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ORIGINAL PAPER

Molecular Changes in Primary Breast Tumors and the Nottingham Histologic Score

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Abstract Pathological grade is routinely used to stratify breast cancer patients into favorable and less favorable outcome groups. Mechanisms by which genomic changes in breast tumors specifically contribute to the underlying components of tumor grade – tubule formation, nuclear pleomorphism, and mitoses — are unknown. This study examined 26 chromosomal regions known to be altered in breast cancer in 256 invasive breast carcinomas. Differences in overall levels and patterns of allelic imbalance (AI) at each chromosomal region were compared for tumors with favorable (=1) and unfavorable (=3) scores for tubule formation, nuclear pleomorphism and mitotic count. Levels of AI were significantly different between samples with high and low scores for tubule formation ($P<0.001$), nuclear pleomorphism ($P<0.001$) and mitotic count ($P<0.05$). Significantly higher levels of AI were detected at regions 11q23 and 13q12 for tumors with reduced tubule formation, chromosomes 9p21, 11q23, 13q14, 17p13 and 17q12 for those with high levels of nuclear atypia, and chromosomes 1p36, 11q23, and 13q14 for those with high mitotic counts. Region 16q11-q22 showed significantly more AI events in samples with low nuclear atypia. Patterns of genetic changes associated with poorly-differentiated breast tumors were recapitulated by the individual components of the Nottingham Histologic Score. While frequent alteration of 11q23 is common for reduced tubule formation, high nuclear atypia and high mitotic counts, suggesting that this is an early genetic change in the development of poorly-differentiated breast tumors, alterations at the other seven loci associated with poorly-differentiated tumors may specifically influence cell structure, nuclear morphology and cellular proliferation.

Keywords Allelic imbalance · Grade · Mitosis · Nuclear atypia · Tubule formation

Abbreviations

AI	allelic imbalance
CBCP	Clinical Breast Care Project
H&E	hemotoxylin and eosin

Background

The most widely used system for histological grading of breast tumors is the Nottingham combined histologic grade, which is based on the classification parameters developed by Bloom and Richardson and modified by Elston and Ellis [1]. Cumulative scores from three components, tubule formation, nuclear pleomorphism and mitotic count, are used to define tumors as well- (grade 1), moderately- (grade 2) or poorly- (grade 3) differentiated. This system of grading has clinical utility in determining patient risk and outcome: patients with

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low-grade carcinomas had a 95% 5-year survival compared to just 50% of patients with high-grade disease [2, 3]. Although useful for risk stratification, assignment of nuclear grade is highly subjective [4], limiting the ability to utilize grade as a reliable prognostic tool.

Breast cancer is a heterogeneous disease, involving multiple molecular pathways. Extensive gene expression analyses have identified patterns of expression that classify breast tumors into five major subtypes [5, 6] as well as predict recurrence and outcome [7–9]. Recently, gene expression profiles have been identified that can successfully discriminate low– from high-grade invasive breast carcinomas [10]. In addition to these gene expression differences, genomic alterations at chromosomes 1p, 1q, 6q, 9p, 11q, 13q, 16q, 17p and 17q have been associated with either low— or high-grade breast tumors [11–13] suggesting that invasive breast cancer may represent two distinct diseases that develop along either high— or low-grade genetic pathways.

Although molecular signatures can be used to discriminate breast carcinomas by grade, how genetic changes at these critical regions contribute to disease pathogenesis remains unknown. To improve our understanding of how genetic alterations contribute to histologic characteristics that define tumor grade, we used a panel of 52 microsatellite markers representing 26 chromosomal regions commonly altered in breast cancer to identify levels and patterns of allelic imbalance (AI) in 256 invasive breast carcinomas. Our objectives were to examine the individual contributions of tubule formation, nuclear pleomorphism and mitoses to overall grade and to identify chromosomal changes associated with each of these pathological components.

Materials and Methods

Paraffin-embedded primary breast tumors from 256 patients were obtained from the Windber Medical Center Pathology Department or the Clinical Breast Care Project (CBCP) Pathology Laboratory. Samples were collected from consecutive patients with sufficient tumor tissue to generate validated genotype data from all markers. Samples from the Windber Medical Center (n=49) were archival in nature and anonymized with no links between the assigned research number and patient identifiers. Clinical information was provided anonymously by the Memorial Medical Center Cancer Registry. Tissue and blood samples from CBCP patients (n=206) 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. Clinical information was collected for all CBCP samples using questionnaires designed by and administered under the auspices of the CBCP. To ensure consistency, diagnosis of every specimen was made by a single, dedicated breast pathologist (JAH) from hemotoxylin and eosin (H&E) stained slides; grade was assigned using the Nottingham Histologic Score [1, 2]. Pathological diagnosis partitioned the samples into grade 1 (n=93), grade 2 (n=83), and grade 3 (n=80). Clinicopathological information for all samples is summarized in Table 1.

