AF122941 | 213 | 57 |
| 321I17-T ₇ | 5'-ACTCAACCTCCTGGGTTCCCT-3'/5'- TCTGAGCTCCTTTCCACGT-3' | AF122942 | 151 | 57 |
| 327I14-Sp ₆ | 5'-TGAAGTCTGCTCAGCACC-3'/5'- CCTGCAAAAAGGCTGACCTAG-3' | AF124292 | 199 | 59 |
| 327I14-T ₇ | 5'-CATCTGTTGATGGGCATTTG-3'/5'- TGGCAATCCCCCTTCTAAG-3' | AF124293 | 138 | 55 |
| 337E21-Sp ₆ | 5'-ATATGTGCACCCAATGCAG-3'/5'- GAGTTCAATTACGGGATAGCC-3' | AF124272 | 174 | 56 |
| 337E21-T ₇ | 5'-TAACCGTGACACTCTACTGCC-3'/5'- TTCATGATCTCAAGGCTGTG-3' | AF124273 | 203 | 53 |

TABLE 3—Continued

| STS | PCR primer | GenBank Accession No. | Product size (bp) | Annealing temperature (°C) |
|------------------------|---|--------------------------|----------------------|----------------------------------|
| 358B10-Sp ₆ | 5'-TGCTGTGCTAGCCAGTTCC-3'/5'- AACAGGTACCACAAGGCAGC-3' | AF124294 | 178 | 60 |
| 358B10-T ₇ | 5'-GCCCTTGCTTAAGAACTCAA-3'/5'- ACCCAGGCATATTGGTGAGT-3' | AF124295 | 205 | 56 |
| 362D2-Sp ₆ | 5'-TGCAGCCTCATTCTGTTGTC-3'/5'- CTCATCAGKGAGTCTGGAGTCG-3' | AF124274 | 133 | 57 |
| 362D2-T ₇ | 5'-ATAGGGTTGACACTGCCACC-3'/5'- TTGGCCAGGCATGGTAGT-3' | AF124275 | 132 | 58 |
| 367I24-Sp ₆ | 5'-AAGGAAGCGCCCAATTC-3'/5'- ACTGGCTTTGCTGGAATTTT-3' | AF124286 | 120 | 56 |
| 367I24-T ₇ | 5'-TGCTGAGCTGGGTGAAATC-3'/5'- CCATGAAGGAAAGGACCTGA-3' | AF124287 | 147 | 56 |
| 426H2-Sp ₆ | 5'-CCCCAAACGTATCAATCCAG-3'/5'- TCCATTTCCAGTGTGTTGGA-3' | AF122945 | 167 | 59 |
| 426H2-T ₇ | 5'-TGACAGAAGGATCTGGGACC-3'/5'- GGAAGTTGAGGCTGCAGAGA-3' | AF122946 | 181 | 60 |
| 432L12-Sp ₆ | 5'-AAAGGGGAGACATTCGGC-3'/5'- TGGGAGGAGAGAGCCAGATA-3' | AF124268 | 149 | 56 |
| 432L12-T ₇ | 5'-AACCTGCACTGCCAATAACC-3'/5'- AAGTTTTTGTGACCTCGGG-3' | AF124269 | 147 | 54 |
| 435N19-Sp ₆ | 5'-CTTTTGGTCAAAGCCCACAT-3'/5'- TTGCAGTGAAGGCTGAAATG-3' | AF124296 | 118 | 54 |
| 435N19-T ₇ | 5'-TGTGTTAGATGATTTTGCCTA-3'/5'- ATATGCCTAGCCTACCAAACA-3' | AF124297 | 100 | 57 |
| 468L5-Sp ₆ | 5'-ACCCAACAACGCCATAAAAC-3'/5'- GTGGGGGTGTTTTTCTTGTC-3' | AF122935 | 162 | 58 |
| 468L5-T ₇ | 5'-TTCCTTCCACTGGATACGC-3'/5'- TTTTCTGTGGTGGGGTAGAC-3' | AF122936 | 141 | 57 |
| 479G14-Sp ₆ | 5'-GCACAGAGGATTTTTACGGC-3'/5'- ACACGTCATCATCACCCAGA-3' | AF124270 | 150 | 60 |
| 479G14-T ₇ | 5'-CACCAGAGAGGTGCAAAAACA-3'/5'- TTGCAGCCAAGTGGTATTG-3' | AF124271 | 156 | 56 |
| 493P15-Sp ₆ | 5'-ACCATCAAGCAACTWGGTTTCCAG-3'/5'- TTCATGMTGACTTGGGGTGC-3' | AF124278 | 145 | 58 |
| 493P15-T ₇ | 5'-CCATGCTACCATTGCACTCCTC-3'/5'- TTGATGGGATGTGGGGCAAAGTGG-3' | AF124279 | 143 | 61 |
| 516O23-Sp ₆ | 5'-TCTGCCACGTTTTTGGTATCAGG-3'/5'- AAATCCCTGGACACACACACCC-3' | AF124280 | 257 | 63 |
| 516O23-T ₇ | 5'-TCTTCAAGACCATGCCTG-3'/5'- GGCATAACATCACCATGCTCA-3' | AF124281 | 278 | 56 |
| 516P13-Sp ₆ | 5'-TGGCTTAGTGCTAACCCCTCA-3'/5'- ATGTTGGAGTCCATAAACCTCG-3' | AF122929 | 101 | 54 |
| 516P13-T ₇ | 5'-CATAGAGGCATTTTGGTTACG-3'/5'- ATGGAAAAGAGATCTAACAGCG-3' | AF122930 | 102 | 54 |
| 525F1-Sp ₆ | 5'-CCTGGGTTCTCTCGTGAATCTTC-3'/5'- TCCTTTTCCACGTTGACAGCC-3' | AF122947 | 136 | 62 |
| 525F1-T ₇ | 5'-GGTACTATCCACACTCGTTGGGGT-3'/5'- AACATGCAATACCAAGGATGCG-3' | AF122948 | 122 | 60 |
| 573G21-Sp ₆ | 5'-CTGCCTCTGCAAAATTTGT-3'/5'- CGAGCACCAGATTTGCTTTT-3' | AF124282 | 196 | 57 |
| 573G21-T ₇ | 5'-ACCCATCATCCCTGGAAG-3'/5'- GTAGTGCCAATTCCTTTCA-3' | AF124283 | 127 | 56 |

mapping and one additional marker, WI-8953, that appeared to fall within the relevant interval. We confirmed the presence of these markers within the YAC

clones 700D3, 710A5, 715C10, 723F10, 729E12, 737E5, 749H6, 770E9, 807F11, 809H8, 894G12, and 915H4 (Fig. 3).

BAC Library Screening

The initial screening of the human BAC library was conducted with 10 markers including 6 microsatellite markers (D8S520, D8S550, D8S1755, D8S265, D8S1695, and D8S1759) and 4 ESTs (T96924, WI-6800, N29512, and Z45810) anchored on the YAC contig (Fig. 3). An average of 5 BAC clones per marker were identified and isolated using PCR assays. After the positive BAC clones were verified, the insert ends of each clone were sequenced. The chromosomal origin of each newly generated STS was verified using a panel of rodent/human hybrids. Only the clones containing chromosome 8-specific STSs at both ends were used for contig construction.

Chromosome Walking

From our initial screening, we were able to build a continuous BAC clone contig from D8S550 to D8S1759, leaving a gap between the markers D8S520 and D8S550 (Fig. 3). To close the gap, STSs developed from T7-end sequences of clones 263K8 and 337E21 were used for secondary BAC library screening. Three clones, 163M3, 321I17, and 525F1, and one clone, 426H2, were identified and isolated from STSs 263K8-T7 and 337E21-T7, respectively. Both insert ends of these newly isolated BAC clones were sequenced, and six chromosome 8-specific STSs were generated. Six STSs (163M3-Sp6, 163M3-T7, 321I17-Sp6, 321I17-T7, 525F1-Sp6, and 426H2-T7) were mapped back onto the YAC/BAC contig using PCR assays, leaving a gap between 525F1-T7 and 426H2-Sp6. From another BAC library screening experiment, five clones were identified and isolated using a PCR assay from the STS 426H2-Sp6; four of them were found to be positive for the STS from 525F1-T7. Among these, four novel STSs (37M9-Sp6, 37M9-T7, 230B4-Sp6, and 230B4-T7) were developed from insert end sequences and mapped back to the contig by overlap analysis (Fig. 3). This completed construction of a continuous BAC clone-based contig of the region.

All of the 27 BAC clones used for contig construction were digested with restriction enzyme *Not*I, from which estimates of the sizes of the clones could be made. This information enabled more precise determination of the size of the region spanned by the contig and of the region containing the putative TSG. Based on the estimated sizes of the BAC clones, the contig spans approximately 1730 kb and the LOH deletion region between D8S520 and D8S265 spans ~1100 kb (Table 4).

TSG Candidate Identification and Database

Homologies

First, databases were searched for candidate genes within the region of chromosome 8p22-p23. We verified by PCR assay the locations of 11 ESTs reported to map within this region. EST A005E28 identified by the

TABLE 4

BAC Clones Mapped on the Contig Spanning the 1.1-cM Deletion Interval

| Clone | Size (kb) |
|--------|------------------|
| 17H6 | 175 ^a |
| 37M9 | 90 ^a |
| 41G3 | — ^b |
| 65L19 | 258 |
| 163M3 | 93 |
| 209H14 | — |
| 230B4 | 143 |
| 258A2 | 130 |
| 263K8 | 178 |
| 269E3 | 172 ^a |
| 271O23 | 135 ^a |
| 321I17 | 100 |
| 327I14 | — |
| 337E21 | 255 |
| 358B10 | 140 ^a |
| 362D2 | 135 ^a |
| 367I24 | 121 |
| 426H2 | 170 ^a |
| 431L12 | 114 ^a |
| 435N19 | — |
| 468L5 | — |
| 479G14 | 125 ^a |
| 493P15 | 170 ^a |
| 516O23 | 175 ^a |
| 516P13 | 121 |
| 525F1 | 127 ^a |
| 573G21 | — |

Note. —, clones not sized.

^a Clones used to construct the minimal clone set for the contig spanning the region between markers of D8S520 and D8S1759.

