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## Breast-Cancer Stromal Cells with TP53 Mutations and Nodal Metastases

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### ABSTRACT

#### BACKGROUND

The importance of cross-talk between a cancer and its microenvironment has been increasingly recognized. We hypothesized that mutational inactivation of the tumor-suppressor gene *TP53* and genomic alterations in stromal cells of a tumor's microenvironment contribute to the clinical outcome.

#### METHODS

We performed *TP53* mutation analysis and genomewide analysis of loss of heterozygosity and allelic imbalance on DNA from isolated neoplastic epithelial and stromal cells from 43 patients with hereditary breast cancer and 175 patients with sporadic breast cancer. Compartment-specific patterns and *TP53* mutations were analyzed. Associations between compartment-specific *TP53* status, loss of heterozygosity or allelic imbalance, and clinical and pathological characteristics were computed.

#### RESULTS

*TP53* mutations were associated with an increased loss of heterozygosity and allelic imbalance in both hereditary and sporadic breast cancers, but samples from patients with hereditary disease had more frequent mutations than did those from patients with sporadic tumors (74.4% vs. 42.3%,  $P=0.001$ ). Only 1 microsatellite locus (2p25.1) in stromal cells from hereditary breast cancers was associated with mutated *TP53*, whereas there were 66 such loci in cells from sporadic breast cancers. Somatic *TP53* mutations in stroma, but not epithelium, of sporadic breast cancers were associated with regional nodal metastases ( $P=0.003$ ). A specific set of five loci linked to an increased loss of heterozygosity and allelic imbalance in the stroma of sporadic tumors was associated with nodal metastases in the absence of *TP53* mutations. No associations were found between any of the clinical or pathological features of hereditary breast cancer with somatic *TP53* mutations.

#### CONCLUSIONS

Stroma-specific loss of heterozygosity or allelic imbalance is associated with somatic *TP53* mutations and regional lymph-node metastases in sporadic breast cancer but not in hereditary breast cancer.

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**D**YNAMIC INTERACTIONS BETWEEN NEOplastic epithelial cells and the surrounding stroma can select stromal cells that modulate tumor behavior.<sup>1-3</sup> Moreover, carcinoma-associated stromal cells can transform normal epithelial cells into neoplastic cells.<sup>4,5</sup> In an animal model, selective mutations in the reactive stroma of a neoplasm accelerated tumor development, a process that was reversed by stromal gain or loss of certain genes, one of which was *TP53*.<sup>6,7</sup>

*TP53* is the most commonly mutated gene in human neoplasms.<sup>8</sup> The p53 tumor-suppressor protein involves the cell cycle, checkpoint control, repair of DNA damage, and apoptosis.<sup>9,10</sup> In whole-tumor material, the frequency of a *TP53* mutation in breast cancers ranges from 20 to 50% and is most common in the hereditary breast-ovarian cancer syndrome that is caused by germ-line mutations in *BRCA1* and *BRCA2*.<sup>11,12</sup> Like p53, *BRCA1* and *BRCA2* proteins regulate cell-cycle control and apoptosis.<sup>11</sup> In vitro work suggests that in cells lacking p53, *BRCA1* and *BRCA2* up-regulate the expression of genes involved in DNA repair.<sup>13</sup> In *BRCA*-associated cancers, not only the frequency but also the spectrum of *TP53* mutations differs from *TP53* mutations in grade-matched sporadic breast cancers.<sup>14-16</sup>

We previously found high frequencies of mutations of *TP53* and phosphatase and tensin homologue (*PTEN*) in neoplastic breast epithelium and the surrounding stroma.<sup>17</sup> In this study of hereditary and sporadic breast cancers, we sought *TP53* mutations and loss of heterozygosity or allelic imbalance in neoplastic epithelial cells and surrounding stromal cells and related them to clinical and pathological features of the disease.

## METHODS

### PATIENTS

We evaluated invasive breast cancers from 218 patients (43 with hereditary breast cancer and 175 with sporadic breast cancer). For both groups, inclusion criteria were a pathological diagnosis of invasive ductal carcinoma. We excluded patients with widely metastatic disease to minimize incomplete ascertainment due to the difficulty of obtaining the original primary carcinoma, on which all analyses were performed.

