Postoperative Prognosis of Breast Cancer Patients Predicted by p53 Gene Mutation in Cancer Cells Obtained by Aspiration Biopsy

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ABSTRACT

The method of cytological examination by fine-needle aspiration biopsy (FNAB) was developed clinically in breast cancer and enabled us to prepare cancer cell nuclei for the detection of p53 gene mutation. In the expectation that this method would improve the prediction of postoperative prognosis, the observation of 10-year survival for breast cancer patients with p53 gene mutations was done. The DNA of the aspirated cells was examined preoperatively for gene alterations in 53 patients with breast cancer. The p53 protein accumulation, DNA ploidy pattern, estrogen receptor (ER) , and clinicopathological factors were examined postoperatively.

The postoperative follow-up was conducted over 10 years and evaluated the status of p53 gene mutation. In 26 patients (49.1%) , 29 p53 gene mutations were shown. p53 protein accumulations and DNA aneuploidy patterns were detected in 33 (62.3%) and 42 (79.2%) cases, respectively, and both significantly correlated with p53 gene mutations. With regard to the postoperative prognosis, in over 10 years of observation, the patients who showed p53 mutations had a significantly worse prognosis in both disease-free survival and overall survival than those showing negative p53 mutation. A similar tendency was also seen in patients with histologic grade 3. Using FNAB, the usefulness of the preoperative detection of p53 gene mutation was revealed, suggesting its clinical benefits for predicting a patient's prognosis.

Key words : Breast cancer, p53 mutation, Aspiration biopsy, Prognosis, 10-year survival

INTRODUCTION

Although several advances have been made in improving the surgical, chemo and/or hormone therapies for regulating breast cancers,

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the mortality rate remains high in Japan and Western countries as well [1, 2]. Therefore, it is important to note that the cure rate for breast cancer patients would improve significantly if breast cancers could be detected during the early stage, and patients can be administered a suitable treatment for better prognoses. Many clinical methods have been developed for the early detection of cancers; mass screening programs are being conducted using mammography, ultrasonography, and needle biopsy combined with physical examination [2]. Most of all, FNAB is regarded as a valuable tool to detect breast cancer cytologically. Moreover, as we have mentioned in our previous reports [3-7], since 1994, FNAB has become a more reliable method to achieve an accurate diagnosis without causing the patient any harm; this is done by detecting p53 gene mutation, using PCR (polymerase chain reaction) combined with SSCP (single strand conformation polymorphism) analysis, and direct nucleotide sequencing.

It has been recently reported that the p53 gene is a multifunctional tumor-suppressor [8] and that its mutations play an important role in the development of many common human malignancies [9, 10]. In case of breast cancer, the frequent genetic alteration of the p53 gene and nuclear accumulation of the p53 protein have been studied in detail [11, 12]. Consequently, in malignant tumor cells, p53 protein localization was first undertaken to elucidate the link between p53 gene mutation and the nuclear accumulation of its protein[13]. According to Allred *et al.*[14], many breast cancers which show nuclear accumulation of the p53 protein are considered to have a poor prognosis. On the other hand, primary breast cancers with a p53 gene mutation are almost always histologically low-grade and show undesirable prognoses, regardless of their p53 protein expression patterns and/or mutational characteristics [15]. Evidence clearly suggests that tumorigenesis results from an accumulation of some genetic abnormalities in potent genes such as p53, BRCA 1 - 3, c-*erb* B-2, and c-*Myc*; therefore, it is logical to evaluate genetic alterations in an effort to predict biologic behavior [7, 8]. Most of all, with regard to the p53 gene, the implication for carcinogenesis is that when p53 function becomes genetically unstable, this event predisposes to gross genomic alterations such as gene amplifications, translocations, and deletions[16]. Thus, the tumor-suppressor gene p53 is now well known to play a crucial role in the development of a wide variety of human cancers. Particularly in breast cancer, most mutations are proved to be located in the evolutionary highly conserved regions of the p53 gene, which span exons 5 to 8 [17, 18].

Since 1992, to clarify the extent of p53 gene mutation involvement in tumor development and to predict the tumor progression, we have investigated p53 gene mutation in the DNA samples of tumor cells extracted preoperatively from breast cancers by FNAB; preoperatively, this resulted in the improvement of diagnostic accuracy [3, 4, 6] and in contributing to the early detection of short-term relapse of disease [7].

