Absence of Genetic Abnormalities in Fibroadenomas of the Breast Determined at p53 Gene Mutations and Microsatellite Alterations

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ABSTRACT

Genesis of breast cancer is a multistage process involving accumulation of genetic alterations, but little is known about the implication of genetic alterations in benign breast disease (BBD) lesions. Among benign lesions of the breast, one of the most common is fibroadenoma. The relationship between fibroadenoma and breast cancer is not clear. Some epidemiological studies show an association with breast cancer risk, whereas recent reports show no increased risk.

In a previous study, we analyzed genetic alterations in a group of BBD lesions composed namely of fibroadenomas unaffected by breast cancer. We reexamined our BBD population for genetic alterations with 13 markers using a PCR-based method. Our results show that no molecular alterations were detected in these BBD lesions composed of fibroadenomas unaffected by breast cancer. Neither p53 gene mutations, determined at exons 5-9, nor microsatellite alterations tested with a very sensitive method were found in these lesions. Therefore, molecular results obtained in our study support recent epidemiological data showing that fibroadenoma does not constitute a significant increase in the relative risk of later contracting breast cancer.

INTRODUCTION

Progressive somatic genetic alterations are associated with the development of breast cancer, and a subset of proliferative breast lesions has been characterized by clonal genetic aberrations. Some of these lesions, including usual ductal hyperplasia and atypical ductal hyperplasia, have been supposed to represent actual precursors of malignancy, although these lesions are histologically considered to be benign. The presence of genetic alterations in histologically benign breast tissue would imply that genetically abnormal clones develop before pathological detection. In addition, genetic alterations determined at the microsatellite sequences have been detected in morphologically normal lobules adjacent to breast cancer. Thus, molecular changes are also detectable in breast tissue that is histologically normal.

BBD lesions are a heterogeneous group of benign breast problems that has been associated with breast cancer risk by several investigators. Among benign lesions of the breast, one of the most common is fibroadenoma. The relationship between fibroadenoma and breast cancer is not clear. Some studies show that surgical removal in the 8–15 years after its initial diagnosis when family history and proliferative changes in the adjacent parenchyma are removed, and the risk rises to greater than three if it has complex histology (3, 5), whereas other reports show no increased risk (6).

Fibrocystic diseases have shown a 2- to 4-fold increase in risk of breast cancer among women with this diagnosis (7). Phyllode tumors of the breast also show the same histological benign characteristics with fibroadenomas, but they have been supposed recently to be a neoplasm of both stromal and epithelial cells (8).

In a previous study, we analyzed genetic alterations in a group of BBD lesions composed of fibroadenomas unaffected by breast cancer, and we found no evident implication of several loci by Southern blot method (9). However, genetic alterations, including p53 gene mutations (10), LOH, MIN, HER-2/neu amplification (12), and cytogenetic chromosomal aberrations (13, 14) including a large 6q24-ter deletion (15), were reported to occur in fibroadenomas.

Taking together, these results prompt us to reexamine our BBD population for p53 gene mutations and for microsatellite alterations. p53 gene mutations were analyzed at exons 5–9, and microsatellite alterations were performed with 13 markers using a PCR-based method. The results were compared with one malignant phyllode tumor (grade II) and a group of 31 breast carcinomas.

MATERIALS AND METHODS

Samples. BBD specimens from 30 patients unaffected by breast cancer including 26 fibroadenomas, 2 fibrocystic diseases, 1 florid adenosis, and 1 benign phyllode tumor, as well as 1 malignant phyllode tumor and 31 infiltrating ductal carcinomas were obtained at the Center Georges François Leclerc between 1991–1993 (Dijon, France). The tissue was frozen in liquid nitrogen immediately after surgical removal and stored at −80°C until DNA extraction. Blood samples were collected from each patient, and peripheral lymphocytes were used as normal controls.

Microdissection providing from the BBD lesions and the malignant phyllode tumor of selected areas from formalin-fixed, paraffin-embedded tissue was performed under direct light microscopic visualization. Cells from epithelium, stroma, and normal adjacent breast tissues were selected from eosin-stained slides and microdissected with a disposable, sterile, 30-gauge needle.

DNA Extraction. DNA was extracted from fresh specimens according to the standard method (16). Briefly, the DNA was treated with 50 μg/ml proteinase K and extracted with phenol-chloroform. DNA extraction from microdissected breast tissues was carried out as described (17). Cells were digested overnight at 37°C with a solution of 0.2 mg/ml proteinase K in 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 0.5% Tween 20. The solution was boiled at 95°C for 10 min, centrifuged for 5 min, and 2 μl of supernatant were used for PCR analysis.

p53 Gene Mutation Detection. p53 mutations were characterized by PCR-single-strand conformational polymorphism of exons 5–9 of the p53 gene. Direct sequencing of the PCR product was performed by fluorescent dye labeled dideoxynucleotides using the BigDye Terminator cycle sequencing kit (Perkin-Elmer Corp. Applied Biosystems) on an automate DNA sequencer (Model 310; Applied Biosystems). All of the suspect samples were analyzed twice by different PCR reactions.

