Jpn. J. Cancer Res. **91**, 293–300, March 2000

Mapping of Target Regions of Allelic Loss in Primary Breast Cancers to 1-cM Intervals on Genomic Contigs at 6q21 and 6q25.3

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Allelic losses on the long arm of human chromosome 6 are frequently observed in cancers of the ovary, prostate, and breast. To identify the locations of putative tumor suppressor genes on 6q, we examined 192 primary breast cancers for patterns of allelic loss at 16 polymorphic microsatellite loci distributed along this chromosome arm. Allelic losses at one or more loci were observed in 105 (55%) of the tumors examined. Detailed deletion mapping with appropriate yeast artificial chromosome (YAC) contigs identified two distinct commonly deleted regions; one was confined to a 1-cM interval at 6q21 flanked by D6S1040 and D6S262 and the other to a 1-cM interval at 6q25.3 flanked by D6S305 and D6S411. Allelic losses at 6q21 were more frequent in invasive solid tubular and scirrhous carcinomas than in tumors of less aggressive histologic types (*P*=0.0006). Allelic loss at 6q25.3 was associated with loss of progesterone receptor (*P*=0.0256). Our results suggest the presence of two tumor suppressor genes for breast cancer on 6q that are likely to be associated with tumor progression and/or loss of hormonal dependency.

Key words: Breast cancer — Loss of heterozygosity — Tumor suppressor gene — Chromosome 6 — Yeast artificial chromosome (YAC)

Breast cancer is the most common malignancy in women. One of nine Caucasian women and one of 40 Japanese women will develop breast cancer in their lifetimes, and the incidence has been increasing worldwide. Solid tumors in humans are now believed to develop through a multi-step process involving activation of oncogenes and inactivation of tumor suppressor genes.[1] Many tumor suppressor genes are inactivated when an intragenic mutation occurs in one allele and a chromosomal region containing the other allele is lost through mechanisms such as aberrant mitotic events. The latter process can be recognized by loss of heterozygosity (LOH) at that locus in tumor cells. In many primary breast cancers LOH has been reported on chromosomal arms 1p, 3p, 8p, 11q, 13q, 16q, 17p, 17q, 18q and 22q.[2–13] Putative tumor suppressor genes are postulated as targets of those cancer-associated events.

We and others have observed frequent LOH involving loci on chromosome 6q in acute lymphocytic leukemia[14,15] and malignant melanoma,[16] as well as in carcinomas of the ovary,[17–19] stomach,[20] prostate,[21] and breast.[22–27] The results of these various molecular genetic studies have indicated that alterations of 6q are important for the development and/or progression of several types of cancer, and that one or more putative tumor suppressor genes lie on this chromosome arm. In the study reported here, we undertook to construct a detailed deletion map of 6q in 192 breast cancers by taking advantage of a high-resolution chromosomal map of the region consisting of 16 loci, and by constructing a physical map based on yeast artificial chromosome (YAC) contigs. In addition, we looked for evidence of correlation between allelic losses on 6q and clinicopathological parameters.

MATERIALS AND METHODS

Samples and DNA preparation Tumors and corresponding non-cancerous tissues obtained from 192 women undergoing surgery for primary breast cancer were excised, frozen immediately, and stored at −80°C. Genomic DNAs were extracted from the frozen materials according to methods described previously.[7] None of the patients had undergone previous radiotherapy or chemotherapy.

LOH analysis LOH was assessed using 16 polymorphic microsatellite markers along the entire length of chromosome 6q: (centromere)–D6S1053–D6S1031–D6S1056–D6S1021–D6S474–D6S1705–D6S1040–D6S262–D6S1009–GATA184–D6S2436–D6S1581–D6S305–D6S411–D6S1277–D6S1027–(telomere). All primer sequences and their locations were obtained from the CEPH/Genethon linkage

