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| AD NUMBER                                                                                                                                                                                                                  |
| ADB282072                                                                                                                                                                                                                  |
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| USAMRMC ltr, 18 Nov 2002                                                                                                                                                                                                   |

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Award Number: DAMD17-97-1-7191

TITLE: Monoclonality and Genetic Instability in Premalignant Breast Tissue

PRINCIPAL INVESTIGATOR: Carol L. Rosenberg, M.D.

CONTRACTING ORGANIZATION: Boston University  
Boston, Massachusetts 02215

REPORT DATE: February 2002

TYPE OF REPORT: Final

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

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**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

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| <b>4. TITLE AND SUBTITLE</b><br>Monoclonality and Genetic Instability in Premalignant Breast Tissue                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                 |                                                                | <b>5. FUNDING NUMBERS</b><br>DAMD17-97-1-7191                          |                                  |
| <b>6. AUTHOR(S)</b><br>Carol L. Rosenberg, M.D.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                 |                                                                |                                                                        |                                  |
| <b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b><br><br>Boston University<br>Boston, Massachusetts 02215<br><br>E-mail: <a href="mailto:crosenberg@medicine.bu.edu">crosenberg@medicine.bu.edu</a>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                 |                                                                | <b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>                        |                                  |
| <b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b><br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                 |                                                                | <b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>                |                                  |
| <b>11. SUPPLEMENTARY NOTES</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                 |                                                                |                                                                        |                                  |
| <b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b><br>Distribution authorized to U.S. Government agencies only (proprietary information, Feb 02). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                 |                                                                |                                                                        | <b>12b. DISTRIBUTION CODE</b>    |
| <b>13. ABSTRACT (Maximum 200 Words)</b> <p>By the time a cancer is detected, its tumor cells already exhibit myriad genetic abnormalities. To gain a better understanding of genetic events that occur early in breast carcinogenesis, this research examined genetic abnormalities 1) in histologically normal tissue from women at low, medium or high degrees of breast cancer risk, using archival specimens of reduction mammoplasties, and of diagnoses of atypical hyperplasia and breast cancers, respectively; and 2) in synchronously occurring putative precursor lesions, including normal-appearing epithelium, simple and atypical proliferative (hyperplastic) lesions and carcinomas themselves.</p> <p>Each specimen is microdissected, its DNA examined using a panel of selected microsatellite markers, and evidence of clonal abnormalities sought, in particular loss of heterozygosity (LOH) and microsatellite instability (MI).</p> <p>Investigation of the project's first goal generated data regarding the timing and sites of early genetic abnormalities. These data raise the possibility that a field defect exists in certain breast tissue. Investigation of the second goal is uncovering the nature of the clonal relationships existing between multiple synchronous putative precursors. These studies are identifying important sites of genetic abnormalities in early breast cancer precursors, and beginning to outline a sequence of acquired genetic abnormalities necessary for precursor lesions to evolve into full-blown malignancies.</p> |                                                                 |                                                                |                                                                        |                                  |
| <b>14. SUBJECT TERMS</b><br>Proliferative, field defect, microsatellite, clonality, loss of heterozygosity, breast cancer                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                 |                                                                |                                                                        | <b>15. NUMBER OF PAGES</b><br>58 |
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| <b>17. SECURITY CLASSIFICATION OF REPORT</b><br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | <b>18. SECURITY CLASSIFICATION OF THIS PAGE</b><br>Unclassified | <b>19. SECURITY CLASSIFICATION OF ABSTRACT</b><br>Unclassified | <b>20. LIMITATION OF ABSTRACT</b><br>Unlimited                         |                                  |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

|                                   |               |
|-----------------------------------|---------------|
| Cover.....                        |               |
| SF 298.....                       | 2             |
| Introduction.....                 | 3             |
| Body.....                         | 4 - 11        |
| Key Research Accomplishments..... | 12            |
| Reportable Outcomes.....          | 12-13         |
| Conclusions.....                  | 13            |
| References.....                   | N/A           |
| Appendices.....                   | 3 pages, 2 MS |

**Introduction:**

Subject, purpose and scope of research:

By the time carcinomas *in situ*, regarded as the earliest human breast cancers, are detected, tumor cells already exhibit multiple recurrent genetic abnormalities. This implies that earlier precursor lesions must exist, and that these precursors likely contain genetic abnormalities critical to the early stages of tumor development. The goal of this research has been to gain a better understanding of the identity of these precursor lesions, and of the genetic abnormalities they contain. Therefore, the research project's two objectives were:

- 1) to determine whether histologically normal breast ducts contain genetically abnormal monoclonal populations of cells by determining whether DNA abnormalities were detectable in histologically normal tissue from women at three different levels of risk for breast cancer (those undergoing reduction mammoplasty (RM); those diagnosed with high-risk proliferative lesions, i.e., atypical hyperplasia (AH); and those diagnosed with breast cancer).
- 2) to investigate the clonal evolution of proliferative lesions by examining the DNA "fingerprint" of multiple synchronous breast tissue samples: normal-appearing epithelial ducts and terminal ductal/lobular units (TDLUs), simple and atypical hyperplastic lesions and malignant tissue from a series of breast cancer specimens. These studies were designed to help elucidate some of the earliest abnormalities in human breast carcinogenesis.

**Body:**

Research accomplishments associated with each task outlined in the approved Statement of Work:

**Task 1.** Months 1-8: Selection of microsatellite primers to be tested, testing combinations for multiplex reactions, choosing final primer combinations.

**Outcome:** This task has been completed. Multiple primers were tested, a panel of 9 were used for Technical Objective 1, an optimized and expanded panel of 18 were used for Technical Objective 2. The panel used for Objective 1 is included in the paper reporting the results (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). The panel used for Objective 2 is shown below.

**Table 1:** Expanded Marker Panel (n=18): Chromosomal Sites and Type of Repeat

| Chromosomal Site | Marker   | Repeat Type |
|------------------|----------|-------------|
| 1q32-42          | D1s549   | tetra       |
|                  | D1s213   | di          |
| 3p24             | D3s1283  | di          |
| 7q31             | D7s486   | di          |
| 11p15            | THO1     | tetra       |
|                  | D11s2071 | di          |
| 11q13            | PYGM     | di          |
| 11q23            | D11s1818 | di          |
|                  | D11s1819 | di          |
| 16q22-24         | D16s402  | di          |
|                  | D16s413  | di          |
|                  | D16s512  | di          |
| 17p13.1          | TP53     | di          |
|                  | D17s796  | di          |
|                  | D17s525  | di          |
| 17q21            | D17s1290 | di          |
|                  | D17s579  | di          |
| Xq11-12          | AR       | tri         |

**Task 2.** Months 1- 8: Identification of specimens. Specimens that belong to the three categories of subjects whose "normal" ducts will be studied will be identified from pathology reports stored in the pathology department archives, specimens for which blocks are not available will be eliminated. Specimens of breast cancer will be identified and reviewed with the pathologist to identify geographically discrete AH lesions.

**Outcome:** This task has been completed. Acquisition of the 20 specimens analyzed in Objective 1 was achieved during months 1-8. Acquisition of the 18 specimens analyzed in Objective 2 was also achieved, but was delayed into years 2 and 3 of the award because genetic analysis of these specimens has taken slightly longer than anticipated.

**Task 3. Months 3-22:** Section acquisition. Review existing slides from all potential specimens with pathologist to confirm histologic diagnoses, if confirmed, have serial sections cut, stain and review again with pathologist, who will assist in marking lesions to be microdissected. Repeat until each subject has an adequate number of ducts/lobules ready for microdissection.

**Outcome:** This task has now been completed. All sections proposed in Objective 1 were performed during the proposed time. A few sections required for the genetic analyses proposed in Objective 2 were acquired during the past year's "no-cost extension".

**Task 4. Months 4-28:** Microdissection and DNA extraction. Perform individual microdissections of demarcated tissue. Extract DNA from each microdissected area.

**Outcome:** This task has now been completed. All microdissections and DNA extractions needed for Objective 1 were completed, analyzed and reported during the original award period (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). Two of 18 cases remained to be analyzed for Objective 2. This work was performed during the past year's "no-cost extension".

**Task 5. Months 6-32:** PCR. Perform multiplex PCR on DNA from each microdissected area. Repeat all abnormal or indeterminate reactions.

**Outcome:** This task has now been completed. All multiplex PCRs for Objective 1's experiments were completed and reported during the original awarded period. (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). The multiplex PCRs remaining from 2/18 cases studied in Objective #2 were performed during the past year's "no-cost extension".

**Task 6. Months 9-34:** Analyze data. Review all gels, tabulate number and type of microsatellite abnormalities, enter data into databank. For Technical objective #2, determine if multiple patterns of monoclonal microsatellite alterations are seen in the

premalignant lesions surrounding breast cancers, and if any share the microsatellite "fingerprint" of the cancer itself.

**Outcome:** This task is complete. The analysis of Objective #1's data indicates that differences exist in the rates of clonal microsatellite abnormalities in histologically normal epithelium among women at no increased risk of breast cancer (those undergoing reduction mammoplasty [RM]) vs. those at increased risk, i.e., diagnosed with the high-risk proliferative lesion atypical hyperplasia (AH) vs. those at highest risk, i.e., diagnosed with breast cancer itself. Analysis comparing all three groups and using the 2-sided Fisher's exact test, which is most appropriate given the small sample size, yield a p value of 0.107, which is suggestive given sample size. Using the same statistical tests, analysis of women <50 years [since age could be an unrecognized factor contributing to the rate of genetic abnormalities], and comparing RM vs breast cancer cases, yields a p value of 0.049.

In addition, we reexamined the likelihood that the distribution of abnormalities we found could be due to chance. We noted 28/35 (80%) abnormalities were at 4 markers located at sites believed important in breast cancer development: 7q31, 11p15, 17p13, 17q21. This result is not likely due to chance, as the increased occurrence of these abnormalities at these sites in comparison with the other 5 sites is statistically significant ( $p < 0.01$ ) (Fisher's exact test). Although these 4 markers were selected because of their chromosomal location, their overrepresentation among all abnormalities indicates that mutations near these sites may predispose to the formation of genetically aberrant clonal populations. In contrast, mutation at arbitrary or neutral sites may not confer a growth advantage, and a detectable mutant clone may not arise. This suggests that the genetic alterations detected are less likely to be random changes and more likely to be relevant to the earliest stages of breast cancer development.

Representative examples of the autoradiography and some of the statistical analyses have been published (Larson et al, Genetically abnormal clones in histologically normal breast tissue. *Amer J Pathol* 1998; 152:1591); others were reported in Annual Report #2. We believe that these results are suggestive but only preliminary. For more definitive analyses, we are accumulating additional subjects and thereby improving the confidence in our results. This work is ongoing, funded by a new grant (NIH/NCI (PHS) RO1 CA081078, "Genetic Abnormalities Early on the Path of Breast Tumorigenesis", Rosenberg (PI) 4/1/01 – 3/31/05), which was awarded based, in part, on the preliminary data generated by the present grant.

Analysis of data for Objective #2 has been completed during the past year's "no-cost extension". Work on this aim was of great importance because although Objective #1 demonstrated that normal-appearing epithelium can be genetically abnormal, the meaning of these abnormal clones is uncertain. They may represent precursor lesions, or clonal "dead-ends". We investigated a total of 232 distinct lesions from 18 cases. To clarify the reporting of results, we have divided the analyses into 2 sections: a) abnormalities in

normal-appearing tissue vs co-existing cancers, and b) abnormalities in hyperplastic lesions vs co-existing cancers.

Analyses of data examining abnormalities in normal-appearing samples have been submitted for publication (see attached MS: Larson, et al, Loss of heterozygosity (LOH), or allele imbalance (AI) in histologically normal breast epithelium is distinct from LOH or AI in co-existing carcinomas). In summary, we examined 109 normal-appearing epithelial samples and 64 co-existing cancer samples from 18 independent breast cancer cases. We found that 14/109 (13%) normal ducts/TDLU, from 8/18 (44%) cancer-containing cases, contained LOH. The location of these 14 ducts/TDLU appeared unrelated to distance from the cancer. LOH in normal-appearing epithelium involved only single markers, whereas LOH in cancers commonly encompassed all informative markers on a chromosome arm. In only 1/14 (7%) ducts/TDLUs with LOH, was the same LOH seen in the co-existing cancer. Global differences in LOH per arm in normal-appearing tissue were not demonstrated, but less LOH was seen at 11q and 17p than at 1q ( $p = 0.002$ ), 16q ( $p = 0.01$ ) and possibly 17q ( $p = 0.06$ ). These results indicate that in a large fraction of women with breast cancer, histologically normal breast epithelium harbors occult aberrant clones. Individual clones rarely are precursors of co-existing cancers. However, they might constitute a reservoir from which cancers develop once additional genetic abnormalities occur, they could contribute to intratumoral genetic heterogeneity, and they are consistent with a role for genetic instability early in tumorigenesis.

