# p53 Point Mutations in Fine-Needle Aspirated Breast Cancer Cells and Their DNA Ploidy Patterns

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## ABSTRACT

Breast cancer cells can be obtained derectly from the patient with minimal damage by fine-needle sampling. The method of aspiration biopsy cytology (ABC) by fine-needle aspiration was developed that enabled us to prepare cancer cell nuclei for detection of p53 gene mutation by PCR-SSCP. Fine-needle sampling was successfully performed on 49 patients with breast tumor. Thirty-one aspirated specimens (63.3%) produced enough malignant material for assessment; 18 were diagnosed as being cytologically benign. In 49 patients, surgical specimens from the same tumors were examined for DNA aneuploid and S-phase fraction using flow cytometry. Twenty-four of the 31 breast cancer specimens (77.4%) were classified as aneuploid pattern. p53 gene mutations were detected in 15 patients (48.4%); all showed point mutations. In this series, no significant correlation was found with respect to the factors such as aneuploidy, p53 mutation, and ER in various tumor size or menopausal status. On the contrary, there was a significant relationship between aneuploidy pattern and p53 mutation; fifteen p53 mutated cancer cell samples showed all aneuploid patterns. Furthermore, these 2 factors showed a tendency of high incidence in advanced clinical stage, histologic grade, and tumor size. Thus, taking ABC into consideration, a combined examination of p53 mutation and DNA histograms derived from flow cytometry in fresh tissue samples could be a valuable tool in clinical use for biological evaluation of breast cancer.

**Key words**: Breast cancer, Aspiration biopsy, p53 mutation, DNA ploidy, PCR-SSCP

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2. The abbreviations used are: ABC, aspiration biopsy cytology; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; ER, estrogen receptor; SPF, S-phase fraction; CV, coefficient of variation; DI, DNA index.

## INTRODUCTION

The prevalence of mass surveys and extended application of operative treatment for breast cancer have come to require the development of simple measures to deny or confirm the presence of this disease(1). In the last two decades, aspiration biopsy cytology (ABC²) has allowed considerable development in the field of diagnosis of breast cancer; it eliminates damage to tissues, offers rapid acquisition of the result and is less expensive compared with open biopsy(2-6).

Recently, we reported that fine-needle aspiration biopsy of the breast was a very useful technique for evaluation of a suspect lesion before surgical operation; using PCR-SSCP analysis for p53 gene mutation, we emphasized that the detection of p53 gene mutation in aspirated tumor cells could be helpful in achieving an accurate diagnosis(7, 8). A clear correlation was demonstrated between the presence 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. The relationship between p53 gene mutation and tumor progression has been stressed in various malignant tumors(9-12). Several reports have addressed whether DNA ploidy is related to the patient's prognosis; Kute *et al.*(13) reported no significant relationship between lymph node involvement and DNA aneuploid incidence, though Hedly *et al.*(14) and Conelisse *et al.*(15) found a high percentage of aneuploidy in patients with lymph node-positive breast cancers. More recently, Lewis(16) reported that precise flow cytometric measurements of DNA ploidy patterns were a good prognostic indicator in patients with node-negative breast cancer. Other current studies using multivariate analyses have indicated that DNA ploidy, DNA index, SPF, ER, and tumor size are powerful prognostic factors in breast cancer patients(14, 16-20).

The purpose of the present study was to clarify the usefulness of DNA ploidy analysis in breast cancer, and in addition, to examine the relationship between p53 gene mutation, cytometric analyzed data, and clinicopathological elements.

## PATIENTS AND METHODS

Forty-nine aspirated cell samples from breast tissue specimens were obtained and examined between 1992 and 1994 from 49 breast tumor patients at the Sapporo Medical University Hospital and its affiliated hospitals. All aspiration biopsied specimens and resected tumor tissue specimens were immediately immersed in liquid nitrogen and stored at −80℃ until use. Thirty-one of the patients were diagnosed as malignant, ranging from clinical stage I to IIIb according to the TNM classification of the Japanese Breast Cancer Society(21); the histologic grade of resected cancers was determined by a system based on a modified WHO

classification(19, 22).

In order to collect adequate cells, at least 2 or 3 trials of aspiration were needed. Consequently, the biopsy specimens used for DNA extraction and DNA flow cytometry consisted of approximately $1\text{-}5\times10^3$ cells. The DNA from each of these was prepared by proteinase K digestion and phenol-chloroform extraction, according to the modified method of Lyons *et al.*(23) PCR-SSCP analysis was then performed, with DNA from normal breast gland tissue showing no malignancy as a negative control. In this study, a conventional PCR-SSCP analysis and direct sequencing was undertaken, as we reported previously(7, 8).