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maps.[28, 29] Microsatellite polymorphisms were amplified by the polymerase chain reaction (PCR) using 10 ng of genomic DNA, 30 m*M* Tris HCl (pH 8.8), 50 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 100 μM dNTPs, 1.6 pmol each of [γ-^{32}P]ATP-end-labeled primer and non-labeled primer, and 0.25 units of *Taq* polymerase in a total volume of 10 μl. Cycling took place in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT), using 10 ng of template DNA in volumes of 10 μl, in 30 cycles at 94°C for 30 s, 52–60°C for 30 s, 72°C for 30 s, and final extension for 10 min at 72°C. PCR products were electrophoresed in 0.3 mm-thick denaturing 6% polyacrylamide gels containing 36% formamide and 8 *M* urea, at 2000 volts for 2–6 h. Gels were transferred to filter papers, which were dried at 80°C and exposed to autoradiographic film at room temperature for 16–48 h.

Definition of LOH Signal intensities of polymorphic alleles were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration using a GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA). The signal intensities of alleles of tumor-tissue DNAs were compared to those of corresponding normal-tissue DNAs. We judged a reduction in signal intensity >50% to be allelic loss, after normalizing each signal to the signal obtained when the same DNA samples were analyzed with markers for loci on other chromosomes.

Preparation of YAC DNA On the basis of information available in the Genome Data Base, we selected nine CEPH YACs (804B5, 958H3, 960H8, 810D7, 934A10, 932F1, 921H3, 956B1, 956F6) and obtained these YAC clones from Genome SystemsTM (St. Louis, MO). Total YAC DNA from each clone was purified according to the protocol supplied by the manufacturer. We mapped each YAC clone by PCR analysis using microsatellite markers D6S1040 and D6S262 at 6q21 or D6S305 and D6S411 at 6q25.3. PCR experiments were performed in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus) using 10 ng of YAC DNA in a volume of 10 μl, with 30 cycles of 94°C for 30 s, 55–60°C for 30 s, and 72°C for 30 s. A 10-μl aliquot of each product was electrophoresed in a 2% agarose gel.

Clinicopathological parameters Tumors were classified by pathologists according to the histologic TNM classification and the histologic typing scheme of the Japanese Breast Cancer Society[30] into the following types: noninvasive ductal (1a), invasive papillotubular (a1), invasive solid-tubular (a2), invasive scirrhous carcinoma (a3), and other specific types (b group). This classification is essentially the same as the World Health Organization scheme for typing breast tumors. Subtypes of the seven DCIS tumors were as follows; four comedo type, one cribriform type, one papillary type, and one Paget disease. Bloom and Richardson's histological grading had not been carried out in our routine pathological examination, thus, it was not available for the present panel of patients. Estrogen receptor (ER) and progesterone receptor (PgR) were measured by radioreceptor assay according to a standard dextran-coated charcoal method, using [^{125}I]estradiol as the labeled ligand and homogenates of fresh-frozen tissue (Otsuka Pharmaceutical, Tokushima). All samples containing >5 fmol/mg ER or >10 fmol/mg PgR protein were considered receptor-positive. The χ^2 test and Fisher's exact test were used for statistical analysis of the results. *P* values of <0.05 were considered statistically significant.

RESULTS

LOH was detected in 105 (55%) of the 192 breast cancers analyzed with 16 polymorphic markers on the long arm of chromosome 6. The marker loci and their frequencies of LOH are listed in Table I in descending order from the centromere to the telomere. The highest frequency of LOH (46%) was detected with D6S305 at 6q25.3. Among the 105 tumors with LOH, 63 had lost alleles at all informative loci; the other 42 showed partial or interstitial deletions, which could be further classified into three groups on the basis of their patterns of LOH. The results of LOH analysis in the 42 tumors with partial or interstitial deletions are summarized as a deletion map in Fig. 1. Fourteen tumors exhibited LOH in a small portion of the q21 region; 17 tumors showed LOH in a more distal portion involving q25.3; and both of these regions were deleted in the other 11 tumors.

