Allelic Imbalance in Primary Breast Carcinomas and Metastatic Tumors of the Axillary Lymph Nodes

Rachel E. Ellsworth, Darrell L. Ellsworth, David M. Neatour, Brenda Deyarmin, Susan M. Lubert, Miranda J. Sarachine, Patrick Brown, Jeffrey A. Hooke, and Craig D. Shriver

Abstract
Axillary lymph node status is the most important prognostic factor in predicting disease outcome in women with breast cancer. A number of chromosomal aberrations in primary breast tumors have been correlated with lymph node status and clinical outcome, but chromosomal changes particular to metastatic lymph node tumors have not been well studied. DNA samples isolated from laser-microdissected primary breast and metastatic axillary lymph node tumors from 25 women with invasive breast cancer were amplified using 52 microsatellite markers defining 26 chromosomal regions commonly deleted in breast cancer. Levels and patterns of allelic imbalance (AI) within and between breast and lymph node tumors were assessed to identify chromosomal alterations unique to primary or metastatic tumors and to examine the timing of metastatic potential. The overall frequency of AI in primary breast tumors (0.24) was significantly greater (P < 0.001) than that in lymph node tumors (0.10), and congruent AI events were observed for <20% of informative markers. AI at chromosomes 11q23.3 and 17p13.3 occurred significantly more frequently (P < 0.05) in primary breast tumors alone; no chromosomal regions showed a significantly higher AI frequency in lymph nodes. Higher rates of AI in primary versus metastatic lymph node tumors suggest that acquisition of metastatic potential may be an early event in carcinogenesis, occurring before significant levels of AI accumulate in the primary tumor. In addition, patterns of AI were highly discordant between tumor types, suggesting that additional genetic alterations accumulated independently in the two cell populations.

Introduction
A number of prognostic factors have been developed to guide treatment choices and predict clinical outcomes for breast cancer, including age, menopausal status, tumor stage, hormonal status, and lymph node involvement. Currently, metastasis to the axillary lymph nodes is the best known predictor of survival; individuals with negative lymph nodes have >90% 5-year survival compared with <70% survival in individuals with positive lymph nodes. Development of a metastatic phenotype requires multiple steps including invasion, angiogenesis, intravasation, extravasation, and metastatic growth. Although the model of clonal evolution in which cells acquire metastatic potential late in the development of the primary tumor, describes the progression of colorectal tumors, recent studies suggest that metastatic potential in breast cancer may be acquired early in the carcinogenic process. Although some investigators have shown that patterns of gene expression and allelic imbalance (AI) in primary breast tumors are similar to those in the corresponding metastases, others have found disparate patterns of genetic change in primary breast tumors and distant/bone metastases. Thus, it is unclear whether cells develop the ability to metastasize with relatively few genetic alterations early in tumor development or whether full metastatic potential is attained after additional genetic changes accumulate in disseminated breast cells.

To identify genetic alterations associated with the timing and acquisition of metastatic potential, we have used a panel of 52 microsatellite markers representing 26 chromosomal regions commonly deleted in breast cancer to identify patterns of AI in primary breast tumors and matched metastatic tumors of the axillary lymph nodes. Our objectives were to examine levels and patterns of chromosomal change both within and between primary breast and lymph node tumors to (a) assess levels of AI commonly associated with lymph node metastasis and (b) identify specific chromosomal regions associated with metastatic potential.

Results
Clinicopathologic Features
All samples were collected from female patients with an average age at diagnosis of 61.7 years; 28% (7 of 25) of patients were premenopausal (<50 years of age). The majority of cases (17 of 25) were diagnosed as invasive ductal carcinoma and represent stages IIA to IIBc. Average disease-free survival time was 40.6 months and 80% of patients are currently living with no evidence of disease.
AI in Primary Breast Tumors and Metastatic Lymph Node Tumors

