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Report

Genomic patterns of allelic imbalance in disease free tissue adjacent to primary breast carcinomas*

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Summary

Mammary stroma plays an important role in facilitating the neoplastic transformation of epithelial cells, modulating integrity of the extracellular matrix, and maintaining genomic stability, but molecular mechanisms by which stroma affects epithelial structure and function are not well-defined. We used laser-assisted microdissection of paraffin-embedded breast tissues from 30 patients with breast disease and a panel of 52 microsatellite markers defining 26 chromosomal regions to characterize genomic patterns of allelic imbalance (AI) in disease-free tissue adjacent to sites of breast disease and to define genomic regions that may contain genes associated with early carcinogenic processes. The mean frequency of AI in histologically normal tissue adjacent to the primary carcinomas (15.4%) was significantly higher than that in distant tissue from the same breast (3.7%). The pattern of AI across all chromosomal regions differed between the adjacent tissue and primary tumor in every case. Unique AI events, observed only in tumor (15% of informative markers) or only in adjacent cells (10% of informative markers), were far more common than AI events shared between tumor and adjacent cells (~4%). Levels of AI characteristic of advanced invasive carcinomas were already present in non-invasive ductal carcinomas *in situ*, and appreciable levels of AI were observed in adjacent non-neoplastic tissue at all pathological stages. Chromosome 11p15.1 showed significantly higher levels of AI in adjacent cells ($p < 0.01$), suggesting that this region may harbor genes involved in breast cancer development and progression. Our data indicate that genomic instability may be inherently greater in disease-free tissue close to developing tumors, which may have important implications for defining surgical margins and predicting recurrence.

Introduction

Several lines of evidence suggest that processes crucial to tumor growth and progression, such as angiogenesis, extracellular matrix modification, and loss of genomic integrity, are influenced by intercellular communication between developing tumors and surrounding stromal tissue [1]. Recent studies examining the functional role of stroma in carcinogenesis reveal that stromal components play an active role in tumor development by initiating abnormal epithelial growth, recruiting cells that activate mitosis, and facilitating the transition from normal to diseased tissue [2, 3]. Investigating interactions between stromal and neoplastic cells and the role of genomic changes in tumorigenesis may provide important insights into the complex molecular processes of tumor growth, invasion, and metastasis.

Breast cancer development and progression are clearly associated with a spectrum of genetic changes in benign and malignant breast carcinomas

* The opinion and assertions contained herein are the private views of the authors and are not to be construed as official or as representing the views of the Department of the Army or the Department of Defense.

[4–6], which are believed to be important to tumor growth and differentiation. Though histologically non-neoplastic in appearance, tissues adjacent to breast tumors have recently been shown to harbor genetic abnormalities characteristic of malignant cells [7–11]. Chromosomal alterations, identified using loss of heterozygosity (LOH) and allelic imbalance (AI) analyses, are now known to occur in normal appearing breast tissue from women with and without breast disease [12, 13].

To further examine genomic changes in tumor and morphologically normal cells, we used laser-assisted microdissection of paraffin-embedded breast tissues from 30 patients with breast disease (Table 1) to obtain tumor cells and disease-free cells adjacent to sites of developing carcinomas. We examined 52 microsatellite markers defining 26 chromosomal regions throughout the genome to: (1) characterize the extent of AI in non-neoplastic cells adjacent to sites of breast disease from pre-invasive ductal carcinomas *in situ* (DCIS) and infiltrating ductal carcinomas, and compare levels of AI in adjacent tissue to levels observed in distant tissue [14]; (2) determine if patterns of AI differ between adjacent tissues and primary tumors; and (3) define genomic regions that may contain genes associated with breast disease. We examined chromosomal regions commonly deleted in breast cancer, rather than markers chosen at random, because we believe these regions will be most informative about molecular events occurring in carcinogenesis. Due to the functional relationship between tumors and surrounding stroma, we hypothesized that AI in adjacent tissue may be associated with tumor development. We inferred that identifying coordinated regions of chromosomal alterations could help elucidate critical molecular changes associated with the development and progression of breast cancer.

