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Advances in Brief

Allelic Loss on Chromosome 17 in Ductal Carcinoma *in Situ* of the Breast[1]

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Abstract

Multiple tumor suppressor genes are implicated in the oncogenesis and progression of invasive carcinoma of the breast. To investigate the chronology of genetic changes we studied loss of heterozygosity on chromosome 17 in ductal carcinoma *in situ*, a preinvasive breast cancer. A microdissection technique was used to separate tumor from normal stromal cells prior to DNA extraction and loss of heterozygosity was assayed mainly using simple sequence repeat polymorphism markers and the polymerase chain reaction. Loss of heterozygosity on 17p was observed in 8 of 28 tumors (29%) when compared with normal control DNA, whereas no loss was seen on 17q, suggesting that at least one locus on 17p is involved early in the development of breast cancer.

Introduction

DCIS[3] of the breast is a preinvasive form of breast cancer in which malignant cells have not breached the basement membrane of the ducts (1). DCIS is a heterogenous group of pathological subtypes comprising comedo, solid, cribriform, papillary, and micropapillary varieties which can also be characterized by nuclear morphology into high and low nuclear grade (1). Biological behavior differs between the subtypes and according to nuclear grade. For example, the comedo, high nuclear grade variety is more likely to recur locally after wide excision than the cribriform or micropapillary low nuclear grade varieties (1). In addition, the comedo subtype is more often associated with areas of microinvasion (1). Tumor suppressor genes have been implicated in the pathogenesis of a variety of cancers including colon cancer (2) and lung carcinoma (3). The loss of function of these genes, due to chromosome deletions or other mutational events, is thought to release cells from normal regulatory controls, thereby resulting in a cancer phenotype. Chromosome deletions can be detected with genetic markers by comparing allelic patterns observed in tumor and normal DNA from the same patient. Sites of putative tumor suppressor genes are revealed as LOH of one of a pair of alleles in the tumor sample. For invasive carcinoma of the breast LOH from a number of chromosomes [1 (4, 5), 3 (6, 7), 6 (8), 7 (9), 11 (6, 7), 13 (6, 7), 16 (10), 17 (6, 7, 11–13), and 18 (6, 12)] has been found. LOH of loci from 17p in the region of the *p53* gene has been observed in up to 67% (7) of invasive breast cancer and LOH at 17q loci has been found in 29–48% (11–13) of cases. Thus far the presence or absence of LOH in DCIS of the breast has not been reported. We investigated allelic loss for several subtypes of DCIS in order to determine the initiation points and chronology of DNA mutations in the development of breast cancer and to ascertain whether differences in biological behavior of the subtypes reflect these molecular changes. These studies demonstrate chromosome deletions from chromosome 17p in the vicinity of the *p53* gene and no observed deletions at a locus on 17q.

Materials and Methods

Subjects. A total of 33 women with DCIS were entered into the study and tumor material collected. Twenty-nine were operated upon at hospitals in St. Louis (Barnes, Jewish, and Deaconess) and four patients underwent surgery at the Edinburgh Breast Unit. Informed consent was obtained prior to entry in the study, which had been approved by the relevant ethics committees. DCIS subtype and nuclear grade were determined by a pathologist (J. R. and by D. Patterson, Department of Pathology, Edinburgh University).

Microdissection and DNA Purification. All samples of DCIS (St. Louis samples) were obtained from archival paraffin-embedded material which had been formalin/alcohol fixed between 1984 and 1992. A microdissection technique was used in order to separate tumor cells from adjacent normal stroma. Twenty-five-μm sections were selected from areas containing DCIS. These unstained sections were overlaid on a hematoxylin and eosin-stained 5-μm section from the same block and areas of DCIS were excised from adjacent stroma using a scalpel blade. Two to four 25-μm sections were processed from each tumor sample. Adjacent normal breast tissue, uninvolved lymph node, or leukocyte DNA were obtained from the same patients to serve as normal control DNA. Samples were deparaffinized with two 800-μl xylene washes at room temperature, followed by three rinses in 100% ethanol (800 μl each). Ethanol was removed under vacuum and the sample was resuspended in 400 μl of 50 mM Tris-0.5 mM EDTA (pH 8)-proteinase K (250 μg/ml) and 1% SDS and incubated at 50°C for 3 to 4 days. If the tissue was not fully digested after an overnight incubation, proteinase K and SDS were added in increments up to a total of 1 mg/ml and 2%, respectively. RNase (150 μg/ml) was added for an additional 30 min at 37°C. Protein extraction was carried out by treatment with phenol/chloroform (3 times) followed by chloroform (once). DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Samples were quantitated using a fluorimeter (Hoefer). The four samples analyzed in Edinburgh were prepared using a similar method except that a second proteinase K/SDS treatment was performed prior to phenol/chloroform extraction.

