p53 Gene Mutations Are Associated with Shortened Survival in Patients with Advanced Non-small Cell Lung Cancer: An Analysis of Medically Managed Patients[1]

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ABSTRACT

Mutations in the *p53* gene are common in many cancers. Nevertheless, the relationship between mutations of this tumor suppressor gene and patient survival in non-small cell lung cancer (NSCLC) remains unclear. Interpretation of prior studies of patient outcomes are complicated by the inclusion of both surgical and nonsurgical patients. To better isolate the potential effects of *p53* gene mutations *per se* on tumor progression, we chose to examine patients with advanced disease in whom surgery was not performed (stages IIIA, IIIB, and IV). We have used PCR-denaturing gradient gel electrophoresis, a sensitive and specific method for the detection of a variety of *p53* mutations in cytology or biopsy specimens, to evaluate the prognostic significance of *p53* gene mutations in nonsurgical patients with advanced NSCLC. In 70 consecutive medical patients, *p53* mutations were found in 29 cases (41%) at the time of initial diagnosis. Followed prospectively, patients with *p53* mutations had a significantly reduced survival time after diagnosis than those without mutations (median survival, 17 *versus* 39 weeks; $P = 0.0003$) independent of other clinical factors. This abbreviated survival occurred in both patients who received chemotherapy ($n = 39$, $P = 0.002$) or best supportive care ($n = 31$, $P = 0.018$). These results indicate that mutations of the *p53* gene in patients with NSCLC who do not undergo surgical resection portends a significantly worse prognosis.

INTRODUCTION

The *p53* tumor suppresser gene is critically involved in the regulation of cell proliferation and cell death. It is commonly mutated in a variety of human tumors including breast, stomach, colorectal, bladder, and NSCLC[3] (1–3). Although many alterations of *p53* including deletions, splicing, and overexpressing mutants have been identified in a variety of tumors, their value in predicting prognosis has been variable (4–8). Previous detailed studies of gene mutations or protein expression in tumor tissues have been performed primarily on surgical specimens because technical limitations precluded analyses of smaller biopsy and cytology specimens. As a result, nearly all studies of *p53* mutations were limited to groups of patients who underwent surgical resection. This significant bias may have partially or completely obscured important biological effects of *p53* mutations on the rate of tumor progression. To prospectively examine the biological effects of *p53* alterations in lung cancer in a nonbiased fashion, we chose to study patients in whom surgery was not performed (stage ≥IIIA). We have reported previously that *p53* gene mutations could be detected in diagnostic cytology or biopsy specimens by PCR-DGGE (9). Using this method, we analyzed *p53* gene mutations in tumors from 70 patients with NSCLC at the time of diagnosis and then prospectively followed their survival.

MATERIALS AND METHODS

Patients. All patients with advanced-stage NSCLC (stage >IIIA) who did not undergo surgical resection but had diagnostic tissue from either the National Hiroshima Hospital or Hiroshima University Hospital from February 1992 to December 1997 were enrolled in the study. All specimens were obtained during diagnostic bronchoscopy, thoracentesis, or percutaneous needle aspiration. Patients gave informed consent before entering the study, and the research protocol was approved by each Institutional Review Board. Tumor stage and progression were classified according to the International Staging System (10).

Tumor Samples and DNA Preparation. All DNA preparation was performed from cytology-positive samples. For specimens obtained by brushing or curetting, a sample of cells was applied directly onto glass slides for diagnosis, and the brush or curette was then placed in a microcentrifuge tube containing 1.5 ml of saline and agitated manually to dislodge the residual cells into the solution. The tube was then centrifuged at

$7000 \times g$ for 5 min. The supernatant was discarded, and the cell pellet was stored at $-80°C$ until DNA extraction. Biopsy samples were pressed onto glass slides for cytological examination (touch preparation), and the remaining tissue sample was transferred into a 1.5-ml tube and stored at $-80°C$ until DNA extraction. Pleural effusions were divided, with half sent for clinical cytopathological examination and half for centrifugation and cell pellet analysis. Peripheral blood samples (2 ml) were taken from each patient to obtain genomic DNA from peripheral blood leukocytes. Genomic DNA was extracted from peripheral blood leukocytes using proteinase K, followed by phenol/chloroform extraction and ethanol precipitation.

