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Detecting p53 Gene Mutation of Breast Cancer and Defining Differences between Silver Staining PCR-SSCP and Immunohistochemical Staining

This study detects and defines the patterns of p53 gene mutations in breast cancers. We analyse p53 gene mutations through comparing the results of single-strand-conformation-polymorphism (SSCP) and immunohistochemistry (IHC), and we try to define the differences between the results of SSCP and IHC. Twenty-seven fresh primary breast cancer tissues and eight normal breast tissues were studied. The IHC was done with the usual streptavidin-biotin peroxidase complement method by using monoclonal antibody DO-7. The results of staining was scored. The SSCP method was done by using Cold SSCP Electrophoresis System. Overexpressions of p53 protein were seven (25.9%) among 27 cancer cases on IHC. Four (57.1%) of seven cases were positive in SSCP. In SSCP, the mutations were detected in 10 (37%) among 27 cancer cases. The mutations were two in exon 5, one in exon 8, and seven cases in exon 7. All of 10 mutations were proved by sequencing analysis. Of them, only four (40%) were positive in IHC. We consider the IHC as a screening method for p53 gene mutations.

Key Words: *Breast Neoplasms; Genes, p53; Mutation; Polymorphism, Single-Stranded Conformational; Immunohistochemistry*

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INTRODUCTION

Breast cancer is the most important malignancy in western society, and approximately 10% of women in the United States will develop the disease during their life. Most *p53* mutations discovered in human cancers are missense changes which occur primarily in four or five highly conserved regions (1-3), and such mutations results in a p53 product with a longer half-life than the wild-type protein which allows immunohistochemical detection of p53 in the nucleus of the affected cell (4, 5). There have been innumerable studies done to evaluate the prognostic significance of p53 alteration in a wide variety of human malignancies including breast cancer. There is evidence that points the development and progression of breast cancer to an accumulation of mutations at the genomic level. *p53*, a tumor suppressor gene, is thought to play a crucial role in the process of breast carcinogenesis (6, 7). Most of these studies have shown an association between *p53* mutations detected by immunohistochemical staining and reduced overall survival of breast cancer (8). Immunohistochemistry (IHC) staining for p53 detects the accumulation for p53 protein in tumor cells as a surrogate marker of loss for p53 function and/or gene mutation. Though there is a close relation between IHC positivity and mutations of the p53 gene, this is not absolute.

Considerable amount of variables affect the IHC results due to differences in technique and interpretation (selection of thresholds of positivity). There have been discrepancies between p53 IHC positivity and poor survival (5). So an alternative approach for examining the prognostic significance of *p53* mutation is to identify gene mutations by molecular analysis (9, 10). It has been noticed that the frequency of *p53* mutations detected by immunohistochemical staining is often twice as high as the results found by polymerase chain reaction (PCR)-based mutation screening procedure such as SSCP or denaturing gradient gel electrophoresis (DGGE) (11). Alternatively, the complementary DNA for the entire coding region can be sequenced, but its use for routine analysis is limited. So, we try to detect the mutations of *p53* using rapid non-isotope PCR-SSCP and compare the results between IHC and SSCP.

MATERIALS AND METHODS

Breast cancers

Tumor specimens from 27 Korean women undergoing

surgery for primary invasive breast carcinoma at the Department of Surgery, Dankook University Medical Center between 1996 and 1997. The control was eight normal breast tissues. The specimens were snap frozen in liquid nitrogen and stored at -80℃ prior to extraction of the DNA. For IHC, formalin-fixed paraffin-embedded blocks of primary tumors were taken from pathological archives of the same 27 patients. The mean age of the patients was 39.8 year-old (ranges from 28 to 49). All of them were in premenopausal states.

Immunohistochemistry

The immunoperoxidase method, using the avidin-biotinylated horseradish peroxidase complex system (DAKO LSAB kit, Los Angeles, CA, U.S.A.) was performed to detect p53 proteins in formalin-fixed paraffin-embedded tissue sections. The anti-p53 mAb (DO-7, Novocastra, Manhasset, NY, U.S.A.) was used to recognize the human p53 molecule. Diaminobenzidine, supplemented with 0.02% hydrogen peroxide, was used as chromogen. A positive control and negative control were used in the each run to monitor the adequacy of immunostaining and possible background staining, respectively. The primary antibody were incubated for 1 hr at room temperature, and the secondary biotinylated rabbit antimouse antibody and avidin combined with biotinylated peroxidase were incubated for 30 min in sequence. The color was developed with diaminobenzidine and was counterstained in hematoxylin.

Scoring methods

The results of immunohistochemistry was quantified as scores. First, percentages of the total number of tumor cells were assigned to one of 6 categories: 0 for <5%; 1 for 5<-<30%; 2 for 30<-<50%; 3 for 50<-<70%; 4 for 70<-<90%; 5 for >90%. Second, the intensity of p53 immunostaining was scored as follows: 1+ for weak; 2+ for moderate; 3+ for intense. Then the scores of percentage and staining intensity were multiplied to produce a weighted score for each tumor, as a score of less than eight was designated as low expression and over as high expression (12).

