Somatic p53 Mutations in Human Breast Carcinomas in an Icelandic Population: A Prognostic Factor

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ABSTRACT

Mutations in the p53 gene are among the most common genetic changes in human carcinomas. They have been found in many tumor types including colon, lung, and breast. We have used constant denaturant gel electrophoresis in order to screen samples from 109 breast carcinomas for mutations in four conserved regions, exons 5, 7, and 8, of the p53 gene. Samples were also analyzed for allelic loss of the p53 gene and of markers more distal on chromosome 17p. Mutations were confirmed by DNA sequencing. Mutations were found in 18 of the 109 samples (16.5%). Loss of heterozygosity at 17p was detected in the majority of informative cases. All cases were also screened for germ line mutations, but none were found. The results obtained were analyzed with respect to clinical parameters and prognosis. There was a significant association between p53 mutation and low content of estrogen receptor protein in the tumors (P = 0.01). An association with poor prognosis was strongly indicated by mortality rates that were 37.5% among the patients with p53 mutation and 9.4% for the control group (mean follow up, 32 months). P53 mutation was found to be the strongest negative factor against survival in a covariate survival analysis (P = 0.001).

INTRODUCTION

The p53 gene is located on the short arm of chromosome 17. It has been classified as a tumor suppressor gene and is thought to require a loss of function mutation for tumor formation. Mutated p53 can transform cells in cooperation with the ras oncogene (1, 2). Normal p53, however, suppresses transformation (3). Alterations within the p53 gene are found in a wide variety of human carcinomas, such as colon, breast, and lung (4–6). Allelic deletions in the p53 region are common in human carcinomas (7, 8), and mutations have frequently been detected in the remaining allele, suggesting that loss of normal p53 plays an important role in the tumor formation.

The p53 protein is a 53-kDa DNA-binding nuclear phosphoprotein which is found in virtually all normal cells at a very low level. The protein is thought to act as a negative regulator of cell proliferation, and its expression is regulated through the cell cycle. The normal protein has a half-life of only 20–30 min, but various oncogenic mutations increase the stability of the protein (9), resulting in an elevated level of the mutated protein in the cell. Mutated p53 protein might sometimes act in a semidominant way by complexing with normal p53 and, thus, interfere with its normal function (3). Mutant forms of the protein have transforming activity and interfere with the cell cycle regulatory functions of the wild-type protein. This could explain why changes in the p53 gene are so often found in malignant cells.

It has been shown that the p53 gene has five evolutionary well-conserved regions (5). The majority of p53 mutations that have been detected, occur in four of these well-conserved regions in exons 5, 7, and 8 (10). In the ras genes, point mutations that can lead to cancer occur in only a few codons, but mutations in the p53 gene have been found in >30 codons that are well conserved in distant species. Therefore, it is likely that mutations in these codons can cause the protein to lose some of its vital functions. The mutational spectra seem to vary between tumor types, but there are also mutational hotspots common to different cancers.

Germ line p53 mutations have been found in families with the heritable cancer syndrome Li-Fraumeni (11, 12). Germ line mutations outside Li-Fraumeni families have also been found in individuals with two different malignancies and in breast cancer families not of the classical Li-Fraumeni type (13, 14). However, germ line p53 mutations are rare, and >98% of p53 mutations found in human cancers are somatic mutations (10).

By using CDGE3 (15, 16), which is based on the DGGE technique, we analyzed breast cancer samples for the presence of mutations in exons 5, 7, and 8 in the p53 gene. This method is based on the melting behavior of DNA strands which is determined by their sequence. In this study, we compared 17p allelic loss in mutated and nonmutated samples and attempted to link p53 mutation to clinical parameters and prognosis in breast cancer.

MATERIALS AND METHODS

Tumor Material. A fresh excision biopsy specimen was obtained from 109 breast carcinomas, 102 primary carcinomas, and 7 local recurrences and metastases. DNA was extracted from peripheral blood and tumor tissue using standard phenol/chloroform extraction. The mean age of patients was 59.3 years (range, 33–94 years) compared with a mean age of 59.4 years for Icelandic breast cancer patients during a period of 30 years (17). Table I shows the clinical parameters for the 102 primary tumors.

PCR Amplification. DNA from tumor and blood samples was PCR amplified using primers covering four regions of the p53 gene as previously described (16). If mutations were found in tumor material, DNA from blood leukocytes was screened to determine whether the mutation was present in the germ line. Four sets of primers were used to amplify the conserved regions of the p53 gene where most of the mutations have previously been found. PCR was performed using 100–400 ng of template DNA in 10 µm Tris-Cl (pH 8.4/8.6), 50 mm KCl, 0.75–2.0 mm MgCl2, 0.2 mm each deoxynucleotide, 50–100 pmol of each primer, and 2.5 units of Taq polymerase (AmpliTaq; Cetus). The total reaction volume was 100 µl. The reaction was amplified on a Perkin-Elmer thermal cycler (Cetus) for 35 cycles consisting of 94°C (45-s segment A), 55°C (45-s segments C and D, 75-s segment B), 55°C (45-s segments A and B), and 72°C for 60 s. The reaction was initiated with a 7-min incubation at 94°C and ended with 10 min at 72°C. The primers were synthesized by Genosys (Houston, TX). In every primer pair, one of the primers had a GC clamp, creating a 60-mer primer (18). Only the 60-mers were ordered.

