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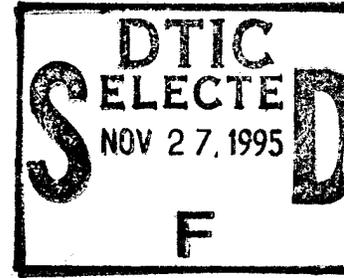
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**Annual Report for Grant Number DAMD17-94-J-4293**

**Tumor Suppressor Genes in Early Breast Cancer and its Progression**

**Diane M. Radford MD**

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## Introduction

### A. Nature of the problem

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

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

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

The chronology of genetic changes leading to invasion has been elucidated for certain cancers, such as the transition from neoplastic

polyp to colon carcinoma (8,9), but has not been established for breast cancer. Little is known about the genetic events which give rise to these hyperplastic and neoplastic conditions. A better understanding of the oncogenesis of breast cancer at the molecular level, and the correlation of this information with clinical data, may aid in treatment choices.

## B. Background of Previous Work

Most solid tumors arise due to the inactivation of tumor suppressor genes and activation of oncogenes. The accumulation of genetic changes is believed to result in the invasive followed by the metastatic phenotypes. Loss of heterozygosity (LOH) of one of a pair of alleles in tumor tissue compared to matched normal control can reveal areas of chromosome deletion which are likely to contain putative tumor suppressor genes. A large number of articles have been published on LOH in invasive breast cancer, and virtually every human chromosome has been shown to exhibit allelic loss (10,11). The most frequent losses are seen on chromosome 7q (0-83%) (10,12,13), 16q (32-63%) (10,11,14-16), 17p (31-75%) (10,11,17-20), 17q (24-79%) (10,11,21-27), and 18q (24-69%) (10,24,28,29). Less frequent losses are found on 1p (3-47%) (10,11,30), 1q (16-32%) (10,11,31,32), 3p (11-47%) (10,11,19), 6q (9-48%) (10,11), 8p (27-33%) (10,18) 11p (10-41%) (10,33) and 13q (16-40%) (11,19).

Tumor suppressor genes thought to be involved with breast cancer include the *p53* gene at 17p13.1 (33,34), *Rb* on 13q (11,19) and *DCC* on 18q (28,29). Schott *et. al.* (35) have discovered another gene (*Brush-1*) on 13q proximal to *Rb*, which is located within the 6 cM (centimorgan) region containing the inherited early onset breast cancer gene *BRCA-2*, located at 13q12-13 (36). A region telomeric to *p53* at 17p13.3 is also lost frequently in invasive breast cancer and is believed to harbor a separate tumor suppressor locus (37,38). Several genes located on 17q are implicated in breast cancer oncogenesis, such as the recently cloned *BRCA-1* gene at 17q21 (39,40) and the metastasis suppressor gene *NM23*, distal to *BRCA-1* (22,23,41). Chromosome 8p is believed to harbor at least two tumor suppressor loci (10,18). Because of the multiple putative tumor suppressor loci which exhibit LOH in invasive breast cancer, it is not clear which loci are involved in oncogenesis and which are lost randomly due to the instability conferred by the malignant state of the genome.

Allelotyping involves the comprehensive screen of the genome for LOH in a particular cancer. Generally an initial screen will involve

assay with at least one marker from each non-acrocentric chromosomal arm. Thereby the average or baseline level of LOH can be determined. This may vary from 5 to 20% depending on the type of cancer. A significant level of LOH, indicating the site of possible tumor suppressor genes involved in oncogenesis, can be ascertained once the background level is known. Regions which show significant LOH can then be analyzed with additional markers to refine the smallest deleted region which may contain the tumor suppressor gene. The analysis of tumors with a number of markers also permits calculation of the fractional allelic loss (FAL) for each tumor. This has been defined as the total number of chromosomal arms which show LOH divided by the total number of informative arms for that tumor (42,43). FAL has been correlated with patient outcome in colon cancer (43), and may correlate with clinical information in other tumor types.

Studies of the molecular changes in DCIS and invasive breast cancer are few. Davidoff et. al. (44) studied 6 examples of synchronous DCIS and invasive cancer for expression of p53 and found the same levels of protein expression in each tissue type. Expression of the oncogenes c-erbB-2 and c-myc is also consistent between coexisting pre-invasive and invasive breast cancer (44,46). Zhuang et. al. studied allelic loss for two loci on 11q13 (INT2 and PYGM). They found that for every case of DCIS which showed LOH (N=15), loss of the same allele was seen in the corresponding invasive tumor (47). O'Connell et. al. (48) studied four loci [TPO (2pter), D4S192 (4q25-34), D16S265 (16q21) and D17S579 (17q21)] and found that 8 of 10 cases of DCIS shared LOH patterns with more advanced lesions for at least one of the 4 loci.

Our studies are directed toward a better understanding of genetic events, particularly inactivation of tumor suppressor genes, in early breast cancer and its progression to invasion.