Detection of LOH. St. Louis-PCR was carried out using 10–50 ng of tumor and corresponding normal control genomic DNA. The SSRPs used were 12G6 (*D17S513*) (14), TP53 CA (*TP53*) (15), and Mfd188 (*D17S579*) (16). Primers were end-labeled with γ-32dATP and reactions were carried out in a buffer containing 10 mM Tris HCl (pH 8.3), 10 mM KCl, 1.0 mM MgCl, 2.5 mM of each deoxynucleotide triphosphate, primer A, Kinase (0.1 pM), primer A unlabeled (1.6 pM), primer B unlabeled (1.7 pM), and 0.05 units Amplitaq (Perkin Elmer). Reactions were cycled in a Perkin Elmer Cetus Thermal cycler as follows: *D17S513* 94°C for 1 min, 57°C for 30 s, 72° for 2 min, total of 28 cycles; TP53CA 94°C for 1 min, 64°C for 30 s, 72°C for 1 min, total of 23 cycles; Mfd188 94°C for 1 min, 57°C for 30 s, 72°C for 2 min, total of 27 cycles. Products were separated on 3M urea denaturing polyacrylamide sequencing gels and dried before exposure to Kodak XAR film for 24 h–5 days at room temperature. For the four samples from Edinburgh, Southern hybridization analysis was performed on tumor and matched lymphocyte genomic DNA after digestion with appropriate restriction endonuclease as previously described (17). Blots were probed with pBH *p53* (*TP53*), MCT 35.1 (*D17S31*), and pYNZ22 (*D17S30*). Two of the four samples were assayed for Mfd188 (*D17S579*) using PCR.

Statistical Analysis. Statistical analysis was performed using contingency tables with the software package Statview (Abacus Concepts Inc., Berkeley, CA).

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; LOH, loss of heterozygosity; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; SSRP, simple sequence repeat polymorphisms.

Chromosome 17 Genetic Linkage Map Construction. Genotypic data available from the CEPH version 6 database were used in addition to genotypes determined in this study for markers 12G6 (*D17S513*), TP53CA (*TP53*), Mfd188 (*D17S579*), and SMSH1 (*D17S844*) to construct a multipoint linkage map with the computer program package CRI-MAP.[4] Unique placement of loci along the map was established using odds for order of at least 1000:1. Cytogenetic localizations of genetic markers have been previously reported (18).

Results

Chromosome 17 Genetic Linkage Map. A comprehensive linkage map for human chromosome 17 has been published recently (18). We constructed a new multipoint genetic linkage map for human chromosome 17 in order to incorporate highly informative microsatellite markers not previously mapped (*D17S513*, 74% HET; *D17S579*, 79% HET; *D17S844*, 80% HET), to substitute more informative genotypic data from the microsatellite polymorphism at the *TP53* locus (90% HET) for the previously published TP53 restriction fragment length polymorphism data, and to correlate the genetic positions with cytogenetic (physical) localizations for these markers. Fig. 1 shows the sex-average map (183 cM in length) which consists of 49 loci, 21 of which have heterozygosities of at least 70%. The order of the markers from our map is in good agreement with the previously published map (18); however, we were able to uniquely localize the *TP53* locus with odds of at least 1000:1. The new map provides the order and genetic spacing of the subset of markers used in the LOH study for 17p13 (telomere - *D17S30* - *D17S513* - *TP53* - *D17S731* - centromere), a region that spans 22.4 cM, and places *D17S579* used for 17q LOH studies within the context of a linkage map.

LOH Studies. From a total of 33 samples of DCIS, 22 were comedo, 5 were cribriform, 3 were mixed, 1 was solid, 1 was papillary, and 1 was a micropapillary subtype. Genomic DNA from the tumors and matched normal controls were tested for chromosome 17 deletions as judged by loss of heterozygosity of alleles from the tumor as compared to normal DNA. Thirty tumors were studied for LOH on the short arm and 28 were informative for at least one of the five markers tested, 12G6 (*D17S513*), PYNZ22 (*D17S30*), MCT35.1 (*D17S31*), TP53CA, and pBH *p53* (*TP53*). As Fig. 2 shows, LOH was observed in 8 of 28 samples (29%). In four cases for which data exist for both 12G6 (*D17S513*) and TP53CA (*TP53*), LOH was observed for both loci which have been physically mapped to 17p13 (19). Fig. 3 demonstrates examples of LOH seen with 12G6 (*D17S513*) and TP53CA (*TP53*). Of the three tumors informative for pYNZ22 (*D17S30*) no LOH was seen. Twenty-seven tumors were analyzed with the SSRP marker Mfd188 (*D17S579*) which mapped to the long arm of chromosome 17. Except for two samples all tumors were informative, and no allelic loss was detected. No statistically significant difference was observed in the frequency of LOH on the short arm when analyzed according to subtype (comedo *versus* non-comedo) or nuclear grade (high *versus* low).

