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# Loss of Heterozygosity on Chromosome Xp22.2-p22.13 and Xq26.1-q27.1 in Human Breast Carcinomas

In an attempt to investigate the X chromosome harboring putative tumor suppressor genes (TSGs) in sporadic breast carcinoma, we performed loss of heterozygosity (LOH) studies on 23 breast carcinomas using 15 polymorphic markers covering the whole X chromosomes. Matched DNA extracted from tumor samples and corresponding normal tissues were analyzed by polymerase chain reactions (PCR) using microsatellite markers. In 10 cases (43.5%), LOH was detected for at least 1 of the 15 polymorphic markers of the X chromosome tested. Four cases carried a LOH at Xp, and three cases LOH on Xp and Xq. Three cases carried a LOH Xq. Percentage of LOH was relatively high in *DXS987* (26.7%), *DXS999* (30.0%), *HPRT* (21.4%), *DXS1062* (23.1%) loci. Common regions of deletions were found on Xp22.2-p22.13 (30% of LOH) measuring about 4.5Mb and Xq26.1-q27.1 (23.1% of LOH) measuring 10 Mb. The deleted allele was an active copy of the X chromosome. The results indicate the TSGs on the X chromosome are involved in breast cancer.

Key Words : *Loss of heterozygosity; Genes, suppressor, tumor; X chromosome; Breast neoplasms*

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## INTRODUCTION

Recent cancer research has focused on delineating the chromosomal location of putative tumor suppressor genes (TSGs) involved in carcinogenesis (1). This work has lead to the discovery of specific TSGs, or regions containing putative TSGs, involved in many cancers including retinoblastoma as well as breast, lung, and colon carcinomas (2-5).

Breast cancer is one of the common malignant tumors in woman. Loss of heterozygosity (LOH), indicating the presence of a TSG, has been reported in breast carcinoma on many chromosome arms including 1p, 3p, 5q, 6q, 7q, 8p, 9p, 11p, 11q, 13p, 13q, 15p, 16q, 17p, 17q, 18q, Xp and Xq.

The X chromosome has been implicated in many genetic diseases and disorders, and an increasing number of genes responsible are being isolated. However, the X chromosome has rarely been mentioned in cancer studies, and significant levels of LOH have not been detected on X chromosome in human tumors (6). A LOH study of a sporadic breast carcinoma has identified at least three independent regions; in the distal portion of the pseudoautosomal region of Xp, Xp pseudoautosomal region close the pseudoautosomal boundary, and distal Xq (7). Two LOH studies of breast cancer have used some probes mapping to the X chromosome in a general attempt to obtain an allelotype, finding 25% LOH on Xq (8) and 16% on Xp and 9% on Xq (9). Of the cell lines derived from six independent metastases from a melanoma patient, all showed the same loss of X chromosome markers (10). Previous studies of ovarian tumors have shown LOH at Xp21 (11, 12), and we found a specific LOH at Xq25-26.1 in 55 cases of ovarian carcinoma (13).

In an attempt to investigate the X chromosome harboring putative TSGs in sporadic breast carcinoma, we performed LOH studies on 23 breast carcinomas using 15 polymorphic markers that cover the whole X chromosome at regular intervals.

## MATERIALS AND METHODS

### Patient materials

Tumor and corresponding normal tissue were obtained from 23 breast cancer patients from Chonnam University Hospital. They consisted of 2 cases of in situ ductal carcinoma, 20 cases of infiltrating ductal carcinoma (not otherwise specified type), and 1 case of medullary type

