Detection of p53 Gene Mutations in Aspiration Biopsy Specimens from Suspected Breast Cancers by Polymerase Chain Reaction-Single Strand Conformation Polymorphism Analysis

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Genomic DNA was extracted from aspiration biopsy specimens taken from 15 suspected cases of breast cancer, including 7 known cases of breast cancer, and the p53 gene was studied for evidence of mutation by using a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. In 5 of the 15 cases (33%), p53 gene mutation was identified and these tumors were subsequently histologically diagnosed as malignant. Further, DNA flow cytometry of the 15 tumors demonstrated that 6 (40%) were aneuploid and malignant, whereas 9 (60%) were diploid and benign. It was also found that the tumor cells in 5 aspirated cases that showed p53 gene mutations were all aneuploid, the p53 protein expression was positive, and the tumors were proved to be histologically malignant. It was thus concluded that the detection of p53 gene mutation by PCR-SSCP analysis of aspirated biopsy specimens from suspected breast cancers is a helpful method for achieving a more accurate diagnosis.

Key words: Breast cancer — Aspiration biopsy — p53 gene — PCR-SSCP

Although remarkable progress has been achieved in improving surgical and radiotherapeutic techniques and chemo-endocrine therapies for managing breast cancers, the breast cancer mortality rate still remains high. The best means of reducing this mortality rate is the early detection of a breast cancer, and fine-needle aspirated breast tissue biopsy is one method to achieve this purpose. However, false-negative or false-positive diagnoses invariably occur and this can detract from the usefulness of the technique. Therefore, to achieve greater diagnostic accuracy with aspirated breast tissue specimens suspected of being cancers, we have evaluated the use of p53 gene mutation to identify cancer cells. In this regard, it has recently been reported that the p53 gene is a tumor-suppressor[1,2] and that its mutations play an important role in the development of many common human malignancies; mutations of the p53 gene have been detected in many types of human cancers, such as breast cancers,[2-6] esophageal cancers,[7] gastric cancers,[8] colon cancers,[9,10] ovarian cancers,[11,12] thyroid cancers,[13] lung cancers,[3,14] hepatocellular carcinomas,[15,16] brain tumors,[3,17] and leukemia.[18,19] The p53 gene lies on the chromosomal locus 17p13 where, on restriction fragment length polymorphism analysis, one allele has been shown to be deleted.[20,21] Thus, p53 is considered to be a tumor-suppressor gene, which is usually inactivated by either of

two events: the mutation of one allele and the loss of the other allele. So far, however, little information is available on the molecular mechanisms underlying such a progression, including the activation of oncogenes and the inactivation of tumor-suppressor genes.

In this study, using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, we examined p53 gene mutations in DNA samples extracted from cells that were obtained from suspected breast cancer by fine-needle aspiration biopsy, in the expectation that this method would improve diagnostic accuracy and also clarify the extent of the p53 gene mutation involvement. Further, various clinicopathological parameters, including the estrogen receptor (ER), the clinical stage, the histologic grade, and the tumor DNA ploidy pattern, were examined in relation to p53 gene mutation, so as to understand the role of p53 mutation in the development of a breast cancer. An immunohistochemical study of p53 protein localization was also undertaken to elucidate the link between p53 mutation and the nuclear accumulation of its protein.

Fifteen aspirated cell samples were examined from breast tissue specimens taken from 15 patients who were all diagnosed as having cytologically suspected breast cancers, which were subsequently resected at the Sapporo Medical University Hospital or its affiliated hospitals between 1992–1994. Prior to this surgery, aspiration biopsies were performed using conventional procedures. In this series, the false-negative case was 1 of the 7 (14%)