In the present study, by using PCR-SSCP with direct DNA sequencing, we detected and analyzed the p53 gene mutations in DNA samples extracted from the cells that were obtained from breast cancers by FNAB. This was done in the expectation that this method would improve the prediction of postoperative prognosis in breast cancer patients; the DFS and OS rates were estimated over 10 years of long-term observation.

PATIENTS AND METHOD

1. Patients and samples

This study comprised 53 randomized women with primary invasive breast carcinoma that was diagnosed and treated between 1992 and 1995 at the Sapporo Medical University Hospital or its affiliated hospitals, without any evidence of distant metastasis at the time of surgery. All patients were preoperatively diagnosed as having malignant tumors by fine-needle aspiration cytology (FNAC), and the p53 gene mutations in the tumor cells were detected by PCR-SSCP and direct DNA sequenc-

ing. The patients' ages ranged from 26 to 72 years, with a mean age of 50.7 years. The patients were either treated by mastectomy (45 patients) or breast conserving surgery (8 patients) . The histological confirmation of nodal status was available in 49 patients. Lymph node involvement was determined in the course of the routine pathological assessment. The resected specimens of each breast cancer were frozen in liquid nitorogen until use. Forty-six patients received adjuvant hormone therapies, and 4 patient received adjuvant chemotherapies, while 3 patients received adjuvant chemo-hormone therapies. Medical records of the patients were reviewed to collect both clinical and pathological data. The postoperative follow-up was conducted at 1～3-month intervals during the first year and at 6～12-month intervals thereafter. The minimal period of follow-up was 10 years until January 2006. The median follow-up time, defined as the median time between the primary operation and the date of evaluation, was 12.9 years.

2. DNA extraction

Fifty-three aspirated biopsy specimens consisting of approximately $1-5\times10^3$ cells were bathed in RPMI-1640 medium and then immediately immersed into liquid nitrogen and stored at −80 ℃ until assayed. The genomic DNA obtained from each of the 53 patients was prepared by proteinase K digestion and phenol/chloroform extraction, according to a modification of the method of Lyons *et al*. [19] .

3. PCR - SSCP analysis

In this study, as was done previously [3-6] , the PCR-SSCP analysis was undertaken for the 53 aspiration-biopsied breast cancer specimens; the mutations in exons 5 to 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 [11, 20] : exon 5, sense 5' - TTCCTCTTCCTTGCACTACTCC - 3' and antisense 5' - CAGCTGCTCACCATCGCTATA-3' ; exon 6, sense 5' - TTGCTCTTAGGTCTGGCCCCTCCTCAG - 3' and antisense 5' - CAGACCTCAGGCGGCTCATAGG - 3' ; exon 7, sense 5' - GTGTTATCTCCTSGGTTGGC - 3' and antisense 5' - CAAGTGGCTCCTGACCTGGA - 3' ; and exon 8, sense 5' - AGTGGTAATCTACTGGGACGG - 3' and antisense 5' -ACCTCGCTTAGTGCTCCCTG-3' . A200-ng genomic DNA sampled from each of the aspirated cells was amplified in 25 μl volumes of the buffer recommended by Perkin-Elmer Cetus (Norwalk, CT) , which contained 1 mM $MgCl_2$, 1 unit of Taq DNA polymerase, and 1 μl of [α-^{32}P] d CTP (3000 Ci/mmol, 10 Ci/ml; Amersham Japan, Tokyo) .

Subsequently, using a thermal programmer (Nippon Genetic Co., Tokyo) , 35 cycles were performed for each sample, each involving denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min. Following this, a 2-μl 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) that was then applied (1μl/lane) to a 6 % neutral polyacrylamide gel. Electrophoresis was performed at 40 W for 3.5 - 5 h with fan cooling. Subsequently, the gel was fixed in acetic acid (10%) , dried, and exposed to X-ray film for 6 -12 h at - 80 ℃.

4. Direct DNA sequencing of PCR products

In order to detect the DNA sequences, the shifted bands in exons 5 to 8 obtained from biopsy specimens, were eluted from the polyacrylamide gel and amplified by a PCR by using the same primers as those used for the PCR products purified with SUPREC™-02 (Takara Shuzo, Kyoto) .

Sequencing was performed by the dideoxy termination method by using a 7-DEAZA Sequencing KIT, Version 2.0 (Takara Shuzo) , as has been described before [4, 5] .