**Table 1.** Clinical and histological data of the case

| No. of case | Age/Sex | Histologic type | Histologic grade | Nuclear grade | Lymph node metastasis |
|---|---|---|---|---|---|
| 1 | 31/F | NOS | PD | II | No |
| 2 | 38/F | Medullary | PD | II | No |
| 3 | 38/F | NOS | MD | II | No |
| 4 | 63/F | NOS | MD | III | Yes |
| 5 | 43/F | NOS | PD | II | No |
| 6 | 34/F | NOS | PD | II | No |
| 7 | 58/F | Intraductal | WD | II | No |
| 8 | 48/F | NOS | PD | III | No |
| 9 | 53/F | NOS | MD | II | No |
| 10 | 38/F | NOS | MD | II | No |
| 11 | 57/F | NOS | MD | II | No |
| 12 | 39/F | NOS | MD | II | Yes |
| 13 | 50/F | NOS | MD | II | Yes |
| 14 | 48/F | NOS | MD | II | No |
| 15 | 59/F | NOS | MD | II | No |
| 16 | 52/F | NOS | MD | II | No |
| 17 | 61/F | NOS | PD | III | Yes |
| 18 | 54/F | NOS | MD | II | No |
| 19 | 37/F | NOS | MD | II | Yes |
| 20 | 31/F | NOS | PD | II | No |
| 21 | 38/F | NOS | PD | II | Yes |
| 22 | 23/F | NOS | MD | II | No |
| 23 | 48/F | Intraductal | MD | II | No |

NOS: infiltrating ductal carcinoma, not otherwise specified
WD: well differentiated; MD: moderately differentiated
PD: poorly differentiated

ductal carcinoma. The histologic grades of the tumors were determined according to Bloom and Richardson (14). Out of 23 breast carcinomas, 1 case was well differentiated type, 14 cases were moderately differentiated, 8 cases were poorly differentiated; 20 cases were nuclear grade II, and 3 were nuclear grade III (Table 1). None of the cases were treated with chemotherapy or radiation before operation.

### DNA extraction

The genomic DNA was extracted from the tumor and corresponding normal tissue from formalin-fixed paraffin-embedded tissue blocks as described (15).

### DNA primers and PCR condition

Each of the matched pairs of normal and tumor DNAs were subject to PCR analysis using 15 microsatellite polymorphic primers on chromosome X: *DXS987, DXS996, DXS999* (16), *KAL* (17), *DXS989, DXS1047, DXS1053, DXS1062, DXS1206, DXS1227, DXS1229, HPRT* (18), *DMD* (19), *MAOA* (20), *AR* (21). Chromosomal position and sequential order on the chromosome are according to Nelson et al. (22). PCR reactions were carried out as described below with the inclusion of one ($\gamma$-$^{32}$P) ATP-end-labeled primer. The PCR was performed in 20 $\mu$l volumes of a mixture containing 1 X PCR buffer [10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$], 1 $\mu$M of each unlabeled and labeled primer, 20 ng template DNA, 0.5 units *Taq* DNA polymerase, and 200 $\mu$M of each deoxynucleoside triphosphates. The reactions were cycled 30 times; each cycle consisted of 1 min at 94℃, 30 sec at 55℃, and 1 min at 72℃, and 10 min at 72℃ for final elongation in a thermal cycler (Perkin Elmer Cetus, Emeryville, CA). The PCR products were denatured and separated on a 6% polyacrylamide gel containing 8.3% urea for 2-3 hr at room temperature. After electrophoresis, the gel was dried and exposed to X-ray film for 12-16 hr.

### LOH analysis

In cases where a particular marker was heterozygous in normal tissue DNA, LOH was assessed in a corresponding tumor sample by two independent observers. When the case was not visually obvious, the intensities of signals were measured by phosphor image analyzer (BAS 1500, Fujifilm, Japan). The ratio of the signal intensity between the two alleles in the tumor DNA was compared with the ratio of signal intensity in corresponding normal tissue DNA. LOH was defined in any case where the value of the tumor allele's ratio, as defined above, was 50% of less than the value of the normal allele's ratio.

### Analysis of X chromosome inactivation

Methylation status near the polymorphic triplet repeats in the androgen receptor gene at Xq21 was examined (23). One hundred ng of genomic DNA from the patient's normal tissue or tumor tissue were incubated at 37℃ for 16 hr in a 10 $\mu$l of appropriate buffer with or without 10 units of *HpaII* (Boehringer Mannheim, Germany). After heat inactivation at 95℃ for 10 min, 1 $\mu$l of this reaction solution was added to PCR reaction to amplify the region containing both the triplets repeats and the *HpaII* sites. The PCR primers and conditions were as described (23). The PCR products were denatured and separated on a 6% acrylamide gel containing 8.3% urea. After electrophoresis, the gel was silver stained (24).

