Presence of Serum Anti-p53 Antibodies Is Associated with Pleural Effusion and Poor Prognosis in Lung Cancer Patients

Chun-Liang Lai, Chun-Ming Tsai,² Tsung-Tsung Tsai, Benjamin Ing-Tiau Kuo, Kuo-Ting Chang, Hsiao-Ting Fu, Reury-Perng Perng, and Jeou-Yuan Chen

Section of Thoracic Oncology [C-M. T., K-T. C., H-T. F.], Chest Department [C-L. L., R-P. P.], and Section of Infectious Diseases, Department of Internal Medicine [B. I-T. K.], Veterans General Hospital-Taipei, Taipei, 11217 Taiwan; Department of Medicine, School of Medicine, National Yang-Ming University, Taipei, 11217 Taiwan [C-M. T., B. I-T. K.]; and Section of Pulmonary Medicine, Department of Internal Medicine, Central Clinic and Hospital [T-T. T.], and Institute of Biomedical Sciences [J-Y. C.], Academia Sinica; Taipei, 11529 Taiwan

ABSTRACT

This study was designed prospectively to evaluate the development of anti-p53 antibodies (Abs) in lung cancer patients in relation to their clinical outcome. Sera, derived from 125 lung cancer patients, consisting of 14 small cell lung cancers (SCLC) and 111 non-SCLCs (NSCLC), were surveyed. The p53-null human NSCLC cell line, NCI-H1299, transfected with a human mutant p53 gene was prepared as the source of p53 antigen for immunoblotting analyses to detect the presence of serum anti-p53 Abs. The control group included sera from 10 healthy adults and 14 patients with benign pulmonary diseases. Clinical data including staging and survival were recorded for statistical analyses. The anti-p53 Abs were found in 8% (10 of 125) of the lung cancer patients studied (8.1% of NSCLC versus 7.1% of SCLC patients), whereas none of the control sera had detectable anti-p53 Abs. The presence of anti-p53 Abs was closely associated with malignant pleural effusions (P = 0.001). The p53 Ab-positive patients had a worse prognosis than the p53 Ab-negative patients (P < 0.02; median survival, 20 versus 41 weeks). In both univariate and multivariate analyses, the tumor extension and probably the presence of anti-p53 Abs were significant predictors for cancer death. The development of anti-p53 Abs (n = 9) was also a predictor for poor survival in patients with malignant effusions (n = 51). In conclusion, the presence of serum anti-p53 Abs is closely associated with malignant pleural effusions in lung cancer patients. It may serve as a negative prognostic factor for survival independent of malignant pleural effusions and tumor staging.

INTRODUCTION

The alteration of tumor suppressor gene p53 is one of the most common genetic abnormalities in human cancers (1–3). Various approaches have been adopted to detect mutations in the p53 gene, such as the molecular analysis by PCR amplification followed by nucleotide sequencing of p53 gene or cDNA fragments, immunohistochemical analysis of p53 gene product, and serological assay of autoantibodies against p53 antigen (anti-p53 Abs) in the sera of cancer patients (4). In lung cancers, p53 mutations are found in 90% of SCLCs (5, 6) and 50–60% of NSCLCs (7, 8). Most of the p53 mutations are point mutations, which often lead to the expression of p53 mutant protein with longer than normal half-life; in many cases, the overexpression of p53 is correlated with a poor patient survival (9). The development of anti-p53 Abs was observed in patients with lung cancer as well as in patients with a variety of other types of cancers (4, 10–14). The clinical implications of anti-p53 Abs have been controversial. In a study including 61 SCLC and 6 NSCLC patients, Winters et al. (10) reported that anti-p53 Abs were observed in 13% of the patients and exclusively found in cases with tumors containing missense mutations in the p53 gene. The presence of anti-p53 Abs was not related to tumor stage, prior treatment, or survival (10). Nevertheless, in their later report (15), the same group also suggested that anti-p53 Abs may have borderline prognostic significance for better survival in SCLC patients. Recently, Rosenfeld et al. (16) showed that the development of anti-p53 Abs in SCLC patients displayed no prognostic significance for survival. On the other hand, Schlichtholz et al. (11) showed that 24% of 42 lung cancer (9 SCLC, 33 NSCLC) patients exhibited serum anti-p53 Abs, and the antibodies were detected before the diagnosis of malignancy in 2 cases; thus, anti-p53 Abs were suggested to serve as an early marker for the tumorigenic development of lung cancer (12). In operated NSCLC cases, anti-p53 Abs were detected in 8.4–15% of sera (13, 14), and the frequency of anti-p53 Abs was higher in squamous cell carcinoma patients than in adenocarcinoma (13). The data concerning the prognostic role of anti-p53 Abs in larger series of lung cancer or NSCLC patients have never been reported.