Patients with hereditary breast cancer had to meet clinical diagnostic criteria<sup>18</sup> and have deleterious

germ-line mutations or unclassified variants of *BRCA1* or *BRCA2*. Two patients whose tumors were wild-type for both genes were included in the hereditary group because they were members of families with a high previous probability of harboring *BRCA1* or *BRCA2* mutations.

The institutional review board at each participating institution approved the study. We used anonymous samples linked only to clinicopathological data. Analyses were performed from September 2005 through May 2007.

### LASER-CAPTURE MICRODISSECTION AND DNA EXTRACTION

Laser-capture microdissection was performed with the use of an Arcturus PixCell II microscope (Arcturus Engineering) to procure epithelium and stroma of the neoplastic tissue.<sup>19,20</sup> This microscope uses a transparent thermoplastic film (also called a standard laser-capture microdissection cap) applied to the surface of the tissue section (5 to 7  $\mu\text{m}$  thick) on standard histopathology slides. The epithelial cells, surrounding stromal cells, and normal cells in the sample were identified and targeted through a microscope, with a relatively narrow (15 to 30  $\mu\text{m}$ ) carbon dioxide laser-beam pulse. The resulting strong focal adhesion allowed selective procurement of only the target cells.

We first removed the neoplastic epithelium and then took the fibroblasts in the stroma. Four to six standard laser-capture microdissection caps (with 8000 to 9000 cells per cap) were procured per compartment, four from the epithelial compartment and six from the stromal compartment (since the stromal compartment has a lower cellular density). We specifically captured stromal fibroblasts residing between aggregations of epithelial tumor cells or no more than 0.5 cm distant from the tumor nodule. This morphological approach allows for replication of the distances between the tumor-stromal and tumor-epithelial fractions for all samples.

DNA for each tumor was also obtained from peripheral-blood leukocytes (in 75% of hereditary tumors) or from normal-appearing cells that were at least 1 cm distant from the tumor in the tissue section. The origin of the normal DNA had no effect on the frequency or pattern of loss of heterozygosity or allelic imbalance. After the performance of laser-capture microdissection, genomic DNA was extracted as described.<sup>17,20</sup>

**Figure 1. Somatic TP53 Mutations in Breast Cancers.**

In Panel A, a representative chromatogram of one sporadic breast cancer shows a GTG173GCG (Val173Ala) mutation of *TP53* in stroma (arrow) but not in epithelium. In Panel B, representative immunohistochemical analysis with the use of anti-p53 antibody reveals increased protein expression in tumor stromal fibroblasts of samples with stromal mutant p53 (Gly325Arg) and no expression in the matched normal stroma, which is wild-type p53. In Panel C, a sequencing chromatogram for the sporadic breast cancer in Panel B shows a GGA325AGA (Gly325Arg) mutation of *TP53* in tumor stroma but not in the matched normal stroma.