Table 1. Clinical characteristics of 30 patients with breast disease

Variable	%
Menopausal status (n = 30)	
Premenopausal	20
Postmenopausal	80
Tumor size (n = 30)	
Tis	27
T1	33
T2	30
T3 or T4	7
Unknown	3
Axillary lymph node status (n = 22)	
Positive	41
Negative	50
Unknown	9
TNM stage (n = 30)	
0	27
I	30
II	30
III	13
Hormonal status[a] (n = 24)	
ER+/PR+	54
ER+/PR−	17
ER−/PR+	8
ER−/PR−	17
Unknown	4
Survival (n = 30)	
Disease-free	64
Recurrence	3
Deceased (cancer)	20
Deceased (other)	13

[a] Includes two ductal carcinoma *in situ* cases.

Materials and methods

Sample collection and processing

Breast biopsies were collected from the pathology archives of Windber Medical Center and Memorial Medical Center, and from patients enrolled in the Clinical Breast Care Project (CBCP), a collaborative breast cancer research program between the Walter Reed Army Medical Center (WRAMC) and the Windber Research Institute. The Institutional Review Boards of the Windber and Memorial Medical Centers and the WRAMC Clinical Investigation/Human Use Committee approved this research, and all subjects enrolled into the CBCP voluntarily agreed to participate and gave written informed consent.

Breast tumors and surrounding margins were surgically removed from patients during diagnostic or therapeutic treatment. Selected tumor sections were embedded in paraffin, then sliced at 4 μm and mounted on foil microscope slides as previously

described [15]. Sixteen serial sections were normally cut from the corresponding blocks, and a single board-certified pathologist viewed the first and last slides, stained with hematoxylin and eosin, to confirm histology and guide microdissection. All tumors were staged using the TNM staging system approved by the American Joint Commission on Cancer (sixth edition) [16]. For the invasive lesions, the degree of tumor differentiation was assessed by Scarff–Bloom–Richardson (SBR) grading, which evaluated tubule formation, nuclear pleomorphism, and mitotic index [17]. Estrogen receptor (ER) and progesterone receptor (PR) status was determined by immunohistochemical staining.

Laser-assisted microdissection

An AS*LMD* Laser Microdissection system (Leica Microsystems, Wetzlar, Germany) and a PixCell® II microdissection apparatus (Arcturus Engineering, Mountain View, CA) were used to extract cells from the paraffin-embedded tissues. Cells were collected from various stages of breast disease, including preinvasive DCIS ($n = 8$), and invasive ductal carcinomas classified as stage I ($n = 9$), stage II ($n = 9$), and stage III ($n = 4$). A band of disease-free tissue, comprised mainly of stromal fibroblasts, immediately adjacent to sites of breast disease (within 850 μm from the nearest diseased cells) was collected separately for each case. Stromal tissue was often fibrous (desmoplastic) in appearance; rarely the stroma appeared hypercellular or edematous. For each sample, normal tissue free from any histological abnormalities, such as nipple tissue or negative axillary lymph nodes, was used as a source of referent DNA.

Genotyping

DNA was extracted from each sample by overnight digestion in Proteinase K (0.4 mg/ml) at 37 °C and purified by centrifugation at 10,000 × *g* through Microcon® YM-50 centrifugal filters (Millipore, Bedford, MA). A review of the breast cancer literature [18] was used to assemble a custom panel of 52 microsatellite markers (Invitrogen, Carlsbad, CA) representing 26 regions throughout the genome that are commonly deleted in breast cancer [19]. We examined DNA from microdissected cells by PCR using puReTaq™ Ready-To-Go® PCR beads (Amersham Biosciences, Buckinghamshire, England) or AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Foster City, CA) in a primer resting thermocycle profile [20] or a modified stepdown protocol [21]. Following amplification, samples were purified using Sephadex G-50 and then genotyped on a Mega-B ACE™1000 capillary electrophoresis DNA analysis system (Amersham Biosciences) using Genetic Profiler software (version 1.5).