The objectives of this study were to examine the frequency and the prognostic role of anti-p53 Abs in human lung cancer

1 The abbreviations used are: Ab, antibody; SCLC, small cell lung cancer; NSCLC, non-SCLC; HRP, horseradish peroxidase.
patients. The relationship between the development of serum anti-p53 Abs and other clinical variables was also evaluated.

MATERIALS AND METHODS

Patients and Serum Samples. One hundred and twenty-five consecutive patients with newly diagnosed lung cancers (17) at the Veterans General Hospital-Taipei from 1995 to 1996 were enrolled in this study. There were 111 cases (88.8%) of NSCLC and 14 cases (11.2%) of SCLC. The case distribution of lung cancer in Taiwan is NSCLC 87-90% and SCLC 10-13% (18). The control group included 10 healthy adults without underlying disease and 14 patients with benign pulmonary diseases (5 cases of tuberculosis, 2 cases of bacterial pneumonia, 2 cases of chronic obstructive pulmonary disease, 2 cases of nonmalignancy, and 1 case of interstitial lung disease).

The staging of the NSCLC was assessed according to the International TNM Staging System (19), and the SCLC was staged according to the recommendation of the International Association for the Study of Lung Cancer (20). The treatment decision was made on the basis of clinical stage, general condition, and the patient’s willingness to participate in the study but independent of the status of serum anti-p53 Abs. For cases of NSCLC with stage I, II, or IIIa, surgery was recommended as the first choice of treatment. Alternatively, local radiotherapy was performed when the patient’s pulmonary function reserve was poor or the patient refused surgery. Adjuvant chemotherapy was suggested depending on pathological staging. For the stages of IIIb and IV of the NSCLC patients, radiotherapy and/or chemotherapy were recommended. The regimen of chemotherapy used for NSCLC were platinum-based, including cyclophosphamide + etoposide + cisplatin and gemcitabine + cisplatin. Other regimens such as regimen of gemcitabine alone were also used when patients were enrolled in the randomized clinical trial (21). For SCLC, chemotherapy with etoposide + cisplatin was the first choice of treatment. In some cases, chemotherapy was also suggested depending on pathological staging. For the stages of IIIb and IV of the NSCLC patients, radiotherapy and/or chemotherapy were recommended. The regimen of chemotherapy used for NSCLC were platinum-based, including cyclophosphamide + etoposide + cisplatin and gemcitabine + cisplatin. Other regimens such as regimen of gemcitabine alone were also used when patients were enrolled in the randomized clinical trial (21). For SCLC, chemotherapy with etoposide + cisplatin was the first choice of treatment. Radiotherapy would be added to cases of limited stage. Informed consent was obtained from each patient. Serum was collected at the time of diagnosis and stored at -70°C until used.

p53 Antigen. We transfected a p53-null human large cell lung carcinoma cell line (NCI-H1299) with pCMV vector containing a human mutant p53 cDNA (22). The mutant p53 cDNA was derived from human colon carcinoma, which encodes human p53 protein with a single amino acid change at codon 143 from valine to alanine (p53V143A) (23). Forty-eight h after transfection, cell extract was prepared and used as the source of p53 protein (antigen) for immunoblotting analyses to detect the presence of serum anti-p53 Abs.

Immunoblotting. Whole cell lysates were prepared from the transfected NCI-H1299 cells and separated on 10% SDS-PAGE using mini-gel apparatus (Hoefer Mighty Small, Pharmacia Biotech). The proteins (100 µg protein/preparative gel) were then transferred to nitrocellulose filters (Schleicher and Schuell), which were then blocked with 5% nonfat dry milk (Anchor Food Product, Inc. Appleton, WI). The dried nitrocellulose filters were cut into 3-mm strips, and each strip was subjected to immunoblotting with one patient’s serum (1:200 dilution) as primary Abs. The secondary Abs that we used were sheep anti-human immunoglobulin HRP conjugates (1:500 dilution, Amershams Life Science). A colorimetric assay was used to detect the presence of HRP by incubating the filter strips with PBS containing 3,3'-diaminobenzidine (0.5 mg/ml) and 0.015% hydrogen peroxide. We used PAB1801 (Ab-2, Oncogene Research Products, Cambridge, MA) at a final concentration of 1 µg/ml as the positive control.

Statistical Analyses. The distribution of anti-p53 Abs was examined using stratification by staging and cell types. The Kaplan-Meier method was used to plot survival curves. The differences among subgroups using survival as end point were compared with the log-rank test. Fisher’s exact test and the Pearson χ² test were used to evaluate the association between anti-p53 Abs and other variables such as pleural effusions, staging, gender, cell types, and metastatic site. Cox proportional hazards models were adopted for multivariate analyses. All Ps reported are two-sided.