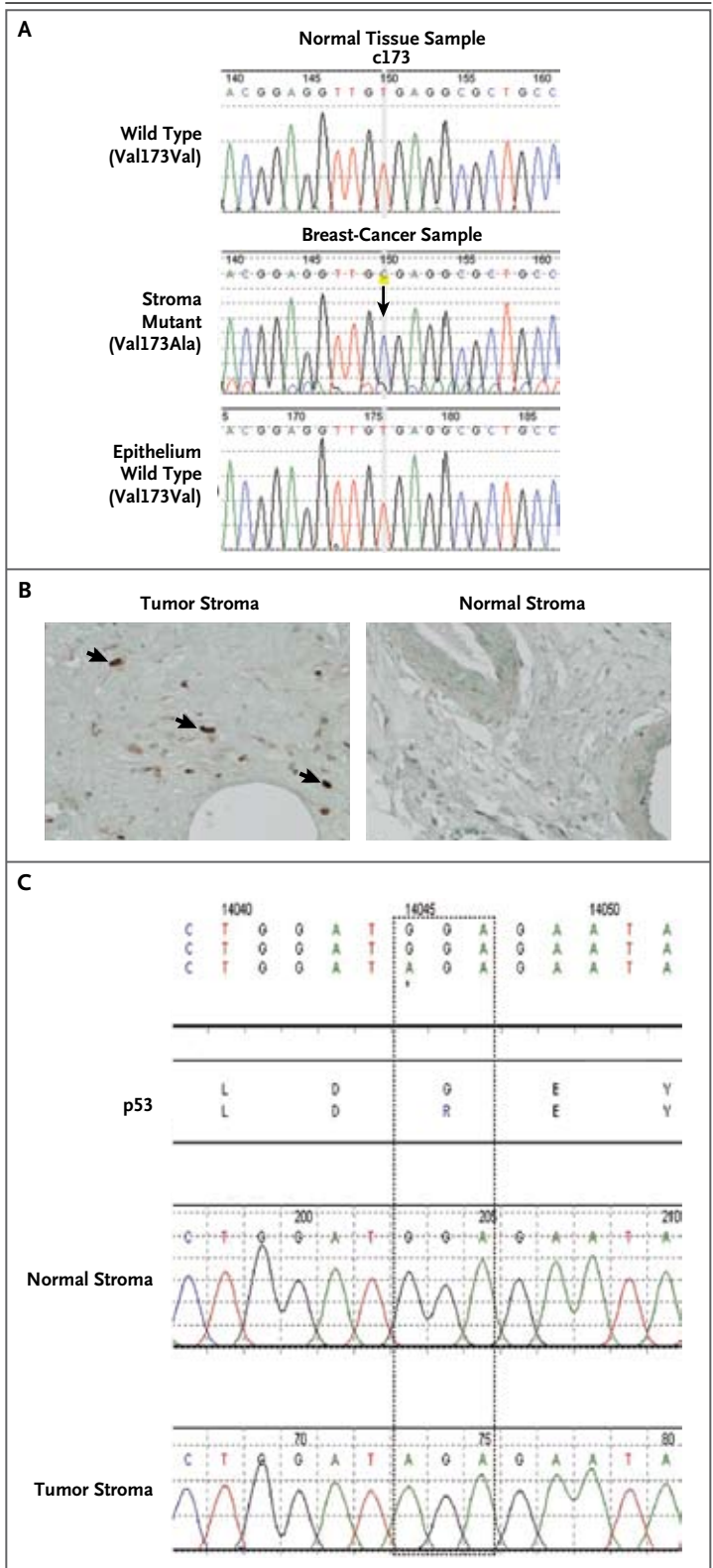
**GENOMEWIDE SCAN**

A polymerase-chain-reaction (PCR) assay was performed on DNA from each compartment (normal, epithelium, and stroma) of each sample and 1 of 72 multiplex primer panels, comprising 372 and 386 fluorescence-labeled microsatellite markers for hereditary and sporadic samples, respectively. These markers are distributed throughout chromosomes 1 through 22 and X and are based on the MapPairs Human Markers set, version 10 (Invitrogen) developed at the Marshfield Institute. This genomewide panel has an average of 16.2 markers per chromosome (ranging from 7 to 29 markers per chromosome), or an intermarker distance of approximately 9 cM.

Genotyping was performed with either the ABI 3700 or the 3730XL semiautomated sequencer (Applied Biosystems). The results were analyzed by automated fluorescence detection with the use of the GeneScan collection and analysis software (Applied Biosystems). Scoring for the loss of heterozygosity or allelic imbalance was performed by visual inspection of the GeneScan output. A ratio of allele peak heights between germ-line DNA and somatic DNA of 1.5 or more was used to define a loss of heterozygosity or allelic imbalance.<sup>21-23</sup> The reliability of such evaluations by multiplex PCR on archived tissue has been extensively validated.<sup>19</sup>

**MUTATION ANALYSIS OF TP53**

Mutation analysis of *TP53* was performed by PCR amplification of exons 4 through 9 of *TP53*, followed by denaturing gradient gel electrophoresis (DGGE) analysis. Fragments showing abnormal migration patterns in the DGGE analysis were re-amplified from the original DNA and sequenced



**Table 1.** Frequency of Loss of Heterozygosity or Allelic Imbalance in 175 Sporadic Breast Cancers and 43 Hereditary Breast Cancers in Epithelium and Stroma.\*

Tissue	Sporadic Group		Hereditary Group		P Value†
	No. of Markers	LOH Frequency	No. of Markers	LOH Frequency	
Epithelium	370		367		<0.001
Median		0.54		0.67	
Interquartile range		0.45–0.64		0.57–0.78	
Stroma	370		368		<0.001
Median		0.51		0.60	
Interquartile range		0.41–0.62		0.50–0.71	

\* LOH denotes loss of heterozygosity or allelic imbalance.

