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Detection of p53 Gene Mutations and Their Protein Overexpression in Fine-needle Biopsy Specimens with False-negative Diagnoses in Breast Cancer

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ABSTRACT

To achieve a more accurate diagnosis in the first aspiration biopsy from breast tumor, p53 gene mutations were detected by PCR-SSCP analysis in aspiration biopsy specimens taken from 26 patients with breast tumors. Of 26 aspirated cell specimens from breast tumors that were all initially diagnosed as being cytologically benign, 2 point mutations of the p53 gene were detected and were subsequently proved to be cancer cells. Further, the p53 protein expression was also examined in the initial aspirated specimens and in the resected tumors that were rediagnosed as being malignant as a result of the second biopsy. Consequently, these p53 gene mutations did not appear to correlate with their protein overexpression in the aspiration biopsy specimens (all cases were negative), however, the specimens from 2 resected tumors that showed p53 gene mutations were positive. In addition, a positive ER level and DNA aneuploidy status were also found only in these two p53 gene mutation cases. Therefore, detection of p53 mutations in aspiration biopsy specimens may prove to be a useful method for detecting breast cancers.

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

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2. The abbreviations used are: PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; ER, estrogen receptor; HSP, heat shock protein.

INTRODUCTION

Recent progress in molecular biology has led to the identification of various oncogenes and tumor-suppressor genes, one such well-known tumor-suppressor gene being p53, which is usually inactivated by two events: the mutation of one allele and the loss of another allele (1-3). Further, it can be speculated that tumor progression is associated with further modification of tumor cell DNA, which, in turn, gives rise to clones with new genetic alterations. This process eventually generates a clone with a metastatic potential.

Most solid tumors are detected only after reaching a size large enough to cause symptoms, and by this time they thus are likely to have accumulated various genetic alterations. Recent studies of colon adenocarcinomas have clarified the interaction between morphologic and genetic alterations(4). In this regard, there is some evidence relatings p53 gene alteration to tumor progression. Fearon and Vogelstein(5) have found that mutational inactivation of the p53 gene is a critical event in the later stages of colorectal tumorigenesis. In case of brain tumors, according to Sidrausky *et al.*(6), the progression of tumors is associated with clonal expansion of cells containing the mutated p53 gene. In addition, Nakamura *et al.*(7) have demonstrated that p53 gene mutation plays a critical role in the progression of a differentiated thyroid carcinoma into an anaplastic carcinoma. Thus, these studies have confirmed a relationship between the p53 gene mutation and tumor progression.

In contrast, however, corresponding alterations that occur in cases of breast cancer still remain obscure, and how genetic alterations of the p53 gene are generated and their accumulatory effect on the development and/or progression of breast cancer have yet to be clarified. Therefore, in this study, in the hope of shedding further light on genetic alterations that occur in breast cancers, a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP[2]) analysis was undertaken for detecting of p53 gene mutations in aspirated biopsy specimens taken from 2 breast tumors that had been diagnosed as cytologically being benign but histologically considered to be in an early cancerous stage. To clarify the processes of genetic mutation in cases of breast cancer may lessen the number of false-negative cytological diagnoses in the first aspiration biopsy, resulting in a more accurate prognosis of such cancers.

MATERIALS AND METHODS

1. *Samples and preparation of DNA*

Studied were 26 aspirated biopsy specimens consisting of approximately 10^3

cells each, and 12 tumor tissues that were obtained between 1992 and 1994 from 26 breast tumor patients at the Sapporo Medical University Hospital and its affiliated hospitals. The 26 aspirated biopsy specimens and the 12 resected tumor tissue specimens were immediately immersed in liquid nitrogen and stored at −80℃ untill use. The DNAs from these specimens were separately prepared by proteinase K digestion and phenol-chloroform extraction, per a modification of a method used by Lyons *et al.*(8).

2. *PCR-SSCP analysis*

PCR-SSCP analysis was performed on the 26 aspirated biopsy breast tumor specimens diagnosed as being cytologically benign. The sequences of the primers used for the PCR were modified, based on the same sequences that other researcher have previously described(9,10), i. e., Exon 5, sense : 5'-TTCCTCTTCCTGCAGTACTCC-3' and antisense : 5'-CAGCTGCTCACCATCG-CTATC-3' ; Exon 6, sense : 5'-TTGCTCTTAGGTCTGGCCCCTCCTCAG-3' and antisense : 5'-CAGACCTCAGGCGGCTCATAGG-3' ; Exon 7, sense : 5'-GTGTTATCTCCTAGGTTGGC-3' and antisense : 5'-CAAGTGGCTCCTGAC-CTGGA-3' ; and Exon 8, sense : 5'-AGTGGTAATCTACTGGGACGG-3' and antisense : 5'-ACCTCGCTTAGTGCTCCCTG-3'.