PCR-DGGE Analysis for *p53* Mutations in Exons 3–9. We examined exons 3–9 in the *p53* gene by the PCR-DGGE method, because previous studies have shown that most of the mutations occurring in NSCLC are found in this region (11, 12). The oligonucleotide primers used to amplify the *p53* genes were synthesized as described previously (9, 13). The genomic DNA (10–100 ng) was amplified in a 50-μl reaction tube containing 200 mM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.25 μM each primer, and 1 unit of *Taq* DNA polymerase (Wako, Osaka, Japan). A programmable temperature control system (TaKaRa, Ohtsu, Japan) was used to subject the DNA to 40 cycles of amplification. DGGE analysis of the PCR-amplified genomic DNA fragments was carried out, as described previously (9). Corresponding peripheral blood obtained from each patient with abnormal mobility shifts of the *p53* gene in the tumor sample was also examined by PCR-DGGE analysis to exclude the possibility of any genomic polymorphism. PCR-DGGE analysis was repeated at least twice to exclude amplification errors (false-positives).

Statistical Analysis. The Fisher's exact test was used to compare the association between the incidence of any *p53* mutation and several clinical and pathological parameters. The Kaplan-Meier method (14) was used to estimate the probability of survival as a function of time (starting from the date of diagnosis to that of death from cancer), and the log-rank test (15) was used to analyze survival differences. The Cox proportional hazards modeling technique (16) was used to identify factors that significantly influenced overall survival, either independently or together. $P < 0.05$ was considered to be statistically significant.

RESULTS

Patients. A total of 70 consecutive patients were entered into the study, and all patients were eligible for *p53* gene mutation analysis. Complete follow-up information was available on all patients. There were 51 men and 19 women, with ages ranging from 37 to 91 years (median, 66 years): 49 patients with adenocarcinoma, 19 with squamous cell carcinoma, and 2 with large cell carcinoma; and 12 patients with clinical stage IIIA, 18 with stage IIIB, and 40 with stage IV at the time of diagnosis. Thirty-nine of the 70 patients received cisplatin- or carboplatin-based chemotherapy, and the other 31 patients were treated with supportive care only. No patient received nonpalliative irradiation. At the time of this report, 67 patients had died of lung cancer, and three patients were alive with survival periods of 98, 109, and 140 weeks.
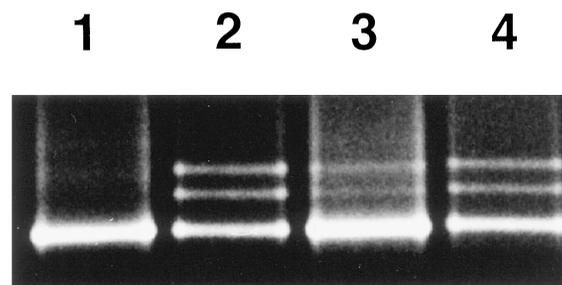
Fig. 1 DGGE analysis of the *p53* gene of clinical specimens. Consecutively obtained samples from a patient with adenocarcinoma of lung were analyzed. The PCR-amplified exons 8–9 were electrophoresed on a denaturing gradient gel and stained with ethidium bromide. Extra bands are seen in *Lanes 2–4*. *Lane 1,* samples from peripheral blood leukocytes as a control. *Lane 2,* transbronchial brushing cytology sample at first diagnosis. *Lane 3,* samples from malignant pleural effusion at recurrence after chemotherapy. *Lane 4,* autopsy sample.

Tissue Analysis. Diagnostic specimens consisted of 5 bronchial biopsies, 45 bronchial brushings or curettetings, 17 pleural taps, and 3 percutaneous needle aspiration biopsy samples. The amount of tissue obtained was sufficient for analysis in all cases. As shown in the Fig. 1, DNA was sufficiently extracted for PCR from any type of samples including transbronchial brushing, pleural effusion, or autopsy tissues as well as control blood cells.