To increase the ability to detect AI within a given chromosomal region, two markers flanking each region were used. All markers had published heterozygosity values of >0.60, although within this data set, heterozygosities ranged from 0.32 (D3S2454) to 0.96 (D7S490). Observed heterozygosities were often lower than published values, but evaluation of each chromosomal region by marker pairs (Table 1) greatly improved informativeness. Five individual markers (D2S442, D16S2624, D16S516, D16S520, and D17S1298) showed AI in >50% of patients. When stratified into primary breast tumor versus lymph node tumor, these five markers showed rates of AI of >40% in the primary breast tumor, whereas the highest frequency of AI in the lymph node tumors occurred at D2S442 for 27% of patients. Analysis of the data by chromosomal region showed that 33% of patients showed AI at chromosomes 2q21-q23, 11q23.3, 16q11.2-q22.1, 16q22.3-q24.3, 17p13.1, and 17p13.3. Rates of AI ranged from 4% at chromosome 7q31 to 57% at chromosomes 16q22.3-q24.3 and 17p13.3. In comparisons of AI in breast versus lymph node tumor, the most frequent AI was seen at chromosomes 11q23.3 (45%), 16q11.2-q22.1 (45%), 16q22.3-q24.3 (52%) and 17p13.3 (48%), and at chromosomes 11q13.4 (25%) and 16q22.3-q24.3 (20%), respectively (Table 1).

Heterogeneity of AI between Primary Breast Tumors and Metastatic Lymph Node Tumors

Rates of discordance between primary breast and lymph node tumors measured as the number of noncongruent AI events over the total number of informative events (by chromosomal region), ranged from 0.05 to 0.59 with an average value of 25% discordance (Fig. 1). The overall frequency of AI in primary breast tumors (146 of 605) was significantly higher than that in metastatic lymph node tumors (63 of 602; P < 0.0001). When AI frequencies for each chromosomal region were considered, rates of AI were significantly higher in primary breast tumors for chromosomes 11q23.3 and 17p13.3. Only chromosome 14q32.11-q31 showed a higher frequency of AI in the lymph nodes than in the primary breast tumor, although this difference was not significant. When the markers were examined individually, only D17S1298 on chromosome 17p13.3, showed significantly more frequent AI (P < 0.03) in primary breast tumors compared with metastatic lymph node tumors. Although the second marker from 17p13.3 (D17S849) showed high levels of AI, these differences did not reach statistical significance (P = 0.0801), suggesting a chromosomal region centromeric to D17S849 is involved in tumorigenesis.

Stratification of the AI by breast tumor only, metastatic lymph node tumor only, or both tumor types (congruent AI), revealed that AI unique to primary breast tumors comprised the majority of AI events (>60%), whereas both AI unique to the lymph node tumors and that seen congruently were less frequent (Fig. 2). When the data were examined by chromosomal region, congruent AI at chromosome 11q13.1 was more frequent than unique AI in either tumor type.

To confirm that congruent AI events represented true ancestral (shared) versus independently derived events, we examined whether the same allele was altered in both the breast and lymph node. Overall, 91% of the shared AI events involved the same allele, supporting the hypothesis that these genomic changes occurred in the primary tumor before departure of metastatic cells.

Based on the patterns of unique and congruent AI events, a model depicting regions that likely play key roles in early and later stages of tumor development is shown in Fig. 3. Six chromosomal regions showed congruent rates of AI of >25% suggesting that these regions may be altered early in the process of tumorigenesis and may contribute to the acquisition of metastatic potential. Conversely, eight chromosomal regions showed high rates (>70%) of unique AI events in the breast, suggesting that alterations of these regions occur in later stages of tumor development, after cells with metastatic potential have dispersed.

Quality Control

The small quantities of DNA isolated after laser microdissection of paraffin-embedded samples precluded replication of all genotype data. Confirmatory analyses, done when AI was observed at only one marker for a given region using DNA isolated from the last serial sections, confirmed AI of ~80% of the time. Genotypes for which AI was not confirmed may reflect PCR variability or intratumoral heterogeneity (8-10).

Discussion

Traditional models of carcinogenesis describe tumor progression as being driven by the sequential accumulation of genetic alterations, with tumors amassing a critical assortment of genomic changes during development of invasive and metastatic capabilities (11). Accordingly, cells that acquire the ability to metastasize have a selective growth advantage and become the predominant cell type in the primary tumor. Whereas this model of clonal evolution may describe molecular events characteristic of colorectal cancer, recent studies have shown that for a number of human cancers, breast included, cells with metastatic potential may not predominate and dissemination of these cells to distant sites may occur early in tumor development (6, 12, 13).

