Society (C.P.G.). rGST was supplied by R. H. Edwards and D. Krantz, and *Bordetella pertussis* from Wyeth Lederle Vaccine and Pediatrics, Pearl River, NY. We thank J. R. Oksenberg for helpful discussions and K. Lovett and L. Brovarney for their dedicated work with the animals.

18 July 1996; accepted 18 November 1996

Loss of Heterozygosity in Normal Tissue Adjacent to Breast Carcinomas

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Loss of heterozygosity (LOH) was detected in morphologically normal lobules adjacent to breast cancers. The most frequent aberration was at chromosome 3p22-25; of ten cases with this LOH in the carcinoma, six displayed the same LOH in adjacent normal lobules. This suggests that in a subset of sporadic breast cancers, a tumor suppresser gene at 3p22-25 may be important in initiation or early progression of tumorigenesis. Among sixteen breast cancers with LOH at 17p13.1 and five breast cancers with LOH at 11p15.5, one case each displayed the same LOH in adjacent normal lobules. Thus the molecular heterogeneity that characterizes invasive breast cancers may occur at the earliest detectable stages of progression.

The mature breast contains lobules, clusters of closed glandular spaces that produce milk during lactation. These lobules are connected to the nipple–areolar complex by a system of branching ducts that are surrounded by varying amounts of fat and connective tissue. Breast cancer is thought to develop within a terminal ductal-lobular unit (TDLU), which includes the lobule and its most proximal ducts (*1*).

Breast cancer evolves by clonal selection of cells that acquire multiple molecular changes. One model suggests that breast cancer, like colon cancer (*2*), develops through a defined progression of morphologically distinguishable stages beginning with benign hyperplasia, which progresses to atypical hyperplasia, then to in situ carcinoma, and finally to invasive cancer (*1*). This sequential progression may not be the only way that breast cancers develop, however. Many small invasive cancers do not have atypical components, which suggests that they may have developed directly from morphologically normal epithelium. If this were true, one might expect to find evidence of a "field effect" in which at least some of the genetic aberrations found in invasive cancers are also present in the morphologically normal epithelium.

To test this hypothesis, we carefully microdissected hematoxylin-eosin–stained sections of breast cancers so as to isolate morphologically discrete regions (Fig. 1A). DNA was prepared from malignant areas of the section and from adjacent normal TDLUs. As a control for each case, DNA was also prepared from normal breast skin (usually from a separate section) that had been similarly microdissected.

We studied LOH at chromosome 3p24, 11p15.5, 13q13, and 17p13.1 because these loci show LOH in a high percentage (~30 to 60%) of invasive ductal breast cancers (*3, 4*). For the carcinomatous regions, the frequency of LOH at 3p24 (48%) and 11p15.5 (29%) was similar to that previously reported (*4*). The frequency of LOH in the invasive components was higher than the literature values for 13q13 (64% here versus ~40%) and for 17p13.1 (80% here versus ~60%). These discrepancies may be due to random variation because our sample size was small.

In 8 of 30 cases we detected LOH in the adjacent morphologically normal TDLUs (Table 1). In all eight cases, the same allele was missing in the adjacent carcinoma (Fig. 2, A and B). LOH in normal TDLUs was seen in 6 of the 10 cases where LOH at 3p24 was found in the carcinoma. LOH at 11p15.5 in the normal TDLUs was seen in one of five cases with this LOH in the carcinoma; LOH at 17p13.1 in the normal TDLUs was seen in 1 of 16 cases with this LOH in the carcinoma. None of 10 cases with LOH at 13q13 in the carcinoma had this lesion in the normal TDLUs. Among tumors with and without LOH in adjacent normal tissues, there was no significant difference in grade, hormone receptor status,