## RESULTS

Adenocarcinomas from 23 patients with breast cancer were examined for LOH using 15 polymorphic markers

**Table 2.** Frequency of LOH at chromosome X in breast carcinoma. The microsatellite markers used in this study and their cytogenetic locations are shown on the left

| Locus symbol | Cytogenetic band | number of LOH cases over number of informative cases | loss (%) |
|---|---|---|---|
| DXS996 | Xp22.3 | 2/15 | 13.3 |
| KAL | Xp22.3 | 3/16 | 18.8 |
| DXS987 | Xp22.2 | 4/15 | 26.7 |
| DXS1053 | Xp22.2 | 3/15 | 20.0 |
| DXS999 | Xp22.1 | 4/10 | 40.0 |
| DXS1229 | Xp22.1 | 0/5 | 0 |
| DXS989 | Xp22.1 | 1/18 | 5.6 |
| DMD | Xp21.2 | 1/12 | 8.3 |
| MAOA | Xp11.4 | 0/11 | 0 |
| ARA | Xp11.2 | 2/20 | 10.0 |
| DXS1206 | Xq25 | 0/14 | 0 |
| DXS1047 | Xq25 | 2/18 | 11.1 |
| HPRT | Xq26.1 | 3/14 | 21.4 |
| DXS1062 | Xq26.2 | 3/13 | 23.1 |
| DXS1227 | Xq27.1 | 2/13 | 15.4 |

mapped to the X chromosome. The use of highly variable microsatellite polymorphism greatly increased the informativeness of the cases and allowed a precise definition of the extent of the deleted region. In 10 cases (43.5%), LOH was detected for at least 1 of the 15 polymorphic markers of the X chromosome tested. Four cases (case 6, 8, 13, 16) revealed LOH at Xq, and three cases (case 9, 18, 21) at Xq, and three cases (cases 2, 11, 17) revealed LOH at Xp and Xq. All of the data are summarized in Table 2 and Fig. 1. Percentage of LOH was relatively high in *DXS987* (26.7%), *DXS999* (40.0%), *HPRT* (21.4 %), *DXS1062* (23.1%) loci.

Two common regions of deletion were identified. Five cases (case 2, 6, 11, 16, 17) were commonly deleted in Xp, and the shortest region of deletion (SRO) was observed in the region defined by loci *DXS1053* and *DXS-1229* (Xp22.2-Xp22.13) measuring about 4.5 Mb. Fig. 2A shows examples of PCR analysis of LOH at *DXS-1053, DXS999,* and *DXS1229*. Case 2 showed LOH at *DXS1053* and *DXS999,* but heterozygosity at *DXS1229*. Case 16 revealed heterozygosity of *DXS1053,* LOH at *DXS999,* and homozygosity at *DXS1229*. And case 2 case 16 define the 4.5 Mb SRO between *DXS1053* (excluded) and *DXS1229* (excluded). Five cases (case 2, 9, 11, 17, 21) were commonly deleted in Xq, and the SRO was observed in the region defined by *HPRT* and *DXS-1227* (Xq26.1-q27.1) measuring 10 Mb. Fig. 2B shows

