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Loss of heterozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer

(tumor suppressor gene/primary breast carcinoma/p53 gene/S-phase fraction)

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ABSTRACT **Loss of heterozygosity (LOH) on the short arm of chromosome 17 (17p) was found in 27 of 52 (52%) previously untreated primary breast cancers. There was a significant correlation between this 17p allelic loss and two parameters associated with aggressive tumor behavior: high cellular proliferative fraction and DNA aneuploidy. These correlations with high cellular proliferative fraction and DNA aneuploidy were not found in tumors with LOH at nine other chromosome locations. The p53 gene, a putative tumor suppressor gene located at 17p13, was examined for aberrations to determine whether it is the target for the 17p LOH in breast cancer. Unlike other types of human cancer, there were no homozygous deletions or rearrangements of the p53 gene, and only 2 of 13 (15%) were mutated in the conserved region where mutational "hot spots" have been previously located. Therefore, we hypothesize that, in breast cancer, either loss or inactivation of gene(s) on chromosome 17p other than the p53 gene or a different mechanism of p53 gene inactivation may be responsible for the observed high labeling index and DNA aneuploidy associated with LOH at 17p.**

Loss of heterozygosity (LOH) at chromosomal locations associated with tumor suppressor genes has recently been implicated in the genesis of many forms of human malignancies (1, 2). LOH at chromosome 17p has been reported in various human cancers, including colorectal carcinomas (3), lung cancers (4), and glioblastomas (5), and in neurofibromatosis (6). In breast cancers, 50–60% of cases have allelic losses at this region (7–10). To date, correlations have not been reported between LOH at 17p and various clinicopathological parameters of breast cancers, including nodal status, presence of hormone receptors, tumor stage, and histology (9, 10).

The p53 gene, a 53-kDa nuclear phosphoprotein (11), has been assigned to chromosome region 17p13 (12). Several studies have indicated that the wild-type p53 gene product functions as a tumor suppressor (13, 14). In experimental systems as well as in human tumors, various mechanisms resulting in loss of the normal p53 gene function have been implicated in tumorigenesis. Observed aberrations of p53 include genomic rearrangement (15), homozygous deletions (15, 16), and LOH with concomitant point mutations in a highly conserved region of the remaining p53 allele (3, 17).

Here we confirm the observation (7, 8) that LOH on the short arm of chromosome 17 is a frequent allelic loss in breast cancers. We found that this loss correlated with high bromodeoxyuridine (BrdUrd) labeling index and DNA aneuploidy, characteristics of aggressive tumor behavior *in vivo* (for review, see ref. 18). However, mutations of the p53 gene were rarely detected in these breast cancers and the few cancers showing mutations did not correlate with the 17p LOH or high labeling index. Therefore, the linkage between LOH at 17p and certain parameters of aggressive disease appears to be independent of DNA aberrations in the conserved region of the p53 gene.

MATERIALS AND METHODS

Human Tissue Samples and Cultured Cells. Human breast tumor samples and skin biopsies were removed surgically. Tumor samples were dissected to remove most of the normal tissue. All samples were stored in liquid nitrogen until use. Whenever the skin tissues were too small to isolate sufficient DNA for analysis, cultured skin fibroblasts from the same patient were used to extract DNA.

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was isolated from cultured fibroblasts, pulverized skin biopsies, and breast tumors as described (19). Restriction endonuclease digestion of DNA samples, agarose gel electrophoresis, Southern transfer, prehybridization, hybridization to DNA probes ^{32}P-labeled by random priming technique (Amersham) (20), and autoradiography were performed as described (21). Quantification of the hybridization signals was performed with a Molecular Dynamics (Sunnyvale, CA) laser scanning densitometer. A reduction of >30% of the normal signal (as adjusted with the signals obtained from normal tissue) was recorded as an allelic loss. All but 3 of the 27 cases with LOH at 17p had a reduction in signal intensity >50%. DNA probe pYNZ22 was kindly provided by Y. Nakamura (Howard Hughes Medical Institute, Salt Lake City). p53 cDNA probe was kindly provided by A. J. Levin (Princeton University).

