Total-Genome Analysis of *BRCA1/2*-Related Invasive Carcinomas of the Breast Identifies Tumor Stroma as Potential Landscaper for Neoplastic Initiation

Frank Weber,[1,2] Lei Shen,[5] Koichi Fukino,[6] Attila Patocs,[1,2] George L. Mutter,[7] Trinidad Caldes,[8] and Charis Eng[1,2,3,4,9]

[1]Genomic Medicine Institute, [2]Lerner Research Institute, and [3]Taussig Cancer Center, Cleveland Clinic Foundation, and [4]Department of Genetics and CASE Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland; [5]Division of Biometrics, School of Public Health, Ohio State University, Columbus; [6]Department of Neurosurgery, Nippon Medical School, Tokyo; [7]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston; [8]Laboratory of Molecular Oncology, San Carlos University Hospital, Madrid; and [9]Cancer Research UK Human Cancer Genetics Research Group, University of Cambridge, Cambridge, United Kingdom

We have shown that the tumor microenvironment of sporadic breast cancer is diverse in genetic alterations and contributes to the cancer phenotype. The dynamic morphology of the mammary gland might be of special interest in hereditary breast/ovarian cancer syndrome (HBOC). We hypothesized that hotspots of loss of heterozygosity or allelic imbalance (LOH/AI) within the tumor stroma of *BRCA1/2*-related breast cancers provide an impaired mammary stroma that could facilitate later malignant transformation of the breast epithelium. We conducted a total genome LOH/AI scan of DNA derived from the epithelium and stroma of 51 *BRCA1/2*-related breast cancers, using 372 microsatellite markers. We compared these data with those from a set of 134 sporadic breast cancers. HBOC-related breast cancers accumulated significantly more genetic alterations than did sporadic breast cancers. *BRCA1/2*-related breast cancer stroma showed LOH/AI at 59.7% of all loci analyzed, similar to the average frequency of LOH/AI observed in the epithelium (66.2%). This is remarkably different from sporadic breast cancers, for which the average epithelial LOH/AI frequency (36.7%) far exceeds the average stromal LOH/AI frequency (28.4%) ($P = .03$). We identified 11 hotspot loci of LOH/AI in the *BRCA1/2* stroma, encompassing genes such as *POLD1*, which functions in DNA replication, and *SDHB*. In a subset of samples, enriched for *BRCA1* cases, we found 45.0% overall LOH/AI in the stroma, which was significantly higher than the 41.8% LOH/AI observed in corresponding epithelium ($P = .04$). Together, our data indicate that, in HBOC-related breast cancers, the accumulation of genomic instability in the cancer stroma coincides with that in the neoplastic epithelium, and we postulate that such a genetically unstable stroma might facilitate a microenvironment that functions as a landscaper that promotes genomic instability in the epithelium and, subsequently, neoplastic transformation.

In 2005, >200,000 new cases of invasive breast cancer were diagnosed, and ~40,410 women died of this disease in the United States alone. Different factors, environmental as well as intrinsic, are associated with an increased lifetime risk of breast cancer, estimated to be 13% in the general female population.[1] This relative risk is increased fourfold if familial disease or germline mutations in breast cancer susceptibility genes are present (breast cancer, familial [MIM #114480]). Germline mutations in one of the two most common breast cancer susceptibility genes—*BRCA1* (MIM +113705) on 17q21 and *BRCA2* (MIM +600185) on 13q12.3—are estimated to occur in 1 in 250 women overall,[2] with the frequency increasing to 5%–10% among women who present with breast cancer.[2,3]

BRCA1 and *BRCA2* have key regulatory roles in crucial cellular events, such as response to DNA damage, cell cycle regulation, and apoptosis.[2] *BRCA* gene participation in the DNA repair machinery is supported by studies showing an elevated incidence of genomic instability in tumor tissue of hereditary breast cancer compared with sporadic breast cancer. While the majority of published reports have focused on selected chromosomal regions, two pangenomic transcriptional and structural studies have been performed.[4,5] These studies identified regions with high frequencies of genomic instability and allowed the differentiation between *BRCA1*- and *BRCA2*-derived cancers based on copy-number changes in a set of genomic loci or gene transcription profiles. These studies, however, only looked at whole tumors, without regard to their components. A few studies even looked at the frequency of loss of heterozygosity at a few selected markers in microscopically normal-appearing breast tissue from a handful of

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Address for correspondence and reprints: Dr. Charis Eng, Genomic Medicine Institute, Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, NE-50, Cleveland, OH 44195. E-mail: engc@ccf.org

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patients with HBOC and found elevated levels of allelic loss.[6,7] Whereas some of the increased breast cancer risk can be attributed to direct effects of germline mutation within breast epithelial cells, it is equally plausible that mutation of adjacent stromal cells creates an abnormal microenvironment permissive of outgrowth of premalignant and malignant epithelial clones. In this model, local stromal-epithelial interactions, as codetermined by both stromal and epithelial genotypes, determine the likelihood of tumor formation.

Why is the tumor stroma important in hereditary breast carcinogenesis? Morphogenesis of the branching tree-like architecture of the breast begins during puberty with first lobule formation, a process that requires coordinated interaction with the intralobular matrix (stroma) comprised of fibroblasts, blood vessels, lymphocytes, and macrophages. Duct branching and acinar growth, which occurs during the first decades of life, is a primarily proliferative process orchestrated in conjunction with changes in the stroma. Cancer-free women who undergo prophylactic mastectomy because of a family history of breast cancer have an altered breast lobular architecture showing less differentiated lobules (termed "Lob 1"), compared with controls with a more dense and fibrotic intralobular stroma that loses its demarcation from the collagenous interlobular stroma.[8] This is direct evidence that a change in epithelial and/or stromal cell function, such as that conferred by heritable mutation, is capable of upsetting the delicate balance of breast tissue morphogenesis. Remodeling of breast lobules continues in adulthood under hormonal control. This is most evident during pregnancy, when the density of acini increases dramatically and epithelial lining cells undergo secretory differentiation.

In this study, we embarked on a whole-genome analysis of breast cancer in patients with and without *BRCA1/2* mutations and sought to determine the extent of genomic instability in the malignant breast epithelium and in the adjacent tumor stroma and how the genomic instability differs from that in sporadic breast cancers. This question is not purely of scientific interest. Discovery of potential hallmarks of stromal cell genomic instability in these heritable breast cancers might provide a means to stratify future cancer risk among patients with familial clustering of breast cancer.