To examine the DNA ploidy patterns, 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 $\mu g/ml$ propidium iodine (Sigma), filtered through nylon mesh, and analyzed immediately by using a FACS-IV (Becton Dickinson, Mountain View, CA). Histograms with a symmetrical $G_0+G_1$ peak were classified as diploid. If two $G_0+G_1$ peaks were present, the histogram was classified as aneuploid, and if more than 2, as multiploid. Examples of different types of histograms are shown in Figure 1. The DNA index (DI) was calculated by dividing the modal channel number of an aneuploid peak by the modal channel number of the diploid peak. By definition, the diploid tumors have a 0.9-1.1 of DI, and the aneuploid shows over 1.1. The mean coefficient of variation (CV) in diploid case was 4.0% (SD, 0.5%). All CV showed under 10%. The SPF was calculated according to the rectilinear method of Baisch *et al.*(24). The SPF could not be calculated in 4 (8.2%) patients (all in papillotubular carcinoma showing an aneuploid) either because of overlapping, small stemlines, or because the presence of large amounts of cell debris interfered with the determination. The height of the SPF was measured near the $G_2+M$ peak to avoid counting cell debris. In aneuploid cases with a large DI ($>1.3$), the SPF was calculated for the aneuploid stemline only.

The ER content of the tissue was determined using dextran-coated charcoal assay (Biomedical Laboratories, Tokyo); a concentration greater than 14 fmol/mg of the ER protein was considered to be positive.

For the statistical analysis, association of the clinicohistological variables, p53 gene mutation, aneuploid patterns, and ER value of the patient was analyzed using the chi-squared test.

## RESULTS AND DISCUSSION

Fine-needle aspiration biopsy has been extensively used for diagnostic biopsies of various tissues, especially of suspicious breast masses(5, 7). It has proven quite effective to reduce the mortality rate of breast cancer patients. Neverthe-

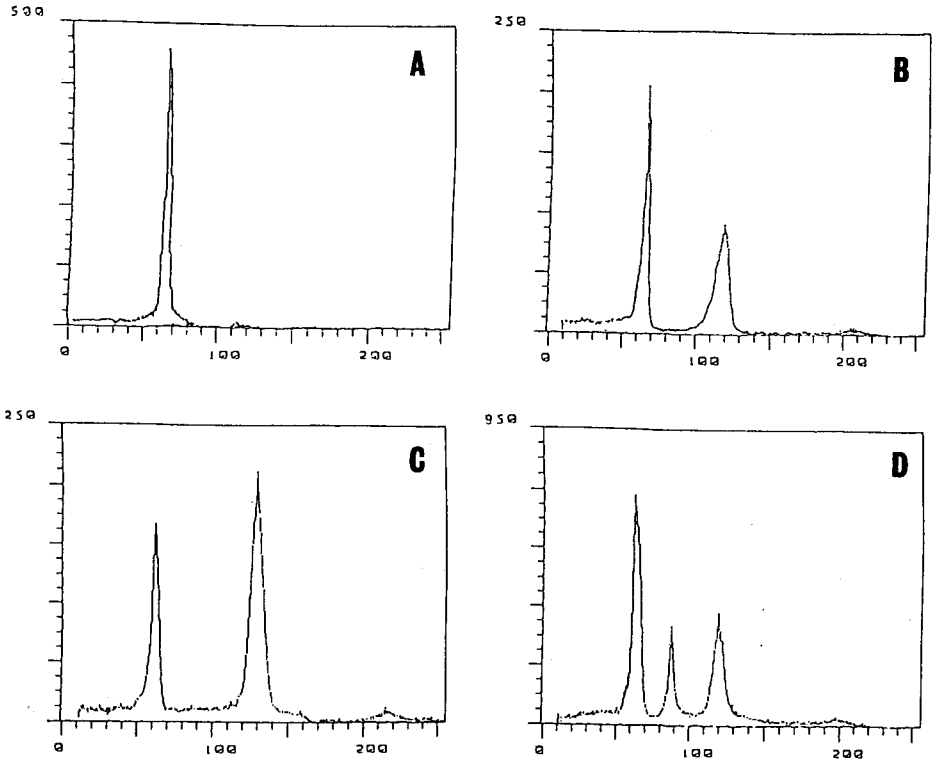


**Fig. 1** Representative DNA histograms obtained from frozen breast cancer specimens. The number of nuclei analyzed is given on the vertical axis, and DNA content as channel numbers on the horizontal axis. The diploid peak is set approximately at channel 60. A shows a typical diploid pattern, SPF 4%, DI 1.00; Aneuploidy patterns are shown in B and C, SPF 22% and 14%, DI 1.78 and 2.10, respectively. D shows a multiploid pattern which is regarded as an aneuploid, SPF 18%, former DI 1.82 and latter DI 2.74.

