Genomic (In)stability of the Breast Tumor Microenvironment

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Abstract

The breast tumor microenvironment plays an active role in tumorigenesis. Molecular alterations have been identified in tumor-associated stroma; however, there is considerable debate as to whether the stroma is characterized by genomic instability or whether detection of chromosomal alterations reflects technological artifact rather than the true genomic content of the tumor microenvironment. Thus, breast stroma specimens from 112 women undergoing reductive mammoplasty ($n = 7$), prophylactic mastectomy ($n = 6$), or mastectomy for a breast disease ($n = 99$) were frozen in optimal cutting temperature medium. Allelic imbalance (AI) analysis was conducted using a panel of 52 microsatellite markers in 484 stromal specimens from 98 women, of which 92% had no detectable AI events. When compared with previously generated AI data from 77 formalin-fixed, paraffin-embedded (FFPE) stroma specimens, 42% of which harbored at least one detectable AI event, the frequency of AI in the FFPE specimens (4.62%) was significantly higher ($P < 0.001$) than that found in frozen specimens (0.45%). This comparison of AI between FFPE and research-grade specimens suggests that past reports of AI in breast stroma reflect artifact in the archival specimens caused by formalin-fixation, paraffin-embedding and tissue storage. Furthermore, SNP data were generated from a subset of 86 stromal specimens using SNP arrays and copy number alterations were identified using Partek Genomics Suite. For 95% of the specimens, no detectable copy number alterations were found and the 11 changes that were detected were small and not shared between specimens. These data, therefore, support a model in which the tumor microenvironment is genetically stable. *Mol Cancer Res; 10(12); 1526–31. ©2012 AACR.*

Introduction

Research in the past decade has increased our understanding of how the breast microenvironment influences tumor development. Stroma, composed of fibroblasts, endothelial, smooth muscle, inflammatory and nerve cells, adipocytes, and macromolecules of the extracellular matrix, serves a supportive role to epithelial cells (1). A number of physiologic changes have been detected in stroma surrounding epithelial neoplasms including altered migratory potential and changes in expression of growth factors and cellular polarity, degradation of the extracellular matrix, and increased angiogenesis (1–5).

One of the hallmarks of breast carcinogenesis is global genomic instability, which may include copy number gains or losses of whole or parts of chromosomes. Over 10 years ago, chromosomal alterations were detected as loss of heterozygosity (LOH) or allelic imbalance (AI) events in morphologically normal terminal ductal lobular units adjacent to breast carcinomas (6). A number of additional studies, the majority of which were conducted using microsatellites markers and formalin-fixed, paraffin-embedded (FFPE) archival specimens, have also detected genomic instability in tumor-associated stroma, furthering the notion that the tumor microenvironment is a dynamic entity that contributes to tumorigenesis through morphologic and molecular alterations (7–12). In contrast, recent studies using DNA from fresh frozen tissues that failed to detect chromosomal changes in stromal cell, leading some investigators to speculate that the LOH/AI studies conducted on DNA from FFPE specimens do not accurately reflect the chromosomal content of cells of the tumor-associated stroma but rather are the result of technical artifacts resulting from the use of microsatellite assays and fixation methods (13–19).

Determination of whether the breast microenvironment is genetically stable is not merely an academic pursuit; genomic instability in tumor-associated stromal cells may impact clinical care for patients with breast cancer. For example, stroma adjacent to breast carcinomas from patients treated with chemotherapy showed increased and persistent LOH posttreatment, especially in those patients who were non-responsive to treatment, suggesting that patients with stable stroma may be more likely to benefit from chemotherapy than those with unstable stroma (20). Likewise, stromal instability may have important ramifications for defining

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surgical margins: the presence of LOH on chromosome 3p in normal terminal ductal lobular units adjacent to early-stage breast carcinomas was associated with a 3.9- to 5.2-fold increased risk of recurrence (21).