A 200 ng DNA sample from each of the aspirated cells was amplified in 25 μl volumes of a buffer recommended by Perkin-Elmer Cetus (Norwalk, CT) that contained 1 m*M* $MgCl_2$, 1 unit of *Taq* DNA polymerase, and 1 μl of [α-^{32}P] dCTP (3000 Ci/mmol, 10 Ci/m*l* Amersham Japan, Tokyo). Next, using a thermal programmer (Nippon Genetic Co., Tokyo), 35 cycles, each consisting of denaturation at 94℃ for 30 s, was performed, followed by annealing at 55℃ for 30 s, and extension at 72℃ for 1 min. Then, 2 μl of each PCR product was diluted 100-fold with a sequencing gel-loading buffer (98% deionized formamide, 10 m*M* EDTA pH 8.0, 0.0025 % xylen cyanol, and 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 at 4℃. After drying, the gel was exposed to a X-ray film at - 80℃ for 6-12 h with an intensifying screen.

3. *Direct DNA sequencing of PCR products*

For direct DNA sequencing, the shifted bands, exon 5 and exon 7, each obtained from 2 biopsy specimens, were eluted from the polyacrylamide gel and amplified by a PCR using the same primers as were used for the PCR products purified with SUPREC™-02 (Takara Shuzo, Kyoto). Sequencing was performed by the dideoxy termination method, using a 7-DEAZA Sequencing Kit, Version 2.0 (Takara Shuzo), as has been previously described(11).

4. *Immunohistochemical analysis*

An antibody against p53 protein, PAb 1801, was used for an immunohistochemical analysis. PAb 1801 (Oncogene Science, Inc., Manhasset, NY) is a murine monoclonal antibody against human p53 that recognizes both the wild-type and mutant forms of the p53 protein. To detect the nuclear accumulation of the p53 protein. PAb 1801 was used in a method that has been previously reported(12). In brief, for immunoperoxidase staining, frozen 6 μm-thick sections from each of the resected tumors were placed onto poly-L-Lysine-coated glass slides and fixed in chilled acetone. The slides were then air-dried for 1 h, after which primary antibodies were applied according to the standard avidin-biotin system as recommended by the vendor (Nichirei, Tokyo). The sections were then stained with 3,3'-diaminobenzidine tetrachloride (Sigma Chemical, St. Louis, MO), and the nuclei were counterstained with methyl green. Phosphate-buffer saline containing 1 % bovine serum albumin was used as the negative control instead of the primary antiserum.

5. *Estrogen receptor (ER) levels*

ER levels in the cells were determined by using a dextran-coated charcoal separation method (Biomedical Laboratories, Tokyo). A concentration greater than 14 fmol/mg of ER protein was considered to be positive.

6. *DNA ploidy pattern*

To examine the DNA ploidy pattern, nuclei were isolated from frozen tissue specimens of resected breast tumors with 0.1 % Triton X-100 (Sigma). They were treated with 0.1 % RNase (Sigma), stained with 50 μg/m*l* propidium iodine(Sigma), filtered through nylon mesh, and analyzed immediately by using a FACS-IV (Becton Dickinson, Mountain View, CA).

RESULTS AND DISCUSSION

Aberrant bands were seen in 2 of the 26 first aspirated biopsy specimens which were both diagnosed as being benign cytologically, in exon 5 from patient 9, and in exon 7 from patient 16, respectively (Table 1, Fig. 1). Subsequently, the character of the p53 gene mutations detected by SSCP analysis were further examined by direct sequencing of the PCR-amplified exons. Both of the p53 mutations detected were consisted of single nucleotide changes, which were point mutations at the second position of codon 176 (TGC to TTC) and codon 251 (ATC to AGC)(Fig. 2). These 2 cases underwent a second aspiration biopsy

Table 1 *The clinical and molecular biological features of 12 patients who underwent breast tumor resections*

