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### Background

The presence of autoantibodies to p53 protein has been associated with the presence of p53 (also known as TP53) gene mutations in primary tumors and with poor prognosis. This study was undertaken to determine the clinical significance of p53 autoantibodies in patients with non-small cell lung cancer (NSCLC). Methods: We studied 188 consecutive patients with NSCLC who underwent pulmonary resection and for whom preoperative serum was available. The presence of p53 autoantibodies, detected by use of two amino-terminal and two carboxy-terminal peptides (20–30 mers) as antigens and an enzyme-linked immunosorbent assay, was related to various clinicopathologic parameters and to overexpression of p53 protein in the primary tumor. For 22 patients who had p53 autoantibodies before surgery, we also examined sera taken during postoperative follow-up. Reported P values are two-sided. Results: Autoantibodies to p53 protein were detected in 38 patients. Patients with squamous cell carcinoma, those with more advanced disease (stage III–IV), and those with tumors that overexpressed p53 had a significantly higher incidence of p53 autoantibodies (P = .05, .0079, and .02, respectively). In all but one of the patients with postoperative serum samples, the antibody titer declined after surgery; however, there was no relationship between clinical course and this change in antibody titer. In addition, there was no relationship between the presence of p53 autoantibodies and overall survival in 171 patients who underwent potentially curative resection (P = .28); however, 13 patients with autoantibodies to amino-terminal peptides had a worse overall survival (P = .02). Conclusions: In NSCLC, the incidence of p53 autoantibodies is associated with histologic type, stage, and p53 overexpression—but not with patient survival. Our data do not support the clinical utility of p53 autoantibodies as diagnostic or prognostic markers in patients with NSCLC. [J Natl Cancer Inst 1998;90:1563–8]

In many types of human cancers, including non-small-cell lung cancer (NSCLC), the p53 (also known as TP53) tumor suppressor gene is completely inactivated when one copy of the gene is mutated and the remaining allele is subsequently deleted (1). p53 protein is thought to act as a negative regulator of cellular proliferation or as an inducer of apoptosis through the transactivation of genes, including p21, BAX, or GADD45 (2). Missense mutation of the p53 gene usually prolongs the half-life of the protein from minutes to hours and results in nuclear accumulation of the p53 protein, which can be detected by immunohistochemistry (2).

A subset of patients with various types of cancer harbor serum autoantibodies, mainly of the immunoglobulin G1 and G2 (IgG1 and IgG2) subclasses, against p53 protein (3). The incidence of p53 autoantibodies is strictly proportional to the frequency of p53 overexpression in various tumors (3). Previous studies reviewed by Soussi (4) have suggested that the presence of p53 autoantibodies is associated with a p53 mutation in the primary tumor,

### References

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See “Notes” following “References.”

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that detection of p53 autoantibodies predicts the diagnosis of cancer, and that the presence of these antibodies is a poor prognostic factor in patients with cancer of the breast, colon, or head and neck. It is also known that most of the p53 autoantibodies react with the amino-terminal (N-terminal) or carboxy-terminal (C-terminal) part of the p53 protein, and usually not with the core domain where most of missense mutations are located (5). In lung cancer, p53 autoantibodies are reported to be detected in 10%–20% of the patients, but their clinical implication remains unclear (6–8).

We previously had studied a cohort of patients with non-small-cell lung cancer (NSCLC) who underwent pulmonary resections consecutively, within a well-defined and reasonably short period of time, and who were examined for abnormal p53 accumulation, cyclin D1, or retinoblastoma (Rb) gene product expression (9,10). In this cohort, p53 overexpression was not a significant prognostic factor when all histologic types were considered, but was a useful prognosticator for patients with adenocarcinoma (9). On the other hand, cyclin D1 overexpression was a good prognostic indicator in NSCLC, but loss of Rb expression was not significantly associated with clinical outcome in any of the subsets of NSCLC (10).

In the present study, we examined sera of these patients for the presence of autoantibodies against peptides derived from either the N- and C-terminal regions of p53 protein. We then investigated the diagnostic, prognostic, or therapeutic implications of the presence of p53 autoantibodies in patients with NSCLC.