Institute for Genomic Research (TIGR) mapped to YAC 770E9. Like STS sJCW, it tested positive for BAC clones 17H6, 65L19, and 516P13. EST T90820 also tested positive for BAC clones 65L19 and 516P13, but not for YAC 770E9 (Table 5). EST T96924 mapped to YACs 770E9, 915H4, 715C10, 737E5, and 729E12. It also tested positive for BAC clones 271O23 and 367I24 identified by D8S265. Two cDNA clones (T96924, 0.8 kb, and W67504, 1.6 kb) were isolated and fully sequenced. The two sequences were compiled into one continuous sequence using ClustalW alignment of MacVector 6.0 program (Oxford Molecular, <http://www.oxmol.com/>). This sequence contains (CA)₂₂ repeats that could be used to develop a microsatellite marker. BAC clones 271O23 and 367I24 were also partially sequenced with a primer selected from the 3' end of cDNA T96924. The sequences from these two BAC clones contain a (CA)₂₂ repeat and match the sequence from cDNA W67504.

A cluster of 6 ESTs that appeared to localize to the region from the UniGene (<http://www.ncbi.nlm.nih.gov/UniGene>) map were placed at the proximal end of our YAC/BAC contig. They all mapped to YAC 809H8. EST R01769 tested positive for BAC clones 358B10 and 435N19. ESTs AA018590, N29512, R01183, and Z45810 tested positive for clone 358B10 only. EST H16027 did not identify any BAC clone on the contig

TABLE 5
Eleven ESTs Mapped to the YAC/BAC Contig

| ESTs | Accession No. | Marker name | UniGene | Mapping data |
|----------|---------------|-------------|---|---|
| R55759 | G26262 | A005E28 | Unidentified, Hs. 26458 | BAC 17H6, 65L19, 516P13 |
| T90820 | G19346 | SHGC-17264 | Unidentified, Hs. 133548 | BAC 65L19, 516P13 |
| R01769 | G28044 | SHGC-33472 | Unidentified, Hs. 119611 | YAC 809H8; BAC 358B10, 435N19 |
| R01183 | R01183 | stSG327 | Unidentified, Hs. 4883 | YAC 809H8; BAC 358B10 |
| T96924 | T96924 | SGC33822 | Unidentified, Hs. 35453 | YAC 715C10, 729E12, 737E5, 770E9; BAC 271O23, 367I24 |
| N29512 | N29512 | SGC30677 | Farnesyl-diphosphate farsyltransferase, Hs. 48876 | YAC 809H8; BAC 358B10 |
| AA018590 | AA018590 | SGC30677 | Farnesyl-diphosphate farsyltransferase, Hs. 48876 | YAC 809H8; BAC 358B10 |
| Z45810 | Z45810 | A005M25 | Unidentified, Hs. 94151 | YAC 809H8; BAC 358B10 |
| W68256 | W68256 | SHGC-24302 | Unidentified, Hs. 27194 | YAC 710A5, 715C10, 729E12, 737E5, 770E9; BAC 516O23, 573G21 |
| AA504989 | AA504989 | | Unidentified | YAC 715C10, 729E12, 737E5, 770E9; BAC 271O23, 367I24, 269E3 |
| H16027 | H16027 | WI-8953 | Cathepsin B precursor, Hs. 84898 | YAC 809H8 |

(Table 5). Eight of these 11 ESTs appeared to be unique since they did not identify homologous sequences in the publicly available databases. ESTs N29512 and AA018590 identified by the marker SGC30677 in UniGene were developed from cDNA clones with sequence homology to human farnesyl diphosphate farnesyltransferase. EST H16027, which is one of the ESTs detected by the marker WI-8953, is derived from a cDNA with homology to human cathepsin B precursor.

In addition to the previously reported ESTs from the region, both end sequences generated from BAC clones were analyzed with BLASTN. Only the Sp6-end sequence from clone 269E3 identified a homologous sequence, Stratagene's fetal retina *Homo sapiens* cDNA clone (AA504989). About 1.5 kb of this cDNA clone has been sequenced. Another cDNA clone (W68256, 0.6 kb) identified by the marker WI-6800 was isolated, and the insert was fully sequenced. A total of nearly 25 kb of sequence was generated from BAC insert ends and four cDNA inserts.

DISCUSSION

DCIS is a preinvasive form of breast cancer that is being detected at an increasing rate due to screening mammography. In some cases it represents a step in the pathway to invasive breast cancer, although it is not always an obligate precursor (Rogers, 1987). In our study of 65 cases of DCIS, using 18 markers on 8p and 1 on 8q, LOH was seen on 8p in 29% of informative cases. Our data identify the smallest common region of LOH to be located at 8p22-p23 in an approximately 1.4-cM region between D8S265 and D8S520. The pattern of LOH for tumor 79 suggests the presence of at least one other tumor suppressor gene proximal to the 8p22-p23 locus.

Other studies have also reported evidence of LOH on 8p in DCIS (Aldaz *et al.*, 1995; Seitz *et al.*, 1997b;

O'Connell *et al.*, 1998; Anbazhagan *et al.*, 1998). Anbazhagan *et al.* (1998) found the most common region of loss in 60 invasive ductal breast tumors to be at 8p21.3-p23.3 between D8S560 and D8S518. The 1.4-cM region of loss we have identified lies within this region. Several studies have attempted to correlate 8p LOH and the ability of a tumor to metastasize (Aldaz *et al.*, 1995; Yaremko *et al.*, 1996; Anbazhagan *et al.*, 1998; Dahiya *et al.*, 1998). Yokota *et al.* (1999) observed a minimal region of LOH on 8p, in a region flanked by markers D8S511 and D8S1991, that was associated with advanced tumor stage and aggressive histologic subtype. This region does not overlap with our minimal region. However, the two studies analyze different tumor types. In our data we observed no correlation between LOH on 8p and tumor grade or the presence of an invasive component. Both observations are thus consistent with the presence of more than one putative tumor suppressor gene on 8p, one associated with early events in breast oncogenesis and one associated with tumor invasion.

Chromosome 8p22-p23 has also been associated with potential tumor suppressor genes involved in other types of human cancers. Wright *et al.* (1998) defined three LOH regions of overlap in ovarian cancer, one at 8p22, near marker LPL, and two at 8p23, near D8S549 and the region distal to D8S1759, with the highest LOH rates at D8S264, D8S550, and D8S1827. Frequent allelic losses at D8S550 have also been observed in hepatocellular carcinoma (Nagai *et al.*, 1997). Thus it is likely that a putative tumor suppressor gene in the region of D8S550 may be involved not only in preinvasive breast cancer but also in other human cancers.

To clone and characterize efficiently the putative tumor suppressor gene in this region of 8p22-p23, we constructed a meiotic linkage map to resolve the ambiguity of marker order within this region. A radiation

hybrid map for the 1.4-cM LOH deletion interval in the region was also constructed. The recently published GeneMap'98 (Deloukas *et al.*, 1998; <http://www.ncbi.nlm.nih.gov/genemap98>) has several positional discrepancies between GB4 and G3 RH maps in the deletion interval. For example, marker WI-6800 was placed distal to D8S520 on the GB4 map while the G3 map placed WI-6800 proximal to D8S550. A direct comparison of the two RH maps reveals a limited number of shared STSs; thus precise marker orders cannot be determined from these data. When comparing markers D8S520, D8S550, WI-6800 (SHGC-24302), and SGC33822 (T96924), our RH data agreed more closely with the locus order on the G3 map than that on the GB4 map. Although our RH map position for the marker D8S1759 is different from that on the G3 map, the position was verified with our BAC clone-based contig.

Previously, physical maps that include part of 8p22-p23 have been constructed based primarily on assembly of existing YAC contigs or RH mapping (Bookstein *et al.*, 1994; Bova *et al.*, 1996; Farrington *et al.*, 1996). However, the calculated order and the distances between loci can be affected by the rearrangements, internal deletions, and chimerisms that can occur in YACs. We have confirmed marker orders with BAC clones, which are known to have low frequencies of these events. Our integrated YAC/BAC contig, extending from the distal marker 65L19-Sp6 (~200 kb distal to D8S520) to the proximal marker WI-8953 (~100 kb centromeric to D8S1759), has enabled a more precise measurement of the physical distance and marker order of the 8p22-p23 region. The contig is also a valuable framework to integrate additional cDNAs, STSs, and ESTs reported in the region. Eleven ESTs (Fig. 3, Table 5) were placed onto the clone contig, and they will be used for preliminary candidate gene analysis.

Additional genes of interest from this region may be found in the literature. Hughes *et al.* (1998) found a novel amplicon at 8p22-p23 resulting in cathepsin B (CTSB), a cancer-related gene that is overexpressed in esophageal adenocarcinoma. The EST H16027, which is similar to the CTSB gene, maps to the proximal boundary of the contig at the marker WI-8953 (Fig. 3, Table 5) and may represent this gene. Harder *et al.* (1997) isolated the gene for human β -defensin-2 (human BD-2, also known as DEFB2), and mapped it to YAC clone 773G4, within the preexisting 8p22-p23 YAC contig WC-1195, which is anchored to our contig by the marker D8S1695.

The STS information and the overlapping BAC clones presented in this report provide the starting material for large-scale sequencing of this genomic region. The contig will also be a useful resource contributing to gene identification and studies of gene expression patterns in the region.

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479

Chromosome 8p deletions in ductal carcinoma *in situ* (DCIS) of the breast. J. C. Wang, D. Radford, M. Holt, C. Helms, W. Brandt, and H. Donis-Keller. Div. Hum. Mol. Genet., Dept. Surgery, Washington University School of Medicine, St. Louis, MO.

In order to efficiently clone and characterize a putative tumor suppressor gene for DCIS that we have mapped within chromosome 8p (Radford et al., 1995, *Can. Res.* 55:3399), we are constructing a fine structure integrated physical and genetic map for this region. A linkage map was first constructed using 23 markers we genotyped through the CEPH reference pedigrees with loci uniquely placed (odds for order 1000:1). The map extends from the telomere of 8p to just beyond the centromere (80.8 cM). The average distance between markers is 3.5 cM.

Based on this map, 18 markers on 8p and two 8q markers were selected for LOH analysis with 65 examples of microdissected DCIS. Of 61 informative specimens, LOH was observed for at least one marker on 8p in 17 tumors (28%). Ten tumors have lost a large portion of the short arm. The smallest common region of deletion localizes to a region of 1.4 cM on 8p22-23. To refine this region, six markers were used to screen Stanford G3 and Genebridge 4 RH panels. The data from the Stanford G3 panel were analyzed and a RH map has been constructed using RHMAPPER. The map spans 17.1 cR in distance with the marker D8S520 placed distal to the centromere and D8S1759 proximal to the centromere. The data from the GB4 panel were submitted to the WICGR Mapping Service center and the order of these six markers has been determined. The locations of D8S520 and D8S1759 were confirmed, however the position of D8S265, D8S1755, and D8S1695 differ from the position found on the G3 RH map. Currently we are using 7 markers and 4 ESTs to screen YAC and BAC libraries for the construction of a contig for this region. A preliminary physical map based on clones isolated thus far confirms the marker order we determined using the Stanford G3 RH panel.

