Combined Total Genome Loss of Heterozygosity Scan of Breast Cancer Stroma and Epithelium Reveals Multiplicity of Stromal Targets

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Abstract

Recent breast cancer studies have highlighted the importance of interactions between cancer epithelium and tumor stroma. Recently, the focus of solid tumor investigations has shifted from mutations in carcinomatous epithelium to disturbances of tissue organization in cancer. The genetic basis of this microenvironment, however, remains to be clarified. To begin to resolve this problem, a total genome loss of heterozygosity (LOH) scan was done on epithelial and stromal DNA from 134 sporadic invasive breast carcinomas. In addition to detecting more frequent LOH at three loci in stroma than in epithelium, we found strong evidence that LOH frequencies were significantly elevated in specific regions of each chromosome. We detected 57 markers, which were preferentially lost either in stroma (n = 38) or epithelium (n = 19), relative to the background LOH frequencies on their respective chromosomes. This multiplicity of stromal cell LOH, and hence loss of genetic material, provides a possible mechanism for interpatient variation in host-stromal response to invading adenocarcinoma cells. This is consistent with a model in which initial, random LOH occurs equally among epithelium and stroma, but subsequent clonal selection is driven by factors, which appear to be distinctly different between malignant epithelial and surrounding stromal cells. Genetic alterations in stroma did not mimic those in epithelium, but they could play a different and parallel role in carcinogenesis and tumor progression, probably by modifying some features specific to breast cancer.

Introduction

Breast cancer is the most common women’s cancer in the Western hemisphere, occurring in one in eight females. Although germ-line mutations in BRCA1, BRCA2, TP53, and PTEN tumor suppressor genes are associated with various inherited breast cancer syndromes, they are rarely mutated in sporadic breast cancers (1). Hemizygous deletion of tumor suppressor genes is an alternative mechanism of tumor suppressor inactivation more commonly invoked in a sporadic disease setting (2). Now it is recognized that dynamic epithelial-stromal interactions in solid tumors may select subsets of stromal cells with the ability to modulate tumor behavior, and the local microenvironment promotes emergence of tumor-associated stromal cells with different functions from normal stroma (3–5). These functional changes of stroma may partly be derived from the changes in secretion of growth factors and in the extracellular matrix (6, 7). Genetic changes in tumor stroma are another mechanism that may create functional divergence of tumor stroma from normal stroma and of interpatient diversity of host response. This possibility has been experimentally validated by several independent studies showing the presence of loss of heterozygosity (LOH) and somatic mutations of tumor suppressor genes such as PTEN and TP53 in tumor stroma (8–12). Because these genetic alterations can be distinct from and, hence, independent of those in adjacent malignant epithelium, the epithelial and stromal elements are clearly not derived from a common progenitor clone but rather undergo similar selective pressures in the tumor microenvironment (9, 13). With laser capture microdissection and multiplex-PCR genome-wide LOH mapping, it is now possible to systematically examine the differences in the extent and pattern of LOH between stroma and epithelium. We therefore used a 381-microsatellite marker whole genome LOH scan to analyze global genomic alterations between carcinomatous epithelium and tumor stroma from 134 sporadic invasive breast carcinoma samples.

Materials and Methods

Breast Carcinoma Samples and Laser Capture Microdissection. A total of 134 samples of clinically sporadic invasive breast cancers was obtained from the Departments of Pathology from The Ohio State University and the Brigham and Women’s Hospital, under approval from their respective Institutional Review Boards. To obtain tumor epithelium and tumor stroma, laser capture microdissection was done with the Arcturus PixCell II microscope (Arcturus Engineering, Inc., Mountain View, CA; ref. 9). Corresponding normal tissues were procured from separate blocks diagnosed as containing no carcinomatous tissue (first choice) and, if not possible, from nonneoplastic tissues at a distance from the cancer, which were separated from the carcinomatous tissues by normal fat-tissue layers.

Total Genome LOH Scan. Genomic DNA was extracted as previously described (9, 13), with the exception that incubation in proteinase K was done at 65°C for 2 days. The primer sets for multiplex PCR defined 386 microsatellite loci in 72 multiplex panels (Research Genetics, Invitrogen, Carlsbad, CA). Genotyping was done with the ABI 377xl or 3700 semiautomated sequencer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT). The results were analyzed by automated fluorescence detection using the GeneScan collection and analysis software (GeneScan; ABI, Norwalk, CT). Scoring of LOH was done by inspection of the GeneScan output. A ratio of peak heights of alleles between germ-line and somatic DNA ≥ 1.9:1 was used to define LOH in this study (9). This process was validated in several ways (see supplement for details).