† P values were calculated with the Wilcoxon rank-sum test.

directly. (A description of the PCR conditions and oligonucleotide primer sequences used for PCR–DGGE and sequencing is available in Table 1 of the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org).) DGGE separation through a 10% polyacrylamide gel containing a 20 to 70% urea–formamide gradient was performed at 120 V and 60°C for 14 hours.<sup>24</sup>

#### IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin sections of breast-cancer specimens were rehydrated and subjected to microwave antigen retrieval for 20 minutes followed by overnight incubation at 4°C with antibodies against p53 from murine clone PAb1801 (Novocastra) at a dilution of 1:300. Slides were washed and incubated with secondary biotinylated antibodies with the use of the Vectastain ABC kit (Vector Laboratories); they were then treated with sequential additions of avidin peroxidase and 3,3'-diaminobenzidine and counterstained by methyl green. The status of p53 was scored visually as positive by a pathologist if the nuclei of stromal cells stained darkly.

#### STATISTICAL ANALYSIS

A total of 372 microsatellite markers from the 43 hereditary cancers and 386 markers from the 175 sporadic breast cancers were analyzed in the samples obtained from the epithelium and stroma. We performed chi-square tests of association between the loss of heterozygosity and *TP53* mutation in these two groups of tumors. The Wilcoxon rank-sum test was applied to compare frequencies of the loss of heterozygosity between each paired group with wild-type *TP53* and with mutated *TP53*.

To identify compartmental hot spots of the loss of heterozygosity associated with mutated *TP53*, the significance of overall frequencies (across all samples), as compared with chromosome-average frequencies, of the loss of heterozygosity was analyzed for each microsatellite marker with the use of logistic regression with *TP53* as a covariate; the significance of the presence of a *TP53* mutation was also tested with the use of analysis of deviance. These statistical methods are meant to identify microsatellite loci with the highest degree of association between a loss of heterozygosity or allelic imbalance and *TP53* mutation. Logistic regression and analysis of deviance were also applied to test the association between loss of heterozygosity or allelic imbalance and each of the clinical and pathological features (pathologically confirmed tumor and nodal status, tumor grade, clinical stage, estrogen-receptor status, and expression of *HER2/neu*). For this analysis, the age at diagnosis was taken into account by its inclusion as a covariate. Adjustment for multiple testing was applied with the use of false positive report probability (FPRP).<sup>25,26</sup> A significant value with a previous probability of 0.01 and an FPRP value of less than 50% is denoted as  $FPRP_{0.01} < 0.5$ .

Among the microsatellite markers that had a significant association with mutated *TP53* in the stroma of the sporadic cancers, linear-by-linear association tests<sup>27,28</sup> were used to identify markers having a significant association with lymph-node metastases. These tests seek associations between lymph-node status and each stromal microsatellite marker, stratified according to *TP53* mutation status. If a significant association was found, we

**Table 2. Frequency of Loss of Heterozygosity or Allelic Imbalance in Stroma and Epithelium of Hereditary and Sporadic Breast Cancers, According to the Type of TP53 Mutation.\***

Tissue	Mutated TP53		Wild-Type TP53		P Value†
	No. of Markers	LOH Frequency	No. of Markers	LOH Frequency	
<b>Hereditary group</b>					
Epithelium	370		367		0.01
Median		0.61		0.67	
Interquartile range		0.50–0.71		0.50–0.80	
Stroma	370		368		<0.001
Median		0.70		0.57	
Interquartile range		0.60–0.78		0.40–0.71	
<b>Sporadic group</b>					
Epithelium	384		385		<0.001
Median		0.62		0.47	
Interquartile range		0.54–0.69		0.40–0.54	
Stroma	384		385		<0.001
Median		0.60		0.42	
Interquartile range		0.53–0.66		0.36–0.50	

\* LOH denotes loss of heterozygosity or allelic imbalance.

† P values were calculated with the Wilcoxon rank-sum test.

then determined which TP53 status (mutation-positive or mutation-negative) was associated with nodal involvement. Multiple-testing adjustments were controlled by a false discovery rate of less than 0.1.

The R package ([www.r-project.org](http://www.r-project.org)) was used for all data mining and statistical analysis.