Statistical analysis

To estimate the degree of allelic imbalance, normalized ratios were calculated using the formula $(T_1/T_2)/(N_1/N_2)$ where T_1 and N_1 are the smaller peak heights in the test and normal referent samples, respectively, and T_2 and N_2 are the larger peak heights in the test and referent samples, respectively [22, 23]. Samples with a normalized ratio of ≤0.35 were considered to show definitive evidence of AI, which indicates that a substantial proportion of cells in the sample contains the same chromosomal alteration compared to normal somatic cells. A normalized ratio of ≤0.35 may be attributable to loss or amplification of a given chromosomal region.

For each microsatellite marker, a value representing the percentage of cells showing AI was determined for tumor and adjacent samples. Hierarchical biclustering was performed using complete linkage and a Euclidean distance metric [24] to identify patterns of similarity or difference in AI between tumor and adjacent samples. The level of AI in distant tissue, calculated for samples from breast quadrants not containing the primary lesion, was compared to that in the primary tumor and adjacent tissue with Fisher's Exact Test (one-sided) under a hyper-geometric distribution. Trends in the frequency of AI by degree of tumor differentiation were assessed using the Jonckheere test for ordered alternatives [25]. Fisher's Exact Test was also used to assess patterns of AI across all markers in both tumor and adjacent tissues.

Quality control

All laser-assisted microdissection was conducted in close collaboration with the pathologist to ensure consistency of clinical diagnoses and to accurately dissect tumor and adjacent cells. The same areas of

tissue were microdissected across multiple slides to minimize differences in patterns of AI in different areas of tissue [26]. Accuracy of allele calling was maintained by setting a minimum acceptable signal intensity of 1000 relative fluorescence units (rfu). Chromosomal regions showing AI were repeated in an independently microdissected sample when sufficient tissue was available. For all replicate samples, the average of the normalized ratios for the independent assays was used in the analyses.

Representative data were rigorously examined for stochastic artifactual variation by testing potential correlations between DNA concentration, percentage of missing genotypes, and percentage of informative markers showing AI. Contingency tables were constructed and Fisher's Exact Test for Independence with a continuity correction was used to determine significance. A binomial probability (two-tailed) test was used to examine ratios of large versus small alleles altered at sites of AI. To establish the background level of AI in normal breast tissue from disease-free patients, paraffin-embedded samples were obtained from three reduction mammoplasty cases and processed as described above. Peak heights from mesenchymal tissue were compared to those in skin or peripheral blood to assess AI.

Results

Global patterns of AI differed between tumor and adjacent cells

The complete genotyping dataset included more than 5200 informative genotypes from tumor, adjacent, and referent samples. Initial inspection of the dataset revealed that the global pattern of AI across all chromosomal regions differed between tumor and adjacent cells in every case. Although tumor and adjacent cells shared common patterns of AI at ~4% of informative markers, unique AI events (observed only in tumor or only in adjacent cells) were far more common (Table 2). Allelic imbalance was observed only in tumor cells at 15% of informative markers, while 10% showed AI events unique to adjacent cells. Hierarchical cluster analysis showed that (1) tumor and adjacent tissues from individual patients rarely clustered together (Figure 1) and (2) neither tumor nor adjacent samples clustered by tumor stage, grade, or ER/PR status.

The frequency of AI was not associated with the degree of tumor differentiation

Trend analyses indicated that the frequency of AI in tumor cells did not increase significantly ($p = 0.448$) with increasing stages of tumor differentiation (Table 3). Likewise, the frequency of AI in adjacent cells was unrelated to tumor stage ($p = 0.320$). These data indicate that (1) in tumor cells, levels of AI characteristic of advanced invasive carcinomas are already present in non-invasive DCIS, and (2) in non-neoplastic tissue adjacent to breast carcinomas, appreciable levels of AI, though generally lower than those in the corresponding tumor, are present at all stages from *in situ* to advanced invasive carcinomas.