All of the SSRP markers used in this study were tested on genomic DNA from the Centre d' Etude Polymorphism Humaine reference pedigree panel to ensure that allele-specific amplification did not occur, since this phenomenon would interfere with LOH detection. In addition, using admixtures of DNA from two individuals who were homozygous for a marker, we determined that detection of LOH was possible by PCR provided that no greater than 20% of contaminating DNA was present in the sample (data not shown).

Discussion

These data demonstrate that LOH of alleles from loci on the short arm of chromosome 17 occurs early in the oncogenesis of breast cancer, whereas LOH from the long arm was not observed in this study. A multistep progression of neoplasia occurs in colon cancer (2) and multiple tumor suppressor genes are likely to be involved in the development of other solid tumors such as breast cancer. Whereas loss of the *p53* gene may occur relatively late in the chronology of genetic changes in colon cancer (2) these data suggest a role for *p53* at a much earlier stage in the development of breast cancer. The possible existence of two suppressor genes on 17p, the *p53* gene at 17p13 and another telomeric to it, has been suggested (13). LOH of these two regions occurs independently of one another in invasive breast cancer. Study of more distal regions of 17p in DCIS is currently limited by the lack of microsatellite markers amenable to LOH studies, although as other markers become available we should be able to determine the smallest common region of deletion in DCIS tumors.

The marker Mfd188 (*D17S579*) is closely linked to the hereditary early onset breast cancer gene described by Hall *et al.* (16). The relatively high frequency of LOH at loci on the long arm in this region which is seen in sporadic invasive breast cancer (6, 11–13), implies

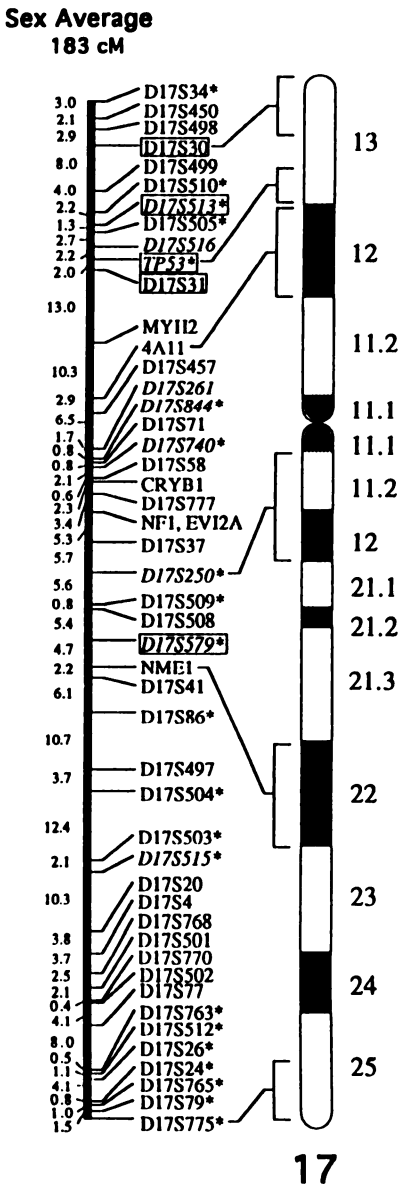

Fig. 1. Multipoint genetic linkage map for human chromosome 17. A sex-average linkage map with genetic loci to the *right of the map line* and intermarker spacing in centimorgans to the *left of the map line*. *Right*, idiogram of the cytogenetic staining pattern for the Giemsa banded metaphase chromosome (400-band resolution) with physical localizations of six selected markers from the genetic map indicated by *tie lines*. *Italics*, SSRP markers (assayed by PCR). *, markers with heterozygosity in excess of 70%. Additional information on marker characteristics can be found in Ref. (18). Markers used for LOH studies in this report are enclosed by a *box*.

[4] P. Green, personal communication.