5. Immunohistochemical analysis

For the immunohistochemical study, Pab 1901, an antibody against the p53 protein was used. PAb 1801 (Oncogene Science, Inc.,

Manhasset, NY) is a murine monoclonal antibody against human p53 and recognizes both the wild-type and mutant forms of the p53 protein. To detect the p53 protein nuclear accumulation, PAb 1801 was used, as we have previously reported [21] ; for immunoperoxidase staining, 6 μm-thick frozen sections from each of the resected tumors were subsequently 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) . The sections were adequately stained with 3, 3-iaminobenzidine (Sigma Chemical, St. Louis, MO) , and the nuclei were 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 50 μg/ml propidium iodide (Sigma) , and filtered through nylon mesh; each sample was immediately analyzed using a FACS-IV cell sorter (Becton Dickinson, Mountain View, CA) .

7. ER levels

The ER content of the cancer tissue was determined by a 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

Fisher's exact tests were used to estimate the association of the clinicopathological variables, p53 gene mutation, DNA aneuploidy patterns, and the ER value. DFS was calculated from the date of operation to the date of recurrence, and OS was calculated from the date of operation to the date of death. Survival analyses were performed according to the Kaplan-Meier method. Comparison of the survival between groups was performed using the log-rank test. Cox proportional hazard analysis was used for univariate and multivariate analysis to explore the effect of variables on survival and estimating the hazard ratio (HR) and 95% confidence interval (CI) . The Statistical Analysis Systems (SAS) software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. A significant difference was determined when the *P*-value was less than 0.05 .

RESULTS

The clinical and molecular biological features of the 53 breast cancer patients are listed in Table 1. Of these patients, 28 were premenopausal patients (mean age, 40.3 years) , and 25 were postmenopausal patients (mean age, 63.0 years) . They were all classified into clinical stages from I to IIIA and into histologic grades from 1 to 3 . In the histological examination, 39 patients had papillotubular carcinomas, 10 had solid-tubular carcinomas, and 4 had scirrhous carcinomas. With regard to the nuclear p53 protein accumulations, positively stained cancer cells were found in 33 patients (62.3% ; 33 of 53), and negatively stained ones, in 20 patients (37.7%) . In hormone receptor investigation, the ER status was available for all patients; ER positive tumors were seen in 33 patients (62.3% ; 33 of 53) , and ER negative tumors, in 20 patients (37.7%) . The examination of the DNA ploidy pattern revealed that there were 11 DNA diploid cancers (20.8% ; 11 of 53) and 42 aneuploid cancers (79.2%) . Regardless of the point mutation pattern, almost all breast cancer patients who had p53 mutations showed DNA aneuploidy patterns (96.2% ; 25 of 26) and tested positive for nuclear p53 protein staining (73.1% ; 19 of 26) as well .

Consequently, p53 protein accumulations and DNA aneuploidy patterns were significantly correlated with p53 gene mutation ($P < 0.01$ and