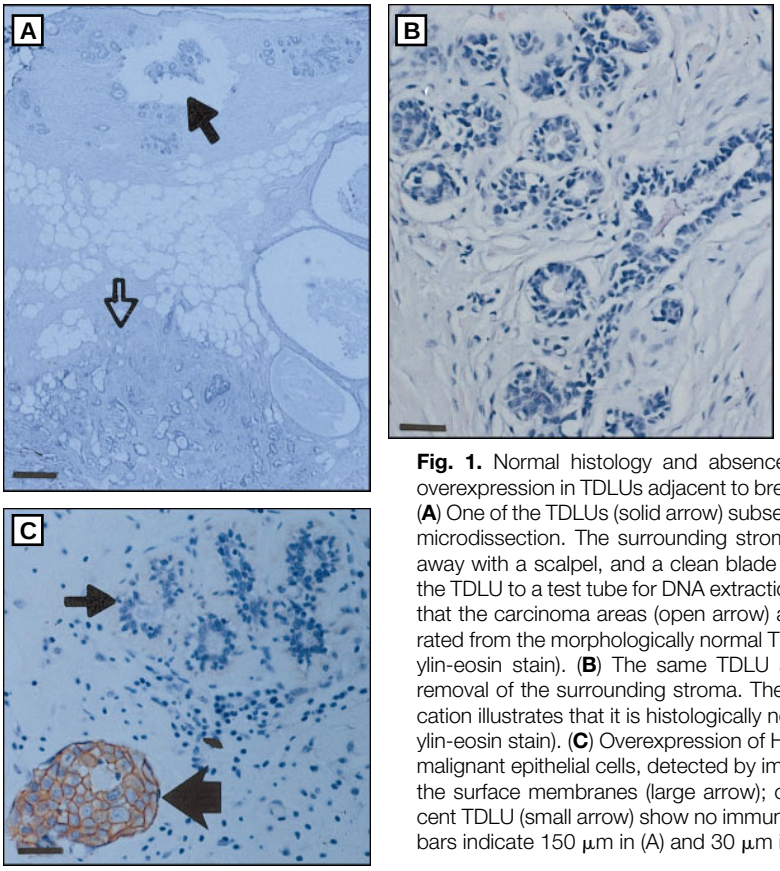


Fig. 1. Normal histology and absence of HER-2/neu overexpression in TDLUs adjacent to breast carcinomas. (**A**) One of the TDLUs (solid arrow) subsequently used for microdissection. The surrounding stroma was scraped away with a scalpel, and a clean blade used to remove the TDLU to a test tube for DNA extraction (*11, 14*). Note that the carcinoma areas (open arrow) are clearly separated from the morphologically normal TDLUs (hematoxylin-eosin stain). (**B**) The same TDLU as in (A) before removal of the surrounding stroma. The higher magnification illustrates that it is histologically normal (hematoxylin-eosin stain). (**C**) Overexpression of HER-2/neu in the malignant epithelial cells, detected by immunostaining of the surface membranes (large arrow); cells in the adjacent TDLU (small arrow) show no immunostaining. Scale bars indicate 150 μm in (A) and 30 μm in (B) and (C).

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degree of differentiation, tumor size, or proliferative fraction, although the number of cases may have been too small to detect minor differences.

We performed several control experiments to show that the polymerase chain reaction (PCR) technique was reproducible and that the normal TDLUs chosen for dissection were free of contaminating cancer cells. To evaluate reproducibility of the assay, we repeated the LOH assays two to five times using the same DNA preparations (documented for cases H12 and H21 in Table 2). Although in some cases, there were variations in the relative intensity of the two bands, in each repeat assay the same allele was lost. If the LOH had been due to random artifacts of assay methodology, one would expect to see either allele lost in repeat experiments. Because only a small amount of DNA was available from the microdissected samples, we used 45 PCR cycles to amplify the DNA before electrophoresis. Skewing of microsatellite markers in favor of the smaller allele was controlled for, as we always expressed allele density relative to that for normal skin from the same person. Furthermore, we showed that the density ratios of the upper to lower alleles were the same after 30 and 45 cycles using microsatellite probe D3S1244 with one tumor DNA sample. For the eight cases reported (Table 1), the lower allele was lost in 6 of 15 assays showing LOH with microsatellite probes, a result consistent with random loss of the upper or lower allele. To investigate possible artifacts related to DNA preparation, we evaluated case H21 by dissecting four individual TDLUs and extracting the DNA separately. Again, in each adjacent TDLU, the same allele was lost (Table 2 and Fig. 2A).