DNA was obtained from homogeneous populations of primary breast tumor cells following laser-assisted microdissection on an AS*LMD* laser microdissection system (Leica Microsystems, Wetzlar, Germany) as previously described [14]. The integrity of multiple serial sections was established by pathological verification of the first and last sections stained with H&E. To avoid PCR artifacts, ≥ 5,000 cells were captured from each of six

Table 1 Clinical and pathological features of 256 invasive breast cancers

	Grade 1 (n=93)	Grade 2 (n=83)	Grade 3 (n=80)	P Grade 1 vs Grade 3
Menopausal Status				
Pre (<50 years)	19%	31%	41%	P<0.005
Menopasual (≥50 years)	81%	69%	59%	
Histology				
IDCA	62%	79%	93%	P<0.0001[b]
ILCA	20%	13%	3%	
Mixed	7%	8%	0%	
Other[a]	11%	0%	4%	
TNM stage				
Stage I	58%	41%	27%	P<0.05[c]
Stage II	29%	34%	43%	
Stage III	12%	20%	20%	
Stage IV	1%	5%	10%	
Lymph node status				
Negative	60%	52%	40%	P<0.05
Positive	40%	48%	60%	
Hormone receptor status				
ER+/PR+	76%	71%	31%	
ER+/PR-	21%	15%	14%	
ER-/PR+	0%	3%	4%	
ER-/PR-	3%	11%	51%	P<0.0001[d]
HER2 status				
Positive	4%	22%	33%	P<0.0001
Negative	96%	78%	67%	

[a] Other histological types include tubular, medullary, apocrine, and mucinous carcinomas

[b] Comparison of frequencies in IDCA

[c] Comparison of stages I and II compared to III and IV

[d] Comparison of frequency of hormone receptor negative (ER-/PR-) compared to ER+and/or PR+

consecutive breast tumor sections, with the sixth section reserved for all confirmatory reruns. Referent DNA samples for the archived samples were extracted from disease-free skin or negative lymph node tissue from each patient using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Referent DNA for the CBCP samples was obtained from blood clots using Clotspin and Puregene DNA purification kits (Qiagen, Valencia, CA).

Microsatellite markers were amplified as previously described [15], 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. AI was detected using methods first described by Medintz et al. [16] and defined by a threshold value of 0.35 which has been shown to have >80% reproducibility when AI events were confirmed on a second aliquot of DNA [17]. The following criteria were used to define AI for each region: when at least one marker for a given region showed an allelic ratio≤ 0.35, the region was considered to show AI; when neither marker had an allelic ratio≤0.35 and at least one marker was informative, the region was considered normal; and when both markers were homozygous, the region was considered uninformative.

Comparison of the clinicopathological factors and levels and patterns of AI by grade were performed using Student's t-tests and Fisher's exact tests. A significance value of P <0.05 was used for all analyses.

Results

Dissection of Grade Components

Low-, intermediate— and high-grade tumors were equally represented in this data set. While the degree of nuclear pleomorphism was broadly distributed across samples from

Table 2 Frequency of AI stratified by low- (score=1) and high- (score=3) grade component at 26 chromosomal regions. Numbers in bold are statistically significant