Table 1 Clinical and biological features of the 53 breast cancer patients

Patient No.	Age ／ Menopause	Clinical stage*	Histology+	Histologic grade§	p53 mutation ／staining	ER	DNA ploidy pattern‖	Relapse**
1	64／−	IIA	Pt	1	−／−	−	A	−
2	49／+	I	Pt	1	−／−	−	D	−
3	59／−	IIA	Sc	2	+／+	+	A	−
4	64／−	IIIA	Pt	3	+／+	−	A	−
5	61／−	I	Pt	1	−／−	+	A	−
6	45／+	IIA	Pt	1	−／+	+	A	−
7	51／+	I	St	1	−／−	+	D	−
8	56／−	IIB	Pt	3	+／+	+	A	−
9	49／+	IIA	Pt	1	−／−	+	A	−
10	43／+	IIIA	Pt	3	+／+	−	A	+
11	67／−	I	Pt	1	−／−	−	D	−
12	30／+	IIIA	Pt	3	+／+	+	A	+**
13	64／−	IIA	Pt	1	−／+	+	A	−
14	44／+	I	Pt	2	−／−	−	A	−
15	50／+	I	Pt	1	−／+	+	D	−
16	66／−	IIIA	Pt	3	+／−	−	A	−
17	26／+	I	St	1	−／+	+	D	−
18	51／−	IIB	Pt	2	+／−	−	A	+
19	61／−	IIB	St	3	+／+	+	A	−
20	48／+	IIA	Pt	3	−／−	−	A	−
21	44／+	IIIA	Pt	2	−／+	+	A	−
22	51／+	I	Pt	1	−／+	−	D	−
23	33／+	I	Pt	1	−／+	+	D	−
24	68／−	IIIA	Pt	3	+／+	−	A	+**
25	45／+	I	St	3	+／+	+	A	−
26	49／+	IIB	Pt	2	+／−	−	A	+
27	72／−	IIB	Pt	2	+／+	−	A	−
28	61／−	I	Pt	1	−／+	+	A	−
29	48／+	IIIA	Sc	2	+／−	+	A	−
30	27／+	I	Pt	1	−／−	+	D	−
31	33／+	IIB	Pt	2	+／+	+	A	−
32	52／−	IIIA	St	3	++／+	−	A	+**
33	66／−	IIIA	St	3	+／+	−	A	−
34	70／−	I	Pt	1	−／−	−	A	−
35	58／−	I	Pt	1	−／−	−	D	−
36	30／+	I	Pt	1	−／+	+	A	−
37	61／−	IIIA	Sc	3	++／+	+	A	+
38	63／−	IIIA	Pt	3	++／+	+	A	+**
39	44／+	I	Pt	1	−／+	+	A	−
40	31／+	I	Pt	1	−／+	+	A	−
41	29／+	I	Pt	1	−／−	+	D	−
42	67／−	I	St	1	+／+	+	A	−
43	58／−	I	Pt	3	+／−	+	A	−
44	37／+	I	St	1	−／+	+	A	−
45	59／−	IIB	St	2	+／+	+	A	−
46	68／−	IIA	Pt	1	−／−	−	A	−
47	36／+	IIA	Pt	2	+／+	+	A	−
48	31／+	IIA	Pt	2	−／+	+	A	−
49	47／+	IIIA	St	3	+／−	−	A	+**
50	71／−	IIIA	Pt	2	+／−	+	D	−
51	35／+	IIA	Pt	2	+／+	+	A	−
52	43／+	I	Pt	1	−／+	+	A	−
53	68／−	IIIA	Sc	3	+／+	−	A	+**

* Clinical stage was determined by the TNM classification of the Japanese Breast Cancer Society [39].

+ PT = papillotubular carcinoma; St = solid-tubular carcinoma; Sc = scirrhous carcinoma.

§ Histologic grade was determined according to the system based on a modified WHO classification [40].

‖ A = aneuploid; D = diploid.

** Death by recurrence of breast cancer.

P <0.001, respectively) . The adjuvant therapy provided to patients as postoperative treatment was decided on the basis of their lymphonodal status, menopausal status, and the level of ER; consequently, 53 patients were administered hormone therapy, chemotherapy, and chemo-hormone therapy for 2 or 3 years after obtaining their informed consent. In all cases of recurrence, regardless of whether patients had p53 gene mutation or were p53 protein positive, they have recieved adequate therapies until their end-stages.

However, no adjuvant therapies significantly affected the favorable prognosis of patients with recurrent cancer (data not shown) . Moreover, of 10 patients with recurrence, 6 patients (60.0% ; 6 of 10) tested positive for p53 mutation and showed DNA aneuploidy pattern as well, besides having advanced clinical stage IIIA, histological stage 3, and p53 protein accumulations (83.3% ; 5 of 6) and being ER positive (83.3% ; 5 of 6) ; these patients reached the terminal stages within 10 years after the initial operation. With respect to the ER status, many recurrent breast cancers were shown to be ER negative (70.0% ; 7 of 10) , and 4 ER negative patients (66.7% ; 4 of 6) died of recurrent cancer.

As shown in Table 2, among 53 breast cancer patients 26 showed p53 gene mutation (49.1% ; 26 of 53) , while no mutation was detected in 27 (50.9%) patients. In total, 26 of the 53 breast cancers revealed 29 mobility shifts in the PCR-SSCP analysis. The characteristics of the p53 gene mutation were identified by the direct sequencing of the PCR-amplified exons from 5 to 8, as listed in Table 2. The positions and incidence of the mutations were distributed as follows: exon 5 (8 samples, 27.6%) , 6 (5 samples, 17.2%) , 7 (10 samples, 34.5%) , 8 (6 samples, 20.7%) ; these included point mutations such as

