Clonal Analysis of Bilateral Breast Cancer

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ABSTRACT

Forty-nine pairs of bilateral breast tumors (41 synchronous and 8 asynchronous cases) were examined for X-chromosome inactivation status and p53 mutations to address the issue of their clonality. Among 12 cases that were informative for the trinucleotide repeat polymorphism in exon 1 of the androgen receptor gene on the X chromosome, 3 cases were found to have different alleles of the locus inactivated in the right and left breast tumors, indicating that the two tumors arose from distinct transformed cells. Thirteen tumors (13%) from 11 women (22%) contained somatic mutations in exons 5–8 of the p53 gene. In two cases, both breast tumors harbored p53 mutations, but the specific mutations were not identical. Seven synchronous and two asynchronous cases had p53 mutations in one tumor only. A germ line p53 mutation at codon 248, one of the most common p53 mutations in Li-Fraumeni syndrome, was observed in one case. Immunohistochemical analysis of p53 protein with a monoclonal antihuman p53 antibody showed concordant positivity between the right and left tumors in three bilateral breast cancer cases. Our results suggest that at least some bilateral breast tumors originate from distinct cells, but that some bilateral breast tumors may be related through a common p53 abnormality.

INTRODUCTION

It remains unclear whether contralateral breast cancer in any given woman represents a totally independent primary breast cancer or that the tumors are related through an altered common progenitor cell or as metastatic disease (i.e., the two tumors are of monoclonal origin). The answer to this question will enhance our understanding of the biological mechanism of this rare condition.

Monoclonality of malignant tumors has been the hallmark of the multistep model of human carcinogenesis, in which genetic alterations in multiple genes (oncogenes and tumor suppressor genes) are required for a single cell to be transformed into cancer (1). The clonality of human tumors has been investigated using approaches based initially on X-chromosome inactivation mosaicism in females (2). Vogelstein et al. (3, 4) devised a refined method of clonal analysis, which uses RFLP in two genes on the X chromosome and two restriction endonucleases. Subsequently, this method has been further modified and applied to clonal analysis of very small samples using PCR (5).

The human androgen receptor gene on chromosome Xq11–12 contains a highly polymorphic trinucleotide (CAG) repeat in the coding region (6, 7). Allen et al. (8) found that the methylation of HpaII and HhaI sites close to this repeat correlates with X-chromosome inactivation and developed a PCR assay that distinguishes between the maternal and paternal alleles based on their size and identifies their methylation status. The technique is applicable using DNA extracted from archival tissue (9, 10).

The p53 gene, which is the most extensively studied tumor suppressor gene, is mutated in about 20% of breast cancers (11). Because of the wide spectrum of mutations in the p53 gene, it is highly likely that two tumors from the same individual containing identical somatic mutations in p53 have a clonal origin. In a recent study by Sozzi et al. (12), two lesions (small cell carcinoma and dysplasia) of the bronchus from one patient had identical p53 mutations, whereas a third lesion (small cell carcinoma) in the lung from the same patient harbored a mutation at a different location of the gene. Zhang et al. (13) reported that only 4 of 16 pairs of mouse squamous cell carcinomas had identical p53 mutations in primary and paired metastatic tumors.

In the present study, we analyzed 49 bilateral breast cancer cases to address the issue of the clonal origin of these tumors. All cases were identified through the Cancer Surveillance Program, the population-based Surveillance Epidemiology and End Results cancer registry of Los Angeles County. The X-chromosome inactivation status was determined by the PCR-based assay of the polymorphic trinucleotide repeat in the human androgen receptor gene. Mutational analysis of exons 5–8 of the p53 gene and immunohistochemical staining of p53 protein were also performed.

PATIENTS AND METHODS

Case Selection and Collection of Tumor Specimens. A list of eligible cases was generated from the computer database of the Cancer Surveillance Program, the population-based Surveillance Epidemiology and End Results cancer registry of Los Angeles County. The criteria for case selection were: (a) tumors in both breasts diagnosed before age 50 years; (b) interval between the two diagnoses either ≤1 month (synchronous
cases) or \( \geq 48 \) months (asynchronous cases); and (c) both tumors diagnosed in the same hospital. Two hundred thirty-eight cases met these criteria. Forty-eight synchronous cases, diagnosed between ages 50 and 60 years, were added later to the list to increase the sample size. All eligible cases were diagnosed and reported between 1972 and 1993.

