Allelic Loss on Chromosome 9q Is Associated with Lymph Node Metastasis of Primary Breast Cancer

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Frequent allelic losses on chromosome 9 are seen in a wide variety of human tumors; moreover, two genes (P16 and PTC) whose mutant alleles confer predispositions to some inherited cancer syndromes have been identified on this chromosome. Using 15 highly polymorphic microsatellite markers distributed on both arms of chromosome 9, we tested 96 primary breast carcinomas for allelic loss in order to define the locations of genes that might be involved in this type of tumor. Allelic loss was observed in 37 of the tumors (39%) and detailed deletion mapping identified target regions at 9p21, 9q22.3 and 9q33. Losses at 9q22.3 and 9q33 were correlated with the presence of lymph node metastasis, and allelic loss at 9q22.3 was observed more frequently in scirrhous tumors than in less aggressive histologic types. Therefore, inactivation of tumor suppressor genes in 9q22.3 and 9q33 regions might play a role in progression of breast cancers, especially in metastasis to lymph nodes and in development of scirrhous tumors.

Key words: Breast cancer — Loss of heterozygosity — Tumor suppressor gene — Chromosome 9

Breast carcinogenesis in humans is considered to require a series of genetic alterations involving dominant oncogenes and tumor suppressor genes.1–6) Inactivation of tumor-suppressor functions usually occurs as a consequence of deletion of one allele followed by mutation of the other. Frequent allelic losses (loss of heterozygosity, LOH) observed at specific chromosomal loci in several types of human cancers have implied the presence of putative tumor suppressor genes in the regions where deletions were detected. In breast carcinomas LOH has been reported on chromosomes 1p,3) 3p,6) 7p,7) 11p,8) 13q,9) 16q,10) 17p,11–13) 18q,14) and 22q15) and putative tumor suppressor genes in these chromosomal regions are postulated to be targets of those cancer-associated events.

Germline mutations of tumor suppressor genes confer predispositions to several dominantly inherited cancer syndromes. Two genes in this category have been localized to chromosome 9. On the short arm, P16, a gene associated with familial malignant melanoma16) was isolated recently from 9p21 near P15. Homozygous or hemizygous deletions of these genes are also common in cell lines or primary tissues derived from various types of cancer17,18) including esophageal squamous cell carcinoma,19) pancreatic adenocarcinoma,20) and squamous cell carcinoma of the bladder.21) On the long arm, a predisposing gene for nevoid basal cell carcinoma (Gorlin) syndrome, PTC, was recently isolated from 9q22.22) Somatic mutations and losses of this gene have also been found in medulloblastoma and meningioma.23) LOH is also frequent in cancers of the urinary bladder at yet another region, 9q32-q33.24) To determine the role of genetic alterations on chromosome 9 in the development and/or progression of breast tumors, we performed LOH analysis of 96 primary breast cancers using 15 microsatellite markers from both arms of chromosome 9, and looked for correlations between LOH and certain clinicopathological parameters.

MATERIALS AND METHODS

Specimens Tumor tissues and corresponding normal tissues were obtained from 96 patients with primary breast cancers during mastectomy at the Cancer Institute Hospital in Japan. Tumors were diagnosed according to the histological typing scheme of the Japanese Breast Cancer Society.25) None of the patients had distant metastasis at the time of surgery. The tumor tissues and non-cancerous tissues were dissected and stored at −80°C until DNA extraction.

DNA extraction Frozen tissue samples were powdered, suspended in lysis buffer, treated with proteinase K and extracted with phenol-chloroform-isooamyl alcohol as described by Sato et al.26) Before polymerase chain reaction (PCR) amplification, paired genomic DNAs were diluted and adjusted individually to 10 ng/µl.
RESULTS

LOH was detected on chromosome 9 in 37 (39%) of 96 breast tumors examined, all of which were informative with at least one of the 15 polymorphic microsatellite markers. The marker loci and their frequencies of LOH in our panel of patients are listed in Table I, in descending order from 9pter to 9qter according to the Généthon human linkage map and comprehensive human linkage map. Among the 23 tumors with LOH on 9p, 12 showed LOH at all informative loci but 11 showed partial or interstitial deletions. Representative autoradiograms for these cases are shown in Fig. 1A, where Tumor 66 showed retention of alleles at D9S157 but LOH at D9S736, and Tumor 25 showed LOH at D9S157 but retention of alleles at D9S736. The deletion map of 9p illustrated in Fig. 2 showed that eight tumors with partial or interstitial deletions were critical in defining a commonly deleted region at 9p21, within a 9-cM interval flanked by D9S157 and D9S736. This interval contains the p16 locus.