Because chromosomal alterations are generally irreversible, genetic changes occurring in precursor cells should also be present in all progeny; therefore, metastatic cells should share many of the genetic abnormalities found in the primary tumor. In this study, overall frequencies of AI were significantly greater in primary breast than in metastatic lymph node tumors, suggesting that (a) metastatic tumor cells diverged early in the primary tumor development with relatively few genetic alterations, and (b) divergent patterns of genetic alterations accumulated independently in the two cell populations after metastatic cells departed from the primary breast carcinoma.

Examination of the chromosomal regions examined here (Fig. 3) also support the theory that metastatic potential may originate early in tumor development. AI at chromosomes such as 6q25-q27, 11q13.1, 13q12, and 16q22.1 has been detected in preinvasive lesions such as ductal carcinoma in situ and atypical ductal hyperplasia (14-17) and associated with early tumor development. On the other hand, AI for chromosomal...
regions such as 8q24, 11p15, 11q23.3, 17p13.3, 17p13.1, and 17q21 has been associated with an invasive phenotype, tumor progression, and poor prognosis (18-21), findings consistent with our data showing higher frequency of AI at these regions only in primary breast tumors. In addition, a number of recent studies using genomic technologies such as AI analysis, comparative genomic hybridization, and gene expression analysis to examine genetic characteristics of primary breast and metastatic tumors have developed models suggesting that metastatic potential is acquired early in tumor development (4, 6, 7, 22). Similar models have been proposed for non–small cell lung carcinoma and melanoma, as well (13, 23).

Many recent publications have proposed that acquisition of metastatic potential is an early-stage event; however, there is debate about whether metastasis-specific genetic alterations exist. For example, Bonsing et al. (4) examined patterns of AI in diploid and aneuploid cells of primary breast and matched metastatic lymph node tumors. Although the rate of AI was increased in aneuploid cells of metastatic lymph nodes, the presence of diploid precursor clones in the metastatic tumors led them to conclude that additional genetic alterations are not needed for metastatic potential. Likewise, microarray studies showing similar gene expression patterns between primary breast tumors and metastases (5) seem to support the hypothesis that exclusive genetic changes are not required for metastasis (22).