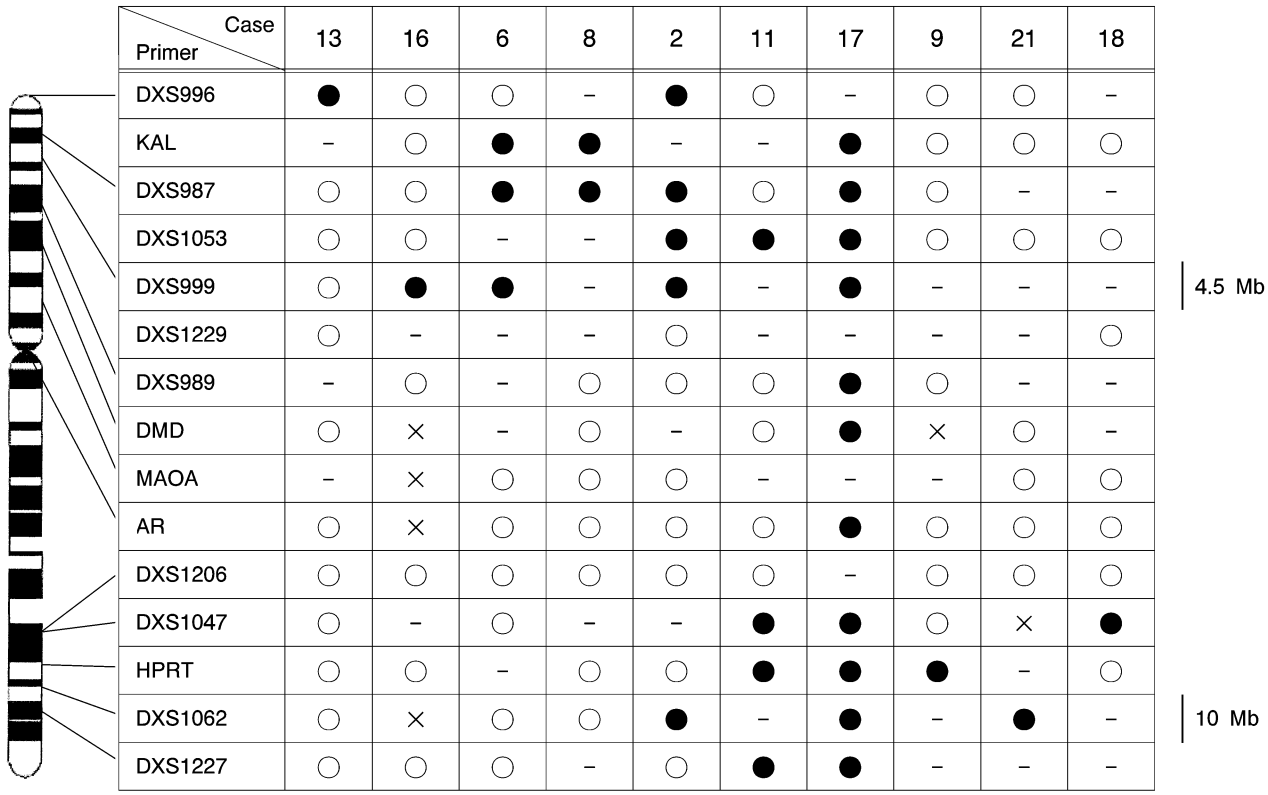


**Fig. 1.** Summary of chromosome X deletion mapping in breast carcinoma. The microsatellite markers used in this study and their cytogenetic locations are shown to the left. Breast tumors that showed partial LOH are labeled across the top. Shortest region of deletions are illustrated by bars on the right. ●, LOH; ○, heterozygosity; −, homozygosity; ×, not amplified.