BrdUrd Labeling and Detection. BrdUrd was given to patients intravenously in normal saline at 200 mg/m^2 over 30 min just prior to tumor excision. Slices 1 mm thick were fixed in 70% ethanol and embedded in paraffin. Alternately, *in vitro* labeling as described by Meyer and McDivitt (22) was used if *in vivo* labeling was not available. A 1-mm-thick tumor slice was incubated in RPMI medium containing 10 μM 5-fluorodeoxyuridine (Sigma) and 100 μM BrdUrd (Sigma) under hyperbaric oxygen for 2 hr at 37°C. Tumors were fixed in 70% EtOH before embedding. Incorporated BrdUrd was detected immunologi-

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Abbreviations: LOH, loss of heterozygosity; PCR, polymerase chain reaction; VNTR, variable number tandem repeat.

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cally with IU4 antibody (Lawrence Livermore National Laboratory) after denaturing DNA in 2 M HCl. For formalin-fixed tissue, crosslinked proteins were cleaved by protease treatment prior to denaturation. The Elite ABC kit for routine indirect immunoperoxidase staining was used to detect incorporated BrdUrd. Light counterstaining with hematoxylin allowed detection of BrdUrd-negative nuclei. Calculation of the BrdUrd labeling index (the number of positively stained cells divided by the total number of cells counted) was done by scoring a minimum of 2000 cells in multiple well-labeled, high-power fields.

DNA Flow Cytometry. DNA content was measured by the detergent/trypsin protocol of Vindelov *et al.* (23). Fresh samples were finely minced to create single cell suspensions and treated with buffers containing detergent and trypsin to produce nuclear suspensions without excess debris. Nuclei were stained with propidium iodide (10 μg/ml) and analyzed for total DNA content using the FACScan flow cytometer (Beckton Dickinson). Normal lymphocytes were used as an internal control to define diploid DNA content when a diploid population was not present in the tumor sample. Chicken erythrocytes were used as a second internal control. DNA index was calculated as the modal channel number for tumor cell DNA content divided by the modal channel number for normal cells. Diploid was defined as having a DNA index of 1.0 ± 0.05, and aneuploid was defined as having a second nondiploid peak that was separable from the diploid populations.

Fluorescence *in Situ* Hybridization. A single cell suspension of tumor cells was fixed in Carnoy's fixative and dropped onto precleaned microscope slides. Cells were hybridized overnight with biotinylated probes specific for pericentromeric sequences on chromosomes 1, 7, and 17 as described (24). The number of hybridization signals for each chromosome was counted in >400 interphase cells.

Polymerase Chain Reaction (PCR). PCR (25) was performed with 200–400 ng of genomic DNA under standard conditions using Amplitaq DNA polymerase (Perkin–Elmer/Cetus). At least two, and in some cases three or four, amplification reactions were performed for each DNA sample. Primer sequences were taken from the published coding and intron sequences for p53 (26). A 2.9-kilobase (kb) genomic DNA fragment containing exons 4–9 was amplified using a 0.5 μM final concentration of primers GACGGAATTCGTCCCAAGCAATGGATGAT and GTCAGTCGACCTTAGTACCTGAAGGGTGA. The amplified 2.9-kb product was electrophoresed on a 0.8% agarose gel, stained with ethidium bromide, and recovered using the "Geneclean" procedure as recommended by the manufacturer (Bio 101, LaJolla, CA). Five to 10% of the yield was subjected to a second "asymmetric" PCR (27) using 0.01 μM primers for exon 5 (TTCCTCTTCCTGCAGTACT and AGCTGCTCACCATCGCTAT), for exon 7 (TGTTGTCTCCTAGGTTGGCT and CAAGTGGCTCCTGACCTGGA), or for exon 8 (CCTATCCTGAGTAGTGGTAA and TCCTGCTTGCTTACCTCGCT). The concentrations of the PCR primers were 0.5 μM and 0.01 μM, respectively. Both strands were sequenced for each exon. The respective primers were then used in the sequencing reactions.

