Comparative Analysis of Molecular Alterations in Fibroadenomas Associated or Not With Breast Cancer

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Hypothesis: The cause of breast cancer is linked to many macroscopic events, including benign breast disease. In this study we asked whether molecular changes could discriminate fibroadenoma, which is one of the most common benign breast disease lesions associated or not with breast cancer.

Design: Retrospective cohort study.

Setting: Anticancer medical center.

Subjects: Archival tissues in 32 cases of fibroadenoma, diagnosed in the same breast as a breast carcinoma, are compared with a control group of 26 cases of fibroadenomas unaffected by breast cancer.

Main Outcome Measures: Histological features are characterized in all samples. The epithelial and stromal components are analyzed for a loss of heterozygosity and a microsatellite instability using a polymerase chain reaction–based method with 11 polymorphic microsatellite markers at 7 chromosomal regions frequently altered in breast cancer. The p53 gene mutations were also determined at exons 5 to 9.

Results: The frequency of complex fibroadenomas was similar in both groups (P=.42). Only in the case group did we observe proliferative lesions confined in fibroadenomas, including atypical ductal hyperplasia (2 cases), lobular neoplasia (3 cases), or low-grade ductal carcinoma in situ (2 cases). There is no significant morphological difference between the 2 groups. Neither microsatellite alterations nor p53 gene mutations are present in the fibroadenoma components. Loss of heterozygosity is found only in the epithelial component of the 2 ductal carcinomas in situ confined in fibroadenomas.

Conclusions: Genetic alterations, which are most frequently involved in malignant breast carcinomas, are not present in fibroadenomas, regardless of their association with breast cancer or their histological complexity. These findings suggest that fibroadenomas are not associated with breast carcinogenesis.


The cause of breast cancer is linked to many macroscopic events that include a positive family history, the number of pregnancies, an exposure to estrogens, type of diet, and benign breast disease. Fibroadenoma is one of the most common benign breast lesions for women who are between 20 and 50 years old that has been associated with a risk of breast cancer by several investigators. The highest peak of incidence occurs at the third decade of life when breast cancer is an unusual event. The reported risk of developing breast cancer for patients previously diagnosed as having fibroadenomas varies from null to 3. The increase depends on the presence of complex features within the fibroadenomas (ie, cysts, sclerosing adenosis, epithelial calcifications, or papillary apocrine changes), a proliferative disease in the parenchyma adjacent to the fibroadenomas, or a family history of breast cancer. However, the family history of breast cancer may cause an overestimation of increased risk associated with fibroadenomas because women with a family history of breast cancer may more readily decide to undergo a surgical procedure. Thus, the association between fibroadenoma and breast cancer is still unclear.

Progressive somatic genetic alterations are associated with the development of breast cancer. Genetic instabilities, manifested by a loss of heterozygosity (LOH) and/or microsatellite instability (MIN), are considered as early events in this type of lesion. Indeed, at least a subset of proliferative breast lesions is characterized by clonal genetic aberrations manifested by LOH and MIN. Some of these lesions, in-
Characterization of the Microsatellite Markers Analyzed in This Study

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide Repeat</th>
<th>Location</th>
<th>Associated Gene</th>
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<tbody>
<tr>
<td>BAT26</td>
<td>A</td>
<td>2p22-21</td>
<td>hMSH2</td>
</tr>
<tr>
<td>D3S1514</td>
<td>GAA</td>
<td>3p21-14.3</td>
<td></td>
</tr>
<tr>
<td>D3S1244</td>
<td>CATT</td>
<td>3p24</td>
<td></td>
</tr>
<tr>
<td>D3S1612</td>
<td>CA</td>
<td>3p24-22</td>
<td></td>
</tr>
<tr>
<td>D6S264</td>
<td>CA</td>
<td>6q27-25</td>
<td></td>
</tr>
<tr>
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<td>CA</td>
<td>6q27</td>
<td>Sen6</td>
</tr>
<tr>
<td>D8S256</td>
<td>CA</td>
<td>8p24.13</td>
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<td>CA</td>
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<tr>
<td>TH01</td>
<td>TCAT</td>
<td>11p15.5</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>D11S2179</td>
<td>CA</td>
<td>11q23</td>
<td>ATM</td>
</tr>
<tr>
<td>TP53</td>
<td>CA</td>
<td>17p13.1</td>
<td>p53</td>
</tr>
<tr>
<td>D17S855</td>
<td>CA</td>
<td>17q21</td>
<td>BRCA1</td>
</tr>
<tr>
<td>AR</td>
<td>CAG</td>
<td>Xq13</td>
<td>Androgen receptor</td>
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</tbody>
</table>

