Characterization of p53 gene mutations in fine-needle aspirated breast cancer : correlation with clinicopathological features and short-term relapse

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ABSTRACT

Mutations of the p53 gene play a key role in the development of common human malignancies. In the case of breast cancer patients, cancer cells can be obtained directly from the patient with minimal damage by fine-needle sampling. Thus, the method of aspiration biopsy cytology (ABC) by fine-needle aspiration biopsy (FNAB) was developed, enabling us to prepare cancer cell nuclei for detection of p53 gene mutation by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. Fine-needle samplings were successfully performed and p53 gene mutations were disclosed in 25 advanced breast cancer patients. We investigated 27 mutations of the p53 gene in these 25 specimens, showing all point mutations including 10 CpG mutations and 3 double mutations in one allele. Of the 25 patients with p53 gene mutations, 16 patients were disease free (64.0%) but 9 had postoperative recurrence (36.0 %) ; in detail, 7 patients (28.0%) experienced short-term relapse of disease, i. e. recurrence within 5 years after operation. In the study using FNAB, we further examined the correlation of p53 gene mutation in breast cancer with clinicopathological features, and especially with short-term relapse of disease. No significant correlation was found with respect to age, menopausal status, histological type, ER status, operative procedures, and postoperative adjuvant therapy in almost all tumors, however there was a significant relationship between p53 gene mutation and short-term relapse ($P<0.01$). Moreover, p53 gene mutation related closely with nuclear p53 protein accumulation ($P<0.01$) and with DNA aneuploidy pattern as well ($P<0.001$). Twelve p53 gene mutations were shown in 9 aspirated biopsy specimens. All were point mutations containing 8 transitions, 2 transversions, and 2 deletions ; the dominant mutations were

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2. The abbreviations used are : PCR-SSCP, Polymerase chain reaction-single strand conformation polymorphism ; FNAB, fine-needle aspiration biopsy ; ABC, aspiration biopsy cytology ; ER, estrogen receptor ; OS, overall survival.

transition at GC base pairs (G to A), constituting 41.7% of all mutations. Typical hot spot codons in the study were codon 175, 248, and 282.

Mutations localizing at the CpG site of the gene were seen in 7 cases (58.3%). Additionally, there was a tendency of high recurrence of disease in advanced clinical stage IIIA and IIIB, lymph node metastatic estimation of N2/N3, and histologic grade 3. These results thus indicate that p53 gene mutations in aspirated breast cancer cells obtained by FNAB reflect the biological characteristics of breast cancer and may be a good indicator of short-term relapse of postoperative patients.

Key words : Breast cancer, p53 mutation, Aspiration biopsy, Short-term relapse

INTRODUCTION

Recently, remarkable advances in molecular genetics have revealed a consistent set of genetic alterations which may be related to complicated multistep carcinogenesis. PCR-SSCP with direct sequencing is recognized as a rapid and highly sensitive method for detecting genetic aberrations of p53 gene. It has been shown that the p53 gene is a tumor suppressor and that its mutations play an important role in the development of many common human malignancies [1-2]. Therefore, frequent p53 gene mutations have been reported in diverse human tumors including those of gastrointestinal tract [3], blood [4], brain [5], thyloid gland [6], and breast [7]. As is well known, breast cancer is the most common malignancy among females world-wide. There have been many reports of genetic abnormalities in breast cancer cells, such as genetic alterations in p53, BRCA1-3, c-mic, and c-*erb* B-2 (HER 2/neu). In human breast cancer to date, p53 gene mutations in particular have been widely studied in order to reveal its function and effect against tumorigenesis. However, few reports have mentioned the clinicopathological significance of mutations of this gene from the perspecting of breast cancer prognosis [8,9]. As for the nuclear accumulation of p53 protein in tumor cells, several studies have demonstrated an association between breast cancer prognosis and p53 protein expression [10,11], and shown that postoperative prognosis was poorer in breast cancer patients with increased p53 protein accumulation [12,13], but other studies have found no difference or even improved survival [14,15]. On the other hand, studies of the association between p53 gene mutations and breast cancer prognosis have yielded more consistent results ; most investigators have reported poor overall and disease-free survival in breast cancer patients with p53 gene mutations [16,17]. In general, a nuclear p53 protein accumulation is considered to reflect a nuclear accumulation of mutant p53 protein, which is coded by the mutated form of the p53 gene that has a prolonged half-life [11-15]. Therefore, it is concluded that there is a highly significant association between the presence of p53 gene mutation and nuclear accumulation of p53 protein, resulting in the poor prognosis of breast cancer patients [16-18]. Regarding the DNA ploidy in breast cancers, we previously reported that an aneuploidy pattern was closely correlated with p53 gene mutations, suggesting that these genetic factors were correlated strongly with tumor progression [19, 20]. This might be supported by the hypothesis that the cancer cells undergo the expected cell cycle change upon induction of p53 gene expression by DNA damage in cells, reflecting tumor DNA ploidy and cell proliferation kinetics.