### **C. Purpose of the Present Work**

As described in our Statement of Work the purpose of our experiments is as follows:

#### **Task 1: The identification and characterization of the extent of chromosomal deletions in DCIS. Months 1-12.**

a. The different subtypes of DCIS will be allelotyped with a panel of microsatellite markers.

- b. The smallest common region of deletion will be refined.

**Task 2: The study of chromosomal deletions in hyperproliferative breast conditions. Months 12-24.**

**Task 3: The study of chromosomal deletions in the progression of DCIS to invasive and metastatic phenotypes. Months 1-36.**

- a. Simultaneous DCIS, invasive cancer and lymph node metastases will be assayed for chromosomal deletions.

**Task 4: Cloning a tumor suppressor gene involved in breast cancer. Months 24-48.**

- a. The smallest common region of deletion will be encompassed with overlapping YAC contigs.
- b. Candidate genes will be identified.

#### **D. Methods of approach**

- a) Accumulation of specimens.

Collaborations have been established with pathologists in St. Louis area hospitals. Archival paraffin embedded material is collected from several hospitals in St. Louis (Barnes, Jewish, Deaconess, St. Louis University, and the Outpatient Surgery Center). Either matched archival normal lymph node DNA or leukocyte DNA is used as control. When it is necessary to draw blood for normal control, informed consent is obtained following Institutional Review Board approval.

- b) Microdissection.

For LOH analysis it is necessary to have a relatively pure tumor sample with little if any contaminating normal stroma. We have been using a microdissection technique in which an unstained 25 micron thick section from a particular block is overlaid on a stained 5 micron thick section. Landmarks such as blood vessels are aligned and the tumor dissected from the unstained section using a scalpel blade.

- c) DNA extraction and LOH analysis.

Following separation of tumor and normal tissue DNA is extracted by digestion with proteinase K, purified with phenol/ chloroform and precipitated with alcohol. DNA is quantified with a fluorimeter. For assay of LOH we have used a panel of highly polymorphic microsatellite markers. Polymerase chain reaction (PCR) is performed in the tumor/normal pairs and the products separated on acrylamide denaturing gels. Reactions have been optimized for 5 to 10 ng of template DNA in order to maximize the number of reactions possible with each tumor. On autoradiography, absence or greatly reduced intensity of one allele in the tumor compared to the heterozygous normal control indicates LOH. We have established that our microdissection technique and method of PCR can result in detection of LOH on chromosome 17 in DCIS (49,50).

d) Generation of genetic linkage maps.

Once a region of chromosomal deletion has been identified it can be narrowed down using a panel of closely linked markers which map to that area. Since new microsatellite markers are becoming available daily, they often do not appear on currently published maps. In order to determine the deletion map in the tumors, it is necessary to know the precise location of the markers being used. A fine structure map can be generated using genotypic data from a number of families made available through the Centre d'Etude Polymorphisme Humaine (CEPH). Having identified a small region of deletion (preferably no larger than 1cM) positional cloning techniques can be undertaken to clone the putative tumor suppressor gene contained within the region.

e) Statistical analysis

Statistical analysis of clinical and histologic information with LOH data is performed with the use of contingency tables and the unpaired *t* test with the software package Statview (Abacus Industries, Berkeley, CA). The null hypothesis for each analysis is that the two variable are independent of each other. At the 0.05 level of significance the null hypothesis is rejected.

## **6. Body: Experimental Methods Used and Results Obtained.**

### **Task 1: The identification and characterization of the extent of chromosomal deletions in DCIS.**

a. The different subtypes of DCIS will be allelotyped with a panel of microsatellite markers.

b. The smallest common region of deletion will be refined.

i) Allelotyping

At the time of submission of the proposal to the Department of the Army allelotyping was underway and 16 chromosomal arms had been assayed. The goal of analysis of all 39 non-acrocentric chromosomal arms was completed by August 1994. The results obtained from that analysis have been published in *Cancer Research* (51) and a reprint of that paper is included in Appendix 1. A total of 61 samples of DCIS were assayed. The average number of informative tumors examined for each marker was 19 (range 8 to 48). The median fractional allelic loss (FAL) was 0.037. The highest % of LOH was shown for loci on 8p (18.7%), 13q (18%), 16q (28.6%), 17p (37.5%) and 17q (15.9%). LOH on 18q was found in 10.7% of informative tumors. FAL was associated with LOH on 17p, with high nuclear grade and with the comedo subtype of DCIS. LOH on 17p correlated with LOH on 17q and on 13q. Additional markers were employed for 16q and 17p to determine the smallest common region of deletion and maps of 17p and 16q were generated.

