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

Isao Murakami, Keiko Hiyama, Shinichi Ishioka, Michio Yamakido, Fumiyoshi Kasagi, and Yasuyuki Yokosaki

Departments of Internal Medicine and Laboratory Medicine, National Hiroshima Hospital, Higashi-Hiroshima 739-0041 [I. M., Y. Y.]; Second Department of Internal Medicine, Hiroshima University, School of Medicine, Hiroshima 734-8551 [K. H., S. I., M. Y.]; and Department of Statistics, Radiation Effects Research Foundation, Hiroshima 732-0815 [F. K.], Japan

**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 suppressor 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 (1–3). Although many alterations of p53 including deletions, splicing, and overexpressing mutations 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 × 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 MgCl₂, 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 palliative 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.

**Tissue Analysis.** Diagnostic specimens consisted of 5 bronchial biopsies, 45 bronchial brushings or curettages, 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, 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 years), 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 for patients with p53 mutations.
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-
DGGE assay. These data confirm the feasibility of detecting the
p53 gene mutations in a nonsurgical diagnostic setting using the PCR-
dGGE 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 bi-
opsy samples (27), because alterations in antibody binding are
not perfectly concordant with genetic alterations (28). Signifi-
cant derangements such as nonsense mutations, splicing muta-
tions and gross deletions are not detectable by immunostaining
(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 sensitiv-
ity 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 pa-

tients (stages IIIA and IIIB) by PCR/single-strand confor-
mational 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 pro-
spective 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 sig-
nificant biological effect of p53 mutations. Differences in pa-

tient selection, surgical approach, in addition to the use of
adjuvant and neoadjuvant radiation and chemotherapy, are ad-
ditional 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 mu-
tations portend poor survival in patients with NSCLC who are
medically managed. Mutations were a negative prognostic fac-
tor 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 prog-
nostic 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.

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

<table>
<thead>
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<th>Population</th>
<th>Hazards ratio</th>
<th>P</th>
<th>95% CI</th>
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<td>All patients (n = 70)</td>
<td>3.43</td>
<td>&lt;0.001</td>
<td>1.99–5.88</td>
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<tr>
<td>Patients received</td>
<td>3.25</td>
<td>0.002</td>
<td>1.55–6.97</td>
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<tr>
<td>chemotherapy (n = 39)</td>
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<tr>
<td>Patients received</td>
<td>4.24</td>
<td>0.018</td>
<td>1.36–15.5</td>
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<tr>
<td>supportive care only (n = 31)</td>
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*Variables used were age, sex, serum LDH level, performance
status, clinical stage, histological type, body weight loss, and p53
mutation.

**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.

**Fig. 3** Kaplan-Meier survival curve in patients with advanced stage
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The number of patients at risk is shown below the graph.
REFERENCES


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