Elevated Frequency and Functional Activity of a Specific Germ-Line p53 Intron Mutation in Familial Breast Cancer

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ABSTRACT

Previous studies have determined that the frequency of germ-line p53 mutations in familial breast cancer patients is 1% or less, but these reports have not investigated the importance of polymorphic intron base changes in the p53 gene. Therefore, we investigated the frequency of both exon and intron germ-line p53 base changes in 42 breast cancer patients with a strong family history of breast cancer. The mean age of presentation of these patients was 44.0 years (range, 29–69), and 12 of 42 (29%) were of known Ashkenazi ancestry. Purified DNA obtained from the 42 index cases was screened for germ-line p53 mutations in exons 2–11 and surrounding introns using a combination of intron based primers for PCR-single strand conformation polymorphism analysis, direct sequencing, and microarray sequencing using the Affymetrix p53 gene chip methodology. Morphological analysis of apoptosis and cell survival determination were performed on EBV-immortalized lymphoblastoid cell lines from two patients with the p53 intron 6 mutation. A germ-line mutation in the p53 gene at nucleotide 13964 with a G to C base change (13964G/C) was identified in 3 of 42 (7.1%) hereditary breast cancer patients. Two patients were heterozygous for this mutation, and one patient had a homozygous mutation. In comparison, 0 of 171 (0%) of sporadic breast cancer patients had the p53 13964G/C mutation (P = 0.0003). We found that 0 of 42 (0%) of these hereditary breast cancer patients had other germ-line p53 mutations in exons 2–11. However, pedigree analysis demonstrated that all three patients had strong family histories of multiple types of cancers consistent with Li-Fraumeni syndrome but with late age of onset. Comprehensive BRCA1 and BRCA2 nucleotide analysis from patients with the p53 13964G/C mutation revealed no concomitant deleterious BRCA1 or BRCA2 mutations, although they were found in the other hereditary breast cancer patients. Functional analysis of two immortalized lymphoblastoid cell lines derived from patients with the p53 13964G/C mutation demonstrated prolonged in vitro survival in response to cisplatinum treatment and showed decreased chemotherapy-induced apoptosis. Immunohistochemical analysis of breast tumors from these patients revealed high levels of mutant p53 protein, suggesting a functional mutation in the p53 gene. In summary, we have identified a single p53 intron mutation in familial breast cancer patients that is present at elevated frequency and has functional activity.

INTRODUCTION

The p53 gene is a tumor suppressor gene localized to chromosome 17 that has been demonstrated to have many important biological functions, including regulation of cellular transformation, cell cycle checkpoint following DNA damage, and DNA repair, and is an important mediator of apoptosis following DNA damage in certain cell lineages (1–7). LFS3 is characterized by germ-line p53 mutations and a clinical phenotype that includes premenopausal breast cancers, childhood sarcomas, brain tumors, leukemias/lymphomas, and adrenocortical carcinomas (8, 9). Approximately 70–80% of LFS patients have germ-line missense mutations in the p53 gene that are generally localized to the conserved exons (9–11). Interestingly, 20–30% of affected LFS families do not have detectable exon mutations, but many studies have not fully examined intron and promoter sites, which may be important regulators of gene expression (10).

p53 intronic base substitutions have been noted in patients including a p53 G to C transversion at base 13964 (p53 13964G/C) in women diagnosed with both breast and ovarian cancer, but this base change has previously been thought to represent a polymorphism of unclear functional significance (12). This is an important because these base changes may result in functional mutations distinct and women with LFS have an 80% lifetime risk of developing breast cancer (10). The clinical significance of germ-line p53 gene mutations in familial breast cancer has not been considered important because less than 1% of all breast cancer patients have germ-line p53 mutations; this percentage is small compared to those with BRCA1 and BRCA2 mutations (13–17).

There is accumulating evidence that novel mechanisms of gene regulation, including mutations in splice donor and acceptor sites, enhancer, intron, and promoter elements, may be important in regulating gene expression, including p53 (18, 19). The identification of these unique mutations that result in aberrant gene expression may provide for the detection of patients at risk for the development of cancer. The identification of these mutations may someday allow physicians to optimize treatment because these genetic changes may alter specific biochemical pathways. We now provide evidence that the p53 13964 base is mutated in patients who have familial breast carcinoma but not sporadic breast cancer. Furthermore, this mutation inhibits apoptosis and prolongs cell survival following DNA damage and thus may indeed affect breast cancer risk. The identification of women at risk for the development of breast cancer will have important implications for the prevention of cancers, treatment strategies, and improved cure rates of these patients.

