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, and we found no evidence of several loci by Southern blot method. However, genetic alterations, including *p53* gene mutations, loss of heterozygosity, microsatellite instability, and cytogenetic chromosomal aberrations, have been reported recently to occur in fibroadenomas. Thus, we reexamined our BBD population for *p53* gene mutations and for microsatellite alterations with 13 markers using a PCR-based method.

Our results show that no molecular alterations were detected in these BBD lesions composed namely 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 (1). 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 (1, 2). 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 (3, 4). Among benign lesions of the breast, one of the most common is fibroadenoma, arising from the epithelium and the stroma of the terminal duct lobular units. The relationship between fibroadenoma and breast cancer is not clear. Some studies show twice the increase risk 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 share 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 namely of fibroadenomas unaffected by breast cancer, and we found no evidence 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-Cl (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-, di-, tri-, 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 D6S264, 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 95°C for 10 min, centrifuged for 5 min, and 2 µl of supernatant were used for PCR analysis.
25-μl reaction volume containing 25 ng of genomic DNA, 0.32 μM of each primer, 200 μM deoxynucleotide triphosphates, 1.5 mM magnesium chloride, and 1.25 units of Gold Taq (PE Applied Biosystems). PCR conditions consisted to an initial denaturation cycle of 5 min at 94 °C followed by 35 cycles of 50 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 with 3 positive cases for MIN and 4 positive cases for LOH served with 35 samples) showed several microsatellite alterations, with, however, more frequent LOH appearance (20 cases that displayed LOH in at least one locus) than MIN (6 cases). The most frequent loci affected by LOH in breast carcinomas were TP53 (23%), AR (23%), D17S855 (22%), D3S1514 (19%), and TH01 (19%; Table 2).

**RESULTS**

*p53 Gene Analysis.* No mutations in the *p53* gene at exons 5–9 were found in the BBD lesions. Only one fibroadenoma 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 fibroadenoma 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 represent chromosomes associated with breast malignancy (18, 19). No MIN or LOH was detected at any of the analyzed loci in our BBD lesions (Table 2). Fig. 1 shows a representative microsatellite analysis of *TP53* locus in both microdissected and fresh-frozen tissue in a fibroadenoma case. In contrast, malignant phyllode tumor showed LOH at five of eight loci; three were affected only in stromal cells and two in both epithelial and stromal cells (Table 2). Breast carcinomas (31 samples) showed several microsatellite alterations, with, however, more frequent LOH appearance (20 cases that displayed LOH in at least one locus) than MIN (6 cases). The most frequent loci affected by LOH in breast carcinomas were TP53 (23%), AR (23%), D17S855 (22%), D3S1514 (19%), and TH01 (19%; Table 2).

**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 fibroadenomas and in 2 of 22 fibrocystic diseases (10). However, 2 of these mutations in each case were silent and do not alter amino acid coding region. In our BBD cases, only a *p53* polymorphism was detected in 1 fibroadenoma, whereas mutations were found in the malignant phyllode tumor as well as in 22% of the carcinomas analyzed.

Microsatellite alterations analyzed with 11 markers have been observed with 3 positive cases for MIN and 4 positive cases for LOH among 39 fibroadenomas (11). Interestingly, no alterations were found at another 8 loci determined in 7 fibroadenomas (18). In our study, 4 markers (D3S1514, TH01, TP53, and D17S855) were identical to those of the first report, in which LOH with D3S1514 and TH01, as well as MIN at TH01 have been found in 2 of 26 and 1 of 32 fibroadenomas, respectively (11). Using microsatellite markers for 3p14.2–21 (D3S1514), 3p22–24 (D3S1612), and 3p24 (D3S1244) regions, no LOH was observed in our BBD lesions, whereas we

### Table 1

| Exons 5–9 p53 gene mutations detected in benign and malignant breast lesions |
|---------------------------|---|---|---|
| Breast lesions            | Exon | Codon | Sequence | Amino acids |
| Benigns                   |     |      |          |            |
| 1 Fibroadenomast          | 6   | 213  | CGA:CGG  | Arg:Arg    |
| Malignants                |     |      |          |            |
| 1 Phyllode grade II       | 6   | 193  | CAT:CGT  | His:Arg    |
| Carcinomas                |     |      |          |            |
| 1                          | 5   | 175  | CGC:CAT  | Arg:His    |
| 2                          | 6   | del 194† | del TAT | del Tyr    |
| 3                          | 7   | 248  | CGG:GAG  | Arg:Gln    |
| 4                          | 7   | 248  | CGG:TGG  | Arg:Trp    |
| 5                          | 7   | 248  | CGG:TGG  | Arg:Trp    |
| 6                          | 7   | del 245 | del GGC | del Gly    |
| 7                          | 7   | 245  | GGC:GAC  | Gly:Asp    |

† 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.

### Table 2

| Microsatellite alteration analyses in benign and breast cancer lesions |
|---------------------------|---|---|---|---|
| Microsatellite markers    | Total | BAT 26 | D3S1514 | D3S1612 | D3S1244 | D6S264 | D6S281 | D8S256 | D10S197 | TH01 | D11S2179 | TP53 | D17S855 | AR |
| Benigns                   |     |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Fibroadenomas             | 26  | 0/24 | 0/16 | 0/14 | 0/23 | 0/17 | 0/23 | 0/16 | 0/22 | 0/17 | 0/22 | 0/23 | 0/23 |
| Fibrocystic diseases      | 2   | 0/2  | 0/1  | 0/1  | 0/2  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/2  | 0/1  | 0/1  |
| Adenosis                  | 1   | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  |
| Phyllode grade I          | 1   | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  |
| Malignants                |     |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Phyllode grade II         | 1   | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  |
| Carcinomas                | 31  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  | 0/1  |

* No. of cases with LOH/no. of informative cases.
† No microsatellite alterations detected.
‡ ni, 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 TP53 was the more frequent alteration (23%) found in carcinomas. In genetic alteration in breast cancer (19), and in our study, LOH at the BBD cases, whereas LOH was detected in 19% of informative

Similarly, no genetic aberrations were observed with TH01 in all of the 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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