In 1994, using PCR-SSCP with direct sequencing method on breast cancer cells obtained by FNAB, we demonstrated that the detection of p53 gene mutation in aspirated cancer cells contributed to ascertaining an accurate diagnosis preoperatively and reported that the existence of p53 gene mutations might help identify a subset of very high risk breast cancer patients with worse prognosis. Thus, p53 gene mutation is considered to be an accurate indicator of postoperative survival. Using FNAB appears to be safer and more convenient than core nee-

dle biopsy or open surgical biopsy, and the detection of p53 gene mutation in minimal specimen obtained from breast cancer mass May be a useful predictive marker of patient's postoperative course.

The aim of this study was to clarify the clinical usefulness of detection of p53 gene mutation in breast cancer cells by preoperative FNAB and to examine the relationship between p53 gene mutation and various histocytological features and short-term relapse of the disease.

MATERIAL AND METHOD

1. Patients and samples

Tumor specimens were obtained from 24 patients with breast cancer who had undergone curative operations at Sapporo Medical University Hospital and its affiliated hospitals and clinics between 1992 and 1994. All patients were preoperatively diagnosed as having malignancy by fine-needle ABC and the p53 gene mutations of tumor cells were detected by PCR-SSCP. Thereafter, the characteristics of the mutations were followed by DNA direct sequencing. The histopathological findings from resected tumors were determined in the course of the routine pathological assessment. Aspirated samples obtained from preoperative cancer tumors and resected tumors were immediately frozen and stored in liquid nitrogen or refrigerator until analysis. The minimal period of follow-up was 10 years.

2. DNA extraction

Twenty-four aspirated biopsy samples consisting of approximately $1-5\times10^3$ cells in number were bathed in a RPMI-1640 medium and then immersed into liquid nitrogen and stored at −80℃ until assayed. The genomic DNA obtained from each of the 24 patients was prepared by proteinase K digestion and phenol/chloroform extraction, according to a modification of the method of Lyons *et al*. [21].

3. PCR-SSCP analysis

In this study, as previously [18-20,22], PCR-SSCP analysis of the 24 aspiration biopsied breast cancer specimens was performed and mutations in exons 5-8 of the p53 gene were examined in each cancer cell. The sequences of the primers used for this PCR were modified, based on the same sequences described in other studies[8,23] :exon 5, sense 5-TTCCTCTTCCTTGC ACTACTCC-3 and antisense 5-CAGCTGCTC ACCATCGCTAT-3 ; exon 6, sense 5-TTGCTC TTAGGTCTGGCCCCTCCTCAG-3 and antisense 5-CAGACCTCAGGCGGCTCATAGG-3 ; exon 7, sense 5-GTGTTATCTCCTSGGTTGGC-3 and antisense 5-CAAGTGGCTCCTGACCTGGA-3 ; and exon 8, sense 5-AGTGGTAATCTACTG GGACGG-3 and antisense 5-ACCTCGCTTAG TGCTCCCTG-3. A 200 ng genomic DNA sampling from each of the aspirated cells was amplified in 25μl volumes of the buffer, recommended by Perkin-Elmer Cetus (Norwalk, CT), which contained 1 mM $MgCl_2$, 1 unit of Taq DNA polymerase, and 1μl of [α-^{32}P] d CTP (3000 Ci/nmol, 10 Ci/ml ; Amersham Japan, Tokyo). Then, using a thermal programmer (Nippon Genetic Co., Tokyo), 35 cyclings of each sampling were perfomed, each consisting of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 1 min; after which a 2μl volume of each PCR product was diluted 100-fold with a sequencing gel-loading buffer (98% deionized formamide, 10 mM EDTA pH 8.0, 0.0025% xylen cyanol, 0.025% bromophenol blue) which was then applied (1μl/lane) to a 6% neutral polyacrylamide gel. Electrophoresis was performed at 40 W for 3.5-5h with fan cooling. Subsequently, the gel was fixed in acetic acid (10%), dried and using intensifying screens, was exposed to X-ray film for 6-12 h at −80℃.

