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Usefulness of Quantitative Analysis for p53 Protein in Non-small Cell Lung Carcinoma using Flow Cytometry

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This study was designed to provide quantitative analysis using flow cytometry (FCM) and immunohistochemical analysis (IH) for p53 protein in 85 patients who underwent pulmonary resection for non-small cell lung carcinoma (NSCLC). We also examined the relationship among numerical aberrations of chromosome 17 and p53 locus, clinicopathological parameters and patient prognosis in NSCLC. PAb 1801 was used as the primary antibody for p53 and the Fluorescence Index (F.I.) was calculated by FCM. Fifty-six patients (66%) showed a higher F.I. (≧0.5), and had a higher rate of lymph node metastasis, more advanced stage and poor survival, while a positive expression of p53 protein by IH was associated with no clinicopathologic factors or patient survival. The F.I. of p53 protein was significantly higher in cases with imbalanced numbers between chromosome 17 and the p53 locus, particularly in patients with higher F.I. This indicated that quantitative analysis by FCM was the most useful method to detect the over-expression of p53 protein compared to that by IH. Higher F.I. (≧0.5) is a prognostic indicator for predicting malignant behavior and poor survival in patients with NSCLC.

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Introduction

It is well known that the p53 gene located in the short arm of chromosome 17 is a tumor suppressor gene.[1] Loss of heterozygosity or mutation of the p53 tumor suppressor gene, and its overexpression has been detected in malignant tumors, including lung carcinoma, which may accelerate the development of tumors.[1-4] Thus, the relationship between p53 abnormalities and patient prognosis has been clarified in malignant tumors. Immunohistochemical analysis has been widely used to detect the overexpression of variant p53 protein without qualitative analysis.[5] With the recent advance of cytometric technology, quantitative analysis of p53 protein using flow cytometry (FCM) is now feasible.[6-8] However, to our knowledge, this analysis has not been performed in lung carcinoma and its relationship to patient survival has not been clarified.[9] The significance of FCM analysis for p53 overexpression compared to immunohistochemical analysis remains controversial.[10]

In this study, we examined the flow-cytometric analysis and immunohistochemical analysis (IH) of variant p53 protein in 85 patients with non-small cell lung carcinomas (NSCLC) who underwent pulmonary resection. Furthermore, numerical aberrations of chromosome 17 and the p53 locus were analyzed using fluorescence in situ hybridization (FISH) with chromosome-specific and cosmid DNA probes. The aim of this study was to clarify the relationship between p53 overexpression, and clinicopathological features, patient prognosis or numerical aberrations of p53 locus.

Materials and Methods

Patient demographics

Data were collected during surgery from 85 patients with NSCLC who were admitted to the Division of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between January 1994 and December 1998. Prior to surgery for NSCLC, no patients received chemotherapy or radiation therapy. They included 67 males and 18 females with a mean age of 63.5± 10.2 years (range, 31-81 years). NSCLC included 44 adenocarcinomas and 41 squamous cell carcinomas. Radical resection was performed to remove the lung tumor, leaving no residual tumor and all pulmo-

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nary tumors were completely resected. After surgery, 13 patients (15%) received adjuvant cisplatin chemotherapy, while it has no definite advantage in prognosis.[11] The minimum follow-up period after pulmonary resection of NSCLC was 5 years. The study design was approved by the Ethics Review Board in the NUGSBS, and written informed consent for treatment was obtained from each patient. Clinical data were collected by the Division of Surgical Oncology in the NUGSBS database. Assessment of each factor was confirmed by histopathological examination of the resected specimen. We used the tumor node metastasis (TNM) classification system of the Japan Lung Cancer Society.[12]

Cell preparation

Tissue samples were obtained from viable portions of lung tumors and were stored at -80°C or fixed in 10% formalin embedded in paraffin. Thin sections (4 μm) were deparaffinized twice with xylene and rehydrated in a series of ethanol solutions (100, 90 and 80%)

Flow cytometry

Immuno-fluorescence stain by FCM was performed using the method described by Morkve et al.[7,9] Three mm fresh frozen tissues were rewarmed and cut into very small pieces using a scalpel or repeated pipetting. Samples were filtered through 50 μm of nylon mesh and the cell pellet was fixed with 0.5% paraformaldehyde at 4°C for 5 minutes. Samples were then treated with 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) at room temperature for 5 minutes. After blocking non-specific binding with 10% normal rabbit serum for 10 minutes, 200 μl of 400 μl of this sample was subsequently incubated for 30 minutes at room temperature with a mouse anti-monoclonal p53 antibody (1:50 dilution; PAb 1801, Oncor Inc., Gaithersburg, MD) as the primary antibody. This was followed by reaction with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:50 dilution, Cappel Co., West Chester, PA) as the secondary antibody for 30 minutes. The nuclei were counterstained with 20 μg/ml propidium iodide (Sigma Chemical Co.)/0.1% RNAse. Nuclear DNA content and fluoresecein-stained PAb1801 of 10,000 nuclei per specimen was simultaneously measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed using Consort 30 software (Becton Dickinson).