To determine whether breast tumor stroma is genetically stable, high-quality breast specimens from mastectomy and reductive mammoplasty were frozen in optimal cutting temperature compound. AI events were detected using the same 52-marker microsatellite panel used in earlier studies that detected AI in FFPE stroma adjacent to and distant from tumors. A subset of high-quality specimens was also subjected to copy number analysis using single nucleotide polymorphism (SNP) array data. These analyses showed that AI levels were significantly higher in FFPE compared with frozen specimens and that stroma from both diseased and nondiseased breasts is genetically stable.

Materials and Methods

Human tissue samples

Enrollment of patients into the Clinical Breast Care Project began in 2001. For inclusion in the Clinical Breast Care Project, all patients must have met the following criteria: (i) adult more than the age of 18 years, (ii) mentally competent and willing to provide informed consent, and (iii) presenting to the breast centers with evidence of possible breast disease, for routine screening mammograms or elective reductive mammoplasty. Tissue and blood samples were collected with approval from the Walter Reed Army Medical Center Human Use Committee and Institutional Review Board (Washington, DC). All subjects enrolled in the Clinical Breast Care Project voluntarily agreed to participate and gave written informed consent. Clinical information was obtained for all samples using questionnaires designed by and administered under the auspices of the Clinical Breast Care Project.

Stroma was collected from patients undergoing surgical procedures, including reductive mammoplasty, prophylactic

mastectomy for *BRCA1* (GenBank:NM_007294) or *BRCA2* (GenBank:NM_000059) mutations and single or double mastectomy after a breast disease diagnosis. Within 5 to 15 minutes of surgical removal, breast tissue was taken on crushed, wet ice to the pathology laboratory where a licensed pathologist or pathologists' assistant conducted routine pathology analyses (gross characterization, margin status assessment, and other indicated purposes). Excess stromal tissues were frozen in optimal cutting temperature medium (Sakura Finetek) on dry ice. Once preserved, frozen tissue samples were stored in liquid nitrogen freezers. All sections in this study were examined by a dedicated pathologist, who diagnosed all regions of disease within the specimen. The integrity of multiple serial sections was established by pathologic verification of the first and last sections stained with hematoxylin and eosin (Fig. 1). Specimens harboring invasive, *in situ*, or atypical ductal hyperplasia were excluded from analysis. Samples with benign diagnoses such as fibrocystic changes, stromal fibrosis, cysts, and microcalcifications were evaluated for AI and/or copy number alterations.

DNA isolation

Excess freezing-medium was trimmed from each specimen before cutting 10–20 twelve micrometer sections on the CM3050 S Cryostat (Leica Microsystems). The 200 to 400 mg of tissue was homogenized using the PRO250 laboratory homogenizer (PRO Scientific Inc.) for 30 to 60 seconds and processed using the Gentra Puregene Tissue kit (Qiagen) according to manufacturer's protocol. Genomic DNA, used as the referent, was isolated from blood clots using Clotspin and Puregene DNA purification kits (Qiagen) according to manufacturer's protocols.

AI analysis

Microsatellite markers were amplified as previously described (22). Genotypes were determined using Genetic
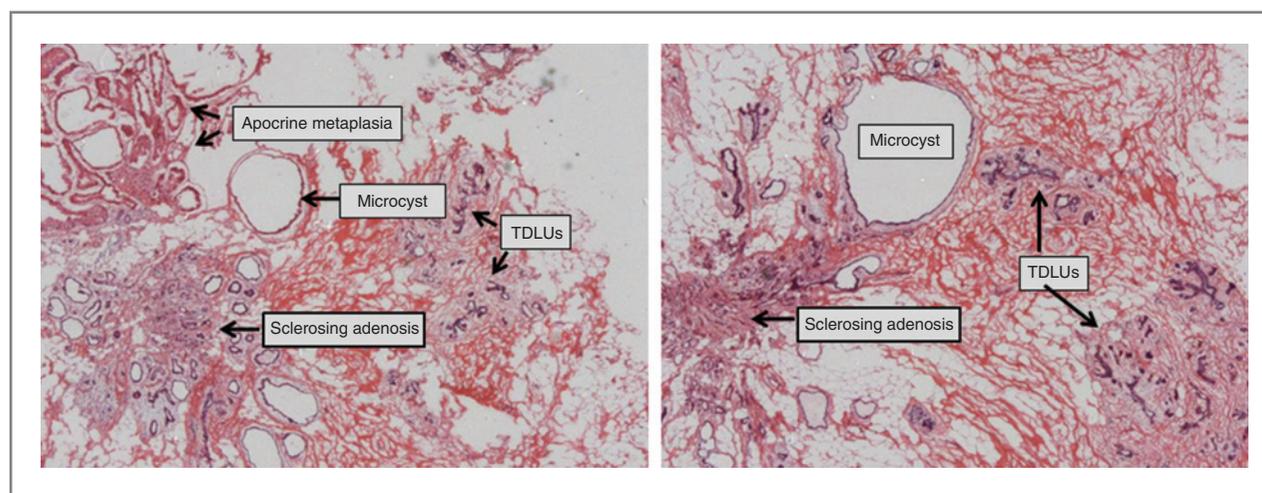