Table I. Frequencies of Loss of Heterozygosity Detected at 16 Polymorphic Loci on Chromosome 6q in 192 Breast Cancers

Locus	Total	LOH/informative (%)
D6S1053	144	18/87 (20.7)
D6S1031	144	17/69 (24.6)
D6S1056	144	26/90 (28.9)
D6S1021	144	10/36 (27.8)
D6S474	192	38/117 (32.5)
D6S1705	192	33/112 (29.5)
D6S1040	192	35/93 (37.6)
D6S262	192	35/95 (36.8)
D6S1009	192	33/96 (34.4)
GATA184A08	192	44/120 (36.7)
D6S2436	192	43/117 (36.8)
D6S1581	192	25/63 (39.7)
D6S305	192	53/116 (45.7)
D6S411	192	29/76 (38.2)
D6S1277	192	23/110 (20.9)
D6S1027	192	34/95 (35.8)
Total	192	105/192 (54.7)

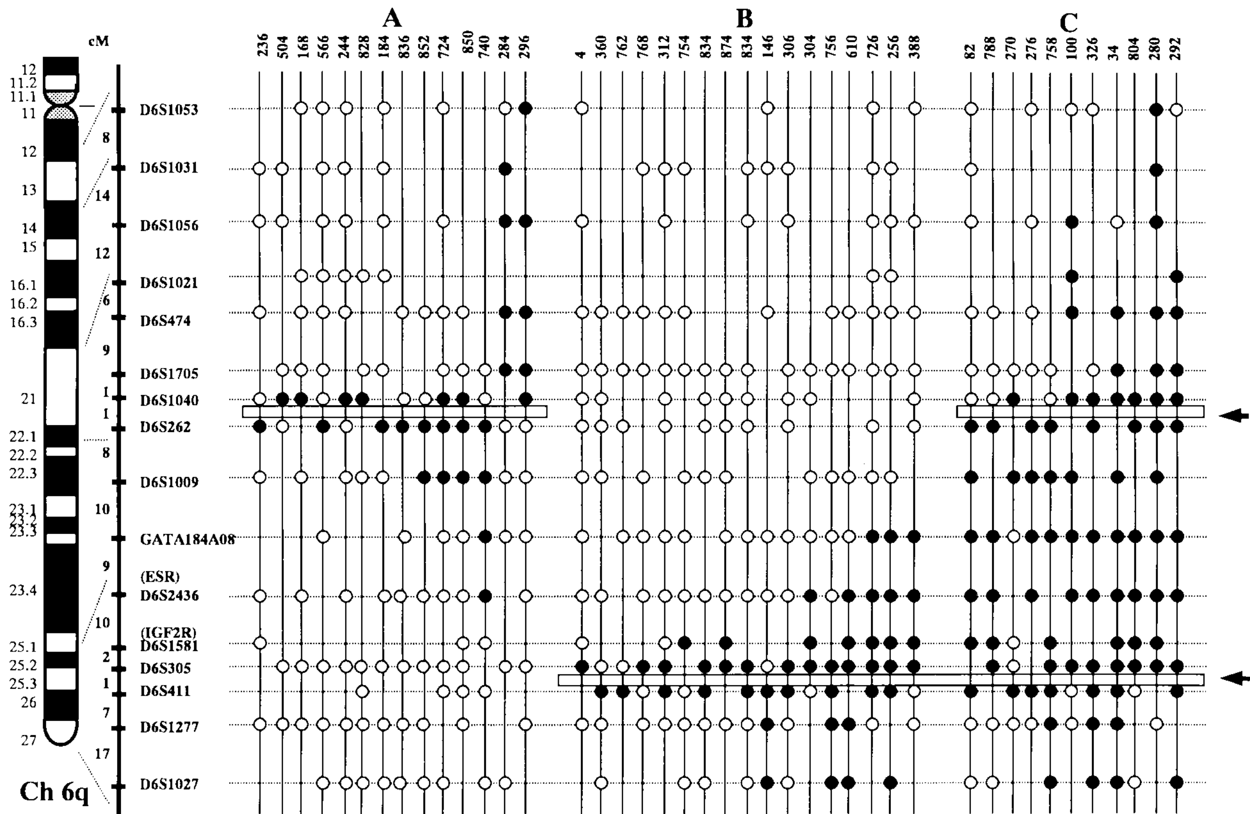

Fig. 1. Schematic representation of deletion mapping in 42 breast cancers with partial or interstitial deletions on 6q. Allelic losses were found in a proximal restricted region in 14 tumors (A), at a more distal region in 17 tumors (B) and at both regions in 11 tumors (C). Tumor identification numbers are at the top of each column. Solid and unfilled circles indicate loss and retention of heterozygosity, respectively. Gaps reflect uninformativeness for specific markers. Commonly deleted regions are shown as rectangles and indicated with arrows.