All samples with a nuclear DNA index not equal to 1 were considered DNA aneuploid. The fluorescein data between samples with the PAb 1801 antibody and without the primary antibody (control) were compared. The Fluorescence Index (F.I.), from the method by Remvikov et al.,[6,8] was evaluated, and calculated as the fluorescence ratio between samples with PAb 1801 and the control (Figure 1). F.I. was considered the quantitative value. F.I. in the DNA aneuploid cells was determined as the representative F.I. of each case.

Immunohistochemical staining[5]

Frozen sections were incubated for 60 minutes at room temperature with a mouse anti-monoclonal p53 antibody (1:100 dilution; PAb 1801, Oncor Inc.) as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) Kit® peroxidase (Daco Co., Carpinteria, CA). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3, 3'-diaminobenzidine using a light microscope (×200). A positive nuclear expression of p53 detected in more than 10% of tumor cells in each 5 high power fields was interpreted as a p53-positive tumor.[13]

Fluorescence in situ hybridization (FISH)

Single cell suspensions were prepared using citrate buffer trypsin (pH 7.6) and isolated cells were fixed with methanol/acetic acid (3:1) solution at -20°C. Fixed cells were dropped onto glass slides coated with poly-L-lysine (Sigma Chemical Co.). The slide was pretreated with 70% acetic acid for 90 seconds, followed by 10-min acetylation in 0.25% acetic anhydrate. FISH was performed using the method described by Pinkel et al.,[14] with modifications reported by Yasutake et al.[15] The glass slides were washed and then heated with 70% foramide/2 × standard saline citrate (SSC) at 70 °C to induce denaturation of the target DNA. The samples were then dehydrated in serial ethanol solutions, and 20 μl of DNA probe, which had been denatured in advance, was placed on each slide. We used a biotinylated chromosome 17-specific DNA probe (Oncor) and a p53 cosmid probe (Oncor). The samples were incubated in a humid chamber to induce hybridization. The hybridized samples were incubated with 5 μg/ml of FITC-avidin DCS (Vector Labo., Burlingame, CA) at 37°C for 30 minutes. The signal was intensified with a biotinylated anti-avidin antibody (Vector) and FITC-avidin DCS. The nucleus was counterstained with 1.0μg/ml of antifade propidium iodide, and 200 nuclei were observed under a fluorescence microscope with a ×1000 oil-immersion lens (LSM-10; Carl Zeiss, Oberkochen, Germany)

Control hybridization to normal peripheral blood lymphocytes and normal lung cells was performed to confirm that the hybridization efficiency of the test and the reference probes was similar. A cell population with a gain (>2) or loss (<2) of copy numbers of chromosome 17 was determined when it constituted 20% or 15%, respectively, as the mean±3SD of gain and loss observed in normal cells was less than 20% and 15% of the total cells. Cell populations with a gain (the copy number of p53 loci was greater than that of chromosome 17) or loss (the copy number of p53 loci was less than that of chromosome 17) of the copy number of the p53 locus were determined when they constituted 30% of the total cells, because the mean±3SD of gain and loss observed in normal cells was less than 30%.[16,17]

Statistical analysis

For univariate analysis, categorical data were analyzed by the chi-

square test or Fisher's exact test. The disease-free interval and overall survival were calculated according to the Kaplan-Meier method,[18] and differences between groups were tested for significance using the log-rank test. A two-tailed p-value less than 0.05 was considered significant. Statistical analyses were performed using STATISTICA™ software (Stat Soft, Tulsa, OK).

Results

Quantitative measurement of p53 protein by flow cytometry

In this present series, 70 patients (82%) had aneuploid DNA by FCM (Table 1). In the aneuploid DNA group, the F.I. of p53 overexpression in the aneuploid area was higher than that in the diploid area in all cases (Figure 1). The F.I. data were divided into two groups; F.I.<0.5 and F.I.≧0.5, and the relationship between the F.I. value and the clinicopathological factors is shown in Table 1. The incidence of F.I.≧0.5 was significantly higher in patients with lymph node metastasis and in advanced stages ($p<0.05$), while the p53 overexpression detected by immunohistochemical stain was not associated with these parameters but only with poor histological differentiation. F.I.≧0.5 was observed in 23 of 44 patients (52%) without p53 overexpression by immunohistochemical stain (Table 2).