Sequencing Reaction. The product of the asymmetric PCR was purified and dissolved in 15 μl of water; 7.5 μl of this was taken for each sequencing reaction. Sequencing was performed by the modification of the method of Sanger *et al.* (28) as optimized for United States Biochemical Sequence. The reaction mixture was then electrophoresed on an 8% polyacrylamide 5 M urea gel for 2 hr with 55 W and exposed to X-Omat AR film (Kodak) overnight.

Data Base Storage and Retrieval. The breast cancer data base is maintained in the Informix relational data base management system (Informix, Belmont, CA), running under Ultrix on a Microvax II computer (DEC, Waltham, MA). Demographic, clinical, and research data from clinical sources and several laboratories are stored in tables in the data base, and the data are linked by patient accession numbers. Data are retrieved using the data base query language SQL (structured query language) or using GQL (graphical query language; Andyne Computing, Ontario, Canada), a Macintosh computer-based program that communicates with the data base and allows access to data using simpler, graphical methods.

RESULTS

Correlation of Pathobiological Parameters with Allelic Loss on Chromosome 17p. Seventy-eight primary breast carcinomas were analyzed for allelic loss on the short arm of chromosome 17 using a variable number tandem repeat (VNTR) probe, pYNZ22, located at 17p13.3. Fifty-two of the cases were heterozygous at this locus and so were informative. Twenty-seven of the 52 informative cases (52%) demonstrated LOH at this locus. Examples of allelic loss at chromosome 17p are illustrated in Fig. 1.

To determine whether those cases with LOH at 17p13.3 constituted a biologically distinct subset of breast cancers, the clinical and biological properties of those cases with LOH were compared with the properties of those cases that were informative but had no loss. A marked difference in BrdUrd labeling index was detected between the two groups (Fig. 2). Among the tumors that retained heterozygosity at 17p, most had low labeling indices. In contrast, many of the cases with LOH at 17p had a labeling index >5%. This difference between the two types of tumors was statistically significant using the Mann–Whitney rank test ($P = 0.0065$; additional statistical tests described below).

There was only one case with a labeling index >10% that did not show LOH at 17p by Southern analysis (*, Fig. 2). However, an existing LOH may not be detected if it occurs in some but not all tumor cells, and/or if there is substantial contamination of the tumor tissue with nonmalignant cells. In this study, a signal reduction of at least 30% was required to define LOH. To further characterize the tumor with high S-phase fraction but no LOH (*, Fig. 2), additional studies were undertaken using fluorescence *in situ* hybridization with pericentric chromosome-specific probes. Although chromo-

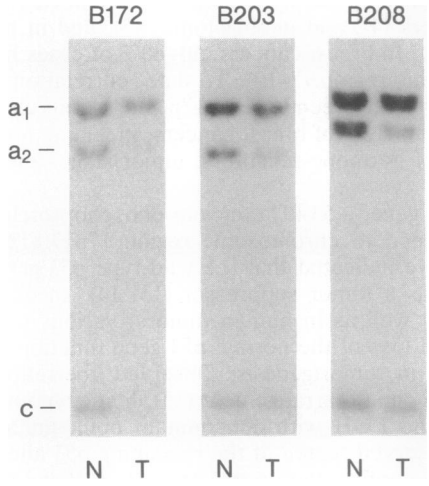

FIG. 1. LOH at loci on chromosome 17p in breast tumors. DNA samples from normal skin tissue (lanes N) and tumors (lanes T) were obtained from patients B172, B202, and B208. The DNAs were digested with *Taq* I and hybridized to ^{32}P-labeled pYNZ22 probe. The allelic designations (a1, a2) are indicated on the left of the autoradiogram. c, Constant band.