Table 2 p53 gene mutation in aspiration-biopsied breast cancer specimens

Patients No.	p53 protein staining	p53 gene mutation Exon／Codon	base change (Amino acid)
3	+	6／220	TAT(Tyr) →TGT(Cys)
4	+	7／248*	CGG(Arg) →CAG(Gln)
8	+	7／234	TAC(Tyr) →TGC(Cys)
10	+	5／175*	CGC(Arg) →CAC(His)
12	+	5／175*	CGC(Arg) →CAC(His)
16	+	8／279	GGG(Gly) →GAG(Glu)
18	+	5／158	CGC(Arg) →CC**
19	+	7／238	TGT(Cys) →TAT(Tyr)
24	+	8／282*	CGG(Arg) →CAG(Gln)
25	+	5／146	TGG(Trp) →TGA(Stop)
26	−	8／275	TGT(Cys) →TTT(Phe)
27	+	7／248*	CGG(Arg) →CAG(Gln)
29	−	7／231	ACC(Thr) →ATC(Ile)
31	+	8／279	GGG(Gly) →GG**
32	+	7／248*	CGG(Arg) →TGG(Trp)
		8／282	CGG(Arg) →CG**
33	+	7／236	TAC(Tyr) →TGC(Cys)
37	+	5／175*	CGC(Arg) →CAC(His)
		7／248*	CGG(Arg) →CAG(Gln)
38	+	6／204	GAG(Glu) →TAG(Stop)
		8／282*	CGG(Arg) →TGG(Trp)
42	+	7／244	GGC(Gly) →GAC(Asp)
43	−	5／176	TGC(Cys) →TTC(Phe)
45	+	6／192	CAG(Gln) →TAG(Stop)
47	+	6／197	GTG(Val) →GAG(Glu)
49	+	7／273*	CGT(Arg) →CTT(Leu)
50	−	6／220	TAT(Tyr) →TGT(Cys)
51	+	5／177	CCC(Pro) →CGC(Arg)
53	+	5／158*	CGC(Arg) →CAC(His)

* CpG
** deletion

20 transitions (69.0% ; 20 of 29) , 6 transversions (20.7% ; 6 of 29) , and 3 deletions (10.3% ; 3 of 29). Of the 26 base-pair substitutions, the more prominent feature was G to A transition (60.0% ; 12 of 20) compared to A to G (20.0% ; 4 of 20) and C to T transitions (20.0% ; 4 of 20) , i. e., 20 transitions (69.0% ; 20 of 29) showed G : C to A : T , A : T to G : C or C : G to T : A patterns. In 6 cases of transverse mutations, the G to T nucleotide change pattern was the predominant substitution (4 samples, 66.7%) , and the others were T to A and C to G. With regard to 3 deletions (10.3% ; 3 of 29) , all were deletions of the nucleotide guanine. Of the 20 transitions, 10 mutations (50.0% ; 10 of 20) occurred in a CpG dinucleotide sequence, which is known to be the hot spot for p53 gene mutations in breast cancers[14] .

Of 10 patients with recurrence, 8 showed methylation at CpG sites (80.0% ; 8 of 10) , and 5 patients who died within 10 years all had CpG mutations.

Our findings from the direct DNA sequencing of the PCR product are consistent with those in a previous report that indicates that most gene mutations are distributed evenly from exons 4 to 8 [22] .