### Table 1. Marker Location, Informativeness, and Frequency of AI

<table>
<thead>
<tr>
<th>CDR Marker Name</th>
<th>Marker Location</th>
<th>Marker Heterozygosity</th>
<th>Pairwise informative rate*</th>
<th>Frequency loss, primary breast tumor</th>
<th>Frequency loss, lymph node metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p36.1-p36.2</td>
<td>D1S468</td>
<td>1p36.32</td>
<td>0.88</td>
<td>0.96</td>
<td>0.17 (4/24)</td>
</tr>
<tr>
<td></td>
<td>D1S1612</td>
<td>1p36.23</td>
<td>0.72</td>
<td>0.82</td>
<td>0.14 (2/2)</td>
</tr>
<tr>
<td>2q21.3-23.3</td>
<td>D2S442</td>
<td>2q21.3</td>
<td>0.64</td>
<td>0.92</td>
<td>0.09 (2/2)</td>
</tr>
<tr>
<td></td>
<td>D2S1353</td>
<td>2q24.1</td>
<td>0.84</td>
<td>0.88</td>
<td>0.26 (6/23)</td>
</tr>
<tr>
<td>3p14.1</td>
<td>D3S1600</td>
<td>3p14.2</td>
<td>0.88</td>
<td>0.88</td>
<td>0.13 (3/24)</td>
</tr>
<tr>
<td>5q21.1-q21.3</td>
<td>D5S1721</td>
<td>5q21.2</td>
<td>0.74</td>
<td>0.92</td>
<td>0.17 (4/23)</td>
</tr>
<tr>
<td>6q15</td>
<td>D6S1043</td>
<td>6q16.1</td>
<td>0.79</td>
<td>0.92</td>
<td>0.17 (4/23)</td>
</tr>
<tr>
<td></td>
<td>D6S300</td>
<td>6q16.1</td>
<td>0.72</td>
<td>0.82</td>
<td>0.22 (5/23)</td>
</tr>
<tr>
<td>6q22.1-q23.1</td>
<td>D6S474</td>
<td>6q21</td>
<td>0.84</td>
<td>0.92</td>
<td>0.26 (5/23)</td>
</tr>
<tr>
<td></td>
<td>D6S242</td>
<td>6q22.3</td>
<td>0.80</td>
<td>0.80</td>
<td>0.36 (9/25)</td>
</tr>
<tr>
<td>6q25.2-q27</td>
<td>D6S441</td>
<td>6q25.2</td>
<td>0.92</td>
<td>1.00</td>
<td>0.13 (3/24)</td>
</tr>
<tr>
<td>7q31.1-q31.31</td>
<td>D7S523</td>
<td>7q31.1</td>
<td>0.80</td>
<td>1.00</td>
<td>0.04 (1/25)</td>
</tr>
<tr>
<td>8p22-p21.3</td>
<td>D8S552</td>
<td>8p22</td>
<td>0.82</td>
<td>0.88</td>
<td>0.14 (3/22)</td>
</tr>
<tr>
<td>8q24</td>
<td>D8S511</td>
<td>8q22</td>
<td>0.72</td>
<td>0.76</td>
<td>0.26 (6/23)</td>
</tr>
<tr>
<td>9p21</td>
<td>D9S523</td>
<td>9p21.3</td>
<td>0.84</td>
<td>0.92</td>
<td>0.26 (6/23)</td>
</tr>
<tr>
<td>10q23.31-q23.33</td>
<td>D10S541</td>
<td>10q23.31</td>
<td>0.71</td>
<td>0.96</td>
<td>0.29 (7/24)</td>
</tr>
<tr>
<td>11q15</td>
<td>D11S1999</td>
<td>11q15.5</td>
<td>0.76</td>
<td>0.88</td>
<td>0.27 (6/22)</td>
</tr>
<tr>
<td>11q13.1</td>
<td>D11S2368</td>
<td>11q15.1</td>
<td>0.75</td>
<td>0.88</td>
<td>0.25 (6/24)</td>
</tr>
<tr>
<td>11q23.3</td>
<td>D11S2371</td>
<td>11q13.4</td>
<td>0.68</td>
<td>0.96</td>
<td>0.25 (6/24)</td>
</tr>
<tr>
<td>13q12.3</td>
<td>D13S1246</td>
<td>13q12.3</td>
<td>0.84</td>
<td>0.96</td>
<td>0.25 (6/24)</td>
</tr>
<tr>
<td>13q14.2-q14.3</td>
<td>D13S153</td>
<td>13q14.2</td>
<td>0.88</td>
<td>0.96</td>
<td>0.25 (6/24)</td>
</tr>
<tr>
<td>14q22.12-q22.1</td>
<td>D14S617</td>
<td>14q22.12</td>
<td>0.71</td>
<td>0.96</td>
<td>0.40 (1/24)</td>
</tr>
<tr>
<td>16q11.2-q22.1</td>
<td>D16S409</td>
<td>16q11.2</td>
<td>0.68</td>
<td>0.88</td>
<td>0.45 (5/22)</td>
</tr>
<tr>
<td>16q24</td>
<td>D16S2624</td>
<td>16q22.2</td>
<td>0.68</td>
<td>0.88</td>
<td>0.45 (5/22)</td>
</tr>
<tr>
<td>17p13.3</td>
<td>D17S489</td>
<td>17p13.3</td>
<td>0.64</td>
<td>0.84</td>
<td>0.52 (11/21)</td>
</tr>
<tr>
<td>17p13.1</td>
<td>D17S849</td>
<td>17p13.3</td>
<td>0.64</td>
<td>0.84</td>
<td>0.52 (11/21)</td>
</tr>
<tr>
<td>17q21.3</td>
<td>D17S250</td>
<td>17q21.3</td>
<td>0.76</td>
<td>1.00</td>
<td>0.24 (6/25)</td>
</tr>
<tr>
<td>18q21.1-q21.3</td>
<td>D18S548</td>
<td>18q21.3</td>
<td>0.76</td>
<td>0.88</td>
<td>0.38 (9/24)</td>
</tr>
<tr>
<td>22q13.2</td>
<td>D22S281</td>
<td>22q13.2</td>
<td>0.92</td>
<td>1.00</td>
<td>0.14 (3/22)</td>
</tr>
<tr>
<td>22q13.1</td>
<td>D22S1045</td>
<td>22q13.1</td>
<td>0.80</td>
<td>1.00</td>
<td>0.20 (5/25)</td>
</tr>
</tbody>
</table>