Allelic Loss and the Progression of Breast Cancer¹Diane M. Radford,² Nancy J. Phillips, Keri L. Fair, Jon H. Ritter, Matthew Holt, and Helen Donis-Keller

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Abstract

To study genetic changes and the evolution of breast cancer, we assayed for loss of heterozygosity (LOH) in 12 sets of synchronous carcinoma *in situ* (CIS) and invasive cancer, compared to normal control DNA. Microsatellite markers were used, which map to each nonacrocentric autosomal arm. Eight tumor sets demonstrated LOH of the same allele in both concurrent invasive cancer and ductal CIS, for a total of 18 chromosomal loci. Three of nine tumor sets showed LOH on 11p. In two of these sets, LOH was seen on 11p only in the invasive tumor, not the corresponding CIS. One of these tumors also exhibited allelic loss in the invasive tumor for 4 loci, all of which were retained in the noninvasive tumor. For two tumor sets, LOH was mirrored in matched ductal CIS, invasive tumor, and lymph node metastasis. The maintenance of LOH for certain loci throughout the stages of breast cancer suggests clonality of the cancer cells.

Introduction

Human solid tumors are believed to arise due to a multistep process involving the activation of oncogenes and the inactivation of tumor suppressor genes. This cascade of genetic events releases the cell from normal regulatory controls, and allows the formation of the malignant phenotype, followed by the development of invasion and metastasis. In certain cancers, a chronology for these events has been determined, such as in the transition from benign polyp to invasive cancer of the colorectum (1). Little is known about the events that are involved in the transition of CIS³ of the breast to invasive cancer. DCIS is a noninvasive carcinoma and is a precursor to invasion in some cases, although it is not an obligate precursor (2). The subtypes of DCIS (such as comedo, cribriform, solid, papillary, and micropapillary) differ in biological behavior. We have studied the allelic loss of chromosomal loci in DCIS (3-5) and have established that the chromosomal arms that show the most frequent allelic losses in DCIS are 8p, 13q, 16q, 17p, and 17q. Other authors have reported LOH in DCIS on chromosomes 11q, 2p, and 4q (6, 7). To determine which chromosomal loci are involved in breast cancer progression, we continued our studies on examples of synchronous CIS and adjacent invasive breast cancer and, when available, lymph node metastases.

Materials and Methods

Twelve samples of tumor and control were obtained; 10 from the archives of the Department of Pathology, St. Louis University, 1 from Jewish Hospital (St. Louis, MO) and 1 from Barnes Hospital (St. Louis, MO). Paraffin-embedded, formalin/alcohol-fixed material was archived between 1988 and 1993. In two of these cases, lymph nodes involved with metastatic tumor were

also obtained for LOH assay. The pathologists (N. J. P. and J. H. R.) determined the subtype of the DCIS component and its nuclear grade (high, intermediate, or low). A microdissection technique was used to separate invasive tumor from CIS and from adjacent normal stroma (3). Uninvolved lymph node DNA from the same patient was used as normal control. DNA was extracted as described previously (3). LOH was assayed using PCR of microsatellite markers. The markers used and their PCR conditions have been described (5). PCR products were separated on 3M urea denaturing polyacrylamide sequencing gels and were dried before exposure to Kodak XAR film. LOH was determined by a combination of visual inspection and scanning densitometry of the autoradiographs. The technique used for scanning densitometry is fully described in Ref. 5. A 3-fold difference in the relative allele intensity ratios between tumor DNA and normal DNA in an informative tumor normal pair was scored as LOH (allele 1/allele 2 in tumor compared to allele 1/allele 2 in normal).

Results

Of the 12 samples of CIS available for study, 7 were comedo, high nuclear grade DCIS (tumors 46, 47, 49, 50, 57, 58, and 69). Tumor 48 was of the cribriform subtype of DCIS, intermediate nuclear grade; tumor 55: cribriform, low nuclear grade; tumor 56: micropapillary, low nuclear grade; tumor 70 terminal duct CIS (a variety of CIS that has histological features that resemble both lobular and DCIS), high nuclear grade; and tumor 72: mixed variety of DCIS, intermediate nuclear grade. In all cases the invasive component was of the invasive ductal variety.

We studied chromosomal deletions by assaying for LOH using 48 microsatellite markers that map to 39 nonacrocentric autosomal arms. The data obtained are summarized graphically in Fig. 1. For a total of 18 chromosomal loci, LOH could be demonstrated in both the CIS and invasive component of the tumor. LOH was observed in both DCIS and invasive tumor for loci on 1p (1 of 8 informative tumor sets), 1q (1 of 7), 7q (2 of 4), 8p (2 of 5), 11p (1 of 9), 13q (2 of 5), 16q (3 of 7), 17p (2 of 5), 17q (2 of 8), 18p (1 of 4), 18q (2 of 8), and 22q (1 of 6). In tumor 47, LOH at loci on 7q and 17p was seen in DCIS and LNM; the invasive tumor could not be assayed due to insufficient sample. Similarly, for tumor 48, LOH was seen at locus *CD3D* (11q) for both DCIS and LNM. At several loci the LOH pattern in DCIS and invasive component was also mirrored in the LNM [e.g., loci *DIS165*, *APOA2*, and *NM23* (tumor 47) and loci *D16S266* and *D16S402* (tumor 48)].

The DNA from two cases (cases 46 and 49) showed LOH on 11p in the invasive tumor but not the DCIS component. Fig. 2 shows examples of LOH for these tumors. Examination of Fig. 2, A-C shows virtually complete loss of an allele in both the DCIS and the invasive component of tumor 46 for loci on 13q, 17p, and 22q. This indicates that the sample was carefully dissected and that significant contamination is not obscuring LOH in the DCIS component. Fig. 2 D shows the pattern of allelic loss for the same tumor sample using a marker on 11p. Two alleles are seen in the DCIS; however, there is a great reduction of the signal from one allele in the invasive tumor. These data were confirmed by densitometry. The fold difference in integrated allele ratios between DCIS and normal for the marker *D11S861* was 1.42 for tumor 46 and 1.74 for tumor 49. These figures do not

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³ The abbreviations used are: CIS, carcinoma *in situ*; DCIS, ductal CIS; LOH, loss of heterozygosity; LNM, lymph node metastases.

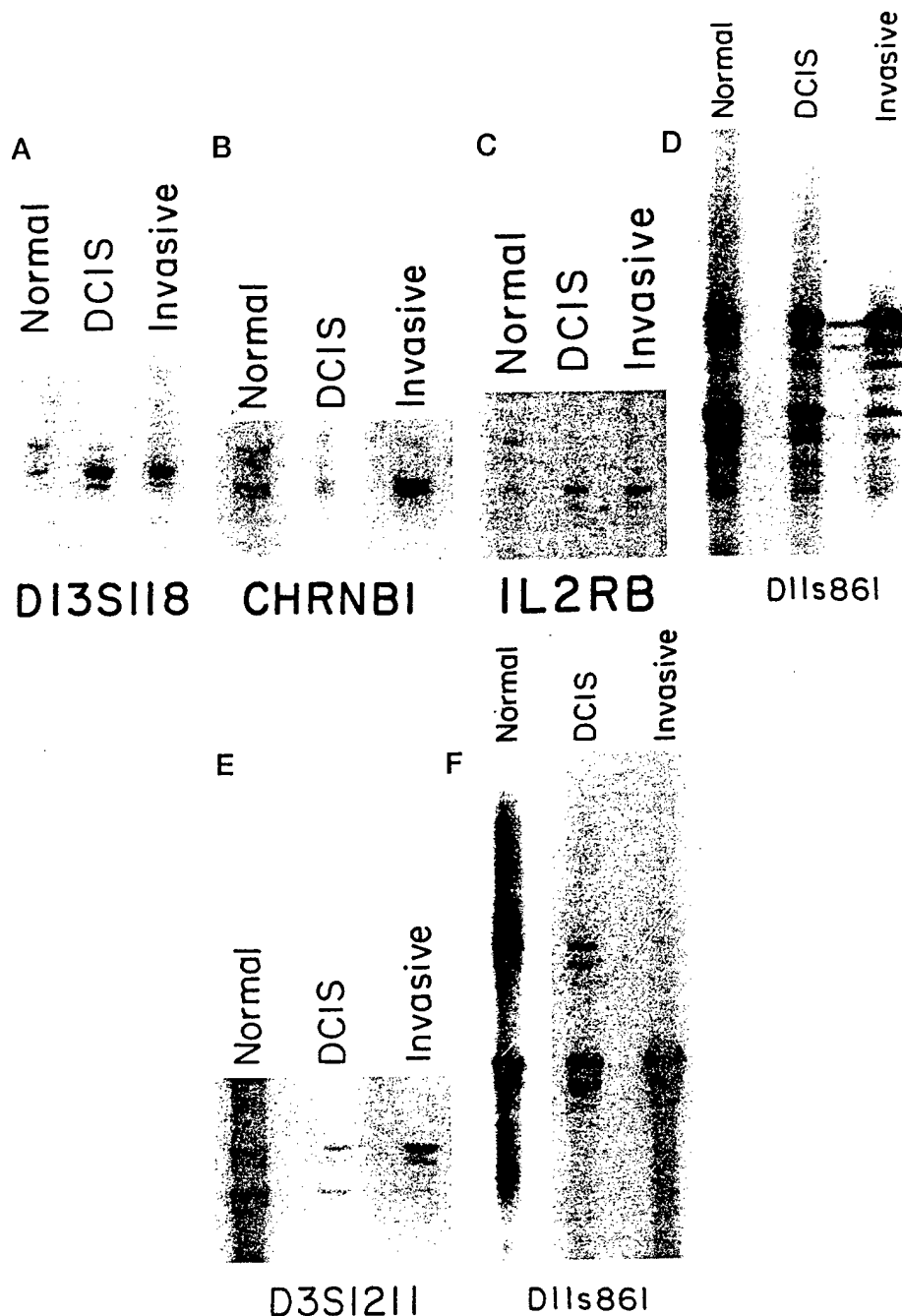


Fig. 2. A, B, and C, tumor 46. Loss of the same allele in both DCIS and invasive component for loci *DI3S118* (13q), *CHRNBI* (17p), and *IL2RB* (22q). The fold difference in integrated allele ratios between tumor and normal are: A, DCIS 4.12, invasive 7.25; B, DCIS 7.78, invasive 9.2; and C, DCIS 6.7, invasive 10.5. D, tumor 46. Loss of one allele in the invasive component but not DCIS for the locus *D11S861* (11p). The fold differences between tumor and normal are DCIS, 1.42 (insufficient to call LOH) and invasive, 5.0 (LOH). E and F, tumor 49. Loss of an allele in the invasive component but not DCIS for loci *D3S1211* (3p) and *D11S861* (11p). The fold differences in integrated allele ratios between tumor and normal are E, DCIS 1.95, invasive 9.7; F, DCIS 1.74, invasive 19.7.

invasive tumor that retains two 11p alleles, although the majority of these cells have lost one allele. In contrast, the loss of one allele is virtually complete in both DCIS and invasive component for this tumor at the other loci shown. According to the theory of Nowell, this suggests that LOH on 11p may be a later event chronologically in the pathway to invasion.

