The Utility of p53 Immunostaining of Transbronchial Biopsy Specimens of Lung Cancer: p53 Overexpression Predicts Poor Prognosis and Chemoresistance in Advanced Non-Small Cell Lung Cancer

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ABSTRACT

There are few reports on the p53 status of small cell lung cancer (SCLC) and advanced non-SCLC (NSCLC) because surgically resected specimens are generally not available. Therefore, we evaluated p53 immunostaining in 175 transbronchial biopsy (TBB) specimens obtained from patients with all stages of lung cancer and retrospectively evaluated the relationship between p53 status and clinical parameters. All of the specimens were obtained prior to therapy. Formalin-fixed, paraffin-embedded TBB specimens were immunostained using an anti-p53 antibody (DO-7). p53 protein was detected in 55% (61 of 111) of NSCLCs and 58% (37 of 64) of SCLCs. The rate of positivity increased significantly with increasing stage (stages I and II, 45%; stage III, 54%; stage IV, 66%), but not with other clinical parameters. Ninety-five patients were evaluated for their response to chemotherapy. Positive staining for p53 correlated significantly with unresponsiveness to chemotherapy. Further, 53% of NSCLCs (8/15) and 68% of SCLCs (6/9) stained positively for p53. p53 immunostaining in TBB specimens predicts poor prognosis and chemoresistance in advanced stage NSCLC.

INTRODUCTION

Lung cancer has become one of the leading causes of death throughout the world (1). The major histological types of lung cancer are SCLC and NSCLC. SCLC frequently is found to be disseminated at presentation, but it is initially highly sensitive to chemotherapy and radiotherapy and is thus treated mainly with these modalities. Nevertheless, median survival with combination chemotherapy is 14-16 months for patients with limited disease and 8-11 months for those with extensive disease (2). In contrast, NSCLC is considered to be chemoresistant and radiation-resistant. Of patients with NSCLC, one-third are treated by surgery, but the long-term survival rate remains unsatisfactory, even in patients who undergo potentially curative resection. Although extensive clinical research has been performed to determine the role of chemotherapy and radiotherapy in the therapeutic management of advanced NSCLC, only small improvements in survival have been reached (3, 4).

Mutation of the p53 gene is one of the most common genetic abnormalities found in various types of human malignancy, and it has been reported to be present in approximately one-half of the lung tumor samples studied (5, 6). Many reports have examined whether the prognosis of NSCLC patients who undergo surgical resection varies according to the p53 status (7-13), using both gene mutation analysis and immunostaining. Still, the relationship between p53 status and prognosis remains controversial. Recently, Rusch et al. (14) reported that p53 overexpression correlates with chemoresistance in operable cases of NSCLC. Loss of wild-type p53 function could lead to relative chemoresistance as a consequence of the abrogation of p53-dependent apoptosis (15).

Because samples are difficult to obtain, there is little data on p53 abnormalities in SCLC (16-18) and advanced NSCLC (8, 10, 12). TBB is an extensively used classic technique (19). In addition to providing a diagnosis. TBB has the benefit of accessing tissue samples that are difficult to obtain, such as those from the peripheral lung. In contrast, NSCLC is considered to be chemoresistant and radiation-resistant. Of patients with NSCLC, one-third are treated by surgery, but the long-term survival rate remains unsatisfactory, even in patients who undergo potentially curative resection. Although extensive clinical research has been performed to determine the role of chemotherapy and radiotherapy in the therapeutic management of advanced NSCLC, only small improvements in survival have been reached (3, 4).

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PATIENTS AND METHODS

Patients and Therapy. Between April 1990 and March 1995, 175 consecutive patients with primary lung cancer, histologically confirmed by TBB at Kyushu University Hospital, were enrolled in this study. The clinical disease stage was...
There were 123 men and 52 women, with ages ranging from 39 to 83 years. Thirty-six of the 39 NSCLC patients received cisplatin-based chemotherapy: 80 mg/m² cisplatin i.v. on day 1, 100 mg/m² carboplatin i.v. on days 2, 3, and 8, and 2 mg/m² vindesine i.v. on days 2, 3, and 8; or 80 mg/m² cisplatin i.v. on day 1 and 3 mg/m² vindesine i.v. on days 1, 8, and 15. Twenty-three patients received the first treatment, and 10 patients received the second treatment. Six patients received other chemotherapy, including four of the 18 patients who received the CAV regimen: 750 mg/m² cyclophosphamide i.v. on day 1, 40 mg/m² doxorubicin i.v. on day 1, and 2 mg/body vindesine i.v. on day 1. Four of the 18 patients who received the CAV regimen could not be evaluated for their response to chemotherapy because they had no measurable disease.