	Tubule formation			Nuclear pleomorphism			Mitosis		
Chromosomal Region	1	3	P 1 vs.3	1	3	P 1 vs. 3	1	3	P 1 vs. 3
1p36.1-p36.2	0.1	0.18	0.3416	0.13	0.21	0.2049	0.12	0.29	**0.0056**
2q21.3-23.3	0.19	0.16	0.6460	0.2	0.14	0.3767	0.16	0.17	0.8325
3p14.1	0.18	0.18	1.0000	0.11	0.17	0.3389	0.18	0.18	1.0000
5q21.1-q21.3	0.12	0.17	0.4886	0.11	0.22	0.1140	0.14	0.24	0.1897
6q15	0.12	0.24	0.1390	0.15	0.19	0.6611	0.23	0.17	0.4391
6q22.1-q23.1	0.16	0.22	0.5318	0.14	0.22	0.2962	0.2	0.19	1.0000
6q25.2-q27	0.1	0.26	0.0542	0.13	0.26	0.0680	0.23	0.28	0.5835
7q31.1-q31.31	0.03	0.11	0.1337	0.05	0.13	0.1004	0.07	0.14	0.1476
8p22-p21.3	0.14	0.25	0.1927	0.15	0.27	0.1065	0.19	0.34	0.0600
8q24	0.1	0.17	0.3404	0.13	0.19	0.3814	0.14	0.22	0.1919
9p21	0.07	0.17	0.1474	0.03	0.22	**0.0015**	0.14	0.22	0.1919
10q23.31-q23.33	0.16	0.15	1.0000	0.18	0.2	0.8353	0.15	0.24	0.1941
11p15	0.3	0.18	0.0923	0.25	0.19	0.3262	0.2	0.22	0.8446
11q13.1	0.21	0.22	1.0000	0.31	0.18	0.0576	0.24	0.17	0.3420
11q23	0.12	0.32	**0.0072**	0.18	0.4	**0.0042**	0.22	0.42	**0.0110**
13q12.3	0.13	0.29	**0.0436**	0.18	0.32	0.0873	0.22	0.35	0.0964
13q14.2-q14.3	0.12	0.25	0.0931	0.08	0.27	**0.0020**	0.17	0.37	**0.0038**
14q32.11-q31	0.21	0.2	1.0000	0.13	0.22	0.2049	0.17	0.24	0.3095
16q11.2-q22.1	0.17	0.3	0.1807	0.34	0.19	**0.0333**	0.32	0.24	0.2904
16q22.3-q24.3	0.18	0.30	0.0768	0.29	0.3	0.8584	0.28	0.32	0.5997
17p13.3	0.21	0.37	0.0830	0.25	0.52	**0.0023**	0.29	0.41	0.1939
17p13.1	0.2	0.34	0.1017	0.23	0.44	**0.0105**	0.25	0.42	0.1634
17q12-q21	0.15	0.2	0.5111	0.08	0.28	**0.0020**	0.15	0.26	0.0608
18q21.1-q21.3	0.1	0.22	0.0830	0.09	0.19	0.0989	0.18	0.2	1.0000
22q12.3	0.08	0.2	0.1009	0.1	0.19	0.2448	0.17	0.19	0.8349
22q13.1	0.29	0.26	0.6941	0.27	0.23	0.5697	0.28	0.25	0.8565

26% with small, uniform nuclei (score=1), to 36% of intermediate size, with moderate nuclear variation (score=2), to 38% with large nuclei with marked variation (score=3), reduced tubule formation (score=3) and low mitotic count (score=1) were the predominant scorings, observed in 71% and 63% of samples, respectively.

Association of Genomic Changes with Tubule Formation, Nuclear Pleomorphism and Mitotic Count

Overall levels of genomic instability were significantly higher ($P<0.005$) in tumors with reduced tubule formation (23%) compared to those without (15%). AI events occurred at significantly higher levels in tumors with reduced tubule formation at chromosomes 11q23 and 13q12 (Table 2). Overall levels of genomic instability were significantly higher ($P<0.001$) in tumors with marked nuclear variation (24%) compared to those without (17%). AI events were significantly more frequent in tumors with marked nuclear variation at chromosomes 9p21, 11q23, 13q14, 17p13.1, 17p13.3 and 17q12-q21, while AI at chromosome 16q11-q22 occurred at a significantly higher frequency in tumors without nuclear pleomorphism compared to those with marked variation. Overall levels of AI were significantly higher ($P<0.05$) in tumors with high mitotic counts (27%) compared to those without (22%). Chromosomes 1p36, 11q23 and 13q14 showed a significantly higher frequency of AI in tumors with high mitotic counts.

Patterns of AI in Intermediate Grade Tumors

Genetic data has supported models in which intermediate-grade breast cancer is not a discrete disease entity, but rather represents a blend between low— and high-grade disease, with some tumors resembling low-grade, some high-grade and some having genetic features of both [10, 11]. Neither overall levels nor patterns of AI for tumors with an intermediate score for tubule formation or for mitosis differed significantly from those with either low or high score. In contrast, tumors with intermediate scores for nuclear atypia had significantly higher ($P<0.05$) levels of AI (21%) than those with low scores (17%) but were not significantly different from those with high scores. AI levels were significantly higher for tumors with intermediate compared to low levels of nuclear atypia at chromosomes 9p21 and 13q14, and were significantly lower than those with high levels of nuclear atypia for chromosomes 17p13.1, 17p13.3 and 17q21 (Table 3). In addition, tumors with intermediate compared to high levels of nuclear atypia had significantly higher levels of AI at chromosome 16q11-q22. Thirty-five percent of tumors with intermediate scores for nuclear pleomorphism had AI at chromosomes 9p21, 13q14, 17p13.1, 17p13.3 and/or 17q12 (high-like), 21% had AI at 16q11-q22 (low-like), 16% had a mix of low— and high-like genetic patterns and 28% had neither low— nor high-like genomic patterns.

