Loss of Heterozygosity on Chromosome 11p15 during Histological Progression in Microdissected Ductal Carcinoma of the Breast

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Microdissection of histologically identifiable components from formalin-fixed, paraffin-embedded tissue sections allows molecular genetic analyses to be correlated directly with pathological findings. In this study, we have characterized loss of heterozygosity (LOH) at chromosome 11p15 at different stages of progression in microdissected tumor components from 115 ductal carcinomas of the breast. Microdissected foci of intraductal, infiltrating, and metastatic tumors were analyzed to determine the stage of progression at which LOH at 11p15 occurs. LOH was detected in 43 (37%) of 115 cases. Foci of intraductal carcinoma could be microdissected from 85 cases, of which 30 (35%) showed LOH at some stage of progression. LOH was detected in the intraductal component in 26 of these 30 cases. Interstitial deletions were characterized by using a panel of 10 highly polymorphic markers. The smallest region of overlap (SRO) for LOH at 11p15 was bounded by the markers D11S4046 and D11S1758. LOH at 11p15.5 showed no correlation with estrogen receptor status, the presence of positive lymph nodes, tumor size, histological grade, or long-term survival. We conclude that 11p15 LOH usually occurs early in breast cancer development but less frequently does not develop until the infiltrating or metastatic stages of tumor progression. (*Am J Pathol* 1998, 153:271–278)

Chromosome 11p15 shows high frequency loss of heterozygosity (LOH) in multiple human malignancies, including tumors of the breast,^{1–7} lung,^{8–12} cervix,¹³ testis,^{14,15} bladder,¹⁶ and stomach¹⁷ and pediatric tumors of the adrenal glands and liver.^{18,19} This region has also been of interest because chromosomal breakpoints associated with the Beckwith-Wiedemann syndrome (BWS) occur at this locus.²⁰ In addition, physical transfer of chromosomal fragments into cell lines has provided functional evidence for one or more tumor suppressor genes in this region.^{21,22} The application of this technique to the breast cancer cell lines MDA-MB-435 and MCF7 has

been shown to result in suppression of metastasis²³ and tumorigenicity,²⁴ respectively.

Previous studies of 11p15 LOH in breast cancer have revealed one smallest region of overlap (SRO) located in 11p15.5 between the markers TH and D11S988 (Figure 1).^{4,6} These studies also provide evidence for additional SROs in this region, one located more centromeric in the 11p15.3 region⁶ and the other more telomeric.⁴ This locus may therefore contain several genes capable of contributing to the initiation or progression of breast cancer. Multiple SROs in this region have also been reported in studies of lung carcinomas^{8,11} as well as in tumors of the adrenals and liver.¹⁸

In this study, LOH at 11p15 was characterized during breast cancer progression. Tumors were examined for the presence of intraductal, invasive, and metastatic foci. Each component present was isolated by microdissection and tested for LOH at a group of markers that span the segment of 11p15 containing the reported SROs for breast cancer. Our results indicate that LOH at 11p15 usually is present at the stage of intraductal carcinoma but occasionally does not occur until later stages of progression.

Materials and Methods

Case Selection

The material used in this study consisted of formalin-fixed, paraffin-embedded tissue from the archives of the Armed Forces Institute of Pathology. The cases analyzed were selected from a group of 964 breast cancer cases submitted to the Armed Forces Institute of Pathology between 1975 and 1982 for which the patient's social security number was available to facilitate determination of vital status. A subset of these cases was selected for LOH analysis on the basis of the availability of normal tissue and the presence of intraductal lesions that were