**Immunostaining.** Immunostaining of the p53 protein was performed using the anti-p53 monoclonal antibody, DO-1 (Ab-6; Oncogene Science, Uniondale, NY). Five-μm sections were cut from paraffin blocks of the TBB specimens and allowed to air-dry. The slides were deparaffinized in xylene and absolute alcohol and autoclaved at 121°C with distilled water for 20 min. After cooling, 10% rabbit serum was placed on the slides to reduce background staining. The slides were then incubated overnight with DO-1 at 4°C in a moist chamber at a concentration of 0.5 μg/ml. Nonspecific mouse IgG was used as the negative control. After washing with PBS, the slides were incubated with secondary antibody for 30 min (biotinylated antimouse IgG; Nichirei, Tokyo, Japan). Following a PBS wash, the slides were incubated with the streptavidin-peroxidase reagent (Nichirei) for 30 min. After another PBS wash, the antigen-antibody complex was visualized using a 0.05% solution of diaminobenzidine tetrahydrochloride in PBS for 5 min. A tumor was considered to be immunopositive for p53 when the number of cells with stained nuclei exceeded 10% (13). All specimens were evaluated without knowledge of the clinical outcome.

**Response Criteria.** We used standard response criteria (22) for evaluating the patients’ responses to chemotherapy. A CR was defined as the disappearance of all clinical and laboratory evidence of malignancy for a minimum of 4 weeks. A PR required a minimum of a 50% reduction in the sum of the products of the greatest perpendicular diameters of all measurable lesions for a minimum of 4 weeks. PD was defined as an increase in disease of greater than 25%, as measured for PR. SD was applied to those patients who did not achieve a PR but did not develop new lesions and whose symptoms did not worsen for a minimum of 8 weeks. The response rate was defined as the total number of cases having a CR plus those having a PR.

**Statistical Analysis.** The χ² test or trend test was used to evaluate the association between p53 immunostaining and several categorical variables. Analyses of survival were performed using the Kaplan-Meier method, and differences between patient groups were tested by the log-rank test. All reported Ps are two-sided. A level of P < 0.05 was accepted as statistically significant.

### RESULTS

**Relationship between p53 Immunostaining and Clinicopathological Parameters.** The numbers of p53-positive cases were 61 of 111 (55%) NSCLC cases and 37 of 64 (58%) SCLC cases. There was no difference in p53 positivity between NSCLC and SCLC (Table 1). There was also no difference among the different histological types of NSCLC: p53 positivity increased significantly with increasing stage (stages I and II, 45%; stage III, 54%; and stage IV, 66%; P = 0.036 by trend test; Table 2). This tendency was greater in SCLC (stages I and II, 38%; stage III, 48%; stage IV, 71%; P = 0.10) than in NSCLC (stages I and II, 46%; stage III, 58%; stage IV, 62%; P = 0.36). However, there was no correlation between p53 positivity and other clinical parameters, including sex, age, smoking history, and performance status. When the data are divided into NSCLC and SCLC groups, there is no correlation between p53 positivity and these factors in either group.

**Effect of p53 Status on Response to Chemotherapy.** Ninety-five patients who received at least two courses of chemotherapy were evaluated for their response to the chemotherapy. Thirty-six of 39 patients with NSCLC received cisplatin-based chemotherapy, and 40 of 56 patients with SCLC received platinum (cisplatin and/or carboplatin)-based chemotherapy. The response rates of NSCLC were 13% in the p53-positive group and 60% in the p53-negative group (Table 3). There was a statistically significant difference between the two groups (P = 0.006). However, in SCLC, the response rate was 80% in the
Table 2 \Relationship between p53 status and clinicopathologic factors\n
\begin{tabular}{|l|c|c|c|c|}
\hline
Factor & No. of cases & Positive (no.) & Negative (no.) & \(P^a\) \\
\hline
All cases & 175 & 98 (56\%) & 77 & \\
Sex & & & & \\
Male & 123 & 70 (57\%) & 53 & 0.83 \\
Female & 52 & 28 (54\%) & 24 & \\
Age (yr) & & & & \\
<60 & 41 & 24 (59\%) & 17 & 0.71 \\
\geq 60 & 77 & 38 (58\%) & 27 & \\
Histologic type & & & & \\
NSCLC & 111 & 61 (55\%) & 50 & 0.83 \\
Squamous cell carcinoma & 39 & 25 (64\%) & 14 & \\
Nonsquamous cell carcinoma & 72 & 36 (50\%) & 36 & \\
SCLC & 64 & 37 (58\%) & 27 & \\
Clinical stage & & & & \\
I + II & 49 & 22 (45\%) & 27 & \\
III & 61 & 33 (54\%) & 28 & 0.036 \\
IV & 65 & 43 (66\%) & 22 & \\
Smoking (cigarettes/day \times yr) & & & & \\
0 & 36 & 17 (47\%) & 19 & 0.49 \\
>0/\leq 1000 & 68 & 40 (59\%) & 28 & \\
>1000 & 71 & 41 (58\%) & 30 & \\
Performance score & & & & \\
0–1 & 134 & 72 (54\%) & 62 & 0.36 \\
2–4 & 41 & 26 (63\%) & 15 & \\
\hline
\end{tabular}