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aspirated specimens from patients with cancer. No false-positive diagnosis was obtained. Therefore, the cytological diagnostic accuracy rate of these breast tumors was 93%. The biopsy specimens used for DNA extraction consisted of approximately 10^3 cells (stored at $-80°C$ till use). The DNA from each of these 15 specimens was prepared by proteinase K digestion and phenol-chloroform extraction, according to the modified method of Lyons et al.[22] PCR-SSCP analysis was then performed, with DNA from normal breast gland tissue showing no malignancy as a negative control. The sequences of the primers used for the PCR were modified, based on the sequences described by others.[6, 23] Exon 5, sense: 5'-TT-CCTCTTCCTGCAGTACTCC-3' and antisense: 5'-CA-GCTGCTCACCATCGCTATC-3'; exon 6, sense: 5'-T-TGCTCTTAGGTCTGGCCCCTCCTCAG-3' and antisense: 5'-CAGACCTCAGGCGGCTCATAGG-3'; exon 7, sense: 5'-GTGTTATCTCCTAGGTTGGC-3' and antisense: 5'-CAAGTGGCTCCTGACCTGGA-3'; exon 8, sense: 5'-AGTGGTAATCTACTGGGACGG-3' and antisense: 5'-ACCTCGCTTAGTGCTCCCTG-3'. Two hundred (200) ng of genomic DNA extracted from the aspirated cells was amplified in 25 μl volumes of a buffer recommended by Perkin-Elmer Cetus (Norwalk, CA) that contained 1 mM MgCl$_2$ and 1 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 10 Ci/ml Amersham Japan, Tokyo). Thirty-five cycles, each consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, were performed using a thermal programmer (Nippon Genetics Co., Tokyo). An aliquot of 2 μl of the PCR product was diluted 100-fold with a se-

quencing gel-loading buffer (98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue) and then applied (1 μl/lane) to a 6% neutral polyacrylamide gel. Electrophoresis was performed at 40 W for 3.5–5 h with fan cooling. The gel was dried and exposed to X-ray film at $-80°C$ for 6–12 h. For direct DNA sequencing, the shifted band obtained from each of 5 biopsy specimens in exon 5 or exon 7 was eluted from the polyacrylamide gel, and amplified by PCR using the same primers that were used for the PCR-SSCP analysis. PCR products were purified with Suprec-02 (Takara Shuzo, Kyoto), and sequenced by the dideoxy termination method with a 7-DEAZA sequencing kit, version 2.0 (Takara Shuzo) as has been described.[24] To examine the DNA ploidy pattern, nuclei were isolated from frozen tissue specimens of the resected breast tumors with 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO), after which they were treated with 0.1% RNase (Sigma), stained with 50 μg/ml propidium iodine (Sigma), filtered through nylon mesh, and immediately analyzed using a FACS-IV (Becton Dickinson, Mountain View, CA).

To detect the nuclear accumulation of p53 protein, the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY), which recognizes both wild and mutant forms of the p53 protein, was used as reported.[25] In brief, for immunoperoxidase staining, frozen 6-μm-thick sections were placed on poly-L-lysine-coated glass slides and fixed in chilled acetone. The slides were then air-dried for 1 h, and primary antibodies were applied according to the standard avidin-biotin system recommended by

Table I. Clinical and Molecular Biological Features of the 15 Patients with Breast Tumor

Patient No.	(Age)	Histological type (tumor size: cm)	Clinical stage[a]	Histologic grade[b]	p53 mutation	p53 nuclear staining	ER	DNA ploidy pattern
1	(39)	M (2.4×2.2)			−	−	−	D
2	(51)	F (2.2×1.8)			−	−	−	D
3	(57)	Pt (1.4×1.1)	I	1	−	−	−	A
4	(45)	M (2.3×1.0)			−	−	−	D
5	(72)	F (1.6×1.2)			−	−	−	D
6	(58)	Pt (2.4×1.5)	II	1	+	+	+	A
7	(45)	F (0.6×0.5)			−	−	−	D
8	(32)	M (2.9×1.1)			−	−	−	D
9	(46)	St (1.8×1.4)	I	1	+	+	−	A
10	(44)	F (1.0×1.0)			−	−	−	D
11	(44)	Sc (2.2×1.8)	II	1	+	+	+	A
12	(70)	Pt (2.8×2.7)	IIIa	3	+	+	+	A
13	(59)	Sc (3.0×2.5)	II	1	−	−	−	D
14	(47)	M (0.9×0.5)			−	−	−	D
15	(51)	Pt (0.9×0.8)	II	3	+	+	−	A

Abbreviations used: Pt, papillotubular ca; St, solid-tubular ca; Sc, scirrhous ca; M, mastopathy; F, fibroadenoma; D, diploid; A, aneuploid.
a) According to the TNM classification of the Japanese Breast Cancer Society.
b) Grading was performed according to the system based on a modified WHO classification (see reference 35).

the vendor (Nichirei, Tokyo). These sections were then stained with 3,3′-diaminobenzidine (Sigma), and the nuclei were counterstained with methyl green. Phosphate-buffered saline containing 1% bovine serum albumin was used as the negative control instead of a primary antiserum. The ER content of the tissue was determined by using dextran-coated charcoal assay (Biomedical Laboratories, Tokyo), and a concentration greater than 14 fmol/mg of the ER protein was considered to be positive.