Rate of *p53* Gene Mutations. *p53* gene mutations were found in 29 of 70 (41%) cases with NSCLC. The locations of the mutations in the gene did not show any predilection among the exons that we studied. Three samples had the mutations in exons 3–4, 6 in exon 5, 6 in exon 6, 6 in exon 7, and 8 in exons 8–9. According to histological typing, one or more *p53* mutations were observed in 18 of 49 (37%) adenocarcinomas, 9 of 19 (47%) squamous cell carcinomas, and 2 of 2 (100%) large cell carcinomas. Four patients of 12 (33%) with stage IIIA, 5 of 18 (28%) patients with stage IIIB, and 20 of 40 (50%) patients with stage IV disease had one or more *p53* mutations in their tumors at the first diagnostic examination.

Association of *p53* Mutations with Clinical Features. We examined the relationship between the presence of any *p53* mutation and several important clinical parameters to test whether clinical features predict the presence of mutations (Table 1). By Fisher exact testing, there was no significant relationship between *p53* mutations and age at diagnosis (>70 *versus* <70 years of age), sex (male *versus* female), serum LDH level (normal *versus* abnormal), Eastern Cooperative Oncology Group scale performance status (0–1 *versus* 2–4), clinical stage (III *versus* IV), histological type (adenocarcinoma plus large cell carcinoma *versus* squamous cell carcinoma), body weight loss (5% or over *versus* under), or smoking history (more *versus* less than 50 pack-years).

Prognostic Value of *p53* Mutations by Univariate Analysis. We analyzed differences in survival in the patients by the presence or absence of any *p53* mutations by univariate analysis. The patients with *p53* mutations survived for a significantly shorter period after diagnosis than those without the mutations ($P = 0.0003$, log rank test; Fig. 2). MSTs were $>$2-fold longer

Table 1 Relationship between *p53* mutation positivity and clinical features with advanced NSCLC
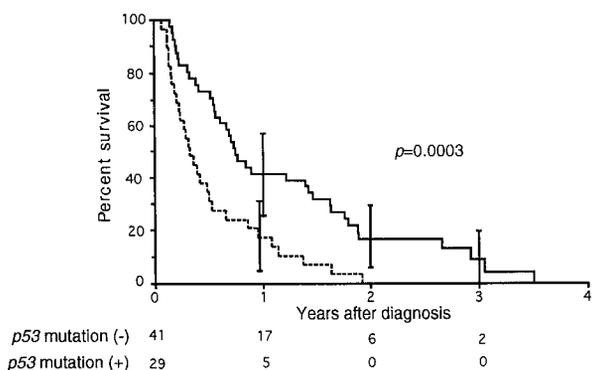
	p53 mutation		
	Positive	Negative	P^a
Sex			
Male	25	26	0.550
Female	4	15	
Age (yr)			
<70	20	19	0.088
≥70	9	22	
Histological type			
Squamous cell carcinoma	9	10	0.538
Adenocarcinoma plus large cell carcinoma	20	31	
Clinical stage			
III	9	21	0.141
IV	20	20	
Performance status			
0–1	20	28	
2–4	9	13	>0.999
Loss of body weight			
<5%	22	35	0.361
>5%	7	6	
Serum LDH			
Normal	7	18	0.129
Abnormal	22	23	
Smoking (pack-years)			
0–49	17	28	0.454
<50	12	13	

a 2×2 Fisher's exact test was used.

Table 2 Univariate analysis of various parameters as prognostic factors in patients with advanced NSCLC

Prognostic factor	No. of patients	Median survival time (weeks)	P^a
p53 mutation			
Negative	41	39	0.0003
Positive	29	17	
Sex			
Male	51	28	0.190
Female	19	35	
Age (yr)			
<70	39	37	0.519
≥70	31	26	
Histologic type			
Squamous cell carcinoma	19	29	0.629
Adenocarcinoma plus large cell carcinoma	51	28	
Clinical stage			
III	30	36	
IV	40	22	0.024
Performance status			
0–1	48	45	<0.0001
2–4	22	11	
Loss of body weight			
<5%	57	34	0.054
≥5%	13	28	
Serum LDH			
Normal	25	50	0.015
Abnormal	45	17	

a Log-rank test was used to analyze survival differences.