RESULTS

The Status of Serum Anti-p53 Abs and Clinical Characteristics of Lung Cancer Patients. In this study, we examined the presence of serum anti-p53 Abs by immunoblot analysis of 125 lung cancer patients including 33 (26.4%) squamous cell carcinomas, 62 (49.6%) adenocarcinomas, 12 (9.6%) large cell carcinomas, and 14 (11.2%) SCLCs. The patient distribution is listed in Table 1. For the NSCLC group, the staging distribution was (a) stage I, 9%; (b) stage II, 3.6%; (c) stage IIIa, 19.8%; (d) stage IIIb, 28.8%; and (e) stage IV, 38.7%; whereas 50% (7 of 14) of the SCLC patients were in limited stage. In our series, there was a relatively high frequency of pleural effusion

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage II</th>
<th>Stage IIIa</th>
<th>Stage IIIb</th>
<th>Stage IV</th>
<th>Limited stage</th>
<th>Extensive stage</th>
<th>Anti-p53 Ab (+) patients/total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC</td>
<td>0/10</td>
<td>0/4</td>
<td>1/22</td>
<td>3/32</td>
<td>5/43</td>
<td></td>
<td>9/111</td>
</tr>
<tr>
<td>SCC</td>
<td>0/2</td>
<td>0/1</td>
<td>1/12</td>
<td>2/8</td>
<td>1/10</td>
<td></td>
<td>4/33</td>
</tr>
<tr>
<td>ADC</td>
<td>0/7</td>
<td>0/3</td>
<td>0/7</td>
<td>1/18</td>
<td>3/27</td>
<td></td>
<td>4/62</td>
</tr>
<tr>
<td>LCC</td>
<td>0/2</td>
<td>0/5</td>
<td>1/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0/1</td>
<td>0/1</td>
<td>0/0</td>
<td></td>
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<td></td>
<td>0/2</td>
</tr>
</tbody>
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* Anti-p53 Ab (+) accounts for 8.1% of NSCLC patients and 7.1% of SCLC patients which represents 8% in total.

SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large-cell carcinoma; ADSCC, adenosquamous cell carcinoma.
The proportional significance of anti-p53 Abs for survival was evaluated by univariate analyses (log-rank test). The entire group of lung cancer patients, both anti-p53 Abs (P = 0.0091) and pleural effusion (P < 0.0001) were negatively prognostic (Table 3). The median survival of anti-p53 Ab(+) patients and anti-p53 Ab(−) patients were 136 days and 287 days, respectively (Fig. 2). In the group of NSCLC patients, the presence of anti-p53 Abs (P = 0.05) along with advanced stage, large tumor size, lymph node involvement, systemic metastasis, impossibility of surgery, and presence of pleural effusion (all, Ps < 0.002), were the factors associated with poor survival. The median survival for anti-p53 Ab(+) NSCLC patients was 141 days compared with 239 days for anti-p53 Ab(−) NSCLC patients.

Multivariate analysis was carried out to further evaluate whether anti-p53 Abs would serve as an independent factor to predict a patient’s outcome. As shown in Table 3, the extension of tumor (stages I, II, and IIIa versus stages IIIb and IV, P = 0.0000), adenocarcinoma (P = 0.017), and probably anti-p53 Abs (P = 0.046, borderline significant) but not pleural effusion were independent prognostic factors for survival. Furthermore, in the subgroup of patients with malignant pleural effusion (n = 51), anti-p53 Abs (n = 9) also served as a poor prognostic factor (P = 0.0467; Fig. 2). Similarly, in patients with advanced NSCLC (stage IIIb and IV), those who had developed serum anti-p53 Abs also showed a shortened survival as compared with those who had not (median survival, 210 days versus 136 days; P = 0.0071).

**DISCUSSION**

Since the first report on serum anti-p53 Abs detected in breast cancer patients by Crawford et al. (24), such antibodies have been demonstrated in many types of cancer patients (4, 25–29). In lung carcinomas, anti-p53 Abs are present in 8–24% patients depending on patient populations, such as in SCLC groups or surgically resected NSCLC groups (10, 13, 14). In this study, we examined a group of 125 lung cancer patients and found that 8% of the patients tested positive for serum anti-p53 Ab. Although the SCLC patients represented a minor group in this study, our data suggested that the development of anti-p53 Abs shows no cell-type difference (8% in NSCLC and 7% in SCLC). Although it has been suggested (26) that the prevalence of anti-p53 Abs in different types of cancer patients correlates to the frequency of p53 gene mutation, such a correlation was not observed in the current series of NSCLC and SCLC patients, in which the reported rates of p53 gene mutation were 45% and 80%, respectively (5–8). Such discordance has also been found in cancers of the head and neck, the liver, and the breast. The frequency of p53 gene mutations in these three types of cancers range within 22–37%, which is rather low when compared with those of other cancer types (>50%; Ref. 3); however, anti-p53 Abs were detected at similar rates (10–19%) in all of the cancer types (28–30).