Patient No.	Age	Histological type (Tumor size : cm)	Clinical[a] stage	Histologic[b] grade	p53 mutation	p53 nuclear stainig	ER	DNA ploidy pattern
1	44	F(1.8 × 1.4)			−	−	−	D
2	55	M(2.3 × 2.0)			−	−	−	D
3	41	F(2.1 × 1.6)			−	−	−	D
4	37	F(3.1 × 2.8)			−	−	−	D
6	51	M(2.0 × 1.7)			−	−	−	D
9	67	St(1.3 × 1.1)	I	1	+	+	+	A
11	47	F(2.3 × 2.1)			−	−	−	D
12	38	M(1.8 × 1.5)			−	−	−	D
13	38	M(2.3 × 2.0)			−	−	−	D
16	58	Pt(1.4 × 1.1)	I	3	+	+	+	A
21	35	F(2.8 × 2.6)			−	−	−	D
25	58	M(1.8 × 1.3)			−	−	−	D

Abbreviations used: Pt, Papillotubular ca ; St, Solid-tubular ca ; M, Mastopathy ; F, Fibroadenoma ; D, Diploid ; A, Aneuploid.

a) According to the TNM classification of the Japanese Breast Cancer Society (20).

b) Grading was performed accoding to the system based on a modified WHO classification (21).

within 6 months, which resulted in the specimens from both cases being diagnosed as being cytologically malignant. Further, these 2 breast cancers immunohistochemically showed a positive nuclear p53 protein accumulation in the resected tumor specimens (Fig. 3) but not in the aspirated specimens. The reason why the specimens of the first aspiration biopsy reacted negatively to a nuclear p53 protein accumulation is not known. However, one possible explanation may be that the number of aspirated cell specimens was few and insufficient for an accurate diagnosis. It should be noted in this regard that the aspirated cells in which mutation of the genes was evident did not necessarily stain positively to the presence of p53 protein (data not shown).

These 2 breast cancers did not present any special histological character but did have significant ER levels and DNA ploidy patterns indicative of an advanced breast cancer (Table 1) ; these 2 breast cancers were histologically diagnosed as being invasive ductal carcinomas that had reacted positively for ER and had an aneuploid DNA pattern. In contrast, the histological diagnosis of the tissue specimens from the 10 other resected tumors of the remaining 24 patients indicated no malignancy and were negative for ER and showed no diploid DNA pattern.

As has been reported in our previous study(12), nuclear p53 immunoreaction is more frequently seen in aggressive breast cancers, e. g., those with an overex-

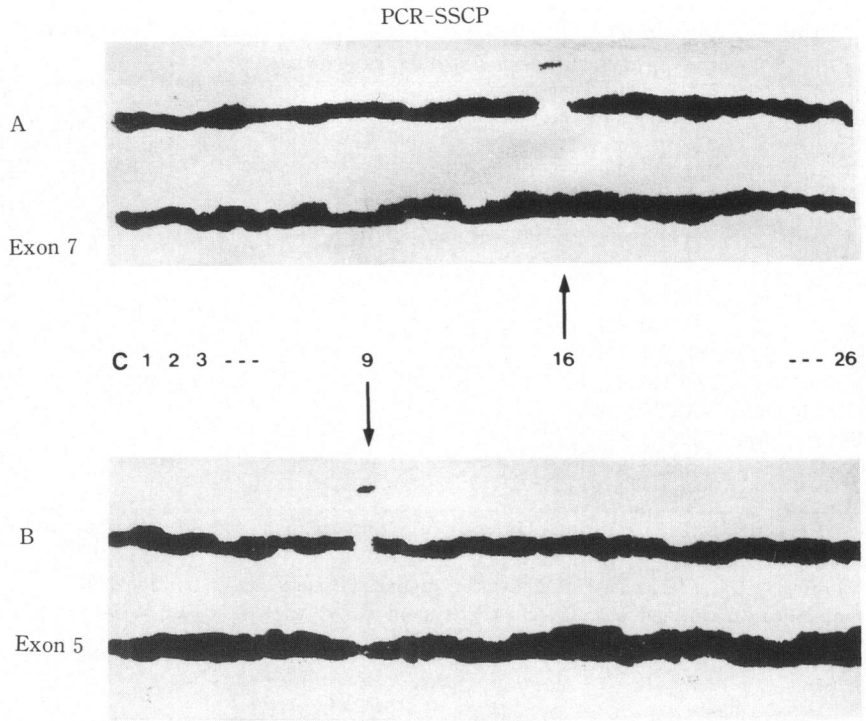

Fig. 1 Detection by a PCR-SSCP analysis of p53 gene mutations in aspirated cells from breast tumors for exon 7 (A) and exon 5 (B). The patient's number is shown at the space between A and B. C: Normal breast gland tissue of a control shows no mobility shifts.