ii) Deletion on 8p

Since September 1994 we have concentrated our efforts on a more detailed analysis of LOH on 8p. In the allelotyping screen we found LOH on 8p with one marker in 3 of 16 informative tumors. A total of 64 cases of DCIS have now been assayed for LOH using 8 markers on 8p and 2 on 8q. PCR and product separation were done as described in D (c). LOH was determined by a combination of naked-eye assessment and scanning densitometry. Densitometry was performed by scanning autoradiographs with a UMAX UC630 color scanner and with the use of the software program Adobe Photoshop 2.5.1 (Adobe Systems, Inc.). The densitometry histograms were analyzed on a Power Macintosh 6100/60 with the use of the public domain NIH image program (Wayne Rasband, NIH). A 3 fold difference in the relative allele intensity ratios between tumor and normal DNA in an informative tumor/normal pair was scored as LOH (allele 1/allele 2 in tumor compared to allele 1/allele 2 in normal). To maintain a conservative scoring approach, marginal allele reduction by inspection was not scored as LOH. All LOH designations were scored by two independent scientists and repeated to verify the result.

Of 55 informative samples, LOH was found for at least one 8p marker in 15 tumors (27.3%). In 6 tumors the deletion appeared to

involve the whole short arm, whereas in the remainder a smaller deletion was seen. The smallest common region of deletion localizes to a 1.3cM region at 8p22-23, between the markers D8S520 and D8S265. No correlation has been found between LOH on 8p and histologic parameters such as subtype, nuclear grade or presence of microinvasion. These data are in press in *Surgical Forum* and will be presented at the American College of Surgeons meeting in October 1995. An example of LOH on 8p is given on Appendix 2 and a copy of the paper in press including the deletion map is given in Appendix 3.

We shall continue to accrue examples of DCIS of all subtypes with or without associated invasion to expand our panel of tumors. We wish to refine the region of deletion on 8p further using numerous closely linked markers. A fine structure map of 8p is required.

### iii) Construction of genetic linkage map of 8p

Simply stated, map construction utilizes patterns of inheritance to determine the order of markers on a chromosome. Differences in the patterns of inheritance from marker to marker represent recombinations and are used to determine the distance between markers measured in centimorgans (cM). Deletion data cannot be interpreted unless a strong unique order exists for the markers used. Genethon utilizes eight of the 61 CEPH reference families (102, 884, 1331, 1332, 1347, 1362, 1413, and 1416) for the construction of framework maps of the human chromosomes (52). The CEPH collaborative mapping groups have made these primary data publicly available. To further strengthen the unambiguous positioning of markers an additional 8 CEPH families (66, 1333, 1334, 1340, 1341, 1345, 1375, 1377) are typed and the data are merged with the available primary data from CEPH V7. Merged data is then processed through the mapping program CRIMAP (P. Green, unpublished) resulting in genetic map with at least 1000:1 odds on order. Primer sequences and reactions conditions for these markers are available from GDB (genome data base, <http://gdbwww.gdb.org>). Primers are synthesized locally (H. Donis-Keller), or ordered from Research Genetics, Inc. Program permutation options "flips2" "flips3" and "flips4" determine the most likely order with the data from the "build" analysis.

The PCR conditions for genotyping are: initial denaturing @ 94° for 30 seconds one cycle, denaturing @ 94° for 30 seconds annealing @ 50-60° for 30 seconds and extension @ 72° for 30 seconds for 25 cycles. After amplification the samples are separated by electrophoresis on a standard 8% polyacrylamide denaturing gel,

dried, and exposed to film for 12-24 hours. Twenty four markers on 8p have been genotyped on the additional 8 families and a map is currently under construction.

Once a marker is uniquely placed on a chromosomal map, that marker will then taken through the DCIS tumor/normal pair panel to determine the percentage of LOH and refine the smallest common region of deletion.

**Task 2: The study of chromosomal deletions in hyperproliferative breast conditions. Months 12-24.**

To date we have assayed 2 examples of LCIS using several markers on 8p. Dr. Michael McDermott (a pathology collaborator) has identified 100 examples of LCIS with or without associated invasive cancer stored in the archives at Barnes Hospital. These slides are being reviewed to assess whether the microdissection technique can be applied. Once DNA been extracted from a panel of tumors the allelotyping strategy already described can be applied.

**Task 3: The study of chromosomal deletions in the progression of DCIS to invasive and metastatic phenotypes. Months 1-36.**

To study genetic changes and the evolution of breast cancer we have assayed for loss of heterozygosity (LOH) in twelve sets of synchronous carcinoma *in situ* (CIS) and invasive cancer, compared to normal control DNA. Microsatellite markers were used which map to each non-acrocentric autosomal arm. Eight tumor sets demonstrated LOH of the same allele in both concurrent invasive cancer and DCIS, for a total of eighteen chromosomal loci. Three of nine tumor sets showed LOH on 11p. In two of these sets LOH was seen on 11p only in the invasive tumor, not the corresponding CIS. One of these tumors also exhibited allelic loss in the invasive tumor for 4 loci, all of which were retained in the non-invasive tumor. For two tumor sets LOH was mirrored in matched DCIS, invasive tumor and lymph node metastasis. The maintenance of LOH for certain loci throughout the stages of breast cancer suggests clonality of the cancer cells. Tumor suppressor loci on 11p may be involved in the invasive phenotype. These data are in press in *Cancer Research* to be published Nov. 15th 1995. A copy of the deletion map for these twelve cases is included as Appendix 4. Examples of synchronous *in situ* cancer and invasive cancer continue to be accrued.