Fig. 2 illustrates two tumors that exhibited partial deletions around 6q21. Tumor 236 showed LOH at D6S262 but retention of alleles at D6S1040 and D6S1009. Tumor 504 showed LOH at D6S1040 but retention of alleles at D6S1705 and D6S262. The proximal limit of the common deletion at 6q21 was defined by D6S1040, on the basis of observations in eight tumors (236, 566, 836, 852, 740, 82, 788, 758) that retained heterozygosity at the D6S1040 locus while showing LOH at more distal D6S262 locus. The distal limit was defined by D6S262; four tumors (504, 244, 284, 296) retained heterozygosity at D6S262 while showing LOH at more proximal D6S1040. To map the physical interval more precisely, we constructed a YAC contig across this restricted region. On the basis of the contig shown in Fig. 2A, we established that the common region of deletion flanked by D6S1040 and D6S262 at 6q21 was a 1-cM interval on two overlapped YACs.

Fig. 3 illustrates three tumors that exhibited partial deletions around 6q25.3. Tumor 768 showed LOH at D6S305 but retention of alleles at D6S2436 and D6S411. Tumor 312 showed LOH at D6S305 and D6S411 but retention of alleles at D6S1581 and D6S1277. Tumor 360 showed LOH at D6S411 but retention of alleles at D6S305 and D6S1277. The proximal limit of common deletion at 6q25.3 was then defined by D6S305, on the basis of observations in four tumors (360, 762, 146, 270) that retained heterozygosity at the D6S305 locus while showing LOH at the more distal D6S411 locus. The distal limit was defined by D6S411; six tumors (768, 754, 304, 388, 100, 804) retained heterozygosity at D6S411 while showing LOH at more proximal D6S305. The YAC contig that we constructed across this commonly deleted region (Fig. 3A) allowed us to define its physical extent, flanked by D6S305 and D6S411 at 6q25.3, as less than 1.5 Mb on a single YAC.

We investigated potential relationships between LOH at 6q21 and 6q25.3 and clinicopathological parameters including tumor size, lymph node metastasis, menopausal status, ER status, PgR status, and histologic type (Table II). LOH at 6q21 was more frequent in tumors of the inva-