Figure 1. Hematoxylin and eosin–stained images of representative breast stroma sections from the upper outer quadrant of patient 19. The image on the left is an image from the original section; that on the right represents the final cut, taken after the intervening 240 μm were used for DNA isolation.

Profiler version 2.0 software and AI 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 (23). Each chromosomal region was represented by 2 polymorphic markers and AI was defined according to the following criteria: (i) when at least one marker for a given region showed an allelic ratio of 0.35 or less, the region was considered to show AI, (ii) when neither marker had an allelic ratio of 0.35 or less and at least one marker was informative, the region was considered normal, and (iii) when both markers were homozygous, the region was considered uninformative. Given the unequal variance in AI levels between the FFPE and frozen groups, differences in AI frequency between groups were assessed using a Wilcoxon rank sum test, using a 2-tailed $P < 0.05$ to define significance.

Copy number analysis

To generate SNP data, 250 ng of stroma or genomic DNA was hybridized to GeneChip Human Mapping 250K Sty arrays (Affymetrix) following manufacturer's protocols. SNP data was imported into Partek Genomics Suite 6.5 (Partek, Inc) as CEL files using default Partek parameters. Copy number was calculated as a paired analysis using the genomic DNA from each patient as the referent sample. Deletions and amplifications were detected using a Hidden Markov Model using a minimum of 5 consecutive altered markers. CEL files and copy number data available at http://www.ncbi.nlm.nih.gov/geo/ accession number GSE38071.

Results

Sample characteristics

Samples were collected from patients undergoing reductive mammoplasty ($n = 7$), prophylactic mastectomy after identification of a deleterious *BRCA1* or *BRCA2* mutation ($n = 6$), mastectomy ($n = 55$), or double mastectomy ($n = 44$) for a diagnosis of invasive or *in situ* breast disease; of the patients with invasive breast cancer, *BRCA1* mutations were identified in 6 patients, *BRCA2* in 4 patients and a seventh patient had a *TP53* (GenBank:NM_000546) mutation resulting in Li-Fraumeni syndrome. For patients undergoing mastectomies, stroma samples from the upper outer, upper inner, lower outer and lower inner quadrants, as well as a piece from the central area of the breast were collected whenever possible. Seven patients with invasive breast cancer were treated with neoadjuvant therapy. Single stromal samples were collected from reductive mammoplasties. In total, 523 breast stroma specimens were subjected to AI ($n = 484$) and/or SNP copy number analysis ($n = 86$).

Patients undergoing reductive mammoplasty and prophylactic mastectomy were significantly younger ($P < 0.0001$) than those with breast disease; the average ages were 37.8 years (range 25–48 years), 36.2 years (range 23–42 years), and 53.51 (range 25–82 years) for reductive mammoplasty, prophylactic mastectomy, and mastectomies, respectively (Table 1). Ethnicity did not differ significantly between groups, with the majority being self-described as white. Within the reductive mammoplasty group, 2 women had fibroadenoma, one had fibrocystic changes, and 4 had no abnormalities detected; none of these women have returned to the breast clinic for subsequent breast disease. Disease progression and breast cancer mortality did not differ significantly between women with prophylactic mastectomy and those undergoing mastectomy.