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Figure 1. Location of markers used in this study and candidate tumor suppressor genes. The upper map shows the position of several genes, including the candidate tumor suppressor genes H19 and KIP2, along the region of approximately 6 Mb analyzed in this study. The lower map shows the positions of the genetic markers used. The map positions of the Genethon markers are from the sex-averaged Genethon Human Linkage Map.²⁸ The distances between Genethon markers are indicated below the map in centimorgans. The marker D11S837 (ST5 gene) has been localized within the centromeric group of BWS breakpoints.²⁶ The locations of the markers D11S988 and TH are from the CHLC/GDB map. The upper and lower maps are shown in approximate alignment. The KVLQT1 gene occupies approximately 300 kb and encompasses the more telomeric group of BWS breakpoints.³⁹ The TH gene is known to be closely linked to D11S1318, but the order of these two loci is not known with certainty. The H19 gene has been identified on a 100-kb bacterial artificial chromosome clone that also contains the marker D11S4046, suggesting close proximity of these loci in the genome.

believed by the initial observers to be separable by microdissection from invasive and metastatic components of the tumor. Each tumor component present was isolated by microdissection from 12- μ m sections that had been deparaffinized with Hemo-De (Fisher, Pittsburgh, PA). Lysates were prepared from these tissue specimens by incubation in 200 μ l of 10 mmol/L Tris, pH 8.0, 50 mmol/L KCl, 0.1 mmol/L EDTA, 0.5% Tween 20, 100 μ g/ml proteinase K for 12 to 16 hours at 55°C followed by a 5-minute incubation at 95°C to inactivate the protease. Insoluble material was pelleted by centrifugation for 5 minutes, and the supernatant was used as the source of DNA template for polymerase chain reaction (PCR).

LOH Analysis

All tumor components were analyzed for LOH at the polymorphic markers D11S922, D11S988, and TH (tyrosine hydroxylase), which lie within or near the boundaries of a previously identified minimal region of overlap at 11p15.5,^{4,6} and D11S837, which maps within a group of potentially growth-regulatory genes at 11p15.3, a region that might represent a distinct SRO in breast cancer.²⁵⁻²⁷ Six additional markers taken from the Genethon panel were used to characterize the SRO in cases with LOH. The order assigned (Figure 1) is based on the Genethon linkage data and physical mapping studies.²⁸⁻³⁰ PCR was performed in the presence of one ³²P-end-labeled primer. Products were resolved on 6% polyacrylamide/7 mol/L urea gels and visualized by autoradiography. The ratio of alleles present was initially evaluated by visual inspection of an appropriately exposed autoradiogram. When allele loss was partial, presumably due to the presence of normal cells in the mi-

crodissected specimens, band intensities were quantitated with a Molecular Dynamics Storm system, and reduction in allele ratio of greater than 50% was scored as LOH. Results were considered uninformative when the normal tissue was homozygous, when the tissue lysate failed to amplify, or when the results could not be interpreted unambiguously. Because nonamplifiable specimens were scored as uninformative, the percentage of cases reported as uninformative for each marker studied is greater than the percentage of homozygotes.

Statistical Analysis

Statistical analysis was performed using the Statistica program package (Release 5.1, StatSoft, Tulsa, OK). Correlations were assessed using contingency table analysis or regression methods, as appropriate. Survival analysis was performed using Kaplan-Meier plots.

Results

Microdissection of Tumor Components

Tumor components used for DNA isolation were identified microscopically on deparaffinized but unstained sections of formalin-fixed, paraffin-embedded tissue. An adjacent section stained with hematoxylin and eosin was examined to confirm the histological identification of the tumor components selected for analysis. Areas of intraductal, infiltrating, and metastatic tumor were dissected from the unstained sections under microscopic observation, and tissue lysates suitable for PCR analysis were prepared as described in Materials and Methods. A total of 115 cases were dissected in this manner. Of these, 8 were intraductal carcinomas without evidence of invasion, 59 had progressed to the invasive stage but were node negative, and 48 had lymph node metastases. In addition, three cases (16, 90, and 103) had carcinomas of the contralateral breast subsequent to the primary tumor, and two cases (77 and 105) had distal metastases from which tissue was available for analysis. All of the tumor components were analyzed for LOH at the markers D11S922, TH, D11S988, and D11S837 to identify cases with 11p15 LOH and to establish the stage of progression at which LOH occurred. Specimens demonstrating LOH with one or more of these markers were further studied with the other six markers in our panel to characterize the SRO.