Requests for archival materials from the eligible breast tumors were sent to 75 hospitals. Specimens from 104 cases were obtained, but some were not usable for a variety of reasons: (a) some hospitals had only permanent histological slides available; (b) some paraffin blocks contained no identifiable tumors; (c) for some, fixation of the specimens was not suitable for DNA extraction and PCR; and (d) material was available from one breast only (therefore, unable to be analyzed as a pair, as required for this study).

Histological sections were prepared from formalin-fixed, paraffin-embedded tumor blocks and stained with either H&E (for histopathological review and the X-chromosome inactivation assay) or ethyl green (for the \( p53 \) mutation assay). All the slides were reviewed by two of the authors (A. S. and M. F. P.) to confirm the diagnosis and to locate tumors to be microdissected for molecular analyses.

Ninety-eight breast tumors from 49 women were analyzed for \( p53 \) mutations. Forty-one pairs were synchronous, whereas eight pairs were asynchronous by the above definitions. The age at diagnosis ranged from 32 to 60 years. Ninety-two percent of the tumors were ductal carcinomas, and 7% were of the lobular type. One tumor was of a mixed type. Forty-four tumors (45%) were localized, and 23 tumors (23%) had metastasized to regional lymph nodes. Nineteen tumors (19%) contained carcinoma in situ only.

**DNA Extraction.** DNA was isolated from archival paraffin-embedded specimens by microdissecting tumor tissues from H&E- or ethyl green-stained sections with a sterile scalpel. Invasive tumors were microdissected and analyzed in the molecular assays, whenever available, except in 19 tumors that contained in situ disease only. Microdissected tissue was incubated with \( 5-30 \mu l \) 10 mm Tris and 5 mm EDTA (pH 8.0) and 1 \( \mu g/\mu l \) proteinase K overnight at 56°C. Phenylmethylsulfonyl fluoride was added to the DNA extract to inactivate proteinase K before use in subsequent PCR analyses.

**X-Chromosome Inactivation Assay.** Five \( \mu l \) of proteinase K extract were quenched with 0.25 \( \mu l \) 30 mm phenylmethylsulfonyl fluoride in DMSO and divided into two aliquots, which were subsequently subjected to either digestion with 1 unit \( HhaI \) endonuclease or mock digestion with restriction enzyme buffer alone for 1.5–2 h at 37°C.

Samples were amplified by two PCR reactions, using nested primer sets specific for exon 1 of the human androgen receptor gene. This gene is located on chromosome Xq11–12 and contains a highly polymorphic trinucleotide repeat (7).

Primary PCR was performed in a total volume of 15 \( \mu l \) and included the digested or mock-digested DNA in 10 mm Tris-HCl (pH 8.3), 1.5 mm MgCl\(_2\), 50 mm KCl, 0.1 mg/ml gelatin, 0.32 \( \mu M \) of each primer, 0.12 \( \mu l \) DMSO, 0.2 mm deoxynucleotide triphosphates, and 0.4 units Taq DNA polymerase. Oligonucleotide primers used in primary amplification were: 1A, 5'-GTGGCGGAAGTGATCCAGAA; and 1B, 5'-TCTGGGACGCAGCCAGAC.'
dilution (mouse clonoPAP; Sternberger Monoclonals, Inc., Baltimore, MD). Slides were rinsed with PBS three times after each antibody treatment. Finally, the site of immunoprecipitate formation was identified by applying diaminobenzidine.

These slides were reviewed by one of the authors (M. F. P.) and scored as positive if >10% of the tumor cells were stained positively.

RESULTS

X-chromosome Inactivation Assay. The X-chromosome inactivation status was determined using the PCR-based assay of the trinucleotide polymorphism in exon 1 of the human androgen receptor gene (Table 1). Twelve cases were informative for this assay; i.e., they were heterozygous at the locus, and the tumors had little contamination with noncancerous cells (infiltrating lymphocytes and fibroblasts). In three cases, different alleles of the locus were found to be inactivated (Fig. 1), indicating that the two tumors arose from distinct transformed cells. The other nine cases shared the same inactivated allele in the right and left breast tumors.

p53 Mutation and IHC of p53 Protein. Table 2 shows the distribution of p53 mutations among the bilateral breast cancer cases. Thirteen tumors (13%) from 11 women (22%) contained mutations in exons 5-8 of the p53 gene. One tumor (right breast tumor of case 1) harbored two mutations; thus, 14 mutations were observed. All the mutations found were single-base changes (i.e., point mutations), three of which did not change the amino acid sequence of the protein. Seven mutations were found in exon 5, three were found in exon 7, and four were found in exon 8. No mutations were identified in exon 6, although two cases showed a previously reported polymorphism at codon 213 of the exon (not included in Table 2).