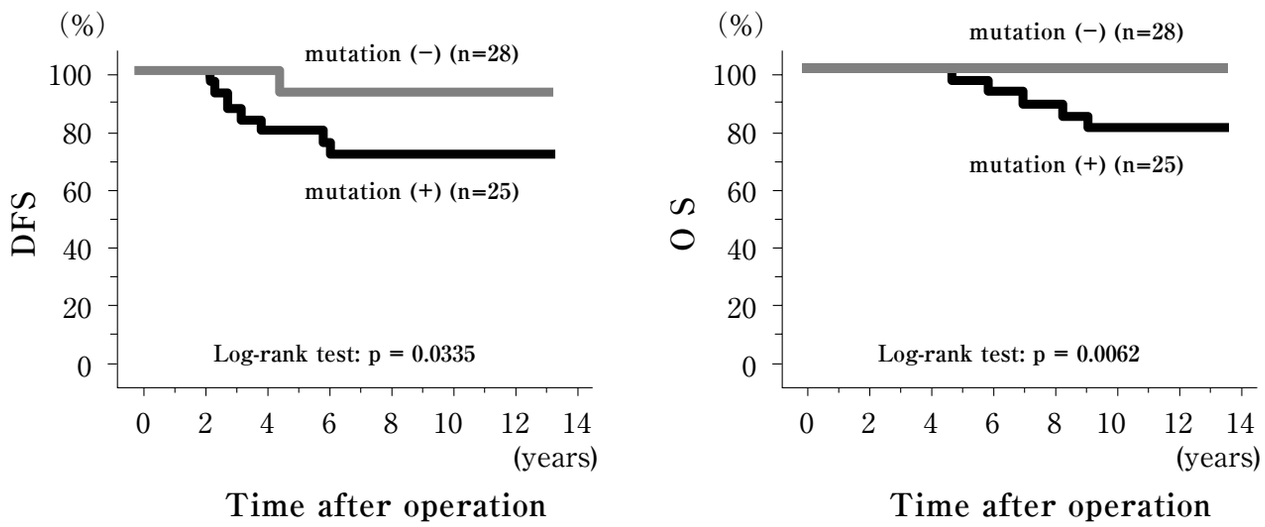

Fig. 1 Kaplan-Meier curves for DFS (left) and OS (right) in breast cancer patients according to p53 genemutations.

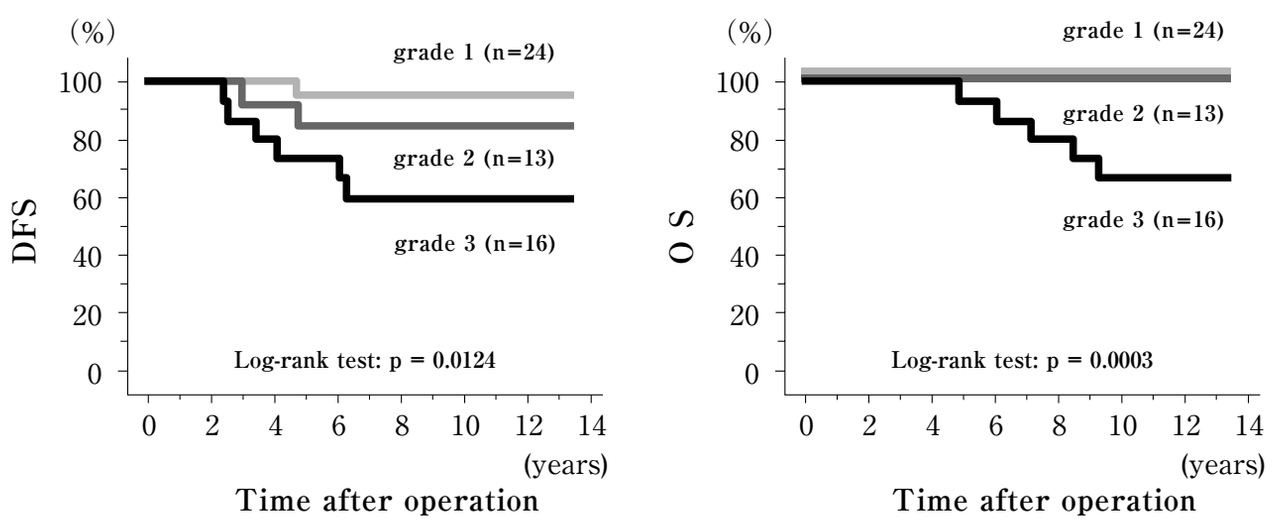

Fig. 2 Kaplan-Meier curves for DFS (left) and OS (right) in breast cancer patients with different histological grades.

As shown in Fig. 1 and Fig. 2, the survival times of breast cancer patients, such as FDS and OS, were proved to be closely related to the presence of p53 gene mutation or an aggressive histologic grade in the cancer cells. After a median follow-up period of 13.6 years, the postoperative 10-year DFS and OS were 64.0% and 76.0% , respectively, in cases with p53 gene mutation. While in the case of the absence of p53 gene mutation, the 10-year DFS and OS were 96.4% and 100% , respectively. Consequently, the patients who tested positive for p53 mutation had a significantly worse prognosis in terms of both DFS and OS (P=0.0335 and 0.0062, respectively) than those who tested negative for it. Furthermore, patients with histologic grade 3 also showed a statistically worse prognosis than those with grade 1 (DFS, P = 0.0124 and OS, P =0.0003, respectively) . On the other hand, there was no statistically significant difference between those who tested positive and those who tested negative for p53 staining, and between those who tested positive and those who tested negative for ER. In addition, the menopausal status of patients also showed no significant relationship with other clinical and cell biological factors. However, in terms of the DNA ploidy pattern and clinical grade, patients with aneuploidy pattern showed the tendency of worse prognosis than those with a diploidy pattern, and those with clinical grade IIIA showed better prognosis than those with grade I and/or IIA. With regard to the prognosis of breast cancer patients, those who showed clinicopathologically aggressive grade such as aneuploidy, histologic grade 2 or 3, and clinical stage IIB or IIIA tested positive for p53 mutaions and had worse prognosis. Meanwhile, the p53 protein accumulation did not affect the patients' survival rate statistically; however, it was present in 7 patients with recurrent cancer and p53 mutation (7 of 10 ; 70.0%) . Thus, the close relationship between p53 mutation and p53 protein accumulation was indeed suggested. However, in this study, although the cancer patients with p53 mutation proved to have a significantly worse prognosis, cases in which the p53 protein accumulated showed mostly good survival rates.