PATIENTS AND METHODS

Patient Population. We identified 42 breast cancer patients with an autosomal dominant pattern of inheritance of breast cancer from the breast cancer database at Yale University School of Medicine. A strong family history of breast cancer was determined by having at least three first- or second-generation family members with a pathological diagnosis of breast cancer or 1 or more family members with premenopausal breast cancer. From this population, we also identified 171 breast cancer patients with no identifiable family history of breast and ovarian cancer, who are thus considered sporadic (Table 1). These patients were all treated with radiation therapy and closely followed in the clinic at Yale University School of Medicine. A protocol for the study was approved by the Human Investigations Committee at the Yale University.

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2 To whom requests for reprints should be addressed, at Bodine Center For Cancer Treatment, Department of Radiation Oncology, Thomas Jefferson University, 111 South 11th Street, Philadelphia, PA 19107-5097. Phone: (215) 955-6706; Fax: (215) 955-0412; E-mail: Bruce.Turner@mail.jwu.edu.
3 The abbreviations used are: LFS, Li-Fraumeni syndrome; SSCP, single strand conformation polymorphism.

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were performed as described previously with the addition of 5 in PCR-SSCP screening assay with intron primers that flank exons 5–9.

chloroform and resuspended in sterile TE. Genomic DNA (100 ng) was used for 7 min. A 10–

94°C for 1 min, 60°C for 1 min, and 78°C for 1 min, with a final cycle of 78°C.

Apoptotic cells were counted by two independent observers and confirmed by

Vogelstein, Johns Hopkins University) using LipofectAMINE (Life Technol-

lymphoblastoid cell lines were treated with 10–

m at position 13964 destroys a

mutation destroys a

p53 INTRON MUTATION IN FAMILIAL BREAST CANCER

RESULTS

Clinical Characteristics of Familial Breast Cancer Patients.

Forty-two breast cancer patients with a strong family history of breast cancer (index cases) consistent with an autosomal dominant pattern of inheritance were included for germ-line p53 analysis. The mean age of presentation of this cohort of patients was 44.0 years (range, 29–69), and 12 of 42 (29%) were of Ashkenazi ancestry. Comprehensive BRCA1/BRCA2 analysis was performed on these patients, and 7 of 42 (17%) were found to have a deleterious BRCA1 or BRCA2 mutation. This finding is consistent with recent reports that suggest that 10–40% of women with strong family histories of breast/ovarian cancer unselected for age have deleterious BRCA1 or BRCA2 mutations (27–29).

Germ-line p53 Mutation Analysis. Previous studies have determined that the frequency of germ-line p53 mutations in familial breast cancer patients is 1% or less, but many of these reports have not investigated the importance of intron base changes in the p53 gene (13–17). Therefore, we investigated the frequency of both exon and intron germ-line p53 base changes in 42 breast cancer patients with a strong family history of breast cancer. Purified DNA obtained from the 42 index cases was screened for germ-line p53 mutations in exons 2–11 and surrounding introns using a combination of intron based primers for PCR-SSCP analysis, direct sequencing, and microarray sequencing using the Affymetrix p53 gene chip methodology. We identified two samples (BT-16 and BT-31) that had intron 6/exon 7/intron 7 gel shift alterations (Fig. 1A, lanes 2 and 10) and a single sample (BT-102) that had a homozygous gel shift alteration by SSCP (Fig. 1A, lane 13). Because examination of exon 7 and the adjoining splice sites revealed no mutation by direct sequencing, we concentrated on sequencing all of the intron 6 and intron 7 regions that were present in the PCR-SSCP fragment. We identified a p53 13964\textsuperscript{GC} intron 6 mutation by direct sequencing which was verified by cloning the PCR products (TA cloning system) and sequencing. We determined that BT-16 and BT-31 were heterozygous (3 of 6 and 4 of 10 clones were mutants), and BT-102 was homozygous for the p53 13964\textsuperscript{GC} base change by automated sequencing (9 of 9 clones were mutants; Fig. 1B). Comprehensive scanning of p53 exons 2–11 was performed by gene array analysis (Affymetrix) and revealed no missense mutations in exons 2–11 of the p53 gene in any of the 42 familial breast cancer samples.

Genotyping Assay for Familial Breast Cancer Patients. Examination of the intron 6 p53 13964\textsuperscript{GC} base change revealed that the mutation destroys a HhaI cleavage site (recognition sequence GCCG), and its presence could thus be detected by restriction enzymatic

num was determined for all immortalized cell lines and stably transfected cell lines.