Non-isotope rapid PCR-SSCP

DNA was extracted from normal and tumor tissues using Qiagen DNA extraction kit (Qiagen, Germany).

Polymerase chain reaction (PCR)

PCR was carried out by thermal cycler (Perkin-Elmer 9600, U.S.A.). The nucleotide sequences of the primers and the amplified sizes were described in Table 1. For each case, the four primer pairs described in Table 1 were used in individual PCRs to amplify p53-coding sequences in genomic DNA from normal and tumor tissues as templates. Ten to 80 ng of genomic DNA was amplified using 20 pmol each of the forward and reverse exon primer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.6), 50 mM KCl and 5 units Taq polymerase (TaKaRa, Japan) in a final volume of 25 μL. Genomic DNA was denatured at 94℃ for 5 min. The 30 cycles consisted of steps listed in Table 1.

SSCP analysis

The PCR product was purified to remove excess salts and primer dimer using QIA quick PCR purification kit (Qiagen, Germany). A 2 μL of PCR product is mixed with 5 μL of formamide solution (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.02% bromophenol blue, 0.02% xylene cyanol).

Denaturation was performed for 5 min at 98℃. Thereafter samples were cooled quickly on ice, and loaded immediately on to the gel. The non-isotope rapid cold SSCP was done using Novex pre-cast TBE gels and Thermo Flow Electrophoresis Temperature Control System (Novex, San Diego, CA, U.S.A.). After electrophoresis, for the band visualization the gels were stained using Silver Xpress (Novex, U.S.A.).

Table 1. Primers used for SSCP studies of exon 5-8

Exon	Sequence of primers Sense (5'→3') Antisense (3'→5')	Size of fragment (bp)	Annealing temperature for 1 min (℃)
5	TGAAAGTTGACAGAGGAAGG GCTGGAGAGACGACAGGGCT	250	60
6	AGTCTATCGCTACCACTCGTCG GGTCTCTGGGAGGAGGGGTTAA	246	60
7	GAACGGTGTCCAGAGGGGTTCC CCTCTTACCGATTTCTTCCATA	266	72
8	ACTAAAGGAATGACGGAGAACG GGAGACCAAGGGTGCAGTTATG	232	64

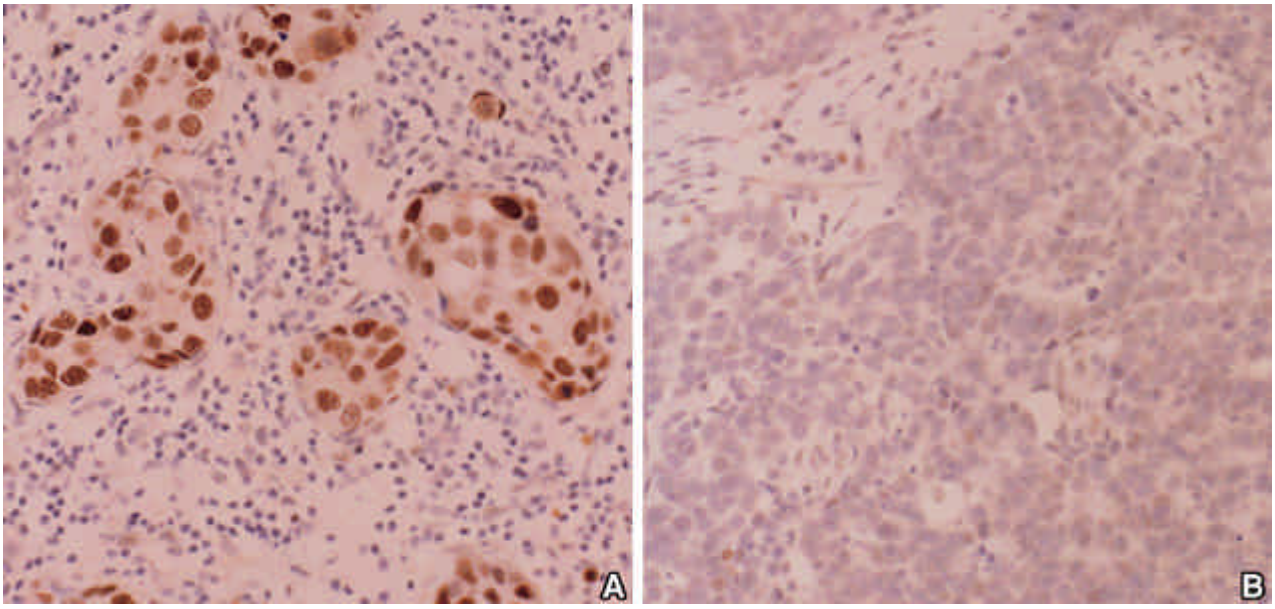

Fig. 1. Immunohistochemical staining of p53 protein in breast cancers. **A:** 70<-<90%, and intense staining; overexpression of p53. **B:** 5<-<30%, and weak staining; low expression of p53.

Sequencing analysis

The results of SSCP were proved by automatic sequencing analysis (ABI PRISM, U.S.A.).