Statistical Analyses. Five markers were excluded from additional statistical analyses because each of these markers was noninformative in all epithelial samples or all stromal samples. For LOH frequencies, we used sample average LOH frequencies and model-based analysis. To account for a number of features such as the intra-sample correlations and varying numbers and sets of samples for which each marker is informative, we used marginal models. The inference resulting from marginal models is robust compared with sample average LOH frequencies. Associations among LOH events were explored using clustering methods. All data analysis was done with the statistical

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Note: K. Fukino and L. Shen contributed equally to this work and should be considered joint first authors. Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

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7231

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Results

Comparison of Sample-Wise Average LOH Frequencies and Overall LOH Frequencies for Each Chromosome between Epithelium and Stroma. Using 381 markers across all chromosomes, ranging from 7 (chromosome 21) to 31 (chromosome 1) markers/chromosome, 43,839 (22,409 for epithelium and 21,430 for stroma) PCR reactions were informative for evaluation of LOH. In total, 14,310 (32.6%) (8,231 in the epithelium and 6,079 in the stroma) LOH events occurred. The average LOH frequency per marker ranged from 3.8 to 77.6% (a median of 42.0%) in epithelium and from 1.5 to 70.0% (a median 34.1) in stroma (Fig. 1A). The average LOH frequencies over entire chromosomes ranged from 30.2 to 43.6% in the epithelium and from 22.9 to 33.4% in the stroma. Moreover, we obtained the estimated LOH probabilities based on a marginal model. Both the sample average LOH frequencies and the model-based LOH probabilities indicated higher values in epithelium than in stroma for all chromosomes at the individual marker level (Fig. 1B), although the differences are not statistically significant at the 0.05 level for chromosomes 2, 3, 6, 7, 10, 14, 19, and 20, i.e., for these latter chromosomes, LOH frequencies were similar in the epithelium and in the stroma at the individual marker level.

Comparison of the Marker-Wise LOH Frequencies between Epithelium and Stroma. For the majority of markers, the average LOH frequencies at each marker were higher in epithelium than in stroma (Fig. 1C). In contrast, the formal marker-level comparisons between LOH probabilities in the epithelium and stroma, by fitting a marginal model for each chromosome, detected three markers representing significantly higher LOH probabilities in stroma (Table 1A), compared with 87 markers in epithelium at the p 0.05 level (Fig. 1D).

Global Comparison of LOH Frequencies among Chromosomes between Epithelium and Stroma. Here, we first performed a simple per marker comparison of LOH frequencies between epithelium and stroma postulating that these data may reflect the cell type-specific differences in rate of cell division, apoptosis, or response to carcinogenic factors. The simple comparison of LOH frequencies between epithelium and stroma may also reflect the chromosome-specific differences such as those of chromosomal size. If the latter were true, then the null hypothesis that the LOH probability would remain constant over each entire chromosome would hold true. However, our global tests on all chromosomes in both epithelium and stroma rejected this null hypothesis, suggesting that LOH across chromosomes was not random in both compartments. To detect LOH hot spots, representing a significantly higher LOH frequency than the rest of the markers on the same chromosome in either epithelium or stroma, we fitted the marginal models that used chromosome-wide LOH averages as a baseline for comparison of single average LOH frequencies. Overall, we obtained 38 markers from 19 chromosomes in stroma and 19 different markers from 15 chromosomes in epithelium, respectively, which may be preferentially lost compared with the other markers on the same chromosomes (Table 1B).

Interactions of LOH Events at Various Loci in Epithelial and Stromal Compartments. To investigate interactions between LOH events, we focused on our 57 LOH hot spot markers (Table 1B) as a first working model. Patterns of association between specific deleted loci are illustrated in a dendrogram resulting from hierarchical agglomerative clustering of the dissimilarity matrix (Fig. 2A) and as the distances in intuitive two-dimensional plot (Fig. 2B). It would be important to note that LOH at any particular marker in epithelium and in stroma did not necessarily cluster together.