Relationship between the fluorescence intensity of p53 in NSCLC and patient survival.

Twenty (24%) of 85 patients died of primary cancer and the mean follow-up period was 460 days. Figure 2 shows the overall survival in patients who underwent pulmonary resection in this study. Patients with F.I.≧0.5 had significantly poor survival after surgery compared to those with F.I.< 0.5 ($p<0.05$). Even in patients without lymph node metastasis, survival in patients with F.I. ≧0.5 was significantly poor than in those with F.I.<0.5 (Figure 2 b) ($p<0.05$), while the p53 status by immunohistochemistry was not associated with patient prognosis.

Relationship between fluorescence intensity of p53 and chromosomal numerical aberrations by fluorescence in situ hybridization.

In normal alveolar cells, disomic cells (2 centromeric spots of chromosome 17) and two signals of p53 locus were observed in 94% and 91% of 200 cells, respectively. FISH analysis was performed in 57 patients with NSCLC. The loss of p53 loci was ob-

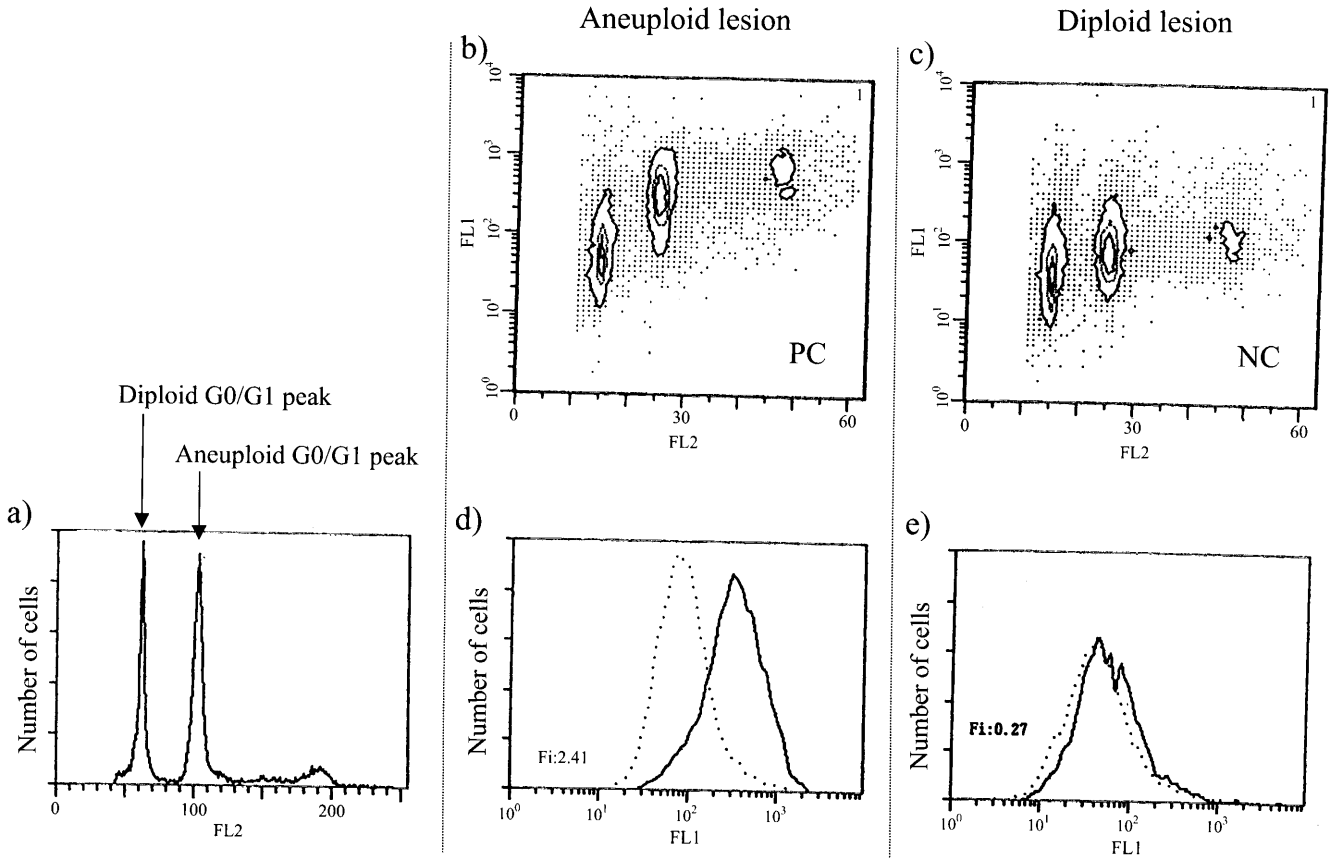


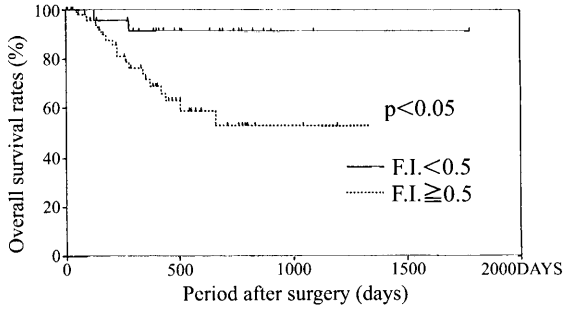
Figure 1. Two-parameter cytogram between nuclear DNA content (FL2; propidium iodide) and PAb 1801(FL1; FITC). a) Histogram of nuclear DNA content. CV (coefficient of variation) =2.8; DI (DNA index) =1.7. b) Cytogram of both FL1 and FL2 in the DNA aneuploid area. c) Cytogram of both FL1 and FL2 in the DNA diploid area. d) Histogram of PAb 1801 in the DNA aneuploid area. e) Histogram of PAb 1801 in the DNA diploid area.