On the long arm of chromosome 9, the observed frequencies of LOH ranged from 10% at D9S61 to 29% at D9S66 (Table I). Among the 32 tumors with LOH on 9q, four showed LOH at all informative loci but the other 28 showed partial or interstitial deletions. For example, Tumor 618 showed LOH at D9S127 but retention of alleles at D9S176 and D9S58, whereas Tumor 1038 showed LOH at D9S1690 but retention of alleles at D9S176 and D9S38 (Fig. 1B). Similarly, Tumor 2170 showed LOH at D9S66 but retention of alleles at D9S113 and D9S158. Tumor 1038 showed LOH at D9S66 but

Table I. Frequencies of LOH at 15 Loci on Chromosome 9

| Marker | Informative cases | LOH | LOH/informative cases (%) |
|--------|-------------------|-----|---------------------------|
| D9S178 | 89                | 10  | 20.8                      |
| D9S157 | 90                | 15  | 21.1                      |
| D9S736 | 90                | 6   | 13.6                      |
| D9S153 | 92                | 13  | 18.3                      |
| D9S180 | 93                | 11  | 20.8                      |
| D9S176 | 90                | 16  | 24.2                      |
| D9S1690| 88                | 16  | 26.7                      |
| D9S127 | 85                | 15  | 26.8                      |
| D9S299 | 78                | 12  | 24.0                      |
| D9S58  | 90                | 17  | 21.3                      |
| D9S302 | 89                | 9   | 12.3                      |
| D9S61  | 92                | 8   | 10.3                      |
| D9S113 | 93                | 16  | 21.3                      |
| D9S66  | 90                | 19  | 28.8                      |
| D9S158 | 88                | 8   | 13.3                      |
| Total  | 96                | 37  | 38.5                      |
Fig. 1. Representative autoradiograms of LOH analysis. Markers are indicated at the bottom of each panel. T and N; paired DNA samples isolated from tumor and normal tissues, respectively. Critical cases that represent each commonly deleted region are shown.

Fig. 2. Deletion map showing partial or interstitial deletions of chromosome 9p in breast cancers. Locations and order of the microsatellite markers were derived from published linkage information; distances between loci are indicated in female centimorgans. Tumor numbers are at the top of each column. White circles indicate retention of heterozygosity, black circles indicate LOH, and gaps reflect un informativeness. The commonly deleted region that contains the p16 gene is indicated by a vertical bar on the far right, between the dotted lines.

retention of alleles at D9S61 and D9S158 (Fig. 1B). Fig. 3 illustrates, as a deletion map, the seven tumors with partial or interstitial deletions that were critical in defining a new commonly deleted region at 9q22.3, within a 17-cM interval flanked by D9S176 and D9S88; this interval contains the PTC locus. Eleven tumors, including five of
Deletion Mapping of Chromosome 9 in Breast Cancer

**Fig. 3.** Deletion map showing partial or interstitial deletions of chromosome 9q in breast cancers. Explanations for symbols are the same as in Fig. 2. Two commonly deleted regions were determined, each outlined by a rectangle.

**Table II.** Correlation of LOH on Chromosome 9 with Clinicopathological Parameters

| t-factor       | No. of samples | 9p21 Statistical significance | 9q22.3 Statistical significance | 9q33 Statistical significance |
|----------------|----------------|-------------------------------|----------------------------------|-------------------------------|
| t1             | 9              | 1 7 NS                        | 2 5 NS                           | 2 7 NS                        |
| t2             | 66             | 10 36 NS                      | 15 43 NS                         | 19 43 NS                      |
| t3             | 15             | 3 8 NS                        | 4 9 NS                           | 3 12 NS                       |
| Lymph node metastasis |        |                               |                                  |                               |
| n (-)          | 35             | 2 21 NS                       | 2 26 P=0.0032                    | 4 29 P=0.0123                 |
| n (+)          | 55             | 13 32 NS                      | 19 31                            | 20 33                         |
| ER             |                |                               |                                  |                               |
| positive       | 34             | 7 21 NS                       | 11 21 NS                         | 10 21 NS                      |
| negative       | 35             | 4 20 NS                       | 6 23 NS                          | 10 24 NS                      |
| PgR            |                |                               |                                  |                               |
| positive       | 46             | 4 31 P=0.0196                 | 12 30 NS                         | 11 31 NS                      |
| negative       | 24             | 7 11 NS                       | 7 14 NS                          | 9 15 NS                       |
| Histological type |            |                               |                                  |                               |
| 1a: intraductal carcinoma | 1     | 0 1 NS                       | 0 1                              | 1 0 NS                       |
| a1: papillotubular carcinoma | 15     | 2 9 NS                       | 2 10 P=0.0343                     | 3 12 NS                      |
| a2: solid-tubular carcinoma | 34     | 5 19 NS                       | 6 24                             | 7 26 NS                      |
| a3: scirrhous carcinoma | 32     | 7 19 NS                       | 12 17                            | 11 19 NS                      |

ER, estrogen receptor; PgR, progesterone receptor; NS, not significant.

*a) a3 vs. others.*
those seven, were critical in defining a different commonly deleted region at 9q33 within a 14-cM interval flanked by D9S113 and D9S158.