Allelic imbalance in research-grade breast stroma specimens

In 484 stromal specimens, 53 AI events were detected; these alterations were detected in 40 of 484 (8%) breast

Table 1. Patient characteristics from 112 women with frozen breast stroma

|	Invasive/*in situ* disesae		Prophylactic ($n = 6$)	Reduction ($n = 7$)
	Mastectomy ($n = 55$)	**Double mastectomy** ($n = 44$)	**Prophylactic** ($n = 6$)	**Reduction** ($n = 7$)
Age diagnosis				
<40 years	0.12	0.16	0.83	0.71
40–49 years	0.24	0.32	0.17	0.29
≥50 years	0.64	0.52	0.00	0.00
Ethnicity				
Caucasian	0.60	0.79	0.67	0.50
African American	0.29	0.16	0.17	0.33
Hispanic	0.06	0.00	0.00	0.00
Asian	0.05	0.03	0.00	0.17
Other	0.00	0.02	0.16	0.00
Disease status				
Disease-free	0.93	0.84	1.00	NA
Progression	0.02	0.09	0.00	NA
Died of disease	0.05	0.07	0.00	NA
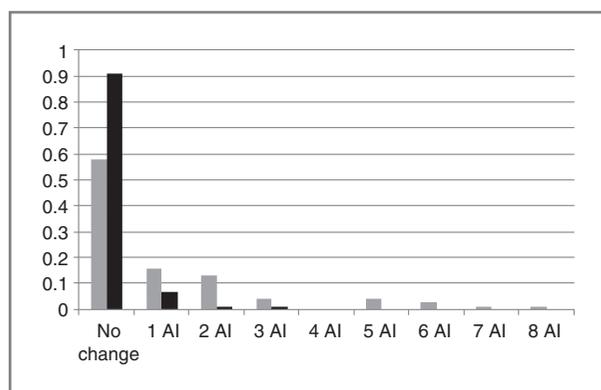
Figure 2. Graph of the number of AI events in FFPE ($n = 77$) compared with frozen ($n = 484$) breast specimens. Gray and black bars represent number of AI events in frozen and FFPE specimens, respectively. The number of specimens with no AI detected was significantly ($P < 0.001$) higher in frozen (91%) compared with FFPE (58%) stromal specimens.

Table 2. Chromosomal regions with altered copy number in breast stroma specimens

Specimen	Copy number	Location	Size
1 upper outer	2.8	1q42.2-1q43	2.3 Mb
1 upper outer	2.6	8p22-8p21.3	2.6 Mb
1 upper outer	5.5	10q26.11-10q26.13	5.1 Mb
1 lower inner	2.6	1q32.1	2.6 Mb
1 lower inner	2.7	9q33.3-9q34.11	2.7 Mb
1 lower inner	3.1	10q22.3	0.7 Mb
1 lower inner	2.6	11p15.5-11p15.4	3.0 Mb
2 upper inner	4.0	10q22.3	0.3 Mb
2 upper inner	2.8	11p11.2	0.9 Mb
2 upper inner	3.1	14q24.1	0.7 Mb
23 reductive mammoplasty	2.7	8q24.3	5.9 Mb

NOTE: One chromosomal region, 10q22.3, was amplified in 2 different stromal specimens. The shared 284 kb region harbors the genes retinoic acid–induced 17 (ZMIZ1), peptidylprolyl isomerase F precursor (PPIF), and Homo sapiens zinc finger, and CCHC domain containing 24 (ZCCHC24).

specimens and the majority (92%) of specimens harbored no detectable AI events. The average AI frequency per specimen was less than 1% and the range was 0 to 25% (median = 0%). In the 5 quadrant specimens with ductal carcinoma *in situ*, a single AI event was detected in 2 specimens with an average AI frequency of 1.6%. In the 7 specimens treated with neoadjuvant therapy, 6 patients had no detectable AI events in any of their quadrants. One patient had a single case of AI at chromosome 18q21 in the upper inner quadrant, although this event was not detected by SNP analysis.

Levels of AI were not significantly different in the breast stroma from patients who died of disease (0.6%), those with recurrence (0.2%), or those who are disease-free (0.5%). Breast stroma from patients with BRCA1 and BRCA2 mutations (0.3%) did not have higher levels of AI than those without known mutations (0.5%). Presence of AI did not differ significantly between quadrants.