Microsatellite Analysis. Microsatellite alterations were analyzed at 13 loci representative of mono-, dinu-, and tetranucleotide repeats using synthesized labeled primers for Bat26, D3S1514, D3S1612, D3S1244, D8S256, TH01, D11S2179, TP53, D17S855, and AR, or provided directly as a set for D6S254, D6S281, and D10S197 (PE Applied Biosystems). The sequences of the synthesized primers used were obtained from the Genome Database.

PCR analyses were performed in a PCR Express Hybrid thermocycler in...
25-μl reaction volume containing 25 ng of genomic DNA, 0.32 μM of each primer, 200 μM deoxyribonucleotide triphosphates, 1.5 mM magnesium chloride, and 1.25 units of Gold Taq (PE Applied Biosystems). PCR conditions consisted of an initial denaturation cycle of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 50 s at the appropriate annealing temperature (55–65°C), 50 s at 72°C, and a final extension at 72°C for 5 min. DNA extracted from microdissections (2 μl) was amplified using the same PCR conditions but carried out for 45 cycles. PCR products were separated under denaturing conditions using an ABI Prism 310 automated fluorescent sequencer. The data collected were additionally analyzed with Genescan and Genotyper softwares (PE Applied Biosystems).

**Determination of Microsatellite Alterations.** Peak heights were compared between breast lesions and corresponding normal cells. MIN was scored when novel alleles were observed in the lesion as compared with correspond-

**RESULTS**

**p53 Gene Analysis.** No mutations in the p53 gene at exons 5–9 were found in the BBD lesions. Only one fibro adenoma case shows a polymorphism in exon 6 at the codon 213. This polymorphism at codon 213 was found in all of the three cellular components of the fibro adenoma case provided from microdissections of epithelium, stroma, and normal adjacent tissues, as well as in lymphocyte blood cells. Although no alteration was detected in one benign phyllode tumor (grade I), p53 gene mutation was found in the malignant phyllode tumor in both epithelial and stromal cells but not in the normal adjacent tissue (Table 1). Mutations in the p53 gene were also detected in 7 of the 31 carcinomas (Table 1).

**Microsatellite Analysis.** Polymorphic microsatellite markers were selected as a mixture of di-, tri-, and tetranucleotide repeats, which were dispersed within the genome on loci that were known to repres-

**Table 1 Exons 5–9 p53 gene mutations detected in benign and malignant breast lesions**

<table>
<thead>
<tr>
<th>Breasts</th>
<th>Exon</th>
<th>Codon</th>
<th>Sequence</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benigns</td>
<td>1 Fibroadenoma</td>
<td>6</td>
<td>213</td>
<td>CGA:CGG</td>
</tr>
<tr>
<td>Malignants</td>
<td>1 Phyllode grade I</td>
<td>6</td>
<td>193</td>
<td>CAT:CGT</td>
</tr>
<tr>
<td>Exons</td>
<td>1</td>
<td>5</td>
<td>175</td>
<td>CGC:CAC</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>del 194</td>
<td>del TAT</td>
<td>del Tyr</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>248</td>
<td>CGG:CAG</td>
<td>Arg:Gln</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>248</td>
<td>CGG:TGG</td>
<td>Arg:Trp</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>248</td>
<td>CGG:TGG</td>
<td>Arg:Trp</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>del 245</td>
<td>del GGC</td>
<td>del Gly</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>245</td>
<td>GCC:GAC</td>
<td>Gly:Asp</td>
</tr>
</tbody>
</table>

* Changes found in epithelium, stroma, and normal adjacent tissues.
* Mutations found in both epithelium and stroma but not in the normal adjacent tissue.
* del, deletion.

**DISCUSSION**

Because of our previous study on the absence of genetic alterations using Southern blot method in BBD lesions (9), several investigations have reported the implication of genetic alterations in these lesions. p53 gene mutations (10), microsatellite alterations (11), HER-2/neu amplification (12), and cytogenetic aberrations (13–15) have been observed. These different observations lead us to investigate p53 gene mutations, and microsatellite alterations in our BBD lesions originated in women unaffected by breast cancer.

