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

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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. 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. 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 >III). We have reported previously that p53 gene mutations could be detected in diagnostic cytology or biopsy specimens by PCR-DGGE. 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 >III) 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 prognosis were classified according to the International Staging System.

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 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).

**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.

**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.
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 cytology 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-

### Table 1  Relationship between \( p53 \) mutation positivity and clinical features with advanced NSCLC

<table>
<thead>
<tr>
<th>( p53 ) mutation</th>
<th>Positive</th>
<th>Negative</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>26</td>
<td>0.550</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>20</td>
<td>19</td>
<td>0.088</td>
</tr>
<tr>
<td>( \geq 70 )</td>
<td>9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>9</td>
<td>10</td>
<td>0.538</td>
</tr>
<tr>
<td>Adenocarcinoma plus large cell carcinoma</td>
<td>20</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>III</td>
<td>9</td>
<td>21</td>
<td>0.141</td>
</tr>
<tr>
<td>IV</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Performance status</td>
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<td></td>
</tr>
<tr>
<td>0–1</td>
<td>20</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>2–4</td>
<td>9</td>
<td>13</td>
<td>&gt;0.999</td>
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<td>Loss of body weight</td>
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<tr>
<td>&lt;5%</td>
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<td>35</td>
<td>0.361</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Serum LDH</td>
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<tr>
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<td>7</td>
<td>18</td>
<td>0.129</td>
</tr>
<tr>
<td>Abnormal</td>
<td>22</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
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<td></td>
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<tr>
<td>0–49</td>
<td>17</td>
<td>28</td>
<td>0.454</td>
</tr>
<tr>
<td>(&lt;50)</td>
<td>12</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) 2x2 Fisher’s exact test was used.

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

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>No. of patients</th>
<th>Median survival time (weeks)</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p53 ) mutation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>41</td>
<td>39</td>
<td>0.0003</td>
</tr>
<tr>
<td>Positive</td>
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<td>Sex</td>
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<td>28</td>
<td>0.190</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>39</td>
<td>37</td>
<td>0.519</td>
</tr>
<tr>
<td>( \geq 70 )</td>
<td>31</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

Histologic type

- Squamous cell carcinoma: 19 patients (29 weeks, MST = 12 weeks, 0.629).
- Adenocarcinoma plus large cell carcinoma: 51 patients (28 weeks, MST = 20 weeks, 0.015).

Clinical stage

- III: 30 patients (36 weeks, MST = 28 weeks, 0.024).
- IV: 40 patients (22 weeks, MST = 18 weeks, 0.0001).

Performance status

- 0–1: 48 patients (45 weeks, MST = 38 weeks, 0.0001).
- 2–4: 22 patients (11 weeks, MST = 7 weeks, 0.0001).

Loss of body weight

- <5%: 57 patients (34 weeks, MST = 28 weeks, 0.054).
- ≥5%: 13 patients (28 weeks, MST = 15 weeks, 0.015).

Serum LDH

- Normal: 25 patients (50 weeks, MST = 45 weeks, 0.0015).
- Abnormal: 45 patients (17 weeks, MST = 10 weeks, 0.001).

\( ^a \) Log-rank test was used to analyze survival differences.
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 the 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 (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 bronchoscope 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 IIA 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 patients 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.

\begin{table}
\centering
\caption{HR of p53 mutation positive versus negative patients with advanced NSCLC by Cox regression analysis\textsuperscript{a}}
\begin{tabular}{llll}
\hline Population & Hazards ratio & \multicolumn{2}{c}{95\% CI} \\
\hline 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 \\
\hline
\end{tabular}
\textsuperscript{a}Variables used were age, sex, serum LDH level, performance status, clinical stage, histological type, body weight loss, and p53 mutation.
\end{table}
REFERENCES


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