Discussion

Although many studies focused on genetic alterations in carcinomatous epithelium, genetic changes in a relatively limited number of tumor-associated genes cannot explain many human in vivo observations such as interpatient variations in tumor behavior and treatment response despite seemingly identical epithelial alterations. It is now slowly being recognized that interactions between carcinomatous epithelium and surrounding tumor stroma can account for such interpatient diversity (14). Very recent in vitro work showed that the tumor stroma is a target of carcinogens and modulates the growth and oncogenic potential of adjacent epithelium (15, 16). Moreover, it was revealed that the corrections of defective signaling in the tumor microenvironment can revert the tumor cells to a normal phenotype (17).

In our attempt to explore the genetic basis of the tumor microenvironment, global genome LOH analysis in the epithelium and stroma of 134 sporadic invasive breast carcinoma analyzed by several different informatic approaches, including a novel model-based algorithm, reveal several interesting human cancer genetic-based insights. First, our LOH data provided strong evidence that LOH, either in stroma or epithelium, was not uniform (i.e., not completely random) along any of the chromosomes. In other words, we are confident that along each chromosome, some regions were preferentially lost compared with the other regions on the same chromosomes. By using chromosome-wide LOH averages as a baseline for comparison of observed single locus LOH frequencies in stroma and epithelium separately, we detected LOH hot spots specific to each of stroma and epithelium. Thus, we obtained 38 markers as LOH hotspots in the stroma, compared with 19 LOH hot spot markers in the epithelium (Table 1B). A recent study on the molecular basis of the tumor microenvironment in breast cancer detected no genetic alteration in tumor “stroma” with the different technologies from ours, i.e., array comparative genomic hybridization (aCGH) and single nucleotide polymorphism array (18). The discrepancy between the results of the previous study (18) and ours may be derived from technology-specific differences. We specifically captured stromal fibroblasts adjacent to malignant epithelium (i.e., the tumor stroma) under direct microscopic observations, whereas the aCGH/single nucleotide polymorphism study analyzed all of the stromal cells in a CD10-enriched fraction following the removal of other cells by using cell-type specific cell surface markers (18). In the latter (18), normal stromal cells intervening among tumor stroma may be included into the stromal samples and/or the very cells that carry the genetic alterations may have been selected out, thus accounting for the false negative results. The differences in the resolutions or the threshold values between the previous aCGH/single nucleotide polymorphism study and ours may also explain the discrepancy between the results. Taken together, this previous study (18) and our current one therefore suggest that stromal LOH must involve region lengths under the resolution of aCGH and that the genetic alterations may only be limited to the true tumor stroma. These are plausible explanations because in addition to the present LOH-based study, previous LOH studies by our group and several independent groups not only show the existence of LOH in the stroma of breast cancer but also in tumor stroma from colon, bladder, and ovarian cancers (8–12, 19), thus validating and reconfirming the existence of genetic alterations in stroma. More importantly, we and other independent groups (10, 12, 13) have showed the existence of somatic mutations in TP53 and/or PTEN in tumor stroma.

For validation of our LOH hot spots, we compared our results and
the raw (not averaged) data from the previous CGH study (20). In that CGH study (20), although without distinction between epithelium and stroma, 27 (87.1%) of 31 cases showing segmental DNA loss revealed that at least one of their lost segments contained the markers belonging to the 57 hot spot markers, suggesting that these markers may be preferentially lost compared with the other markers on the same chromosome in breast cancer, even if they may not be distinguished by LOH frequency with the conventional simple average comparisons.

Moreover, our results suggested, surprisingly, that the stromal LOH
A. Information on three markers that show significantly higher LOH probabilities in the stroma than in the epithelium (Table 1).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Proportion of LOH in EP</th>
<th>Proportion of LOH in ST</th>
<th>Number of LOH in both EP and ST</th>
<th>Number of LOH in EP but not in ST</th>
<th>Number of LOH in ST but not in EP</th>
<th>Number of LOH in neither EP nor ST</th>
<th>P value for difference between EP and ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATA79C10</td>
<td>1p35.1</td>
<td>0.229</td>
<td>0.400</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>0.0262</td>
</tr>
<tr>
<td>D6S594</td>
<td>6p25.3</td>
<td>0.250</td>
<td>0.469</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>0.0365</td>
</tr>
<tr>
<td>DSS1136</td>
<td>8q13.1</td>
<td>0.310</td>
<td>0.517</td>
<td>10</td>
<td>5</td>
<td>19</td>
<td>20</td>
<td>0.0085</td>
</tr>
</tbody>
</table>