Analyses of data examining abnormalities in 58 hyperplastic lesions vs 48 co-existing cancers are nearing completion and a MS will be submitted shortly. **Table 1** (below) lists the number of samples per case and per histology. In contrast to what was found in the normal-appearing samples, a higher proportion of hyperplastic lesions contained LOH (25/58, or 43%) and most lesions with LOH contained the same LOH as the coexisting cancer (18/25, or 72%). **Table 2** summarizes these data and **Figure 1** illustrates representative examples. Nevertheless, more than half of hyperplastic lesions did not contain LOH (despite a fairly comprehensive "LOH fingerprint"), and more than a quarter of hyperplastic lesions with LOH did not share their LOH with co-existing cancers (see **Table 3**). This implies that considerable genetic heterogeneity still exists in hyperplastic lesions. We are currently performing statistical analyses to determine whether certain chromosomal sites undergo LOH more frequently than others.

**Table 1.** Microdissected specimens: number and histology

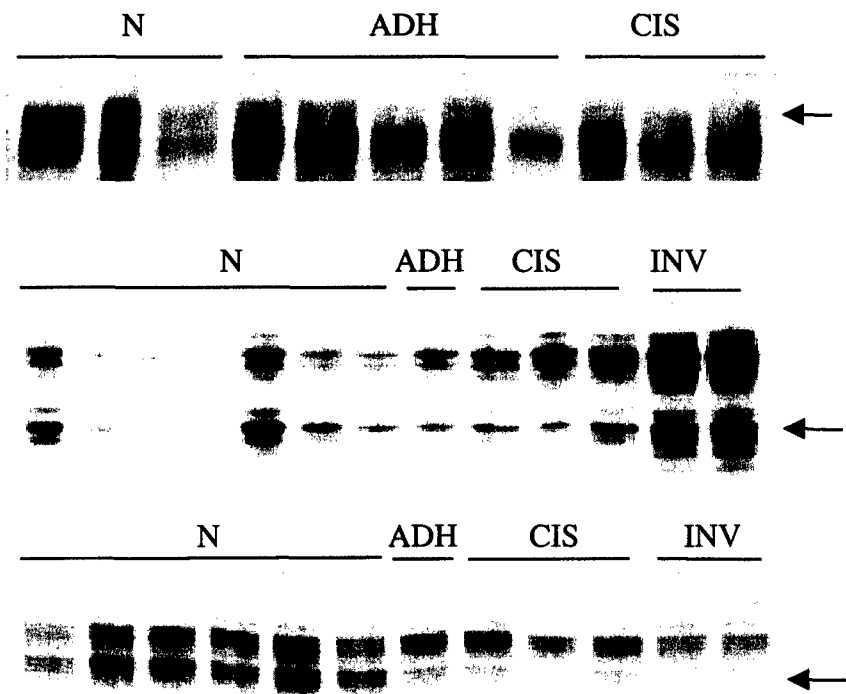
| Case   | Number of specimens<br>per histology |     |     |     |
|--------|--------------------------------------|-----|-----|-----|
|        | DH                                   | ADH | CIS | Inv |
| 2004   | 1                                    | 3   | 3   | -   |
| 2012   | -                                    | 4   | -   | 2   |
| 2014   | -                                    | 1   | 1   | -   |
| 2028   | -                                    | 1   | 1   | -   |
| 2031   | -                                    | 1   | 3   | 2   |
| 2032   | 2                                    | -   | -   | 2   |
| 2034   | -                                    | 1   | 1   | -   |
| 2044   | -                                    | 2   | -   | 3   |
| 0038   | -                                    | 3   | 3   | 5   |
| 0039   | 2                                    | 8   | 4   | -   |
| 0052   | 1                                    | 2   | 2   | 1   |
| 0053   | 2                                    | 2   | 2   | 1   |
| 0070   | -                                    | 5   | 3   | -   |
| 0071 R | 1                                    | 5   | 2   | 1   |
| 0071L  | 2                                    | 1   | -   | 1   |
| 0072   | 1                                    | 2   | 3   | -   |
| 0074   | 1                                    | 4   | 2   | -   |
| Total  | 13                                   | 45  | 30  | 18  |

**Table 2.** LOH in hyperplastic samples and co-existing cancers

| Samples | No. (%) cases<br>with LOH | No. (%) samples<br>with LOH | No. (%) samples with<br>same LOH as in<br>cancer | Extent of LOH on<br>chromosome arm: |                  |
|---------|---------------------------|-----------------------------|--------------------------------------------------|-------------------------------------|------------------|
|         |                           |                             |                                                  | single<br>locus                     | multiple<br>loci |
| DH      | 2/9 (22%)                 | 2/13 (15%)                  | 1/2 (50%)                                        | 100%                                | -                |
| ADH     | 12/17 (71%)               | 23/46 (50%)                 | 17/23 (74%)                                      |                                     | 44%              |
| CIS     | 13/13 (100%)              | 28/30 (93%)                 | -                                                |                                     | 67%              |
| Inv     | 8/9 (89%)                 | 17/18 (94%)                 | -                                                |                                     | 100%             |

**Table 3:** Sites of LOH among hyperplastic samples and co-existing cancers.

| Case  | # samples with LOH/ all samples: |     | Site of LOH in DH, ADH and cancers                                                                                                                                      |
|-------|----------------------------------|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|       | DH                               | ADH |                                                                                                                                                                         |
| 2004  | 0/1                              | 0/3 |                                                                                                                                                                         |
| 2012  | -                                | 0/4 |                                                                                                                                                                         |
| 2014  | -                                | 1/1 | ADH has same LOH at 3p, 11q, 17p as CIS.                                                                                                                                |
| 2028  | -                                | 1/1 | ADH has same LOH at 11p, 11q, 17p as CIS.                                                                                                                               |
| 2031  | -                                | 1/1 | ADH has same LOH at 1q, 11q, 16q as CIS & Inv.<br>This ADH also has LOH at another 11q site not seen in cancers.                                                        |
| 2032  | 0/2                              | -   |                                                                                                                                                                         |
| 2034  | -                                | 1/1 | ADH has same LOH at 1q as CIS.<br>ADH also has LOH at 11p, 11q not seen in cancer.                                                                                      |
| 2044  | -                                | 2/2 | 2 ADH have same LOH at 1q, 16q as Inv.                                                                                                                                  |
| 0038  | -                                | 1/3 | 1 ADH has LOH of opposite 16q allele as cancers.                                                                                                                        |
| 0039  | 1/2                              | 0/8 | 1 DH has LOH of opposite 17p allele as CIS.                                                                                                                             |
| 0052  | 0/1                              | 0/2 |                                                                                                                                                                         |
| 0053  | 0/2                              | 1/2 | 1 ADH same LOH at 1q, 16q as CIS & Inv.<br>This ADH also has LOH at 11q not seen in cancers.                                                                            |
| 0070  | -                                | 5/5 | 5 ADH have same LOH at 1q, 16q as CIS.                                                                                                                                  |
| 0071R | 0/1                              | 5/5 | 3/5 ADH have same LOH at 1q as CIS & Inv.<br>2/5 ADH also have same LOH at 17p as CIS & Inv.<br>1 ADH has LOH of opposite 1q allele as in other ADH, CIS & Inv lesions. |
| 0071L | 0/2                              | 1/1 |                                                                                                                                                                         |
| 0072  | 0/1                              | 2/2 | 2 ADH have LOH of opposite 16q allele as CIS.                                                                                                                           |
| 0074  | 1/1                              | 2/4 | DH and 1 ADH and 1 CIS have same LOH at 16q<br>1 ADH has LOH at 11q not seen in CIS.                                                                                    |



**Figure 1:** Atypical ductal hyperplastic lesions (ADH) have LOH of the same alleles as co-existing cancers. Arrows indicate the allele undergoing LOH.

Top panel: LOH of the larger allele at marker D16s413 is seen in all 5 ADH and 3 CIS from case 0070.

Middle panel: LOH of the smaller allele at marker D1s213 is seen beginning in the ADH and with progressively greater intensity in the 3 CIS and 2 INV samples from case 2031.

Bottom panel: LOH of the smaller allele of marker PYGM is seen beginning in the ADH lesion and with progressively greater intensity in the 3 CIS and 2 INV samples from case 2031.

**Task 7.** Months 12 and 24: Statistical analysis. For Technical Objective #1, preliminary statistical analysis will be performed to determine rate of microsatellite abnormalities, differences between groups, significance and power of the findings. The preliminary analysis may indicate a need for additional samples from one or another of the subject groups in order to achieve statistically meaningful results.

**Outcome:** This task is completed. The statistical analyses have been presented above (see Task 6). These analyses indicate significant differences between the rate of microsatellite abnormalities in normal appearing breast epithelium in the controls (RM) vs the breast cancer group. Consistent with these findings, subjects diagnosed with AH (i.e., those at intermediate risk) had an intermediate rate of microsatellite abnormalities. Therefore, a new research grant was submitted to the NIH to confirm these differences and examine them in greater detail and utilizing additional groups of subjects (i.e., those with constitutional mutation of BRCA1 or BRCA2). This grant (NIH/NCI (PHS) RO1 CA081078, "Genetic Abnormalities Early on the Path of Breast Tumorigenesis", Rosenberg (PI) 4/1/01 – 3/31/05), was awarded based, in part, on the preliminary data generated by the present DAMD grant.

**Task 8.** Months 30-36. Clinical correlation and follow-up. Review medical records to determine if presence of microsatellite alterations in histologically normal breast tissue is correlated with clinical features. Determine if presence of multiple, monoclonal, genetically distinct premalignant lesions is associated with clinical features of the breast cancer.

**Outcome:** This task is completed. We reviewed the clinical data available and determined that the presence of clonal microsatellite alterations in normal-appearing breast epithelium is linked to breast cancer risk, particularly in women < 50 yrs (see Task 6, above). No other associations could be found, but the numbers of subjects available for analysis was small. The role that age plays is unclear. Based on the preliminary results from these studies, we have proposed that subjects < 50 and > 50 be analyzed separately.

**Task 9:** Months 34-36. Final statistical analysis.

**Outcome:** This task is completed. The statistical analyses for Objective #1 were completed during months 12 and 24. The statistical analysis for Objective #2 was completed during this year's "no-cost extension".

### Key Research Accomplishments:

1. Demonstration that monoclonal microsatellite DNA abnormalities are detectable in histologically normal breast epithelium from women at all degrees of breast cancer risk. The rate of abnormalities increases with increasing risk, especially among women < 50 years ( $p < 0.05$ ).
2. Low frequency but fairly high prevalence of abnormal clones in histologically normal breast tissue from women < 50 years with breast cancer. However these clones are rarely linked clonally to synchronous cancers. Because their sites of LOH do not appear to be random, however, they may constitute a reservoir from which cancers may arise, identify a high-risk group of women, and suggest the location of tumor-suppressor genes important to the earliest stages of tumorigenesis.
3. In contrast to the data in histologically normal epithelium, high-risk proliferative lesions (particularly atypical ductal hyperplasia) demonstrate LOH much more frequently, and lesions with LOH are commonly clonally linked to co-existing cancers. Considerable genetic heterogeneity does exist even among the highest risk lesions, however.

### Reportable Outcomes:

#### Manuscripts:

1. Genetically Abnormal Clones in Histologically Normal Breast Tissue. PS Larson, A de las Morenas, LA Cupples, K Huang, **CL Rosenberg**. Am J Pathol 1998; 152:1591.
2. Loss of heterozygosity (LOH) or allele imbalance (AI) in histologically normal breast epithelium is distinct from LOH or AI in co-existing carcinomas. PS Larson, A de las Morenas, SR Bennett, LACupples, **CL Rosenberg**. MS in revision.
3. Genetic changes in co-existing human breast hyperplasias and carcinomas. PS Larson, A de las Morenas, SR Bennett, LACupples, **CL Rosenberg**. MS in preparation.

#### Abstracts:

1. Clonal Progression of Premalignant Breast Cancer Precursors. Larson PS, de las Morenas A, **Rosenberg CL**. Proceedings AACR, 4/2000 #3277.

**Presentations:**

1. Genetically Abnormal Clones in Histologically Normal Breast Tissue. **CL Rosenberg**. Gordon Research Conference: DNA Alterations in Transformed Cells. Colby - Sawyer College, New London NH, 8/11/98. (Abstract also)
2. Clonality and Genetic Instability in Premalignant Breast Tissue. **CL Rosenberg**, invited platform presentation in the Molecular Epidemiology program, Department of Defense Breast Cancer Research Program meeting, Atlanta GA, June 8-11 2000. (Abstract also)
3. Genetic Abnormalities Early in Breast Tumorigenesis. **CL Rosenberg**, invited speaker, Ruth Sager Memorial Symposium on breast cancer. Boston Cancer Research Association, Harvard Medical School, Boston MA, 4/25/01.
4. Genetic Abnormalities Early in Breast Tumorigenesis. **CL Rosenberg**, invited speaker, University of Connecticut Health Sciences Center, Farmington, CT 5/2/01.

Funding awarded based on work supported by this award:

1. NIH R01CA81078 (funding began in 4/01).

**Conclusions:**

We conclude that histologically normal breast epithelium harbors genetic abnormalities. The rate of abnormalities increases with increasing risk of breast cancer, especially among women < 50 years ( $p < 0.05$ ). This has implications for our understanding of the earliest steps of breast tumorigenesis, which may begin before any pathologic changes are evident. Investigation of more subjects, and subjects from additional risk groups, should confirm and expand these findings.