\textsuperscript{a}Numbers in parentheses are percentages of total.
\textsuperscript{b}\(\chi^2\) test.

\textsuperscript{c}NSCLC vs. SCLC.

\textsuperscript{d}Adenocarcinoma plus large cell carcinoma.

\textsuperscript{e}Trend test.

Table 3 \Response to chemotherapy according to p53 status\n
\begin{tabular}{|l|c|c|c|c|c|}
\hline
 & CR & PR & SD & PD & RR\textsuperscript{f} & \(P^a\) \\
\hline
NSCLC & & & & & & \\
p53-positive & 0 & 3 & 16 & 5 & 13\% (3/24) & 0.006 \\
p53-negative & 0 & 9 & 5 & 1 & 60\% (9/15) & \\
SCLC & & & & & & \\
p53-positive & 6 & 22 & 5 & 2 & 80\% (28/35) & 0.22 \\
p53-negative & 1 & 11 & 7 & 2 & 57\% (12/21) & \\
\hline
\end{tabular}

\textsuperscript{f}RR, response rate.

\textsuperscript{g}\(\chi^2\) test, responder (CR + PR) vs. nonresponder (SD + PD).

p53-positive group and 57\% in the p53-negative group, and there was no difference between the two groups \((P = 0.22)\). The same tendency was observed among the patients who received only platinum-based chemotherapy. Positive staining for p53 correlated significantly with unresponsiveness to cisplatin-based chemotherapy in NSCLC \((\text{response rate, 14 versus 60\%; } P = 0.012)\), but not in SCLC \((82 \text{ versus 83\%})\). Fourteen patients with SCLC received the CAV regimen. Four of the six patients in the p53-positive group responded to chemotherapy, and two of the eight patients in the p53-negative group responded. There was no difference in the response to CAV. Six patients with NSCLC and four patients with SCLC developed progressive disease. Five of the six NSCLC samples were p53 positive, and two of the four SCLCs were p53 positive (Table 3).

Effect of p53 Status on Prognosis. We analyzed the effect of p53 positivity on prognosis, stratified by stage, because p53 overexpression increased with increasing stage. The overall mortality rate was 63\% (111 of 175 patients), with a median follow-up of 22 months for the patients who were treated surgically and 7 months for the patients who were not treated by surgery. Overall survival did not differ between the p53-positive and -negative groups for stage I and stage II NSCLC \((P = 0.79; \text{Fig. 1A})\). There were no significant differences between these two groups with respect to sex, age, histological type, stage, or performance status. Of these cases, 16 of 19 p53-positive and 16 of 21 p53-negative patients were treated by surgery. However, for stage III and stage IV NSCLC, the median survival of the p53-positive
Fig. 1 Kaplan-Meier curve for overall survival, with respect to p53 immunostaining in patients with stage I and stage II NSCLC (A), stage III and stage IV NSCLC (B), and in all patients with SCLC (C).

group was significantly shorter (6.6 months) than that of the p53-negative group (12.1 months; \( P = 0.02 \); Fig. 1B). There was also no significant difference in clinical parameters or therapy between these two groups (Table 4). When the analysis was restricted to the NSCLC patients who received chemotherapy \( (n = 35) \), the survival difference was not statistically significant \( (P = 0.09) \). However, among the patients who were not treated by chemotherapy \( (n = 31) \), the survival difference was statistically significant \( (P = 0.04) \).

On the other hand, there was no survival difference between p53-positive and p53-negative patients with SCLC \( (P = 0.35; \text{Fig. 1C}) \). There were no significant differences between the two groups of SCLC patients with respect to age, sex, stage, or performance score.

**DISCUSSION**

p53 immunostaining is an attractive alternative to the molecular biological detection of p53 mutations because it is inexpensive, less time-consuming than molecular biological detection, and feasible in most clinical laboratories. Although there are still some problems with p53 immunostaining, including differences in monoclonal antibodies, methods of antigen retrieval, and discordance with gene studies, it is used widely. TBB has the benefit of providing untreated tumor specimens. By combining these two methods, our first purpose was to analyze the p53 status of samples of SCLC and advanced NSCLC, reports of which are few.