B. Information on 19 markers in the epithelium and 38 markers in the stroma that show elevated LOH probabilities compared with the other markers on the same chromosome.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Proportion of LOH in EP</th>
<th>Proportion of LOH in ST</th>
<th>Model-based estimate for LOH probability</th>
<th>P value for difference between EP and ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM11532</td>
<td>1q21</td>
<td>0.440</td>
<td>0.591</td>
<td>4.42E-04</td>
<td></td>
</tr>
<tr>
<td>D2S15790</td>
<td>2p11.2</td>
<td>0.418</td>
<td>0.587</td>
<td>7.88E-05</td>
<td></td>
</tr>
<tr>
<td>D2S1790</td>
<td>2p11.2</td>
<td>0.418</td>
<td>0.587</td>
<td>7.88E-05</td>
<td></td>
</tr>
<tr>
<td>D2S1776</td>
<td>2q24.3</td>
<td>0.313</td>
<td>0.588</td>
<td>2.97E-04</td>
<td></td>
</tr>
<tr>
<td>D3S2398</td>
<td>3q28</td>
<td>0.485</td>
<td>0.608</td>
<td>4.21E-06</td>
<td></td>
</tr>
<tr>
<td>D4S2368</td>
<td>4q23.3</td>
<td>0.358</td>
<td>0.587</td>
<td>2.18E-03</td>
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<tr>
<td>D5S1050</td>
<td>5q23.1</td>
<td>0.582</td>
<td>0.548</td>
<td>8.29E-04</td>
<td></td>
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<tr>
<td>D9S930</td>
<td>9q32</td>
<td>0.410</td>
<td>0.641</td>
<td>2.97E-05</td>
<td></td>
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<tr>
<td>D10S1423</td>
<td>10p12.31</td>
<td>0.381</td>
<td>0.567</td>
<td>1.01E-03</td>
<td></td>
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<tr>
<td>D11S2002</td>
<td>11q14.1</td>
<td>0.396</td>
<td>0.598</td>
<td>1.77E-04</td>
<td></td>
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<tr>
<td>D11S1986</td>
<td>11q23.1</td>
<td>0.478</td>
<td>0.548</td>
<td>1.40E-03</td>
<td></td>
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<tr>
<td>D12S297</td>
<td>12q13.13</td>
<td>0.425</td>
<td>0.540</td>
<td>8.56E-04</td>
<td></td>
</tr>
<tr>
<td>D13S894</td>
<td>13q13.3</td>
<td>0.500</td>
<td>0.628</td>
<td>7.37E-06</td>
<td></td>
</tr>
<tr>
<td>D13S793</td>
<td>13q21.1</td>
<td>0.231</td>
<td>0.748</td>
<td>1.26E-03</td>
<td></td>
</tr>
<tr>
<td>D15S822</td>
<td>15q21</td>
<td>0.493</td>
<td>0.570</td>
<td>1.02E-03</td>
<td></td>
</tr>
<tr>
<td>D17S1298</td>
<td>17p13.2</td>
<td>0.373</td>
<td>0.639</td>
<td>1.53E-03</td>
<td></td>
</tr>
<tr>
<td>D17S874</td>
<td>17q13.1</td>
<td>0.545</td>
<td>0.621</td>
<td>3.69E-04</td>
<td></td>
</tr>
<tr>
<td>GATA178F11</td>
<td>18p11.32</td>
<td>0.329</td>
<td>0.549</td>
<td>3.82E-03</td>
<td></td>
</tr>
<tr>
<td>D19S714</td>
<td>19p13.12</td>
<td>0.373</td>
<td>0.637</td>
<td>1.50E-04</td>
<td></td>
</tr>
<tr>
<td>D20S480</td>
<td>20q13.2</td>
<td>0.343</td>
<td>0.479</td>
<td>3.53E-03</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. The last column shows the P value in the comparison between this marker and the other markers on the same chromosome.