**Task 4: Cloning a tumor suppressor gene involved in breast cancer. Months 24-48.**

Once an implicated chromosomal region is less than 1 cM various methods can be employed to further analyze the region. Clones can be obtained through various libraries (YAC, MegaYAC, P1) from collaborators or from Research Genetics, Inc. New markers can be developed from simple sequence repeats (di-nucleotide, tri-nucleotide and tetra-nucleotide sequence repeats) contained in these clones resulting in further refinement of both the genetic and deletion maps. These markers can be used as entry points for chromosomal walking ultimately leading to the cloning of the putative suppressor gene. All the tumors can then be analyzed for any mutations existing for that gene and a consensus determined as to the significance of these mutations in the development and progression of breast cancer.

**Conclusions**

Multiple tumor suppressor loci on 5 chromosomal arms (8p, 13q, 16q, 17p and 17q) are involved in pre-invasive breast cancer. We have found LOH on 8p in 27% of informative samples of DCIS, suggesting that one or more tumor suppressor loci on 8p play a significant role in breast oncogenesis. The regions of deletion on 8p are being refined and a fine structure map generated. Study of synchronous *in situ* and invasive breast cancer reveals that, when invasive cancer is found to co-exist with DCIS, it has most likely arisen by clonal expansion of the pre-invasive cells. Loss of additional loci such as 11p may be involved in the invasive phenotype. We continue to accrue examples of *in situ* tumor to expand our tumor panel. Our initial studies of DCIS show that some genetic information may be correlated with histopathologic features (comedo, high nuclear grade tumors have a higher FAL). This information may be of value in determining treatment choices. We shall continue to perform statistical analyses with the larger tumor panel, including examples of LCIS, and plan to include other information in the analyses, such as the presence of local recurrence and patient outcome.

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## Allelotyping of Ductal Carcinoma *in Situ* of the Breast: Deletion of Loci on 8p, 13q, 16q, 17p and 17q<sup>1</sup>

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### ABSTRACT

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

### INTRODUCTION

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

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

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

### MATERIALS AND METHODS

#### Subjects

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

#### Microdissection, DNA Purification, and Allelotyping

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

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<sup>3</sup> The abbreviations used are: DCIS, ductal carcinoma *in situ*; GDB, Genome Database; FAL, fractional allelic loss; LOH, loss of heterozygosity; CEPH, Centre d'Etude Polymorphisme Humain.

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

### LOH on Chromosome 17

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

### Statistical Analysis

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

### Genetic Map Construction

**Chromosome 17p.** Nineteen loci were incorporated into a linkage map for a portion of the short arm of chromosome 17. Five microsatellite markers (*c1541/D17S260*, *AFM234wg3a/3m/D17S849*, *Mfd152/D17S578*, *ut20/D17S654*, and *ut269/D17S695*) were not part of our previous 17p map (38). Genotypes for *D17S513* (12G6), *D17S260* (c1541), *D17S578* (Mfd152), *D17S849* (AFM234wg3a/3m), *CHRN1* (IMG4125/26), *D17S379* (DL1), *TP53* (TP53CA), *D17S643* (ut18), *D17S654* (ut20), and *D17S695* (ut269) were produced in our laboratory with the use of the CEPH 40 family reference pedigrees. Genotypic data for *D17S31* (pMCT35.1M*spl* and pMCT35.2M*spl*), *D17S28* (pTB7c-2.1), *D17S30* (pYNZ22*Rsa*I), *D17S499* (cLS17.6P*vul*I), *D17S510* (fLB17.3A*lu*I), *D17S505* (fLB17.8A*lu*I), *D17S506* (fLB17.9P*st*I), *MYH2* (p10.5H*ind*III), and *D17S34* (p144-D6*Rsa*I) were obtained from the CEPH database (version 6). Oligonucleotide primer sequences and reaction conditions for ut markers were obtained from the Utah Genome Center (40), and several of these have since been published (41). PCR primer sequences and reaction conditions for *D17S379* were provided by Dr. D. Ledbetter and have subsequently become accessible via GDB (42). In our previous paper it was arbitrarily referred to as DL1 because it had not been published at that time (39). Primer sequences and reaction conditions for the other markers used in the map are available from the GDB (<http://gdbwww.gdb.org>). The CRIMAP program package was used to construct the map with odds for loci order of at least 1000:1. "flips4" permutations of marker order was used to test for the most likely order with the use of data from the "build" analysis. Two additional markers from chromosome 17, ut9, and ut69 (made available from the Utah Genome Center) were also genotyped in this study, but two-point analysis indicated that they mapped to 17q, and they were not studied further.