Further, we conclude that only a small subset of these aberrant clones in normal-appearing tissue are clonally linked to synchronous breast cancers. Thus, multiple aberrant clones can coexist. Their eventual fate cannot be determined, but it is of interest that the same sites of LOH are detected in normal-appearing, proliferative and malignant tissue, suggesting that LOH at these sites may inactivate a tumor suppressor gene and lead to clonal expansion. In contrast to normal-appearing clones, most high-risk atypical hyperplastic lesions are clonally linked to synchronous breast cancers, indicating that these lesions are more likely to be true precursors rather than markers of increased risk.

Future work will involve confirmation of the findings in normal tissue by examining additional subjects from different risk groups, and identification and testing of candidate tumor-suppressor genes important in the earliest stages of tumor progression.

**Appendix:**

1. Manuscript (Larson et al, AJP 1998)
2. Manuscript (Larson et al, under review 2002)
3. 3 Abstracts :

8/11/98:

**Genetically Abnormal Clones in Histologically Normal Breast Tissue**

CLRosenberg, Boston University Medical Center, Boston MA 02118.

Breast cancer is a genetic disease, but little is known about the genetic abnormalities that are central to the earliest steps of tumorigenesis. Identifying these abnormalities may be critical to understand breast cancer risk and development, and to create new detection and treatment strategies. To elucidate what these important early abnormalities might be, we have developed a system to investigate small quantities of archival human breast tissue specimens. This system utilizes ~20 highly heterozygous microsatellite markers (mono, di, tri and tetranucleotide repeats), located at ~11 chromosomal regions (including some potentially relevant to breast tumorigenesis), multiplexed into ~5 PCRs. With these combinations we can reliably examine nanogram quantities of DNA from microdissected tissue sections.

Using this system, we found monoclonal, genetically abnormal populations of cells in "benign" proliferative lesions (atypical hyperplasia), and in histologically normal breast ductal tissue. Both LOH and microsatellite instability were detected. The abnormalities in normal-appearing tissue were more common in women with cancer than in control (reduction mammoplasty) subjects, and were detected more frequently at chromosomal regions implicated in breast tumorigenesis, compared with randomly selected or neutral sites. These data lead us to hypothesize that certain individuals' breast tissue may contain widespread genetic abnormalities, i.e., "field cancerization". Affected tissue, although normal or benign appearing, would contain a pool of genetically abnormal precursor lesions which could result in increased susceptibility to malignancies, or in malignancies that are distinctive in genetic, clinical or other features.

To investigate these possibilities, we are using our system to compare normal-appearing ductal tissues from several groups of women: those with sporadic cancer vs. those with an hereditary predisposition vs. reduction mammoplasty controls. We are also examining normal-appearing and malignant breast tissues from women exposed prenatally to the potent estrogen compound diethylstilbestrol (DES) vs. controls. We speculate that there will be increased genetic abnormalities in all three groups, compared to controls, and that the pattern of abnormalities may indicate genes or pathways important to the earliest steps of breast tumorigenesis.

4/00:

**Clonal Progression of Premalignant Breast Cancer Precursors.** Larson PS, de las Morenas A, Rosenberg CL. Boston University Medical Center, Boston MA 02118.

The earliest recognized breast malignancies, carcinomas in situ (CIS), contain multiple abnormalities, suggesting that precursor lesions exist. Hyperplastic lesions are candidate precursors, since epidemiological evidence links them to increased breast cancer risk, and genetic data indicate they can contain clonal abnormalities. However, their relation to malignancies remains unknown. To determine whether ductal hyperplasias are precursors of ductal malignancies, we multiplexed ~20 microsatellites, from 9 chromosomal arms, and examined loss of heterozygosity (LOH) in DNA from multiple lesions microdissected from single specimens. From 14 specimens, 83 controls (stroma, node or epithelium), 9 simple hyperplasias, 38 atypical hyperplasias (AH), 27 CIS and 17 invasive carcinoma (IC) samples were examined. We find 1) in 6/14 subjects (43%) one or more AH shares site(s) of LOH with CIS and/or ICs in the same specimen. Other, histologically identical, AH may not contain the same LOH. 2) LOH seen in histologically normal tissues is not always detected in synchronous hyperplastic or malignant lesions. These data suggest 1) AHs are genetically heterogeneous but at least a fraction are clonally related to cancers, either as direct precursors or by sharing a common precursor. 2) Aberrant clones in normal-appearing tissue are not obligate cancer precursors. These results should help define sequences of genetic abnormalities that result in breast cancer development.

6/00

### **CLONALITY AND GENETIC INSTABILITY IN PREMALIGNANT BREAST TISSUE**

**Carol L Rosenberg MD, Antonio de las Morenas MD  
L Adrienne Cupples PhD, Pamela S Larson PhD**

Boston University Medical Center, Boston MA 02118

[croseberg@med-med1.bu.edu](mailto:croseberg@med-med1.bu.edu)

Multiple genetic abnormalities characterize the earliest recognized breast malignancy, carcinoma in situ (CIS), implying that premalignant precursors exist. Recent data demonstrate hyperplastic lesions, considered benign but associated with increased risk of breast cancer, can contain clonal genetic abnormalities, in particular, loss of heterozygosity (LOH). We hypothesized: 1) LOH might be detectable earlier, perhaps even in histologically normal breast epithelium. If so, aberrant clones might represent very early stages in breast tumorigenesis, and their genetic alterations implicate loci critical to the beginning of cancer development; 2) we could explore breast cancer

progression by examining patterns of LOH in synchronous normal-appearing, hyperplastic and malignant tissues.

To investigate, we used a panel of 10-20 microsatellite markers selected for chromosomal location at known or putative tumor suppressor (ts) genes, % heterozygosity, and size of amplified product, to examine DNA from histologically defined lesions. 95 normal-appearing samples of breast epithelium were microdissected from archived blocks of 6 consecutive cases of reduction mammoplasty, 9 of atypical hyperplasia and 5 of sporadic breast cancer. From these and additional cancer specimens, 83 more normal, 47 hyperplastic, 27 CIS and 17 invasive carcinoma lesions were also microdissected.

We found clonal abnormalities, primarily LOH but occasional microsatellite instability, in 22% (21/95) of histologically normal samples; in women <50 yrs trends towards increased abnormalities were noted with increased breast cancer risk ( $p = 0.05$ ). Abnormalities clustered at sites of known or postulated ts genes vs at more random or neutral sites: 80% (28/35) were at 7q, 11p, 17p, 17q, vs. 20% (7/35) at 1p, 1q, 2p, 18q, Xq ( $p = 0.05$ ). Preliminary investigations into the progression of aberrant clones suggest that multiple independent clones can exist within a single breast, some of which are related, and other unrelated, to the cancer that is present.

Thus, genetic abnormalities are present even in histologically normal breast tissue and genetic instability characterizes certain premalignant breast tissues. Future studies should help distinguish clones that are likely to progress and from those that are not.

# Genetically Abnormal Clones in Histologically Normal Breast Tissue

Pamela S. Larson,\*<sup>†</sup> Antonio de las Morenas,<sup>†</sup>  
L. Adrienne Cupples,<sup>‡</sup> Katie Huang,\* and  
Carol L. Rosenberg\*<sup>†§</sup>

From the Cancer Research Center\* and the Departments of  
Pathology,<sup>†</sup> Epidemiology and Biostatistics,<sup>‡</sup> and Medicine,<sup>§</sup>  
Boston University School of Medicine, Boston, Massachusetts

**Breast cancer is believed to develop as multiple genetic abnormalities accumulate, each conferring some growth advantage, but the timing and nature of the earliest steps in this progression are not yet elucidated. Proliferative breast lesions, associated with an increased risk of breast cancer although considered benign, recently were shown to contain clonal genetic abnormalities. Therefore, we hypothesized that clonal genetic abnormalities might be detectable before any phenotypic abnormalities are evident, ie, in histologically normal breast tissue. We examined DNA extracted from 95 normal-appearing breast ducts or terminal ductal-lobular units from 20 individuals at varying degrees of risk (those undergoing reduction mammoplasties, those with atypical hyperplastic proliferative lesions, and those already diagnosed with breast cancer). Using nine microsatellite markers, we sought evidence of genetic instability or of allelic imbalance (most likely representing loss of heterozygosity). We found genetically abnormal clones in 21/95 (22%) seemingly normal samples from 10/20 (50%) women from all three risk groups. In women under age 50, trends toward increased rates of abnormalities were noted with increased cancer risk. The abnormalities identified were more likely to be at sites of known or postulated tumor suppressor genes rather than at random or neutral loci. Our data indicate that genetic abnormalities potentially critical to breast tumorigenesis accumulate before pathological detection even of high-risk lesions and are detectable in tissue that is not only histologically benign but also completely normal. (*Am J Pathol* 1998, 152:1591-1598)**

Breast cancer is believed to develop as multiple genetic abnormalities accumulate, each conferring some growth advantage. Aberrations of oncogenes, loss of genetic material, and some degree of genomic instability can be detected in breast cancers of all stages.<sup>1-9</sup> Recent evidence indicates that at least a subset of proliferative

breast lesions may also be characterized by clonal genetic aberrations, including loss of heterozygosity (LOH) or some type of microsatellite instability.<sup>10-17</sup> Histologically, these lesions are considered to be benign, although epidemiologically they are associated with increased risk of cancer development. Thus, some of these lesions could represent actual precursors of malignancy. Based on these findings, we hypothesized that some genetic abnormalities may have occurred even earlier, ie, before the development of histologically abnormal tissue and, therefore, might be detectable in normal-appearing breast ductal tissue. Finding genetic abnormalities in histologically normal breast tissue would imply that genetically abnormal clones develop and can be identified much earlier than has been appreciated. In addition, the nature of any identified abnormalities could indicate events important to the initial steps of breast tumorigenesis.

To investigate this hypothesis, we examined multiple samples of histologically normal breast ductal tissue from 20 individuals' archival specimens. Both single ducts and the larger terminal ductal-lobular units (TDLUs) were examined, as it was recently demonstrated that TDLUs are likely to represent the progeny of a single breast ductal precursor or stem cell.<sup>18, 19</sup> Although this was a pilot study examining specimens from 20 individuals, we selected breast tissue from three distinct groups of subjects: 1) those at no increased risk of breast cancer (reduction mammoplasties), 2) those without a history of breast cancer but who are at a four- to five-fold increased risk of developing the disease because of a biopsy revealing an atypical hyperplastic (AH) lesion, and 3) those already diagnosed with breast cancer (lumpectomies and mastectomies).

Each sample of normal tissue was examined with a panel of nine highly informative microsatellite markers. The markers were selected so that approximately one-half were situated at chromosomal regions known to be lost or mutated in breast cancer (and therefore might represent sites of tumor suppressor genes important to breast tumorigenesis); the other half were at genes or anonymous sequences not known to be relevant to breast tumorigenesis. Additionally, loci were selected to

Supported by NIH PHS grant CA62179, by a Massachusetts Department of Public Health Breast Cancer Research Grant, and by the Boston City Hospital Fund for Excellence.

Accepted for publication March 6, 1998.

Address reprint requests to Dr. Carol L. Rosenberg, Boston University Medical Center, 80 East Concord Street, R908, Boston MA 02118. E-mail: crosenberg@med-med1.bu.edu.

achieve a mixture of di-, tri-, and tetranucleotide repeats. In most breast cancers, the microsatellite instability seen is not the widespread dinucleotide repeat alterations characteristic of tumors lacking normally functioning mismatch repair genes. Instead, changes are seen in more often in tri- or tetranucleotide repeats, and at comparatively lower frequency, suggesting a more subtle defect maintaining genomic integrity.<sup>3, 8</sup>

Using these specimens and this panel of markers, we determined the incidence and pattern of genetic abnormalities in multiple independent histologically normal samples of breast ductal tissue.

## Materials and Methods

### Selection of Samples

All samples were reviewed by a single breast pathologist to identify histologically normal tissue. In total, 95 samples of histologically normal single ducts or TDLUs were obtained from 20 subjects; an average of 5 ducts or TDLUs were examined per subject. In addition, lymph node and stromal tissues were examined when available. Six cases of reduction mammoplasties were identified at random from the pathology department archives; candidates were screened before surgery to eliminate those in whom there is a personal or familial history of breast cancer. Nine cases of AH had been identified previously.<sup>14</sup> Five cases of breast cancers diagnosed in pre- or perimenopausal women were selected at random from the pathology department archive. Breast cancer cases were selected in an attempt to match the uniformly young age of the reduction mammoplasty subjects and the relatively young age of those with AH. Most specimens dated from 1994 or 1995; a few were older. Initial diagnostic or therapeutic specimens were used, and therefore, no subjects had received prior chemotherapy or radiation. Tissue from both right and left sides was identified in all reduction mammoplasty and in breast cancer subject 34, who had bilateral disease. Except in this case, no subject with breast cancer was aware of a positive family history. Tissue from only the affected side was available in the remaining cases.