Material and Methods

Sample Population

A total of 51 invasive breast cancers from 51 patients with hereditary breast and/or ovarian cancer (HBOC) were accrued for this study, and a meticulous family history of all 51 patients was obtained and documented. We will refer to this series as "*BRCA1/2*-related"; it comprises patients with germline deleterious mutations in *BRCA1* ($n = 22$) or *BRCA2* ($n = 13$), patients with germline unclassified variants in *BRCA1* (*BRCA1uv*; $n = 6$) or *BRCA2* (*BRCA2uv*; $n = 8$), and two patients with HBOC who are wild type for both genes (*BRCAwt*) (tables 1 and 2). Besides mutation analysis, the diagnosis of HBOC was determined on the basis of HBOC criteria and clinical practice guidelines for all families.[9] The human subjects review boards of the respective participating institutions approved this study. One patient, harboring a *BRCA2* deleterious mutation, underwent prophylactic mastectomy. To compare data from the *BRCA1/2*-related cancers with data from sporadic breast cancers, our previously reported[10] data set comprising data from 134 clinically sporadic stage 1–3 invasive breast carcinomas was reanalyzed.

Laser Capture Microdissection and DNA Extraction

Laser capture microdissection was performed using the Arcturus PixCell II microscope (Arcturus Engineering) to isolate the two compartments of neoplastic tissue (epithelium and stroma) separately.[10,11] We specifically captured stromal fibroblasts adjacent to malignant epithelium (i.e., the tumor stroma), under direct microscopic observation. These stromal fibroblasts resided either in-between aggregations of epithelial tumor cells or no more than 0.5 cm distant from a tumor nodule. Unlike enrichment procedures, such as cell-type specific separation of cells previously dissociated from large tissue samples, microscopic dissection is able to control for proximity of stroma to cancer cells among all samples. Corresponding normal DNA for each case was procured from peripheral blood leukocytes (possible for 63%) or, if this was not possible, from normal tissue, obtained a large distance from the tumor site or from a different tissue block containing only normal tissue. The different origins of the corresponding normal DNA had no effect on the frequency or pattern of loss of heterozygosity or allelic imbalance (LOH/AI)

Genomewide LOH/AI Scan

Genomic DNA was extracted as described elsewhere,[10–12] with the exception that an incubation with proteinase K was performed at 65°C for 2 d. PCR was performed using DNA from each compartment (normal control, tumor epithelium, and tumor stroma) of each sample and one of 72 multiplex primer panels, which comprised 372 fluorescent labeled microsatellite markers. These 372 markers map to chromosomes 1–22 and X and are based on the MapPairs genomewide Human Markers set, version 10 (Invitrogen), developed at the Marshfield Institute. This whole-genome panel has an average of 16.2 markers per chromosome (range 7–29 markers per chromosome), or an ~9-cM intermarker distance.

Genotyping was performed with the ABI 377xl or 3700 semiautomated sequencer (Applied Biosystems, Perkin-Elmer). The results were analyzed by automated fluorescence detection using the GeneScan collection and analysis software (Applied Biosystems). Scoring of LOH/AI was performed by manual inspection of the GeneScan output. A ratio of peak heights of alleles between germline and somatic DNA of ≥ 1.5 was used to define LOH/AI, as described elsewhere by us and others.[13–15] As described elsewhere, the methodological veracity of LOH/AI by use of multiplex PCR on archived tissue has been extensively validated.[10]

Table 1

Mutation Spectra of Samples from 35 Patients with HBOC and Deleterious Mutations

Sample	Mutation	Domain	*D17S1299*		*D13S1493*	
			EP	ST	EP	ST
1	*BRCA1* 4020delAG	SCD	…	…	…	…
4	*BRCA1* IVS4−1G→T	…	…	…	…	…
5	*BRCA1* 185delAG	RING finger	X	X	…	…
6	*BRCA1* 1135insA	…	X	…	X	…
7	*BRCA1* 185delAG	RING finger	…	…	…	…
8	*BRCA1* 2530delAG	DNA binding	…	…	…	…
9	*BRCA1* 3600del11	…	…	X	…	X
10	*BRCA1* IVS18−2delA	…	X	X	…	…
11	*BRCA1* 1240delC	…	X	X	…	…
12	*BRCA1* 589delCT	…	…	X	…	…
17	*BRCA1* IVS18+3A/C	…	…	…	…	…
19	*BRCA1* 1389insAG	…	…	X	…	X
20	*BRCA1* IVS6−1C/T	…	X	…	X	…
21	*BRCA1* del ex23–24	BRCT	X	X	X	…
22	*BRCA1* IVS18+3A/C	…	X	X	X	X
23	*BRCA1* del ex23–24	BRCT	…	X	…	…
24	*BRCA1* 157delCT	RING finger	…	…	…	…
25	*BRCA1* 4229insATCT	SCD	X	X	…	…
37	*BRCA1* 5385insC	BRCT	…	…	…	…
50	*BRCA1* 2552delC	DNA binding	X	…	…	X
51	*BRCA1* C61G	RING finger	X	…	X	…
52	*BRCA1* IVS5−11T→G	…	…	X	X	…
2	*BRCA2* 2567delC	…	X	…	X	…
3	*BRCA2* 6174delT	…	X	…	…	…
13	*BRCA2* Y1894X	…	X	X	…	…
14	*BRCA2* 6503delTT	…	…	…	…	…
15	*BRCA2* 6503delTT	…	…	…	…	X
16	*BRCA2* 8294insTT	…	…	…	…	X
26	*BRCA2* 5804delAAAA	…	…	…	…	…
29	*BRCA2* 8234delTT	…	…	…	…	…
36	*BRCA2* 5578delAA	…	…	…	X	X
44	*BRCA2* 8234del23	…	…	…	…	…
45	*BRCA2* 5804delAAAA	…	…	…	X	…
46	*BRCA2* 8765delAG	…	…	…	…	…
49	*BRCA2* 3036delACAA	…	…	X	…	…

Note.—*D17S1299* is a marker at the *BRCA1* locus, and *D13S1493* is a marker at the *BRCA2* locus. BRCT = C-terminal portion of the *BRCA1* gene; EP = epithelium; SCD = stromalin conservative domain; ST = stroma. "X" indicates LOH/AI for that sample in the corresponding compartment.

Statistical Analysis

Of the 372 markers, 1 was excluded from further statistical analyses because it was noninformative in all epithelial samples or all stromal samples. Statistical analysis was performed for samplewise, chromosomewise, and markerwise LOH/AI frequencies. Comparisons made included those between normal control and tumor epithelial and stromal samples, between different chromosomes, and between markers on the same chromosome. Two levels of analyses were employed: simple averaging of observed LOH/AI frequencies and model-based analysis. The former is straightforward and provides useful data summaries as well as suggests interesting patterns and differences. For the latter, we employed marginal models that extend generalized linear models to correlated data. The resulting inference is robust because a sandwich-type estimator for the variance matrix was used. We have reported this approach in detail elsewhere.[10] Hierarchical clustering and multidimensional scaling were performed using the proportion of discordant LOH/AI events between a pair of samples as the dissimilarity measure. All data analysis was performed using the statistical package R, version 1.8.1 (The R Foundation for Statistical Computing). For comparisons between groups, the χ^2 tests with Yate's correction and the nonparametric Spearman rank correlation test were used.