Fragment A (exon 5) sense primer was (5'-TTCCTCTTCTCGACTAATCT-3') and antisense primer was (5'CGCCCCGCGCCCCCGCCCGCTCC-CCGGCCCCCGCCCGTGGCGGCGGACGCGGGTGCCG-3'); fragment B (exon 5) sense primer was (5'CGCCCCGCGCCCCCGCCCGCTCC-CGGCCCCCGCCCGTGGCGGCGGACGCGGGTGCCG-3') and antisense

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3 The abbreviations used are: CDGE, constant denaturant gel electrophoresis; DGGE, denaturant gradient gel electrophoreses; PCR, polymerase chain reaction; LOH, loss of heterozygosity; TAE buffer, 40 mm Tris acetate, 1 mm EDTA, pH 8.0.

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primer was (5'-GCCCGACGCTGCTCAACTCATC-3'); fragment C (exon 7) sense primer was (5'-CACCATCCACTACAACCT-3') and antisense primer was (5'-GGGGCGGGCGCGGGCGGCGGGCGGCGGCGGGGCGGGGGGCGGGC-3'); and fragment D (exon 8) sense primer was (5'-GTGTTAGACTGGAAACCTTT-3') and the sequence primer was 5'-AGGGGTCAGCGGCAAGCA-3'. In exon 8, PCR primers were 5'-Biotin-TTGGGAGTAGATGGAGCCT-3' and 5'-AGGTGTAGACTGGA4AAACCTTT-3', and the sequence primer was 5'-AGGGGTCAGCGGCAAGCA-3'.

**RESULTS**

**Mutations in the p53 Gene.** A total of 109 breast tumor samples were screened for mutations in four "hotspot" regions corresponding to most of the evolutionary conserved areas of the p53 gene with the CDGE method. Using this method, we found mutations in 17 samples: 3 in hotspot A (exon 5, codon 155–185), 7 in hotspot B (exon 5, codon 155–185), 4 in hotspot B (exon 5, codon 155–185), 7 in hotspot C (exon 7, codon 237–253), and 3 in hotspot D (exon 8, codon 265–301) (Table 2). All except one mutation were confirmed by direct sequencing. As a control, 7 randomly selected CDGE mutation-negative tumor samples were sequenced, and no mutations were detected. Mutations were found in 16 of 102 primary carcinomas (15.7%) and 2 of 7 metastases and local recurrences (28.6%). Sequencing results showed that in 13 samples a single nucleotide substitution leading to an amino acid change occurred. In 3 cases, there was a single base deletion leading to a frame shift mutation. Ten of the base substitutions were transitions, and three were G→T transversions. A wide range of mutations were found, but the distribution of mutations corresponded to previously published results (26). Matching blood samples of tumor DNA showing a mutation in the p53 gene were analyzed to determine whether there was a germ line change in the gene. No such changes were detected.

In one tumor (tumor 42), a complete loss of heterozygosity was detected with probe BHP53, but no changes were found on the CDGE gels. When sequenced, a 14-base deletion was detected in the 5' end of exon 8, PCR primers were 5'-Biotin-CTTGGGAGTAGATGGAGCCT-3' and 5'-AGGTGTAGACTGGA4AAACCTTT-3', and the sequence primer was 5'-AGGGGTCAGCGGCAAGCA-3'. In exon 8, PCR primers were 5'-Biotin-CTTGGGAGTAGATGGAGCCT-3' and 5'-AGGTGTAGACTGGA4AAACCTTT-3', and the sequence primer was 5'-AGGGGTCAGCGGCAAGCA-3'.
of exon 8 (codon 262), resulting in a frameshift mutation. The deletion was within the PCR primer region of hotspot D, and therefore, only the wild-type allele was amplified. A big deletion starting in this region has been found in other tumors (6). This tumor was also informative for the p53 gene, and 5 of 8 (62.5%) (Table 4) but not with respect to progesterone receptor (P = 0.3). No significant association was found between p53 mutation and absence of estrogen receptor protein in the tumors (P = 0.01) but not with respect to progesterone receptor (P = 0.3). No statistically significant association was found between p53 mutation and tumor type, tumor size, node status, age at diagnosis, or family history. A highly significant association was, however, observed between p53 mutation and survival (Fig. 2). Among the patients with primary tumors, 6 of 16 patients with p53 mutation had died of breast cancer by the end of the study period as compared with 8 of 85 of the control group. Women with the p53 mutation had a 3.3-fold higher risk of dying during the study period (P = 0.001) than those without the mutation, after taking into account the effects of nodal status, tumor size, and age at the time of diagnosis. Follow-up time was 3–56 months (mean, 32 months).