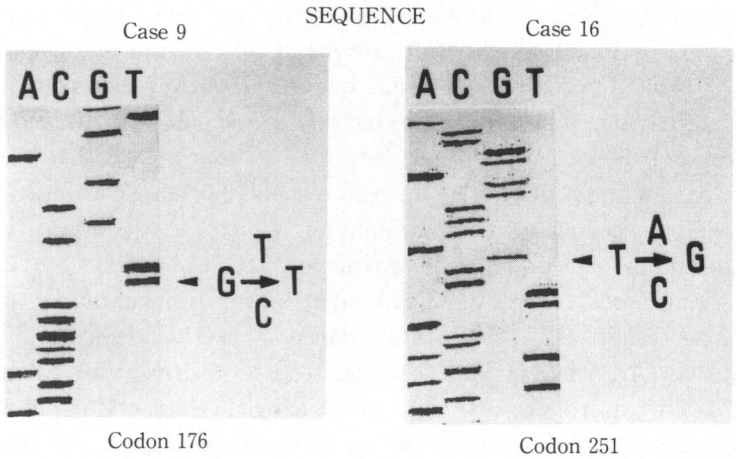

Fig. 2 Identification of p53 mutations in aspiration biopsy specimens from breast cancers by direct DNA sequencing. Shown are the sequences of the coding strands for exon 5 (patient 9) and exon 7 (patient 16).

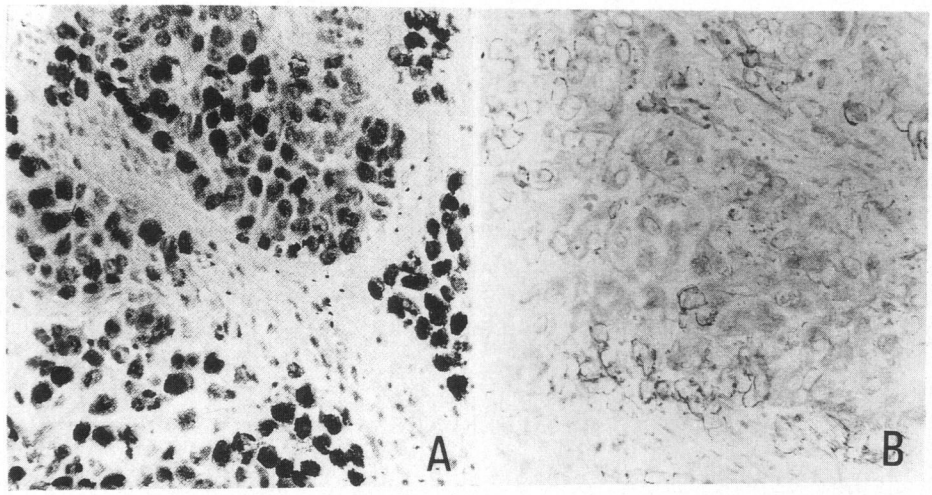

Fig. 3 Immunohistochemical staining of breast cancer cells in patient 9 by the anti-p53 monoclonal antibody PAb 1801. Positive staining is limited to the nuclei of cancer cells (A). No nuclear staining is detectable in a negative case (B).

pression of c-*erb*B-2 oncoprotein, heat shock protein (HSP) 70, but with a negative ER status. Generally, a nuclear p53 immunoreaction is considered to reflect a nuclear accumulation of mutant p53 protein, which is coded by the mutated form of the p53 gene and has a prolonged half-life. As for HSP 70, in experiments using a human cancer cell line(14,15), mutant p53 protein was found to form a complex with HSP 70; certain forms of HSP 70 are associated with mutant p53 (14,16,17). Thus, it is supposed that the expression of p53 as well as HSP 70 is thought to play a role in the progression of breast cancer and this is consistent with our previous report in 1994(12). In the present study, however, a HSP 70 expression was not seen in the 2 aspirated specimens taken from the 2 breast tumors diagnosed as being malignant (data not shown). On hte other hand, a negative ER level has also been found to be usually associated with aggressive breast cancers and the relationship between p53 mutation and low ER value has been seen in invasive carcinomas with a high histologic grade(18). In our previous data, however, biopsies of aspirated specimens from suspected breast cancers did not shown a positive ER response, so that the ER level in aspirated specimens had little importance in achieving the diagnosis(19).

To conclude, this is the first report regarding the detection of p53 gene mutations in aspiration biopsy specimens from breast tumors, and it is speculated that the detection of such p53 gene mutation in the initial aspirated specimens may be

of importance in avoiding false-negative diagnosis and achieving a clinical acurate breast cancer diagnosis. However, further studies are required and now are in progress to ascertain the consistency of these initial findings.

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