Results

Of the 51 patients with HBOC, 2 are wild type for *BRCA1/2* but show a strong family history of breast and ovarian cancers (fig. 1). Among the 49 patients with

germline variants in either *BRCA1* or *BRCA2*, 35 have mutations considered deleterious on the basis of the Breast Cancer Information Core database and recent publications (table 1). The analyses of the demographic data show that the average age at onset for the *BRCA1* group was 39.1 years, significantly lower than that for the *BRCA2* group (50.8 years; $P = .031$). Our study also showed a strong positive correlation between estrogen (ER) and progesterone (PR) receptor status and type of mutation ($P = .003$ and .013, respectively), with a higher frequency of ER-positive and PR-positive cases in the *BRCA2* group (37.5% ER positive; 50% PR positive) than in the *BRCA1* group (17.6% ER positive; 17.6% PR positive; $P = .0496$), as expected. High-grade tumors (grade 3) occurred more frequently in the *BRCA1* group (66.6%) than in the *BRCA2* group (37.5%). Forty-eight tumors were classified as invasive ductal carcinoma, one as an invasive lobular carcinoma (*BRCA1uv*), and one as a ductal carcinoma in situ (*BRCA1uv*). In our data set, we found LOH/AI at 17q21 (*BRCA1* locus) significantly more often in patients with a *BRCA1* mutation than in those with a *BRCA2* mutation ($P = .0318$) (table 1). Interestingly, while 68.2% of all *BRCA1* cases show LOH/AI in either the neoplastic epithelium and/or stroma, in five cases (22.7%), the allelic loss is confined to the stromal compartment (table 1). Thus, overall, our sample set is consistent with other reports, showing a lower frequency of ER/PR-positive status, lower age at onset, higher frequency of grade 3 tumors, and loss of one functional *BRCA1* allele among *BRCA1* mutation–positive patients.[16]

Frequency and Pattern of LOH/AI in Breast Carcinoma Epithelium and Surrounding Stroma from HBOC Cases

Genomic instability, as manifested by LOH/AI, was a frequent event in our series of *BRCA1/2*-related (HBOC) breast cancers, occurring in 63.0% of all informative markers, exceeding the overall frequency of 32.6% LOH/AI found in sporadic breast cancers ($P < .0001$).

Table 2

Samples from 16 Patients with HBOC and without the Deleterious Mutations in *BRCA1/2*

Sample	Genotype	Effect
27	*BRCA1uv* N1236K	Unclassified
28	*BRCA1uv* S1040N	Unclassified
39	*BRCA1uv* A1623G	Unclassified
41	*BRCA1uv* IVS2−14T→C	Unclassified
43	*BRCA1uv* S1040N	Unclassified
47	*BRCA1uv* S1623G	Unclassified
30	*BRCA2uv* A2466V	Unclassified
31	*BRCA2uv* IVS8+56T→C	Unclassified
32	*BRCA2uv* IVS21−11A/C	Unclassified
33	*BRCA2uv* A2951T	Polymorphism
35	*BRCA2uv* I3412V	Unclassified
38	*BRCA2uv* K1057R	Unclassified
40	*BRCA2uv* A2466V	Unclassified
42	*BRCA2uv* A2951T	Polymorphism
34	*BRCA1/2wt*	Wild type
48	*BRCA1/2wt*	Wild type

The frequency of LOH/AI is approximately equal in the neoplastic epithelium (66.2%) and corresponding surrounding stroma (59.7%; $P = .17$) of HBOC cases. In contrast, sporadic breast cancers show a significantly higher frequency of LOH/AI in the epithelium (36.7%) than in the stromal compartment (28.4%; $P = .037$)[10] (fig. 2). Notably, breast cancer epithelial and stromal cells from individuals with deleterious mutations in *BRCA1/2* and those with *BRCA1/2uv* showed a similar degree of overall genomic instability.

To evaluate for any specific patterns of LOH/AI, we employed an unsupervised hierarchical cluster analysis based on 371 microsatellite markers and 51 breast cancers from probands with HBOC. For each patient, the epithelium and stroma of breast carcinoma was considered separately (i.e., as two samples for each patient), giving us 102 end branches (fig. 3). Because the analysis was performed without any presumption about the grouping of these samples, the global pattern of LOH/

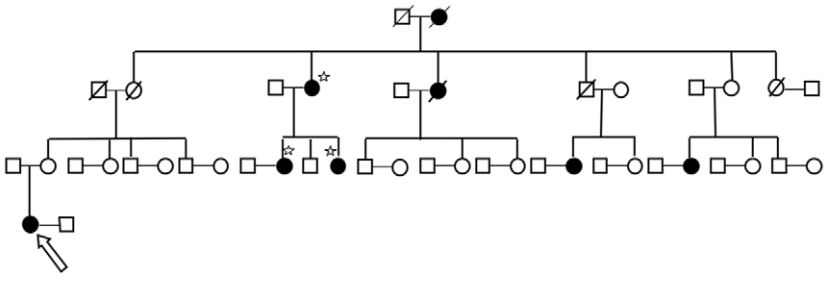

Figure 1 Pedigree of a family with HBOC segregating *BRCA* variants. The pedigree is shown across 4 generations, with affected members (with breast cancer) indicated by blackened circles. The proband (*arrow*) was diagnosed with breast cancer at age 49 years, and testing showed her as wild type for both *BRCA* genes. Other affected family members (*stars*) tested positive for the *BRCA1* Ser1040Asn and *BRCA2* Ser2483Gly variants.