less, false-negative or false-positive diagnoses invariably occur and detract from the usefulness of the technique. Therefore, in an attempt to improve the accuracy of this technique, we recently have evaluated the use of p53 gene mutation to identify cancer cells in aspirated biopsy cells; 33.3% (5 of the 15 suspected breast cancer cases) patients showed p53 gene mutation and these tumors were subsequently histologically diagnosed as being malignant(7). Furthermore, in the case of 26 aspirated cell specimens taken from breast tumors which were initially diagnosed as being cytologically benign, 2 point mutations of the p53 gene were detected and were subsequently proved to be cancer cells(8). Thus, p53 mutations in aspiration biopsied specimens proved to be a useful method for detecting breast cancers. The incidence of mutation of the p53 gene has been reported as

13-32%. Fifteen (out of 31; 48.4%) breast cancer patients with p53 mutation were detected in the present study (Table 1).

As for DNA ploidy in breast cancer, several investigators have found DNA aneuploidy to have a poorer prognosis as compared with DNA diploid cancers, and suggested measures to identify additional biologic factors(15, 25, 26). A more recent study has enabled development of a new method for the analysis of DNA ploidy pattern with small, fresh samples(27-29). DNA index and SPF are considered to be meaningful prognostic factors(14, 30). Flow cytometric measurement of DNA content is a rapid and reliable technique which gives information about the potentially important variables of tumor ploidy and proliferative activity. Its precise role in routine clinical practice is uncertain, but the large number of patient's adequate follow-up may reveal some conclusions about the clinical and biological significance of DNA aneuploidy and percentage of SPF in breast cancer.

In the present study, of the 49 samples studied, 18 were benign and 31 were

**Table 1** *Relationship between Ploidy Pattern, p53 mutation, ER, and Clinicopathological Variables of the 31 Patients with Breast Cancer*

| Variables | No.of patients | Aneuploid (%) | p53 mutation (%) | ER positive (%) |
|---|---|---|---|---|
| Clinical stage[a] | | | | |
| I | 12 | 6 (50.0) | 3 (25.0) | 6 (50.0) |
| II | 13 | 12 (92.3) ** | 7 (53.8) * ** | 8 (61.5) * |
| IIIa | 4 | 4 (100) | 3 (75.0) *** | 3 (75.0) ** |
| IIIb | 2 | 2 (100) | 2 (100) | 0 ( 0 ) |
| Histologic grade[b] | | | | |
| 1 | 17 | 10 (58.8) | 4 (23.5) * | 10 (58.8) |
| 2 | 4 | 4 (100) ** | 2 (50.0) *** | 2 (50.0) |
| 3 | 10 | 10 (100) | 9 (90.0) | 5 (50.0) |
| Tumor size | | | | |
| <2 cm | 13 | 9 (69.2) * | 6 (46.2) | 4 (30.8) ** |
| ≧2 cm | 18 | 15 (83.3) | 9 (50.0) | 13 (72.2) |
| Menopausal status | | | | |
| pre | 11 | 9 (81.8) | 4 (36.4) * | 6 (54.5) |
| post | 20 | 15 (75.0) | 11 (55.0) | 11 (55.0) |

a) According to the TNM classification of the Japanese Breast Cancer Society (21).

b) Grading was performed according to the system based on a modified WHO classification (19, 22).

Statistical significance were calculated using the chi-squared test between each clinical variable; *P<0.05, **P<0.005, ***P<0.001.

malignant aspirates. All contained sufficient cells to provide good quantities of mutant p53. From a histological point of view, mean SPF in the 18 diploid benign tumor showed 7.5%, which was lower than that in cancers (15.5%), but no significant difference was shown in histopathologically different cancers (Table 2). According to Hedley *et al.*(14), SPF of over 10% was strongly correlated with high tumor grade and abnormal DNA index but weakly correlated with hormone receptor and menopausal status. In this study, mean SPF of cancer was closely correlated with aneuploidy pattern and p53 mutation (Table 2).