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

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

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

## RESULTS

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

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

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

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

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

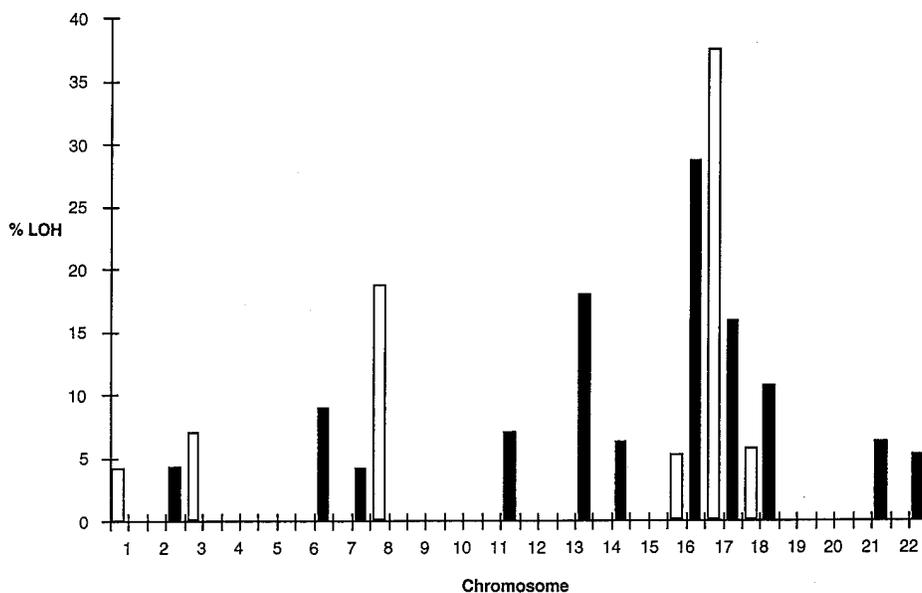


Fig. 1. DCIS allelotyping. □, p arm; ■, q arm.