**PATIENTS AND METHODS**

**Patients and Sera**

During a 5-year period from 1984 through 1988, 224 consecutive patients underwent pulmonary resection for the treatment of NSCLC in our department as a routine clinical practice. The availability of preoperative sera limited the number of patients included in this study to 188. Patients’ blood was drawn for a routine preoperative clinical examination and excess sera were kept frozen at −70 °C and were used for the present analysis. There were 128 male and 60 female patients, with an age ranging from 36 to 81 years (median, 63 years). The patients had 96 adenocarcinomas, 75 squamous cell carcinomas, seven large cell carcinomas, and 10 lung cancers of other types. Preoperative staging procedure included chest and abdominal computerized tomography scans, bone scans, and computerized tomography or magnetic resonance imaging scans of the brain. Either a lobectomy or pneumonectomy was performed on all patients but one.

Postoperative staging was done in conjunction with the mapping of regional lymph node metastases and the determination of tumor size; involvement of visceral pleura was staged according to the international staging system published in 1986 (11). Ninety-four patients had stage I disease, while 25 had stage II, 46 had stage IIIa, six had stage IIIb, and 17 had stage IV disease. Excluding 17 patients with incomplete resection, 171 patients were subjected to survival analysis. Most of these patients (161 [94%] of 171) did not receive postoperative adjuvant treatment (chemotherapy and/or radiotherapy). For a subset of patients who had p53 autoantibodies before surgery, sera that were taken at various postoperative follow-up times (up to postoperative year 9) were analyzed for autoantibody titer. Because archived specimens were used, patient informed consent was not required.

Sera from 84 subjects from the general population, obtained at the time of a health checkup with appropriate informed consent, were used for determination of the cutoff value of the p53 antibody titer. Sera from 12 Japanese patients with lung cancer that had been tested for p53 autoantibodies by an enzyme-linked immunosorbent assay (ELISA) system, provided by Dr. Thierry Soussi, were used for validation of our ELISA system (Mitsudomi T, Nishida K, Oyama T, Yasumoto K, Sugimachi K, unpublished observation).

**Western Blotting**

Cell pellets of ACC-LC-172, a human lung cancer cell line that harbors a missense mutation of the p53 gene and overexpresses p53 protein (Takahashi T, Takahashi T, Ueda R: unpublished observation), were suspended and sonicated in ice-cold lysis buffer (150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 50 mM Tris-HCl [pH 7.4]). The protein concentrations of the cell lysates were measured by Bradford analysis (Bio-Rad protein assay, Bio-Rad, Tokyo). Whole-cell lysates were mixed with 2x loading buffer and boiled for 5 minutes, and 50 μg of lysate was separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel and electrophoretically transferred to a polyvinylidenedifluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). After blocking the membranes by Block Ace (Dainippon Seiyaku, Osaka, Japan), the membranes were incubated at room temperature for 1 hour, sequentially, with patient serum diluted 1:100 in Block Ace and a horseradish peroxidase-conjugated anti-human immunoglobulin G (H+L) (MBL, Nagoya, Japan) diluted 1:100 in PBS containing 1% BSA at 25 °C for 1 hour, and with horseradish peroxidase-conjugated human immunoglobulin G (H+L) (MBL, Nagoya, Japan) (100 μL/well), diluted 1:10000 in PBS containing 1% BSA and washed with PBS–0.05% Tween 20 five times. Western blotting was performed using 10μL of tetrathymabenzidine solution. The reaction was stopped by adding 100 μL of N H₂PO₄, and the optical density (OD) at 450 nm was measured with an ELISA microplate reader (model 3550, BioRad). In each plate, positive controls (serum #268 for peptides C1 and C2 and serum #126 for peptides N1 and N2) and negative controls (no serum) were included for calculation of antibody indices (see below). These sera gave a strong band at 53 kd by western blotting and the intensity of the band significantly diminished when the sera were absorbed by excess peptide solution (100 μg/mL of each peptide) (not shown).