LOH on 11p has been correlated with low estrogen receptor protein and tumor size in invasive breast cancer, both of which are indicators of poorer prognosis (17). Concordance of loss of 11p and 17p in invasive breast cancer is more frequently associated with the development of lymph node metastases (18). One invasive cancer (tumor 49) also showed loss of loci on 1p, 2q, 3p, 6q, 7q, and 17p, whereas the corresponding DCIS displayed loss only at the locus on 17p. Loss at all these sites has been reported for invasive breast cancer (8, 9) and may indicate a more aggressive phenotype. For example, LOH on 1p has been correlated with the

presence of lymph node metastases, larger tumor size, and non-diploidy (19), and LOH of loci on 7q with impaired survival (20). The high fractional allelic loss of the invasive component of tumor 49 suggests that multiple genetic events have taken place in this tumor.

In summary, these data suggest that the subtypes of DCIS represent precursor lesions of invasive ductal carcinoma of the breast. LOH of regions most commonly involved in DCIS (8p, 13q, 16q, 17p, and 17q) is maintained in the concurrent invasive component. Loss of alleles at other loci such as 11p may be later events which are a feature of the invasive phenotype.

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Allelotyping of Ductal Carcinoma *in Situ* of the Breast: Deletion of Loci on 8p, 13q, 16q, 17p and 17q¹

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ABSTRACT

In order to determine which tumor suppressor loci are involved in preinvasive breast cancer, we have assayed for loss of heterozygosity (LOH) in ductal carcinoma *in situ* (DCIS). Areas of DCIS were microdissected from archival paraffin-embedded tissue. DNA was extracted, and LOH was determined by PCR of microsatellite markers that map to 39 autosomal arms. Either uninvolved lymph node or white cell DNA was used as normal control. A total of 61 samples of DCIS were assayed. The average number of informative tumors examined for each marker was 19 (range, 8-48). The median fractional allelic loss was 0.037. The highest percentage of LOH was shown for loci on 8p (18.7%), 13q (18%), 16q (28.6%), 17p (37.5%), and 17q (15.9%). LOH on 18q was found in 10.7% of informative tumors. Fractional allelic loss was associated with LOH on 17p, with high nuclear grade and with the comedo subtype of DCIS. LOH on 17p correlated with LOH on 17q and on 13q. Additional markers were used for 16q and 17p to determine the smallest common region of deletion. These data provide evidence that tumor suppressor loci that map to these regions are involved in the oncogenesis of breast cancer before progression to the invasive phenotype. Our findings provide additional support that multiple loci on 17p and 16q are involved in the development of breast cancer.

INTRODUCTION

DCIS³ of the breast is a preinvasive form of breast cancer in which malignant cells have not penetrated the basement membrane (1). The histopathological diagnosis encompasses a heterogeneous group of subtypes, including comedo, cribriform, solid, papillary and micropapillary, some of which may differ in biological behavior. The potential for associated microinvasion and likelihood of recurrence after breast conservation therapy are higher with the comedo subtype (2, 3). As with invasive breast cancer, DCIS can also be stratified by nuclear grade: high, intermediate, and low. A large number of articles have been published on LOH in invasive breast cancer, and virtually every human chromosome has been shown to exhibit allelic loss (4, 5). The most frequent losses are seen on chromosome 7q (0-83%) (4, 6, 7), 16q (32-63%) (4, 5, 8-10), 17p (31-75%) (4, 5, 11-14), 17q (24-79%) (4, 5, 15-21) and 18q (24-69%) (4, 18, 22, 23). Less frequent losses are found on 1p (3-47%) (4, 5, 24), 1q (16-32%) (4, 5, 25, 26), 3p (11-47%) (4, 5, 13), 6q (9-48%) (4, 5), 8p (27-33%) (4, 12), 11p (10-41%) (4, 27), and 13q (16-40%) (5, 13). Tumor suppressor genes thought to be involved in breast cancer include the *p53* gene at 17p13.1 (27, 28), *Rb* on 13q (5, 13) and *DCC* on 18q (22, 23). Recently, Schott *et al.* (29) have discovered another gene on 13q proximal to *Rb* (*Brush-1*), which is located within the 6-cM region

containing the inherited early onset breast cancer gene *BRCA-2*, located at 13q12-13 (30). A region telomeric to *p53* at 17p13.3 is also lost frequently in invasive breast cancer and is believed to harbor a separate tumor suppressor locus (31, 32). Several genes located on 17q are implicated in breast cancer oncogenesis, such as the recently cloned *BRCA-1* gene at 17q21 (33, 34) and the metastasis suppressor gene *NM23*, distal to *BRCA-1* (16, 17, 35). Because of the multiple putative tumor suppressor loci, which exhibit LOH in invasive breast cancer, it is not clear which loci are involved in oncogenesis and which are lost randomly due to the instability conferred by the malignant state of the genome.

The chronology of genetic changes leading to invasion has been elucidated for certain cancers, such as the transition from neoplastic polyp to colon carcinoma (36, 37), but has not been established for breast cancer. Our study is the first reported allelotype of DCIS and expands our knowledge of the genetic events leading to invasion in breast cancer.

MATERIALS AND METHODS

Subjects

A total of 61 women with the diagnosis of DCIS were entered into the study. Archival paraffin-embedded material for 57 cases was collected from the pathology department of several hospitals in St. Louis: Barnes Hospital, Jewish Hospital, Deaconess Hospital, St. Louis University Hospital, and the Outpatient Surgery Center, Inc. (St. Louis, MO). Data on 4 patients were provided by A. M. Thompson of the University of Edinburgh. Either matched archival normal lymph node DNA or leukocyte DNA was used as control. Examples of DCIS were categorized by the pathologists (J. H. R., N. J. P., K. DeSchuyver, and R. Brangle). When it was necessary to draw blood for normal control, informed consent was obtained following Institutional Review Board approval. Histological subtype and nuclear grade were: 42 comedo, 3 solid, 2 micropapillary, 1 papillary, 10 cribriform, and 3 mixed; 45 high nuclear grade, 14 low nuclear grade, and 2 intermediate.

Microdissection, DNA Purification, and Allelotyping

Tumor microdissection, DNA purification, and buffers for allelotyping have been described previously (38). The microsatellite markers used for these experiments are listed in Table 1. The PCR conditions for these markers was as follows all denaturation steps were at 94°C for 1 min and the majority of extension steps were performed at 72°C for 1 min. Annealing temperature ranged from 52 to 64°C, annealing time, from 30 s to 2 min, and number of cycles ranged from 22 to 30. The majority of markers chosen had a heterozygosity $\geq 70\%$. LOH was determined by a combination of naked-eye assessment and scanning densitometry. The tumors scored as showing LOH by visual inspection were subjected to densitometry. Densitometry was performed by scanning autoradiographs with a UMAX UC630 color scanner and with the use of the software program Adobe Photoshop 2.5.1 (Adobe Systems, Inc.). The densitometry histograms were analyzed on a Power Macintosh 6100/60 with the use of the public domain NIH image program (written by Wayne Rasband, NIH; available from the Internet by anonymous FTP from zippy.nimh.nih.gov.). A 3-fold difference in the relative allele intensity ratios between tumor DNA and normal DNA in an informative tumor normal pair was scored as LOH (allele 1/allele 2 in tumor compared to allele 1/allele 2 in normal). We have performed admixture experiments with the use of the DNA from two homozygous individuals and have established that LOH can be determined by the naked eye provided that

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The abbreviations used are: DCIS, ductal carcinoma *in situ*; GDB, Genome Database; FAL, fractional allelic loss; LOH, loss of heterozygosity; CEPH, Centre d'Etude Polymorphisme Humain.

"contamination" does not exceed 20% (data not shown). Samples with marginal allele reduction by inspection were not scored as LOH; thus, our method of scoring is conservative.

LOH on Chromosome 17

The markers used previously (38, 39) and two additional markers, *D17S578* and *D17S849*, were applied to a panel of 61 examples of DCIS.

Statistical Analysis

Statistical analysis was performed with the use of contingency tables and the unpaired *t* test with the software package Statview (Abacus Industries, Berkeley, CA). The null hypothesis for each analysis was that the two variables were independent of each other. At the 0.05 level of significance the null hypothesis was rejected.

Genetic Map Construction

Chromosome 17p. Nineteen loci were incorporated into a linkage map for a portion of the short arm of chromosome 17. Five microsatellite markers (*c1541/D17S260*, *AFM234wg3a/3m/D17S849*, *Mfd152/D17S578*, *ut20/D17S654*, and *ut269/D17S695*) were not part of our previous 17p map (38). Genotypes for *D17S513* (12G6), *D17S260* (c1541), *D17S578* (Mfd152), *D17S849* (AFM234wg3a/3m), *CHRN1* (IMG4125/26), *D17S379* (DL1), *TP53* (TP53CA), *D17S643* (ut18), *D17S654* (ut20), and *D17S695* (ut269) were produced in our laboratory with the use of the CEPH 40 family reference pedigrees. Genotypic data for *D17S31* (pMCT35.1MspI and pMCT35.2MspI), *D17S28* (pTB7c-2.1), *D17S30* (pYNZ22Rsal), *D17S499* (cLS17.6PvuII), *D17S510* (fLB17.3AluI), *D17S505* (fLB17.8AluI), *D17S506* (fLB17.9PstI), *MYH2* (p10.5HindIII), and *D17S34* (p144-D6Rsal) were obtained from the CEPH database (version 6). Oligonucleotide primer sequences and reaction conditions for ut markers were obtained from the Utah Genome Center (40), and several of these have since been published (41). PCR primer sequences and reaction conditions for *D17S379* were provided by Dr. D. Ledbetter and have subsequently become accessible via GDB (42). In our previous paper it was arbitrarily referred to as DL1 because it had not been published at that time (39). Primer sequences and reaction conditions for the other markers used in the map are available from the GDB (<http://gdbwww.gdb.org>). The CRIMAP program package was used to construct the map with odds for loci order of at least 1000:1. "flips4" permutations of marker order was used to test for the most likely order with the use of data from the "build" analysis. Two additional markers from chromosome 17, ut9, and ut69 (made available from the Utah Genome Center) were also genotyped in this study, but two-point analysis indicated that they mapped to 17q, and they were not studied further.