Table 1 Relationship between p53 overexpression detected by flow cytometry and immunohistochemistry, and clinicopathological parameters in NSCLC

Clinicopathological parameters	FCM[b]		IHC[c]	
	F.I.≧0.5 (n=56)	F.I.<0.5 (n=29)	Positive (n=41)	Negative (n=44)
T factor				
T1 (n=23)	15	8	11	12
T2 (n=40)	25	15	18	22
T3 (n=11)	8	3	6	5
T4 (n=11)	8	3	6	5
Lymph node metastasis				
N0 (n=46)	25[d]	21	22	24
N1 (n=9)	7	2	6	3
N2 (n=30)	24	6	13	17
Distant metastasis				
M0 (n=80)	52	28	40	40
M1 (n=5)	4	1	1	4
TNM stage[a]				
I (n=31)	15[d]	16	14	17
II (n=7)	5	2	5	2
IIIA (n=31)	24	7	16	15
IIIB (n=11)	8	3	5	6
IV (n=5)	4	1	1	4
Histological differentiation				
Well (n=24)	16	8	7	17
Moderately (n=38)	21	17	19	19
Poorly (n=17)	14	3	13[d]	4
DNA ploidy				
Diploid (n=15)	10	5	4	11
Aneuploid (n=70)	46	24	37	33
Histological typing				
Squamous cell carcinoma (n=41)	24	17	18	23
Adenocarcinoma (n=44)	32	12	23	21

[a]According to *Classification of Lung Cancer* by The Japan Lung Cancer Society.[12]
[b]Flow cytometry.
[c]Immunohistochemistry.
[d]$p<0.05$ by chi-square test.

a) Overall survival in all patients



b) Overall survival in patients without lymph node metastasis

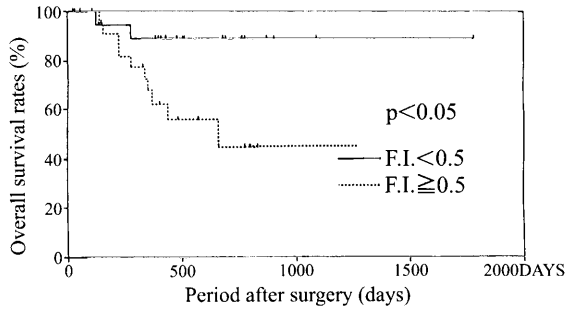


Figure 2. Overall survival of patients who underwent pulmonary resection for NSCLC. a) All patients. b) Patients without lymph node metastasis. Comparison of F.I. (≧0.5) and (<0.5).