We attempted to correlate LOH at loci on chromosome 9 with clinical parameters (histologic diagnosis, clinical classification, lymph node metastasis, and hormone receptor status) in all tumors for which clinicopathological data were available. Table II summarizes the results of these comparisons. While chromosome 9 loss had no particular association with tumor size, it did exhibit a significant association with lymph node metastasis. Of the 21 tumors that showed LOH at 9q22.3, 19 (90%) had metastasized to lymph nodes, whereas only 31 (54%) of the 57 tumors that showed retention of both alleles at 9q22.3 had metastasized (P=0.0032). Similarly, of the 24 tumors that showed LOH at 9q33, 20 (83%) had metastasized to lymph nodes whereas only 33 (53%) of the 62 tumors that showed retention of both alleles at 9q33 had metastasized (P=0.012). We also identified a significant correlation between 9q alleleic status and histological type; i.e., LOH at 9q22.3 was observed more frequently in scirrhous type (12/29, 41%) than in other types (8/43, 18%) (P=0.034). Also, a significant correlation was identified between 9p alleleic status and hormonal status: of the 11 tumors that showed LOH at 9p21, 7 (63%) were PgR-negative, whereas only 11 (26%) tumors of the 42 tumors that showed retention of both alleles at 9p21 were PgR-negative (P=0.0196). No other regions on chromosome 9 showed a significant correlation with any other clinicopathological parameter examined.

DISCUSSION

In the present study we detected frequent LOH (39%) on chromosome 9 in a large panel of primary breast cancers, though LOH frequencies of less than 10% (3–8%) had been observed when the same panel was studied with markers from 1q21-32, 2q13-21, 19q13, 20p12-13, and other sites (unpublished data). Our observations with regard to chromosome 9 therefore reflect non-random genetic alterations associated with breast carcinogenesis. We constructed a high-resolution deletion map using 15 microsatellite markers along the entire length of this chromosome, and identified three distinct commonly deleted regions: 9p21, 9q22.3 and 9q33. When a particular type of cancer exhibits frequent LOH in a specific chromosomal region, one can infer that a tumor suppressor gene important in the genesis of that tumor is likely to be present there. This idea received experimental support when LOH studies revealed 5q21 and 17p13 as targets of frequent LOH in colon cancers(25) and further investigations led to identification of the APC(26) and p53(27) genes, respectively, as the mutated tumor suppressors in the regions indicated. The frequent allelic losses at multiple sites on chromosome 9 (9p21, 9q22.3 and 9q33) and on other chromosomes in studies of breast cancers reported by us and others(6–12, 26) have suggested that numerous mutant genes can participate in breast carcinogenesis.

The commonly deleted region we defined in a 9-cM interval at 9p21 contains the p16 gene, a known tumor suppressor. LOH at 9p21-22 has been observed in 38–58% of breast cancers examined(30–33) but is also frequent in cancers originating in a variety of tissues: nasopharyngeal carcinoma,(34) malignant mesothelioma,(35) bladder cancer,(36) renal cell carcinoma,(37) non-small cell lung cancer(38) and head and neck cancer.(39) Homozygous deletion of the p16 gene has been observed in esophageal squamous cell carcinoma,(39) pancreatic adenocarcinoma,(40) squamous cell carcinoma of the bladder(41) and a small proportion of breast-cancer cell lines.(42) However, specific mutations of p16 have been documented in only a few cases of sporadic breast cancer.(30, 40)

The commonly deleted region at the 17-cM interval on 9q22.3 contains the PTC gene. PTC was recently isolated as a predisposing gene for nevoid basal cell carcinoma (Gorlin) syndrome.22) Somatic mutations and loss of this gene have also been found in some cutaneous basal cell carcinomas, medulloblastomas, and meningiomas, but seldom in breast cancers.23)

Frequent LOH on 9q33 (14-cM) has not been described previously in breast cancers, although it has been observed in bladder cancer.36 We believe this chromosomal region to be another locus containing a candidate gene for breast cancer. We further suggest that p16, PTC, and the locus on 9q33 are independent targets of allelic loss in breast cancer, and that these genes might play a role in the genesis of some proportion of breast cancers. However, since somatic mutation seldom occurs on the remaining copy of these genes, the classical “two hit” theory of Knudson defining tumor suppressor genes does not apply in these situations. Elucidation of mechanisms whereby these genetic alterations contribute to breast carcinogenesis awaits further investigation.

Ito et al.(20) previously showed associations of LOH at 17q21 and 17p13.3 with the loss of ER and/or PgR. In the present study, we detected an association between LOH at 9p21 and PgR-negative status. The presence of ER and PgR in breast cancers is a recognized indicator for responsiveness to hormone therapy: absence of these receptors usually predicts non-responsiveness or loss of hormone dependency.(43)

We identified a significant association of LOH at both 9q22.3 and 9q33 with lymph node metastasis, as well as an association between LOH at 9q22.3 and scirrhous histologic type. These data imply that alterations of one or more tumor suppressor genes at the 9q22.3 and 9q33 region play significant roles in the development and/or progression of breast cancer.
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