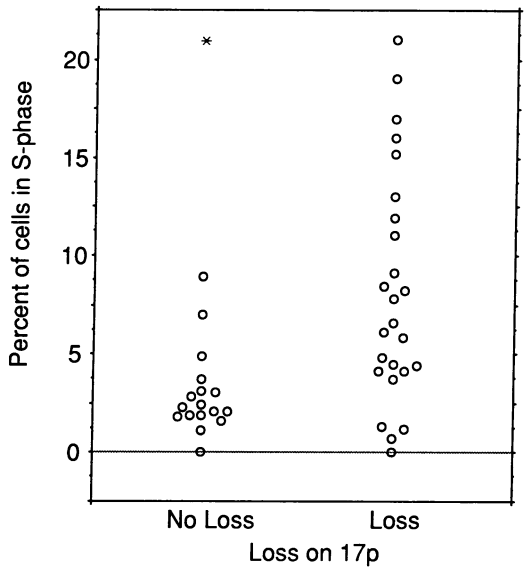

FIG. 2. Correlation between LOH at 17p13.3 and S-phase fraction of primary breast cancer. LOH at 17p13.3 was defined using pYNZ22 probe by Southern blot analysis. S-phase fraction (%) was obtained by BrdUrd labeling. Each circle represents a case of breast cancer. The asterisk (*) represents the single case where LOH was absent by Southern blot analysis but was shown to be 40% monosomic for chromosome 17 using *in situ* hybridization with a pericentrimeric probe on chromosome 17.

somes 1 and 7 were predominantly disomic, 40% of the interphase cells in the same preparation were monosomic for chromosome 17 (Table 1). It is likely that the p arm is also monosomic in cells with centromeric monosomy; therefore, 40% monosomy together with some normal cell contamination could readily explain the scoring of this case as informative with no LOH at 17p.

To determine whether the correlation between LOH at 17p and high S-phase fraction was specific for LOH at 17p, data were collected on LOH at nine other loci: 1p, 1q, 2q, 4p, 11p, 11q, 17p, 18p, and X. For each locus, the samples were divided into two classes: those with and those without LOH, and the frequency of cases with S-phase fraction ≥5% was determined. Because the various probes differed in degree of polymorphism, LOH was not equally ascertainable at all chromosomal loci. Only for chromosomes 1q and 17p were there sufficient data for statistical testing. As an additional comparison with results for 17p, the data of LOH at all chromosomes except 17p were pooled.

The association between allelic loss and high S-phase fraction and five other markers associated with poor prognosis are summarized in Table 2. The number of informative cases and the fraction of total cases with S phase ≥5% for samples with and without LOH are indicated (see "S-phase fraction ≥5%"). The odds ratio (29) was calculated at 7.5, indicating that when LOH at 17p is present, it is 7.5 times more likely that the S-phase fraction will be ≥5% than when LOH at 17p is absent. The precision of this statistic is indicated by the 95% confidence interval whose lower boundary was >1, indicating that it is highly likely that S-phase fraction ≥5% and LOH at 17p are related.

Table 1. Distribution of chromosome copy number per cell

Signals, no. per cell	Chromosome, %		
	No. 1	No. 7	No. 17
0	2	0.3	0.8
1	11.3	1.9	40.6
2	76.6	89.2	54.6
3	9.96	7.0	2.8
4	0	0	1.2
Total *n*	291	315	249

Statistical significance also was evaluated based on χ^2 test formed by the 2 × 2 table relating LOH to S-phase fraction ≥5%. One-sided tests were used because it was expected, *a priori*, that LOH would represent a deviation from normality and thus be associated with markers of poorer prognosis. One-sided *P* values for the χ^2 evaluation are indicated in Table 2.

The data in Fig. 2 and Table 2 indicate that there is a significant correlation between high S-phase fraction and LOH that is specific for 17p. When either the pooled data minus LOH at 17p or LOH at another individual locus, 1q, are examined, there is no longer a significant correlation with high S-phase fraction.

A similar statistical evaluation with performed for the five other pathobiological indicators of poor patient prognosis. As with high S-phase fraction, there was a very strong correlation between loss at 17p and aneuploid DNA content (odds ratio, 15.4; one-sided *P* value, 0.0016). However, there was no correlation between aneuploidy and LOH at the other loci examined. LOH at any of the chromosomal loci tested did not significantly correlate with positive axillary nodes, advanced T stage, hormone receptor status, age of the patient, or histological type of breast cancer (data not shown).