Cutoff values were determined by examining sera from 84 healthy subjects. The reactivity indices of sera against each of the four p53 peptides were calculated as the percentage of net OD relative to that for the corresponding positive controls by the formula: I = (ODsample − ODblank)/(ODpositive control − ODblank) × 100. Sera with indices greater than the mean value observed in the 84 healthy subjects plus two standard deviations were considered to be positive for p53 autoantibodies against that particular peptide. By this procedure, we determined cutoff values of 23.7 for C1, 4.89 for C2, 8.08 for N1, and 10.92 for N2. To ensure the reliability of our results, we compared the results obtained with our ELISA system with those from western blotting (not shown). Nine of 12 control sera were found to be positive for p53 autoantibodies by western blotting, however, two of these nine failed to give positive results in our ELISA. All three sera that appeared negative by western blotting were also negative by ELISA. Overall concordance between western blotting and ELISA was 10 (83%) of 12.

**Statistical Analysis**

A comparison of proportion was performed using the chi-squared test and the relationship between differences in tumor size and p53 antibody positivity was analyzed by Mann–Whitney U test. The
Results

Relationship Between the Presence of p53 Autoantibodies and Clinicopathologic Features

In 188 patients with NSCLC, 14 (7%) had autoantibodies that reacted to at least one of N-terminal peptide (N1 and/or N2), whereas 27 (14%) had autoantibodies that reacted with a C-terminal peptide (C1 and/or C2). Only three sera reacted to both C- and N-terminal peptides. Overall, p53 autoantibodies were detected in 38 (20%) patients. Table 1 shows the relationship between the presence of p53 autoantibodies and various clinical/pathologic characteristics. There was no association with such factors as sex, age, or smoking status of the patients. However, p53 autoantibodies were significantly more prevalent in patients with squamous cell carcinoma (27%) than in those with adenocarcinoma (15%) (P = .05). There was also a statistically significant difference in the incidence of p53 autoantibodies between the early disease group (stage I and II) (14%) and the advanced disease group (stage IIIa–IV, 30%) (P = .0079). The size of tumors from antibody-positive patients (mean, 40.6 mm; standard deviation [SD] = 18.9 mm) was significantly larger than that from antibody-negative patients (mean, 34.8 mm; SD = 16.6 mm) (P = .037, Mann–Whitney U test). We also analyzed our data in terms of epitopes of autoantibodies (i.e., N-terminal or C-terminal). There was a statistically significant tendency for histologic types to be associated with N-terminal—but not C-terminal—autoantibodies (histology versus N autoantibodies: P = .046; histology versus C autoantibodies: P = .19; chi-squared test). The relationships between stage and N autoantibodies (P = .099; chi-squared test) or stage and C autoantibodies (P = .078; chi-squared test) (not shown) were not statistically significant.

Relationship Between the Presence of p53 Autoantibodies and Nuclear Accumulation of p53 Protein

In our previous study (9), we showed that the nuclear accumulation of p53 protein, examined by immunohistochemistry, was observed in about half of the patients with NSCLC and that p53 accumulation is an independent indicator of a poor prognosis in a subset of patients with adenocarcinoma. In this study, we quantified nuclear accumulation of p53 protein on a four-point scale as follows, 0 = no staining at all, 1 = less than 10% p53-positive cells present, 2 = at least 10% and less than 66% p53-positive cells, and 3 = at least 66% p53-positive cells present. Ten percent p53 positivity was chosen as an optimized cutoff, since this gave a good separation between p53-positive and -negative cases as well as the best concordance between immunohistologic and molecular biologic analyses (9). In the 188 patients studied here, overall p53 positivity was 87 (46%) of 188, with the score distribution shown in Table 1. The incidence of p53 autoantibodies in patients whose tumors showed overexpression of p53 protein (score 2 and 3; 28%) was significantly higher than that in patients whose tumor did not have p53 overexpression (score 0 and 1; 14%) (P = .02, chi-squared test). The incidence of p53 autoantibodies was 15%, 11%, 26%, and 29% in patients with p53 immunohistochemistry score 0, 1, 2, and 3, respectively. Of 82 patients whose tumors were completely negative for p53 overexpression, seven (9%) were still positive for p53 autoantibodies when the cutoff value of the p53 antibody titer was elevated to mean plus four SDs (not shown).