Six (40%) of the 15 aspirated cell samples were found to be aneuploid; the remainder were diploid (Table I). Of the 6 aneuploid cancer cell populations, a mobility shift in SSCP analysis was detected in 5 cases; 3 in exon 5 and 2 in exon 7 (Fig. 1 and Table I). The position and type of the p53 gene mutation were identified by direct sequencing, and are listed in Table II. The position and incidence

of the mutations were distributed as follows: exon 5 (3 samples) and exon 7 (2 samples) and all were point mutations. Case 11 contained a deletion of a glutamine nucleotide at codon 176 (TGC to TC) in exon 5. The other 4 mutations were transitions such as G:C to A:C or A:T to G:C, and occurred in exon 5 and exon 7. Representative results are shown in Fig. 2; in case 9, a C-to-T transition was revealed at the first position of codon 248 and in case 12, a G-to-A transition was revealed at the second position of codon 175. Of the 5 point mutations, 2 (40%) were in a CpG dinucleotide sequence, which frequently occurs in breast cancers.[26]

Our findings, obtained by direct DNA sequencing of PCR products, are consistent with previous reports that
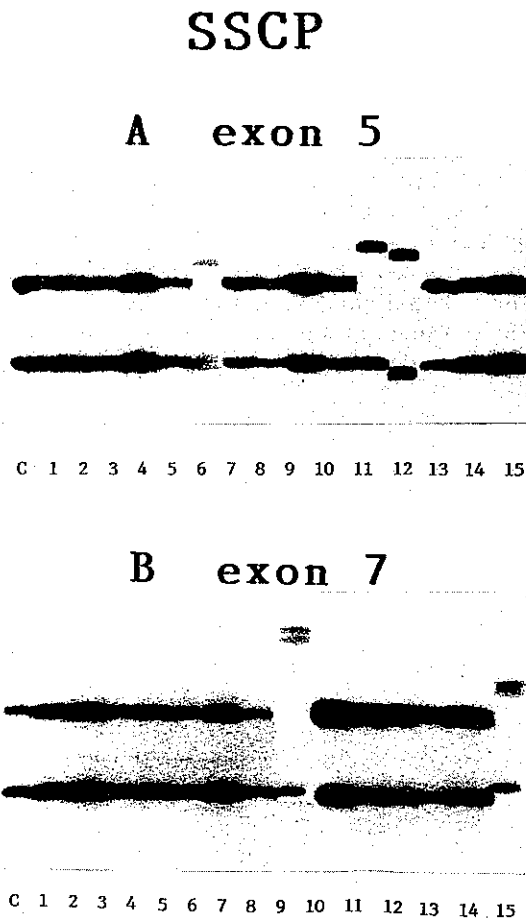

Fig. 1. Detection of p53 gene mutations in aspirated cells from breast tumors by PCR-SSCP analysis for: A, exon 5 and B, exon 7. The patient's number is shown at the bottom of each lane. C: Normal breast gland tissue (control) shows no mobility shifts.

Table II. List of the p53 Gene Mutations in Aspiration Biopsy Specimens from Breast Cancer

Patient No.	Exon	Codon	Base change (amino acid)
6	5	165	GAC (Asp)→AAC (Asn)
9	7[a]	248	CGG (Arg)→TGG (Trp)
11	5	176	TGC (Cys)→TC[b]
12	5[a]	175	CGC (Arg)→CAC (His)
15	7	234	TAC (Tyr)→TGC (Cys)