The stromal specimen with the highest level of AI (25%) was from the upper outer quadrant of patient 1 who was diagnosed at age 47 with a stage II, poorly differentiated, basal-like breast tumor. The other 3 quadrants did not have detectable AI. The tumor for this patient was located in the mid-outer region. Tumor was not detected for this patient in either the first or last section; and the section was composed primarily of adipose cells. After 2 years of follow-up, this patient was disease-free.

Frequencies of AI from these research-grade frozen stromal specimens were compared with data previously generated in our laboratory from 77 FFPE archival breast quadrants (9). Forty-two (42%) archival quadrants had at least one detectable AI event and the frequency of AI in the FFPE specimens (4.62%) was significantly higher ($P < 0.001$) than that found in frozen specimens (0.45%; Fig. 2).

Copy number alterations in research-grade breast stroma

SNP array data was generated from 27 women undergoing mastectomy ($n = 18$), prophylactic mastectomy ($n = 2$), or reductive mammoplasty ($n = 7$). Of the 86 breast specimens

assayed, no copy number alterations were detected in 82 of 86 (95%). The 11 copy number changes detected in the 4 stroma specimens from 3 patients are listed in Table 2. Amplification of chromosome 10q22.3 was detected in the lower inner quadrant from patient 1 and the upper inner quadrant from patient 2.

SNP data were available from the tumor component of patient 1. In contrast to the stromal specimens that had little to no copy number alterations, the tumor was characterized by numerous amplification and deletion events detected throughout the genome (Fig. 3).
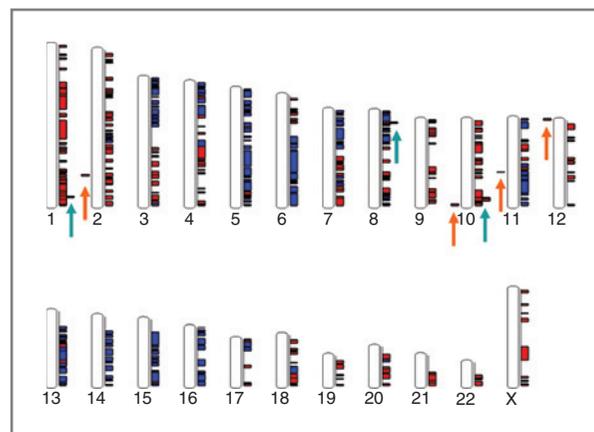

Figure 3. Chromosomal map of copy number alterations detected in tumor and stroma components from patient 1. Amplifications are depicted in red, deletions in blue. Teal and orange arrows mark copy number alterations detected in patient 1 top outer and bottom inner quadrants, respectively.

Discussion

One of the earliest studies that reported chromosomal changes in the breast tumor microenvironment assessed LOH/AI in DNA isolated from tumor epithelial cells and adjacent normal terminal ductal-lobular units from FFPE specimens. LOH/AI was detected in terminal ductal-lobular units adjacent to the tumor but not in those distant to the tumor (6). These results led to the idea that molecular data may be more accurate than surgical or pathologic data in defining clean margins. Additional data from breast stroma close to and distant from the tumor have led to clinically important conclusions such as the precession of molecular changes in stroma compared with epithelial cells, possibly facilitating tumor invasion (7, 9), identification of an array of hotspots in the stromal cells that influence variability among patients in tumor biology, response to treatment and outcome (8, 12), and that *BRCA1/2* mutations may lead to genomic instability in the stroma, promoting neoplastic transformation (11). Together, these data suggest that the tumor microenvironment is genetically unstable and this instability contributes to risk and prognosis of breast pathogenesis.