STUDY DESIGN AND POPULATION

All cases of fibroadenoma analyzed in this study were identified from the surgical pathology file of the Centre Georges François Leclere, Dijon, France, between April 1, 1989, and June 30, 2000. This work was done with the approval of the local boards governing research on human subjects. Fibroadenoma samples came from patients diagnosed as having breast cancer and who had previously undergone surgical resection of a fibroadenoma or had presented a concurrent fibroadenoma at the time of the diagnosis of breast cancer. They constituted the case group (n = 32). This group was divided into 2 subgroups: concurrent cases (n = 24) and those preceding breast cancer by 2 to 9 years (n = 8). In both cases, fibroadenomas had developed in the same breast as the following or concurrent cancerous tissue. The second group was used as controls and included patients diagnosed as having fibroadenomas with no clinical manifestation of breast cancer at the date of diagnosis, not even in the follow-up period (mean follow-up, 10 years). The mean age of the patients was 56.6 years (age range, 38-78 years) for the case group and 39.5 years (age range, 16-63 years) for the control group. For the case subgroups, the mean ages were 56.0 and 57.2 years, respectively.

PATHOLOGIC AND TISSUE REVIEW

For each formalin-fixed, paraffin-embedded fibroadenoma, a 3-µm-thick section was stained by hematoxylin-eosin and safron for histological analysis and 5 to 10 subsequent 5-µm-thick sections were prepared for microdissection. Histological characteristics were reviewed by 2 pathologists (L.A. and F.M.) independently. Fibroadenomas were classified as complex if they contained either 1 or more of the following features: cysts larger than 3 mm in diameter, sclerosing adenosis, epithelial calcifications, or papillary apocrine changes. The occurrence of UDH without atypia, of ADH, of lobular neoplasia (LN), or ductal carcinoma in situ (DCIS) within each fibroadenoma was also checked for each case. Parenchyma adjacent to the fibroadenoma was assessed by the presence of these proliferative diseases, that is, UDH, ADH, LN, or DCIS.

Manual microdissection of epithelial and stromal components of fibroadenomas, as well as adjacent healthy tissue, was performed using a 30-gauge needle under microscopic visualization with direct light. The cases in which the fibroadenoma was inside or in contact with the breast cancer were excluded from the study. All selected cases for this study were either distant or separated from the associated carcinoma by at least a small rim of healthy tissue. Microdissection of the epithelial component was performed and included complex or proliferative lesions confined in the fibroadenoma, when present.

MOLECULAR ANALYSES

All molecular analyses were done without the knowledge of the fibroadenomas’ histological characteristics. Microdissected cells were kept in 70% alcohol at 4°C until DNA extraction was performed; the cells were then washed with ultrapure water. Cells were incubated for 18 to 26 hours in 0.5% polysorbate 20 (Tween 20), 1mM EDTA (pH 8.0), 50mM Tris hydrochloride (pH 8.5), and 500 µg/mL of proteinase K at 37°C, followed by a 10-minute incubation at 95°C for proteinase K inactivation. Tissue-frozen fibroadenomas and matched leukocytes stored at -80°C were also available in 19 cases in the control group. DNA extraction was performed according to a standard method. Briefly, tissues were treated with 30 µg/mL of proteinase K and DNA was extracted with phenol-chloroform.

