Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis

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INTRODUCTION

Breast cancer is both genetically and clinically a heterogeneous and progressive disease. The severity of disease may be determined by the accumulation of alterations in multiple genes that regulate cell growth and proliferation. The inactivation of tumor suppressor genes, by a two-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the genetic evolution of breast carcinomas (1). Several chromosome arms, including 1p, 1q, 3p, 11p, 11q, 13q, 16q, 17p, 17q and 18q, have been reported to show moderate (20–40%) to high (>50%) frequencies of LOH in breast tumors (1). This implies that multiple tumor suppressor genes are likely to be involved in the development and progression of breast cancer.

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer (2–17). Several childhood tumors demonstrate LOH for 11p, including rhabdomyosarcoma (7,8), adrenocortical carcinoma (9), hepatoblastoma (10), mesoblastic nephroma (11) and Wilms’ tumors (WT) (12). Recurrent LOH at 11p is also observed in adult tumors including bladder (13), ovarian (14), lung carcinomas (15), testicular cancers (16), hepatocellular carcinomas (17) and breast carcinomas (2–6), suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies.

Birch et al. (18) have reported an increased risk of breast cancer among mothers of children with embryonal rhabdomyosarcoma, providing genetic evidence for the apparent high-risk association between these two tumor types. The familial association between breast cancer and rhabdomyosarcoma and the other childhood tumors may well be the consequence of alterations in chromosome 11p15. The ability of a tumor suppressor gene(s) on chromosome 11 to re-establish control of the malignant phenotype has been demonstrated by transfer of a normal human chromosome 11 to the breast cancer cell line MDA-MB-435 (19). However,
positioned cloning efforts to identify the target genes on 11p15 have been complicated by the large size of this region (≈10 Mb) and the complexity of LOH at 11p15.

With the goal of identifying the putative tumor suppressor gene(s) on chromosome 11p15, we have refined the minimal regions of LOH in this region, using a high-density marker analysis of 94 informative primary breast tumors and paired normal breast tissue. We have defined precisely and identified two distinct regions of chromosome 11p15.5–11p15.4 that frequently are deleted in breast cancer. The association of LOH with clinical and histological parameters reveals the biological role of the putative tumor suppressor genes in the etiology of breast cancer.

RESULTS

Refinement of the tumor suppressor loci on chromosome 11p involved in breast cancer

Fluorescent PCR semi-automated genotyping (5) was used to detect and analyze allelic losses on chromosome 11 using a panel of 17 microsatellite markers. Previous studies have determined that this technique is more rapid and sensitive compared with the classical radioactive method in determining LOH in tumor DNAs (20). To identify the smallest common deleted region on chromosome 11p15 in breast tumors, 94 paired normal–tumor DNAs were assessed for LOH at 17 chromosome 11p15-specific microsatellite markers. These markers encompass the chromosomal sub-regions 11p15.5–11p15.4, estimated to be ≈8–10 Mb (21,22) (Fig. 1). The results indicate that the loss of all or part of chromosome 11p is a more common event in human breast cancer than previously appreciated (3,4). LOH occurred in at least one marker on the short arm of chromosome 11 in 56 of 94 (60%) informative tumors. The overall frequency of LOH for each marker varies from 16 to 60%, with two peaks seen at markers D11S1318 (45%) and D11S1338 (60%) (Fig. 1). In addition to the 23% LOH at the D11S988 locus (Fig. 1), there was a high incidence of microsatellite instability (MSI) at this marker as we had described earlier (5). Therefore, the possibility that MSI obscures the accurate determination of LOH at the D11S988 locus in some of these tumors cannot be ruled out.

Tumors 57, 94, 6 and 24 (Genescans; Fig. 2) are illustrative examples of LOH patterns seen on chromosome 11p15 and provide a critical description of the LOH regions. Interstitial deletions, examples of which are seen in tumors 57, 94, 6 and 24, were observed more commonly than loss of the entire chromosomal arm as seen in tumor 7 (Fig. 3). In some cases, an example of which is seen at the marker D11S1997 in tumor 24 (Fig. 2), it was observed that the peak for the allele which loses heterozygosity does not change between normal and tumor tissues. Rather, the peak for the other allele increases by several fold in the tumor.
Figure 2. LOH studies of normal (N) and tumor (T) breast cancer pairs. Genescans of samples 57 (D11S1318, D11S4088, D11S2078, D11S988, D11S1758 and D11S1760), 94 (TH, D11S1318, D11S4088, D11S860, D11S988 and D11S1758), 6 (D11S860, D11S988, D11S1758, D11S1760, D11S1338 and D11S1323) and 24 (D11S1760, D11S1338, D11S1323, D11S1997, D11S866 and D11S1331) are shown. Arrows represent allelic loss. LOH represents samples that exhibit loss of heterozygosity and was calculated as described in the text.