### Preparation of DNA

Histologically normal single ducts or TDLUs, stromal tissue and lymph nodes, were identified by hematoxylin and eosin (H&E) staining of the top and bottom of seven consecutive sections cut from a tissue block; the five intervening unstained sections were then microdissected as previously described.<sup>14</sup> DNA was extracted using standard techniques.<sup>20, 21</sup> To make sure abnormalities were not artifacts due to small amounts of template DNA,<sup>22</sup> we performed serial dilution experiments. Using normal lymphocyte DNA, we determined the concentration at which the pattern of the products amplified by the AR primers was no longer reproducible. In each case, the amplified products were identical in size and intensity when 100 pg or more of template DNA was used, but with

10 pg, the results were inconsistent (data not shown). To approximate the minimal DNA concentration in each reaction using DNA from ducts or TDLUs, we determined that the number of cells microdissected from a normal duct varied between 250 and 1000; a TDLU contains far more cells. Assuming 6.5 pg of DNA per cell and 50% loss of DNA during extraction, and given that 1/10 of the final volume of DNA solution was used per reaction, we estimate that a minimum of between 80 and 325 pg of template DNA was available per reaction.

### Microsatellites

After DNA extraction, each sample was examined using nine microsatellite markers at nine genomic loci. The nine microsatellite sequences examined were MYCL1 (1p), D1S549 (1q), D2S123 (2p), D7S486 (7q), THO1 (11p), TP53 (17p), D17S579 (17q), D18S34 (18q), and AR (Xq). Primers were purchased from Research Genetics (Huntsville, AL) or synthesized commercially.

### Polymerase Chain Reaction and Data Analysis

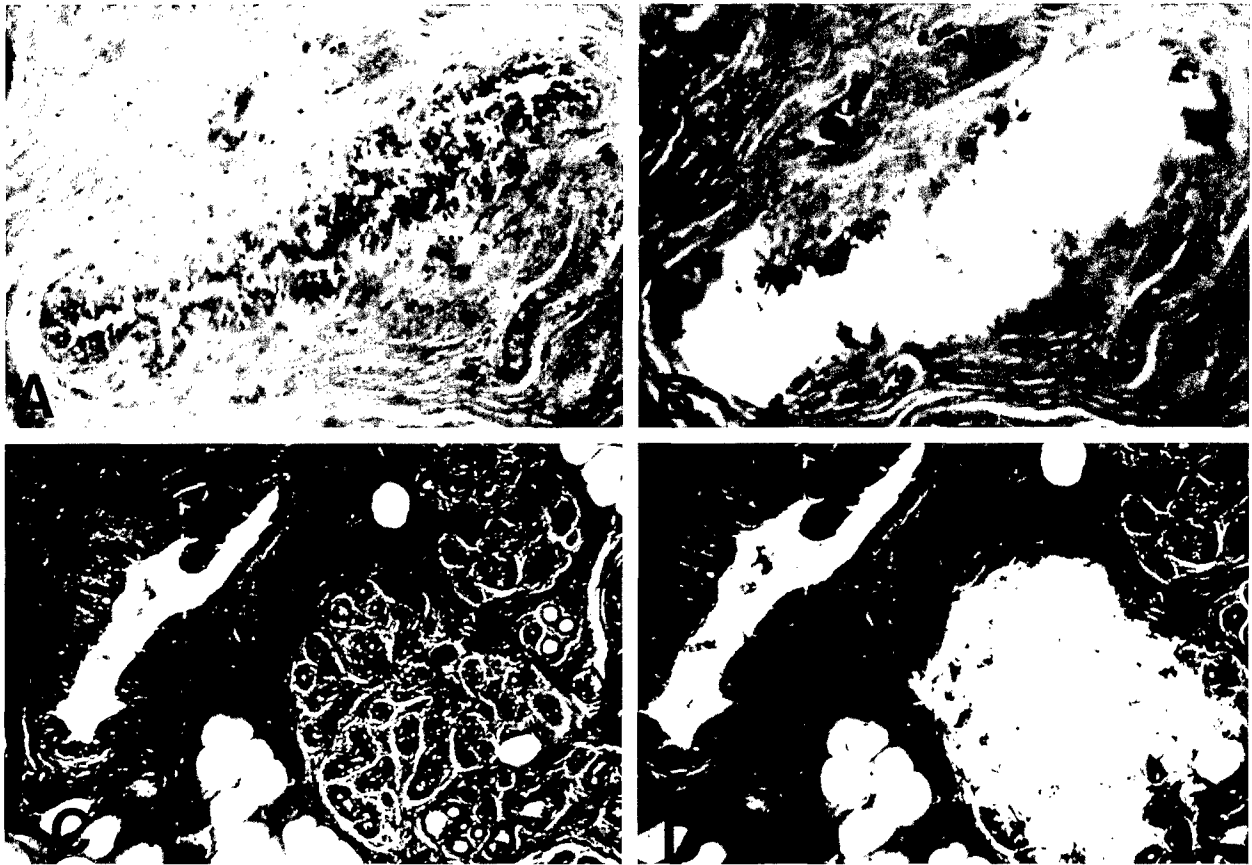
We performed multiplex polymerase chain reactions (PCRs) as described elsewhere.<sup>14</sup> Briefly, 1/10 of the DNA solution served as a template in a 50- $\mu$ l reaction volume. After 40 cycles of amplification incorporating [ $\alpha^{32}$ P]dCTP, with annealing temperatures of 55°C, 58°C, or 60°C, one-fifth of the amplified products were electrophoresed through 7% denaturing gels. Microsatellite changes were scored by visual inspection as instability (when a novel-sized amplified product was present) or as allelic imbalance suggestive of LOH when unequivocal loss of intensity of one allele was seen at heterozygous loci. To be scored as abnormal, demonstration of instability or LOH needed to be reproduced at least twice with identical results. Because of the limited quantities of DNA available, unequal amplification in early PCR cycles could lead to inaccurate relative allele intensities; therefore, ratios of relative allele intensities at heterozygous loci based on densitometry were not calculated, and relative allele imbalance was scored as no loss. After scoring, the total number of abnormal samples and of abnormal alleles, and the nature of the abnormalities, were determined.

### Statistical Analysis

We compared the three groups on the following measures: 1) the proportion of subjects with at least one abnormality, 2) the mean percentage of abnormal loci, and 3) the mean percentage of abnormal ducts. The data were examined for all subjects and, separately for women under age 50, by analysis of variance (weighting by the number of ducts or TDLUs).

## Results

Histologically normal breast ducts and TDLUs (samples) were microdissected carefully (see Figure 1). We de-



**Figure 1.** Examples of tissue before and after microdissection. Slides were stained with H&E for the purpose of illustration. Subject 35's sample 6 (duct) is shown before (A) and after (B) microdissection. Magnification,  $\times 200$ . Subject 35's sample 2 (TDLU) is shown before (C) and after (D) microdissection. Magnification,  $\times 100$ . Amplification of the DNA extracted from these two samples is shown in Figure 3D.

tected genetically abnormal clones of cells in these normal-appearing samples in 10/20 (50%) subjects studied; 21/95 (22%) ducts or TDLUs were involved. The data are presented in Table 1. Alterations were present in subjects from all three groups, ie, in 2/6 (33%) women without apparent increased risk of breast cancer (reduction mammoplasty), in 4/9 (44%) women with an increased risk of breast cancer (AH), and in 4/5 (80%) women who already had developed the disease. More than one independent abnormal clone was detected in 1/6 (17%) subjects who had undergone reduction mammoplasty (subject 32), in 1/9 (11%) subjects with AH (subject 19), and in 4/5 (80%) subjects (subjects 34, 35, 36, and 37) with breast cancer.

Samples characterized by microsatellite changes were histologically indistinguishable from those without (see Figure 2), and no preference for abnormalities in single ducts compared with TDLUs was seen. Somewhat more microsatellite instability (22 examples) than LOH (13 examples) was evident, but this may have been skewed by one subject (12) with nine instances of instability and only one of LOH. In addition, because relative allele imbalance was scored as no loss (due to the small quantity of template DNA; see Materials and Methods), it is possible that we underestimated the number of cases characterized by LOH. However, as each duct or TDLU likely represents the progeny of a single ductal stem cell,<sup>18</sup>

allelic alterations should usually affect at least a substantial fraction of the cells comprising the sample and therefore should, generally, be detectable.

From six subjects undergoing reduction mammoplasties, who had no increased risk of breast cancer, 32 histologically normal samples were examined. Five ducts from two individuals contained clonal genetic changes. Subject 24 had a single abnormality identified in one of five samples (data not shown). In subject 32 (see Figure 3A), 4 of 10 samples, all from the right breast, had evidence of five microsatellite abnormalities, four of which involved a single microsatellite locus. In this subject, more than one abnormal clone was present. To determine with certainty this subject's germline configuration at these loci, three geographically distinct samples of stromal tissue were microdissected from the same blocks. All three demonstrate a single pattern, identical to that seen in the majority of the ducts (see Figure 3A).

From nine subjects diagnosed with high-risk AH lesions (some of which have been shown to contain clonal abnormalities<sup>14</sup>), a total of 26 histologically normal samples were examined. Because the diagnosis of AH is usually made from a biopsy, the amount of tissue available for investigation is much smaller than from reduction mammoplasty or cancer specimens, and it is always unilateral. Despite the smaller number of samples exam-

**Table 1.** Microsatellite Alterations in Histologically Normal Breast Tissue Samples

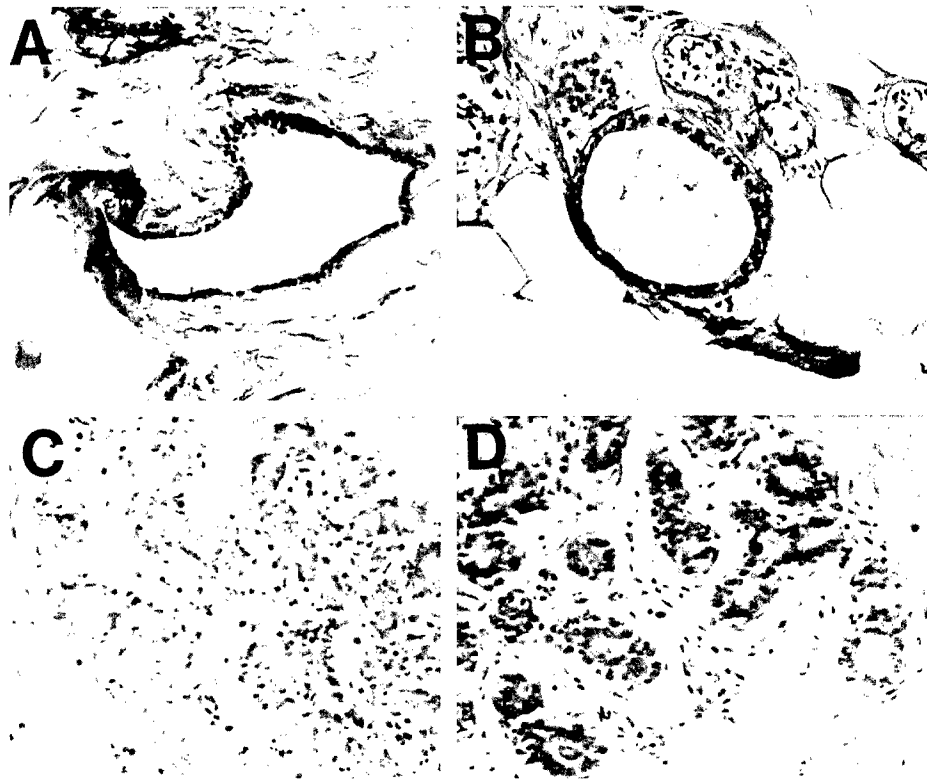
| Subject                                                                                   | Age | Number of microsatellite loci | Number of altered samples/total samples | Number of altered alleles/total alleles examined* | Type of alteration                |
|-------------------------------------------------------------------------------------------|-----|-------------------------------|-----------------------------------------|---------------------------------------------------|-----------------------------------|
| Histologically normal ducts or TDLUs from reduction mammoplasty specimens ( <i>n</i> = 6) |     |                               |                                         |                                                   |                                   |
| 24                                                                                        | 34  | 9                             | 1/5                                     | 1/90                                              | 1 instability                     |
| 25                                                                                        | 25  | 9                             | 0/4                                     | 0/72                                              |                                   |
| 26                                                                                        | 24  | 9                             | 0/3                                     | 0/54                                              |                                   |
| 29                                                                                        | 42  | 9                             | 0/3                                     | 0/48                                              |                                   |
| 31                                                                                        | 23  | 7                             | 0/7                                     | 0/92                                              |                                   |
| 32                                                                                        | 36  | 8                             | 4/10                                    | 5/138                                             | 1 instability, 4 LOH              |
| Subtotal                                                                                  |     | 9                             | 5/32 (15.6%)                            | 6/494 (1.2%)                                      | 2 instability, 4 LOH              |
| Histologically normal ducts or TDLUs from AH biopsies ( <i>n</i> = 9)                     |     |                               |                                         |                                                   |                                   |
| 10                                                                                        | 58  | 9                             | 0/3                                     | 0/50                                              | 9 instability, 1 LOH <sup>†</sup> |
| 12                                                                                        | 63  | 8                             | 1/6                                     | 10/92                                             |                                   |
| 13                                                                                        | 37  | 8                             | 0/2                                     | 0/32                                              | 2 instability, 2 LOH, 1 LOH       |
| 17                                                                                        | 51  | 8                             | 0/3                                     | 0/48                                              |                                   |
| 18                                                                                        | 31  | 8                             | 0/2                                     | 0/32                                              |                                   |
| 19                                                                                        | 41  | 8                             | 2/2                                     | 2/32                                              |                                   |
| 20                                                                                        | 74  | 7                             | 1/3                                     | 2/40                                              |                                   |
| 21                                                                                        | 59  | 5                             | 1/3                                     | 1/26                                              |                                   |
| 23                                                                                        | 63  | 8                             | 0/2                                     | 0/32                                              |                                   |
| Subtotal                                                                                  |     | 8                             | 5/26 (19.2%)                            | 15/384 (3.9%)                                     | 11 instability, 4 LOH             |
| Histologically normal ducts or TDLUs from subjects with breast cancer ( <i>n</i> = 5)     |     |                               |                                         |                                                   |                                   |
| 34                                                                                        | 39  | 7                             | 3/7                                     | 3/84                                              | 1 instability, 2 LOH              |
| 35                                                                                        | 38  | 7                             | 2/11                                    | 4/154                                             | 4 instability <sup>†</sup>        |
| 36                                                                                        | 39  | 7                             | 3/8                                     | 3/98                                              | 2 instability, 1 LOH              |
| 37                                                                                        | 38  | 8                             | 3/6                                     | 4/74                                              | 2 instability, 2 LOH <sup>†</sup> |
| 38                                                                                        | 53  | 6                             | 0/5                                     | 0/60                                              | 9 instability, 5 LOH              |
| Subtotal                                                                                  |     | 7                             | 11/37 (29.7%)                           | 14/470 (3.0%)                                     |                                   |
| Total                                                                                     |     | ~8                            | 21/95 (22.1%)                           | 35/1348 (2.6%)                                    | 22 instability, 13 LOH            |

\*Occasional amplifications either were not successful or did not yield reproducible results; in these cases, the alleles were not scored, and hence, the actual number of alleles examined is in some instances slightly smaller than the maximum possible number would be. The maximum possible number of evaluable alleles equals: (number of primers) × (number of ducts) × (two alleles per locus).