Change of Titer of p53 Autoantibodies After Surgery

For 22 of 38 patients who had p53 autoantibodies preoperatively, sera taken at various postoperative follow-up times up to postoperative year 9 were also available. In most cases, the antibody titer declined after surgical resection. However, there was no clear relationship between change in titer and clinical course of the patients as shown in Fig. 1.

Table 1. Relationship between incidence of p53 autoantibodies with various clinicopathologic features

<table>
<thead>
<tr>
<th>Feature</th>
<th>No. of cases examined</th>
<th>p53 antibody-positive cases, %</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>128</td>
<td>26, 20</td>
<td>.96</td>
</tr>
<tr>
<td>Female</td>
<td>60</td>
<td>12, 20</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤63</td>
<td>97</td>
<td>22, 23</td>
<td>.38</td>
</tr>
<tr>
<td>&gt;63</td>
<td>91</td>
<td>16, 18</td>
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</tr>
<tr>
<td>Smoking status</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>53</td>
<td>10, 19</td>
<td>.77</td>
</tr>
<tr>
<td>Smoker</td>
<td>135</td>
<td>28, 21</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>96</td>
<td>14, 15</td>
<td>.05†</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>75</td>
<td>20, 27</td>
<td></td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>7</td>
<td>1, 14</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10</td>
<td>3, 30</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>I–II</td>
<td>119</td>
<td>17, 14</td>
<td>.0079</td>
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<tr>
<td>III–IV</td>
<td>69</td>
<td>21, 30</td>
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<tr>
<td>Nuclear p53 score‡</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>82</td>
<td>12, 15</td>
<td>.02§</td>
</tr>
<tr>
<td>1</td>
<td>19</td>
<td>2, 11</td>
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<tr>
<td>2</td>
<td>39</td>
<td>10, 26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>14, 29</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>188</td>
<td>38, 20</td>
<td></td>
</tr>
</tbody>
</table>

*All P values were two-sided and statistically significant for P<.05.
†P value for squamous cell carcinoma versus adenocarcinoma.
‡p53 immunohistochemistry score was defined as follows; 0 = no staining at all, 1 = less than 10% of nuclei were positive; 2 = 10% or more and less than 66% nuclei were positive; and 3 = 66% or more nuclei were positive.
§P value for immunohistochemically positive cases (score 0 and 1) versus immunohistochemically negative cases (score 2 and 3).
Effect of p53 Autoantibodies on Patient Survival

We analyzed the association between p53 autoantibodies and overall survival of 171 patients who underwent potential curative resection (Fig. 2, A). Although there appeared to be some difference after 5 years in these curves, this was not statistically significant (P = .28). We also drew survival curves with respect to epitopes of the p53 protein (Fig. 2, B). Patients who had autoantibodies against the N-terminal region of the p53 protein had a poorer overall survival (P = .02, N-peptide-specific versus other autoantibodies). When a multivariate analysis was performed using age, sex, histologic type, stage, tumor positivity for p53 protein by immunohistochemistry, and the type of p53 autoantibodies present as potential prognostic factors, the independently significant parameters were found to be stage and the presence of autoantibodies to an N-terminal peptide (Table 2).

DISCUSSION

In the present study, we detected anti-p53 autoantibodies in 38 (20%) of 188 sera taken preoperatively from Japanese patients with NSCLC. This incidence is generally in accordance with previous reports from Western countries. Schlichtholz et al. (8) reported that they detected p53 autoantibodies in the sera of 10 of 42 patients with lung cancer, while Wild et al. (7) found that the incidence was 16 of 136. In our series, autoantibodies against N-terminal epitopes were detected in 14 (7%) patients (five for N1 and 12 for N2), while antibodies against C-terminal epitopes were more prevalent and were found in 27 patients (14%) (eight for C1 and 19 for C2). This was in contrast with the report by Wild et al., who found that 15 of 16 sera from patients with lung cancer reacted with N-terminal peptides. In terms of histologic types, Wild et al. (7) and our group found that p53 autoantibodies were more frequent in squamous cell carcinoma than adenocarcinoma, probably reflecting the higher incidence of p53 overexpression in squamous cell carcinoma than in adenocarcinoma (9). In this context, patients with small-cell lung cancer (SCLC) might be expected to have higher incidence of p53 autoantibodies, since the incidence of p53 mutation in SCLC is even higher than in squamous cell carcinoma (1). However, they were only detected in 27 (16%) of 170 patients with SCLC according to Rosenfeld et al. (15).
were positive for p53 autoantibodies. von Brevern et al. (16) suggested the possible presence of a second, undiagnosed cancer or of cellular or viral proteins that bind to wild-type p53 to form complexes that may elicit a B-cell response. These researchers also found that, even with the very same mutational base change, some patients develop an autoantibody while others do not, reflecting variations in host immune response (16).