![Diagram of LOH studies](image-url)

Since the surrounding markers show LOH, we believe that this allelic imbalance represents LOH and not gene amplification.

The genotypes of the 13 representative breast tumors described in Figure 3, along with other tumors analyzed (data not shown), serve to refine and identify two distinct regions of LOH on 11p15. Region 1 is encompassed by markers D11S1318 and D11S4088 and is defined by the LOH break points in tumors 57 and 94. Tumor 57 retained heterozygosity for the markers D11S2071, D11S1984, D11S1363, D11S222, TH and D11S1318, but showed LOH for the markers D11S4088, D11S2078, D11S860, D11S988 and D11S1758. This tumor also retained heterozygosity for all the remaining proximal markers. Tumor 94 showed LOH at markers D11S2071, D11S1984, D11S222, TH and D11S1318. This tumor was non-informative for the marker D11S1363 and retained heterozygosity at all the proximal markers. Tumors 42, 57 and 94 are examples of tumors that contain interstitial deletions exclusively in region 1 (Fig. 3).

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 6 and 24 (Figures 2 and 3). Tumors 6, 24, 35, 45 and 76 are examples of tumors that harbor interstitial deletions in region 2 (Fig. 3). Tumor 6 showed LOH for the markers D11S988, D11S1758, D11S1760 and D11S1338, but retained heterozygosity at all the markers distal to D11S988 and at all the markers proximal to D11S1338. Tumor 24 was heterozygous for all the markers proximal to D11S1338 but showed LOH at D11S1323, D11S1997, D11S866 and D11S1331. It is notable that tumors 6 and 24 exhibit LOH at either D11S1338 or D11S1323, while the other locus retains heterozygosity. This clearly indicates that region 2 is within the interval that spans the markers D11S1338–D11S1323, a distance of ~336 kb based on the estimate of James et al. (22) and the sequence map of chromosome 11 (http://mcdermott.swmed.edu/). The yeast artificial chromosome (YAC) 847a12 that contains the markers D11S1338, D11S1323 and D11S1997 is ~1.4 Mb in length and is non-chimeric (STS-based map of the human genome; http://www-genome.wi.mit.edu). We have identified integrin-linked kinase (p59ILK)
as a candidate gene for this locus. p59ILK previously was mapped to the CALC–HBBC region on chromosome 11p15 (24). We have refined the map location of p59ILK and placed it on the YAC 847a12. PCR amplification of DNA from the YAC 847a12 with several different p59ILK primers produced the expected length fragments. No products were seen from a BAC DNA specific for the marker D11S1323 or from yeast DNA (data not shown).

A total of five tumors, examples of which were seen in tumors 7, 20, 26, 30 and 34 (Fig. 3), appeared to have lost both of the regions on the chromosome 11p arm. In tumor 7 (Fig. 3), 14 of the 17 markers analyzed showed LOH. This tumor was non-informative for the markers D11S2071, D11S1363 and D11S1758. The probability of three or more allelic losses in the same fragment being caused by independent events is small, and a series of LOH in contiguous markers is more likely to be due to deletion of the entire segment. In most instances, however, LOH on 11p15 appeared to be interstitial (e.g. tumors 20, 26, 30 and 34) and, therefore, restricted to relatively small chromosomal regions.

These data attest the presence of two distinct regions of LOH within 11p15.5–15.4. Region 1 lies between markers D11S1318 and D11S4088 (~500 kb) and region 2 lies between markers D11S1338 and D11S1323 (~336 kb). As described in Figure 3, the two regions were lost in different tumors, although in some tumors both of these regions appeared to be lost due either to interstitial deletions or to the loss of the entire 11p arm.

Negrini et al. (4) previously have reported a third LOH region, towards the telomere, between the markers D11S576 and D11S1318. The percentage LOH that we observe for the telomeric markers D11S2071, D11S1984, D11S1363 and D11S922 (16–22%) is consistent with the observations of Negrini et al. (4). However, the percentage LOH for these markers is well within the background LOH seen at the remaining 11p markers (Fig. 1). In addition, we did not identify tumors that showed LOH exclusively in the telomeric markers D11S2071–D11S922. In our tumor panel, LOH at the distal markers occurred in concert with LOH at region 1. We therefore did not represent the distal region as an independent and third region of LOH.