Univariate analysis by Cox proportional hazards model revealed that in the DFS of postoperative breast cancer patients, the prognosis was significantly worse for patients with p53 mutation (HR 5.183, P = 0.0375) and histologic grade 3 (HR 3.516, P = 0.0076) (Table 3) . However, the result of the multivariate analysis for the clinicobiological factors of patients showed that although there was a tendency of unfavorable prognosis in patients with p53 mutation, aggressive histologic grade and clinical stage, and DNA aneuploidy pattern, there was no statistically significant evidence of this in DFS and OS (data not shown) .

DISCUSSION

Since first reported by Martin and Ellis [23] , FNAB has been well established as a conven-

Table 3 Univariate analyses of DFS in breast cancer patients

Variables	Categories	HR*	95 % CI **	P value
menopause	+ / −	1.022	0.973 − 1.022	0.3785
ER	+ / −	0.377	0.106 − 1.338	0.1312
DNA ploidy	A / D	ND +	ND +	0.9939
Histologic grade	1, 2 / 3	3.516	1.397 − 8.850	0.0076
Clinical stage	I , IIA, IIB/ IIIA	0.991	0.598 − 1.642	0.9731
p53 protein staining	+ / −	1.059	0.299 − 3.755	0.9290
p53 gene mutation	+ / −	5.183	1.100 − 24.425	0.0375

* hazard ratio.

**confidence interval.

+ ND = not determined because no patients with diploid patterns reccured.

ient and reliable method for the diagnosis of breast cancers.

Furthermore, the cytological information by FNAC provides useful preoperative information unlike pathological information, which is obtained after surgical excision [24]. If the information concerning the risk of recurrence, in terms of chemosensitivity or drug resistence, could be obtained preoperatively, it would be of great clinical value to indicate the optimal adjuvant and/or neoadjuvant treatment. Although the prognostic value of microscopic information provided by FNAB has been emphasized, the much valuable additional advantage of gene mutation in aspirated cells was considered to be a good prognostic value [25]. Thus, with the introduction of PCR, which has become applicable to small DNA samples obtained by FNAB [3, 4, 6], p53 gene mutation is regarded as a potent predictor of poor postoperative prognosis in breast cancer patients [8]. In particular, in our previous paper[5, 6], we emphatically reported that the detection of p53 gene mutations in aspirated biopsy specimens of breast cancers was helpful for an accurate and rapid cytological diagnosis, which might reflect the prognosis.

Mutant p53 was clearly known to be the most commonly altered proto-oncogene in breast cancer [26]. Recently, abnormal levels of the p53 gene product have been found by routine immunohistochemical methods in 19% − 58% of breast cancer tissues [27, 28], and the molecular analysis of samples with immunodetectable protein confirmed in most cases the presence of point mutations within the gene. In general, p53 mutations are present in 20% − 40% of all breast cancers [11, 13-15, 29]. In this study, the frequency of mutation that we found is comparable to the rate of p53 overexpression observed immunohistochemically. Therefore, p53 overexpression was rarely observed in the patients with no p53 mutation.

On the other hand, according to Kalogeraki *et al*. [30], in case of FNA specimens, p53 overexpressions were found in 45% of breast cancer patients. In the present study, p53 overexpressions were shown in 62.3% patients.