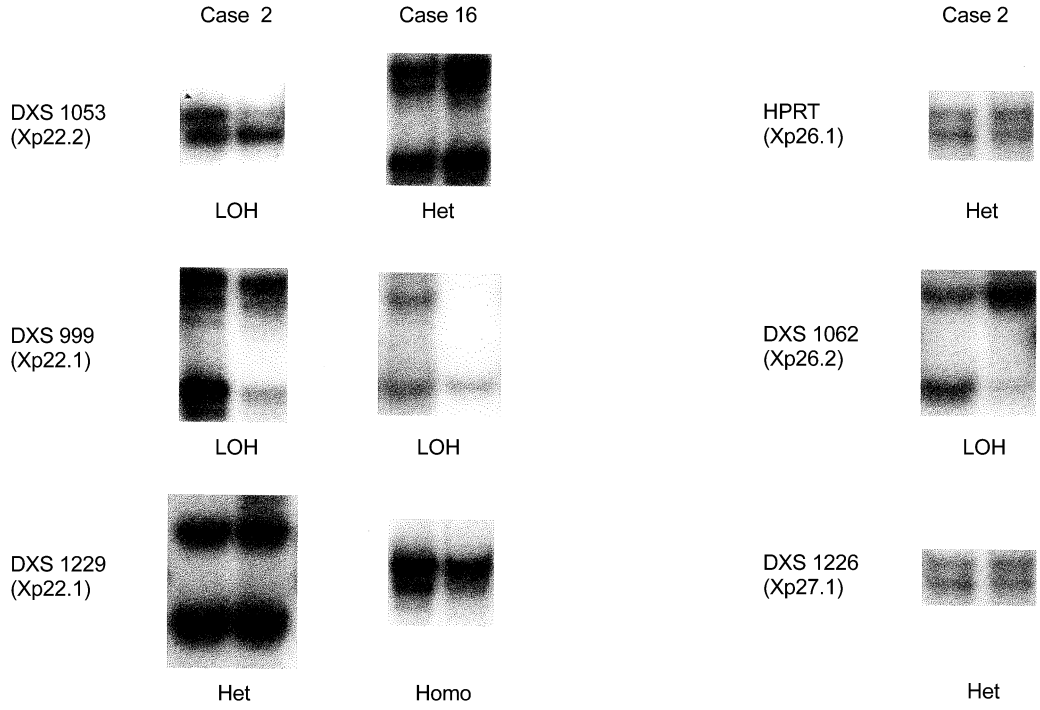


**Fig. 2.** Autoradiograph of LOH data in tumors defining the SRO. A, Minimum overlapping Xp LOH flanked by *DXS1053* at case 16 and *DXS1229* at case 2 is demonstrated. B, Minimum overlapping Xq LOH flanked by *HPRT* and *DXS1227* at case 2 is demonstrated. Het, heterozygosity; Homo, homozygosity.

autoradiogram of case 2 at *HPRT* and *DXS1227* loci, but LOH at *DXS1062* locus.

To determine whether the deleted allele is an active X or an inactive X, we analyzed the methylation status of a *HpaII* restriction site 5' to the (CAG)-repeats in AR

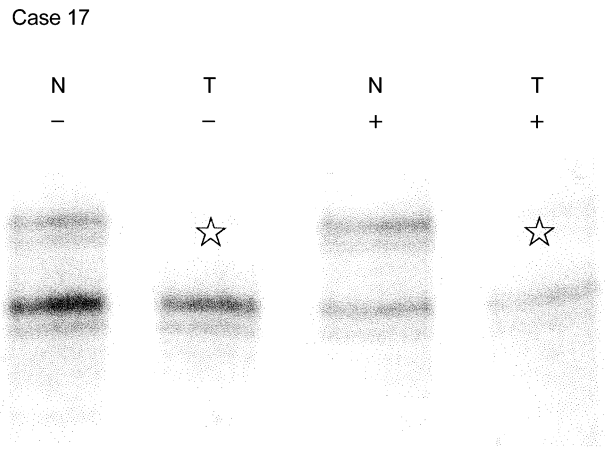


**Fig. 3.** Analysis of X chromosome inactivation. *Hpall*-digested (+) and non-digested (−) DNA from either normal (N) or tumor tissue (T) from case 17 was subject to PCR-amplification of the locus near the AR gene. Upper allele is lost in tumor(☆). Bottom alleles are retained in a tumor and resistant to *Hpall* digestion, indicating that this allele is on inactive X chromosome and that lost allele in tumor is on the active X chromosome.

gene for the carcinoma that showed LOH at Xp, Xq and *AR* loci. The region on the active X chromosome is unmethylated, thus the *HpaII* restriction site of this locus is sensitive to *HpaII* digestion in case 17, suggesting that the remaining chromosome is the inactive X chromosome (Fig. 3).