Sixteen of the 19 loci, including all 9 markers used for the LOH analysis, were uniquely placed with odds for order of at least 1000:1. The sex average map extends a total of 47.1 cM. *CHRN1* has now been uniquely localized at 1000:1 odds, whereas in a map reported previously the odds were less than 50:1 (39). Genotypes used to construct the chromosome 17p map, map graphics, and associated data reported here are available through the GenLink resource (<http://www.genlink.wustl.edu>).

Chromosome 16. A human chromosome 16 linkage map constructed previously, which included all 11 markers used for our LOH analysis was available from the Cooperative Human Linkage Center along with the genotypic data from the CEPH database (version 6; Ref. 43; <ftp.chlc.org>). We used the "fixed" option of CRIMAP to calculate the genetic distances between the markers and the "all" option to verify the placement of 3 nonuniquely placed markers (*D16S423*, *D16S405*, and *D16S261*). It is apparent that *D16S423* is the most distal 16p marker used for LOH studies and that *D16S405* is the penultimate LOH marker. Nonuniquely localized LOH marker *D16S261* is flanked by LOH markers *D16S403* and *D16S541* on the linkage map.

Cytogenetic Tie Points. All physical localizations that tie the genetic to the cytogenetic maps for chromosomes 16 and 17 were taken from information available from the GDB.

RESULTS

Allelotyping. A total of 61 microdissected cases of DCIS were assayed for LOH with the use of a panel of microsatellite markers

Table 1 Polymorphic markers and percentage LOH observed in DCIS allelotyping. The combined LOH for markers on 16p was 1 of 19 (5.2%), 16q 8 of 28 (28.6%), and 17p 18 of 48 (37.5%)

| Chromosome | Marker | LOH (%) |
|--------------|----------------|--------------|
| 1p36 | <i>D1S165</i> | 1/24 (4.1) |
| 1q21-23 | <i>APOA2</i> | 0/23 |
| 2p25-24 | <i>TPO</i> | 0/19 |
| 2q33-37 | <i>D2S128</i> | 1/23 (4.3) |
| 3p24.2-22 | <i>D3S1211</i> | 2/28 (7.1) |
| 3q27 | <i>D3S1314</i> | 0/17 |
| 4p | <i>D4S403</i> | 0/8 |
| 4q | <i>D4S426</i> | 0/9 |
| 5p | <i>D5S392</i> | 0/19 |
| 5q | <i>D5S429</i> | 0/18 |
| 6p24.2-23 | <i>F13A1</i> | 0/10 |
| 6q21-23.3 | <i>D6S225</i> | 2/22 (9.0) |
| 7pter-p15 | <i>D7S481</i> | 0/16 |
| 7q | <i>D7S466</i> | 1/24 (4.2) |
| 8p23 | <i>D8S262</i> | 3/16 (18.7) |
| 8q | <i>D8S373</i> | 0/23 |
| 9p23-22 | <i>D9S157</i> | 0/14 |
| 9q34.1 | <i>D9S214</i> | 0/15 |
| 10p | <i>D10S172</i> | 0/17 |
| 10q11.2-qter | <i>D10S109</i> | 0/12 |
| 11p15.2 | <i>D11S861</i> | 0/31 |
| 11q23.3 | <i>CD3D</i> | 1/4 (7.1) |
| 12p13 | <i>D12S94</i> | 0/17 |
| 12q14-24.33 | <i>D12S101</i> | 0/13 |
| 13q14.1 | <i>D13S118</i> | 4/22 (18) |
| 14q | <i>D14S543</i> | 1/16 (6.3) |
| 15q | <i>D15S87</i> | 0/21 |
| 16p13.3 | <i>D16S423</i> | 0/15 |
| 16p13.1 | <i>D16S405</i> | 1/8 (12.5) |
| 16p12 | <i>D16S403</i> | 0/13 |
| 16q12.1 | <i>D16S261</i> | 3/13 (23) |
| 16q12.1 | <i>D16S541</i> | 3/10 (30) |
| 16q12.1-12.2 | <i>D16S415</i> | 3/14 (21.4) |
| 16q21 | <i>D16S265</i> | 3/23 (13) |
| 16q23 | <i>D16S266</i> | 2/8 (25) |
| 16q24.2 | <i>D16S402</i> | 5/20 (25) |
| 16q24 | <i>D16S539</i> | 4/12 (33.3) |
| 16q24.3 | <i>D16S413</i> | 4/19 (21) |
| 17p | <i>D17S643</i> | 3/22 (13.6) |
| 17p | <i>D17S849</i> | 5/14 (35.7) |
| 17p | <i>D17S379</i> | 3/17 (17.6) |
| 17p | <i>D17S513</i> | 13/34 (38.2) |
| 17p | <i>D17S578</i> | 1/10 (10) |
| 17p | <i>CHRN1</i> | 6/20 (30) |
| 17p13.1 | <i>TP53</i> | 8/26 (30.7) |
| 17q11.2-12 | <i>D17S250</i> | 1/13 (7.7) |
| 17q | <i>D17S579</i> | 4/38 (10.5) |
| 17q21-22 | <i>NM23</i> | 2/16 (12.5) |
| 18pter-11.22 | <i>D18S59</i> | 1/17 (5.8) |
| 18q23 | <i>D18S70</i> | 3/28 (10.7) |
| 19p13.3 | <i>D19S177</i> | 0/12 |
| 19q13.2 | <i>APOC2</i> | 0/14 |
| 20p | <i>D20S59</i> | 0/11 |
| 20q13 | <i>D20S102</i> | 0/11 |
| 21q22.2 | <i>D21S167</i> | 1/16 (6.3) |
| 22q13 | <i>IL2RB</i> | 1/19 (5.2) |

mapping to 39 non-acrocentric autosomal arms. Frequency of LOH is summarized in Table 1 and depicted graphically in Fig. 1. Examples of loci exhibiting LOH are shown in Fig. 2. The average number of informative loci/tumor was 19 (range, 8-48). Range of percentage LOH was from 0 to 37.5 with a mean of 5.2%. Significant LOH was arbitrarily chosen to be a value above the mean (or background) percentage LOH plus 1 SD. Chromosomal arms that showed LOH >14% were 8p (18.7%), 13q (18%), 16q (28.6%), 17p (37.5%), and 17q (15.9%). LOH on 18q was found in 10.7% of informative cases. FAL, defined as the number of arms showing allelic loss divided by the total number of informative arms/tumor, was calculated for a total of 24 tumors with 15 or more informative arms (44, 45). The range of FAL was from 0.00 to 0.333 with a median of 0.037 and a mean of 0.063.

The unpaired *t* test and contingency tables were used to determine if there was any significant correlation among variables such as LOH

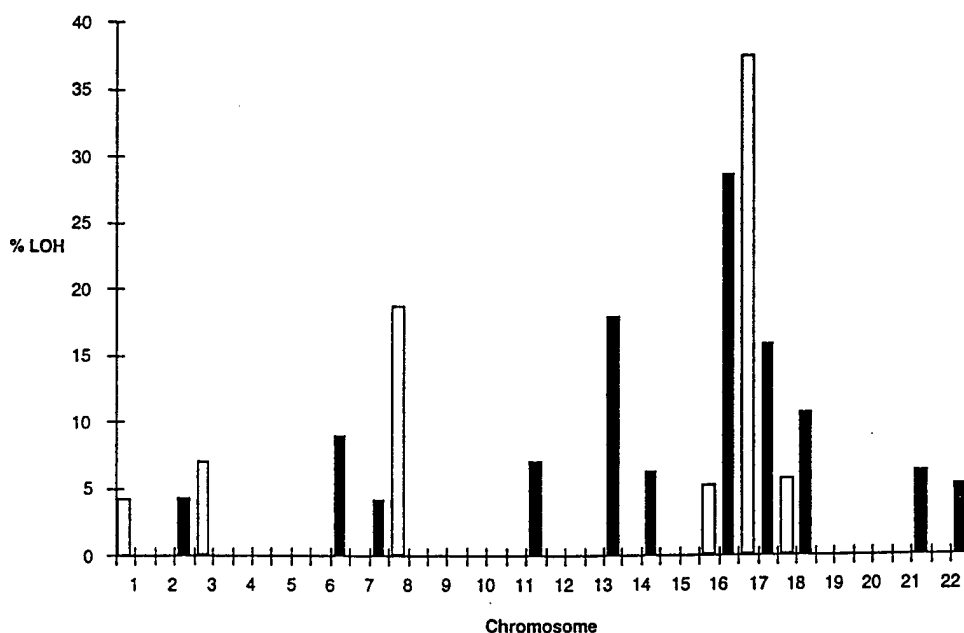


Fig. 1. DCIS allelotyping. □, p arm; ■, q arm.

on 17p, 17q, 16q, 13q, and 8p, nuclear grade, subtype (comedo versus noncomedo) and FAL. Due to the small sample size, the subtypes were not statistically evaluated separately. Significant associations were found between LOH on 17p and LOH on 17q ($P = 0.006$), LOH on 17p and LOH on 13q ($P = 0.0055$), and LOH on 17p and FAL greater than median ($P = 0.0038$). The mean FAL for comedo tumors was 0.087 versus 0.016 for noncomedo tumors ($P = 0.0326$), and the mean FAL for tumors of high nuclear grade was higher than those of low nuclear grade ($P = 0.0338$).

LOH on Chromosome 17. Fig. 3 depicts the LOH data for chromosome 17 with the use of a panel of 53 tumors. Of a total of forty-eight 17p arms, for which markers were informative, LOH was seen in 18 (37.5%). In three tumors, the deletion appeared to involve the entire short arm (numbers 19, 23, and 41), and this may also be the case for tumors 46 and 47. In tumor 11 the deletion did not extend to the most telomeric marker *D17S643* (ut18). Tumor 1 exhibits two separate areas of deletion, one encompassing the *TP53* and *CHRN1* loci and the other extending from *D17S513* to *D17S849*, with retention of the intervening locus *D17S578*. Three tumors showed a smaller deletion: in tumor 27, the only locus showing loss was *D17S513*; tumor 38 had LOH at *D17S849*; and tumor 39 had a loss of an allele at *TP53*. Tumor 10 had deletion at the *D17S513* locus with retention of *TP53*. The two smallest common regions of deletion on 17p are depicted by vertical lines on the right of Fig. 3. One region measuring 11.6 cM encompasses the markers *D17S849* through *D17S379* at 17p 13.3, and its boundaries are established by the deletion pattern seen in tumors 1 and 38. The other deleted region is localized to 17p 13.1 and includes the *TP53* locus and *D17s31* and measures 3.4 cM.