Fig. 2 Kaplan-Meier survival curve in all patients with advanced stage NSCLC with *p53* gene mutations (*dashed*) and without mutations (*solid line*). *Bars,* 95% CI at each year. The number of patients at risk is shown *below* the graph.

in patients without any mutation (17 *versus* 39 weeks in positive *versus* negative patients; Table 2). Both chemotherapy and supportive-care cases with *p53* mutations showed significantly worse survival than those without *p53* mutations (chemotherapy cases: *n* = 39, MST = 28 *versus* 47 weeks, *P* = 0.017; supportive care cases: *n* = 31, MST = 10 *versus* 21 weeks, *P* = 0.032; Fig. 3).

Cox Multivariate Regression Analysis. To confirm that the prognostic value of *p53* mutations was independent of other clinical factors, we performed Cox multivariate regression analysis using age, sex, serum LDH, performance status, clinical
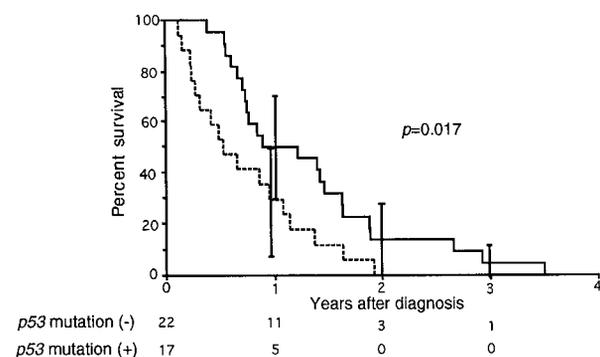
stage, histological type, body weight loss, and *p53* mutations as variables. The presence of any *p53* mutation was an independent prognostic factor with a HR of 3.43 *(*95% CI, 1.99–5.88, *P* < 0.001; Table 3). The only other independent prognostic factor was performance status (HR, 5.38; 95% CI, 2.73–10.6; *P* < 0.001). To exclude the possibility that the survival disadvantage of *p53* gene mutations in NSCLC simply predicted a poor response to chemotherapy, we separately analyzed treated and nontreated cases. The existence of any *p53* mutations was again an independent poor prognostic factor in both the chemotherapy cases (*n* = 39; HR, 3.25; 95% CI, 1.55–6.97; *P* = 0.002) and in patients who received supportive care only (*n* = 31; HR, 4.24; 95% CI, 1.36–15.5; *P* = 0.018; Table 3).

DISCUSSION

We have reported previously that *p53* gene mutations could be detected in extremely small clinical samples, such as cytopathology or biopsy specimens, after diagnostic procedures such as flexible fiberoptic bronchoscopy, thoracentesis, and percutaneous needle aspiration using PCR-DGGE (9). This approach allows us to examine specimens obtained from nonsurgical patients. Little is known about *p53* gene mutations in advanced NSCLC, where surgically resected specimens are not available. Our data from 70 patients with advanced NSCLC indicate that *p53* gene mutations at the time of diagnosis portend a poor prognosis after adjustment for other clinical factors.

In this report, *p53* gene mutations were detected in 41% of the patients with NSCLC. This is consistent with previous reports of prevalence rates of 35–40% (6, 17–20). By histological subtype, *p53* mutations were detected in 37% of adenocar-

A) Chemotherapy cases (n=39)
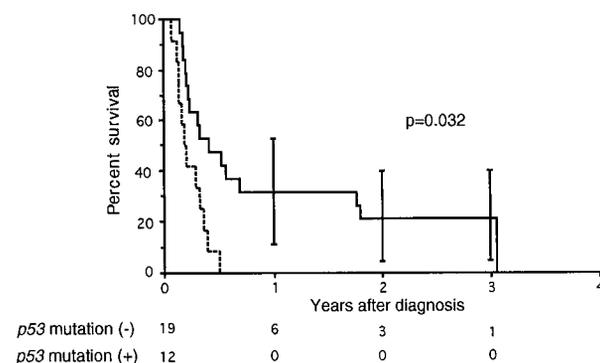

B) Supportive care cases (n=31)

Fig. 3 Kaplan-Meier survival curve in patients with advanced stage NSCLC by treatment. *Dashed curves,* survival of patients with *p53* gene mutation; *solid curves,* without mutation. *Bars,* 95% CI at each year. The number of patients at risk is shown *below* the graph.