Contamination of the adjacent TDLUs with cancer cells was excluded by two experiments. First, the TDLUs used for each microdissection appeared morphologically normal by the following criteria: They contained clusters of small ductules composed of a single myoepithelial basal layer and a single cuboidal luminal layer with clear and prominent lumina, and the cells had uniform, small nuclei with diffusely distributed chromatin (Fig. 1B). Second, for seven of the eight cases, we demonstrated that the cancer cells contained additional molecular aberrations not detected in the adjacent normal TDLUs (Table 1). These aberrations included LOHs at other chromosomal loci and abnormal immunopositivity for the tumor suppressor protein p53 or the oncoprotein, HER-2/neu. For example, DNA from the normal TDLUs of cases H12, H21, H37, and H40 showed LOH at 3p24 but not at 17p13.1 even though the carcinomas showed LOH at both loci (Table 1). DNA from the normal TDLUs of case H5 showed LOH at 11p15.5 and case H6 showed LOH at 17p13.1; both cases showed LOH at 13q13 in the carcinomas but not in adjacent TDLUs (Table 1). If tumor DNA contaminated the normal material, one would expect the same LOH profile in the normal and tumor material. Additionally, in cases where the tumor components contained cells immunopositive for HER-2/neu (cases H22, H37, and H40) or p53 (cases H12 and H40), there were no immunopositive cells in the adjacent normal TDLUs (Fig. 1C and Table 1).

To determine whether the LOH at 3p was present in all of the normal mammary epithelium or only in the normal TDLUs adjacent to the carcinoma, we evaluated mastectomy tissue (available from four of the six cases) showing LOH at 3p in ad-