**DISCUSSION**

Mutations in the p53 gene are common in breast cancer. In our study, we examined unselected consecutive samples. Mutations were found in 15.7% of primary carcinomas and 28.6% of metastatic tissue. Other investigators have reported higher frequencies of mutations in the p53 gene in breast tumors. There is, however, considerable variation in reported frequencies. Runnebaum et al. (27) studied p53 mutations in breast cancer cell lines and primary tumors and found p53 mutations in exons 5–9 in 17% (10 of 59) of primary tumors by

![Fig. 2. Relationship between p53 mutations and breast cancer survival. a. survival of patients without p53 mutations. b. survival of patients with p53 mutations.](image-url)
single strand conformation polymorphism analysis. Thompson et al. (28) found mutations in 28% (17 of 60) of tumors studied in exons 5–9 using the amplification mismatch technique. A Norwegian study, in which investigators studied exons 5–8 using exactly the same method and conditions as in the present study, detected p53 mutations in 21% (35 of 163) of primary breast tumors.4

The sensitivity of the CDGE method has been tested in a comparative study involving three laboratories where it was found to detect 100% of mutants under optimal conditions in exons 5–8 of the p53 gene (29). In a collaborative study of germ line mutations in breast cancer patients from three different populations, 40 CDGE mutation-negative samples from early-onset breast cancer cases were verified by sequencing (13). Randomly selected CDGE mutation-negative samples from our study were also found to be negative by sequencing.

It is difficult to compare different studies. Our samples came from a completely unselected series of patients. Most of the tumors were small due to intensive screening. Overexpression of p53 protein has been correlated with a higher stage of the disease. If p53 mutations are more often detected in larger and more advanced tumors as indicated by some studies (9), this might partly explain the slightly lower proportion of p53 mutations detected in this population. In addition, this study did not include exons 6 and 9 of the p53 gene.

The distribution of mutants within the p53 gene is in agreement with other published data (6, 10, 26). More than 80% of p53 mutations found have been in exons 5, 7, and 8, but mutations have also been found in other regions of the gene, mainly in exon 6 (10). Extending the screening area might add to the mutations detected in our samples. The p53 mutations found in the present series were mainly transitions, but 3 were transversions and 4 were deletions.

In samples with p53 mutation, loss of heterozygosity at the p53 locus was found in 62.5% of informative cases as compared with only 44.8% of samples that were not mutated. Although this is based on only a few informative samples, it supports the idea that loss of a functional p53 plays a part in the formation of the tumors.

Many investigators have found loss of heterozygosity at 17p in breast tumors (8, 20, 21, 30), indicating that this region is important for the formation of tumors in the breast. It has been suggested that there might be two frequently lost regions on 17p, one at 17p13.1 (the p53 locus) and the other at 17p13.3 (probes pYNZ22, pYNH37.3, and p144D6) (31). In our series, a loss of heterozygosity with all of the probes at 17p13.3 was found more often in samples with p53 mutations (73.3%) compared with nonmutated samples (45.3%). Our data indicate that the 144D6 probe recognizes a site closer to the p53 gene than do pYNZ22 and pYNH37.3. All of the mutants that do have a deletion with p144D6 also have LOH within the p53 gene (Table 4). Because of lack of informative samples, this finding is based on only a few samples, but it might indicate that there is another gene in the 17p13.3 region.

Changes at more than one site frequently occur in the same tumor. Amplification of the erbB2 oncogene is another common alteration in breast cancer and has been associated with poor prognosis (32). We analyzed amplification of the erbB2 oncogene in these same samples (data not shown). Amplification at the erbB2 locus was found in 47% of samples with p53 mutation compared with 24% of nonmutated samples.5 Altered cell cycle arrest and gene amplification potential has been associated with loss of wild-type p53 in recent studies (33, 34). Livingstone et al. (33) found that loss of wild-type p53 involving both copies of the gene, i.e., one mutated copy and loss of the other, is necessary for normal cells to be able to undergo N-(phosphoryl)-L-aspartate (PALA)-selected amplification. Interestingly, in our study, all of the tumors that had p53 mutation and erbB2 amplification also had a deletion on 17p.

All patients were checked for family history of breast cancer. No association was found between family history and p53 mutations, which agrees with a lack of germ line changes in our samples. A significant association was found between p53 mutation and low levels of estrogen receptor protein in tumors (P = 0.01). This has been reported in other studies (28, 35). Other factors indicative of the seriousness of the disease, such as tumor size and node status, did not show a significant difference between patients with and without a p53 mutation.

The patients in this study have been under observation for 3–56 months. When we look at mortality rates for this period, there is a striking difference between patients with mutation and the controls. Of the patients with primary tumors, a total of 14 had died by the end of the study period: 6 (37.5%) of the patients with p53 mutation and 8 (9.4%) of the control group. According to this, p53 mutation is a strong prognostic factor, apparently, irrespective of other factors.

In conclusion, in this study, we found p53 mutations in exons 5, 7, and 8 in 16% of unselected primary breast tumors. Allelic loss around the p53 locus was found more often in samples with p53 mutation, supporting the idea that inactivation of both alleles of the gene can lead to tumorigenesis. Amplification of the erbB2 oncogene was also found to occur more frequently in p53-mutated tumors than controls. Our data indicate that p53 mutation may be an important prognostic indicator of short-term survival for breast cancer patients.

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