Microsatellite markers are listed in Table. The sequences of the synthesized primers used were obtained from the Genome Database. Polymerase chain reactions (PCRs) were carried out in a 25-µL reaction volume containing 1.5 µL of extracted DNA, 0.32µM fluorescence-labeled primers, 200µM deoxynucleotide triphosphates, 2mM magnesium chloride, and 1U of AmpliTaq Gold in 100mM Tris hydrochloride (pH 8.3) and 500mM potassium chloride (1X GeneAmp PCR Buffer II; Perkins-Elmer Inc, Applied Biosystems, Foster City, Calif). Polymerase chain reaction was carried out in a thermocycler (Hybaid PCR-Express Thermocycler Q; Ashford, England) and consisted of 10 minutes at 95°C, 45 cycles of 50 seconds at 94°C,
of the cases vs 3 (11%) of 26 of the controls (F. plex features frequency considered between the 2 groups (42%) of 26 fibroadenomas were classified as complex.

within the concurrent subgroup. Within the control group, 11 (53%) of the 32 samples from the case group, among which only UDH was detected in 7 (22%) of the 32 cases and 4 (15%) of the 26 controls (P = .70). The histological distribution of the associated breast cancers, concomitant with or following the diagnosis of fibroadenomas, was as follows: 27 invasive ductal carcinomas, 4 invasive lobular carcinomas, and 1 high-grade DCIS.

MOLECULAR STUDY FINDINGS

In the hypothesis of an evolution of the fibroadenomas toward malignancy, it is important to determine which of the 2 cellular components (stromal or epithelial), if not both, is more prone to malignant transformation. The microdissection approach used was well adapted to the separate characterization of the distinct components of fibroadenoma.

Thus, DNA from the epithelial, stromal, and adjacent normal tissue components was subjected to microsatellite analysis on 8 microsatellite loci (BAT26, D3S1514, D3S1244, D6S264, D10S197, D11S2179, TP53, and D17S855) for the case group. When sufficient material was available for the 3 components (16 of 32 cases), 2 additional loci were also analyzed (AR and TH01). D6S281 was analyzed only in noninformative cases for D6S264 since both markers screened the same region of interest (6q25-ter). All microsatellite loci were analyzed for the control group. All loci were highly informative except for BAT26, which was used as a marker of MIN.

Loss of heterozygosity was detected in the epithelial component of only 2 fibroadenomas from the case group. Both fibroadenomas concern the 2 cases containing the low-grade DCIS, and they belonged to the subgroup concurrent to carcinomas. In these 2 cases, DCIS constituted an independent entity from the invasive carcinoma present in the same breast and were not an extension of the fibroadenomas (Figure 1). Two different loci, D3S1514 and TP53, were found altered in the epithelial component of these DCIS confined in the fibroadenomas (Figure 2). No MIN was detected in either the BAT26 locus or in the remaining loci of any of the analyzed samples. All of the other fibroadenomas from the case and the control groups showed normal patterns.

No mutation in the p53 gene at exons 5 through 9 was found in either the case or in the control groups. Only 1 fibroadenoma from the control group showed a polymorphism in exon 6 at the codon 213 (CGA/Arg:CGG /Arg). This polymorphism was found in the 3 cellular components obtained by microdissection (epithelium, stroma, and healthy adjacent tissues), as well as in lymphocyte blood cells.

COMMENT

Strong evidence proves that an essential feature of breast cancer malignancy resides in the accumulation of multiple genetic alterations. A subset of these aberrations may occur at early stages and some others at later stages of the disease. Thus, it is important to understand the
normalities, microsatellite alterations, and fibroadenomas, including cytogenetic chromosomal aberrations. However, several genetic alterations were reported to occur in fibroadenomas, including cytogenetic chromosomal abnormalities, microsatellite alterations, and p53 gene mutations. In this study we investigated whether fibroadenomas arising in a context of breast cancer could be distinguished from those not associated with any breast malignancy by molecular analyses determined at microsatellite alterations and at p53 gene mutations.