Table 2. Genomic patterns of AI in breast tumors (T) and adjacent tissue (A) stratified by tumor stage

Stage (*n*)	No AI		Shared AI		Unique AI				Total
	Markers with T – normal A – normal		Markers with T – AI A – AI		Markers with T – AI A – normal		Markers with T – normal A – AI		
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	
0 (8)	182	(72.2)	11	(4.4)	40	(15.9)	19	(7.5)	252
I (9)	222	(72.1)	13	(4.2)	43	(14.0)	30	(9.7)	308
II (9)	227	(71.6)	18	(5.7)	39	(12.3)	33	(10.4)	317
III (4)	84	(61.3)	3	(2.2)	31	(22.6)	19	(13.9)	137
Total (30)	715	(70.5)	45	(4.4)	153	(15.1)	101	(10.0)	1014

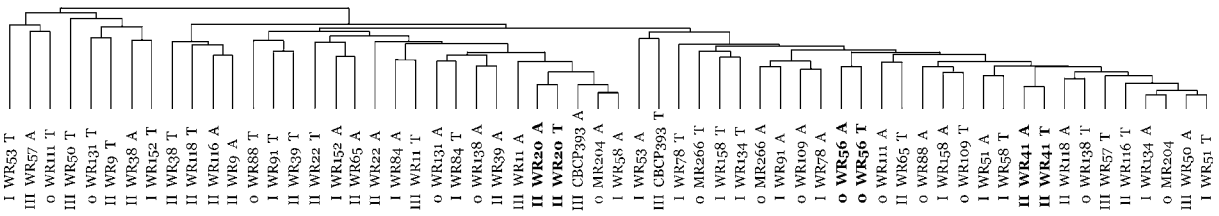

Figure 1. Hierarchical cluster analysis of primary breast tumors (T) and disease-free tissue (A) immediately adjacent to the primary tumors based on global patterns of AI. The pathological stage of the primary carcinoma is provided as a prefix to the specimen identification number.

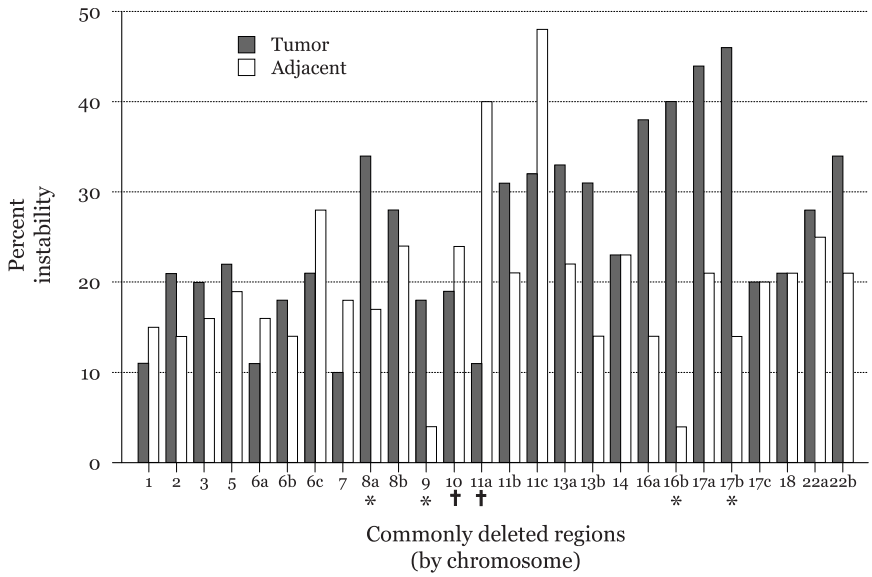

Figure 2. Levels of AI across 26 chromosomal regions commonly deleted in breast cancer for primary tumors and adjacent tissues. Markers in regions indicated by (*) showed a significantly higher frequency of AI in the tumors versus adjacent tissue; markers in regions denoted by (†) showed a significantly higher frequency of AI in adjacent tissues compared to the primary tumors.

AI was more frequent in tissue adjacent to, compared to tissue distant from, breast tumors

The frequency of AI in the primary tumor ($p < 0.0001$) and adjacent tissue ($p < 0.05$) was significantly higher than that in the distant tissue. The average frequency (%) of AI events per patient was 19.6% in the primary tumor, 15.4% in adjacent tissue, and 3.7% in distant tissue. The overall patterns of AI were unique to the adjacent or distant tissue and differed from those observed in the primary tumor.