Futher, in a complicated genetic implication, a clear correlation was shown between the presence of p53 gene mutation and an aneuploidy pattern of tumor DNA, which has been shown to be a marker of tumor's aggressive biological behavior. This supported the hypothesis that p53 gene mutation is involved in tumor progression which has been bolstered by several recent studies of various malignant tumors(9-12). In this stydy, p53 gene mutations were produced in advanced clinical stage and histologic grade and aneuploidy patterns as well. Aneuploidy patterns were also shown in over 2cm sized tumors predominantly (Table 1). In terms of DNA ploidy status, aneuploidy status of the tumor DNA is considered to occur through a marked enhancement of chromosamal instability, and some researchers have suggested that p53 gene mutation is either directly or indirectly related to chromosomal instability in the tumor cells(19, 29).

In the present study, 24 aneuploid breast cancers (77.4%) were obtained.

**Table 2** *Aneuploidy Status, SPF, and p53 mutation in Histopathology of Benign and Malignant Breast Tumor from 49 Patients*

| Histopathology | No. of patients | Aneuploid (%) | p53 mutation (%) | Mean SPF (%±SD) in aneuploid |
|---|---|---|---|---|
| Benign tumor | 18 | 0 | 0 | |
| Mastopathy | 9 | 0 | 0 | |
| Fibroadenoma | 9 | 0 | 0 | |
| Malignant tumor | 31 | 24 (77.4) | 15 (48.4) | 15.5±6.7* |
| Papillotubular ca | 23 | 19 (82.6) | 10 (43.5) | 15.0±6.7** |
| Solid-tubular ca | 5 | 3 (60.0) | 3 (60.0) | 14.7±8.1 |
| Scirrhous ca | 3 | 2 (66.7) | 2 (66.7) | 21.0±4.2 |

No significant differences between 3 histopathologically different cancers or in p53 gene mutations.

* The mean SPF (%±SD) of cancers was larger in aneuploid than in diploid 18 benign tumors (7.5±3.6, chi-squared test; $P<0.001$).

** In the case of papillotubular carcinoma, the SPF could not be calculated in 4 (8.2%) patients.

Their mean DNA index was 1.72 (mean SFP was 15.5%) and all p53 mutated cancer cells showed aneuploidy patterns, whereas 9 aneuploidy patterns were detected in the cancers with no mutations. In 18 benign tumors, no p53 mutations or aneuploidy patterns were shown (Table 2). In comparison between cytological findings and DNA ploidy patterns, definitive malignant cytology (Class IV and V) has a significant value in aneuploidy pattern. In case of benign and borderline tumors (Class II, IIIa, and IIIb), each tumor with an aneuploidy pattern proved histopathologically to be malignant (Table 3). As for the ER, a tendency to a negative receptor pattern was shown in advanced clinical stage, while the cancers over 2 cm in size tended strongly to be positive, but were not distinctive in terms of histologic grade or menopausal status (Table 1). According to the multivariate analyses by Kallioniemi *et al.*(26), the content of progesterone receptors (PR) which related to the ER was a better prognostic indicator. They also reported a highly significant association between DNA ploidy and absence of PR, which may be a useful prediction for the response to endocrine cancer treatment. As Hedly *et al.*(14) found that DNA ploidy was not significantly related to menopausal status, this relationship remains controversial.

Joensuu *et al.*(18) have demonstrated in a multivariate analysis that the combination of DNA ploidy, DNA index and SPF is an independent prognostic factor in ductal breast carcinoma, but few if any researchers have taken this concept up. It remains to be determined how DNA ploidy is related to various clinicopathological parameters and what the prognostic value of DNA ploidy after a long follow-up period is. Further accumulation of clinical data along this line is necessary to improve assessment in patients with breast cancer.

To conclude, our study showed that aspiration biopsied specimens used for PCR-SSCP analysis of nuclear DNA yield satisfactory accuracy in evaluation of

**Table 3** *Comparison between Cytological Findings and DNA Ploidy Patterns*

| ABC | | DNA ploidy pattern | | Total |
|---|---|---|---|---|
| | | Diploid | Aneuploid | |
| Class | I | 4* | 0 | 4 |
| | II | 6* | 2 | 8 |
| | IIIa | 5* | 1 | 6 |
| | IIIb | 4 | 5 | 9 |
| | IV | 3 | 6** | 9 |
| | V | 3 | 10* | 13 |

Statistical significances were calculated using the chi-squared test between diploid and aneuploid; *$P<0.001$, **$P<0.005$.

the prognosis of breast cancer. In addition, the assessment of DNA ploidy status using flow cytometry and the estimation of ER content were important to clarify tumor's biological behaviour.

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