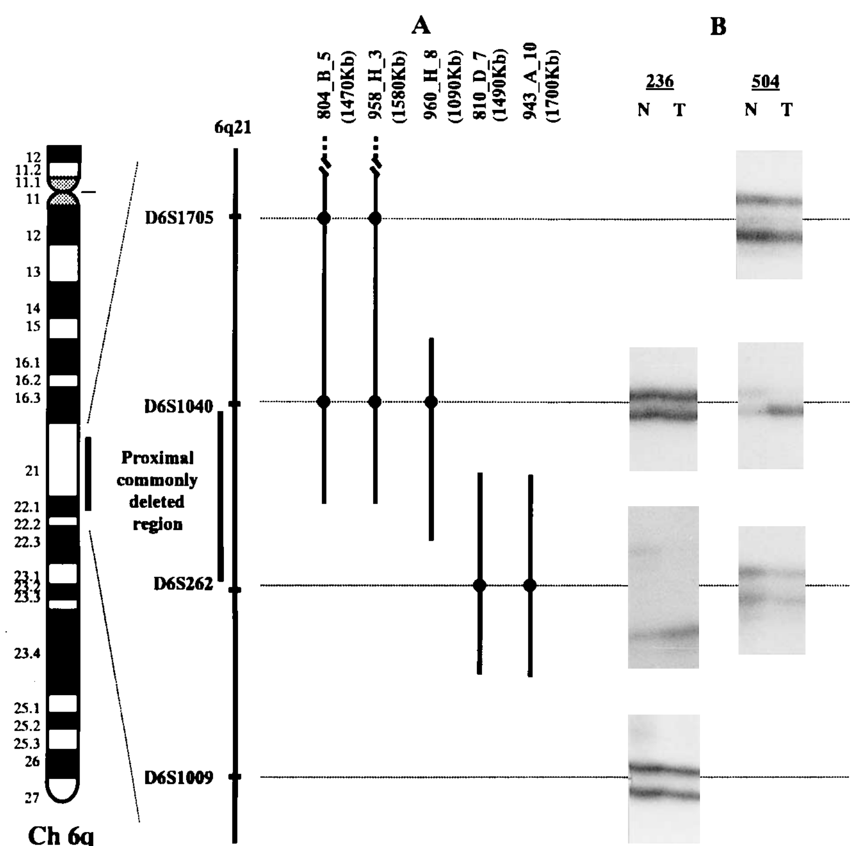

Fig. 2. A: Schematic representation of five contiguous YACs spanning the commonly deleted region on 6q21. The clone name and size of each YAC appear above the schema. Each clone contained one or more of the relevant microsatellites (closed circles). B: Representative autoradiograms used for determining the commonly deleted region at 6q21. N and T, matched DNA samples isolated from normal and tumor tissues, respectively. Tumor 236 retained heterozygosity at D6S1040 and D6S1009, but showed LOH at D6S262; tumor 504 retained heterozygosity at D6S1705 and D6S262 but showed LOH at D6S1040.

sive solid tubular and scirrhous types (43 of 82; 52%) than in less aggressive types (4 of 27; 15%) (P=0.0006). With respect to hormone-receptor status, we detected an association between LOH at 6q25.3 and PgR; PgR-negative status, defined as a PgR level <10 fmol/mg protein, was more frequent in tumors that had lost heterozygosity at 6q25.3 (23 of 59; 39%) than in tumors that had retained both alleles of this locus (16 of 75; 21%) (P=0.0256). ER status showed no significant correlation with LOH at 6q25.3; tumor size and lymph node metastasis had no significant associations with LOH at either 6q21 or 6q25.3.

DISCUSSION

Observations of frequent LOH at several chromosomal locations in breast cancers have suggested that multiple tumor suppressor genes may play carcinogenic roles in mammary tissue.[2–13] Through analysis of patterns of LOH involving partial and interstitial deletions, we identified commonly deleted regions, each a 1-cM interval, on YAC-contig maps of 6q21 and 6q25.3. Thus, the present study has defined two new chromosomal locations as candidates harboring putative suppressor genes for breast cancer. We can conclude that the frequency of LOH (55%) we observed on 6q reflects non-random genetic alterations associated with breast carcinogenesis.

Deletions in the 1-cM interval between D6S1040 and D6S262 at 6q21 were associated with tumors of aggressive histologic types. In previous LOH studies, deletions at 6q21 were found in a variety of neoplasms including acute lymphoblastic leukemia,[14, 15] prostate cancer[21] and breast cancer.[25–27] The region in question was usually defined by D6S287–D6S407, centromeric of the deleted region reported here. However, detection of common deletions on the same band in multiple types of malignancy might reflect inactivation of common tumor suppressor genes.

The deletion region we defined at 6q25.3 in the present study lies within a 1.5-Mb interval between D6S305 and