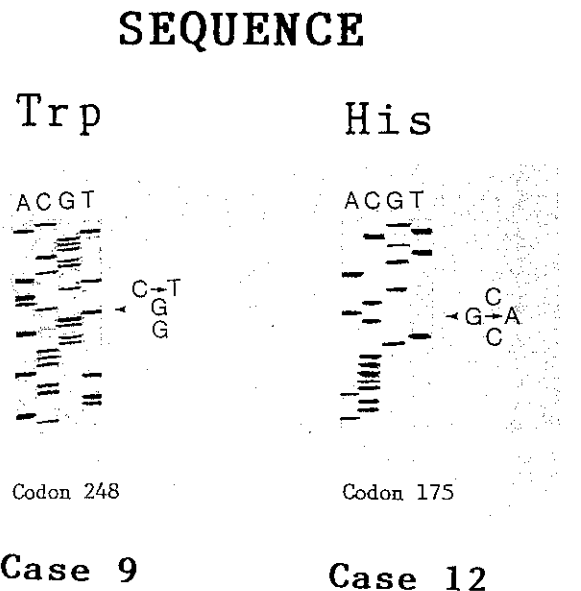a) Mutations at CpG sites.
b) 1 bp deletion of G.

Fig. 2. Identification of p53 mutations in aspiration biopsy specimens from breast cancers by direct sequencing. The sequences of the coding strands in patients 9 and 12 are shown as representative cases. Data are summarized in Table II.
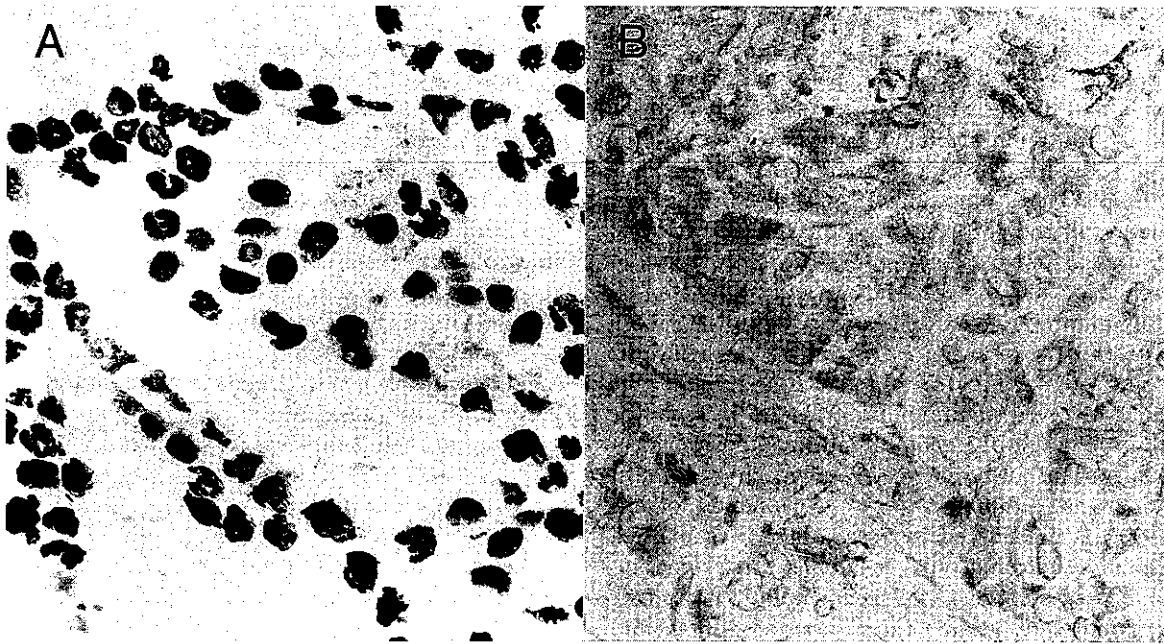
Fig. 3. Immunohistochemical staining of breast cancer with anti-p53 monoclonal antibody PAb 1801. A: Cancer cells were stained in their nuclei. B: The p53 expression in a negative control. No cells were stained.

most point mutations are distributed evenly from exon 4 to 8.[27] All resected tumors corresponding to the 5 aspirated cell samples with gene mutations showed an accumulation of p53 protein and were recognized as breast cancers; in the resected tumor specimens there was a strong association between the presence of p53 gene mutation and a nuclear accumulation of the p53 protein (Fig. 3). In contrast, the nuclear accumulation of the p53 protein in aspirated cell specimens was not detectable clearly enough to allow a diagnosis, because of the insufficient number of aspirated cells. Indeed, a positive staining for p53 protein in the aspirated cells did not necessarily reflect a mutation of the gene. In a case of repeated biopsy, an adequate amount of cells had been obtained to enable the detection of a positive nuclear accumulation of the p53 protein (data not shown), but the procedure caused psychosomatic damage to the patient.