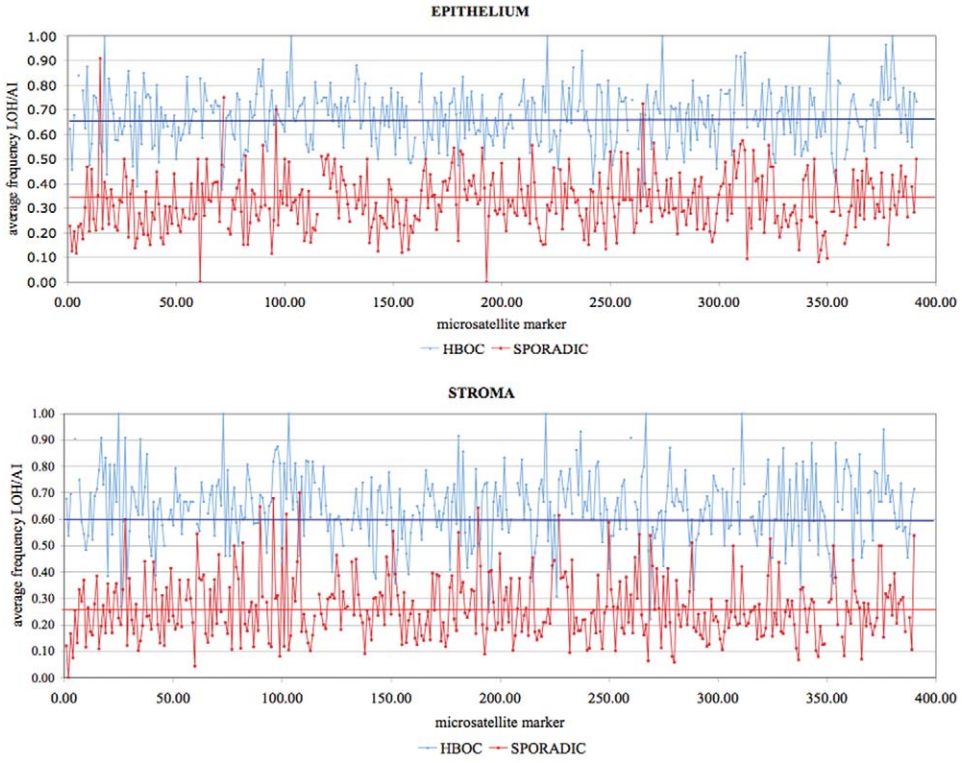

Figure 2 Frequency of LOH/AI observed in the epithelium and stroma in HBOC-related breast cancer compared with sporadic breast cancers. Frequency of LOH/AI (*Y*-axes) is plotted on a markerwise level (*X*-axes). The average LOH/AI frequency of markers in the epithelium (*top panel*) and stroma (*bottom panel*) are shown for 51 cases of HBOC-related breast cancer (*blue dots*) and 134 cases of sporadic breast cancer (*red dots*). The bold horizontal lines indicate LOH/AI frequencies averaged over all markers.

AI would cluster similar samples close to each other on the basis of the pattern of LOH/AI observed for all 371 loci. The analysis reveals no distinct separation of *BRCA1* and *BRCA2* samples nor of the *BRCA1/2* variants considered deleterious and those of unknown effect (*BRCA1/2uv*) into individual clusters. Also, no separation of tumor epithelium and stromal samples occurred. However, we noted a subset of *BRCA1* samples that clustered close together (fig. 3). Part of this subset consists of 15 patients whose epithelium and matching stroma cluster directly together. This means that, for these 15 patients, the pattern of LOH/AI in the epithelium is more similar to the pattern of LOH/AI observed in the corresponding stroma of the same patient than to any other sample. Among this group of 15 patients, *BRCA1* cases are significantly overrepresented (10 of 15 cases; $P = .028$). In contrast to almost half (45.5%) of all *BRCA1* patients, only 2 (15.4%) of 13 *BRCA2* samples showed this similarity in LOH/AI between epithelium and stroma ($P = .07$). Because the hierarchical cluster analysis is limited to one dimension, we also used two-dimensional scaling to visualize the relation between samples (fig. 4). Here, we note that both *BRCA1/2* samples and those from patients with *BRCA1/2uv* are contained in a similar region within this two-dimensional space. In addition, the location and therefore the pattern of LOH/AI of the two clinical HBOC cases with wild-type *BRCA1/2* overlaps with all the other HBOC cases. However, using two-dimensional scaling, we again noted that a subset of predominantly *BRCA1* samples grouped in a distinct region (fig. 4), similar to what we observed using unsupervised hierarchical clustering (see above and fig. 3).

Hotspots of LOH/AI in the Epithelium and Stroma of BRCA1/2-Related Breast Carcinomas

We then set out to detect nonrandom (hence, hotspot) LOH/AI occurring in *BRCA1/2*-related breast cancer epithelium and stroma and to correlate these with presenting clinico-pathologic features. Potential hotspots of LOH/AI were identified for 11 loci on six chromosomes in the epithelium and in 10 loci on six chromosomes in the stroma (table 3). These hotspot loci showed significantly elevated LOH/AI frequencies compared with those of the rest of the chromosome (table 3). Of note, the hotspot LOH/AI loci in *BRCA1/2*-related breast cancer epithelium and stroma are distinct from those we

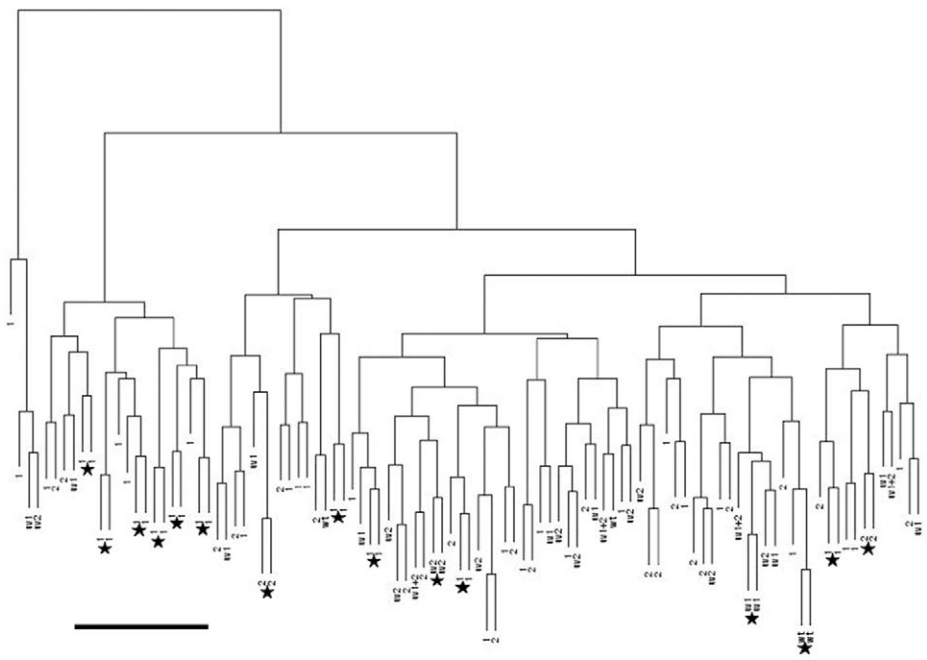

Figure 3 Unsupervised hierarchical cluster analysis. Average linkage and the dissimilarity measure of proportion of discordant LOH/AI between samples are used. The analysis was based on the presence or absence of LOH at 371 informative loci for 51 HBOC-related breast carcinoma epithelial samples and the matching 51 stromal samples (for a total of 102 end branches). The numbers at each end branch indicate germline deleterious mutations in *BRCA1* (1) or *BRCA2* (2) or unclassified variants of these genes (uv1 and uv2, respectively). The stars indicate those samples for which the stroma and epithelium of one case cluster directly together. Note the clustering of *BRCA1* samples near the left (*black bar*; see text for details). wt = Wild type.

previously identified in sporadic breast cancers.[10] For example, in sporadic breast cancer, hotspots of genomic instability were found at 11q22.3 in the epithelium, compared with 11q24.1 in *BRCA1/2*-related breast cancer epithelium. Interestingly, while, in sporadic breast cancer, this 11q22.3 hotspot was tightly flanked by regions that retained heterozygosity, in *BRCA1/2*-related breast cancers, we commonly find that the 11q24.1 LOH/AI hotspot extends some distance centromerically to 11q22.3.