*Values combine data from markers for a given chromosomal region.
Conversely, discordant patterns of genetic change between primary breast tumors and distant metastases detected using comparative genomic hybridization suggest that metastatic cells may acquire a growth or survival-promoting array of alterations after dissemination (6, 7). In accordance with these studies, our observations of highly discordant patterns of AI between the primary breast and metastatic lymph node tumors supports the independent evolution of genomic alterations in the two tumor types and suggest that patterns of AI in the primary tumor may not predict metastatic tumor development and behavior.

In the data presented here, patterns of AI in the lymph node metastases were less complex than the primary tumors. In the study by Bonsing et al. (4), sorting of diploid and aneuploid cells allowed, in some cases, a more accurate detection of AI. Because we did not sort cells by karyotype, it is possible that rates of AI are underrepresented in our data set, although this underrepresentation should also be present in the primary tumors. To examine whether low levels of AI in the metastatic tumors reflected a sampling artifact, three patients with multiple positive nodes were identified and tumor cells from each node were microdissected and subjected to AI analysis. The number of microdissected nodes ranged from two to seven, and in all cases, the frequency of AI was lower in the lymph node tumors than in the primary breast tumors, suggesting that the lower frequency of AI in the lymph node tumor was not reflective of the specific node dissected.

Patterns of AI consistent with a metastatic phenotype were not observed in this study, which may be due to the low levels of alterations in the lymph nodes detected with this marker panel. Twenty-six chromosomal regions commonly deleted in breast cancer (24) were examined; these regions which are important to primary breast cancer development may not be relevant to the acquisition of full metastatic capabilities. It is, therefore, possible that an independent set of chromosomal alterations, not assayed here, is critical to metastatic tumor growth and survival. Whereas both primary and metastatic tumors share a number of common features, such as uncontrolled proliferation and escape from cell cycle checkpoints, metastatic tumors represent specific cell types transplanted to a distant tissue microenvironment, with unique challenges to survival and growth. In addition, metastatic tumor growth and propagation may follow pathways quite different from that of the primary tumor (6, 7). The use of a global profiling approach may identify a set of chromosomal alterations important for survival and growth of metastatic lymph node tumors.

In conclusion, AI data from primary breast and metastatic lymph node tumors support an emerging model of acquisition of metastatic potential early in the development of the primary tumor. In contrast with other studies that observed similar or higher levels of genomic instability in breast metastases, the discordant patterns of genetic alterations detected by AI supports a model in which additional genetic alterations may be necessary for disseminated breast cells to reach their full metastatic potential. As many genomic changes in the primary tumor may evolve independently after metastatic cells have departed, genetic profiling of the primary tumor may not adequately predict metastatic behavior. The application of global profiling technologies may identify specific molecular alterations that confer upon metastatic breast cells the ability to thrive in a foreign environment, and allow for development of novel diagnostics targeted to metastatic tumors.

Materials and Methods

Paraffin-embedded primary breast and matched axillary lymph node tumor tissues from 25 patients were obtained from the Windber Medical Center Pathology Department or the Clinical Breast Care Project (CBCP) Pathology Laboratory. Samples from the Windber Medical Center (n = 8) were archival in nature and anonymized with no links between the assigned research number and patient identifiers. Clinical information including age at diagnosis, estrogen receptor/ progesterone receptor status, disease recurrence, and cause/date of death was provided anonymously by the Memorial Medical Center Cancer Registry. Tissue and blood samples from CBCP patients (n = 17) were collected with approval from the Walter Reed Army Medical Center Human Use Committee and Institutional Review Board. All subjects enrolled in the CBCP voluntarily agreed to participate and gave written informed consent to participate and gave written informed consent to participate.