As for ER levels, a positive result was seen in only 2 cancers that were over 2.0 cm in size but had a low histologic grade (Table I). According to Tsuda et al.,[27] there is a relationship between p53 mutation and a low ER value in invasive carcinomas with a high histologic grade. However, these aspirated specimens from suspected breast cancers did not appear to have a high histologic grade. Therefore, the detection of ER levels in aspirated specimens from suspected breast cancers seems to be of little value in achieving a definitive diagnosis.

PCR-SSCP analysis is considered a very sensitive method for detecting gene mutations such as in the tumor-suppressor gene p53. As many reports have concluded,[10, 12, 20, 21] owing to the frequent loss of heterozygosity of chromosome 17p, it is highly probable that the p53 gene is frequently inactivated by point mutation on one allele and by the loss of a gene on the other allele, and that its inactivation plays an important role in the development of human cancers, e.g., esophageal cancers[7, 28] and ovarian cancers.[12, 21, 29] Recently, mutation of the p53 gene has also been reported in cases of breast cancers (13–32%).[3–6, 27, 30, 31] Compared with the percentage of p53 gene mutation in breast cancer reported by other investigators, our result is rather high (71%). However, this high percentage might be a consequence of the examination of a small number of selected patients. Further studies are required. According to Yamashita et al.,[32] the correlation between the incidence of p53 gene mutation in breast cancers and clinicopathological findings of breast cancer cases suggests that p53 gene mutation is involved in the progression of cancer to a higher grade of malignancy. On the other hand, to clarify the clinical significance of a breast tumor, the immunohistochemical p53 protein expression is important,[33] but such expression is rarely found in tumors of less than 2 cm,[32] and this expression might depend on the short half-life of a wild-type p53 gene.

In the case of ovarian cancers, mutation of the p53 gene has been found on histological examination to be more frequent in moderately or poorly differentiated carcinomas and absent in borderline cases.[34] However, in this study, mutation in the p53 gene was seen in some borderline cases on cytological examination and all the cases with p53 gene mutations also showed a nuclear accumulation of the p53 protein in the resected tumors. Further, a clear correlation was demonstrated between the presence of p53 gene mutation and an aneuploid pattern of tumor DNA, which has been shown to be a marker of the tumor's aggressive biological behavior, thereby also supporting the hypothesis that p53 gene mutation is involved in tumor progression. In fact, the relationship between p53 gene mutation and tumor progression has been bolstered by several recent studies of various malignant tumors.[13 17, 18, 34] Aneuploidy of the tumor DNA is considered to occur through a marked enhancement of chromosomal instability, and some authors have suggested that p53 gene mutation is either directly or indirectly related to chromosomal instability in the tumor cells. Based on studies of p53 gene mutation and the loss of heterozygosity of chromosome 17p in colorectal carcinomas, Vogelstein et al.[9, 10] maintain that the rate-limiting step in p53 inactivation is point mutation. Also, as Kihana et al.[34] have demonstrated in their study of ovarian carcinomas, positive nuclear staining of p53 protein is an indication that p53 gene mutation has

occurred during the process of malignant transformation. Taking these findings into account, we consider that the detection of point mutations in p53 genes of aspirated tumor cells could be of clinical utility in achieving a diagnosis, even though mutation in other exons was not analyzed and certain types of mutation are not detectable by PCR-SSCP analysis.

To conclude, the present findings strongly suggest that in cytological examinations of a suspected breast cancer, the detection of mutation in the p53 gene can be helpful in achieving a diagnosis. However, further investigation of breast cancer cases is required to elucidate the involvement of multiple genes, oncogenes, and tumor-suppressor genes in the early and late stages of carcinogenesis and to understand their biological and clinical significance.

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