We examined whether the presence of LOH/AI at these hotspot loci in the epithelium and/or stroma correlated with presenting clinico-pathologic status or germline mutation status. LOH/AI at 12q23.2 in the epithelium occurred significantly more often in tumors of stage pT2–4 than in pT1 tumors ($P = .043$). In addition, LOH/AI at 20p11.2 in stroma appears to be associated with the presence of unclassified variants ($P = .004$).

LOH/AI in the Epithelium and/or Stroma of BRCA1/2-Related Breast Cancer Compared with Sporadic Breast Cancer

In the next step, we focused on those cases with deleterious *BRCA1/2* germline mutations. To elucidate the relationship of *BRCA1* and *BRCA2* to each other as well as to sporadic breast cancers, we performed multidimensional scaling, looking separately at the neoplastic epithelium and at the stromal compartment (fig. 5). We found that *BRCA1* and *BRCA2* samples grouped, to some degree, in a similar space in this two-dimensional plot. The LOH/AI pattern of *BRCA1/2* neoplastic epithelium overlapped, to a great extent, with that observed in the epithelium of the sporadic counterparts (fig. 5). In striking contrast, however, we found that there was a stronger separation between *BRCA1/2*-related samples and sporadic ones, when looking at the stromal compartment (fig. 5).

Knowing that stroma and epithelium have a close interaction during carcinogenesis, we then combined both LOH/AI data sets (from epithelium and stroma) of 51 cases of HBOC and 134 cases of sporadic breast cancer and performed unsupervised cluster analysis (fig. 6). On the basis of this combined epithelium and stroma data set, we noted a strong clustering of HBOC cases. However, we found that 11 cases with germline *BRCA1/2* mutations grouped outside the strong HBOC cluster. This effect was also observed, to varying extents, in the previous analysis (as illustrated in figs. 3 and 4). The uniqueness of these samples is revealed when we look

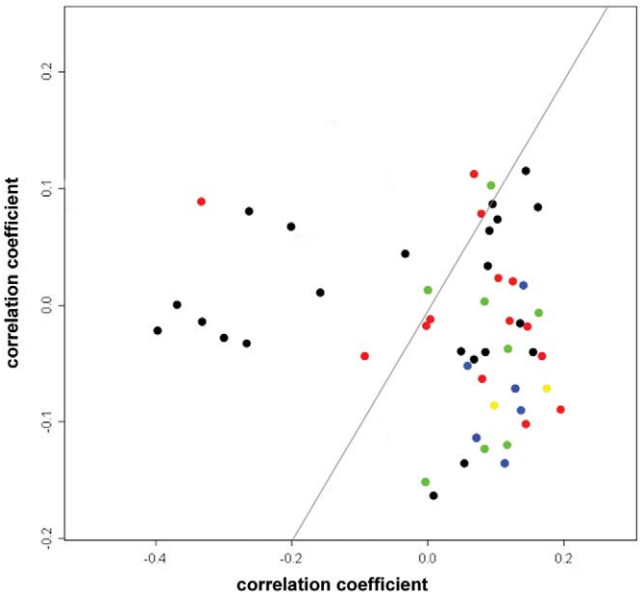

Figure 4 Two-dimensional scaling of 51 HBOC-related breast cancer cases. Each case is represented by combining the stroma and malignant epithelium from the same patient. Samples were obtained from individuals with germline mutations in *BRCA1* (*black dots*) or *BRCA2* (*red dots*), with variants in *BRCA1* (*BRCA1uv* [*green dots*]) or *BRCA2* (*BRCA2uv* [*blue dots*]), or without mutations or variants in either gene (*yellow dots*). Note the clustering of samples with *BRCA1* mutations to the left of the diagonal line.

at the overall frequency of LOH/AI in this group of 11. We found that the samples that are consistently outliers (denoted as "group 1") showed an average LOH/AI frequency of 41.8% in the epithelium and 45.0% in the stroma, compared with LOH/AI frequencies of 72.9% and 64.0% in the epithelium and stroma, respectively, of the remaining group of HBOC cases (denoted as "group 2"). Although the frequencies of LOH/AI in these two groups are significantly different in the epithelium ($P = .046$), the difference was not statistically significant for the frequencies observed in the stroma ($P = .25$).

Discussion

The elucidation of the functional properties of *BRCA1/2* germline mutations and their role in carcinogenesis of HBOC syndrome allowed genetic testing for cancer susceptibility in members of affected families. However, classic HBOC-affected families exist that lack germline deleterious mutations in the *BRCA1/2* genes or other breast cancer susceptibility genes. This implies that other factors, yet unknown to us, are contributing to breast cancer risk. Therefore, one of the questions to be answered is, Are breast cancers derived from individuals with *BRCA1* or *BRCA2* mutations and non-*BRCA1/2* cases of breast cancer distinct diseases at the somatic genetic level, or do they share key genetic aspects in carcinogenesis?

The important role of the tumor microenvironment, both at the genetic and cell biological level, in the initiation and progression of sporadic breast cancer has been shown elsewhere by us and others.[10–12,17–20] In fact, a recent animal model suggests that the process of carcinogenesis can be normalized through manipulation of stromal-mediated mechanisms.[19] In our current study, we isolated the malignant breast epithelium and its surrounding stroma separately, which allowed us, for the first time, to portray a comprehensive genomic picture of the tumor microenvironment. Our data suggest that, indeed, in patients with *BRCA1/2*-related breast cancers, genomic alteration in the stroma coexists equally with that in the epithelium, and, thus, the genetically unstable stroma might provide for a microenvironment that functions as a landscaper that positively selects for genomic instability in the epithelium, thereby promoting neoplastic transformation.