4. Direct DNA sequencing of PCR products

For the examination of DNA sequences, the shifted bands in exon 5 to exon 8, each obtained from biopsy speciemens, were eluted from the polyacrylamide gel and amplified by PCR using the same primers as used in prior PCR-SSCP analysis. The PCR products were purified with SUPREC^{TM}-02 (Takara Shuzo, Kyoto). Sequenc-

ing was performed by the dideoxy termination method, using a 7-DEAZA Sequencing KIT, Version 2.0 (Takara Syuzo), as has been described before [3-6].

5. Immunohistochemical analysis

In p53 staining, antibody PAb 1801 (Oncogene Science, Inc., Manhasset, NY) against p53 protein was used. This antibody recognizes both wild-type and mutant forms of the p53 protein. To detect the p53 protein nuclear accumulation, PAb 1801 was used as previously reported [24] ; for immunoperoxidase staining, 6μm-thick frozen sections from each of the resected tumors were placed onto poly-L-lysine-coated glass slides and fixed in chilled acetone. The slides were air-dried for 1 h, after which the primary antibodies were applied in accordance with the standard avidin-biotin system recommended by the vendor (Nichirei, Tokyo). Sections were stained with 3,3-diaminobenzidine (Sigma Chemical, St. Lowis, MO) and the nuclei counterstained with methyl green. Phosphate-buffered saline, containing 1% bovine serum albumin, was used as the negative control instead of the primary antiserum.

6. DNA ploidy pattern

To examine the DNA ploidy pattern, nuclei from the surgically resected breast cancers were isolated from each of the frozen tissue specimens with 0.1% Triton X-100 (Sigma). The isolated nuclei were treated with 0.1% RNase (Sigma), stained with 50μg/ml propidium iodine (Sigma), filtered through nylon mesh, and each sample was immediately analyzed by use of a FACS-IV (Becton Dickinson, Mountain View, CA).

Table 1 Histological and molecular biological characteristic of 9 breast cancer patients with recurrence

Case	Age	Menopausal status	Tumor size (cm)	Histology a)	Lymph node	Clinical stage b)	Histologic grade c)
1	43	pre	1.8 x 1.5	Pt	N3	IIIA	3
2	30	pre	8.0 x 8.0	Pt	N2	IIIB	3
3	51	post	2.8 x 2.7	Pt	N1	IIB	2
4	68	post	5.5 x 4.5	Pt	N2	IIIA	3
5	48	pre	2.5 x 2.5	Pt	N1	IIB	2
6	52	post	2.0 x 1.7	St	N2	IIIA	3
7	61	post	2.8 x 2.0	Sc	N2	IIIA	3
8	63	post	3.5 x 2.8	Pt	N2	IIIA	3
9	47	pre	2.4 x 1.8	St	N2	IIIA	3

Case	ER receptor	p53 staining	p53 mutation d)	DNA ploidy pattern	Disease free interval
1	−	+	+	A	4Y10M
2	+	+	+	A	2Y 6 M
3	−	−	+	A	3Y 1 M
4	−	+	+	A	3Y 6 M
5	−	−	+	A	4Y 1 M
6	−	+	++	A	6Y 1 M
7	+	+	++	A	2Y 8 M
8	+	+	++	A	6Y 4 M
9	−	−	+	A	4Y 1 M

a) Abbreviations used : Pt , Papillotubular ca ; St , Solid - tubular ca ; Sc , Scirrhous ca; A, aneuploid.
b) According to the TNM classification of the Japanese Breast Cancer Society [45].
c) Grading was performed according to the system based on a modified WHO classification [46].
d) +, single mutation ; ++, two mutations in one allele.