Table 3 HR of *p53* mutation positive *versus* negative patients with advanced NSCLC by Cox regression analysis[a]

Population	Hazards ratio	*P*	95% CI
All patients ($n = 70$)	3.43	<0.001	1.99–5.88
Patients received chemotherapy ($n = 39$)	3.25	0.002	1.55–6.97
Patients received supportive care only ($n = 31$)	4.24	0.018	1.36–15.5

[a] Variables used were age, sex, serum LDH level, performance status, clinical stage, histological type, body weight loss, and *p53* mutation.

(29), making this technique insensitive for mutational analysis. In contrast, PCR-DGGE is sufficiently sensitive for detailed analysis of cytological as well as biopsy samples. This sensitivity allowed us to study patients who did not undergo surgery at any point during the course of their illness.

To our knowledge, there have been only two other reports from groups describing *p53* mutation in samples obtained in a nonsurgical setting (30, 31). Mitsudomi *et al.* (30) obtained bronchial biopsy samples ($n = 4$) using a fluorescent broncho-scope system and detected *p53* gene mutations using PCR/single-strand conformation polymorphism assay. Fluorescent bronchoscopy, however, is applicable only to patients with endobronchial lesions. Safran *et al.* (31) detected *p53* mutations in paraffin-embedded tumor tissues of advanced NSCLC patients (stages IIIA and IIIB) by PCR/single-strand conformational polymorphism assay. Samples from 30 patients (47%) were analyzed in this study. Both studies are limited, however, by their retrospective design. In contrast, the PCR-DGGE assay used in this study facilitates analysis of either cytological or biopsy specimens from patients with NSCLC and allowed prospective correlation with clinical outcomes.

We have also chosen patients with advanced stage NSCLC, who were managed medically to avoid the biases of many previous studies in which only surgical patients were studied. Survival periods of these patients may be significantly and systematically altered by clinical management masking a significant biological effect of *p53* mutations. Differences in patient selection, surgical approach, in addition to the use of adjuvant and neoadjuvant radiation and chemotherapy, are additional important potential confounding factors in many of these studies (6, 18). In this context, gene mutation analysis on nonsurgical subjects is most likely to shed light on the true biological importance of *p53* mutations on the growth and lethality of the primary tumor.

In summary, we have observed that a variety of *p53* mutations portend poor survival in patients with NSCLC who are medically managed. Mutations were a negative prognostic factor in both univariate or multivariate analysis. This effect was equivalent in patient who received chemotherapy or supportive care alone. These results show that evaluation of *p53* mutations at the time of diagnosis is feasible and carries important prognostic information. Thus, further analysis of the effects of the *p53* gene mutations on tumor responses to chemotherapy could provide further insights into individual tumor biology, allowing customization of the treatment of advanced NSCLC.

cinomas, 47% of squamous cell carcinomas, and 100% of large cell carcinomas. These frequencies are also comparable with previous reports: 18–45% in adenocarcinoma, 29–58% in squamous cell carcinoma; and 17–85% in large cell carcinoma. These data confirm the feasibility of detecting the *p53* gene mutations in a nonsurgical diagnostic setting using the PCR-DGGE assay.

Although *p53* gene mutations are common in lung cancer, the importance of these mutations in the clinical course of patients has been unclear. Many authors have examined whether the prognosis of NSCLC patients varies according to *p53* status. Although studies describing worse prognosis in patients with abnormal *p53* predominate (4, 6, 17, 21–23), others have reported the opposite effect (7, 24). Still other studies have noted no effect of *p53* mutations of prognosis (5, 20, 25, 26).

In this study, we have used a gene mutation analysis technique, PCR-DGGE, which we have shown works at high sensitivity in extremely small diagnostic samples (9). We have chosen this method over immunostaining of transbronchial biopsy samples (27), because alterations in antibody binding are not perfectly concordant with genetic alterations (28). Significant derangements such as nonsense mutations, splicing mutations and gross deletions are not detectable by immunostaining

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