Allelic Imbalance and Histological Subtypes

IDCA was the predominant tumor type, representing more than 75% of all tumors studied. While the number of mixed and other tumor types was insufficient to generate statistically significant data, chromosomal changes were examined in ILCA ($n=31$) and IDCA ($n=196$) separately. Patterns of AI detected with all tumor types were maintained in the IDCA specimens. Because lobular carcinomas do not form tubules, tubule formation was not assessed for the ILCA samples. The majority of ILCA had a score of 1 for nuclear atypia (54%) and mitosis (94%), thus differences in patterns of AI were examined between IDCA and ILCA with nuclear atypia and mitotic scores = 1. The frequency of AI was significantly higher ($P<0.05$) in ILCA at chromosome 11q13.1 for both nuclear atypia (47%) and mitosis (36%) compared to IDCA (18% and 11%, respectively).

Discussion

Histological grading of primary breast carcinomas has become widely accepted as an important predictor of outcome [18]. Recent molecular studies have identified specific chromosomal alterations and gene expression profiles that correlate with tumor grade, as well as an array of candidate genes that may contribute to tumor behavior and phenotypic characteristics [10, 11]. Despite the impor-

Table 3 Identification of loci that differ significantly for tumors with intermediate scores for nuclear pleomorphism compared to those with low— and high-scores

Chromosomal region	Score =1	Score =2	Score =3	*P* 1 v 2	*P* 2 vs. 3
9p21	0.03	0.15	0.22	**0.0297**	0.3292
13q14.2-q14.3	0.08	0.25	0.27	**0.0091**	0.7356
16q11.2-q22.1	0.34	0.37	0.19	0.8617	**0.0127**
17p13.3	0.25	0.22	0.52	0.6827	**0.0001**
17p13.1	0.23	0.22	0.44	0.8461	**0.0016**
17q12 -q21	0.08	0.14	0.28	0.4334	**0.0181**

tance of molecular changes in shaping tumor growth and differentiation in patients with breast cancer, genetic contributions to physiological and structural attributes that define histological grade have not been well-defined.

Previous research from our laboratory identified molecular signatures that were associated with low- (loss of chromosome 16q11-q22) and high-grade (AI at chromosome 1p36, 9p21, 11q23, 13q14, 17p13.1 and 17q12-q21) invasive breast tumors [11]. These molecular signatures persisted when components defining tumor grade were examined independently. Therefore, overall histologic grade may be determined by genetic changes that influence nuclear structure and rates of cellular proliferation.

Significantly higher levels of AI at chromosome 11q23 were found for all grade components, suggesting that changes in this region are associated with the development of poorly-differentiated breast carcinomas. FRA11G is a common fragile site located on 11q23.3, rendering this region susceptible to frequent genomic instability [19]. Deletion of the H2A histone family, member X (H2AFX) gene on chromosome 11q23.3 has been associated with genomic instability and tumorigenesis; recently, Srivastava et al. found that deletion of H2AFX was associated with high-grade tumors and suggested that deletion of H2AFX may be an early event, leading to uncontrolled cellular proliferation and initiation of tumor development [20]. Alterations of 11q23 may, therefore, serve as an initiating event in the development of high-grade tumors.

In contrast to the shared alteration of 11q23, each component of the Nottingham Histologic Score was associated with unique array of chromosomal alterations, allowing us to further dissect genetic contributions associated with tumor growth and differentiation. Genomic alterations at chromosomes 1p36 and 13q14 were associated with high mitotic count, a measure of proliferation. Based on current knowledge of functional attributes, several candidate genes in these regions may influence mitotic count and thus contribute to histologic grade. The chromodomain helicase DNA binding protein 5 (CHD5), located at chromosome 1p36.31, serves as a tumor suppressor by controlling cellular proliferation [21], while genomic instability near the tumor suppressor gene RB1 on chromosome 13q14 has been associated with high-grade invasive and *in situ* carcinomas [11, 22] and underexpression of RB1 has been correlated with increased cellular proliferation [23]. Thus, changes at each of these chromosomal regions may contribute to the increased mitotic counts associated with high-grade breast tumors.