All of the p53 genetic changes that we observed were mainly missense mutations; 26 were point mutations, with the exception being 3 one-base pair deletions (10.3%) that were detected in 24 breast cancers. Among point mutations, a stop codon was produced in only 3 cases (10.3%). Of 29 p53 gene alterations in exons 5 to 8, 20 were missense mutations, and 3 patients showed nonsense mutations, which involved an in-frame deletion of one base pair. Of the 20 transition mutations, 16 were changes from G : C to A : T. Such a prevalence of G : C to A : T substitutions in breast cancers was also observed by other researchers [12]. On the whole, as shown in table 2, the mutation spectrum we found in breast cancer appears more complex compared to other tumor types [17, 31]. The occurrence of spontaneous base substitutions may contribute to the appearance of various mutation patterns. In the present study, the prominent mutations were the association of G : C to A : T and C : G to T : A transitions (20 of 29 ; 69.0%), and these occurred frequently (9 of 10 ; 90.0%) at the CpG dinucleotide sites (3 at codon 175, 4 at codon 248, and 2 at codon 282), which are known to be hot spots for mutations in the p53 gene for breast cancer.

However, the mutation at codon 158, known as a novel hot spot, was not detected in this study. Spontaneously occurring deamination at methylated CpG sites is a general mechanism accounting for C to T and G to A changes [32] and has been indicated as an endogenous cause of somatic mutations in the p53 gene [33]. As Esteller *et al*. [34] demonstrated, CpG island hypermethylation has been described in almost every tumor types; this hypermethylation is found in the promoter regions of tumor-suppressor genes and is now established as an important mechanism for gene inactivation.

With regard to the change in the p53 gene of the nuclei in cancer cells, the association of mutations in the p53 gene and DNA aneuploidy pattern was found in 25 breast cancer patients (47.2% ; 25 of 53), while only one case of DNA

ploidy pattern with the p53 mutation was detected (1.9% ; 1 of 53) , suggesting a complementary effect of p53 gene alterations in breast cancer tumorigenesis. DNA aneuploidy, which was predominant in various malignant tumors[35] , might represent an early and critical event in such a process. According to the NIH consensus conference [36] , the DNA ploidy status was considered to be one of the most significant prognostic factors of the early stage in breast cancer patients. In addition, p53 gene alteration appears to be particularly involved in more severe tumor cell transformation. On the other hand, in the present study, almost all patients with clinical stage I cancers tested negative for p53 mutations (19 of 22 ; 86.4%) and 92.9% (13 of 14) of the patients with stage IIIA tumors tested positive for p53 mutations, suggesting that these may be relevant to the progression of malignancy to a higher clinical grade. Furthermore, the histologic grade also appears to be related to the characteristics of tumor malignancy, indicating that the morphology and biology of breast cancer are closely linked. Such a tendency was observed in our study, e.g., DNA diploidy patterns were mainly shown in clinical stage I (10 of 22 ; 45.5%) and also in histologic grade 1 (10 of 24 ; 41.7%) . In fact, 42 patients with DNA aneuploidy patterns showed 25 p53 gene mutations (59%) , while 1 patient showed DNA diploid status with p53 mutation, clinical stage IIIA, and histologic grade 2 and did not experience recurrence. In spite of adequate adjuvant therapies, patients with p53 mutation showed worse prognosis.

In the observation of 10-year survival for breast cancer patients with p53 gene mutations in cancer cells aspirated from their tumors, postoperative prognosis was significantly correlated with p53 gene mutation and with aggressive histologic grade 3. Furthermore, advanced clinical stage, such as IIIA, was closely related with p53 gene mutation, ER negative status, DNA aneuploidy pattern, and histologic grade 3. These results were consistent with those of other reports in case of various solid tumors[37, 38] . With regard to the ER status in patients, regardless of the p53 gene status and other clinicohistological factors, the positive receptor status is particularly related with a better postoperative prognosis in DFS rather than OS. The better DFS rate might be attributed to the effect of hormonal adjuvant therapy.

FNAC is a very useful examination and is routinely performed for the diagnosis of breast tumors and provides suitable material for both cytologic examination and DNA analysis. Recently, there appears to be a growing movement in favor of core needle biopsy (CNB) over FNAC in detecting breast cancer. However, FNAC has some advantage over CNB, including simplicity, cost-effectiveness, and lower invasiveness. Moreover, FNAC permits numerous multidirectional passes through the mass, but a CNB device allows for only 1 monodirectional sample; FNAC was the more sensitive method for detecting cancer cells. Consequently, the genetic analysis of FNAB materials reveals important information such as the risk of recurrence, particularly short-term relapse, and the prognosis. Further long-term investigations concerning these p53 gene mutations and the determination of their biological and prognostic significance in breast cancer are necessary in a large number of examined cases.

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