Association of AI in commonly deleted regions with tissue type

Certain chromosomal regions showed significantly higher levels of AI ($p < 0.01$) in tumor cells, while other regions showed higher levels of AI ($p < 0.01$) in adjacent cells (Figure 2). For example, chromosome 8p22-p21.3 near marker D8S511 showed higher AI in tumor cells (37.5% of informative markers) from all stages of differentiation, but rarely showed AI in adjacent cells (4.2%). Conversely, the chromosomal region near marker D11S2368 (11p15.1) showed frequent AI in adjacent cells (66.7% of informative markers), but infrequent AI was observed in the corresponding tumors (13.6%).

Quality control

Results of the quality control analysis suggest that no systematic artifactual variation was present in the AI data. No statistical correlations were detected between the DNA concentration of each

Table 3. Average frequency (%) of AI per patient in breast tumors and adjacent tissue stratified by tumor stage

Stage (*n*)	Mean	Range
In situ carcinomas		
0 (8)		
Tumor	20.0	0–48.4
Adjacent	12.9	6.5–18.8
Invasive carcinomas		
I (9)		
Tumor	19.0	0–37.5
Adjacent	15.3	0–36.0
II (9)		
Tumor	17.6	2.4–34.2
Adjacent	16.7	2.6–33.3
III (4)		
Tumor	24.5	4.5–46.3
Adjacent	17.4	0–45.7
Invasive total (22)		
Tumor	19.4	0–46.3
Adjacent	16.3	0–45.7
Overall total (30)		
Tumor	19.6	0–48.4
Adjacent	15.4	0–45.7

sample and the percentage of missing genotypes ($p = 0.927$) or the percentage of markers showing AI ($p = 0.326$). Allele size was not associated with the probability of AI ($p = 0.226$), which means large alleles were not altered significantly more often than small alleles. The average frequency of AI per patient in the reduction mammoplasty samples was <0.8%, demonstrating that our methods detect a very low level of AI in disease-free breast tissues.

Discussion

Genomic instability is a landmark feature of most human cancer cells. Impaired stability is often associated with distinctive oncogenic phenotypes, such as uncontrolled proliferation, invasion, angiogenesis, and the ability to metastasize [27], but causal relationships between genetic changes and tumor development remain unclear [28]. Although a spectrum of genetic alterations is common in both benign disease [29] and malignant breast carcinomas [30], AI has recently been demonstrated in histologically non-neoplastic tissues adjacent to, and distant from, sites of breast disease [31, 32]. Because AI in non-neoplastic breast tissues is now known to be more heterogeneous and widespread than previously believed, it is important to understand the role of genetic alterations in normal tissues in breast cancer development and progression.

When examining many markers throughout the genome, patterns of AI emerge that may have important implications for tumor development and may indicate the locations of genes involved in tumorigenic processes. Observations that global patterns of AI across 26 chromosomal regions differed between tumor and adjacent cells in each case, and that unique AI events were detectable in tumor and adjacent cells at all pathological stages, confirm previous findings that independent mutational events occur in tumor and adjacent tissues, even at early *in situ* stages of breast disease [31–33]. Our data suggest that independent AI events are not sporadic genetic anomalies because they occur in appreciable frequency in tumor (15% of informative markers) as well as non-neoplastic cells (10% of informative markers).

Studies of gene expression have shown that expression profiles from different stages of breast cancer are all highly similar, supporting the hypothesis that tumor progression is, in part, a product of molecular events occurring in preinvasive stages and is not solely dependent on sequential genetic changes [34]. The AI data showed no correlation between the frequency of AI in tumor cells or in adjacent cells and the degree of tumor differentiation, suggesting that progression of breast disease may be influenced by a variety of genomic alterations in both tumor and adjacent tissue, which are present in early preinvasive stages.