Nuclear pleomorphism measures changes in cell size and uniformity. Previous studies have shown that AI at chromosome 16q11-q22 is associated with the development of low-grade invasive breast cancer [11, 13, 24, 25]. Because genomic changes in this region are specifically associated with preservation of nuclear structure, alterations of 16q11-q22 may confer a protective advantage against nuclear damage. Although several genes on chromosome 16q have been investigated for a possible role in low-grade disease [25, 26], specific genetic changes have not been identified.

In contrast, a number of candidate genes have been identified that may contribute to the development of nuclear pleomorphism. Structural changes at chromosome 9p21, including the CDKN2A, CDKN2B and MTAP genes, have been associated with poor prognosis in high-grade breast tumors [27]. Similarly, loss of the 17p13 region is commonly observed in large, poorly-differentiated tumors [28, 29], and, in particular, loss of the p53 gene strongly correlates with marked nuclear atypia [30]. The 17q12-q21 region, which includes both the HER2 and BRCA1 genes is frequently altered in tumors with nuclear pleomorphism [31, 32]. Thus, changes at several chromosomal regions may contribute to nuclear instability and the resulting poor outcomes often associated with high-grade tumors.

Markers on chromosome 13q12 span a small region flanking the breast cancer 2 (BRCA2) gene. BRCA2 is a DNA repair enzyme, disruption of which is associated with increased genomic instability [33], and may contribute to the significantly higher levels of allelic imbalance in high-grade tumors. Furthermore, disregulation of BRCA2 is associated with failure of cytokinesis, resulting in binucleated cells [34]. These changes may lead to the decreased cellular organization and reduced tubule formation found in poorly-differentiated tumors.

Moderately-differentiated invasive breast carcinomas present a particular challenge as 30–60% of carcinomas have mixed features of high— and low-grade disease. Because tumor grade has prognostic power and can be used in treatment decision making, improved characterization of intermediate grade disease is critical. The data presented here supports earlier results from Sotiriou et al. in which gene expression data could characterize intermediate grade breast carcinomas as low-, high— or mixed low— and high-grade [10]. Thus, while molecular analysis may be useful in identifying low-like and high-like breast carcinomas, a significant number of tumors will remain classified as a mixed, or intermediate-grade disease.

Previous studies have investigated whether a two-tiered scoring system, excluding tubule formation, is more accurate than the current tripartite system. In the study of Rank et al. nuclear pleomorphism was superior to tubule formation and mitotic counts in predicting survival which may suggest the traditional tripartite histologic scoring system should be reconsidered [35]. Likewise, a two-tiered scoring system involving nuclear pleomorphism and mitotic counts could effectively discriminate high— from low-risk, node-negative breast cancer patients [36]. Finally, Komaki et al. compared a two— and three-tiered scoring system for

predicting disease recurrence and found that a two-tiered system comprised of mitotic counts and nuclear pleomorphism was significantly more accurate [37]. In contrast, genetic data supports a tripartite system as tubule formation was associated with AI at chromosomes 11q23 and 13q12; elimination of tubule formation may obscure the contribution of molecular changes, especially at 13q12, to the development of high-grade disease.

IDCA and ILCA are thought to represent distinct diseases, with ILCA characterized by loss of expression of E-cadherin (CDH1) on chromosome 16q [38]. Evaluation of AI data in this study failed to reveal significant differences in allelic imbalance at chromosome 16q22.1, the site of the CDH1 gene, between IDCA and ILCA. ILCA with low scores for nuclear atypia and mitotic count, however, did have significantly higher levels of AI at chromosome 11q13. A recent study found common loss of chromosome 11q13 between classical ILCA and pleomorphic lobular carcinomas, which are marked by more conspicuous nuclear atypia and pleomorphism than classical ILCA [39]. Because the frequency of AI was significantly higher ($P<0.05$) in ILCA (35%) compared to IDCA (18%) regardless of grade, it is possible that alterations of 11q13 are, like loss of CDH1 expression, a general hallmark of lobular carcinomas.

In conclusion, patterns of AI that discriminate high— from low-grade invasive breast tumors are recapitulated in the individual components of the Nottingham Histologic Score. Alteration of 11q23 may occur early in tumorigenesis, serving as a critical step in the development of high-grade disease while changes at chromosomal regions 1p36, 9p21, 13q12, 13q14, 17p13 and 17q12-q21, which harbor candidate genes involved in increased cellular proliferation, nuclear changes, genomic instability and poor prognosis, contribute specifically to tubule formation, nuclear atypia or mitotic counts. These data provide new insights into the genetic basis of the development and progression of high-grade breast cancer.

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