The mechanism of the development of autoantibodies against p53 protein in cancer patients was intriguing. Accumulated evidence suggested that the generation of anti-p53 Abs is very likely a self-immunization process resulting from the ac-
Serum Anti-p53 Abs in Lung Cancer

The wild-type and mutant conformations of p53 protein (10, 32) are frequently found in human cancers (3, 31). Such antibodies are not specific for a particular p53 mutant and can recognize both the wild-type and mutant conformations of p53 protein (10, 32).

It is, therefore, suggested that the development of anti-p53 Abs is attributed to the increased level of p53 protein rather than to the change of its molecular conformation resulting from missense mutation. The process of immunization may occur while the gene mutates, which always precedes the development of neogrowth (11, 12, 28). Not unexpectedly, anti-p53 Abs have been shown to be present during the tumorigenic process in persons who are at high cancer risk and even before the manifestation of the disease in lung cancer patients (11, 12). Similarly, the early diagnosis of malignancy by the serological test of anti-p53 Abs was also demonstrated in cases of angiosarcoma of the liver, which tends to develop in high-risk groups who have been exposed to vinyl chloride (28). Under these circumstances, anti-p53 Abs are useful as an early diagnostic marker to identify subsets of patients who are at higher cancer risks. In this study, we showed that anti-p53 Abs were closely associated with malignant pleural effusion, which has never been reported before. Because the majority of malignant pleural effusions are known to exhibit a differential count of a predominance of small lymphocytes (33), it is possible that the power of massive lymphocytes into the pleural cavity may augment the immuno-reactivity to p53 antigen, which facilitates the detection of anti-p53 Ab. Nevertheless, in the context of this study, our qualitative results in accordance with the report of Winter et al. (10) showed that the development of anti-p53 Abs did not correlate to disease stage (P = 0.1540) nor to metastasis (P = 0.2780). Because of this and of their low frequency in lung cancers (8%), serum anti-p53 Abs do not serve as a useful diagnostic marker for lung cancer screening.

Studies on cancers of the head and neck, the breast, and the gastrointestinal tract have shown that patients who developed serum anti-p53 Abs have a poorer prognosis (29, 30, 34). In lung cancers, however, the prognostic significance of serum anti-p53 Ab has remained controversial. Although it has been reported that patients positive with anti-p53 Abs have a (borderline

Table 2 Association of anti-p53 Abs in lung cancer patients with malignant pleural effusion

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<tr>
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<th>Multivariate P (odds ratio)</th>
</tr>
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<tbody>
<tr>
<td>Advanced stage (IIIB+IV)*</td>
<td>&lt;0.0001</td>
<td>0.0000 (4.1356)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
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<td>0.0168 (1.8029)</td>
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<tr>
<td>Anti-p53 Abs</td>
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<td>0.0455 (2.1436)</td>
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<td>&lt;0.0001</td>
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* Patients in advanced stage were thought to be surgically inoperable.

Table 3 Statistic analysis of the prognostic factors in lung cancer patients

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better survival rate in SCLC (15), this survival advantage could not be confirmed by other studies (16). The prognostic role in NSCLC patients and in general groups of lung cancer patients has never been reported. In the present study, we demonstrated that the presence of anti-p53 Abs predicted a poorer prognosis in a general group of lung cancer patients (P = 0.009), as well as in the group of NSCLC patients (P = 0.05). Multivariate analyses further revealed that anti-p53 Abs—indeed of cell type, tumor extension, and the presence of malignant pleural effusion—may be a negative prognostic factor in this series of lung cancer patients. Despite the fact that there is a close association between malignant pleural effusions and the presence of serum anti-p53 Abs, our data clearly demonstrated that the poor-prognosis role of anti-p53 Abs was preserved in patients with malignant effusions. In the subgroup of patients with advanced NSCLC, the presence of anti-p53 Abs also predicted a poorer prognosis. Davidoff et al. (35) have shown that only p53 with mutations localized in sequences encoded by exons 5 and 6 elicited p53 Abs, whereas mutations localized in sequences encoded by exons 7 and 8 did not, suggesting that the type of mutation can influence the production of Abs against p53. This finding offers an explanation for the low frequency of p53 antibodies in patients with p53-overexpressing tumors. In addition, Merlo et al. (36) have shown that mutations in exons 5 and 6 are associated with a high S-phase fraction in breast cancer. Thus, it may be postulated that the presence of anti-p53 Abs reflects p53 mutations that give rise to a more aggressive tumor phenotype.

In conclusion, anti-p53 Abs were found in 8% of patients with lung cancer. It was not cell-type-dependent. The presence of anti-p53 Abs was closely associated with malignant pleural effusions. Furthermore, serum anti-p53 Abs may serve as a negative prognostic factor for survival that is independent of tumor extension and malignant pleural effusions.

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