## RESULTS

### PATIENTS

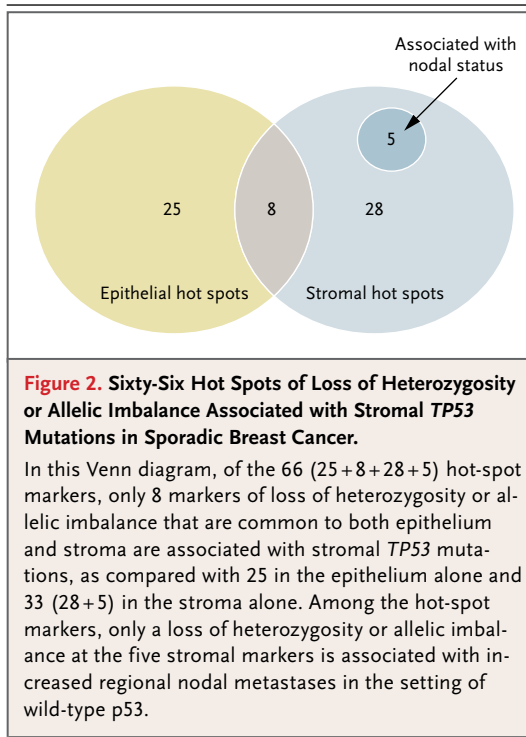
Table 2 of the Supplementary Appendix summarizes the clinical and pathological features of all patients. The mean age at diagnosis was 42.6 years (range, 23 to 86) in the group with hereditary cancers (hereditary group) and 52.3 years (range, 25 to 82) in the group with sporadic cancers (sporadic group) ( $P=0.002$ ). Positivity for either the estrogen receptor or the progesterone receptor was less frequent in the hereditary group than in the sporadic group (64% vs. 41%,  $P=0.02$ ). There were no significant differences in tumor stage and nodal status between the two groups. The patients' demographic and clinical characteristics and pathological features of the tumor samples are consistent with those of results reported previously.<sup>29</sup>

### TP53 MUTATIONS

A total of 32 of 43 samples from the hereditary group (74.4%) and 74 of 175 samples from the sporadic group (42.3%) had TP53 mutations ( $P<0.002$ ) (Fig. 1, and Table 3 of the Supplementary Appendix). Of the samples in the hereditary group, 11 (25.6%) had mutations in epithelium alone, 11 (25.6%) had mutations in stroma alone, and 10 (23.3%) had mutations in both compartments. Of the samples in the sporadic group, 26 (14.9%) had mutations in epithelium alone, 34 (19.4%) had mutations in stroma alone, and 14 (8.0%) had mutations in both compartments, similar to our data published previously<sup>17</sup> (Fig. 1A). Of 45 breast cancers with sequence-confirmed TP53 missense mutations, 2 samples showed nuclear staining for p53 in tumor stroma but not normal stroma (Fig. 1B).

### LOSS OF HETEROZYGOSITY OR ALLELIC IMBALANCE

The frequency of loss of heterozygosity or allelic imbalance in epithelium and stroma in the hereditary group was higher than in the sporadic group. The median frequency of loss of heterozygosity or allelic imbalance in the neoplastic epithelium was 67% in the hereditary group and 54% in the sporadic group ( $P<0.001$ ). The median frequency of the



loss of heterozygosity or allelic imbalance in stroma was 60% in the hereditary group and 51% in the sporadic group ( $P < 0.001$ ) (Table 1). We found that a *TP53* mutation in epithelium or stroma was associated with an increased frequency in loss of heterozygosity or allelic imbalance in both the hereditary group and the sporadic group, but the association was more pronounced in the sporadic group (Table 2). There was no significant difference in overall loss of heterozygosity or allelic imbalance in breast cancers from patients with deleterious *BRCA1* or *BRCA2* mutations or those with unclassified variants in *BRCA1* or *BRCA2* (data not shown).

We tested the hypothesis that compartment-specific *TP53* mutations are associated with loss of heterozygosity or allelic imbalance at specific microsatellite markers. Markers with a significantly higher frequency of loss of heterozygosity or allelic imbalance than all other markers on the same chromosome are considered to be hot spots.<sup>19,30</sup> Among all samples in the sporadic group, we identified 66 hot-spot loci linked to a loss of heterozygosity or allelic imbalance that were associated with a compartmental *TP53* mutation (at  $P < 0.05$  and  $FPRP_{0.01} < 0.5$ ) (Fig. 2). Thus, these 66 loci harbored a loss of heterozygosity or allelic imbalance at frequencies significantly high-