Table 2 p53 status according to flow cytometry and immunohistochemical stain

p53 status	F.I.≧0.5 (n=56)	F.I.<0.5 (n=29)
Immunohistochemical stain		
p53 positive (n=41)	33	8
p53 negative (n=44)	23	21

Table 3 Relationship between p53 overexpression by flow cytometry and numerical aberrations of chromosome 17 and p53 locus by fluorescence in situ hybridization

Aberration	F.I.≧0.5 (n=37)	F.I.<0.5 (n=20)
Ch 17 centromere = p53 locus[a]	2	7
p53 loss	33	12
p53 gain	2	1

[a]The copy number of chromosome 17 and p53 loci had the same predominance.

served in 45 patients (79%), the gain of p53 loci was observed in 3 (5%), and no imbalance between chromosome 17 and p53 loci was observed in 9 (16%). Table 3 shows the relationship between F.I. and FISH analysis. The incidence of p53 loss or gain in patients with F.I.≧0.5 was significantly higher than that in patients with F.I.<0.5 ($p=0.014$), while the incidence of p53 overexpression by immunohistochemical stain was not associated with these p53 locus abnormalities detected by FISH (data not shown).

Discussion

In the 1990s, Morkve et al. and Remvikovs et al. reported quantitative analysis of the overexpression of variant p53 protein using FCM,[6-8] although the immunohistochemical study was widely used for the detection of p53 overexpression. Remvikos et al. reported that F.I.≧0.5 was significantly associated with poor survival in patients with colorectal carcinoma.[6,8,19] Our results also showed that F.I.≧0.5 was related to the malignant behavior of lung cancers and poor patient survival. These results suggested that the overexpression of variant p53 protein may be associated with tumor aggression in solid cancers. Carbone et al. and others reported a significant relationship between p53 overexpression or mutation and poor survival in NSCLC patients.[20-22] However, Westra et al. reported that p53 abnormality was not associated with survival in NSCLC patients by immunohistochemical analysis.[23-25] Thus, the relationship between p53 and patient survival in NSCLC is still controversial, and therefore, more sensitive and quantitative analytical methods than immunohistochemical stain are necessary. In this study, the quantitative measurement by FCM showed a difference in results compared with immunohistochemical stain, and the F.I. of p53 overexpression by FCM had more relationship significance with tumor aggression. Higher F.I. was observed in the patients with negative p53 expression

by immunohistochemistry. This indicated that FCM analysis can detect subtle expressions of the target protein more sensitively. Another advantage of FCM analysis is the measurement of target proteins in each DNA diploid and aneuploid cell in a specimen. It is possible to measure the protein expression only in cells with malignant behavior using FCM. Our results showed that the F.I. of p53 protein in DNA aneuploid cells was higher compared to DNA diploid cells. The overexpression of variant p53 protein may be more active in DNA aneuploid cancer cells. Cancers without lymph node metastasis have lower malignancy.[26] However, interestingly, the prognosis of patients with higher F.I. was very poor even in patients without lymph node metastasis. The role of variant p53 protein in NSCLC may be important for tumor progression as well as TNM factors.

Chromosome alterations are variously observed in NSCLC.[27] The overexpression of p53 protein is caused by genetic abnormalities of the p53 gene located on chromosome 17p13.1.[1-4] This locus of the p53 gene can be clearly detected by the FISH method.[16,17] At this stage, the relationship between p53 overexpression and numerical aberrations of the p53 locus or chromosome 17 has not been clarified, and we hypothesized this relationship. Tsuji et al. reported that loss of the p53 locus on chromosome 17 was related to p53 point mutation or the loss of heterozygosity of chromosome 17.[28] Nanashima et al. reported that the loss or translocation of the p53 locus was closely related to malignant potential in patients with colorectal carcinomas.[16] In this study, numerical aberrations of p53 locus were observed in 86% of patients and p53 loss was a predominant alteration. Loss of p53 was frequently observed in breast cancer,[29] colorectal cancer16 and hepatocellular carcinomas.[17] Furthermore, higher F.I. of p53 overexpression detected by FCM was significantly associated with an imbalance of the copy number between chromosome 17 and the p53 locus. This result indicated the relationship between p53 overexpression and numerical alterations of the p53 locus, while the p53 status by immunohistochemistry could not show such a relationship.

In conclusion, the detection of p53 overexpression using flow cytometry was significantly superior to the immunohistochemical method because of the increased sensitivity for detecting subtle abnormalities of the target proteins. Compared to immunohistochemical analysis, the results of p53 overexpression by FCM were more significantly associated with tumor aggression and poorer prognosis in patients who underwent pulmonary resection for NSCLC. Overexpression of p53 was significantly associated with imbalance of the copy number between chromosome 17 and p53 locus. Quantitative analysis of p53 overexpression by FCM is a useful tool for predicting malignant behavior and poor survival in patients with NSCLC.

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