Genetic Aberrations of the p53 Gene Occurs Infrequently in Primary Breast Cancers. A potential target of the LOH at 17p in breast cancer is the p53 gene, located at chromosome 17p13.1. To determine whether the p53 gene is the target for LOH at 17p in breast cancer, we analyzed the tumor DNA for evidence of homozygous deletions, rearrangements, or mutations of the p53 gene. DNA from 50 breast cancers was digested with *Eco*RI and examined by Southern blot analysis using a p53 gene cDNA probe, pc53-5N. There was no evidence for either homozygous deletions or abnormally migrating bands indicative of gene rearrangements.

Eight breast cancers with allelic loss at 17p (including five with high S-phase fraction) and five cases that were informative with no loss were selected for further analysis. Genomic DNA from these cases was used to sequence an evolutionarily conserved region of the p53 gene that includes exons 5, 7, and 8 where mutations of the p53 gene have been detected in other tumors (17, 26). A 2.9-kb DNA fragment for sequencing was generated by PCR. Only two mutations were detected in all 13 DNA samples examined. One was from a case with allelic loss at 17p and high S-phase fraction; four other similar cases were negative. The mutation, located at codon 149, was specific for the tumor since it was not found in DNA from skin fibroblasts of the same patient (Fig. 3). Mutations were not detected in three additional cases with LOH at 17p13.3 but with low proliferative index. The other tumor with a p53 mutation at codon 175 was one of the five cases examined with a low S-phase fraction that retained heterozygosity at 17p (data not shown).

For 9 of 11 cases without p53 mutations, LOH was detected in one or more chromosomal loci. Therefore, these DNA preparations were predominantly derived from tumor cells. Since the same DNA samples were used for p53 sequence analysis, any p53 mutations in the tumor DNA would have been detected. For these studies, the sensitivity of detecting p53 mutations was such that 1 mutant allele in 10 would have been detected (30). Thus, only if a mutant allele was present in <10% of the DNA, would it have been missed in this analysis. The above analyses show that the correlations of LOH at 17p with high S-phase fraction and with DNA aneuploidy cannot be explained by mutations or other genetic aberrations of the p53 gene.

Table 2. Association between pathobiological markers and allelic loss for primary breast cancer

Marker	Chromosome locus	Informative	No LOH	LOH	Odds ratio	95% confidence interval for odds ratio		One-sided *P* value*
						Lower boundary	Upper boundary	
S-phase fraction ≥5%	17p	43	3/18	15/25	7.50	1.72	32.80	0.0049
	Any except 17	69	20/45	12/24	1.25	0.46	3.37	0.33
	1q	47	16/31	5/16	0.43	0.12	1.52	0.09
DNA aneuploid	17p	32	5/16	14/16	15.40	2.49	95.06	0.0016
	Any except 17	59	18/36	16/23	2.29	0.76	6.88	0.069
	1q	42	12/27	10/15	2.50	0.67	9.31	0.084

*Based on χ^2 distribution.

DISCUSSION

We have demonstrated that allelic loss on chromosome 17p correlates with high labeling index and DNA aneuploidy. These biological correlations are specific for LOH at chromosome 17p since significant correlations were not observed in breast cancers with LOH at other analyzed chromosomal locations. Even though our studies utilized only probes on the short arm of chromosome 17, others (8) demonstrated that the q arm was rarely involved in tumors with LOH on chromosome 17p. Therefore, we hypothesize that loss of function for a gene(s) located on chromosome 17p results in a phenotype characterized by DNA aneuploidy and rapid cellular proliferation.

Several studies have indicated that the wild-type p53 protein functions as a tumor suppressor and that inactivation of the p53 gene results in malignant transformation (13, 14). Inactivation of the p53 gene may be accomplished by deletion, rearrangement, and/or mutation; all of these have been found with high frequency in the p53 gene of primary colon carcinomas (3, 17), lung carcinomas (15, 17), glioblastomas (17), sarcomas (16), and neurofibromatosis (6). Mutations of p53 have also been found in human breast cancer cell lines (31).