![FIGURE 1. Patterns of AI in primary breast (PBT) and matched metastatic lymph node (MLN) tumors from two patients with stage IIIA invasive ductal carcinoma. Black squares, AI; white squares, normal chromosomal content. Patient 5 showed one of the highest rates of discordance (59%) in this data set. Patient 9 showed a similar overall AI frequency as patient 5; however, the frequency of discordant events was much lower (20%).](mcr.aacrjournals.org)
Demographic and clinical information was provided for all CBCP samples using questionnaires designed by and given under the auspices of the CBCP. Diagnoses of all tumor samples were made by one pathologist from H&E-stained slides using the new guidelines defined by the AJCC Cancer Staging Manual (25). Specimens included stage IIA (n = 6), stage IIB (n = 7), stage IIIA (n = 9), and stage IIIC (n = 3) infiltrating ductal or lobular carcinomas. In addition, five stage I to IIA primary breast tumors with negative lymph nodes were collected as control cases. Clinical information for all samples with positive lymph nodes is summarized in Table 2.

DNA was obtained from homogeneous populations of primary breast and metastatic axillary lymph node tumor cells following laser-assisted microdissection on an ASLMD laser microdissection system (Leica Microsystems, Wetzlar, Germany; ref. 26). All microdissected sections were examined by the CBCP pathologist who identified and marked regions of tumor before dissection. The integrity of multiple serial sections was established by pathologic verification of the first and last sections stained with H&E. Because small quantities of DNA isolated from formalin-fixed paraffin-embedded specimens have been associated with PCR artifacts (27), a minimum of 250 cells were used in every PCR reaction. To achieve this cell count, 5,000 cells were captured from each of six consecutive primary breast tumor sections, with the sixth section reserved for all confirmatory reruns. To achieve a similar cell count from the lymph nodes, cells were isolated and pooled from four consecutive sections, with sections 21 to 24 reserved for confirmations. Reference DNA samples for the archive samples were extracted from disease-free skin (nipple) tissue from each patient using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Reference DNA for the CBCP samples was obtained either from blood clots using Clotspin and Puregene DNA purification kits (Gentra, Minneapolis, MN), or from microdissected disease-free breast tissues.

Microsatellite markers (Table 1) were amplified as previously described (28), purified using Sephadex G-50 resin and genotyped on a MegaBACE-1000 capillary electrophoresis apparatus (Amersham Biosciences, Piscataway, NJ) following standard protocols. Genotypes were determined using Genetic Profiler version 2.0 software with alphabetical bin labels that facilitated accurate allele calling (Fig. 4). AI was detected using the formula (T1 / T2) / (N1 / N2) where T1 and N1 represent the peak heights of the less intense alleles and T2 and N2 represent the peak heights of the more intense alleles of the tumor and referent samples, respectively (29).

Threshold values for determining AI in the literature vary widely from 20% to 75% change in tumor allele intensity.
Data were analyzed using peak height ratios of <0.50 and <0.35, and although <0.50 identified more AI events, the threshold value for detecting AI was set at 0.35 as this more stringent ratio was more robust to confirmatory analyses.

Each chromosomal region was represented by two polymorphic markers, permitting analysis by marker and by chromosomal region. When analyzed by region, AI was defined according to the following criteria: (a) when at least one marker for a given region showed an allelic ratio of <0.35,
the region was considered to show AI; (b) when neither marker had an allelic ratio of $<0.35$ and at least one marker was informative, the region was considered normal; and (3) when both markers were homozygous, the region was considered uninformative.

Comparison of the overall frequency of AI between breast and lymph node tumors was done using Student’s $t$ test; all other analyses used the Exact Unconditional Homogeneity/Independence Tests for $2 \times 2$ tables (http://www4.stat.ncsu.edu/~berger/tables.html). A significance value of $P < 0.05$ was used for all analyses.

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References
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