LOH at 11p15 during Breast Cancer Progression

A total of 43 of the 115 cases (37%) analyzed demonstrated LOH in at least one tumor component. Samples of intraductal carcinoma were available for analysis in 30 of the 43 cases (70%) showing LOH at 11p15. Three of the eight pure intraductal carcinomas (37.5%) had LOH. LOH was detected in 19 of the 59 (32%) node-negative cases that had progressed to the invasive stage. Intra-

Table 1. Summary of LOH Data during Histological Progression

Histologic diagnosis	Number of cases	LOH at 11p15 by stage of progression			
		Any component	Intraductal	Invasive	Metastatic
Intraductal carcinoma only	8	3 (38%)	3/3 (100%)	NA	NA
Invasive carcinoma without metastases	59	19 (32%)	13/15 (87%)	19/19 (100%)	NA
Invasive carcinoma with metastases	48	20 (42%)	10/12 (83%)	15/18 (83%)	18/20 (90%)

NA, not applicable.

ductal components could be identified and dissected from 15 of these 19 cases, and LOH was detected in 13 of the 15 intraductal specimens. Of the 48 cases with lymph node metastases, 21 (44%) demonstrated LOH in at least one tumor component. Twelve cases in this group had dissectable intraductal components, with 10 showing LOH. Fifteen of 18 specimens of invasive carcinoma in this group and 18 of the 21 metastases demonstrated LOH. The results are summarized in Table 1.

LOH at 11p15 was therefore most commonly observed in the intraductal component of these tumors and maintained throughout subsequent stages of progression. However, several exceptions were observed (Figure 2). Of the cases with LOH, 27 had a dissectable intraductal component in addition to material representing later stages of progression. Of these cases, four (72, 78, 83, and 93) did not show LOH in the intraductal carcinoma. Similarly, there were three cases (78, 93, and 112) with LOH in the metastatic component but not in the corresponding specimen of invasive tumor. Interestingly, two cases (50 and 105) showed LOH in the intraductal and invasive specimens that was not detected in the lymph node metastasis. Of three cases with asynchronous contralateral tumors, two cases (16 and 90) demonstrated LOH in the primary tumor but not in the subsequent tumor in the opposite breast. In case 77, a pleural recurrence 6 years after excision of the primary tumor demonstrated LOH, whereas the primary tumor did not.

Inferences regarding the development of LOH during breast cancer progression could potentially be inaccurate because of contamination of the microdissected tumor components with normal cells, thereby obscuring the LOH present in certain tumor components. To rule out this possibility, lysates from cases showing different patterns of LOH at different stages of progression were tested for LOH at various other loci. As shown in Figure 2, the detection of LOH at loci on chromosome 17q in two of these cases (50 and 72) demonstrates that the dissected material was of sufficient purity to detect LOH if it were present. Therefore, in a minority of cases, 11p15 LOH does not occur until progression beyond the intraductal stage.

Analysis of the SRO for LOH at 11p15

Cases showing 11p15 LOH were further analyzed with a panel of 10 loci spanning the region from 11p15.3 to 11p15.5, previously shown to contain SROs relevant to breast cancer (Figure 3). Of 43 cases with LOH, 19 showed LOH at all informative markers, whereas 24 pro-

vided evidence of interstitial deletions. Overall, the markers TH and D11S1318 appeared to lie within a major SRO, with each marker detecting LOH in 100% of informative cases whereas markers flanking this region on either side showed reduced sensitivity for the detection of LOH. Twenty of the twenty-four cases with interstitial deletions yielded results consistent with loss of a continuous segment of the chromosome. Data from four cases (41, 93, 101, and 103) suggest two regions of LOH, one centered on the markers TH and D11S1318 and the second located more proximally at 11p15.3–15.4.