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

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

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

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

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

## DISCUSSION

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

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

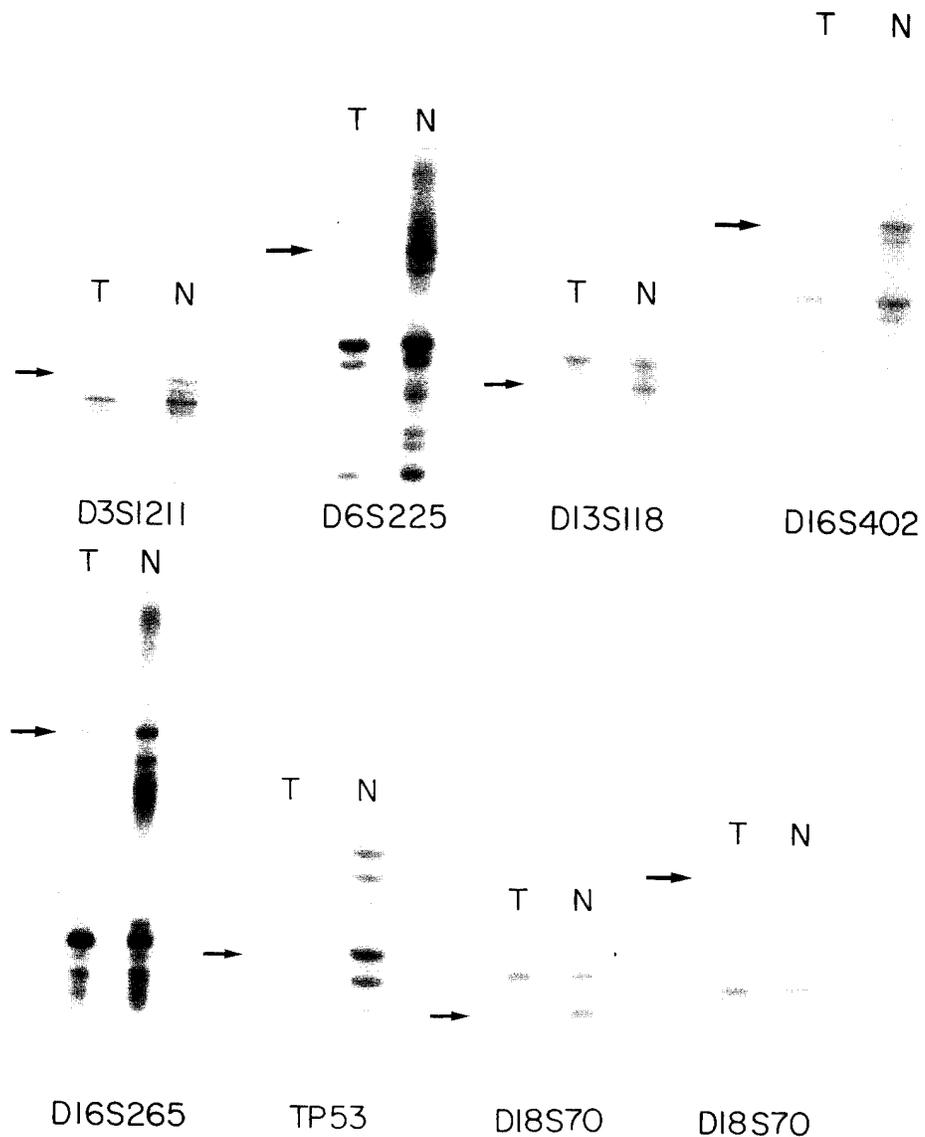


Fig. 2. LOH at multiple loci in DCIS. For each autoradiograph the locus, the tumor number, and the fold-difference in integrated allele ratios between tumor and normal are listed: *D3S1211*, tumor 37, 4.8-fold; *D6S225*, tumor 23, 3-fold; *D13S118*, tumor 58, 5.2-fold; *D16S402*, tumor 19, 7.6-fold;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

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

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

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

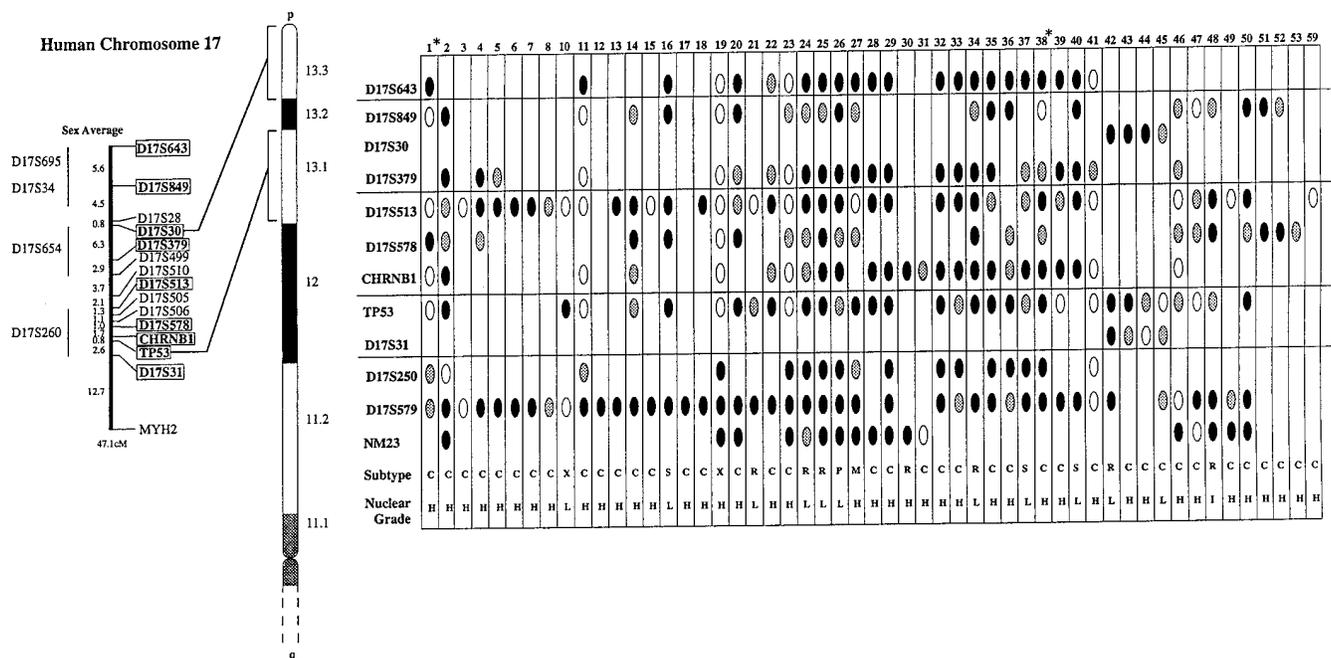


Fig. 3. Sex average multipoint linkage map for distal 17p including cytogenetic tie points and cumulative data for chromosome 17 deletions observed in 53 DCIS tumors. Map, genetic loci uniquely placed with odds for order of at least 1000:1 are shown to the right of the vertical line of the linkage map and nonuniquely localized markers are shown to the left of the map line with the location intervals indicated by thin vertical bars. No recombinants were detected between D17S695 and D17S34 (p144-D6Rsa1). Genetic distance in cM is given to the left of the linkage map line. Markers used for LOH studies are enclosed by a box. Genetic markers for which physical localizations were available are tethered to the cytogenetic map. D17S250, D17S579, and NM23 are located on 17q. LOH data: top, DCIS samples; bottom, DCIS subtype and nuclear grade. Genetic markers are listed in the order determined from the linkage map. ●, no loss; ○, LOH; ⊙, noninformative, H, high nuclear grade; L, low nuclear grade; I, intermediate nuclear grade; C, comedo; R, cribriform; S, solid; M, micropapillary; P, papillary; X, mixed. Horizontal lines within the diagram and vertical lines on the right, common areas of deletion. Tumors which limit these boundaries are marked with an \*.