The incidence of p53 positivity in SCLC was 58%, which
was approximately the same rate as in NSCLC. The rate of p53 mutations in SCLC has been reported to be 73–80% by gene analysis (16, 17) and 61% by immunohistochemistry (18). Although these previous studies were relatively small (n = 17–28), our results from 64 samples were similar. The rate of positivity increased with increasing disease stage in our study. The incidence of p53 positivity by immunostaining in resected NSCLC was 45% (22 of 47) in our study, which is equivalent to the results of others who used resected samples (7, 9, 11, 13). Positivity increased to 62% in stage IV NSCLC in our study. This tendency was greater in SCLC than in NSCLC.

We examined the effect of p53 positivity on the response to chemotherapy. Positive staining for p53 was significantly correlated with unresponsiveness to chemotherapy in NSCLC, but not in SCLC. Lowe et al. (15) have demonstrated that cells lacking wild-type p53 are resistant to both ionizing radiation and cancer chemotherapy in vitro, whereas cells that express wild-type p53 are sensitive and exhibit cell death by apoptosis. The therapeutic effect of several antineoplastic agents may be mediated through DNA damage and the secondary induction of apoptosis (23). In general, mutant p53 suppresses the function of wild-type p53, which arrests the cell cycle at the G1 phase during DNA repair (24, 25). These observations therefore suggest that p53 mutations may potentially provide a genetic basis for drug resistance and radioresistance (26, 27). However, little clinical data have been reported to support this idea (28). Rusch et al. (14), who used resected samples, have reported that aberrant p53 expression correlates with resistance to cisplatin-based chemotherapy in NSCLC. Most of the patients with NSCLC enrolled in our study also received cisplatin-based chemotherapy. In vitro studies indicate that cisplatin inhibits DNA synthesis by causing double-stranded breaks (29), leading to apoptosis at the G2-M transition (30). A link between wild-type p53 and chemotherapy-induced apoptosis has been reported for cisplatin in NSCLC cell lines (31). Cisplatin may be one of the important drugs that induce apoptosis of cancer cells during chemotherapy.

The tendency toward chemoresistance in p53-positive tumors was not observed in the patients with SCLC in our study who also received cisplatin-based chemotherapy. This may be due to differences in tumor types or drug combinations. Recent reports have suggested that p53 immunostaining may predict the response to chemotherapy in breast cancer and ovarian cancer (28, 32). It is not known which drugs can induce p53-dependent apoptosis in cancer cells. In contrast, it has recently been reported that the loss of normal p53 function confers sensitivity to Taxol by increasing G2-M arrest and apoptosis (33).

Many reports have examined whether p53 abnormalities affect the prognosis of patients with resected NSCLC (stages I–IIIA; 7, 9, 11, 13). However, the clinical importance of p53 mutation as a prognostic factor remains controversial. Using resected samples, Mitsudomi et al. (8) reported that p53 mutations are a poor prognostic factor in patients with advanced disease (stages IIIA–IV) but not in those with early-stage disease (stages I–II). Our data are derived from greater numbers of patients and gave similar results. In general, due to the longer overall survival and fewer deaths among the resected patients, the effect of p53 mutations on prognosis may be difficult to detect in early-stage disease. In our study, p53 positivity was a statistically significant negative prognostic factor for patients with stage III or stage IV NSCLC (P = 0.02). This may be attributable to the relationship between p53 positivity and chemoresistance. However, the prognosis of the advanced-stage patients who did not receive chemotherapy was affected more by the p53 status than was the prognosis of all patients with stage III or stage IV NSCLC. Current chemotherapy regimens do not make a significant impact on the survival of patients with NSCLC (34). Also, in our study, we saw no difference in prognosis between responders (n = 12) and nonresponders (n = 27; P = 0.12; data not shown). Thus, we postulate that p53 mutations influence the prognosis of patients with advanced NSCLC, regardless of their response to chemotherapy.

In conclusion, we have shown the utility of p53 immunostaining of TBB specimens. Our findings indicate that p53 immunostaining of TBB samples from patients with advanced NSCLC may be clinically useful in predicting resistance to chemotherapy and prognosis. However, these results were obtained from a relatively small number of patients. In addition, the chemotherapy regimens were not uniform. Thus, a prospective clinical study using one uniform regimen is called for to compare the therapeutic response and the prognosis of patients with and without p53 mutations.

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