Cytogenetic studies based on fibroadenoma cultures enriched by either epithelial or stromal cells have reported chromosomal aberrations in both tissues, suggesting that the 2 components may involve neoplastic changes. In addition, alterations recurrently found in breast carcinomas have been detected exclusively in epithelial-enriched fibroadenoma cultures. These results suggest that breast cancer and fibroadenoma may follow the same genetic route. However, among the wide variety of chromosomal rearrangements detected in fibroadenomas by cytogenetic studies, no recurrent aberrations have emerged. The only exception has been the report of a common region of deletion on chromosome 6q spanning 6q25 to 6qter in 23 cases classified as complex. Thus, 2 markers of this region (D6S254 and D6S281 located at 6q25-27 and 6q27, respectively) were included in our study. Our results showed no LOH within these 2 loci, neither in the control nor in the case groups. Since the cytogenetic method requires a cellular incubation that may result in a cell selection during culturing, a comparison with cytogenetic results should be made cautiously. Cell cultures from tissues are made of different cell types. A bias of selection, which does not reflect the in vivo situation, may then be introduced.

The microsatellite analysis has reported LOH at D3S1514 in 1 of 39 cases analyzed, as well as 1 MIN and 1 LOH occurring at TH01 (11p15.5). In contrast to this, no alterations were found in another 8 loci determined in 7 fibroadenomas. A molecular-based study has also shown the presence of p53 gene mutations in some fibroadenomas. Three microsatellite loci spanning the 3p region, the TH01 marker, and the p53 gene mutations were analyzed in our study. Because MIN at BAT26, reflecting a dysfunction of the DNA mismatch repair system, was found in early stages of colon cancer progression, for example, in benign adenomas, we have also analyzed this marker. These data suggested a possible DNA mismatch repair system–defective mechanism in the origin of proliferative benign lesions. In addition, MIN was reported in fibroadenomas at breast cancer–related loci.

The results showed that neither microsatellite alterations (LOH or MIN), at the tested loci, nor p53 gene mutations were present in any of the fibroadenoma components regardless of their association with breast cancer or their histological complexity. A recurrent criticism in molecular studies is that the presence of normal tissue in the sample may mask abnormal clones. In our study, the fact that samples were obtained from microdissection, excludes risk of contamination with normal tissue.

Recently, it has been shown that atypia (atypical lobular or ductal hyperplasia) confined in fibroadenomas does not lead to an elevation of long-term breast cancer risk compared with fibroadenomas in general. The fact that the presence of complex features, including atypical hyperplasia, within fibroadenomas in our population did not correlate with an increased occurrence of genetic alterations at the examined loci is in agreement with this study. Ac-
ually, the only 2 alterations observed in the case group were related to the presence of a DCIS component within these fibroadenomas. However, although our results showed no genetic alterations in fibroadenomas at these loci, they did not exclude the fact that there may be some genetic changes, not analyzed in this study, leading to the initiation of these lesions.

The presence of neoplasia (LN, DCIS, and invasive carcinomas) within fibroadenomas is rare and is observed in patients 20 years older than patients without cancer. As our study found, LN is more frequent than DCIS, and invasive lesions confined in fibroadenomas are exceptional.13 Since such lesions are not frequent, there is no significance of these neoplasias confined in fibroadenomas because the risk of developing invasive cancer in other areas of the breast is still unknown, and their gathering in the same area seems to be a fortuitous event.14 Although the 2 cases of DCIS in fibroadenomas belong to the group with carcinomas, our series is too small to conclude that these DCIS increase the risk of subsequent breast cancer. Loss of heterozygosity has been interestingly reported in DCIS at several loci, including 3p21-14.3 and 17p13.1.36,37 Because no data on histological features were available,11,15 molecular changes attributable to fibroadenomas may be due to the presence of these neoplasias confined in fibroadenomas.

Together, our results suggest that genetic alterations, most frequently involved in malignant breast carcinomas, were not identified in fibroadenomas, regardless of their association with breast cancer or their histological complexity. These findings support the concept that fibroadenomas are not associated with breast carcinogenesis.

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