It has been reported (17,18) that for some tumors, detection of p53 autoantibodies may predate diagnosis of cancer and therefore facilitate identification of early lesions. Similarly, it could be expected that following the titer of the p53 autoantibodies might be useful for early detection of tumor relapse or diagnosis of a second primary cancer. However, as shown in Fig. 1, there was no clear association between the clinical course and p53 antibody titer in the present study.

p53 overexpression is usually associated with poor overall survival of patients with NSCLC, especially those in the adenocarcinoma subset (9,19), although there is still a considerable controversy regarding this point (20). Similarly, in such tumors as cancers of breast (21), colon (22), or head and neck (23), the presence of p53 autoantibodies has been reported to be a marker of poor prognosis. However, in our study of a cohort of patients with NSCLC treated consecutively within a well-defined and reasonably short period of time, detection of p53 autoantibodies was not predictive, in general, of poor overall survival. Of interest, however, was that detection of p53 autoantibodies against N-terminal peptides was an independent marker of poor prognosis. We have no explanation for this discrepancy and this remains the subject of future study.

In conclusion, examination of 188 patients with NSCLC for anti-p53 autoantibodies showed 20% to be positive for such antibodies. These antibodies were mainly found in patients with tumors overexpressing p53. Our data, however, did not provide support for the usefulness of p53 autoantibodies in diagnosis or for predicting relapse or prognosis of patients with NSCLC. Recently, therapeutic implications of p53 autoantibodies have been suggested. Tilkin et al. (24) reported that the proliferative T-cell response to p53 protein parallels the presence of p53 autoantibodies in patients with breast cancer. It has also been reported that p53 is able to elicit a cytotoxic T-cell response in vitro (25). Therefore, it is tempting to regard p53 autoantibodies as a marker of patients who would be likely to benefit from a new modality of cancer immunotherapy that targets p53.

**References**


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**Table 2. Cox’s proportional hazards model for factors associated with the overall survival of patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>Unfavorable/favorable</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>0.95 (0.62–1.48)</td>
<td>&lt;=63/&gt;63†</td>
<td>.83</td>
</tr>
<tr>
<td>Sex</td>
<td>0.75 (0.44–1.25)</td>
<td>Female/male</td>
<td>.28</td>
</tr>
<tr>
<td>Histologic types</td>
<td>0.80 (0.48–1.33)</td>
<td>Squamous/nonsquamous</td>
<td>.39</td>
</tr>
<tr>
<td>Stage‡</td>
<td>2.28 (1.45–3.61)</td>
<td>III-IV/III–II</td>
<td>.0004</td>
</tr>
<tr>
<td>Nuclear p53 staining</td>
<td>0.90 (0.58–1.39)</td>
<td>Positive/negative</td>
<td>.63</td>
</tr>
<tr>
<td>Ab to N peptide</td>
<td>1.96 (1.01–3.83)</td>
<td>Positive/negative</td>
<td>.048</td>
</tr>
<tr>
<td>Ab to C peptide</td>
<td>0.92 (0.47–1.83)</td>
<td>Positive/negative</td>
<td>.82</td>
</tr>
</tbody>
</table>

*Ab = antibody; N = amino-terminal; C = carboxy-terminal.

*All P values were two-sided and statistically significant for P<.05.

†The median age was chosen as the cut point.

‡Stage according to (11).


NOTES

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