For loci on the long arm (*D17S250*, *D17S579*, and *NM23*), a total of 44 arms were informative with LOH being seen on 7 (15.9%). For the individual loci, LOH was as follows: *D17S250*, 1 of 13 (7.6%); *D17S579*, 4 of 38 (10.5%); and *NM23*, 2 of 16 (12.5%). Both *D17S250* and *D17S579* are linked with the hereditary early-onset breast cancer gene *BRCA-1* (33). The combined LOH for these two markers was 5 of 40 (12.5%).

LOH on Chromosome 16. Fig. 4 shows the cumulative data for LOH on chromosome 16 with the use of the markers listed in Table 1. Allelic loss was found in 8 of 28 q arms for which markers were informative (28.6%). In 3 tumors the deletion was large (tumors 4, 19, and 23); however, in 3 tumors a smaller deletion was seen. In tumor

2 only the *D16S413* locus was lost; in tumor 38 only the *D16S402* locus was lost. The full extent of the deletion in tumor 48 cannot be determined currently as the markers flanking *D16S266* and *D16S402* were noninformative. The smallest common areas of deletion on 16q are shown by vertical lines. One region of deletion shown by tumor 20 includes the markers *D16S261* through *D16S415*. This region measures from 8.5 to 17.2 cM and is located at 16q12.1. A precise size cannot be given because *D16s261* is not uniquely placed on the map. This tumor also shows a separate region of deletion from *D16S402* distally. Tumors 20 and 2 limit the other common region of loss to a 26-cM distance below *D16S402* (at 16q24.2). All of the microsatellite markers used in the above experiments were tested on DNA from the CEPH reference panel to ensure that allele-specific amplification did not occur.

DISCUSSION

In our allelotyping study of DCIS we have shown that some of the earliest changes in the progression to malignancy in the breast are loss of alleles on 8p, 13q, 16q, 17p, and 17q. It is presumed that most solid tumors in humans arise through a cascade of genetic events involving oncogenes and tumor suppressor genes that results in decreasing stability of the genome and ultimately leads to the malignant phenotype. As additional mutations and/or deletions occur, these malignant cells may progress to invasion and later metastasis. The majority of LOH studies on breast cancer reported to date have concentrated on the invasive varieties of breast cancer and have found significant LOH on multiple chromosomal arms (4, 5). DCIS is a noninvasive precursor (though not an obligate precursor) of breast cancer (46) and, therefore, studies of allelic loss in this condition will help to determine which are some of the early events in oncogenesis. Our data indicate that the number of chromosomal arms that show LOH in DCIS is considerably fewer than in invasive cancer. This would be expected if one considers DCIS to be a preinvasive landmark on one of the pathways to invasion. The accumulation of additional mutations and deletions on other chromosomal regions may then result in the invasive phenotype.

Both 16q and 17p have been reported to harbor more than one tumor suppressor locus (8–11). On 17p one locus is the *TP53* gene at 17p13.1 with an additional independent locus situated more telomeric

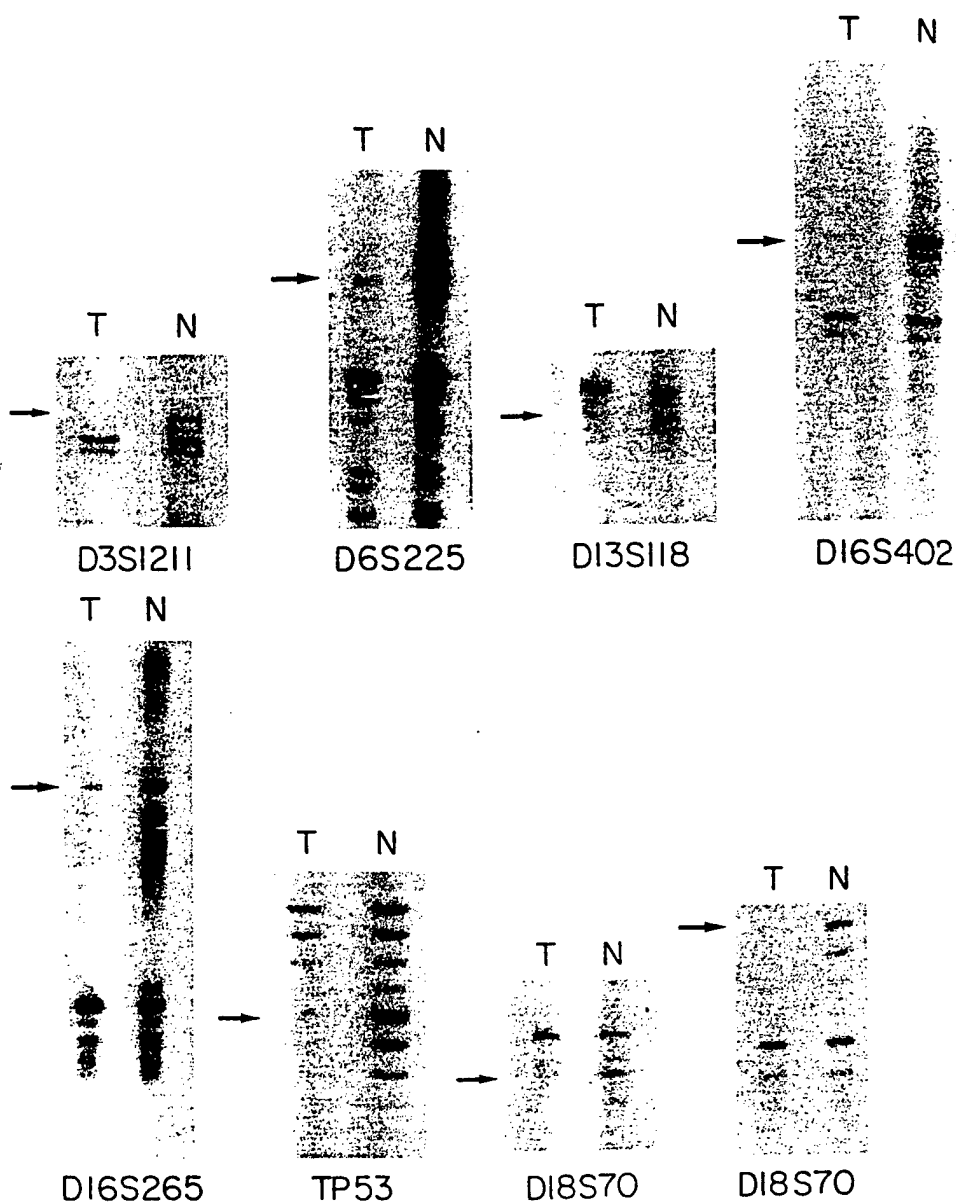


Fig. 2. LOH at multiple loci in DCIS. For each autoradiograph the locus, the tumor number, and the fold-difference in integrated allele ratios between tumor and normal are listed: *D3S1211*, tumor 37, 4.8-fold; *D6S225*, tumor 23, 3-fold; *D13S118*, tumor 58, 5.2-fold; *D16S402*, tumor 19, 7.6-fold; *D16S265*, tumor 23, 4-fold; *TP53*, tumor 41, 4.1-fold; *D18S70*, tumor 37, 6.3-fold; *D18S70*, tumor 46, 4.3-fold. In each case PCR products from tumor (T) and normal (N) genomic DNA from an individual with DCIS are shown. The loss of one allele in the tumor lane is seen in all photographs. →, allele loss.

at 17q13.3 (18). Our deletion map of 17p supports the existing evidence for two separate loci. The isolated deletion of *D17S513* in tumor 27 raises the possibility of a third tumor suppressor locus on 17p. Similarly, in invasive breast cancer, three regions on 16q may contain the loci for tumor suppressor genes; one region near the telomere at 16q24.3, one at 16q22-23, and another at 16q21 (8-10). Our data also support the existence of multiple tumor suppressor loci on 16q that appear to be involved in the early stages of breast cancer. Two regions of loss are located at 16q12.1 and 16q24.2, respectively. Tumor 38 lost the *D16S402* marker at 16q24.2. One tumor also lost the most telomeric marker *D16S413*, at 16q24.3, but although the more proximal marker was noninformative, the extent of deletion in this tumor is unknown. We did not find an overlapping region of deletion at 16q21 as others have reported. The marker *D16S265*, which maps to this area, was informative in 23 cases and showed LOH in 3 of them (13%). Tumor 20 excludes this area from the common regions of deletion. Tsuda *et al.* (10) noted LOH at 16q12 in 36% of invasive breast cancers, with an overall rate of LOH on 16q of 52%. The highest rate of loss seen by these investigators was at 16q24.3. This group analyzed 27 cases of intraductal and predominantly intraductal cancers and found LOH on 16q in 8 of them (35%). It is not

stated how many of the tumors that showed LOH were purely intraductal without an invasive component.

LOH on 17q occurs in a large proportion of invasive breast cancer, both familial and sporadic (15, 18-21). At least three separate regions of deletion have been reported 17q12-21, 17q22-23, and 17q24-25 (15, 47). The hereditary early onset breast cancer gene *BRCA-1* at 17q21 has been identified recently (34) and appears to act as a tumor suppressor in inherited breast cancer. However, Futreal *et al.* (48) have found few mutations in the remaining *BRCA-1* allele in what was believed to be sporadic breast cancer. Of the 32 cases of invasive breast cancer that showed LOH of the *BRCA-1* region only, 3 patients had *BRCA-1* mutations in their tumors. All of these patients were found to have germline mutations. It has been suggested that the chromosomal deletions at 17q21 in sporadic breast cancer unmask another tumor suppressor locus distinct from *BRCA-1*. We found the frequency of allelic loss of loci linked to *BRCA-1* to be 12.5% in DCIS lesions (a figure which did not meet the value of 14%, which we considered to be significant). Another tumor suppressor locus, which may be unmasked by the usually large deletions of 17q found in invasive breast cancer, is *NM23* at 17q22 (35). *NM23* appears to be involved in both progression and metastasis of breast cancer (49).