er than could be obtained by chance. This association holds across all tumors and compartments but cannot be ascertained for a single tumor. Of these 66 hot-spot markers, 8 — D2S2944 (2q34), D3S1262 (3q27), D5S1462 (5q15), D7S1818 (7p12), D15S128 (15q11), D16S2616 (16p13), D18S1390 (18q23), and D20S103 (20p13) — occurred in both epithelium and stroma, and each was associated with the presence of a *TP53* mutation (Fig. 2, and Table 4 of the Supplementary Appendix). Of the remaining 58 hot spots, 25 were found only in epithelium and 33 only in stroma (Fig. 2, and Tables 5, 6, and 7 of the Supplementary Appendix). We also found that stromal *TP53* mutations were associated with loss of heterozygosity or allelic imbalance at 41 stromal hot-spot markers (Fig. 2). In the hereditary group, there was only one hot spot of loss of heterozygosity or allelic imbalance associated with a *TP53* mutation, specifically at D2S1400 (2p25.1) in the stroma (at  $P < 0.05$  and  $FPRP_{0.01} < 0.5$ ) (Table 5 of the Supplementary Appendix).

#### ASSOCIATION WITH CLINICAL AND PATHOLOGICAL FEATURES

We found a significant association between the *TP53* mutation status in stroma and lymph-node status ( $P = 0.003$ ) only in the sporadic group (Table 8 of the Supplementary Appendix). Moreover, *TP53* mutations in stroma were associated with nodal metastases only in the sporadic group (Fig. 3). We also identified loss of heterozygosity or allelic imbalance at five stromal hot-spot regions — D7S821 (7q21), D10S677 (10q23), D15S128 (15q11), D16S3401 (16p), and D17S2193 (17q24) — that were associated with nodal metastases in the absence of *TP53* mutations in the tumor stroma (at  $P < 0.05$  and a false discovery rate  $< 0.1$ ) (Table 3 and Fig. 3). Thus, stromal *TP53* mutation alone and stromal loss of heterozygosity at five hot-spot loci alone were both associated with nodal metastases. There was no additive effect of the presence of *TP53* mutation and loss of heterozygosity or allelic imbalance at these five loci ( $P > 0.05$ ) (Table 2 and Fig. 3).

#### DISCUSSION

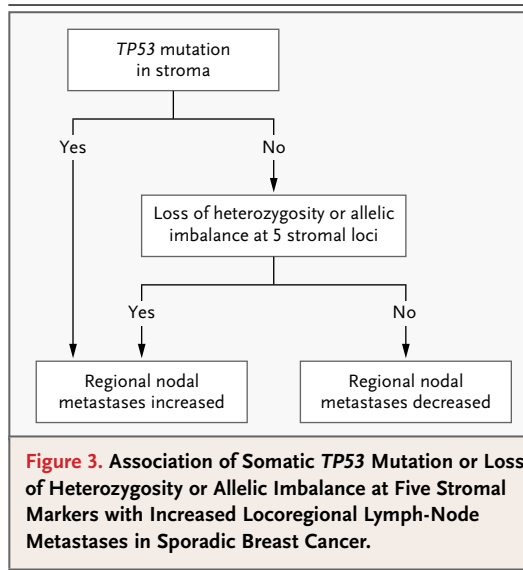
The results of the few studies that have investigated the prognostic value of *TP53* mutations in breast and other cancers are contradictory.<sup>31-33</sup> In our study, we evaluated the associations between the presence of *TP53* in the tumor, genomic altera-

tions in the tumor microenvironment, and presenting clinical and pathological findings in two groups of tumors: hereditary breast cancers associated with *BRCA1* or *BRCA2* mutations and sporadic breast cancers.

In studies of epithelium and stroma from hereditary breast cancers with germ-line *BRCA1* or *BRCA2* mutations, frequencies of loss of heterozygosity or allelic imbalance were higher than those in sporadic breast cancers.<sup>30</sup> The *TP53* mutations in familial breast cancers often retain their activities that induce apoptosis, up-regulate genes, and inhibit growth.<sup>15</sup> In most cases, however, the *TP53* mutations in hereditary and sporadic breast cancers differ in their position along the gene. We found that *TP53* mutations in the stroma of hereditary and sporadic breast cancers were associated with an increased frequency of loss of heterozygosity or allelic imbalance across all microsatellite markers. Despite this overall increase, we found only one marker in the 43 hereditary breast cancers that was identified as a hot spot associated with mutant *TP53*: marker D2S1400 on 2p25.1, containing E2F6, a transcription factor that targets *BRCA1* and has an important role in the regulation of apoptosis.<sup>34</sup>