In two patients (patients 1 and 74), both breast tumors harbored p53 mutations, but the mutations were not identical. Seven synchronous bilateral breast cancers had p53 mutation in single tumors only (Fig. 2). Two women with p53 mutations had their two tumors staged differently (patients 11 and 13); in one, a mutation was found in the in situ tumor but not in the more advanced tumor; in the other, the more advanced tumor harbored the mutation. The two asynchronous cases with p53 mutations (patients 8 and 68) had mutations only in the more recent tumors.

One woman (patient 15), who happened to be the youngest of the study subjects at the time of her breast cancer diagnosis, was found to carry a mutation at codon 248, the most common p53 mutation in Li-Fraumeni syndrome, in both right and left breast tumors. This mutation was confirmed to be a germ line mutation by analyzing DNA from a peripheral blood sample.

Immunohistochemical analysis of p53 protein with a monoclonal antihuman p53 antibody was performed for a subset of the bilateral breast cancer specimens (31 pairs or 62 tumors; Table 3). Among these 62 tumors, 18 tumors (29%) were positive for IHC, only 7 of which also contained detectable p53 mutations. Of the 11 tumors in this group with p53 mutations, 4 were negative for IHC. Although the correlation between the results of IHC and mutational assays was statistically significant (two-sided $P < 0.01$, Fisher’s exact test), Spearman’s rank correlation coefficient was only moderate ($r = 0.354$; 95% confidence interval, 0.091–0.617). Three cases showed concordant positivity in IHC between the right and left breast tumors.

DISCUSSION

Bilateral breast cancer is a rare disease; the proportion of women with breast cancer who develop second tumors in the contralateral breast is reported to be between 3 and 9% (16–22). About 1.3% of >88,000 breast cancer patients reported to the Cancer Surveillance Program of Los Angeles County between 1972 and 1993, from which the study subjects were selected, have had tumors diagnosed in both breasts.

Women with bilateral breast cancer have been found to be younger (23) and to have more frequent family histories of breast cancer (16, 21) than unilateral breast cancer cases. These
Table 2 Bilateral breast cancer cases with somatic p53 mutations

<table>
<thead>
<tr>
<th>Tumor 1</th>
<th>Tumor 2</th>
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<tbody>
<tr>
<td>Patient</td>
<td>Age at diagnosis</td>
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<tr>
<td>1</td>
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<td>102</td>
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Synchronous cases

| Asynchronous cases |
|                   |
| 8                  | 43        | 2      | −   | Wild type  |
| 68                 | 43        | 2      | +   | Wild type  |

Table 3 Results of IHC and mutational analyses of p53 in bilateral breast cancer

<table>
<thead>
<tr>
<th>p53 mutation</th>
<th>Detected</th>
<th>Not detected</th>
<th>Total</th>
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<tr>
<td>IHC</td>
<td>7</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>40</td>
<td>44</td>
</tr>
<tr>
<td>Total</td>
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<td>51</td>
<td>62</td>
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The origin of contralateral breast cancer in a woman with primary breast cancer is unclear. Establishing the clonality of bilateral breast cancer would help us understand the biological mechanism of this uncommon disease. If the two tumors do not share a common origin (i.e., a common progenitor cell), there likely exists a susceptibility factor (which could be either genetic or environmental in nature), which puts multiple cells at an elevated risk of transformation in such women.