RESULTS

Immunohistochemistry of p53 protein

Overexpressions of p53 protein were recognized in seven (25.9%) of 27 cancer cases (Fig. 1). In four (57.1%) of seven cases, p53 mutations were detected by both IHC and PCR-SSCP. There were no overexpressions in eight normal tissues.

p53 mutations in PCR-SSCP analysis and sequencing

PCR-SSCP analysis of p53 exons 5-8 revealed abnormal band migration indicative of a mutation in 10 (37.0%) of the 27 cases. Two mutations were found in exon 5 (Fig. 2), one in exon 8, and seven in exon 7. All of 10 cases proved as mutation by sequencing analysis (Fig. 3). Only the four (40%) of 10 cases were defined as mutations in IHC. There were no abnormal band migration in eight normal tissues.

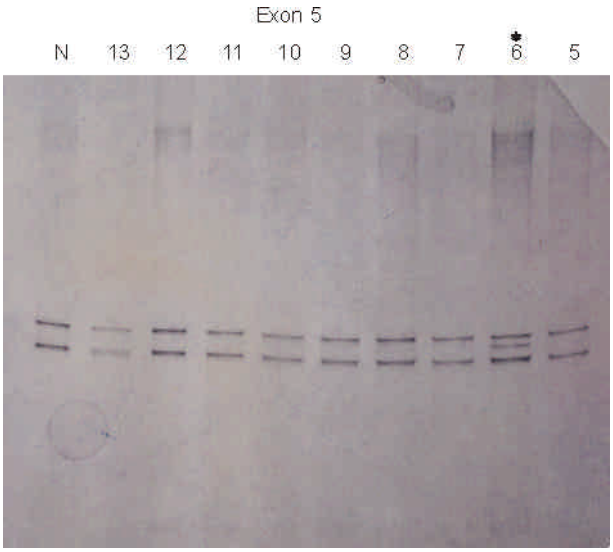

Fig. 2. PCR-SSCP analysis of the *p53* gene. Two of the 10 band shifts recognized by PCR-SSCP are shown here. DNA samples of breast tumors and normal tissue were amplified using PCR primers for the exon 5. Samples with shifted band (*) is seen in the tumor samples from patient 6. N, normal control tissue.

DISCUSSION

Abnormal expression of p53 protein as a prognostic parameter has been widely studied in human breast cancer, and most investigators conclude an association with

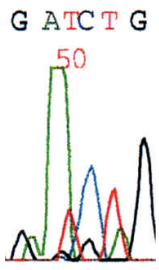

Fig. 3. Automatic sequencing analysis: point mutation in exon 5. GACCTG→GATCTG, C→T transition change.

aggressive forms of the disease (13, 14), though this has not been a consistent finding. Difficulties in standardizing IHC methodology and interpretation of IHC staining results may explain some of the discrepancies between different laboratories, and potentially limit the clinical application of IHC, leading to the possibility that the presence or absence of IHC staining is not a reliable indicator of an underlying p53 gene mutation (15). p53 positivity on immunostaining may mean accumulation of wild-type as well as mutant protein, while the absence of staining does not always indicate the absence of mutation. More significant information may be derived from direct molecular analysis of the p53 gene for mutation (9), and there are many studies which found a strong association between mutation identified by molecular analysis and poor survival (16).

In Korea, investigators have reported mutation ranges of p53 protein from 30% to 50% in IHC staining (17-21). Recently, comparative study between PCR-SSCP and IHC staining of p53 protein reported that the mutation rates were 30% in PCR-SSCP and 37% in IHC staining (21).

In this study, we observed that 25.9% of the tumors were overexpressed in the IHC scoring method. Our result is similar with those reported by You et al. (17), but relatively low when compared with those of other investigators in Korea (18-21). Variations might reflect heterogeneity between the tested cohorts of tumor, but technical conditions and interpretation methods could also account for these disparities. In fact, the fixation of tissues (type and duration of the process) and the antibody used in IHC have been shown to influence greatly the numbers of positive cases and cytoplasmic positivity (22, 23). If more than 5% of staining was regarded positive, the positive rate was more than 50%.

These variations in staining patterns as well as reports on possible differences between immunohistochemistry and analyses for mutations at the DNA level led us to perform a comparative search for mutations in the p53 gene using PCR-SSCP (24, 25). Our data suggest that there is a poor concordance between the mutation level and positive immunostaining.

In silver staining SSCP, the mutations of p53 gene were 10 cases (37%) among 27 breast cancer cases. The incidence of p53 mutation in our study is higher than others who reported 19% and 22% (16, 26). In the 10 cases, overexpressions by IHC were only 4 (40%) cases. Borresen-Dale (27) explained that the IHC miss nonsense and all types of frameshift mutations. In a study of p53 mutation in breast cancer in Korea, 41.7% (5/12) were reported as frameshift mutation (28), while our result was 40% (4/10) (data not shown). We suggest that this may be caused by differences of biologic behaviors of breast cancer. So, the application of IHC as a screening method of p53 mutation will be needed to assess by accepted criteria, and evaluate through large-scale prospective data in Korea.

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