7. Estrogen receptor (ER) levels

The ER content of the cancer tissue was determined by dextran-coated charcoal assay (Biomedical Laboratories, Tokyo), and a concentration greater than 5 fmol/mg of the ER protein was considered to be positive.

8. Statistical analysis

Chi-square tests and Fisher's exact tests were used to estimate the associations of p53 gene mutation, short-term relapse, clinicopathological variables, DNA aneuploidy patterns, and ER value. A significant difference was determined when the P value was < 0.05.

RESULTS AND DISCUSSION

Of the 25 patients diagnosed as having malignancy by FNAB, 16 patients were disease free (16 of 25 ; 64.0%) and 9 had recurrences (9 of 25 ; 36.0%) after operation. Of particular note, 7 patients (7 of 25 ; 28.0%) recurrence within 5 years, which was regarded as a short-term relapse of disease (Table I). Twelve p53 gene mutations were found in 9 patients including 7 short-term relapse patients, and a multiplicity of p53 gene mutations were seen in 3 breast cancers. The 9 recurrence patients were ranged from 30 to 68 years old (average 51.6 years old) and 4 of them were premenopausal (44.4%) and 5 were post menopausal (55.6%). In terms of the clinical stage, 7 of the 9 (77.8%) patients were over stage III, and 2 of the 9 (22.2%) were stage IIB, regarding advanced breast cancers as almost histologic grade 3 (7 of 9 ; 77.8%) and N2/N3 lymph node positive status (7 of 9 ; 77.8%). In addition all of the 9 recurrent disease patients had DNA aneuploidy patterns.

As we demonstrated previously [18-20,22], tumor malignancy had a significant correlation with DNA aneuploidy pattern ; each tumor with an aneuploidy pattern proved histopathologically to be malignant. Therefore, as Czuba *et al*. mentioned [25], DNA aneuploid breast cancers were characterized by increased aggressiveness which manifests itself through rapid local progression and metastatic spread. In the present study, supported by our previous findings, a clear correlation was also shown between the presence of p53 gene mutations and DNA aneuploidy patterns, which was consistent with other data in different malignancies [25], suggesting that DNA aneuploidy patterns are a potent marker of a tumor with aggressive biological behavior. Thus, in this study, aneuploid DNA patterns proved to be correlated positively with advanced clinical stage and histologic grade. In terms of DNA ploidy status, not diploidy but aneuploidy patterns of the tumor DNA are considered to occur through a marked enhancement of chromosomal instability, and some researchers have suggested that p53 gene mutation is either directly or indirectly related to chromosomal instability in the tumor cells [1,2,25].

With regard to the hormonal parameters, negative ER receptor patients in this study were dominant (6 of 9 ; 66.7%) and showed a slight tendency to have a worse DFS. According to the report by Kallioniemi *et al*. [26], the content of progesterone receptors which related to the ER was an accurate prognostic indicator, and Saimura *et al*. [27] reported that patients with positive hormone receptor status, estrogen and / or progesterone receptor positive, tended to have better OS rates than those negative for both hormone receptors. In fact, there is a great deal of data concerning the relationship between ER receptor and clinicopathological valiables [28,29], and above all else, a highly significant association between DNA aneuploidy pattern and abscence of ER receptor was revealed to be a useful predictor of the response to endocrine cancer treatment [26].