The clonality of malignant tumors has long been of interest. The concept that malignant tumors arise from single cells is well accepted (24), but only recently have advances in molecular biology made it possible to actually test this notion (3) and to determine whether multiple tumors in the same individual share a common origin (25). Studies using the phosphoglycerokinase gene on the X chromosome as a marker have clearly demonstrated that multiple tumors present concurrently in the bladder (25) or breast (26) of individual female patients share the same inactivated X chromosome, suggesting that all such tumors developed from a common transformed cell.
Few studies have examined the clonal origin of bilateral breast cancer, either by histocytochemical or molecular biological techniques. Dawson et al. (27) compared the immunoreactivity of paired breast tumors from 31 patients using six different immunocytochemical markers. No concordance between bilateral breast tumors was observed, suggesting independent origins of those tumors. Noguchi et al. (28) reported a patient whose contralateral breast cancer was shown to be an independent primary tumor using the RFLP method to study the X-chromosome-linked phosphoglycerokinase gene.

Three cases of bilateral breast cancer included in the present study showed different X-chromosome inactivation patterns in the right and left breast tumors. This disproves the hypothesis that all bilateral breast tumors develop from a single transformed cell. For the other nine cases analyzed that shared the same inactivated X chromosome, we could not make any conclusion about their clonal origins, because there is a 50% chance of observing concordance between two tumors even if they are independent in origin under the assumption of random inactivation of X chromosomes.

The p53 tumor suppressor gene has been found to be altered in many different types of human cancer (11). Although germ line mutations in the p53 gene seem to play a major role in Li-Fraumeni syndrome, one of the familial cancer syndromes, previous studies indicate that the great majority of p53 gene mutations occurring in human tumors represent somatically acquired changes. The reported frequency of p53 mutations in sporadic breast cancer (mostly unilateral) averages 22% (11), precisely the percentage observed here. The percentage is somewhat lower in our study if individual tumors (13%) are counted instead of patients. A lower frequency of p53 mutations in bilateral breast cancer, when compared with sporadic breast cancer, is surprising and suggests that the p53 gene may play a lesser role in breast cancer development than widely believed.

Two patients had p53 mutations in both breast tumors, but the mutations in the right and left breast were not identical in either case. The results suggest that, in those two cases, the p53 mutations were acquired independently. In an additional nine patients, a p53 mutation was found in one breast tumor, but not in the other. There are at least two possible explanations for the discordance of p53 mutations between two breast tumors in these 11 women: (a) the two tumors developed from independently transformed cells; therefore, they do not share genetic alterations; and (b) the two tumors developed from a single transformed stem cell early in the course of development, but the p53 mutations occurred much later in the course of disease progression in one or both breasts, after daughter cells of the transformed cell diverged into opposite breasts. It is not possible to determine which explanation is more plausible with our results alone. However, our data on the X-chromosome inactivation suggest that at least some bilateral breast cancer cases are compatible with the first explanation. The very low frequency of bilateral breast cancer also suggests that development of two tumors from a single transformed cell would be very rare.

Immunohistochemical staining of p53 protein using monoclonal antibodies is designed to detect protein abnormalities. Those cells that contain stabilized protein (i.e., a prolonged half-life) are stained positively when histological sections are treated with specific antibodies. Because point mutations in the p53 gene are thought to cause stabilization of the protein, IHC has been used as a rapid screening method or a substitute for identifying p53 mutations.

Although a statistically significant association has been observed between IHC of p53 protein and mutational analysis of the p53 gene in bladder tumors (29), the correlation is rather low in breast tumors. Hurliman et al. (30) reported that only 50% of IHC-positive tumors also had p53 mutations in exons 5–9 identified by SSCP. The positive predictive value of IHC (i.e., the probability of identifying a mutation when IHC is positive) was higher (79%) in another study (31) but lower (39%) in the present study. It is possible that the false-positive tumors in this study contained unidentified mutations outside of exons 5–8 of the p53 gene. However, a recent study by Bergh et al. (32), in which the entire coding sequence was screened for mutations in 316 breast tumors, showed that the majority of mutations were located within the region that we screened. Positive IHC results in some breast tumors without p53 mutations may be due to changes in other genes and/or altered cellular environment which stabilize p53 protein.

Our results of the X-chromosome inactivation assay indicate that some, if not all, bilateral breast tumors are independent of each other in their clonal origin. Our data also suggest that the concordance of somatic p53 mutations in bilateral breast cancer is very low. Further investigation of the underlying susceptibility that causes transformation of multiple cells in breast (and eventually the development of multiple breast tumors) is needed.

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Clonal Analysis of Bilateral Breast Cancer

748 Clonal Analysis of Bilateral Breast Cancer


Clonal analysis of bilateral breast cancer.
A Shibata, Y C Tsai, M F Press, et al.