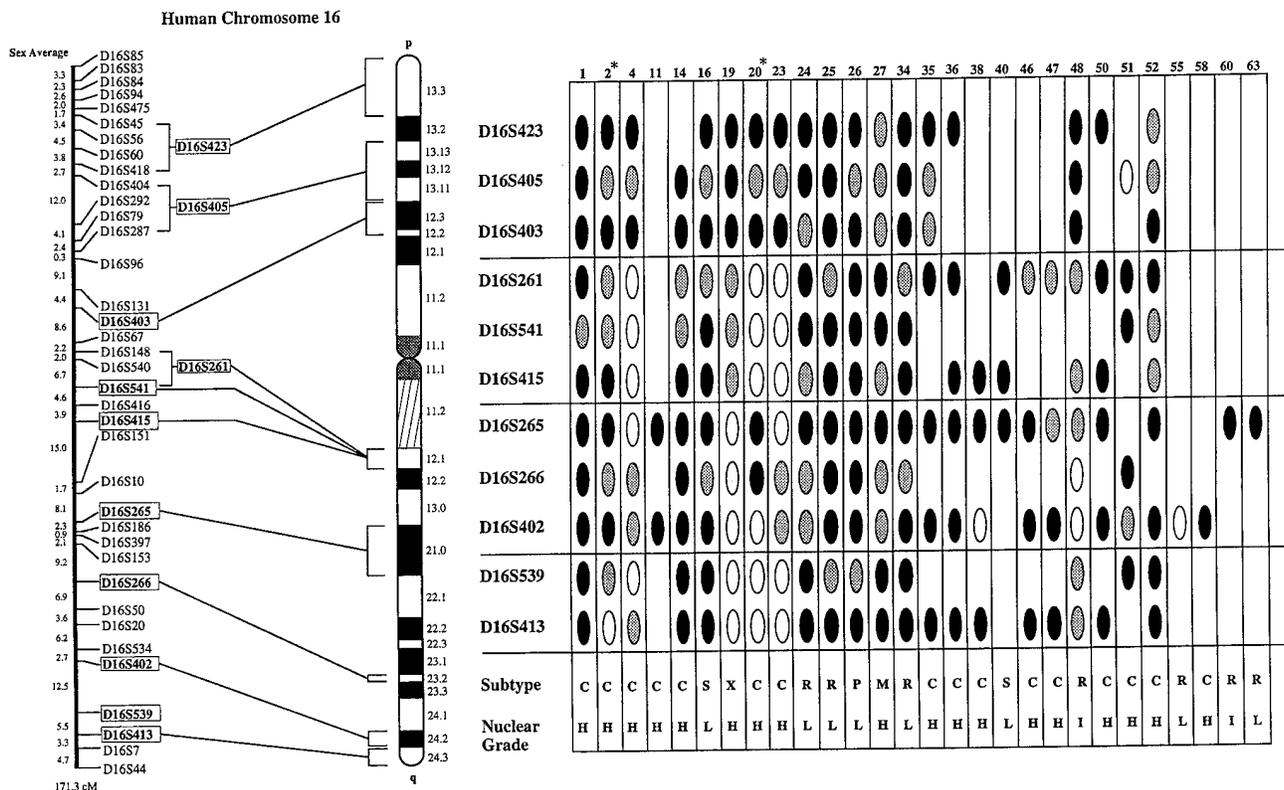


Fig. 4. Sex average multipoint linkage map for chromosome 16 and cumulative data for chromosome 16 deletions observed from 28 DCIS tumors. Marker designations are as indicated for Fig. 3. Nonuniquely and uniquely placed markers are on the right of the vertical line and genetic distances on the left.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

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

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

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

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

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Appendix 2.

Loss of heterozygosity on 8p in an example of DCIS (T) compared with normal control (N).

TUMOR 41

T N

-

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D8S261

1201X

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

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

### MATERIALS AND METHODS

Sixty-four cases of DCIS were microdissected from paraffin embedded, archival material to remove the adjacent normal stroma. DNA was extracted and polymerase chain reaction (PCR) carried out as described previously<sup>3</sup>.

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PCR products were separated on denaturing polyacrylamide DNA sequencing gels. Either uninvolved lymph node or white cell DNA was used as normal control. PCR was carried out using eight microsatellite markers which map to 8p and two markers which localize to the long arm of the chromosome. The order of the 8p microsatellites from telomere to centromere is D8S262- D8S277- D8S520- D8s265- D8S552- D8S261- LPL- NEFL.

### RESULTS

Of 55 cases of DCIS for which the assays were informative, LOH on 8p was seen in 15 tumors (27.3%). In 6 tumors the deletion appeared to involve the whole short arm, whereas in the remainder a smaller deletion was seen. The smallest common region of deletion localizes to a 1.3 cM region at 8p22-23, between the markers D8S520 and D8S265 (Figure 1).