The correlation of the presence of p53 gene mutations with various clinicopathological findings is shown in Table 2. There was a higher incidence of advanced lymph node metastases (N2/N3 ; 77.8%) and histologic grade (3 ; 77.8%), resulting in a significant correlation with p53 gene mutations ($P<0.01$ and $P<0.01$, respectively). Additionally, a close relationship was also shown between N2/N3 metastases and histological grade 3 ; though 2 N1 patients were

Table 2 Correlation of p53 gene mutation with histological findings, DNA ploidy pattern, and short-term relapse of the disease

Parameters	p53 gene mutation	
	n = 9	(%)
Lymph node metastasis		
N 1	2	(22.2)
N 2 / N 3	7	(77.8)
Clinical stage		
IIB	2	(22.2)
IIIA / IIIB	7	(77.8)
Histological type		
Papillotubular	6	(66.7)
Solid-tubular	2	(22.2)
Scirrhous	1	(11.1)
Histologic grade		
1	0	(0)
2	2	(22.2)
3	7	(77.8)
p53 staining		
Positive	6	(66.7)
Negative	3	(33.3)
DNA ploidy pattern		
Diploid	0	(0)
Aneuploid	9	(100)
ER receptor		
Positive	4	(44.4)
Negative	5	(55.6)
Short-term relapse a)		
Relapsed	7	(77.8)
Not relapsed	2	(22.2)

a) Relapse within the 5 year postoperative period.

grade 2, but 7 N2/N3 patients were grade 3. DNA flow cytometry of the recurrent 9 breast cancers demonstrated that tumor cells of these 9 patients with p53 gene mutations were all aneuploid. In contrast, diploid tumor with p53 gene mutations was not seen. The DNA ploidy pattern in malignant tumor in our data was consistent with other researchers' findings [30,31], and DNA aneuploidy patterns were closely correlated with tumor progression and poor prognosis.

Indeed, although there was only a small number of patients in this study, the data indicated that the p53 gene mutation in aspirated biopsy cells correlated significantly with their DNA aneuploid ($P<0.001$), demonstrating poor prognosis. Furthermore, the tendency of high recurrence in advanced tumor status such as clinical stage IIIA and IIIB was also significantly associated with p53 gene mutation ($P<0.01$). However, the relationship between histological type of tumor and short-term relapse of disease was not significant even if p53 gene mutation was present. Moreover, no significant correlation was found with such factors as age, menopausal status, ER status, operative procedures, and postoperative adjuvant therapy. In this study, the close correlation of p53 gene mu-

tations with short-term relapse of disease was due to clinicopathological characteristics and / or genetically aggressive tumorigenesity. Many researchers have investigated the clinicopathological significance of p53 gene mutations[15,32-34]. We noted a higher incidence of lymph node metastasis, poorly differentiated tumor and DNA aneuploidy in cases with a mutated p53 gene in this study, regardless of whether ER receptor was positive or negative and adjuvant therapy was often effective but not always effective. The relationship between the patients with recurrence and their postoperative adjuvant therapy, affected by clinicopathological factors, such as age, menopausal status, histological findings, clinical stage, and ER receptor, was not statistically significant, and this was also true for operative procedures as well (data not shown).

As shown in Table 3, 12 p53 gene mutations in 9 patients were found. The positions and types of gene mutations were as follows : exon 5 (3 samples), 6 (2 samples), 7 (4 samples), 8 (3 samples) and they were all point mutations. The base transitions at G C base pairs accounted for 41.7% (5 of 12) of all mutations. Five-eighths of these transitions were G to A transitions (5 of 8 ; 62.5%) followed by C to T transitions (3 of 8 ; 37.5%) in C G base pairs. Other studies of various cancers have also demonstrated similar transition patterns and an even higher number of transitions [35,36]. Generally, this type of mutation is typically found in breast cancers [37] and is also frequent in other types of malignancy, suggesting that this type of mutation may be very common as a result of spontaneous deamination of 5-methylcytosine and is most frequently observed in colorectal cancer in particular [38]. On the other hand, 2 transversion mutations (2 of 12 ; 16.7%) were found at G C and T A base pair, respectively, while another 2 cases had deletions of single nucleotide (2 of 12 ; 16.7%). Among the 12 point mutations only one case produced a stop codon and 3 cases had 2 mutations in one allele. The majority of mutations were found in common typical hot spot codons of breast cancers such as codon 175 (2 of 12 ; 16.7%), 248 (3 of 12 ; 25.0%), 282 (3 of 12 ; 25.0 %). Three mutations in codon 248 were all transitions in a CpG doublets, and two-thirds of the mutations in codon 282 had CpG. In our data, of 7 CpG mutations, 5 cases had base change G to A and 2 had C to T. Spontaneously occurring deaminatation at methylated CpG site is a general mechanism accounting for G to A and C to T changes [39] and has been indicated as an endo-