High Frequency of LOH/AI in Stroma Suggesting Landscaping Role

We noted the overall high frequency of genomic instability in the epithelium and stroma of all samples with

Table 3

Hotspots of Genomic Instability in the Epithelium and Stroma of *BRCA1/2*-Related Breast Carcinomas

Tissue and Locus	Marker	*P*	Gene(s)
Epithelium:			
1p36.13	*D1S3669*	.0185	*TP73, SDHB*
1p21.1	*ATA42G12*	.0234	
1q23.1	*D1S1653*	.0135	*FCRL2*
1q42.3	*D1S235*	.0402	*GNG4*
11q24.1	*D11S4464*	.0108	*LOH11CR2A, ETS1, NFRKB*
11q24-25[a]	*D11S4463*	.0062	
12q23.2	*PAH*	.0149	*PAH, ASCL1, IGF1*
14q23.1	*D14S592*	.0274	*SIX1*
20p12.1-11.23	*D20S1143*	.0097	*RBBP9, SNRPB2, PCSK2*
20q13.32	*D20S164*	.0196	*RAB22A*
Xp22.2	*DXS9902*	.0038	*FANCB, BMX, STS*
Stroma:			
1p36.13	*D1S3669*	.0447	*TP73, SDHB*
1p21.1	*ATA42G12*	.0193	
1q42.3	*D1S235*	.006	*GNG4*
7p14.1	*D7S2846*	.0178	*SFRP4*
10p11.21	*D10S1208*	.0268	*CUL2, CREM*
18q21.32	*D18S1357*	.0397	*BCL2, DCC*
18q23	*D18S1390*	.0237	
19q13.33	*D19S246*	.0031	*POLD1*
20p13	*D20S482*	.0326	*SMOX, RASSF2, SLC23A2*
20q13.32	*D20S164*	.0492	*RAB22A*

[a] Mapped between *D11S4464* and *D11S1304*.

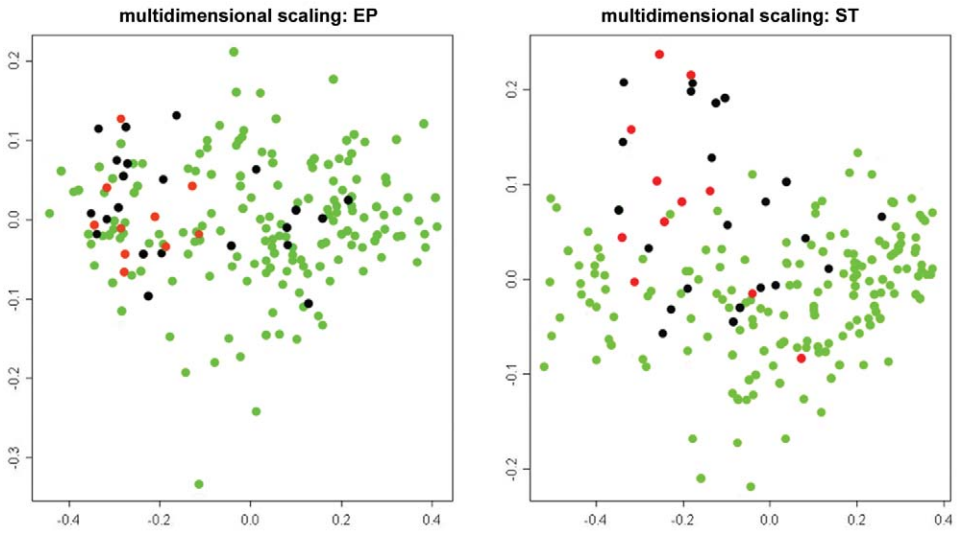

Figure 5 Multidimensional scaling of LOH/AI patterns from 35 patients with deleterious *BRCA1/2* mutations. The LOH/AI patterns from epithelium (EP; *left panel*) and stroma (ST; *right panel*) of individuals with germline mutations in *BRCA1* (*black dots*) and *BRCA2* (*red dots*) were analyzed separately in the context of the pattern of LOH/AI observed in sporadic breast cancers (*green dots*). Note that the stromal LOH/AI pattern differentiates *BRCA1* and *BRCA2* mutation-positive breast cancers from sporadic breast cancers.

HBOC, regardless of the germline *BRCA1/2* mutation status. In comparing them with our cases of sporadic breast cancer, we therefore conclude that predisposition to genomic instability, as manifested by high frequencies of LOH/AI, is a unifying factor in *BRCA1/2*-related and non–*BRCA1/2*-related HBOC breast cancers. Interestingly, in HBOC breast cancers, we note that, not only the malignant epithelium, but also the stroma harbors a similarly high frequency of genomic instability. The potentially important role of the stroma in HBOC carcinogenesis is corroborated by morphological observations during mammary development. The process of mammary differentiation requires not only extensive cell proliferation but also penetration of the breast epithelium at the ductal end buds into the stroma. On the basis of clinical epidemiologic observations, one can hypothesize that, as the mammary mesenchyme proliferates during puberty, it is already affected by the impaired DNA repair mechanism resulting from defective *BRCA* genes, and so somehow predisposes to the highly susceptible, proliferating mammary epithelium during pregnancy.[21] This idea is supported by the data from our patient with a germline *BRCA2* mutation who received prophylactic mastectomy. Histological examination by two pathologists confirmed that the breast tissue analyzed in our study did not contain any signs of atypia. We already found LOH/AI in 31.5% of all informative markers in the microscopically normal-appearing breast epithelium (compared with germline/blood DNA). Such a high frequency of genomic alterations in normal-appearing tissue might be surprising at first but can be explained by the impaired DNA repair machinery. This finding is supported by a report by Cavalli et al.,[6] who analyzed normal tissue of five patients with *BRCA* mutations and found LOH/AI in 50% of the 15 loci analyzed. Interestingly, the stromal fibroblasts of the prophylactic mastectomy specimen showed LOH/AI in >65.5% of all informative markers, which was not different from the frequency of LOH/AI observed in the tumor stroma of breast cancers among the other HBOC cases. A similar observation is made when we look at the subset of cases (group 1) that showed a lower overall frequency of genomic instability in the neoplastic epithelium; their corresponding stroma showed a higher overall LOH/AI frequency, statistically similar to that observed in group 2 (which had higher overall LOH/AI frequencies in both stroma and epithelium). Such an observation could be interpreted in two ways—either that stromal genomic instability precedes that in the epithelium in some *BRCA1/2*-related breast cancers, and therefore stroma accumulates more genetic alterations, or that at least two distinct progression pathways exist, leading to two distinct phenotypes, one with high-level and the other with low-level genomic instability within the breast cancer epithelium.