Immunohistochemistry for Somatic p53 Mutations. Following identification of the patients with the p53 intron 6 13964\textsuperscript{GC} base change, the individual 10% formalin-fixed and paraffin-embedded blocks were processed for immunohistochemical staining as described previously (26). After blocking 5-\textmu m sections with goat suppressor serum, p53 protein levels were determined by using the DO1 monoclonal antibody (Oncogene Science, Uniondale, NY) to mutant p53 protein at a 1:1000 dilution. Positive staining was defined by nuclear staining only within the invasive breast cancer component. No antigen retrieval was used to amplify the immunohistochemical signal. All samples were stained with positive and negative controls that included staining of tumors with known p53 mutations and absence of primary p53 antibody.

Statistical Analysis. The frequency of base changes in the p53 alleles between the patients with familial and sporadic breast cancer were compared using the Fischer’s exact test. Differences in categorical variables between the patient groups has been compared using the Spearman $\chi^2$ test. A $P$ of less than or equal to 0.05 (two-tailed) was considered to indicate statistical significance.
digestion of PCR amplified products analyzed on 4% NuSieve/agarose gels (Fig. 2). Using the genotyping assay, we screened the 42 familial breast cancer patients and identified only those patients previously found on SSCP and sequencing to have the heterozygous (BT-16 and BT-31) and homozygous (BT-102) mutations at base 13964 of the p53 gene (Fig. 2). Two samples (BT-36 and BT-52) that contained p53 wild-type sequences were used as controls and produced the expected 172- and 24-bp fragments on HhaI digestion (Fig. 2).

Genotyping Assay for Sporadic Breast Cancer Patients. The HhaI genotyping assay was used to screen 171 sporadic breast cancer patients, 43 of whom (25%) were of Ashkenazi ancestry but none of whom had a family history of breast/ovarian cancer (Table 1). The sporadic and familial breast cancer cohorts were found to be matched for histopathology (adenocarcinoma), stage (I/II), and Ashkenazi ancestry as determined by χ² analysis (Table 1). We found the age of presentation of familial breast cancer patients to be 44 years compared to 55 years for sporadic breast cancer patients (P < 0.001; Table 1). We identified 0 of 171 sporadic breast cancer patients to have the p53 13964GC base change compared to 3 of 42 (7%) index cases (Fisher’s exact test, P = 0.0003). Thus, there was a highly statistically significant association between the intron 6 p53 13964GC mutation and familial breast cancer.

Clinical and Molecular Characteristics of Patients with the p53 13964GC Base Change. The ages of presentation of the three patients were 30, 42, and 59 years; all three patients presented with stage I or II invasive ductal carcinoma, and none developed metastatic disease (Table 2). We found that both patients with the heterozygous p53 13964GC base change had a family history of pre- and post-menopausal breast cancers and that both had bilateral breast cancer.
consistent with the inheritance of a dominant gene (30). Interestingly, both of these patients were of Ashkenazi ancestry, although no previously defined relationship between p53 gene mutations and Ashkenazi ancestry has been described. We were able to obtain the paraffin-embedded breast tumor blocks from a proband’s (BT-16) mother and maternal aunt, who had a history of breast cancer diagnosed at ages 42 and 55, respectively. Genotyping revealed the p53 13964GC base change in both affected relatives, demonstrating that the genetic change appears to track with the cancer-prone family members. Tissue from other family members to evaluate for the p53 13964GC mutation was not available. The single patient with the homozygous p53 13964GC mutation has a strong family cancer history, including postmenopausal breast cancers, early and late onset leukemias, brain tumors, and lung cancers from both maternal and paternal lineages with no evidence of consanguinity within this family (Fig. 3). We performed complete sequencing of all coding exons and intron/exon junctions of BRCA1/BRCA2 genes in these patients and found all patients with the p53 13964GC mutation to have wild-type BRCA1/BRCA2 genes.