Frequent LOH at specific chromosomal regions in certain cancers implies the presence of tumor suppressor genes (Table 4). The region at chromosome 8p22-p21.3, which showed frequent AI in tumor cells, appears to be one of the most frequently altered chromosomal regions in breast cancer [35]. Genomic instability at chromosome 8p22-p21.3 is common in premalignant *in situ* carcinomas [36], but this region has not been well

Table 4. Genes with a putative role in breast cancer development on chromosome 8p22-p21.3 and chromosome 11p15.1[a]

Gene name	Symbol	Known or putative function	Reference
8p22-p21.3			
Rho-related BTB domain-containing protein 2	RHOBTB2	Expression inhibits growth of breast cancer cells	44
PIN2-interacting protein 1	PINX1	Potent telomerase inhibitor	45
Deleted in liver cancer 1	DLC1	Inhibits proliferation/tumorigenicity of cancer	37
Tumor suppressor candidate 3	TUSC3	Silenced by methylation in colon cancer cell lines	46
Leucine zipper, putative tumor suppressor 1	LZTS1	Inhibits cancer cell growth via regulation of mitosis	47
Platelet-derived growth factor receptor-like	PDGFRL	Mutations/deletion associated with various cancers	48
11p15.1			
Extracellular link domain containing 1	XLKD1	Lymphangiogenesis and marker of tumor metastasis	49
Murine retrovirus integration site 1 homolog	MRVI1	Similar to mouse myeloid leukemia tumor suppressor	50
Tumor susceptibility gene 101	TSG101	Cell differentiation and growth regulation	51
HIV-1 Tat interactive protein 2, 30 kDa	HTATIP2	Metastasis suppression/apoptosis induction	52

[a] From http://www.ncbi.nlm.nih.gov/LocusLink/.

studied in earlier stages of breast disease such as atypical hyperplasia. Several genes have been localized to the 8p region with a putative role in breast cancer. Of note, the DLC-1 gene shows reduced levels of expression in 70% of breast carcinoma cell lines and has a significant inhibitory effect on tumorigenicity in mice [37]. Chromosome 11p15.1 demonstrated frequent AI in adjacent stroma and has been shown to exhibit LOH/AI in preinvasive breast lesions including various components of fibrocystic change and hyperplasia [38]. Tumor susceptibility gene 101 (TSG101; GenBank accession NM_006292), located at chromosome 11p15.1, has been implicated in early tumorigenesis because mutations in TSG101 occur at high frequency in breast cancer [39], TSG101 acts as a negative regulator of cell growth and differentiation, and a functional knockout of *tsg101* has been shown to promote transformation and metastatic tumor formation in mice [40]. Further study is needed to determine if genetic alterations in one or more genes located at 8p22-p21.3 and 11p15.1 contribute to breast carcinogenesis.

The role of genetic alterations in normal-appearing tissues in breast cancer development and progression remains largely unknown. Two observations from this study: (1) unique genetic alterations are common in disease-free tissues adjacent to breast lesions as early as *in situ* stages; and (2) the frequency of AI in tissue adjacent to breast carcinomas is significantly higher than in tissue distant from sites of disease, suggest that genomic instability may be inherently greater in cells close to developing tumors. Although it is unclear at present whether genetic alterations in non-neoplastic tissues comprise a "field" of genomic instability that increases the propensity for tumor development, the presence of AI in tissue adjacent to preinvasive DCIS suggests a potential role in tumor development and/or progression. Recent findings indicate that AI in pre-neoplastic oral squamous cell carcinoma lesions is predictive of progression to malignancy [41] and that genetic alterations in stromal cells may represent molecular events crucial to tumorigenesis and progression rather than nonspecific or artifactual events [42, 43].

Breast tissues that appear non-neoplastic pathologically often harbor genetic changes that may be important to understanding the local breast environment within which cancer develops. Our data suggest that genetic alterations may occur more frequently in tissue close to sites of breast disease than in distant tissues, but patterns of genomic instability in the breast appear to be complex and not a simple function of distance from a developing tumor [14]. These findings have implications for defining surgical margins and predicting local recurrence. Novel avenues for breast cancer treatment that specifically target normal tissue in addition to developing tumors have exciting potential for regulating cancer growth.

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