Clonal Patches in Genetically Unstable Stroma and Epithelium

Our data show that genetically unstable cell populations collected over large expanses of microdissected tissue (e.g., epithelium or stroma) share LOH/AI patterns

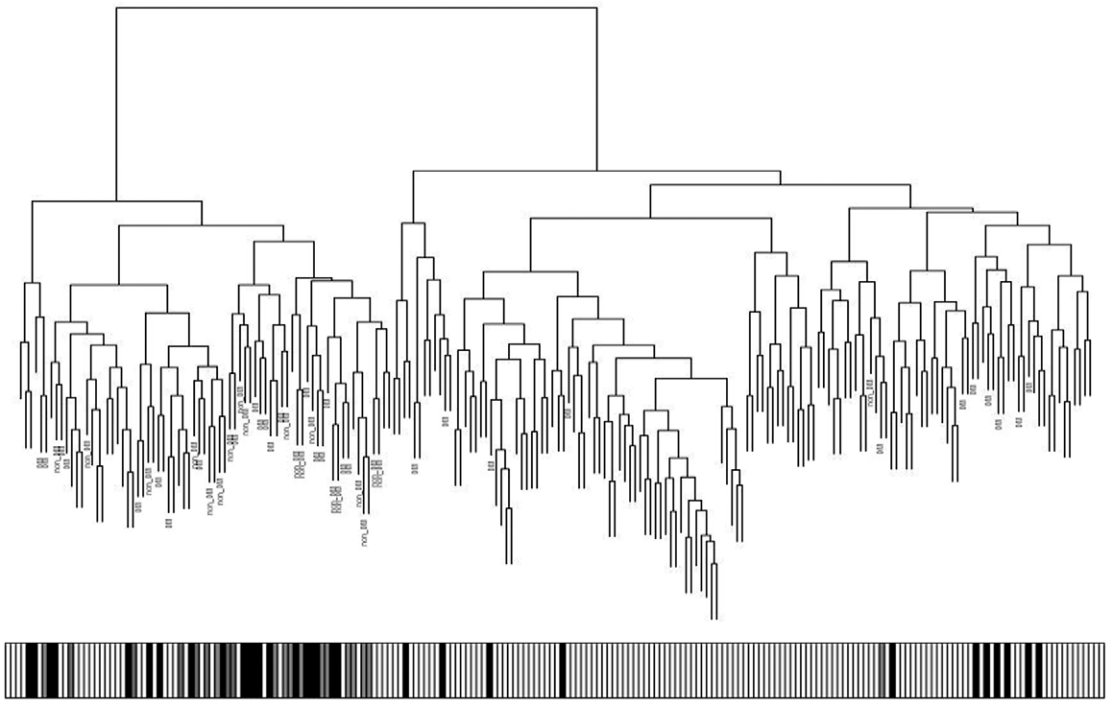

Figure 6 Unsupervised cluster analysis by LOH/AI status in a combined stromal and epithelial data set for HBOC-related breast cancers and sporadic breast cancers. HBOC breast cancers are labeled at the end branches with "DM" (deleterious mutation) and "non-DM" (comprising those with variants of unknown significance and those without detectable mutations). The bar below the cluster plot visualizes the separation of HBOC breast cancers (*black* and *gray bars*) from sporadic breast cancers (*white bars*).

among cells, suggesting clonal expansion of the affected cells. Much of this conclusion is based on the technical limitations of LOH/AI analysis, which is not capable of detecting allelic imbalance without a majority (>50%) of the sample contributing to the shared altered genotype. Such conservation of specific genetic alterations across many spatially distributed cells is a characteristic feature of (benign and malignant) neoplastic processes in which a geographic clonal expansion of mutated cells occurs, with exclusion of unaffected wild-type cells.[22] The monoclonal character of tumor stroma, as documented by conserved locus-specific LOH/AI, is compelling evidence that the stroma itself is neoplastic and capable of overrunning, or outcompeting, genetically intact stromal cells. This is one mechanism for local enrichment of genetically altered stromal cells, which may increase the magnitude of their functional impact on adjacent breast epithelium.[22] Alternatively, one might propose that stromal fibroblasts could be subjected to apoptosis as a result of an increased rate of DNA damage due to loss of BRCA1/2 function. Those cells evading apoptosis might, therefore, gain a growth advantage; thus, the consequent clonal expansion could be viewed as a result of a repair mechanism.

Hotspots of Genomic Instability in the Epithelium and Stroma

In addition to a general genomic instability, we identified a similar number of LOH/AI hotspots in the epithelium (11 loci) and stroma (10 loci) of HBOC breast cancers, which contrasts our observations in sporadic breast cancer, for which we identified 19 loci in the epithelium and 38 loci in the stroma (table 3). In sporadic breast carcinomas, there is an overall higher frequency of LOH/AI in the epithelium than in the stroma, which suggested that epithelial genetic events initiate sporadic breast carcinogenesis and, perhaps, that stromal genetic events lend biological diversity.[10] This is also reflected by the diversity of genetic alterations. While *BRCA1/2*-related stromal hotspot LOH/AI loci are more limited (10 loci), each locus bears a significantly higher frequency of LOH/AI (average 77.35% ± 7.95%), compared with sporadic breast cancer stromal hotspots, which are diverse (38 loci) but possess a lower frequency of LOH/AI (53.22% ± 7.36%). Notably, there is virtually no overlap in the epithelial and/or stromal hotspot loci observed in the HBOC breast cancers compared with those observed in sporadic breast cancers.

We hypothesized, as outlined above, that the impaired

stroma might have a landscaping role for the neoplastic transformation of mammary epithelium. On the basis of our data, we speculate that regulatory genes affecting the microenvironmental organization, the cell-cell interaction, or the response to paracrine stimuli might be altered and provide a basis for such an hypothesis. For instance, we observed frequent loss of the *SDHB* locus in the stroma and epithelium. Succinate dehydrogenase complex, subunit B (SDHB [MIM *185470]), is involved in mitochondrial electron transport and lies at the juncture of the Krebs cycle, and loss of SDHB results in the disruption of mitochondrial complex II and subsequent transcription factor hypoxia inducible factor 1 (HIF1α [MIM *603348]) activation. Increase in HIF1α in turn leads to activation of the cell proliferation pathway and to an increase in paracrine-acting growth factors, such as vascular endothelial growth factor (VEGF [MIM *192240]).[23]

In our series, we were able to associate genomic instability at the PAH locus (12q23.2) in the epithelium with higher T stage (T1 vs. T2, T3, and T4). The insulin-like growth factor 1 gene (*IGF1* [MIM *147440]) is among the genes that map to this locus. IGF1 plays an important role in the development of the differentiated mammary gland, and elevated levels of IGF1 have been associated with an increased risk of early breast cancer. However, the absence of the common *IGF1* 19-repeat allele (a CA repeat in the promoter region that occurs in most white women) has been identified as a high-risk genotype.[24] Loss of this protective allele might contribute to advanced disease or contribute to the Lob 1 seen in HBOC cases.