<sup>†</sup>One or more biallelic changes noted.

ined per subject in this group than in either of the others, we identified evidence of microsatellite alterations in 5 of 26 histologically normal breast samples from 4 of 9 subjects in this high risk group (subjects 12, 19, 20, and 21). Subject 12 demonstrated 10 microsatellite alterations, all present in one of six samples (see Figure 3B). (This subject had colon cancer diagnosed at age 60, 3 years before breast biopsy, and 9 years later remains free of disease. It is possible that she represents a case of hereditary nonpolyposis colon carcinoma). Subject 19 demonstrated microsatellite instability in each of the two samples examined. A different locus was altered in each sample; thus, this subject had more than one abnormal clone. In subjects 20 and 21, one of three samples was abnormal, each with evidence of LOH (data not shown). Interestingly, the AH lesions from these four subjects were not found to have allelic alterations.<sup>14</sup> Similar dissonance between genetic abnormalities in AH and in simple hyperplastic lesions from the same subject has been reported recently.<sup>17</sup>

The third group studied consisted of five subjects with breast cancer, from whom a total of 37 histologically normal ducts and TDLUs were microdissected. Seemingly normal tissues from four of five subjects contained genetic abnormalities. In all four subjects, multiple abnormal clones were found. Three of seven samples from subject 34 contained genetic alterations at three different loci (see Figure 3C). Samples from both left and right breast were abnormal. As a control to confirm the subject's germline configuration at the apparently altered loci, DNAs from three lymph nodes pathologically free of tumor were examined. Their microsatellite patterns were the same as the predominant pattern seen in the ducts or TDLUs (see Figure 3C). Two of eleven samples from subject 35 contained four genetic alterations involving two microsatellite loci (see Figure 3D). As a control, lymphoid tissue from three separate nodes was examined; no microsatellite alterations were detected. Three of eight samples from subject 36 demonstrated three genetic alterations at three separate loci (see Figure 3E). Finally,



**Figure 2.** Normal-appearing ducts and TDLUs. Those with or without genetic alterations are histologically indistinguishable. **A:** From subject 12, sample 6, a genetically normal duct. **B:** Sample 3, which is genetically aberrant. **C:** From subject 32, sample 3, a genetically normal TDLU. **D:** Sample 2, which is genetically aberrant. Magnification,  $\times 100$ .

three of six samples from subject 37 revealed four abnormalities at three microsatellite loci (data not shown). The remaining subject (38), with five samples examined, demonstrated no abnormalities.

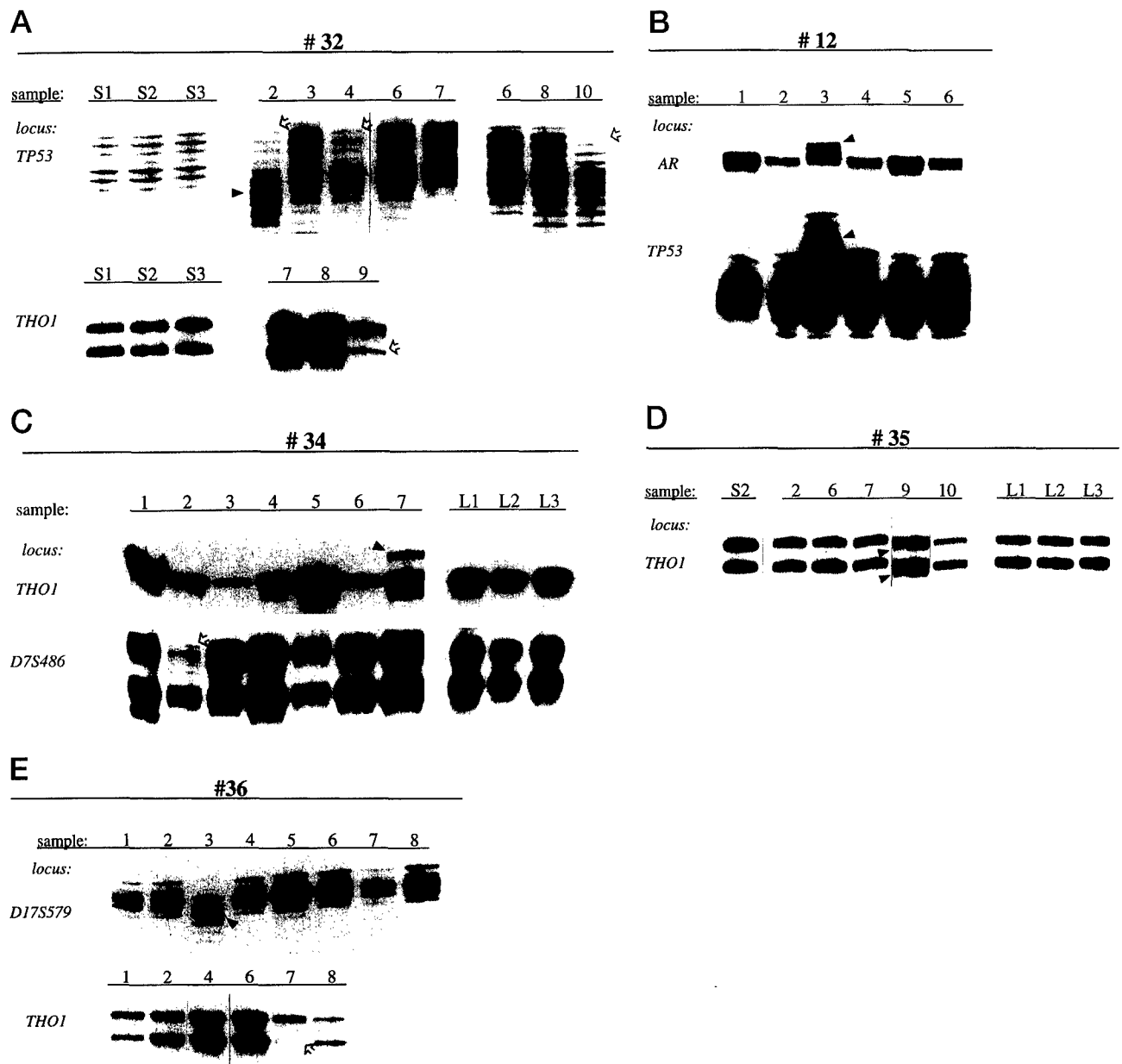
Overall, a total of 35 clonal alterations were detected among 1348 alleles examined, yielding a mutation rate of 2.6%. There were suggestions that as the risk of breast cancer increased so did the number of alterations, particularly for women less than 50 years old. For example, the percentage of all subjects with any abnormality increased from 33.3% to 44.4% to 80.0% across the three groups, and the mean percentage of abnormal alleles rose from 1.2% in women with reduction mammoplasties to 3.9% in women with AH and to 3.0% in women with breast cancer. Similarly, the mean percentage of abnormal ducts increased with risk of breast cancer, from 15.6% in the reduction mammoplasty group to 19.2% in the AH group and to 29.7% in the breast cancer group. However, perhaps due to the relatively small sample size of this pilot study, these observations did not achieve statistical significance. When women under age 50 were examined, the trends were more pronounced and reached significance when comparing mean percentage of abnormalities between the subjects with reduction mammoplasty (1.2%) and those with breast cancer (3.4%;  $P = 0.049$ ; see Table 2).

Certain microsatellite loci were altered much more commonly than others. Four of the nine loci examined accounted for 28/35 (80%) abnormalities. In contrast, the remaining five loci accounted for only one-fourth as

many: 7/35 (20%) abnormalities. When LOH alone was considered, the results were similarly skewed; 11/13 examples of LOH were at these four loci, whereas only 2/13 examples of LOH were at the other five loci. The four frequently altered loci are all situated near sites of known or putative tumor suppressor genes postulated to be relevant to breast tumorigenesis: 7q31,<sup>23</sup> 11p15,<sup>24, 25</sup> 17p13,<sup>26</sup> and 17q21.<sup>27, 28</sup> In contrast, the five less frequently altered loci are situated at genes or sites less commonly associated with breast cancer. When microsatellite instability was considered, we found that 12/22 abnormalities were seen at the five dinucleotide repeat markers and 10/22 at the four tri- and tetranucleotide repeat markers. This pattern reflects that reported in breast cancer.<sup>3, 8, 9</sup>

## Discussion

We have found multiple genetically abnormal clones existing in breast tissue, although that tissue looks not only benign but also histologically completely normal. These data indicate that genetic abnormalities that may be critical to breast tumorigenesis start accumulating far before pathological detection even of high-risk lesions. The eventual fate of a given clone is unknown, as is the risk to a woman whose breast contains these occult lesions. It is noteworthy that we find mutant clones both in women at low and at high risk of developing breast cancer, because the majority of women who develop breast cancer



**Figure 3.** Representative examples of genetic abnormalities seen in histologically normal ducts or TDLUs from five subjects. **A:** LOH and microsatellite alteration at two loci in subject 32, who had a reduction mammoplasty. LOH at the upper allele of the TP53 microsatellite is seen in samples 2, 4, and 10 (open arrows). In addition, in duct 2, the lower TP53 allele is smaller than normal (closed arrow). The bottom part of the panel demonstrates LOH at the lower THO1 allele in duct 9 (open arrow). S1, S2, and S3 are three separate stromal specimens, each demonstrating the same unaltered pattern at both loci. **B:** Microsatellite alterations at two loci in subject 12, diagnosed with AH. A larger band, representing a novel allele, is seen at both the AR and TP53 microsatellites in sample 3 (closed arrows) but in none of the other five ducts. **C:** LOH and microsatellite alteration at two loci in subject 34, with breast cancer. A larger band, representing a novel allele, is seen in sample 7 at the THO1 microsatellite (closed arrow), and LOH of the upper allele at the D7S486 microsatellite is seen in sample 2 (open arrow). L1, L2, and L3 are three separate lymph nodes, each demonstrating the same pattern of microsatellite bands, representing the germline pattern. **D:** Biallelic alterations (closed arrows) at the THO1 locus in sample 9 from subject 35. One sample of stromal tissue (S2) and three lymph nodes (L1, L2, and L3) were also examined and demonstrated no alterations. (Photographs of samples 2 and 6 before and after microdissection are shown in Figure 1). All ductal samples were amplified and electrophoresed simultaneously, but different exposures have been placed adjacently. **E:** LOH and microsatellite alteration at two loci in subject 36, with breast cancer. At the D17S579 microsatellite, a shortened allele replacing the upper allele is seen in duct 3 (closed arrow). At the THO1 microsatellite, LOH of the lower allele is seen in duct 7.

have no identifiable risk factors. Clinical follow-up of the individuals in this study is not currently available.

It is possible that the mutant clones we detect are relevant to the earliest stages of breast tumorigenesis. Several observations support the speculation that these clones may indicate tissue at increased risk of cancer development. First, 4 of 5 women with breast cancer had

multiple abnormal clones, whereas only 2 of 15 women without the disease had more than a single abnormal clone. Second, 80% of the microsatellite abnormalities were at four loci believed to play a role in breast tumorigenesis. LOH, suggesting the presence of a tumor suppressor gene, has been found at 7q31<sup>23</sup> in a subset of breast cancers; the recently identified *TSG101* putative

**Table 2.** Rates of Genetic Abnormalities in Normal-Appearing Breast Tissue from Subjects <50 Years Old in Three Breast Cancer Risk Groups

| Group         | % subjects with abnormality | Mean % abnormal alleles | Mean % abnormal ducts |
|---------------|-----------------------------|-------------------------|-----------------------|
| Mammoplasty   | 33.3                        | 1.2                     | 15.6                  |
| AH            | 33.3                        | 2.1                     | 33.3                  |
| Breast cancer | 100.0*                      | 3.4†                    | 34.4‡                 |

\*P = 0.076 versus mammoplasty group.