Examples of the LOH data from cases critical for establishing the SRO or for demonstrating two regions of LOH are presented in Figure 4. Case 38 demonstrates LOH at the more telomeric of the markers analyzed but shows retention of heterozygosity over the centromeric segment of this region. Case 93 is an example of a case with two regions of LOH separated by a region of retention of heterozygosity. In this case, the allele ratios calculated for the proximal markers D11S988 and D11S837 were significantly less than those for the more telomeric markers TH and D11S1318, suggesting that LOH at these two regions may have occurred at different times in the evolution of the tumor. Such disparities in allele ratio were observed only infrequently in this study. Case 124 shows retention of heterozygosity at D11S4046, LOH at D11S1318, and retention of heterozygosity at more proximal markers.

Analysis of patterns of LOH in cases with interstitial deletions suggests a minimal region of overlap between D11S4046 and D11S1758. Two cases retained heterozygosity at D11S4046, indicating that the minimal region of overlap does not extend telomeric to this marker. At the centromeric border of this region, one case (38) retained heterozygosity at D11S1758 and two (38 and 101) at D11S4146, suggesting that these markers lie proximal to a minimal region of overlap.

Clinicopathological Correlations

Survival curves for cases with and without LOH at 11p15 demonstrate no significant difference between the two groups (Figure 5). Other clinical and pathological data on these cases were reviewed. No relationship was found between LOH at this locus and estrogen receptor status, the presence of positive lymph nodes, S phase fraction, tumor size, or histological grade.

Figure 2. LOH occurring at different stages in the progression of breast cancer. Alleles showing loss in tumor specimens are indicated by arrows. Allele ratios were determined by quantitating the bands on a Molecular Dynamics Storm system, calculating the ratio of (lost allele)/(retained allele) and dividing by the same ratio obtained with the normal tissue. In case 72, LOH was first seen in invasive tumor at 11p15 but in intraductal at 17q. In case 112, LOH was first detected in the metastasis. In case 77, LOH was not seen in primary tumor but was present in a pleural metastasis 6 years later. In case 90, LOH was present in primary tumor but not in a contralateral tumor (labeled recurrence) 4 years later. In case 50, LOH was detected at 11p15 and 17q in invasive tumor but only at 17q in metastasis.

Discussion

Microdissection of histological sections permits the isolation of tissue samples representing progressive stages in the evolution of breast cancer. We have applied this method to the analysis of LOH at chromosome 11p15, a locus known to show a high frequency of genetic alterations in breast cancer. Our results demonstrate several variant patterns for the occurrence of LOH at this locus during breast cancer progression. Most commonly, we observed LOH in the earliest stage available for analysis. In four cases, however, LOH was absent from the intra-

ductal tumor but present in later stages of progression. Interestingly, in two cases, LOH seen in an invasive tumor was not detected in synchronous lymph node metastases. The latter observation indicates that invasive and metastatic components of an individual tumor can, at least in a small proportion of cases, represent genetically divergent clones rather than progressive stages in the clonal evolution of the tumor. Although the frequency with which genetically divergent clones arise during breast cancer evolution cannot be determined from the present study, this phenomenon has implications for the development of therapies targeted at specific growth-regula-

Figure 3. Sublocalization of the minimal region of LOH at 11p15 in breast cancer. Results obtained with the 43 cases showing LOH with at least one marker are presented. **Solid circles**, LOH; **shaded circles**, retention of heterozygosity; **open circles**, uninformative (homozygous or nonamplifiable); M, microsatellite instability. The **solid bar** to the right of the figure indicates the minimal shared region of LOH inferred from this data set.

tory signaling pathways and therefore merits further investigation. Studies of microdissected multifocal prostate cancers have similarly demonstrated evidence for genetic heterogeneity.³¹⁻³³

Our observation that 11p15 LOH is usually present by the time the tumor has progressed to the intraductal carcinoma stage is of interest with respect to the finding that LOH can be observed in morphologically benign terminal duct lobular units (TDLUs) adjacent to foci of intraductal carcinoma.³⁴ In that study, only one of five cases with LOH at 11p15.5 was found to have LOH in the adjacent TDLU whereas chromosome 3p markers showed LOH with a much higher frequency in such morphologically normal specimens. This observation, together with our finding that LOH at this locus is usually present in the intraductal carcinoma, suggests that loss of function of the tumor suppressor gene at this locus may often be an event involved in the progression from morphologically benign epithelium to intraductal carcinoma.