### CONCLUSIONS

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

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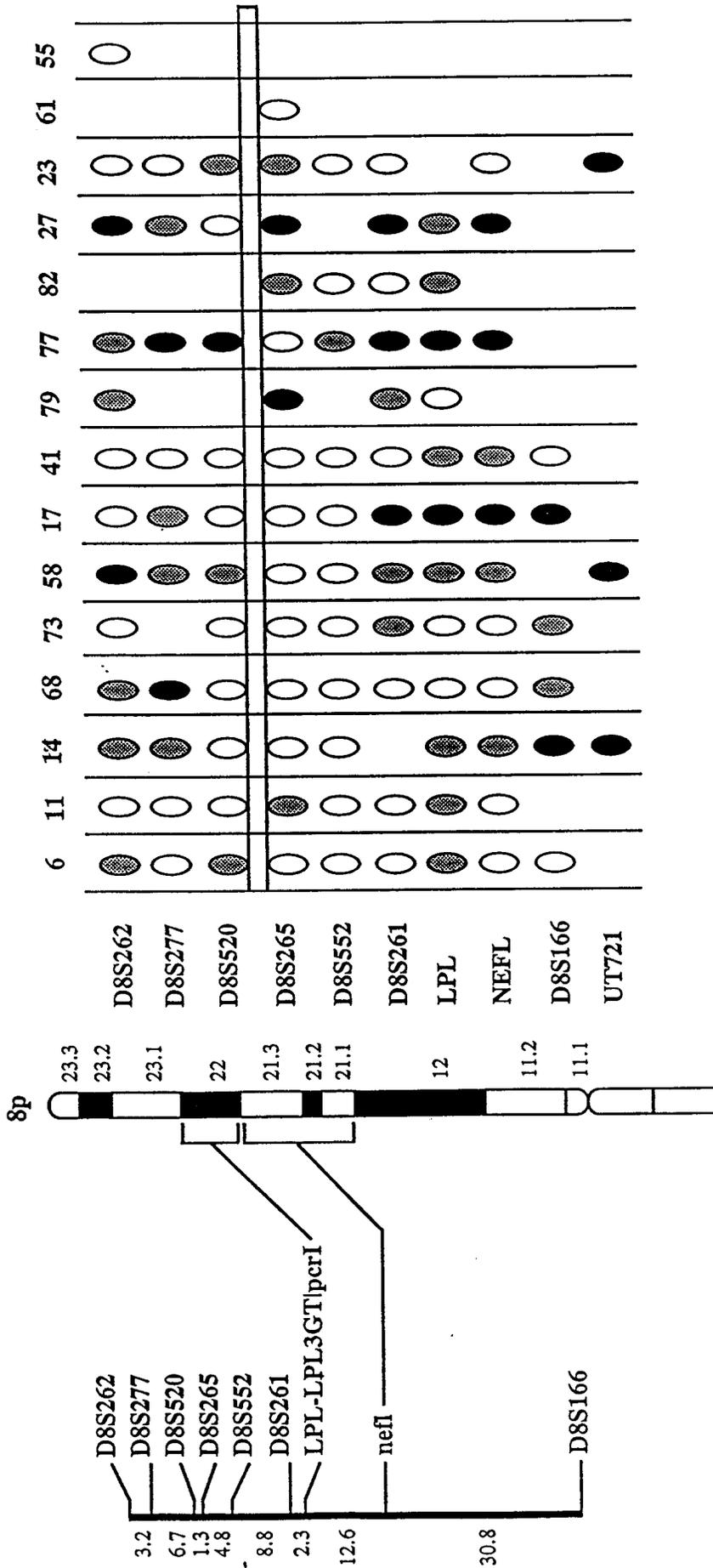
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## LEGEND

**Figure 1.** Chromosome 8 deletions observed in 15 DCIS tumors. DCIS samples are numbered at the top. Genetic markers are uniquely placed with odds of 1:1000 and are listed in the order determined. The genetic map and distances are shown on the left of the figure. Cytogenetic localization is shown by tie bars. D8S166 and UT721 map to 8q. ● no loss; ○ LOH; ⊙, noninformative. A vertical line on the right of the figure and horizontal lines within the figure show the smallest common region of deletion.

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## Appendix 4.

## LEGEND

Summary of LOH data for 10 sets of synchronous CIS and adjacent invasive cancer and for two sets of DCIS, invasive cancer and lymph node metastasis. Chromosomal arm is listed on the left, the marker used is on the right and tumor number on the top. ● indicates LOH; ○ no LOH; ◐, noninformative. INV, invasive carcinoma; LNM, lymph node metastasis. C, comedo subtype; R, cribriform; M, micropapillary, TD, terminal ductal; X, mixed. H, high nuclear grade; L, low nuclear grade, I, intermediate.