†P = 0.049 versus mammoplasty group.

‡P = 0.123 versus mammoplasty group.

tumor suppressor gene, located at 11p15,<sup>24, 25</sup> is mutated in a fraction of human breast tumors; mutations of the *P53* tumor suppressor gene, located at 17p13,<sup>26</sup> are the most frequently identified genetic abnormalities in breast cancer; and the breast cancer susceptibility gene, *BRCA1*, and possibly other relevant tumor suppressor genes, are found at 17q21.<sup>27, 28</sup> Although *BRCA1* itself has not been found to be mutated in a significant percentage of sporadic human breast cancers,<sup>29</sup> LOH in the region of the gene is detectable.<sup>27, 28</sup> Thus, another mechanism of *BRCA1* inactivation or another gene may be playing a role.

Although these microsatellite loci were selected because of their chromosomal location, the overrepresentation of abnormalities at these sites indicates that they may predispose to the formation of genetically aberrant clonal populations. In contrast, mutation at arbitrary or more neutral sites may not confer a growth advantage, and a detectable mutant clone may never arise. This would suggest that the genetic alterations we have detected are less likely to be random changes and more likely to be relevant to the earliest stages of breast cancer development. Finally, it is noteworthy that the pattern of microsatellite instability seen in normal-appearing tissues is similar to the type of instability reported in breast cancers, ie, overall, a low level of microsatellite alterations, with a substantial proportion of changes seen in tri- and tetranucleotide repeat markers.<sup>3</sup> Microsatellite instability has been detected in all stages of breast cancer, and consequently, it has been postulated that this abnormality occurs early in the course of disease development.<sup>2, 4, 6-9</sup>

Our findings in subjects with breast cancer are consistent with the limited data available from studies in other tissues indicating that histologically normal tissue at increased risk for the development of cancer can contain specific clonal genetic abnormalities. For example, clones of p53 mutated keratinocytes occur in sun-exposed normal-appearing human skin<sup>30, 31</sup> and in normal-appearing mucosa from patients with cancers of the upper aerodigestive tract.<sup>32</sup> Microsatellite alterations have been seen in normal-appearing colonic mucosal epithelium of patients with chronic ulcerative colitis, who are at increased risk of developing colon cancer.<sup>33</sup> Finally, LOH at chromosome 3p has been reported recently in breast cancers and in directly adjacent, but not more distant, histologically normal breast tissue.<sup>19</sup> In contrast, cytogenetic studies examining macroscopically normal breast tissue surrounding breast cancers<sup>34</sup> and investigations of

LOH and/or microsatellite instability in breast cancers have not reported abnormalities in normal-appearing tissues.<sup>1-9</sup> This may be due, in part, to the relatively large amount of normal tissue generally used as a control, making detection of small abnormal clones difficult.

Even if only a rare abnormal clone expands by acquiring additional mutations and the others represent dead ends (ie, they would involute or remain stable), these data could help explain the genetic heterogeneity noted in many breast cancers. Multifocal breast cancers can represent independent, not metastatic, malignancies,<sup>35</sup> single breast malignancies can contain karyotypically unrelated clones,<sup>36, 37</sup> and heterogeneous patterns of allelic loss have been reported in ductal carcinoma *in situ* tumors.<sup>38</sup> It is unclear how all the distinct clones could represent outgrowths from a single original population. The presence of multiple genetically distinct abnormal clones, several of which could progress independently and simultaneously, could represent one explanation.

Finally, it is notable that a trend may exist in the rate of abnormalities among the three groups of women studied, particularly when in women <50 years of age. Several factors may explain the absence of statistical significance associated with most of these associations. First, the baseline rate for somatic mutation in normal breast tissue may be relatively high, even in women at no identifiable increased risk of breast cancer. We obtained a rate of 1.2% in women with reduction mammoplasties and an overall rate of 2.6%, both of which are higher than the baseline rate of somatic mutation estimated to be <0.5% in clones derived from normal T cells.<sup>39</sup> The rate is suspected to be low in other normal tissue but, as far as we are aware, has not been measured. In addition, the data from the reduction mammoplasty group could have been skewed by the presence of subject 32; this individual had quite a few clonal abnormalities. One could speculate about whether women with abnormalities similar to those of subject 32 could be at higher risk for the eventual development of breast cancer. Second, although the number of alleles examined was large, the number of individuals in each of the three groups may have been too small to detect small but significant differences. Examination of additional specimens from more subjects could clarify this important point. Third, the rate of abnormalities seen in each group may not be related to risk but may reflect the effects of aging. The average age was lowest in the reduction mammoplasty group (31 years) with the lowest rate of abnormalities, intermediate in the breast cancer group (41 years) with the highest rate of abnormalities, and highest in the AH group (53 years) with an intermediate rate of abnormalities; but in this last group, more abnormalities were seen in specimens from older women. Examination of specimens from a larger group of women of varied ages may answer this question. Finally, it is possible that the critical event is mutation of a postulated breast-tissue-specific gatekeeper gene, without which progression of any nascent clone does not occur.<sup>40</sup> Thus, the observed mutation rate might not be the key factor.

## Acknowledgments

We acknowledge the helpful support of Dr. Douglas V. Faller, thank Drs. Alan Fine and Charles A. Powell for thoughtful commentary on the manuscript, and appreciate the technical assistance of Ms. Susie Smith.

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**Loss of Heterozygosity (LOH), or Allele Imbalance (AI),  
In Histologically Normal Breast Epithelium  
Is Distinct From LOH or AI in Co-Existing Carcinomas.**

Pamela S Larson<sup>1</sup>, Antonio de las Morenas<sup>1</sup>, Sheila R Bennett<sup>2</sup>, L Adrienne Cupples<sup>3</sup>

Carol L Rosenberg<sup>1,2</sup>.

Departments of <sup>1</sup>Pathology and Laboratory Medicine, and <sup>2</sup>Medicine, Boston University  
School of Medicine and Boston Medical Center, and <sup>3</sup>Department of Epidemiology and  
Biostatistics, Boston University School of Public Health, Boston, MA. USA 02118.

1. # text pages: 15      # tables: 4      # figures: 3
2. Running Title: LOH in normal breast epithelium and cancer.
3. Funding from: Department of Defense Breast Cancer Research Program (DAMD 17-97-7191); the Massachusetts Department of Public Health Breast Cancer Research Program; and the National Institutes of Health (CA81078).
4. Correspondence/reprint requests to:  
  
Carol L Rosenberg MD  
  
Boston University Medical Center  
  
650 Albany Street, EBRC-4  
  
Boston, MA 02118.  
  
Tel: 617-638-5611  
  
Fax: 617-638-7530  
  
e: [croseberg@medicine.bu.edu](mailto:croseberg@medicine.bu.edu)
5. Abbreviations: AI: allele imbalance; DCIS: ductal carcinoma *in situ*; FAL: fractional allele loss; Inv: invasive carcinoma; LOH: loss of heterozygosity; TDLU: terminal ductal lobular unit

## ABSTRACT:

To better understand early steps in human breast carcinogenesis, we examined allele imbalance (AI) or loss (loss of heterozygosity (LOH)), in coexisting normal-appearing breast epithelium and cancers. We microdissected a total of 173 histologically normal ducts or terminal ducto-lobular units (TDLU) and malignant epithelial samples from 18 breast cancer cases, and examined their DNA for LOH at 21 microsatellite markers on 10 chromosome arms. Fourteen of 109 (13%) normal ducts/TDLU, from 8/18 (44%) cases, contained LOH. The location of these 14 duct/TDLU appeared unrelated to distance from the cancer. LOH in normal-appearing epithelium involved only single markers, whereas LOH in cancers commonly encompassed all informative markers on a chromosome arm. In only 1/14 (7%) ducts/TDLUs with LOH, was the same LOH seen in the co-existing cancer. Global differences in LOH per arm in normal-appearing tissue were not demonstrated, but less LOH was seen at 11q and 17p than at 1q ( $p = 0.002$ ), 16q ( $p = 0.01$ ) and possibly 17q ( $p = 0.06$ ). These results indicate that in a large fraction of women with breast cancer, histologically normal breast epithelium harbors occult aberrant clones. Individual clones rarely are precursors of co-existing cancers. However, they might constitute a reservoir from which cancers develop once additional genetic abnormalities occur, they could contribute to intratumoral genetic heterogeneity, and they are consistent with a role for genetic instability early in tumorigenesis.

## INTRODUCTION:

Breast cancers, even carcinoma in situ (CIS), the earliest recognized breast malignancy, contain numerous genetic abnormalities (1, 2). No signature abnormality characterizes breast cancers, but allele imbalances (AI) or loss (commonly described as loss of heterozygosity (LOH)), are especially frequent. Recurrent sites of LOH are thought to identify the location of genes important to tumorigenesis. Cancer precursors must exist, and identification of these precursors and delineation of their genetic abnormalities is important to elucidate critical early steps in breast cancer development, to determine targets for chemopreventive agents, and to identify lesions destined to progress to invasive disease.

Candidate precursors include proliferative lesions; depending on their histology, varying proportions of these lesions demonstrate LOH, often at the same sites as breast carcinomas (reviewed in ref (3)). Given the frequency of LOH in proliferative lesions, it is not surprising that LOH has been detected recently in histologically normal epithelium, i.e., ducts and terminal ducto-lobular units (TDLUs) (4-7). LOH, often at sites implicated in breast carcinogenesis, has been found in several small series examining normal-appearing breast epithelium from women with and without breast cancer, in tissue both adjacent to and distant from the primary tumor, and with an incidence possibly increasing with cancer risk. Reproducible LOH indicates that a substantial proportion of the sample's cells contain an identical DNA abnormality, compared to the individual's normal somatic pattern. Therefore, samples with LOH likely contain a genetically aberrant, clonal

population. It is unclear whether histologically normal epithelial samples with LOH represent early precursors in breast cancer, markers of increased risk, or “background” abnormalities.

To begin to clarify this issue, we evaluated LOH in histologically normal human breast epithelium and co-existing cancers. We microdissected multiple normal epithelial samples (primarily TDLUs) and co-existing *in situ* and invasive malignant lesions from 18 breast cancer cases. We examined their DNA for LOH at 21 microsatellite loci selected primarily for their location at chromosome regions that undergo LOH frequently in breast cancer. We speculated that within a given case, we could identify normal and malignant samples with a possible precursor-product relationship, when the samples had LOH at the same locus (particularly if they were adjacent). In contrast, normal and malignant samples with different LOH should represent distinct clones. We also conjectured that determining the sites and extent of LOH in morphologically normal epithelium should help elucidate early genetic events contributing to the development of sporadic breast cancer.

#### **METHODS:**

**Specimen acquisition:** 18 consecutive lumpectomy or mastectomy specimens were selected at random from Department of Pathology archives. Demographic characteristics of the catchment area suggest few cases would represent familial breast cancer. Review of pathology findings also suggested a non-selected population: 17/18 cases were ductal carcinomas, 1 case (2008) was lobular; 2 cases (0071R, 0071L) represented synchronous

bilateral disease (analyzed separately); 80% of CIS had some evidence of high-grade disease (high nuclear grade, comedo histology or necrosis) and 66% of invasive cancers were grade III/III.

Existing slides were reviewed by a single experienced breast pathologist (AdelasM) who identified multiple examples of normal ducts/TDLU, and *in situ* and invasive carcinoma. Stroma or nodal tissue was available in 7/18 cases.

**Microdissection and DNA extraction and quantitation:** After identifying areas of interest on the hematoxylin and eosin (H + E) stained slides, 7 serial sections were cut from the corresponding blocks, and the top and bottom sections were stained with H + E. After reconfirming histology in these stained slides, they were used to guide microdissection of areas of interest from the unstained sections. Microdissection was performed using a laser capture microdissection apparatus (Arcturus Engineering, Mountain View, CA) (8). By counting nuclei and considering a cell to be 20 $\mu$  in diameter, we estimated that we obtained 200-1000 cells per normal-appearing sample, and considerably more cells per tumor sample. DNA was extracted using standard techniques we have described previously (5, 9). *DNA for control reactions was quantitated fluorimetrically (PicoGreen dsDNA Quantitation Kit, Molecular Probes, Eugene, OR).*

**Microsatellite selection:** 21 microsatellite markers, located on 10 chromosomal arms, were selected for utility in fixed breast tissue based on the following criteria: a) location at regions relevant to breast tumorigenesis (i.e., regions of LOH in early-stage carcinomas, or

at sites of identified or putative tumor suppressor genes). Markers at regions not believed relevant to breast tumorigenesis were also included; b) size of amplified fragment < 200 bp for reliable use in fixed tissue, which produces fragmented template DNA; c) highly polymorphic (ideally > 75% heterozygosity); d) ability to be multiplexed together without adverse interaction. Chromosomal regions and markers used were as follows: 1p: D1s468; 1q32-42: D1s549, D1s213; 3p24: D3s1283; 7q31: D7s486; 11p15: THO1, D11s2071; 11q13: PYGM; 11q23: D11s1818, D11s1819; 16q22-24: D16s265, D16s402, D16s413, D16s512; 17p13.1: TP53, D17s796, D17s525; 17q12-21: D17s1290, D17s579, D17s855; Xq11-12: AR. Primers were purchased from Research Genetics (Huntsville, AL) or synthesized commercially.