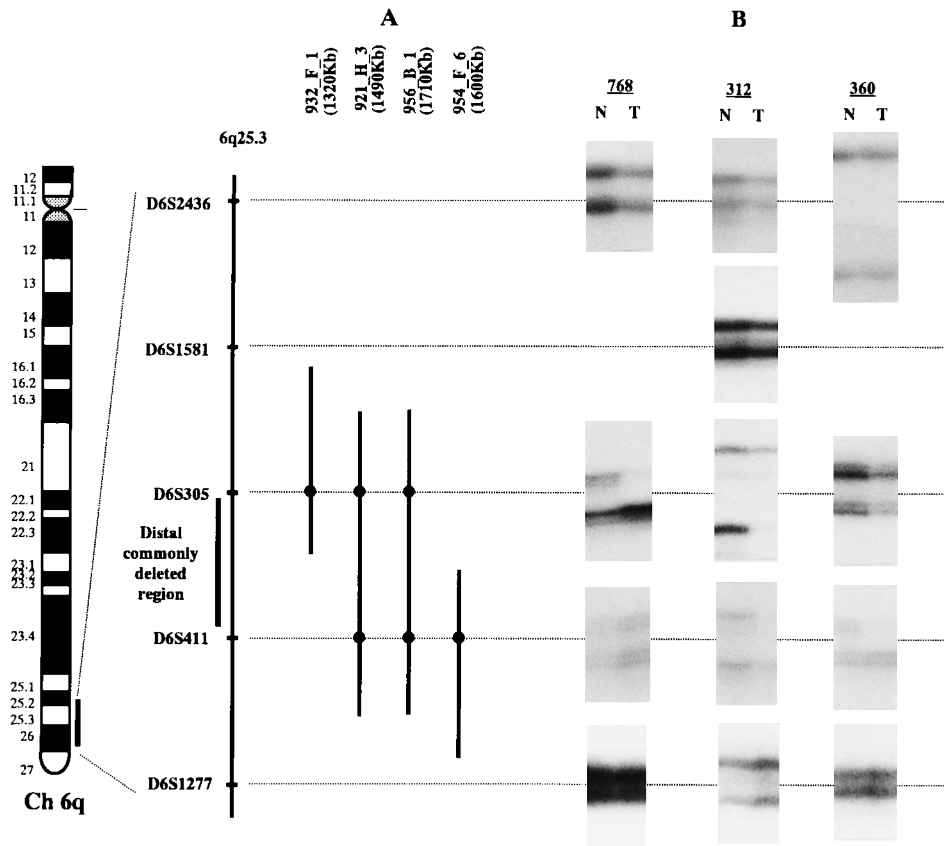

Fig. 3. A: Schematic representation of the YAC contig spanning the commonly deleted region on 6q25.3. B: Representative autoradiograms used to determine the distal commonly deleted region at 6q25.3. Tumor 768 retained heterozygosity at D6S2436 and D6S411, but showed LOH at D6S305. Tumor 312 retained heterozygosity at D6S1581 and D6S1277 but showed LOH at D6S305 and D6S411. Tumor 360 retained heterozygosity at D6S305 and D6S1277, but showed LOH at D6S411.

D6S411. Cytogenetic abnormalities and allelic losses involving the distal part of 6q have already been described in breast cancers. For example, comparative genomic hybridization (CGH) analyses carried out by Tirkkonen *et al.*[31] detected frequent losses at 6q22-qter in breast cancers, and Devilee *et al.*[22] reported frequent allelic losses at two markers on 6q (D6S37 at 6q26-q27 and the MYB locus at 6q23.3-q24). Orphanos *et al.*[23] detected LOH at 6q13, 6q16.3-q21, and a region at 6q25.2-q27 defined by markers D6S220 and D6S193. Fujii *et al.*[24] described a commonly deleted region encompassing band 6q23 to q25.2, within a 12-cM interval flanked by D6S310 and D6S255, which lies centromeric to the common region described here. Noviello *et al.*[25] detected frequent LOH between markers D6S411 and D6S281; that segment partially overlaps with the region we have described at 6q25.3. Chappell *et al.*[32] detected frequent LOH with markers located in bands 6q25.1-q27 in breast cancers.

In other types of tumor, De Souza *et al.*[33] described frequent LOH at 6q26-27 in hepatocellular carcinomas; Saito *et al.*[17] defined a commonly deleted region in ovarian cancers to a 0.3-Mb region of 6q27; and Colitti *et al.*[19] recently described a commonly deleted region at 6q25.1-25.2 in ovarian cancers. Taking advantage of a panel of microsatellite markers localized on a YAC contig map, we were able to define a region that is commonly deleted in breast cancers to a 1.5-Mb interval on a single YAC lying at 6q25.3. Although clarification of a precise positional relationship among the variously reported regions of deletion awaits further refinement of genetic and physical maps of distal 6q, the combined results indicate that at least one tumor suppressor gene is present on distal 6q and raise the possibility that a single gene may be involved in tumors originating from breast and some other tissues.