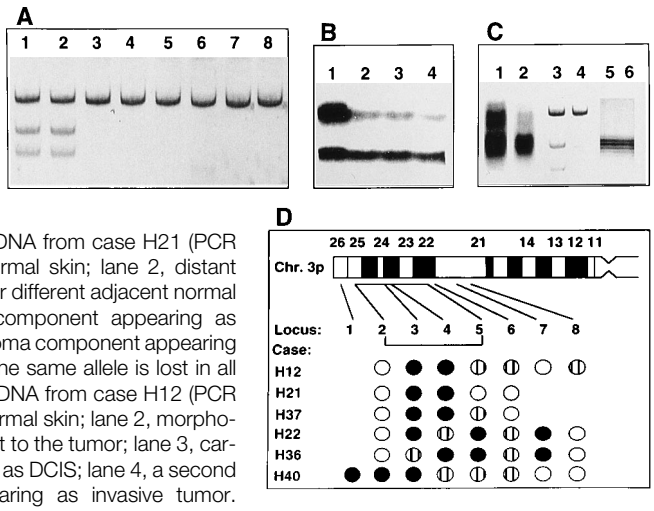


Fig. 2. LOH at different loci on chromosome 3p in breast cancer and adjacent normal lobules. The density of each allele was determined and the relative densities of the two alleles in each lane were compared to their relative densities in skin (*14, 15*). (**A**) LOH at 3p24 of DNA from case H21 (PCR primers EABMD). Lane 1, normal skin; lane 2, distant normal TDLU; lanes 3 to 6, four different adjacent normal TDLUs; lane 7, carcinoma component appearing as DCIS; lane 8, a second carcinoma component appearing as invasive tumor. Note that the same allele is lost in all samples. (**B**) LOH at 3p24 of DNA from case H12 (PCR primers D3S1244). Lane 1, normal skin; lane 2, morphologically normal TDLU adjacent to the tumor; lane 3, carcinoma component appearing as DCIS; lane 4, a second carcinoma component appearing as invasive tumor. Note that the same allele is lost in all samples. (**C**) LOH at various loci on chromosome 3p in morphologically normal TDLUs from case H40. Lanes 1 and 2, chromosomal locus 3p25 (PCR primers D3S1597); lanes 3 and 4, chromosomal locus 3p24 (PCR primers EABMD); lanes 5 and 6, chromosomal locus 3p21 (PCR primers D3S1573; for this locus, the two alleles are separated by only 2 base pairs, but are readily distinguished on the film). Note that LOH is detectable in adjacent normal TDLU at 3p25 and 3p24, but not at 3p21. Lanes 1, 3, and 5 contain normal skin samples; lanes 2, 4, and 6 contain samples from morphologically normal TDLUs adjacent to the tumor. (**D**) LOH was measured at various loci on chromosome 3p and the results summarized for each case where the adjacent normal TDLUs had LOH at chromosome 3p. The loci tested (numbered 1 to 8 in the figure) are: 1, 3p26 (D3S2397); 2, 3p25 (D3S1597); 3, 3p24 (EABH or EABMD); 4, 3p24 (D3S1244); 5, 3p22 (D3S1612); 6, 3p22 (D3S1582); 7, 3p21 (D3S1573); and 8, 3p21 (D3S1295). The common region of LOH falls within the region 3p25 to 3p22 as indicated by the solid line. Black spheres indicate LOH present; open spheres indicate no LOH; striped spheres indicate not informative.

Table 1. Analysis of breast cancers and adjacent normal TDLUs for LOH and for p53 and HER-2/neu immunopositivity. The samples were a sequential series of infiltrating ductal carcinoma from mastectomies in which the carcinomatous elements contained at least 10% ductal carcinoma in situ (DCIS) and invasive cancer in the same tissue section (*14*). N, normal TDLU adjacent to carcinoma; C, cancer; +, LOH; −, no LOH; NI, not informative; NT, not tested; *Fractions are the number of cases with LOH over the number of informative cases; †Indicated range reflects the variability seen in different areas of the malignant component. HER-2/neu was more heterogeneous than p53 in its immunostaining pattern; the DCIS components showed more staining than the invasive components.

Case	Loss of heterozygosity								Immunopositive (%)			
	3p24		11p15.5		13q13		17p13.1		p53		HER-2/neu	
	N 6/17*	C 10/21	N 1/16	C 5/17	N 0/11	C 10/14	N 1/19	C 16/20	N	C	N	C
H12	+	+	−	+	NI	NI	−	+	0	1	0	0
H21	+	+	NI	NI	NI	NI	−	+	0	0	0	0
H22	+	+	−	−	NT	+	NI	NI	0	0	0	30–60†
H36	+	+	−	−	NI	NI	NI	NI	0	0	0	0
H37	+	+	NI	NI	NT	+	−	+	0	0	0	5–10
H40	+	+	−	−	−	−	−	+	0	10	0	30–70
H5	NI	NI	+	+	−	+	NI	NI	0	0	0	0
H6	NI	NI	−	−	−	+	+	+	0	0	0	0

jacent normal TDLUs. In all four cases, TDLUs taken from blocks distant to the carcinomas showed no LOH (Table 2). Thus the 3p LOH was present only in a localized region of normal epithelium near the carcinoma rather than in the entire mammary tree.

To evaluate the extent of the LOH, we characterized each case with LOH at 3p24 in the normal TDLUs for LOH at additional loci on 3p. The size of the chromosomal region showing LOH in the morphologically normal TDLUs differed among the cases. For example, case H40 showed LOH at 3p26 (Fig. 2D), 3p25, and 3p24 (Fig. 2C) but not at 3p21 (Fig. 2, C and D). In contrast, cases H12 and H21 both showed LOH at 3p24 (Fig. 2, A and B), but not at 3p25 (Fig. 2D). However, all cases shared a region of LOH within 3p25 (locus D3S1597) to 3p22 (locus D3S1612) (Fig. 2D).