Correlation between loss of heterozygosity at 11p and pathological features of breast tumors

Conflicting clinical data and clinical correlations of 11p LOH in breast cancer exist in the literature. This study was initiated with those concerns in mind. To examine the role of 11p LOH in breast cancer and to determine if the two regions are involved differentially in predicting the clinical course of this disease, we correlated our LOH data with the various clinical and histological parameters (Table 1).
none of the tumors with LOH in region 2 contained a DCIS component \( (P = 0.016) \). DCIS of the breast is considered a pre-invasive stage of breast cancer and may be a precursor of infiltrating breast cancer (26). Although the number of tumors analyzed is small, the statistically significant association between LOH in region 1 and such tumors suggests the involvement of a target gene in this region with early events in malignancy or invasiveness. The statistical analysis showed a significant association between 11p LOH and tumor ploidy. The majority of tumors (16/24) with region 1 LOH were either diploid or near diploid \( (P < 0.001) \). In contrast, the majority of tumors with region 2 LOH, were aneuploid \( (P < 0.001) \).

A trend was also observed between LOH at 11p and S-phase fraction (SPF). Fifty four percent of tumors with LOH in region 2, had a high SPF (>10% of cells in S-phase), compared with only 32% tumors with LOH in region 1. However, due to the small number of tumors in each category, statistical significance could not be established. It has been suggested that abnormal ploidy or elevated SPF identifies patients with shorter survival, and worse metastatic disease-free survival, as well as being associated with poor outcome in locoregional control of the disease (27). The association between LOH at region 2 and tumors with high SPF and abnormal ploidy, that we observe, is therefore very relevant.

A striking correlation was observed between loss of region 2 and lymphatic invasion. Importantly, 69% of patients with 11p LOH in region 2 showed lymphatic invasion, whereas this infiltration was present in only 29% of patients with region 1 LOH. Thus, tumors that had lost region 2 reveal a significantly higher incidence of metastasis to a regional lymph node(s) \( (P = 0.012) \) than tumors that had lost region 1. Tumors that had lost the entire 11p arm, or had lost both regions, showed the clinicopathological features of tumors that had lost region 2. We also observed the trend that LOH in region 2 occurs more frequently in higher grade (grade III) tumors than LOH in region 1. Thus, LOH at region 2 may be a late event in mammmary tumorigenesis, potentially enabling a clone of previously transformed cells to exhibit greater biological aggressiveness.

**DISCUSSION**

We have identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis. The high frequency of somatic loss of genetic information and the striking clinical correlation observed suggest their role in the pathogenesis of breast cancer.

We have defined precisely and narrowed the location of the putative tumor suppressor gene in region 1 from ~2 Mb (3,4) to ~300 kb. The critical region appears to extend between the markers D11S1318 and D11S4088 at 11p15.5. Previous studies (3,4) had only been able to place the putative gene in the larger overlapping area between \( TH \) and D11S988 (Fig. 4). Although LOH frequencies for this region are consistent (24–45%, this report; 35%, ref. 3 and 22%, ref. 4), the peak incidence of LOH in this report is highest at D11S1318, ~1 Mb distal to the peak at D11S860 reported by Winquist et al. (3) and Negrini et al. (4).

This discrepancy may reflect the characteristics of the tumor samples analyzed or a difference in interpretation of the corresponding allelic patterns. LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (7,8), WT (7), ovarian carcinoma (14), stomach adenocarcinoma (28) and with a region conferring tumor suppressor activity

### Table 1. 11p LOH and clinico-pathological features of sporadic breast tumors

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>LOH in region 1 N</th>
<th>LOH in region 2 N</th>
<th>P-value</th>
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<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>If ductal</td>
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<tr>
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<td>71.4</td>
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*Statistically significant \( (P < 0.05) \).
previously identified by genetic complementation experiments (29). Reid et al. (30) have used a functional assay to localize a 11p15.5 tumor suppressor gene that maps to this region in the G401 cell line. It is interesting to note that the physical map and 11p15.5 tumor suppressor gene that maps to this region in the IGF2 insulin-like growth factor II gene (31) and in stomach adenocarcinoma (28).

be expressed monoallelically in adult tissues (33,34), suggesting that genomic imprinting may be maintained in adult tissues. As illustrated in Figure 4, several genes that map to the LOH region 1 are subject to imprinting. It has been suggested that deregulation of imprinting may play a role during tumorigenesis (35,36). One model proposes that inappropriate methylation (hypermethylation) silences one copy of a tumor suppressor gene (36). This could be due to inappropriate activation of, or mutations in imprintor genes (37). If the first 'hit' represents the non-expression of one of the alleles due to the imprinting process, the second 'hit' may be mutational or may result from loss of all or part of the chromosome carrying the remaining functional tumor suppressor allele, thereby fulfilling Knudson's "two-hit" hypothesis (38). Hypermethylation as an alternative pathway for tumor suppressor gene inactivation has been demonstrated elegantly for the retinoblastoma (Rb1) (39), the von HippeL Landau (VHL) syndrome (40), and the p16 tumor suppressor genes (37).