Another particular hotspot locus, at 19q13.33, is worthy of mention. This region harbors *POLD1* (MIM *174761), which codes for a primary replicative enzyme with proofreading capabilities.[25] Its function during DNA duplication at the replication fork creates single-strand DNA regions.[25,26] Impaired POLD1 can therefore lead to single-strand gaps and double-strand breaks.[26] The defective $POLD1^{D400A}$ is associated with cancer susceptibility,[27] and a recent kin-cohort study found that a variant of $POLD1^{R119H}$ (0.06% allelic frequency) was associated with an approximately twofold increase in the relative risk for sporadic breast cancer ($P = .058$).[28] These observations together with our findings of significantly elevated LOH/AI at the *POLD1* locus are important, since they suggest a possible functional reason for the high frequency of genomic instability observed in the stromal compartment. This mechanism involving *POLD1* is restricted to carriers of deleterious *BRCA1/2* mutations as well as HBOC cases (with *BRCA1/2uv* or no mutation), such that no LOH/AI hotspots at 19q13.33 were found in sporadic breast cancer stroma.

Previous studies have addressed LOH/AI frequencies in breast cancers derived from HBOC cases and may have reported relatively high frequencies of LOH/AI at loci distinct from our hotspots. There are several straightforward explanations for these apparent discrepancies. First, it is important to note that our operational definition of a hotspot is based on our model-based approach: a hotspot is defined as having a significantly high frequency of LOH/AI, compared with all other loci along the same chromosome.[10] Thus, it is possible that other studies using a small set of markers might find an apparently high frequency of LOH/AI in one marker and label this locus significant[29]; however, other loci along the same chromosome, which may not have been examined, might have LOH/AI at a similar or even elevated level than the selected marker. Second, studies using array-based comparative genomic hybridization, although they have the advantage of differentiating between allelic gain and loss, usually detect losses and/or gains of larger genomic regions, spanning several BAC clones.[5,16,30] Finally, another important factor that allowed us to identify previously unrecognized hotspots is the separation of neoplastic epithelium and stroma. The previous studies have looked at the admixed (epithelium and stroma) tumor tissue. Since our findings indicate a high level of genomic instability in the stromal compartment of HBOC-related breast cancers, we can only assume that, in previous studies, only regions with concordant allelic imbalance in epithelium and stroma would have been identified as hotspots.

Epithelial-to-Mesenchymal Transition in a Subset of BRCA1-Related Breast Cancers?

It has been shown recently that *BRCA1*-related breast cancers commonly present with a "basal type" phenotype, identified by the expression of myoepithelial markers.[16] Interestingly, a "basal type" gene-expression pattern was associated with a subset of sporadic breast cancers as well,[31] implying that a subset of sporadic breast cancers follow an oncogenic pathway similar to that *BRCA1/2*-related cancers. In our study, we found that the pattern of genomic instability observed in the epithelium and stroma in a subset of 15 HBOC cases (group 1) is so similar that the two compartments (epithelium and stroma) from a given individual cluster directly together. The majority of these samples are from *BRCA1* cases, commonly associated with the "basal type" phenotype. We addressed the issue that such similarity can potentially arise from admixed tissue (e.g., contaminating epithelial cells in the stroma fraction or vise versa). Besides the great caution that was used during the laser capture microdissection procedure, other observations provide conclusive evidence against an erroneous finding. For one, in this set of 15 HBOC cases, we noted markers with opposing LOH/AI calls in each compartment of a given tumor (e.g., LOH/AI observed

in epithelium but not stroma, and vice versa). In addition, in some cases with concordant LOH/AI calls, we find that different alleles are lost in a compartment-specific manner. Third, we identified somatic mutations in some of these 15 cases that were confined to either the epithelium or the stroma compartment.[32] Since all analyses were performed from the same pool of extracted DNA, such observations rule out the possibility of tissue admixture or intercompartmental contamination.

In our previously reported analysis of sporadic breast cancer, we noted a pattern in which the corresponding epithelium and stroma cluster together in a few cases.[10] For sporadic breast cancers, we believe that the small subset of cases may suggest an epithelial-to-mesenchymal transition. Whether the observation of tight epithelium-corresponding stroma clustering in the subset of *BRCA1* cases also reflects epithelial-to-mesenchymal transition is not known. Another hypothesis that might warrant further exploration in this context is the concept of cell fusion.[33,34] In this theory, cell fusion is assumed to be essential for the development and maintenance of a clinically significant tumor.[33] Advanced breast cancer is commonly associated with aneuploidy and thus a labile genome, whereas cell fusion is thought to produce polyploid cells that ultimately end up as small cells with scarce or sparse cytoplasm.[33] Interestingly, Roy et al.[35] reported that 30.64% of all tumor metaphases of HBOC cases were hyperdiploid. We therefore might hypothesize that, in this subset of HBOC cases, the malignant breast epithelial cells fuse with the stromal cells and, in rejuvenating the labile genome, result in further enhanced genomic instability in both the epithelium and stroma. Although this hypothesis is consistent with our current observations, further proof will clearly require confirmation by use of functional modeling. Similarly, while our observation of the frequency and distribution of genetic alterations in sporadic and HBOC breast cancers is straightforward, the conclusion that the HBOC mammary stroma has a landscaping role would require functional modeling. With expansion of the approaches reported by Shakar et al.[20] and others,[19] one intriguing approach would be to obtain stromal fibroblasts of HBOC breast cancer and prophylactically operated HBOC cases and to investigate their influence on mammary epithelial cells in coculture. In addition, such an in vitro model would allow testing of the co-contribution of other paracrine-acting factors, such as IGF2 (MIM 147470) or estrogens.

Summary and Conclusion

In summary, our data show that, in contrast to stroma of sporadic breast cancers, genomic alterations in the stroma of *BRCA1/2*-related breast cancers are an important, unifying, and potentially driving mechanism in the pathogenesis of breast cancer. We identified several potential hotspots of genomic instability that occur not only in carriers of deleterious *BRCA1/2* mutations but also in HBOC cases without obvious pathogenic mutations (mutation-negative cases and cases with variants of unknown significance). These hotspots are distinct from those identified in sporadic breast cancers,[10] and so our observations suggest that these HBOC-related breast cancer hotspots are specific to the pathogenesis of breast cancer in those with germline *BRCA1/2* mutations and in those whose clinical picture is consistent with *BRCA1/2* disease, even in the absence of pathogenic mutations or the presence of only variants of unknown significance. How these findings might improve the sensitivity of early diagnosis needs further elucidation, but they may suggest novel therapeutic approaches, since normalization of an impaired stroma can alter and potentially reverse preneoplastic or maybe even neoplastic breast epithelium.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for familial breast cancer, *BRCA1, BRCA2,* SDHB, HIF1α, VEGF, *IGF1, POLD1,* and IGF2)

The R Foundation for Statistical Computing, http://www.r-project.org/

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