ALLELOTYPING OF DCIS

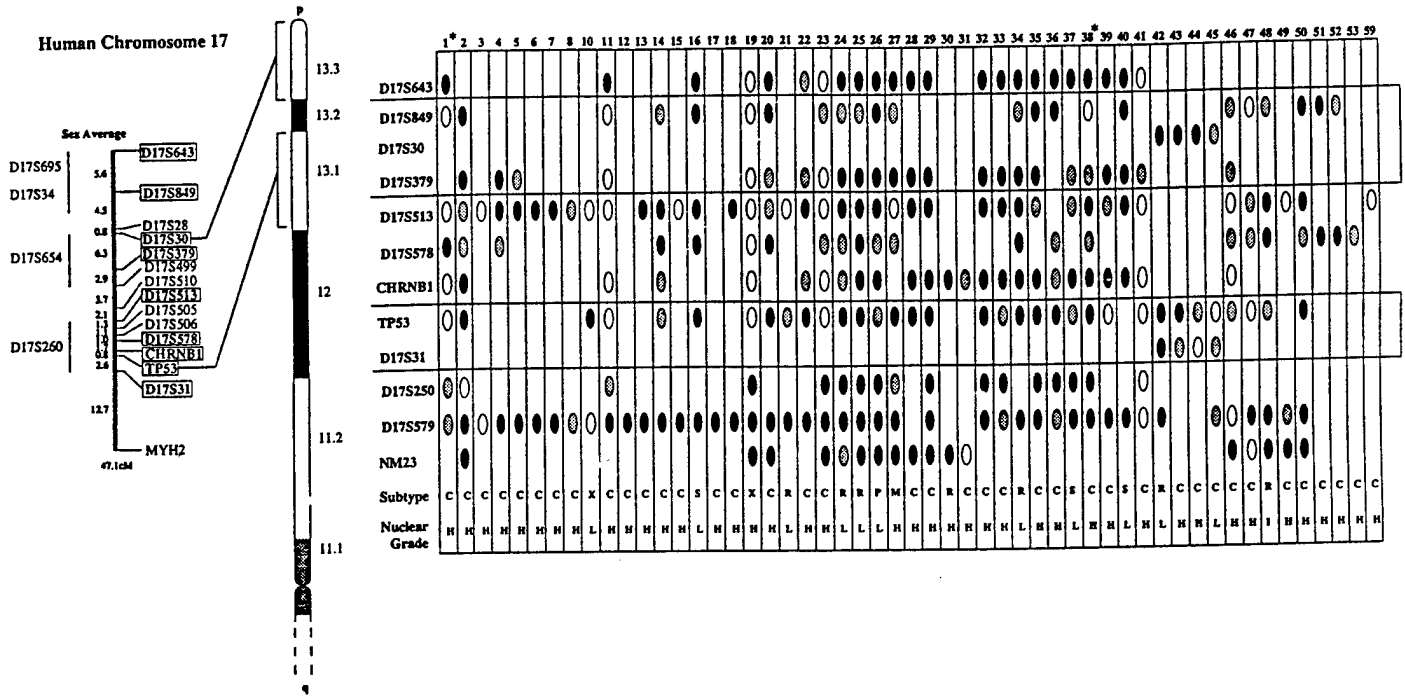


Fig. 3. Sex average multipoint linkage map for distal 17p including cytogenetic tie points and cumulative data for chromosome 17 deletions observed in 53 DCIS tumors. Map, genetic loci uniquely placed with odds for order of at least 1000:1 are shown to the right of the vertical line of the linkage map and nonuniquely localized markers are shown to the left of the map line with the location intervals indicated by thin vertical bars. No recombinants were detected between *D17S695* and *D17S34* (p144-D6Rsa1). Genetic distance in cM is given to the left of the linkage map line. Markers used for LOH studies are enclosed by a box. Genetic markers for which physical localizations were available are tethered to the cytogenetic map. *D17S250*, *D17S79*, and *NM23* are located on 17q. LOH data: top, DCIS samples; bottom, DCIS subtype and nuclear grade. Genetic markers are listed in the order determined from the linkage map. ●, no loss; ○, LOH; ⊙, noninformative, H, high nuclear grade; L, low nuclear grade; I, intermediate nuclear grade; C, comedo; R, cribriform; S, solid; M, micropapillary; P, papillary; X, mixed. Horizontal lines within the diagram and vertical lines on the right, common areas of deletion. Tumors which limit these boundaries are marked with an *.

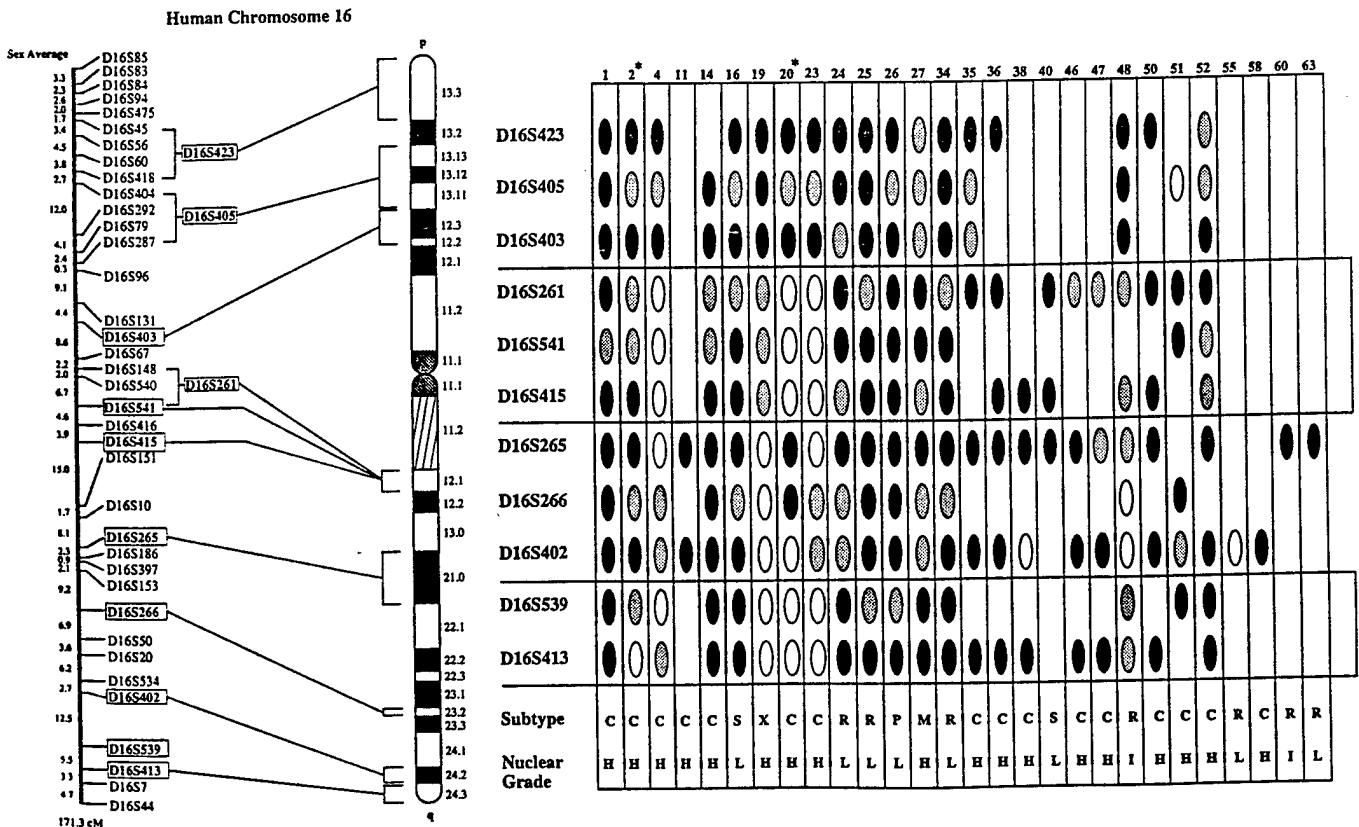


Fig. 4. Sex average multipoint linkage map for chromosome 16 and cumulative data for chromosome 16 deletions observed from 28 DCIS tumors. Marker designations are as indicated for Fig. 3. Nonuniquely and uniquely placed markers are on the right of the vertical line and genetic distances on the left. Abbreviations are the same as for Fig. 3. Vertical lines, common areas of loss.

In the past LOH assays on 13q in invasive breast cancer have concentrated on the region around the *Rb* gene at 13q14 (13). Recently, Schott *et al.* (29) have reported a separate tumor suppressor gene on 13q, *Brush-1*, which maps proximal to *Rb* at 13q12-13. They found that rates of LOH in invasive breast cancer at the *Rb* locus and at 13q13 were similar. A second hereditary breast cancer gene *BRCA-2* was recently mapped to 13q12 and *Brush-1* is contained within the 6 cM region containing *BRCA-2* (30). The marker we used for 13q, *D13S118*, maps to 13q14.1. Whether the *Brush-1* gene is inactivated in DCIS is yet to be determined.

Our data showed allelic loss in 18.7% of informative tumors at 8p23. LOH on 8p has been seen in several tumor types, including colorectal and hepatocellular carcinoma (50, 51). 8p also harbors at least two tumor suppressor loci which map to 8p23.1-pter and 8p21 respectively (50).

The relationship between FAL and clinical behavior of cancer was first noted by Kern *et al.* (45) who found FAL greater than the median in colorectal cancer to be associated with distant metastases (45). We have established that the median FAL in DCIS is 0.037. The FAL reported for invasive breast cancer is 0.05 (5). Our finding that the comedo subtype and high nuclear grade DCIS are associated with higher FAL may be correlated with the more aggressive clinical behavior of these phenotypes. Comedo high nuclear grade tumors are more likely to recur locally after resection and to progress to invasive cancer (52). They have a higher incidence of aneuploidy (53) and higher thymidine-labeling indices (54). Several authors have found that genetic abnormalities in breast cancer correlate with clinicopathological parameters. For example, LOH on 7q is associated with decreased survival (7). LOH at 17p13 and at 16q24.3 have been reported to be associated with more aggressive disease in invasive breast cancer (8, 55-57). In the accumulation of LOH which contributes to cancer formation, LOH of certain regions appear to act in a cooperative manner. In invasive breast cancer, LOH on 13q and 17p is associated as is LOH on 17p and 17q (13, 14). As Kern *et al.* (45) noted in colorectal cancer, we also found an association between FAL and LOH of 17p.

In summary, significant LOH occurs in DCIS for loci on 8p (18.7%), 13q (18%), 16q (28.6%), and 17p (37.5%), regions that also exhibit high rates of loss in invasive cancer. Our data imply that inactivation of tumor suppressor genes at these sites are early events in the tumorigenesis of breast cancer, and that LOH of other loci may be involved in progression and metastasis. Of note, even in this early noninvasive stage of breast cancer, at least 7 chromosomal regions, harboring tumor suppressor genes, are already implicated in oncogenesis. These data support the epidemiological hypothesis that from 3 to 8 genetic abnormalities must accumulate over time for malignancy to develop (58). We plan to refine further the regions of loss on 16q, 17p, and 8p with fine structure mapping and to apply positional cloning techniques to identify the tumor suppressor genes in these areas of interest.