Functional Importance of the p53 Intron 6 13964GC Base Change. We immortalized lymphoblastoid cells from patients BT-16 and BT-102 and created cell lines containing the heterozygous and homozygous p53 13964GC base change, respectively. The cell lines did not contain any exons 2–11 mutations, as determined by PCRSSCP and p53 gene chip analysis. Functional p53 studies were carried out to determine the importance of the p53 intron base change at nucleotide 13964. We examined whether cells with the heterozygous or homozygous p53 intron 13964GC base change would be resistant to chemotherapy-induced apoptosis, as demonstrated previously for p53 missense mutations (5, 31, 32). BT-16 and BT-102 lymphoblastoid cells containing the p53 13964GC base change and two p53 wild-type lymphoblastoid cell lines were treated with varying doses of cisplatinum, and cell survival was determined at 48 h (Fig. 4A). The survival of BT-16 and BT-102 cells at 48 h following treatment with 5, 10, and 20 μg/ml cisplatinum was 79 ± 2, 75 ± 2, and 62 ± 1% for BT-16 and 89 ± 2, 82 ± 2, and 76 ± 2% for BT-102, respectively, compared to 42 ± 3, 31 ± 3, and 18 ± 2% for the p53 wild-type lymphoblastoid cell line VDS (Fig. 4A). The survival of p53 wild-type 012 cells demonstrated similar survival characteristics to VDS cells and generally had poor survival following chemotherapy treatment compared to BT-16 and BT-102 cells (Fig. 4A). Chemotherapy-induced apoptosis was determined in two p53 wild-type lymphoblastoid cell lines and was found to be maximal at 48 h following drug treatment (data not shown). Treatment of BT-16 and BT-102 lymphoblastoid cells with 10 μg/ml cisplatinum for 48 h resulted in apoptosis in 15 ± 3 and 10 ± 3%, respectively. In comparison, p53 wild-type cells VDS and 012 (data not shown) demonstrated 47 ± 5 and 38 ± 5%, respectively (Fig. 4B). Transfection assays with the pc53-SN3 plasmid containing wild-type p53 into VDS, BT-16, and BT-102 cells demonstrated partial restoration of normal chemotherapy-induced apoptosis in the BT-16 cells containing the homozygous p53 13964GC base change but not in BT-16 cells containing the heterozygous base alteration. Following treatment with cisplatinum, apoptosis was found in 30 ± 4 of BT-102/p53T cells compared with only 10 ± 3% of untransfected BT-102 cells (Fig. 4B). The morphological changes of nuclear blebbing and membrane fragmentation characteristic of apoptosis are shown by staining of cells with bis-benzimide Hoechst stain are prominent in p53 wild-type cell lines VDS and 012 (Fig. 5, C and D) but poorly demonstrated in the BT-16 and BT-102 lymphoblastoid cell lines (Fig. 5, A and B).

Somatic p53 Mutations. Because previous investigators have identified increased mutant p53 protein in tumor specimens from familial breast cancer patients without germ-line p53 missense mutations, we evaluated for mutant p53 protein expression in tumor specimens from patients with the p53 13964GC mutation (33). We did not use an antigen retrieval technique because this has been associated with increased background signal, making the interpretation of the staining pattern difficult. Mutations in the p53 gene have previously been shown to prolong protein stability, thus allowing the detection of mutant p53 protein by using an antibody specific to this protein.

We obtained the paraffin-embedded breast tumor blocks from patients BT-16, BT-31, and BT-102, which were found to have the

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Table 2 Clinical, pathological, and molecular characteristics of breast cancer patients with germ-line p53 13964GC mutation

<table>
<thead>
<tr>
<th>Identification no.</th>
<th>Age at diagnosis (yr)</th>
<th>Family history of multiple cancers</th>
<th>Bilateral breast cancer</th>
<th>Ashkenazi ancestry</th>
<th>BRCA1 and BRCA2 mutations</th>
</tr>
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<tbody>
<tr>
<td>BT-16</td>
<td>42</td>
<td>Strong</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>BT-31</td>
<td>30</td>
<td>Strong</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>BT-102</td>
<td>59</td>
<td>Strong</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

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Fig. 3. Pedigree of patient with the homozygous p53 13964GC germ-line mutation (BT-102). Br, breast; Leuk, leukemia, BT, brain tumor.
germ-line p53 13964GC mutation. Immunostaining of breast tumor specimens from BT-102 (Fig. 6A), BT-31 (Fig. 6B), and BT-16 (Fig. 6C) revealed strong nuclear staining within the invasive ductal component of all three patients. Previously, LFS patients with p53 mutations have been shown to have high levels of mutant p53 protein in benign tissue containing only the germ-line heterozygote p53 mutation, but there was little benign tissue on these sections, and expression of mutant p53 in benign tissue could not be evaluated. Staining of sections with a secondary antibody alone revealed no nuclear reactivity, demonstrating the lack of nonspecific cross-reactive proteins (Fig. 6D).