Table 3 Characteristics of p53 gene mutations in 9 aspiration biopsied breast cancer specimens

	p53 gene mutation			
Case	Exon / codon	Nucleotide mutation	Base change	Amino acid change
1	7 / 248	CGG → CAG	G → A	Arg → Gln
2	5 / 175	CGC → CAC	G → A	Arg → His
3	5 / 158	CGC → CC**	G delete	Arg
4	8 / 282	CGG → CAG	G → A	Arg → Gln
5	7 / 231	ACC → ATC	C → T	Thr → Ile
6	7 / 248	CGG → TGG	C → T	Arg → Trp
	8 / 282	CGG → CG**	G delete	Arg
7	5 / 175	CGC → CAC	G → A	Arg → His
	7 / 248	CGG → CAG	G → A	Arg → Gln
8	6 / 204	GAG → TAG	G → T	Glu → Stop
	8 / 282	CGG → TGG	C → T	Arg → Trip
9	6 / 197	GTG → GAG	T → A	Val → Glu

* Mutation at CpG site.

** Deletion of single nucleotide.

genous cause of somatic mutations in p53 gene[40]. Three codon 248 in exon 7 encoded arginine in this study. As Kalogeraki and colleague [41] mentioned, such a residue may be critical in the binding of p53 to specific DNA sequences of activated genes, resulting in the arrest of the cell cycle. It is thought that the resulting genetic instability increases the probability of the tumor cells being unable to regulate exessive growth[42].

Thus, the present data demonstrated that p53 gene mutations in aspirated cancer cells occur more frequently in aggressive cancers. In addition, a short-term relapse of the malignant tumor also occurred more frequently in the cases which carried a mutated p53 gene. In the present study, however, the differences of p53 gene mutation did not affect the prognosis of patients. In a recent report on p53 immunohistochemistry, not in resected tumors specimens but in fine-needle aspirated specimen of tumors, a significant correlation between the overexpression of p53 and prognosis was identified in breast cancer [43]. As a genetic basis for p53 overexpression in human breast cancer, overexpression of an activated form of p53 protein may be involved in neoplastic transformation. In fact, accumulation of mutant p53 protein has been correlated with poor patient prognosis and p53 gene mutation as well, suggesting that these two genetical factors are important biomarker for malignancy. Therefore, overexpression of p53 protein was considered to be almost always associated with mutation of p53 gene [44]. p53 protein was positive in 6 of the 9 patients with p53 gene mutations who had postoperative recurrent disease (6 of 9 ; 66.7%). However, in this study, there was no correlation between either p53 staining or p53 gene mutation and the disease free interval of patients ; there was no significant relationship between 4 p53 protein positive and 3 negative patients in terms of short-term relapse of disease.

In conclusion, p53 gene mutations obtained by FNAB were frequently detected by PCR-SSCP analysis in breast cancer, and this mutation may be a potent prognostic indicator in terms of the short-term relapse of postoperative patients. Moreover, assessing the relationship between p53 gene mutation and clinicopathological factors, such as lymph node metastasis, clinical stage, histologic grade, and DNA aneuploid was very useful. Therefore, p53 gene mutation is considered to be an important determinant of tumor behavior and aggressiveness and is thus an important prognostic factor. Because our study is based on a small number of mutations, further studies of a large number of tumors are required to confirm the data from a more detailed molecular approach. These studies are currently being undertaken.

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