p53 gene point mutations have been reported in 2 of 8 fibroadeno-

**Table 2 Microsatellite analysis in benign and breast cancer lesions**

<table>
<thead>
<tr>
<th>Microsatellite markers</th>
<th>Total</th>
<th>BAT 26</th>
<th>D3S1514</th>
<th>D3S1612</th>
<th>D3S1244</th>
<th>D6S264</th>
<th>D6S281</th>
<th>D8S256</th>
<th>D10S197</th>
<th>TH01</th>
<th>D11S2179</th>
<th>TP53</th>
<th>D17S855</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benigns</td>
<td>26</td>
<td>2</td>
<td>0/0</td>
<td>0/16</td>
<td>0/14</td>
<td>0/23</td>
<td>0/23</td>
<td>0/23</td>
<td>0/16</td>
<td>0/22</td>
<td>0/17</td>
<td>0/22</td>
<td>0/23</td>
<td>0/23</td>
</tr>
<tr>
<td>Fibrocytic diseases</td>
<td>2</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Adenosis</td>
<td>1</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Phyllode grade I</td>
<td>1</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Malignants</td>
<td>1 Fibroadenoma</td>
<td>31</td>
<td>1</td>
<td>6/31</td>
<td>3/17</td>
<td>3/16</td>
<td>3/23</td>
<td>0/1</td>
<td>1/2</td>
<td>1/2</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Phyllode grade II</td>
<td>1</td>
<td>2</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
<td>2 Min</td>
</tr>
</tbody>
</table>

* No. of cases with LOH/no. of informative cases.
* No microsatellite alterations detected.
* Not informative; nd, not determined.
* LOH found only in stroma.
* LOH found in both epithelium and stroma.
* Min, microsatellite instability shown by the presence of new alleles as compared to corresponding normal cells.
detected LOH in 19% and in 18% of informative breast carcinomas. Similarly, no genetic aberrations were observed with TH01 in all of the BBD cases, whereas LOH was detected in 19% of informative carcinomas and MIN in 1 case. LOH of the p53 gene is a frequent genetic alteration in breast cancer (19), and in our study, LOH at the TP53 was the more frequent alteration (23%) found in carcinomas. In contrast, no microsatellite alterations appear in the BBD lesions, consistent with result found with D17S559 surrounding the p53 gene region (11). D17S855, localized at intron 20 of BRCA1, is also frequently affected by LOH (22%) in the group of carcinomas, but no loss was detected in our BBD lesions, consistent with previous results found in fibroadenomas with this marker (11). Nevertheless, malignant phyllid tumor showed p53 gene mutation and LOH in epithelial and stromal cells, consistent with recent reports demonstrating genetic changes in both components of these tumors (8, 20). However, although our results show no genetic alterations in fibroadenomas at the analyzed loci, it is not excluded that they may still have some genetic changes not analyzed in this study leading to the initiation of these lesions.

Cytogenetic and fluorescence in situ hybridization studies have also reported many chromosomal aberrations in fibroadenomas (13–15). Recently, a large deletion spanning 6q25 to 6qter has been reported in 84% of benign breast tumors (15). In our study, 2 markers localized at 6q25.2–27 (D6S264) and 6q27 (D6S281) were analyzed, and no loss was found in the BBD lesions. It will be interesting to examine chromosome 6q by detailed LOH mapping to determine whether there are any chromosomal deletion/LOH in this region.

The discrepancies between our results and those reported recently may be explained by several points. Firstly, one of the most important points is the selection of benign lesions. Our group of BBD lesions belong to women unaffected by breast cancer with an average follow-up of 10 years. Unfortunately, data on individual breast cancer development were not available for cases showing p53 gene mutations (10), for those reported with microsatellite (11), or cytogenetic alterations (13–15). Thus, the different cohorts analyzed might be a reason for the differing result leading to the fact that certain fibroadenomas might show some genetic alterations. Another important point concerns microsatellite alteration detection that could be regarded with strong precaution. Indeed, recent studies demonstrated the existence of PCR artifacts in LOH and MIN determinations (21–23). Finally, comparison with cytogenetic results should be also made with precaution, because this method requires cellular incubation that may result in cell selection during culturing. In addition, the large 6q deletion reported recently has been found in a majority of fibroadenomas classified as complex (15). Complex histology is characterized by any combination of changes including apocrine metaplasia and/or sclerosing adenosis within fibroadenomas (24). Therefore, cytogenetic alterations found in complex fibroadenomas (15) may be attributable to the presence of these components in which genetic alterations have been reported recently (25).

Epidemiological data have shown that ~70% of women with fibroadenomas are without increased risk (24) and that fibroadenoma per se were not associated with a relevant increased risk of subsequent breast cancer (26). Recently, it has been also shown that even atypical (atypical lobular or ductal hyperplasia) confined to fibroadenomas does not incur an elevation of long-term breast cancer risk greater than that of fibroadenomas in general (27).

Thus, our results show an absence of molecular alterations determined at several loci in BBD lesions composed namely of fibroadenomas unaffected by breast cancer. Neither p53 gene mutations nor microsatellite alterations tested with a very sensitive method were found in these lesions. Therefore, taken together, epidemiological data and molecular results obtained in our study support the concept that fibroadenoma does not constitute a significant increase in the relative risk of later contracting breast cancer.

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