In summary, we have shown that the normal mammary TDLUs near a breast cancer contain genetic aberrations that have previously been seen only in hyperplastic (*5*), premalignant (*5–7*), and malignant breast epithelium (*3, 4*) as well as colonic adenomas (*2*), Barrett's esophageal metaplasia (*8, 9*), and lung hyperplasias (*10*). The presence of the LOH only in TDLUs near the cancer suggests that the entire mammary gland is not affected. Because regions with LOH are thought to contain recessive genes relevant to malignancy, LOH in the normal adjacent lobules may define a localized predisposed region from which the cancer arises. Conceivably, this predisposed region may be present prior to mammary gland differentiation, as single stem cells form localized regions of the mammary gland (*11*). Epidemiologic evidence supports the notion that breast cancer initiation can occur prior to mammary gland differentiation: for example, breast cancer risk was found to be high in women who were in the first decade of life at the time of exposure to atomic bomb irradiation and in women who had undergone thymus irradiation in infancy (*12, 13*).

If LOH in normal lobules defines a localized region of increased risk, its presence may be clinically important. Because the adjacent mammary epithelium is not completely removed during lumpectomy for invasive carcinoma or ductal carcinoma in situ (DCIS), remaining cells with the LOH may subsequently progress to form another carcinoma. Thus, additional studies should be undertaken to determine if patients with LOH in their normal TDLUs are more likely to have a tumor recurrence than patients whose normal TDLUs are not genetically aberrant. If there is a correlation, analysis of the adjacent normal TDLUs might help to identify patients who would benefit from more aggressive local therapy or who should be counseled about their higher risk for local failure.

Table 2. LOH at 3p24 in normal mammary TDLUs adjacent to carcinoma.

Case	Density of upper allele: density of lower allele*	
	DNA from distant normal TDLUs	DNA from adjacent normal TDLUs
H12		
Pooled TDLUs	1.0 : 0.95	0.51 : 1.0
Pooled TDLUs	NT	0.31 : 1.0
Pooled TDLUs	NT	0.42 : 1.0
Pooled TDLUs	NT	0.13 : 1.0
H21		
Pooled TDLUs	1.0 : 0.92	1.0 : 0.03
Pooled TDLUs	NT	1.0 : 0.05
TDLU 1	NT	1.0 : 0.05
TDLU 2	NT	1.0 : 0.1
TDLU 3	NT	1.0 : 0.05
TDLU 4	NT	1.0 : 0.05
H22	1.0 : 0.97	1.0 : 0.56
H37	1.0 : 1.0	0.25 : 1.0

*Values were calculated as in Fig. 2. NT, not tested.