DISCUSSION

We now present evidence that the germ-line p53 13964GC base substitution is identified in familial breast cancer patients but not in sporadic breast cancer patients and that the base change alters normal gene function. The functional studies support our theory that the p53 13964GC base change functions as a dominant mutation similar to the more common missense, nonsense, and splice-site mutations described previously for LFS. BRCA1 and BRCA2 mutations have been thought to account for 10–40% of hereditary breast cancers, but there are approximately 200–300 different mutations with different phenotypes, and detection of these mutations is often difficult and expensive. The p53 13964GC mutation represents a single noncoding base change that is easily detected and may be responsible for a significant number of hereditary breast cancers; this mutation is the first demonstration of the importance of intronic sequences in regulating p53 function, as suggested by other studies (18).

In a cohort of breast cancer patients with a strong family history of breast cancer, 3 of 42 (7%) have the specific p53 13964GC mutation compared with 0 of 171 sporadic breast cancer patients (P = 0.003). A detailed analysis of the pedigrees of these patients reveals a pattern of late-onset cancers that appear to be inherited in an autosomal dominant pattern. Late-onset Li-Fraumeni cancers have been described previously in families with exon 4 mutations and indicate that some germ-line p53 mutations may allow for partial function of the protein, which results in the late-onset phenotype of tumors (19, 34). Genetic analysis of one of the proband’s mother and maternal aunt, both of whom developed breast cancer, demonstrated that they carried the p53 13964GC mutation. Examination of the pedigree from the patient with the homozygous 13964GC substitution is striking, revealing many early and late-onset LFS types of cancers including breast...
cancers. It is noteworthy that there are both maternal and paternal relatives with late onset breast cancers and other Li-Fraumeni-like cancers. Two of the breast cancer patients with the $p53_{13964\text{GC}}$ mutation were of Ashkenazi ancestry, suggesting that this may represent a founder mutation.

It was important to establish whether this intron base change represented a functional mutation. Therefore, we established an EBV immortalized lymphoblastoid cell line from two patients with the $p53_{13964\text{GC}}$ base change. The $p53$ protein is a transcription factor which is a downstream regulator of many signal transduction pathways,
especially those that mediate an apoptotic response to chemotherapy and ionizing radiation. By using p53 knockout and transgenic mice that overexpress dominant negative p53, several independent groups have shown that p53 is essential for apoptosis following treatment with ionizing radiation, chemotherapy, or serum withdrawal (4, 7). A report from another group describes lymphoblastoid cell lines from LFS patients with heterozygous p53 germ-line mutations at codons 282 and 286 to be associated with resistance to radiation-induced apoptosis (35). Our in vitro experiments demonstrating that the p53 13964GC mutation results in prolonged cell survival and inhibits chemotherapy-induced apoptosis is consistent with these findings.

The p53 13964GC base substitution was first reported by Buller et al. (12), who found the p53 13964GC base change in 3 of 73 (4%) patients with both breast and ovarian cancer. The authors concluded that the p53 13964GC base change represents a nonfunctional polymorphism because there was no direct functional activity to implicate it in malignant transformation. Furthermore, a recent study examining children from Belarus with possible radiation-induced thyroid tumors demonstrated that 5 of 70 (7.1%) had the p53 13964GC base change, whereas 1 healthy child was also found to carry this base change (36). The family history of these patients were not available, so no firm conclusions were made from this study with regard to associations with inherited cancers, although many childhood malignancies are often due to inherited genetic mutations. Other p53 intron 6 base substitutions have also been identified that may be associated with tumor development. A substitution at nucleotide 13961GA was identified in 6 children with the Li-Fraumeni spectrum of tumors, whereas only 1 of 184 healthy controls was found to have this base change (P = 0.036; Ref. 37). Tissue specimens from these patients were found to contain mutant p53 protein immunoreactivity in both tumor and normal tissue. Recently, investigators have identified another p53 intron 6 CpG-rich Alu repetitive element adjacent to the p53 13964 base may explain the frequency of mutation at this site (41). The detection of patients at risk for malignancies is critical in identifying candidates for screening and prevention programs and someday may allow optimal treatment. Genetic testing to identify these subgroups of patients at risk for familial breast cancer and to determine their respective outcome to modern treatments has only just begun. Our finding that BRCA1 and BRCA2 wild-type familial breast cancer patients have the germ-line p53 13964GC mutation is one step toward identifying genetic mutations that predispose women to breast cancer. Larger epidemiological studies are now warranted to further define the frequency of this mutation and perhaps detect other important intronic mutations.

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