**PCR/electrophoresis:** Six multiplexed PCRs were performed using ~ 1/10 volume of the DNA solution as a template in a 50µl reaction, 30-35 cycles of amplification, incorporation of  $\alpha^{32}\text{PdCTP}$ , and annealing temperatures between 55-60°C. One-fifth of the amplified products was electrophoresed through 7% non-denaturing gels that were then exposed to autoradiography film.

**Determination of allele imbalance:** The normal pattern at each microsatellite in each individual was defined as the pattern in stroma and nodes, or the predominant pattern in normal epithelium. LOH was defined at heterozygous loci as an imbalance of allele intensities greater than 25%, i.e., when  $(n_1)(t_2)/(n_2)(t_1) > 1.33$  or  $< 0.75$ , where  $n_1$  = normal samples' larger allele,  $n_2$  = normal samples' smaller allele,  $t_1$  = test sample's larger allele,  $t_2$  = test sample's smaller allele. This degree of allele imbalance indicates that a substantial

proportion of the cells within a sample contains the same DNA abnormality and likely represents the presence of a clonal population. Abnormal results were demonstrated at least twice with equivalent results. At certain loci allele imbalance probably reflects increased copy number, rather than loss of an allele. Distinguishing between these possibilities is important conceptually, but would not change data analysis. Therefore, all allele imbalances were labeled as LOH.

The proportion of LOH for each arm, or fractional allele loss (FAL), was calculated in each case as: # of LOH / # of observations per arm. This adjusts for multiple samples that may have separate patterns of LOH, which was common in normal-appearing samples. For each histology, overall FAL was calculated as the mean of all 10 arms' FAL.

**Statistical tests:** The exact Wilcoxon test was used to assess differences in median sample number between cases with vs. without LOH in normal-appearing tissue. *A t-test assessed differences in mean allele imbalance in control reactions vs abnormal duct/TDLUs.* To assess global differences in FAL across arms within each histologic type of tissue, we employed analysis of variance methods that accounted for the correlated multiple observations coming from the same individual and weighted for the number of observations on which the calculation of FAL was based, using Proc Mixed in SAS (10). We also employed this strategy to evaluate *specific hypotheses, i.e., that* different levels of LOH *existed* between specific arms in normal-appearing samples. Because of the weighting strategy, this approach adjusts for different numbers of samples per case and observations per sample.

## RESULTS:

**Samples:** From 18 independent breast cancer-containing lumpectomy or mastectomy specimens, we microdissected 173 lesions including normal-appearing terminal ductal-lobular units (TDLU), (or, rarely, simple ducts), carcinomas in situ (CIS) and invasive carcinomas (Inv) and when available, uninvolved lymphoid tissue (L) or stroma (S).

**Figure 1** illustrates a representative microdissection. **Table 1** lists the number of samples per histology and specimen. Samples were taken from all available blocks: 28/109 ducts/TDLU located on the same block as the cancer, the remainder came from elsewhere in the specimen.

***LOH in controls:** To establish the rate at which LOH might mistakenly be identified in normal-appearing duct/TDLUs (i.e., a false positive rate), we performed 40 independent control PCRs. Each reaction contained 125 pg of lymph node DNA that had been microdissected and extracted using the same conditions as the breast samples. This template (reflecting the DNA content of ~20 cells, our lowest estimated cell number per reaction), was amplified at 2 markers (D1s549 and D17s579). We found limited variation in allele ratios among these 40 reactions, with the mean allele ratio = 1.06, sd = 0.25 (see **Figure 2**). Eight/40 (20%) reactions had allele ratios outside our predetermined cutoff values ( $>1.33$ ,  $<0.75$ ). Because our criteria for LOH require at least 2 independent demonstrations of abnormal allele ratios, this indicates that our estimated rate of false positives is, at most,  $(0.2) \times (0.2) = 0.04$ , or (4%). It is probably closer to 2%, since artifactual imbalances should affect each allele half the time.*

**LOH in breast samples:** Using multiplex PCR, DNA from each microdissected breast sample was analyzed at 21 microsatellite markers on 10 chromosome arms. On average, 11 markers were informative and interpretable per case. Some LOH could have been missed due to admixture of truly normal cells. **Table 2** summarizes these data.

*Histologically normal epithelium:* Eight of 18 (44%) cases contained 1 or more duct/TDLU with LOH. Overall, 14/109 (13%) normal-appearing samples contained 14 LOH, consistent with proportions reported previously (5). *Since the confidence interval for this 13% rate is 7% - 19%, but the maximal estimated rate of artifactual LOH is 4%, these abnormalities are unlikely to be due to chance. In addition, the magnitude of allele imbalance in these 14 duct/TDLUs was greater than in the 8 control reactions whose ratios outside the cut-off (mean allele ratio: 2.15 [53% imbalance] vs 1.52 [34% imbalance],  $p = 0.03$ ).*

*Ten cases contained no abnormalities; 4 cases contained a single duct/TDLU with LOH; in 3 cases, separate duct/TDLUs contained distinct sites of LOH. In 2 cases, 2 normal-appearing samples had LOH of the same marker and allele (see Table 3). These were considered independent, but could have represented a single, convoluted duct. There was a trend towards examination of more ducts/TDLU in cases with LOH than cases without LOH (7.5 vs. 4.5,  $p = 0.10$ ), suggesting that detection of abnormalities may be related to the number of ducts/TDLU examined. Detection of LOH in normal-appearing epithelium was not influenced by the subject's age at diagnosis (45 yrs in women with LOH vs. 46 years in women without LOH). All 14 samples contained a single abnormality.*

To confirm each case's constitutional pattern at each marker, stromal or nodal tissue was examined in the 7 cases where the tissue was available. Although LOH has been reported in stroma (11, 12), we microdissected multiple samples to obtain each case's normal somatic pattern. In all 7 cases, the stromal or nodal pattern at each marker was the same as the predominant pattern in normal-appearing breast epithelium (see section below). In the 11 cases that lacked stroma or lymph nodes, an average of 5.7 (range: 4 - 11) normal ducts/TDLUs were available to determine each marker's normal pattern. Therefore, LOH in a single, normal-appearing sample is less likely to represent an aberration occurring early in breast development and more likely to represent a later genetic event.

*Cancers:* All cancers contained genetic abnormalities, usually multiple (see **Table 2**). Overall, 29/32 (91%) CIS samples from 14/14 (100%) evaluable cases contained 124 LOH; and 19/20 (95%) Inv samples from 9/10 (90%) cases contained 98 LOH. The pattern of LOH among all CIS, or all Inv, samples within a case was usually identical, implying that they derived from a single clone (data not shown). When they differed, it was most commonly due to the presence of a normal pattern, suggesting contamination with normal cells.

*Stroma and lymph node:* No LOH was seen in 9 samples from 3 subjects with stroma examined. In 1/4 (25%) cases (0072), 1 out of 6 pathologically uninvolved LNs demonstrated 1 LOH. This may represent the baseline rate of mutation in lymphoid

tissue, although an occult metastasis cannot be ruled out since the cancer contained the same abnormality.

**LOH in relation to distance between ducts/TDLU and cancer:** We asked whether LOH in ducts/TDLU was related to a sample's distance from the cancer. Twenty-eight of 109 (26%) duct/TDLUs examined were located on the same block as cancers. Four of these 28 (14%) (all from case 2034), had LOH, but none of these 4 LOH were present in adjacent cancers. LOH was also seen in 10/81 (12%) ducts/TDLU located on blocks not containing cancer. As described above, in only 1 case was a precursor-product relationship possible. Thus, although duct structure is convoluted, it appears that ducts/TDLU more distant from the cancer were equally likely to contain abnormalities as samples nearer to the cancer. In fact, normal-appearing ducts/TDLU adjacent to cancers did not contain abnormalities present in the malignancy.

**Extent of LOH: single vs multiple loci:** As shown in Table 2, LOH in normal-appearing ducts/TDLU encompassed only single markers, i.e., additional informative loci on the same chromosome arm showed no LOH. In contrast, LOH in cancers usually encompassed all informative markers on a chromosome arm. Overall, LOH at all informative loci on 1 or more arms was seen in 0/18 (0%) histologically normal specimens, 10/14 (71%) CIS and 9/9 (100%) Inv cancers.

**Chromosome arms demonstrating LOH:** LOH in normal-appearing tissue could indicate chromosome regions harboring tumor suppressor genes important early in breast

carcinogenesis (13). To identify these regions, we determined the proportional LOH on each chromosome arm for normal and malignant epithelial samples. The mean proportional LOH (or FAL) was 0.01 for normal epithelium, 0.27 for *in situ* cancer and 0.30 for invasive cancers; the values for cancers are consistent with previous reports (14, 15). Results are shown in **Table 4**.

Perhaps because the overall number of LOH was relatively small, no significant global differences in proportional LOH between arms were detected in normal tissues ( $p = 0.39$ ). (Global differences were suggested in *in situ* ( $p = 0.09$ ) and detected in invasive cancers ( $p = 0.02$ )). However, we observed that arms with frequent, or rare, LOH in malignant tissue often showed the same relative frequency of LOH in normal-appearing tissue. For instance, in both normal and malignant tissue frequent LOH was seen on 1q and 16q, and infrequent LOH on 1p, 3p, Xq and probably 7q. However, 2 arms had inconsistencies: LOH at 11q and 17p were common in cancer but completely absent in normal tissue. More focused analyses, *comparing specific arms using the same analytical strategy*, indicated significantly less LOH in normal tissue at sites on 11q and 17p than at sites on 1q ( $p = 0.002$ ), 16q ( $p = 0.01$ ) and possibly 17q ( $p = 0.06$ ).

**LOH in co-existing normal-appearing and malignant epithelium:** We evaluated whether identical abnormalities were present in morphologically normal ducts/TDLU and co-existing cancers. If so, then the normal-appearing sample could represent a clonal precursor of the cancer. In contrast, LOH in normal but not malignant tissue suggests the presence of 2 independent clones. We found only 1/14 (7%), ducts/TDLU with LOH,

from 1/8 (13%) cases, had the same LOH as the co-existing cancer (see **Table 2**).

Representative examples are presented in **Figure 3** and **Table 3** summarizes the results.

In the single case (0053) with LOH of the same site and allele in a normal-appearing duct/TDLU and the cancer, it is possible that the 7q LOH was coincidental, because this LOH was not seen in proliferative lesions that shared other LOH with the cancer (data not shown). We did find LOH of a marker's opposite alleles in ducts/TDLU compared with co-existing cancers (see **Figure 3b**). Thus, the great majority of ducts/TDLU with LOH (13/14, or 93%) were clonally distinct from co-existing cancers.

#### **DISCUSSION:**

This study reports the largest investigation to date of LOH, or allele imbalance, in histologically normal breast epithelium. The results demonstrate a consistent, low level of LOH (14/109 [13%] samples, overall proportional LOH = 0.01) in normal-appearing ducts/TDLUs from a large subset (8/18 [44%]) of breast cancer cases. *The frequency and the magnitude of LOH/AI are greater than expected by chance.* Ducts/TDLU with LOH were found throughout the breast specimens. All LOH in normal-appearing epithelium involved single markers, whereas LOH in cancers commonly encompassed all informative markers on a chromosome arm. Remarkably, ducts/TDLU with LOH were rarely implicated as cancer precursors, because their LOHs involved different markers, or different alleles of the same marker, as the coexisting cancer. Global differences in LOH per arm could not be demonstrated in normal-appearing tissue (perhaps because the overall number of LOHs is low), but sites of LOH did not appear to be completely

random. *Although the number of observations is small, we found less LOH at 11q and 17p than at 1q, 16q and possibly 17q.*

These results raise two primary points for consideration. First, what roles do ducts/TDLU with LOH play? The ability to detect LOH indicates that a substantial proportion of the sample's cells contain the identical genetic abnormality, i.e., represent the progeny of a single cell. LOH at various sites might occur in individual cells, but it will be detected only if the cell subsequently undergoes clonal expansion. Clonal expansion can result from a growth advantage conferred on the cell by loss of a critical gene. (Although the function of the putative critical gene's remaining allele is unknown, haploinsufficiency alone is capable of generating a phenotype). Alternatively, clonal expansion could reflect normal mammary development, since a genetic change occurring in the breast prior to puberty would probably be manifest as a detectable mutation in normal adult breast tissue. We favor the former explanation because LOH was found in scattered, single ducts/TDLU, whereas the mammary gland's stem-cell derived monoclonal patches probably encompass larger areas and contain multiple TDLUs (16-18).