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14. Formalin-fixed, paraffin-embedded histologic sections stained with hematoxylin-eosin were reviewed to identify slides containing both carcinoma and morphologically normal TDLUs on the same section. Selected areas of sections from 30 such cases were microdissected using a light microscope (*11*). A similarly stained section of normal breast skin from the same patient was microdissected as a control. Adjacent 4-μm sections were immunostained for p53 and HER-2/neu as in H. B. Muss *et al*. [*N. Engl. J. Med.* **330**, 1260 (1994)]. Microdissected tissue was extracted with 100 μl of xylene, washed with 100 μl of 95% ethanol, and the DNA extracted (*11*). The DNA (4 μl) was amplified by PCR in 50 μl of 10 mM tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 200 μM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphospate (dTTP), 0.1 μM upstream and downstream primers, 1% dimethyl sulfoxide (DMSO), 0.44 μg *Thermus aquaticus* (Taq) antibody (TaqStart antibody, Clontech), and 2 units of Taq DNA polymerase C [D. K. Wright and M. M. Manos, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innes, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA, 1990), pp. 153–158]. The PCR reaction consisted of 45 cycles of denaturing (94°C for 30 s), annealing (56 to 66°C for 30 sec.), and chain extension (72°C for 30 s). The PCR products were separated by electrophoresis [G. Deng *et al*., *Cancer Res.* **54**, 499 (1994)]. Each allele of the PCR product was measured by densitometry of x-ray film exposed to the gel or of a negative photographic film of the ethidium bromide–stained gel. The density ratios of the two alleles (the undigested allele: the digested allele, or the top allele: the bottom allele) for different tissues were calculated. The density of each allele was determined (using a Computing Densitometer by Molecular Dynamics, Model 300A) and the relative densities of the two alleles in each lane were compared to their relative densities in skin. Samples were scored as positive for LOH if the calculated value was <0.70 according to the following equation: ‖density of weaker allele of sample : density of stronger allele of sample/density of weaker allele of skin : density of stronger allele of skin‖. This calculated ratio for each lane in Fig. 2 relative to skin (lane 1) is as follows: Fig. 2A, lane 2, 0.95; lanes 3 to 8, <0.10; Fig. 2B, lane 2, 0.31; lane 3, 0.29; lane 4, 0.37; Fig. 2C, lane 2, 0.15; lane 4, 0.25; lane 6, 0.93.
15. The primer pairs included restriction fragment length polymorphisms (RFLPs), C-A repeat, and tetranucleotide microsatellite polymorphic loci. The sequences of the PCR primers for LOH analysis are as follows (in each case, the forward primer is followed by the reverse primer): 3p26 (*16*) D3S2397: ATAGAGCCACACTTTGTCTCA: TCTTTGAGAACCACTGTCTCC; 3p25 (*16*) D3S1597: AGTACAAATACACACAAATGTCTC: GCAAATCGTTCATTGCT; 3p24 (*17*) EABH: CATCTGAAATGCTGACCTGTT: AGCTGTCAGAACTAAGTGCTT; 3p24 (*18*) EABMD: AACGTTGGACCTCAAGCCCAT: AGAATGCCAAGGAAGGGTGCA; 3p24 (*19*) D3S1244: GTGCCCTTCCAGGAGTT:AGTGAGGCATCCACTACC; 3p22 (*16*) D3S1612: TCTTTTAGTCAGCAGTTATGTC: CCCATTAAGAAATGTTACTCTAC; 3p22 (*16*) D3S1582: AGCAGGTACTATGAAAGCCTGT: GGAACAGCCCAT G GTTCAC; 3p21 (*16*) D3S1573: TTCATTTTTGCTTATTAAGATATGC: CCAGTAAATNACAGGGCTAT; 3p21 (*16*) D3S1295: TGTAGTAATGGTTTCATGGATACAC: ATTTTATAAGTTTTGATACCCTCCC; 11p15.5 (*20*) TH2:CAGCTGCCCTAGTCAGCAC: GCTTCCGAGTGCAGGTCACA; 13q13 (*21*) D13S218: GATTTGAAAATGAGCAGTCC: GTCGGGCACTACGTTATCT; 13q13 (*21*) D13S219: AAGCAAATATGCAAAATTGC: TCCTTCTGTTTCTGACTTAACA; 17p13.1 (*22*) TP53.5: CAATGGATGATTTGATGCTG: TGGTAGGTTTTCTGGGAAGG; 17p13.1 (*23*) TP53.6: AGGTCTGGTTTGCAACTGGG: GAGGTCAAATAAGCAGCAGG; 17p13.1 (*24*) TP53.8:T: CAGAAGGAAGTAGGAAGGACTCAG: GAAGAGCCTCGGTTATGGGTATACA.
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25. Supported by National Cancer Institute SPORE grant 2P50CA58207.

31 May 1996; accepted 18 November 1996