Technological improvements in identifying copy number changes and access to non-FFPE tissue specimens has generated new data that questions the conclusions, and indeed, the validity of the data generated from AI/LOH approaches. Gene expression differences were detected in purified stromal cell types (e.g., epithelial, myoepithelial and endothelial cells, leukocytes, myofibroblasts, and fibroblasts) from normal and diseased breasts; copy number alterations, while detected in the tumor epithelial cells, were not detected in stromal cells (13). In a follow-up study, stromal cells were found to be hypomethylated compared with their normal counterparts (14). In the absence of chromosomal alterations, epigenetic, rather than chromosomal, changes in stromal cells may alter the tumor microenvironment and contribute to breast tumorigenesis (15, 18). Finally, copy number analysis conducted on DNA from microdissected epithelial and cancer-associated fibroblasts failed to detect any chromosomal alterations. In addition, microsatellite analysis from a region of chromosome 11 considered a hotspot for LOH in breast stroma from earlier studies was also conducted using these DNA samples and LOH was not detected (16). The inability of these studies to detect copy number changes in breast stromal cells has led to the suggestion that the LOH/AI events detected in stroma from FFPE specimens represent technical artifact.

The quality of DNA in FFPE samples is affected by the fixation and embedding processes as well as long-term storage. Formalin causes chemical modification and cross-linking of nucleic acids, whereas high temperatures used during paraffin-embedding can lead to fragmentation of DNA, resulting in low yields of degraded DNA (24). As a consequence, DNA isolated from FFPE specimens is associated with a higher frequency of mutation artifacts and successful amplification of PCR products more than 300 bp is difficult (24, 25). Thus, to determine whether AI events detected in archival stroma specimens using PCR-based

microsatellite analysis represents technical artifact, a direct comparison of technology and/or tissue types must be conducted. In our study, we compared AI detected in research-grade stroma specimens compared with that detected in FFPE stroma specimens; both data sets were generated with the same set of 52 microsatellite markers using the same amplification conditions, with the same criteria to define AI (9, 22). Of note, the use of highly multiplexed PCR, failure to optimize PCR conditions when amplifying DNA of low quantity and quality from FFPE samples, and lack of replicate PCR reactions have been proposed as contributions to the erroneous detection of chromosomal alterations in breast stroma (18). All 52 microsatellite markers used were optimized to robustly amplify DNA from FFPE specimens and multiplexed PCR was not conducted. In addition, all AI events detected were confirmed using a second microsatellite marker or by conducting a second amplification on a separate DNA aliquot from the same specimen (17). Despite these precautions, levels of AI were more than 10-fold higher in stroma from FFPE specimens compared with those from frozen stroma specimens. Given that the 52 microsatellite markers represented small and defined regions of the genome, the use of global SNP arrays provided an enhanced coverage as well as an agnostic approach to identify chromosomal alterations in breast stroma. Although DNA from FFPE stroma specimens was not assayed on SNP arrays, the paucity of copy number changes in DNA from frozen stroma specimens suggest that the tumor microenvironment is genetically stable.

Given the complexity of breast stroma, it is possible that chromosomal alterations present in certain stromal cell types are masked by the genomic content of other components. Elevated frequencies of AI/LOH in fibroblasts laser-microdissected from patients with BRCA1/2 mutations (59.7%) and sporadic tumors (36.7%) suggest that the fibroblast component may contribute to the instability detected in whole stroma (11), yet assessment of fibroblasts isolated from frozen breast stroma did not support these findings (16). In addition, evaluation of the stromal cell types individually failed to detect copy number alterations (13). Paradoxically, while evaluation of DNA from isolated cell types should provide the most accurate estimation of genomic alterations, the majority of copy number changes were detected in whole stroma FFPE specimens.

Conclusions

The data presented here support a model in which the tumor microenvironment is genomically stable. The direct comparison of copy number alterations between FFPE and frozen research-grade specimens using identical methodologies suggests that past reports of significant AI/LOH in breast stroma, both adjacent to and distant from the tumor, reflects artifact in the archival specimens caused by formalin-fixation, paraffin-embedding and tissue storage. Importantly, these data suggest that the tumor microenvironment from patients with aggressive tumors or inherited susceptibility to breast cancer does not harbor significant instability. Finally,

this stability suggests that therapeutics targeting the tumor microenvironment may be effective tools in the treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

Conception and design: C.D. Shriver, R.E. Ellsworth
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.L. Valente, J. Kane, R.E. Ellsworth
Study supervision: A.L. Valente, R.E. Ellsworth

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