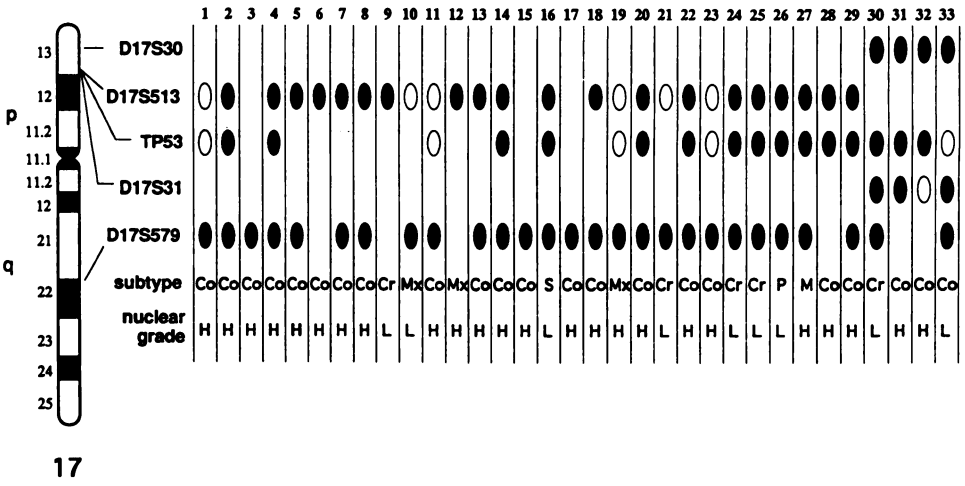

Fig. 2. Summary of chromosome 17 deletions observed from 33 DCIS samples. *Top*, DCIS samples (1–33), *bottom*, DCIS subtype and nuclear grade. Genetic markers are listed in the order determined from the multi-point map (see Fig. 1). ●, no loss; ○, LOH; ⬮, noninformative. *H*, high nuclear grade; *L*, low nuclear grade; *Co*, comedo; *Cr*, cribriform; *S*, solid; *M*, micropapillary; P, papillary; *Mx*, mixed.

that a tumor suppressor gene on 17q is also involved in progression of the disease. We found no LOH with Mfd188 (*D17579*) in DCIS; therefore, genetic changes at this locus do not appear to be involved in the initiation of DCIS but may confer an invasive phenotype.

Detection of LOH using PCR has potential pitfalls such as amplification of contaminating stromal DNA and allele-specific amplification. The microdissection technique we used enabled us to keep stromal contamination to a minimum and thereby facilitate LOH detection. The markers chosen for this study did not show allele specific amplification in normal Centre d' Etude Polymorphism Humaine DNA, making them suitable markers for LOH detection. PCR uses much smaller amounts of tumor DNA (10–50 ng) than the amount necessary for restriction fragment length polymorphism anal-

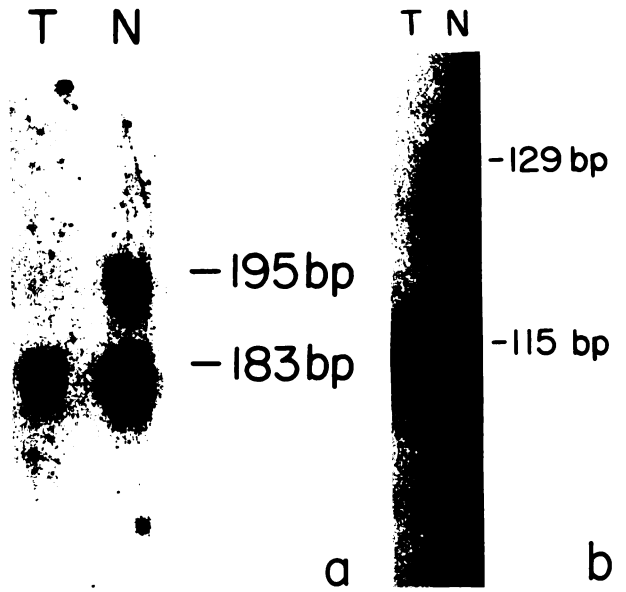

Fig. 3. LOH at 17p in DCIS. *a*, LOH at 17p locus D17S513 in sample number 10. PCR products from tumor (*T*) and normal (*N*) genomic DNA from an individual with DCIS are shown. Loss of one allele is seen in the tumor sample. *b*, LOH at 17p locus TP53CA in sample number 19. Similarly, *Lane T* contains the products from PCR of tumor DNA and *Lane N* is the PCR product from corresponding normal genomic DNA of the same patient. Loss of one allele is seen in the tumor. *bp*, base pairs.

ysis by Southern hybridization (5 μg). From the microdissection DCIS samples we routinely isolate 200 ng to 3 μg of DNA; therefore, PCR is the preferred technique for LOH detection, especially when a number of chromosomal sites are to be examined. The construction of a chromosome 17 map was necessary in order to determine the order and spacing of the markers used for the LOH study. As new markers become available they will be incorporated into the map, enabling selection of additional markers for deletion studies in DCIS.

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