Several genes important in cellular growth and regulation are present on distal 6q; among them are the mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) gene and the estrogen receptor (*ESR*) gene. The *M6P/IGF2R* gene, located on chromosome 6q26-27,[34] is required for activation of transforming growth factor-β.[35]

Table II. Loss of Heterozygosity on 6q and Clinicopathological Factors

	At q21		At q25.3	
	LOH/informative cases (%)	Statistical significance	LOH/informative cases (%)	Statistical significance
Tumor size				
T1	9/17 (52)		11/22 (50)	
T2	38/96 (40)	NS	40/97 (41)	NS
T3	6/9 (67)		8/15 (53)	
Lymph node metastasis				
negative	24/62 (39)	NS	25/64 (39)	NS
positive	29/60 (48)		34/70 (49)	
Menopausal status				
pre.	31/67 (46)	NS	31/74 (42)	NS
post.	22/55 (40)		28/60 (47)	
ER status				
negative	19/47 (40)	NS	26/55 (47)	NS
positive	34/74 (46)		33/79 (42)	
PgR status				
negative	16/30 (53)	NS	23/39 (59)	P=0.0256
positive	37/91 (41)		36/95 (38)	
Histological type[a]				
1a	2/7 4/27 (15)		1/7 (14)	
a1	2/20	P=0.0006[b]	9/25 (36)	NS
a2	17/37 43/82 (52)		22/43 (51)	
a3	26/45		20/42 (48)	

a) 1a, noninvasive ductal carcinoma; a1, papillotubular carcinoma; a2, solid tubular carcinoma; a3, scirrhous carcinoma.
b) 1a, a1 vs. a2, a3 (P=0.0006).

This gene is associated with a 70% frequency of LOH, and a 25% incidence of point mutations in the remaining allele, in hepatocellular carcinomas[33]; in view of its location, this gene would be a candidate for involvement in breast cancer as well. Although mutations within the *IGF2R* gene are seldom found in breast cancers (only two of 62 tumors in a study reported by Hankins *et al*.[36]), fully a third of breast cancers appear to have lost heterozygosity at this locus.

We did not detect a significant association between LOH on 6q regions and loss of estrogen receptor in the present study. We have previously observed significant correlations between loss of estrogen receptor and LOH of some specific chromosomes, such as 3p, 11p, 13q, and 17q,[5, 6, 8, 11] which justify the conclusion that measurement of estrogen receptor by standard radioreceptor assay used in the present study was no less sensitive than other methods. In accord with our finding, Iwase *et al*. studied the relationship between LOH at *ESR* gene and estrogen receptor status measured by enzyme immunoassay, and found no association between them.[37] Although the estrogen receptor plays a central role in hormonal control of cellular proliferation in normal breast epithelium, no predisposing mutation of *ESR* gene, either germline or somatic, has been described to date in breast cancers[22, 37] or ovarian cancers.[38] Furthermore, the *ESR* gene, located in band 6q25.1,[39] lies outside the common region of deletion observed in this study in band 6q25.3. Our results suggest that a tumor suppressor gene or genes, distinct from ESR, plays an important role in the carcinogenesis of breast cancers.

ACKNOWLEDGMENTS

The authors thank Dr. Futoshi Akiyama for pathological examinations, Drs. Masataka Yoshimoto and Takashi Tada for sample preparation, and Drs. Toyomasa Katagiri, Yousuke Harada, Isao Ito and Kenji Kobayashi for preparing tumor DNAs. This work was supported by Grants-in-Aid for the priority areas of "Cancer Research" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan; by a Research Grant for Cancer Research from the Ministry of Health and Welfare of Japan; and by a grant from the Vehicle Racing Commemorative Foundation.

(Received August 30, 1999/Revised December 1, 1999/Accepted December 8, 1999)

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