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ALLELIC LOSS ON CHROMOSOME 8p OCCURS EARLY IN
THE DEVELOPMENT OF BREAST CARCINOMA

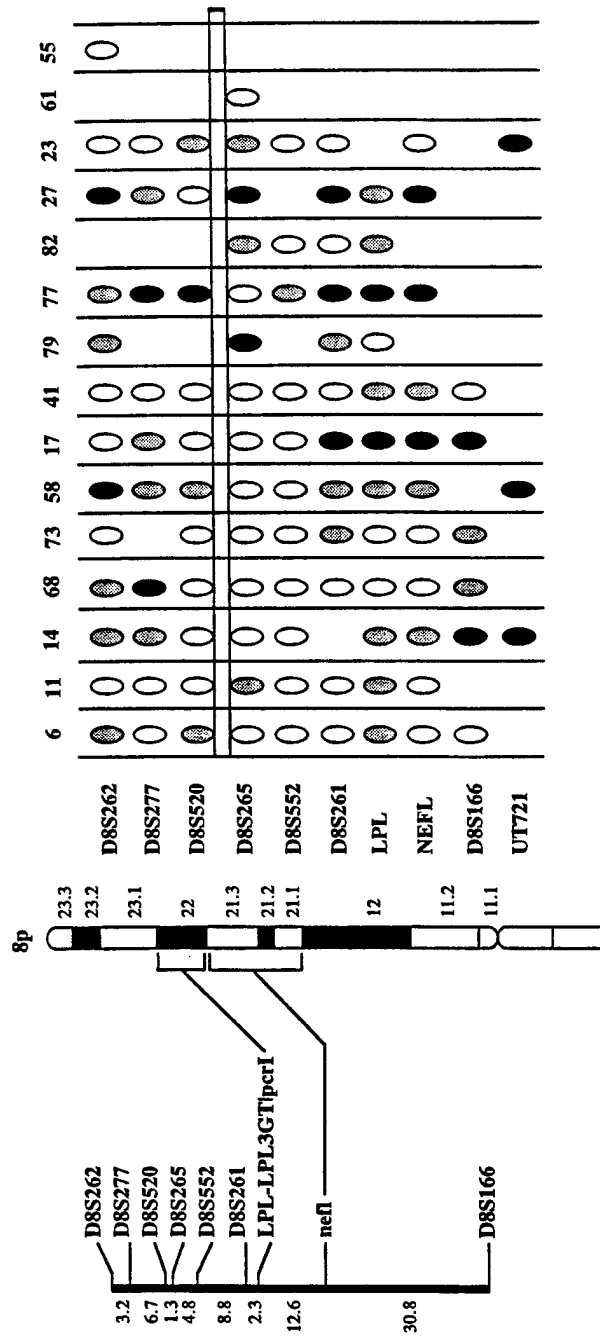
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MANY CHROMOSOMAL REGIONS harboring tumor suppressor genes are implicated in the cascade of genetic events that leads to invasive breast cancer.¹ Loss of activity of a tumor suppressor gene is revealed as loss of an allele in the tumor DNA when compared to matched normal control DNA. We have presented data previously that loss of heterozygosity (LOH) on 17p occurs in the preinvasive lesion ductal carcinoma in situ (DCIS)² and is therefore an early event in breast cancer progression. We have assayed samples of DCIS for LOH at other chromosomal loci, including 8p, to determine the sites of other putative tumor suppressor genes involved in breast cancer oncogenesis.

MATERIALS AND METHODS

Sixty-four cases of DCIS were microdissected from paraffin-embedded, archival material to remove the adjacent normal stroma. DNA was extracted and polymerase chain reaction (PCR) carried out as

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Chromosome 8 deletions observed in 15 DCIS tumors. DCIS samples are numbered at the top. Genetic markers are uniquely placed with odds of 1:1,000 and are listed in the order determined. The genetic map and distances are shown on the left of the figure. Cytogenetic localization is shown by tie bars. D8S166 and UT721 map to 8q. ● = no loss; ○ = LOH; ⊙ = noninformative. A vertical line on the right of the figure and horizontal lines within the figure show the smallest common region of deletion.

described previously.³ PCR products were separated on denaturing polyacrylamide DNA sequencing gels. Either uninvolved lymph node or white cell DNA was used as normal control. PCR was carried out using eight microsatellite markers that map to 8p and two markers that localize to the long arm of the chromosome. The order of the 8p microsatellites from telomere to centromere is D8S262, D8S277, D8S520, D8S265, D8S552, D8S261, LPL, NEFL.

RESULTS

Of 55 cases of DCIS for which the assays were informative, LOH on 8p was seen in 15 tumors (27.3%). In six tumors the deletion appeared to involve the whole short arm, whereas in the remainder a smaller deletion was seen. The smallest common region of deletion localizes to a 1.3-cM region at 8p22-23, between markers D8S520 and D8S265 (Fig).

CONCLUSIONS

Little is known about the chronology of genetic events leading to invasion. Our data suggest that LOH of loci on 8p occurs before the development of the invasive phenotype in the progression of breast cancer. At least one tumor suppressor gene in the 1.3-cM region identified at 8p23 is implicated.

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CHROMOSOMAL REGIONS IMPLICATED IN THE DEVELOPMENT OF BREAST CANCER

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MULTIPLE CHROMOSOMAL REGIONS harboring tumor suppressor genes are implicated in the oncogenesis and progression of invasive carcinoma of the breast.¹ Loss of activity of suppressor genes, allowing tumor formation to occur, can be detected by loss of heterozygosity (LOH) of the tumor DNA compared to normal control DNA. To identify initiating events and investigate the chronology of genetic changes in the development of breast cancer, we assayed LOH in ductal carcinoma in situ of the breast (DCIS) mainly using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Because contamination of tumor DNA by surrounding normal cells can interfere with the detection of LOH, we separated tumor from stroma by microdissection of archival tissue.¹ DNA was extracted and PCR carried out as previously described.¹ The microsatellite markers used, which map to chromosome 17, were 12G6 (D17S513), TP53CA (TP53), Mfd188 (D17S579), UT18, DL1, CHRNB1, Mfd15 (D17S250), and NM23. For four tumors Southern analysis was performed with restriction fragment length polymorphism (RFLP) markers PYNZ22 (D17S30), MCT35.1 (D17S31), and pBHp53 (TP53). PCR products were separated on denaturing polyacrylamide DNA sequencing gels.

RESULTS

The markers 12G6 (D17S513), TP53CA (TP53), Mfd188 (D17S579), UT18, and DL1 were ordered uniquely within a multipoint linkage map. CHRNB1 maps closely to TP53 with odds of 47:1. On the short arm of chromosome 17, the order from telomere to centromere is UT18-D17S30-DL1-D17S513-TP53-CHRNB1-D17S31. On the long arm, from telomere to centromere lie D17S250-D17S579 and NM23. A total of 45 tumors have been examined to date. Of 39 tumors informative for markers on the short arm, LOH is seen in 10 (26%). In two cases, the length of

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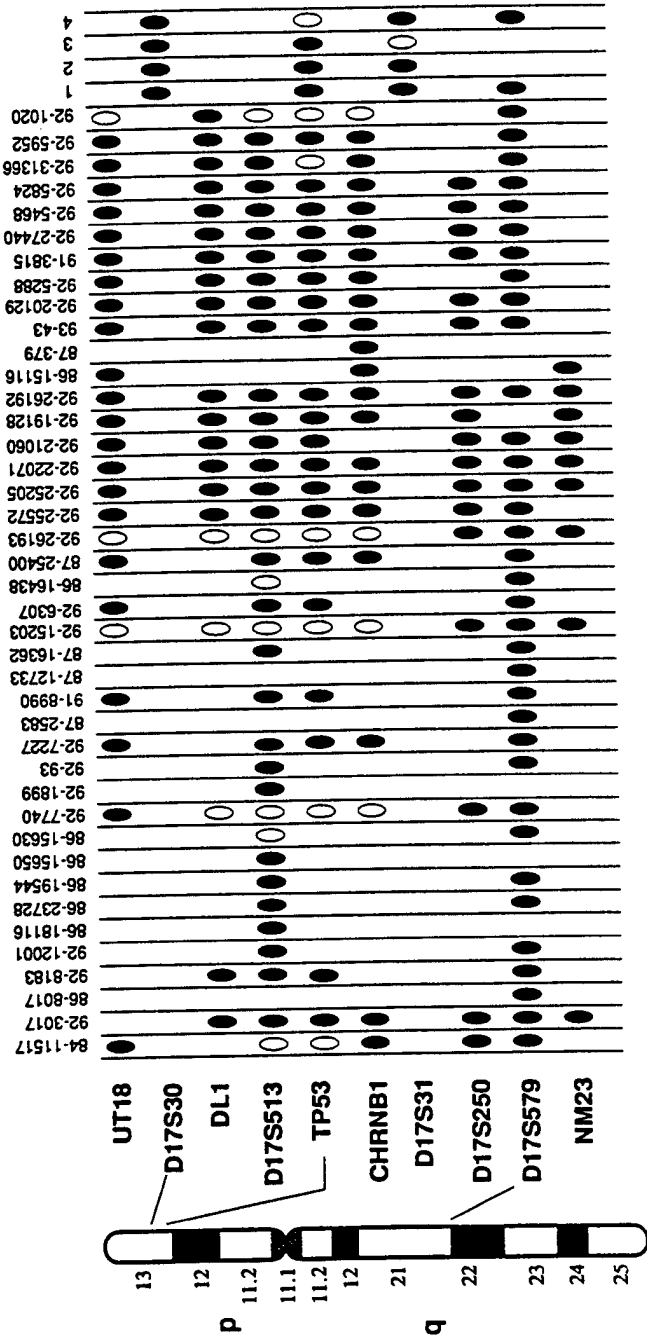


Fig 1—Summary of chromosome 17 deletions observed from 45 DCIS samples. DCIS samples are indicated along the top of the figure. Genetic markers are listed in the order determined from the multipoint map. ● = No loss; ○ = LOH; ● = noninformative.

deletion does not extend to the most telomeric marker (UT18). As shown in Figure 1, the smallest common region of deletion involves the TP53 and D17S513 loci. LOH has not been seen with any of the markers from the long arm.

CONCLUSIONS

Two tumor suppressor genes have been proposed to reside on 17p¹ and two on 17q.² One 17q locus is thought to include the hereditary early-onset breast cancer gene (BRCA1) to which D17S579 is closely linked.³ We have demonstrated significant LOH on 17p (26%) at loci near the p53 gene, which does not involve a telomeric locus in all cases. These data suggest that at least one locus on the short arm of chromosome 17 is involved early in the development of breast cancer, whereas inactivation of a gene or genes on 17q may be involved in later stages of progression to the invasive phenotype.

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