Pathological data and long-term clinical follow-up for as long as 25 years following the initial diagnosis was available for most of the cases analyzed in this study. This information was used to evaluate 11p15 LOH as a potential prognostic marker. We observed no significant correlation between 11p15 LOH and survival, tumor stage, grade, or presence of metastases. Presumably, the significant genetic alteration at this locus occurs before the cell has acquired the aberrant growth characteristics necessary for invasion and metastasis. Although some studies have reported associations between LOH at 11p15 and clinical parameters, one report has suggested that these correlations may actually reflect LOH at 11q, which is believed to contain a distinct tumor suppressor gene, rather than at 11p.⁶ Other investigators have also observed a lack of correlation between 11p15 LOH and clinical parameters.⁴ The reasons for the apparent discrepancy between this study and others that find no correlation between 11p15 LOH and the development of metastases^{4,6} and a report that such a correlation exists⁵ are not clear but may reflect differences in the specific markers used or in the population of breast cancer patients analyzed.

This report provides confirmatory evidence for the existence of an SRO distal to D11S988 at 11p15.5. In our

study, LOH was identified in 43 (37%) cases, of which 24 had interstitial deletions. The overall percentage showing LOH is comparable to the 35% previously reported in breast cancer by Winqvist et al,⁶ who also noted that LOH at this locus is most often interstitial. Similar percentages of LOH have been reported in other tumors, including lung (43%),¹¹ bladder (30%),¹⁶ stomach (41%),¹⁷ and testis (59%).¹⁴ The boundaries of the SRO appeared to be similar when data from each stage of tumor progression were analyzed separately; that is, we did not observe a widening of the SRO when the data from only infiltrating or only metastatic tumor components were analyzed, as might have been expected if additional genes from this region were involved in later stages of tumor progression. The four cases in which LOH was not observed in the intraductal component but was detected at a more advanced stage of tumor progression also yielded evidence of a similar SRO. These results are consistent with the possibility that the same gene can be involved at different stages of tumor progression.

The SROs at 11p15 in breast cancer inferred from this and previous studies include a major region of LOH centered on or near the marker D11S1318 plus one or more secondary regions. LOH at these secondary regions seems to occur only in those tumors that demonstrate LOH of the major region. The results of Winqvist et al⁶ identified the markers TH and D11S988 as boundaries of one minimal region of overlap. A second SRO was identified more proximally, an observation similar to our finding of several cases with two distinct regions of LOH. Data from another study⁴ supported two independent regions of LOH at 11p15.5, one between D11S1318 and D11S988 and the other located distal to D11S1318. The major SRO defined by our data set may therefore encompass more than one gene important in the etiology of breast cancer. Although several studies have now reported cases with distinct regions of LOH at 11p15, no cases have been reported in which LOH involves the proximal (to D11S988) or distal (to D11S1318) regions without also involving the major SRO between D11S988 and D11S1318. It remains unclear whether this implies that genes in the proximal and distal SROs become important only after inactivation of a gene in the central region or, perhaps, that the mechanism of genetic loss may occasionally permit retention of a chromosomal seg-

A. Case 38

B. Case 93

C. Case 124

Figure 4. Patterns of LOH at 11p15 in breast cancer. **A:** Case 38, showing LOH at the distal group of markers, retention of heterozygosity at D11S1758, D11S4146, and D11S988, defining centromeric border of SRO. **B:** Case 93, with LOH at proximal and distal markers with retention of heterozygosity between these two regions. **C:** Case 124, with retention of heterozygosity at D11S4046, defining telomeric border of SRO. N, normal tissue; T, tumor. **Arrowheads** indicate the allele lost in assays showing LOH. When LOH was present, quantitation was as in Figure 2. In cases where there was no LOH, the numerator and denominator of the reported allele ratio were chosen so as to yield a number less than the ideal value of 1.0.

ment when flanking sequences on both sides are deleted.