Because of its involvement in so many other human malignancies and breast cancer cell lines, it has been proposed that the p53 gene, which is located at chromosome region 17p13, is the target for the 17p LOH in primary breast cancer. However, none of 50 cases analyzed had a homozygous deletion or gene rearrangement and only 2 of 13 cases (15%) had mutations in the conserved region of the p53 gene

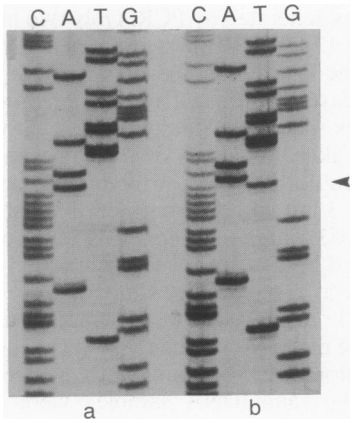

FIG. 3. Mutation in breast carcinoma specimen as detected by direct PCR sequencing. (*a*) Specimen 187, the wild-type sequence of the p53 gene within the fifth exon using skin fibroblast DNA. (*b*) Specimen 187, a C → T mutation at codon 149 changing a Pro to a Ser in the breast carcinoma DNA. The wild-type sequence is still present in this tumor due to contaminating normal DNA. The sequence is read from a 5′ → 3′ direction starting from the top to the base of the gel. The arrowhead indicates the site of C → T mutation in the primary breast carcinoma DNA.

even though ≈50% of breast cancers had LOH at chromosome 17p. Of the two primary breast cancers with mutated p53, one had a high S-phase fraction and LOH at 17p, whereas the other was heterozygous at 17p13.3 and had a very low S-phase fraction. Thus we conclude that neither mutations in the conserved region nor deletions in the remaining allele of the p53 gene are responsible for the pathobiological properties associated with 17p LOH in breast cancers. Although a mutated p53 gene does occur in a small proportion of primary breast cancers, the role of these mutations remains to be elucidated.

In breast cancer, *in vitro* immortalization has been associated with late stages of malignant progression (32). Therefore, the high incidence of p53 mutations seen in breast cancer cell lines relative to primary breast cancer suggests that mutation of the p53 gene may be a late event in breast cancer malignant progression. Similar observations have been made for another oncogene, *ras*. Activating *ras* mutations have been detected in a high proportion of colon (33, 34) and lung (35) cancers and in breast cancer cell lines but not in uncultured primary or metastatic breast cancers (36). These observations suggest that various types of human cancers may differ in the sequence of acquiring genetic aberrations or in the specific genes involved.

Our results suggest that inactivation of a gene on chromosome 17p other than p53 may be responsible for conferring a high S-phase fraction and DNA aneuploidy in primary breast cancers. There is precedence for the hypothesis that two suppressor genes may be located on the same chromosome arm. For example, two loci on the short arm of chromosome 11 (11p13 and 11p15) have been implicated in the development of Wilm tumor and hepatocellular carcinomas (37). Furthermore, Coles *et al.* (38) recently identified two distinct regions of loss of heterozygosity on chromosome 17p, located in bands p13.3 and p13.1. An alternate explanation consistent with the results is that the 17p loss occurs in conjunction with loss at another, as yet unidentified, region where the gene in question is actually located. Whichever explanation proves correct, a deletion-mapping study using a panel of polymorphic markers to define the smallest common region of deletion will be helpful in identifying the region on chromosome 17p associated with high S-phase fraction and DNA aneuploidy in breast cancer.

In summary, we have shown that LOH of 17p is significantly associated with high S-phase fraction and DNA aneuploidy, indicators of aggressive tumor behavior. However, LOH was not associated with mutation of the p53 tumor suppressor gene. These results suggest that the 17p13 region contains gene(s) whose function is important in the suppression of breast cancer and that this activity may involve another gene besides p53. Alternately, if p53 is the target gene, its inactivation must occur by a mechanism other than mutation of the conserved region where mutational "hot spots" have been previously located.

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