The other mechanism of altered imprinting that may affect tumorigenesis involves a gene activation hypothesis (36) that results in the reactivation of the silent allele due to the relaxation or loss of imprinting (LOI). LOI mutations have been detected at H19, IGF2, and to a lesser extent at p57KIP2 in WT and BWS (35). LOI of IGF2 has been observed in benign and malignant breast tumors, in other adult and childhood tumors, and in a subgroup of patients with BWS (35,41). IGF2 is a potent mitogen with autocrine and paracrine effects, and the biallelic expression and possible increased dosage of the growth factor may explain the somatic overgrowth characteristics found in BWS patients and may play a role in tumor development. Although IGF2 maps outside the LOH region 1, the presence of novel genes that map to region 1 that may have similar mitogenic effects cannot be ruled out. p57KIP2 (42) is a potential tumor suppressor candidate gene that maps to region 1. However, single strand conformation analysis and direct sequencing of 20 breast tumors (with LOH) and 15 breast tumors (without LOH) failed to reveal mutations in IGF2.

Figure 4. Schematic representation of regions on chromosome 11p15.5–15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in breast cancer (2–5), Wilms tumor (7), non-small cell lung carcinoma (43), rhabdomyosarcoma (7), Beckwith–Wiedemann syndrome (31) and in stomach adenocarcinoma (28).

Importantly, we have refined this region from 5–10 Mb described earlier (2,43) to ∼336 kb, with the highest incidence of LOH at the marker D11S1338. Previous studies (2,43), have only analyzed a few markers, sparsely distributed in the region proximal to HBB. Our study, therefore, is the first report of a detailed analysis of markers proximal to HBB that has considerably refined the boundaries of LOH in region 2.

We observed a significant correlation between LOH at the two chromosomal regions and the clinical and pathological parameters of the breast tumors. LOH in region 1 correlated with tumors that contain ductal carcinoma in situ synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. LOH at region 2 correlated with clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. The association between 11p LOH, tumor progression and metastasis that we describe is analogous to the observations made...
in other epithelial tumors including breast cancer (3,6). For example, LOH at 11p correlated with advanced T stage and nodal involvement in non-small cell lung carcinoma (44) as well as subclonal progression, hepatic involvement (45) and poor survival in ovarian and breast carcinomas (3,46). Phillips et al. (19) have shown that micro-cell-mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435 had no effect on tumorigenicity in nude mice, but suppressed metastasis to the lung and regional lymph nodes. This further supports the observation that chromosome 11 harbors a metastasis suppressor gene. The integrin-linked kinase gene (24) has been shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents. We have refined the map location of p59ILK, and placed this gene on the YAC 847a12 that spans the markers D11S1338 and D11S1323. Thus, p59ILK is a tumor suppressor candidate for region 2.

It is not clear if LOH involving regions 1 and 2 act independently or synergistically in breast tumors. The identification of two subsets of tumors that have lost either region 1 or region 2 suggests that LOH at the two regions occurs independently and perhaps at different time points during breast tumor progression. This is consistent with the possibility that at least two tumor suppressor genes involved in the progression of breast cancer are located on the chromosome 11p15.5–15.4. These genes may function at distinct stages in the development and progression of breast cancer; alternatively, different target genes may be inactivated in different tumors. It is possible that specific subsets of tumors are defined by the particular set of mutations that they contain, which results in the clinical heterogeneity that is frequently seen in breast cancer.

Chromosome 11p15 contains several imprinted genes and two or more tumor suppressor genes. The fine mapping of this intriguing chromosomal region should facilitate the identification of novel genes, the evaluation of candidate genes and the establishment of the mechanisms whereby they contribute to the evolution of adult and childhood cancers.

**MATERIALS AND METHODS**

**Patient materials and preparation of genomic DNA**

Primary tumor and adjacent normal breast tissue samples were obtained from 94 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding non-involved tissue from each patient were collected at the time of surgery, snap-frozen and transferred to –80°C. Clinical and histopathological features of the tumors described in Table 1 were performed by the Pathology Department at CCF and were revealed only after the of the tumors described in Table 1 were performed by the Pathology Department at CCF and were revealed only after the of the tumors described in Table 1 were performed by the Pathology Department at CCF and were revealed only after the.

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**LOH analysis with Genescan**

Fluorescent technology (5) was used to detect and analyze CA repeat sequences. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. T1:T2/N1:N2, where T1 and N1 are the area values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. We assigned a ratio of 0.70 or less to be indicative of LOH on the basis that tumors containing no normal contaminating cells and showing complete allele loss would theoretically give a ratio of 0.0, but because some tumors in this series contained an estimated 30–40% normal stromal cells (interspersed among the tumor cells), complete allele loss in these tumors would give an allele ratio of only 0.70. At least three independent sets of results were used to confirm LOH in each tumor.

**Statistical analysis**

Clinical features of breast tumors are summarized as frequencies and percentages, separately for each LOH region. The chi-square test was
used to compare these features between regions 1 and 2. All statistical tests were performed using a 5% level of significance.

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