Abbreviations: LOH, loss of heterozygosity; EP, epithelium; ST, stroma.

hot spots were more numerous and widely distributed compared with the epithelial ones. Therefore, LOH in the epithelial cells may be intensely concentrated in a smaller number of loci, whereas in the stromal cells, the pattern was more spatially complex, being distributed over a larger number of loci. This suggests that clonal selection in malignant epithelial cells is strongly biased toward a smaller subset of loci, compared with stromal cells. These observations allow us to propose a model of carcinogenesis by a limited set of genetic alterations, perhaps targeting a limited number of tumor suppressor genes, in the epithelium (Fig. 2C). These would cause transformation. Then, interpatient diversity by the multiplicity and larger variety of stromal LOH targets would lead to both biological and clinical inter-patient variations (Fig. 2C).

The individual marker-wise analysis between stroma and epithelium, and the global LOH comparison along chromosomes in either stroma or epithelium address different aspects of carcinogenesis with respect to the two compartments. The basis of greater LOH frequencies in epithelium than in stroma at each marker is not known, but possibilities include cell type-specific differences in de novo deletion rates, rate of cell division, or response to clonal selection factors.

The LOH noted on 11q are worthy of comment. Chromosome arm 11q contained six LOH-hot spot markers (four in stroma and two in epithelium), suggesting the existence of three distinct deleted regions (two in stroma and one in epithelium; Fig. 3). The 44-Mb deleted region in epithelium intervened between the proximal 29-Mb- and the distal 11-Mb–deleted regions in stroma. Each of these deleted regions included some known tumor suppressor genes (Fig. 3). In particular, the matrix metalloproteinase (MMP) gene cluster was included in the epithelial deleted region. As MMPs regulate the microenvironment of breast cancer by degrading extracellular matrix, epithelial LOH in the MMP cluster region could perturb the microenvironment.

According to the results of cluster (Fig. 2A and unpublished data) and other analyses, the differences of LOH patterns between matched epithelium and stroma were greater (e.g., different allele lost between epithelium and stroma or LOH in one but not the other) than what would be expected from epithelial-mesenchymal transition and exclude the passive transfer of naked DNA between epithelial and stromal cells. Our data, therefore, lend human genetic evidence that the epithelial and stromal cells can originate along two independent paths.
In summary, this study revealed that LOH hot spots in stroma are more numerous and widely distributed than those in the neoplastic epithelium. In other words, stroma is rich in the multiplicity of genetic-alteration targets compared with the epithelium. We suggest that it is the diversity and multiplicity of stromal targets that may contribute to interpatient diversity in biological behavior and clinical outcome in breast cancer. Furthermore, the genetic alterations in stroma did not necessarily mimic those in epithelium but could play a different and parallel role in carcinogenesis and tumor progression. We suspect that global genetic alterations in solid tumor stroma will be an important universal mechanism for tumor invasion and progression as evidenced by independent preliminary data documenting somatic mutations and/or LOH in the stroma of colon cancer (10), bladder cancer (12), and ovarian cancer (19).
The cluster of in stroma (f/18554) and one in epithelium (existence of three distinct target regions of genetic alterations were suggested, with two stroma (in solid-line boxes) and two in epithelium (in dotted-line boxes)) and two in epithelium (in breast cancer suppressor candidate 1), PPP2R1B (serine/threonine protein phosphae 2A b-subunit), SDHD (succinate dehydrogenase subunit D), PGR (progesterone receptor), MMP (matrix metalloproteinase) genes. See text for details.

Fig. 3. Three distinct regions of LOH of markers in 11q, two in stroma, and one in the epithelium. Chromosome arm 11q contained six multiplicity-identified markers, four in stroma (in solid-line boxes) and two in epithelium (in dotted-line boxes), respectively. The existence of three distinct target regions of genetic alterations were suggested, with two in stroma ( and one in epithelium ( ), respectively. The cluster of MMP genes was not located in the stromal deleted region but in the epithelial deleted region. MEN1 (susceptibility gene for multiple endocrine neoplasia type 1), RARRES3 (retinoic acid receptor responder 3), BCSC-1 (breast cancer suppressor candidate 1), THY1 (Thy1; a T-cell marker), ATM (ataxia-telangiectasia mutated), PPP2R1B (serine/threonine protein phosphatase 2A b-subunit), SDHD (succinate dehydrogenase subunit D), PGR (progesterone receptor), MMP (matrix metalloproteinase) genes. See text for details.

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