Unlike hereditary breast cancer, sporadic breast cancer does not have an underlying generalized genomic instability.<sup>30</sup> Nevertheless, we found that *TP53* mutations in sporadic breast cancer were associated with 66 hot-spot markers of loss of heterozygosity or allelic imbalance. The eight hot spots associated with *TP53* mutations in both epithelium and stroma map to regions that encode proteins in p53-related pathways. For example, 3q27.3 (D3S1262) contains *TP73L*, a member of the *TP53* gene family; and p63, encoded by *TP73L*, is expressed exclusively in the myoepithelial cells of normal breast tissue, and its decreased expression in breast cancer is associated with disease progression.<sup>35</sup> In addition, certain markers identified as hot spots only in *TP53*-mutated epithelium map to chromosomal regions containing genes that encode p53 targets. Thus, sporadic breast cancers must have multiple mechanisms that disrupt normal cellular regulation, such as cell-cycle progression and checkpoints, DNA repair, and apoptosis. Our data suggest that these mechanisms, whether in play in stroma or epithelium, involve p53.

The overall frequency of loss of heterozygosity or allelic imbalance was similar in the epithelial and stromal compartments of sporadic breast cancers. Somatic *TP53* mutations in the stroma were



associated with loss of heterozygosity or allelic imbalance of chromosomal regions harboring p53-related genes. The significant association between stromal *TP53* mutations and nodal metastases in sporadic breast cancers suggests that such mutation-bearing stromal cells provide a favorable microenvironment for tumor spread (Fig. 3). We also found that even in the absence of *TP53* mutations in stroma of sporadic breast cancers, loss of heterozygosity or allelic imbalance at five microsatellite markers was associated with nodal metastases. The D10S677 marker (10q23.3) maps to a region containing the gene that encodes phospholipase C, epsilon 1 (*PLCE1*); this enzyme catalyzes the hydrolysis of polyphosphoinositides and thereby initiates the growth and differentiation of cells. *PLCE1*, the corresponding protein, mediates the effects of the small guanosine triphosphatases belonging to the Ras superfamily on the actin cytoskeleton and membrane protrusion.<sup>36</sup> Another marker, D16S3401 (16p13.3), maps to a region containing the gene encoding nonmetastatic cells 4 (*NME4*), a member of the nucleoside diphosphate kinase family. The function of *NME4* protein is unknown, but another *NME* family member, *NME1*, is regulated by p53 and decreases metastatic potential.<sup>37</sup>

Our observations suggest that *TP53*-mutated stroma or loss of heterozygosity or allelic imbalance at five specific stromal markers accelerates tumor progression. If our results are corroborated in a large prospective study, then analysis of breast-tumor stroma for the presence of *TP53* mu-

**Table 3. Associations among Hot Spots of Loss of Heterozygosity or Allelic Imbalance in Stroma, TP53 Mutation Status, and Nodal Status in Sporadic Breast Cancer.\***

Marker	Locus	LOH Frequency in Wild-Type TP53				LOH Frequency in Mutated TP53			
		Nodal Status			P Value†	Nodal Status			P Value†
		0	1	2 or 3		0	1	2 or 3	
D7S821	7q21.3	0.39	0.41	0.88	0.04	0.60	1	1	0.06
D10S677	10q23.3	0.24	0.48	0.83	0.004	0.64	0.60	0.75	0.77
D15S128	15q11.2	0.19	0.41	0.67	0.02	0.50	0.25	1	0.12
D16S3401	16p13.3	0.22	0.36	0.83	0.007	0.63	0.40	1	0.18
D17S2193	17q24.2	0.32	0.48	0.88	0.01	0.63	0.50	0.83	0.46

\* LOH denotes loss of heterozygosity or allelic imbalance.

† P values are for the overall comparisons among the nodal-status subgroups. Multiple-testing adjustments were controlled so that a false positive finding would occur less than 10% of the time.

tations and loss of heterozygosity or allelic imbalance at the five markers might help predict nodal status (Fig. 3). Although the procurement of stroma by laser-capture microdissection is currently complex and perhaps beyond routine clinical use, analysis of TP53 mutation and of the five microsatellite markers for loss of heterozygosity or allelic imbalance involves techniques that are routinely used in clinical laboratories.

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