Minimal regions of overlap for LOH at 11p15 have been described in several tumor types other than breast cancer. In non-small-cell lung cancer, one study reported

evidence for a telomeric SRO distal to TH, centered on D11S922, and a second region proximal to D11S988, centered on D11S909.¹¹ The latter marker is close to D11S837, the most proximal marker used in the present study. As observed in other studies of LOH at 11p15.5,

Figure 5. Cumulative proportion surviving (Kaplan-Meier). Survival curves were generated for cases with (—) and without (---) LOH at 11p15.

no cases demonstrated LOH exclusively at the more proximal locus. These results suggest that the major SRO observed in breast cancer (D11S988-TH) may not play a role in non-small-cell lung cancer. However, studies of several other tumor types point to minimal regions of LOH that contain the chromosomal segment identified as the major SRO for breast cancer. Characterization of 100 carcinomas of the bladder suggested a minimal region of overlap between D11S922 and D11S569,¹⁶ a region that includes the D11S988-TH chromosomal segment. A study of 13 hepatoblastomas suggested a significant region of LOH distal to the HBB locus.¹⁹ In an analysis of 60 adenocarcinomas of the stomach,¹⁷ most cases were found to have LOH of all informative markers, but two cases suggested a minimal region of overlap bordered by D11S1318 and D11S988.

The chromosomal segment containing the major breast cancer SRO has also been implicated as the locus of genes involved in cancer by other methodologies. Direct transfer of chromosomal fragments allowed the mapping of a tumor suppressor activity for the cell line G401 to 11p15.5.^{21,22} Using subchromosomal transferable fragments, a growth-inhibitory activity for the rhabdomyosarcoma cell line RD was mapped to a similar region.³⁵

Studies of chromosomal translocations in several human malignancies have mapped breakpoints within the common region of LOH at 11p15, providing additional evidence for the presence of one or more tumor suppressor genes in this region. A breakpoint in a rhabdoid tumor was localized approximately 60 kb centromeric to the TH gene.³⁶ In a myeloid leukemia, an 11p15 breakpoint was found to represent a fusion between the Nup98 gene on 11p15 and the homeobox gene HOXA9 on chromosome 7.³⁷ Studies of BWS translocations have led to the identification of at least 11 genes within a 320-kb segment containing five of eight mapped breakpoints.³⁸ The potassium channel gene KVLQT1, which spans all five BWS breakpoints localized within these 320 kb, is strongly implicated in BWS.³⁹ The gene encoding the cyclin-dependent kinase inhibitor p57Kip2 maps within this region and may also play a role.⁴⁰ This segment, extending to a point approximately 100 kb centromeric to the insulin/TH/

IGF2 gene cluster, lies within the major SRO observed in breast cancer.

The remaining three mapped BWS breakpoints occur within a 1.2-Mb region of band 11p15.3, approximately four Mb centromeric to the cluster of five breakpoints. This region contains three genes potentially involved in growth regulation: rhombotin, WEE1, and ST5.²⁵⁻²⁷ Another candidate tumor suppressor, H19, lies less than 200 kb telomeric to IGF2. This gene lies telomeric to the D11S988-TH segment but maps within the boundaries of the more telomeric SRO identified by Negrini et al⁴ as well as within the SRO defined by the present study. The *tsg101* gene, which was found to undergo aberrant splicing in several breast carcinomas, localizes to 11p15.1, a locus centromeric to the region containing the BWS breakpoints and the common region of LOH identified in multiple tumor types.^{41,42}

In summary, characterization of LOH at 11p15 in a panel of breast carcinomas demonstrated that LOH usually occurs at this locus by the time the disease has developed to the intraductal carcinoma stage, but may occur at a later stage of progression. Analysis of the SRO at this locus supports localization of a tumor suppressor gene involved in breast cancer to a region similar to loci identified by chromosome transfer studies and analysis of BWS breakpoints, suggesting that the same gene or genes at 11p15 may be involved in BWS, growth suppression detected by chromosome transfer, and breast cancer.

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