There was no correlation between the LOH of X chromosome and age of the patients, histological type, grade, and lymph node metastatsis (data not shown).

## DISCUSSION

The establishment of a common region of deletion restricted to the X chromosome in sporadic breast carcinoma raises questions about gene dosage. According to Lyon's hypothesis, the extra copy of the X chromosome in female is randomly inactivated. If one copy of the TSG(s) located in the X chromosome is inactivated in female and only a single copy is present in male, the remaining copy of the gene would be a target requiring only a single "hit" rather than two "hits" hypothesized by Knudson (25). This would be unfavorable for the cells, with random (nonspecific) loss of genetic material will easily lead to tumorigenesis. Therefore, it is reasonable that a "normal" TSG(s) requiring "two hits" were situated on X chromosome, then it should escape from X

inactivation. If this is the case, there should be a corresponding gene on the Y chromosome. A number of genes shown to escape X inactivation are *MIC2* (the cell surface antigen), *XE7*, *XG* (X-linked blood group gene), *sulfatase genes (ARSD, ARSE), STS (steroid sulfatase gene), KAL1 (Kallman syndrome gene), XE113, ZFX (zink-finger protein, X-linked), XE59, UBE1 (ubiquitin activating enzyme E1), PCTK1 (PCTAIRE-1), DXS423E, XE169, RSP4X (ribosomal protein S4, X-linked), XIST (X inactive specific transcript)*, and several genes located to the pseudoautosomal region of X chromosome. Among the genes known to escape X inactivation, XE59 that is mapped to Xp21.1-22.1 can be a candidate for the TSG in Xp. So far no genes at Xq26.1-27.1 have been known to escape X inactivation. Searching for the expressed genes for the LOH overlapping region of Xp and Xq on an inactive X chromosome might be a feasible approach to identify the gene(s).

Loupart et al. (7) reported 30-33% of allelic imbalance on Xp22.2-p22.1 (*DXS278* and *DXS9*) in breast carcinoma, which overlaps with the SRO in this study. In ovarian carcinoma, other groups have found 40% of LOH on *DXS538* (Xp11.21-p21.1) (26) and 28% of LOH on *DXYS20* (Xp22.32) (27). A common region of deletion has been established between *DXS84* and *DXS7* on Xp21.1-11.4 (11, 12). Other study using comparative genomic hybridization has shown frequent loss of the Xp region in ovarian cancer (28). Those studies support the possibility that there might be a TSG common to breast and ovary on Xp.

The SRO on Xq26.1-q27.1 of this study overlaps with the common region of deletion (27.3-33.3%) on Xq25-26.1 found in ovarian carcinoma (13). It also overlaps with the common region of deletion (35-38%) on Xq26.1-ter in breast carcinoma (7). A significant LOH (26%) at *DXS3* locus (Xq21.3) has been reported for uterine cervical carcinoma (29). Xq LOH has also been demonstrated in renal oncocytomas (30). *MCF2* oncogene has been localized in this region (31) and recurrent X chromosome rearrangements have been observed on Xq26 in cemento-ossifying fibroma (32). Because the frequency of LOH on Xq in this study is higher than 30%, it may not be a nonrandom change of the gene.

Sandberg (33) and Moertel et al. (34) have found that the entire X chromosome (presumably inactive copy) is lost in numerous cancers. Wang et al. (35) have found two identical active X chromosomes in Elco and MCF-7 human breast cancer cell lines, suggesting that alteration of X chromosome is important to breast carcinogenesis. The transfer of the X chromosome to rodent or human tumor cells have induced senescence-like tumor growth arrest (36, 37), which suggest that the X chromosome contains the genes that are important to mammalian carcinogenesis. It would be interesting to determine whether introduction of the X chromosome via microcell-mediated chromosome transfer has any effect on in vitro or in vivo cell growth or on the degree of differentiation of breast cancer cells that have lost a portion of X chromosome. A combined approach, using a genetic complementation study and positional cloning, may facilitate isolation of the gene.

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