Also suggestive that at least some ducts/TDLU with LOH result from loss of a critical gene, and may be meaningful clones, is that the sites of LOH were not entirely random. We noted LOH at 1q and 16q, and perhaps 17q, relatively frequently in both normal and malignant tissue, whereas LOH at 11q and 17p were noted only in malignancies. *These results suggest that LOH at certain sites may be associated with clonal expansion,*

*whereas LOH at other sites may be associated with later steps in tumor development.*

*Based on a small number of LOH events, these results warrant further examination in a larger study. However, they are consistent with data suggesting that genes important early in breast tumorigenesis may be located on 1q and 16q (14, 19), whereas genes acting later may be on 17p (for instance, p53 (20-22)) or 11q (22, 23). We did not examine abnormalities of specific genes, but microsatellites were selected to be in the vicinity of several genes implicated in breast carcinogenesis, i.e., p53 (on 17p13) CCND1 (cyclin D1, on 11q13), or ATM (on 11q23).*

Thus, although the majority of normal-appearing clones may not be destined to evolve into invasive disease, we speculate that ducts/TDLU with LOH, particularly at 1q or 16q, might constitute a reservoir from which more advanced lesions develop. This would be consistent with a previous report noting the same LOH in a cancer and adjacent TDLU (4). In the present study, ducts/TDLU with the same LOH as the co-existing cancer may have been missed due to sampling, (we did not enrich for ducts/TDLU located near cancers), or due to obliteration of the original aberrant duct/TDLU by tumor growth. Their absence does not alter the conclusion that most ducts/TDLU with LOH appear unrelated to co-existing cancers. Progression of more than one unrelated clone might contribute to the intratumoral heterogeneity that can be seen in breast cancer (24, 25).

An unanswered question that should help determine the roles played by ducts/TDLU with LOH is whether the number of samples with LOH, or the fraction of cases with LOH in histologically normal epithelium, is increased in these cancer-containing breasts

compared to presumed, but undefined, normal "background" rates. Additional studies will address this question more conclusively, but preliminary evidence has suggested that abnormalities increase in histologically normal epithelium as breast cancer risk increases (5). This view would be consistent with breast cancer patients' increased risk of cancer in the contralateral breast (26, 27). Since detection of LOH in the present study may have been related to the number of ducts/TDLU examined ( $p = 0.10$ ), if more samples were examined, an even larger proportion of cases might contain abnormalities. Similarly, since the location of ducts/TDLU with LOH seemed unrelated to their distance from the cancer, if more samples were examined, more aberrant ducts/TDLU might be found in each breast.

The second point for consideration is whether these results provide support for particular mechanisms implicated in human breast carcinogenesis. A majority (13/14 [93%]) of normal-appearing epithelial clones detected in the present study were distinct from, and thus were not precursors of, the co-existing cancer. The presence of multiple, distinct co-existing clones (i.e., the cancer plus any ducts/TDLU with LOH) is consistent with the hypothesis that some form of genetic instability, manifest in this study as LOH, may begin very early in breast tumorigenesis, while tissue is histologically normal. The geographic extent of any such area of instability is uncertain. "Field" abnormalities have been proposed previously in cancer-containing breasts (28-30).

The present results also suggest that the predominant mechanism(s) leading to LOH may change during tumor progression. LOH of limited chromosome regions, as we found in ducts/TDLU, is consistent with mitotic recombination (although other mechanisms are

plausible) (31-33) and might occur first. In contrast, LOH at all evaluable markers along an arm, as we found in cancers, would more likely result from loss of an entire chromosome (chromosome non-disjunction) (33). Chromosome number instabilities are characteristic of human cancers (34) and may be due to malfunction of the mitotic chromosome segregation apparatus (35). Thus, mechanisms leading to LOH in limited chromosomal regions may contribute early to breast tumorigenesis, whereas mechanisms leading to whole chromosome abnormalities may contribute to late events. Consistent with this, a recent study posits aneuploidy as a late event in breast carcinogenesis (36), and only rare cytogenetic abnormalities have been reported in normal-appearing tissue adjacent to cancers (19, 37).

In summary, the current data indicate that clonal, genetically abnormal ducts/TDLU are scattered throughout normal-appearing epithelium of cancerous breasts. These clones are distinct from the co-existing cancer, and could be a consequence either of normal development or of pathologic events. It is possible that ducts/TDLU with LOH form a reservoir from which cancers may develop if sufficient additional abnormalities accumulate. The presence of multiple clones (i.e., the cancer plus any duct/TDLU with LOH) suggests that some type of genetic instability, affecting an undefined area of breast tissue, may contribute early to tumorigenesis.

**Table 1: Microdissected samples**

| Case         | No. samples |               |            |            |
|--------------|-------------|---------------|------------|------------|
|              | $S^a, L^b$  | <i>normal</i> | <i>CIS</i> | <i>Inv</i> |
| 2004         | -           | 5             | 3          | -          |
| 2008         | -           | 4             | 2          | 2          |
| 2012         | -           | 4             | -          | 2          |
| 2014         | 1           | 1             | 1          | -          |
| 2028         | 3           | 7             | 1          | -          |
| 2031         | 2           | 5             | 3          | 2          |
| 2032         | 3           | 6             | -          | 2          |
| 2034         | -           | 9             | 1          | -          |
| 2044         | -           | 8             | -          | 3          |
| 0038         | -           | 5             | 3          | 5          |
| 0039         | -           | 4             | 4          | -          |
| 0052         | 1           | 8             | 2          | 1          |
| 0053         | 1           | 9             | 2          | 1          |
| 0070         | -           | 4             | 3          | -          |
| 0071R        | -           | 5             | 2          | 1          |
| 0071L        | -           | 4             | -          | 1          |
| 0072         | 1           | 10            | 3          | -          |
| 0074         | -           | 11            | 2          | -          |
| <i>Total</i> | <i>12</i>   | <i>109</i>    | <i>32</i>  | <i>20</i>  |

<sup>a</sup> *S* = stroma, <sup>b</sup> *L* = lymph node

**Table 2.** LOH in histologically normal ducts/TDLU vs. co-existing cancers.

| Samples               | No. (%) cases with LOH | No. (%) samples with LOH | No. (%) cases with same LOH in normal and cancer | No. (%) samples with same LOH in normal and cancer | Extent of LOH      |
|-----------------------|------------------------|--------------------------|--------------------------------------------------|----------------------------------------------------|--------------------|
| ducts/TDLUs           | 8/18 (44%)             | 14/109 (13%)             | 1/8 (13%)                                        | 1/14 (7%)                                          | Single locus       |
| <i>in situ</i> cancer | 14/14 (100%)           | 29/32 (91%)              | <i>NA</i>                                        | <i>NA</i>                                          | Multiple >> single |
| invasive cancer       | 9/10 (90%)             | 19/20 (95%)              | <i>NA</i>                                        | <i>NA</i>                                          | Multiple >> single |

**Table 3:** Heterogeneous LOH in histologically normal epithelium (N) vs. co-existing cancers

| Case  | LOH                                                                                                                                         |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------|
| 2004  |                                                                                                                                             |
| 2008  |                                                                                                                                             |
| 2012  |                                                                                                                                             |
| 2014  |                                                                                                                                             |
| 2028  | 1 N has LOH at 16q, not seen in CIS<br>1 N has LOH at 1q, not seen in CIS                                                                   |
| 2031  | 1 N has LOH at 16q site not seen in CIS & Inv                                                                                               |
| 2032  |                                                                                                                                             |
| 2034  | 1 N has LOH at 17q, not seen in CIS<br>1 N (N7) has LOH at 11p, not seen in CIS<br>2 Ns have LOH at 11p (distinct from N7), not seen in CIS |
| 2044  | 1 N has LOH of opposite 1q allele as Inv                                                                                                    |
| 0038  |                                                                                                                                             |
| 0039  | 2 Ns have LOH at 17q site, not seen in CIS                                                                                                  |
| 0052  |                                                                                                                                             |
| 0053  | 1 N has LOH at 7q which is lost in CIS & Inv<br>1 N has LOH of opposite 1q allele as CIS & Inv                                              |
| 0070  |                                                                                                                                             |
| 0071R | 1 N has LOH of opposite 1q allele as CIS & Inv                                                                                              |
| 0071L |                                                                                                                                             |
| 0072  | 1 N has LOH of opposite 16q allele as CIS                                                                                                   |
| 0074  |                                                                                                                                             |

**Table 4.** Proportional LOH in chromosome arms

| Arm          | Proportional LOH |             |             |
|--------------|------------------|-------------|-------------|
|              | <i>Normal</i>    | <i>CIS</i>  | <i>Inv</i>  |
| 1p           | 0                | 0           | 0           |
| 1q           | .02              | .48         | .50         |
| 3p           | 0                | .19         | 0           |
| 7q           | .01              | .07         | .06         |
| 11p          | .02              | .35         | .23         |
| 11q          | 0                | .32         | .41         |
| 16q          | .02              | .65         | .79         |
| 17p          | 0                | .38         | .64         |
| 17q          | .02              | .29         | .37         |
| Xq           | 0                | 0           | 0           |
| <i>Mean:</i> | <i>0.01</i>      | <i>0.27</i> | <i>0.30</i> |

## FIGURE LEGENDS:

**Figure 1. Laser Capture Microdissection (LCM) of fixed, unstained morphologically normal breast epithelium.** A: H + E stained section showing a normal-appearing TDLU; B: adjacent unstained section; C: after microdissection; D: tissue on cap.

*Figure 2. Reproducibility of PCR. Representative examples from 40 independent control reactions, each amplifying marker D1s549 from 125 pg of template DNA. Normalized allele ratios, indicated below each lane, all fall within normal limits.*

**Figure 3. Morphologically normal ducts/TDLU have LOH distinct from LOH in co-existing cancers.** Examples from 3 cases (A & D: 2034, B: 2044; C: 039) demonstrating that genetically abnormal ducts/TDLUs do not commonly share LOH with co-existing cancers. Arrows indicate lost alleles in duct/TDLUs, arrowhead indicates lost alleles in cancers, markers are listed at each panel's left, lesions are indicated by lettering across the top of each panel. (N = normal, CIS = carcinoma in situ, INV = invasive tumor).

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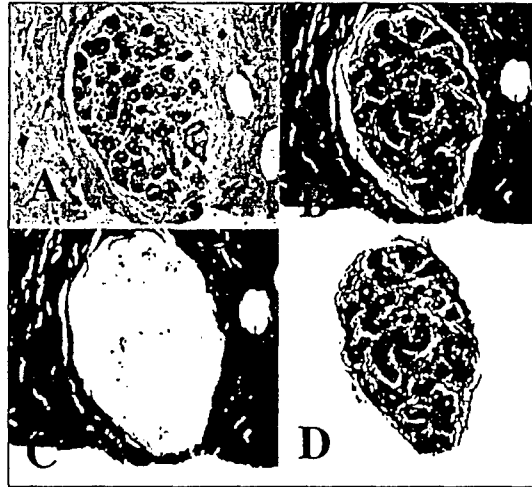
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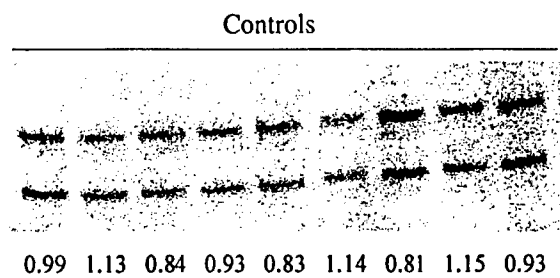
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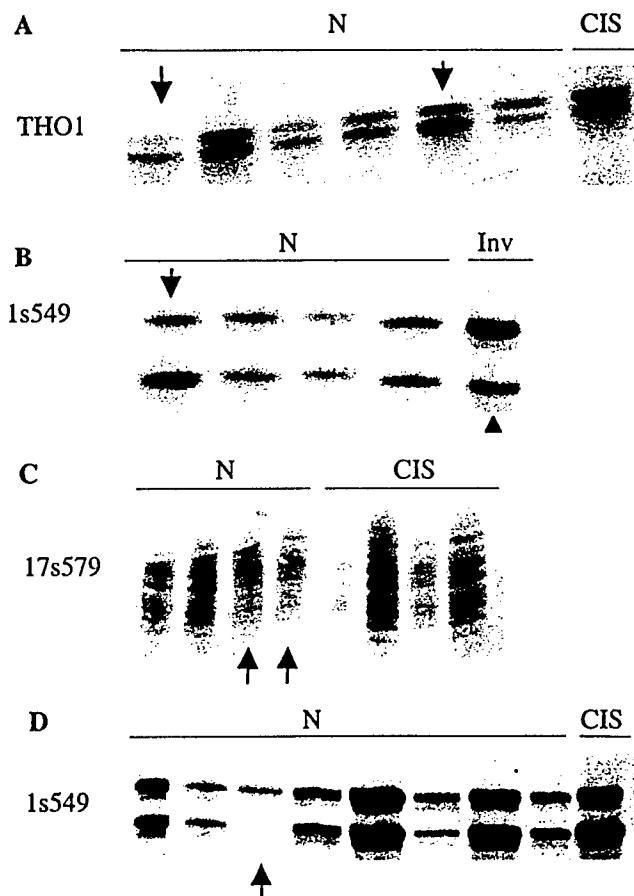
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**Figure 1.** Laser Capture Microdissection (LCM) of fixed, unstained morphologically normal breast epithelium.



**Figure 2.** Reproducibility of PCRs.



**Figure 3.** Morphologically normal ducts/TDLU (N) have LOH distinct from LOH in co-existing cancers (CIS, Inv).



DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

18 November 2002

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-97-1-7191. Request the limited distribution statement for Accession Document Numbers ADB262448, ADB265603 and ADB282072 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management