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Serum anti-p53 autoantibodies in primary resected non-small-cell lung carcinoma

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Abstract Mutated p53 proteins accumulate in the nuclei of tumor cells, and anti-p53 autoantibodies are found in the sera of patients with non-small-cell lung carcinoma (NSCLC). We analyzed the correlation among serum anti-p53 autoantibodies, immunohistochemical staining for p53, and clinical features (age, gender, smoking history, histological type, differentiation, stage, T factor, tumor size, and N factor) in resected non-small-cell lung carcinomas. A total of 62 cases of resected NSCLC were studied (43 men and 19 women; 33 adenocarcinomas, 21 squamous cell carcinomas, 8 large-cell carcinomas). Preoperative serum titers of anti-p53 autoantibodies were detected in 13/62 cases (21.0%). A correlation between histological type and positive titers of serum p53 autoantibodies was seen (large-cell carcinoma versus squamous cell carcinoma and adenocarcinoma, $P = 0.031$, χ^2-test). Out of 25 cases, 10 (40%) with positive immunohistochemical staining for p53 had positive titers, whereas 3 positive titers were found in 37 patients with negative immunohistochemical staining for p53 ($P = 0.0025$, χ^2-test). Serum titers of anti-p53 autoantibodies were present in approximately 20% of the cases of NSCLC, and overexpression of p53 protein in tumor cells was detectable in approximately 40%. Serum anti-p53 autoantibodies may be a clinical parameter for the presence of p53 mutations and p53 overexpression in NSCLC patients.

Key words Lung cancer · p53 · Autoantibody · Immunohistochemistry · Tumor markers

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Introduction

The tumor suppressor gene, *p53*, is involved in the regulation of the cell cycle. *p53* mutations are frequent in malignant neoplasms, including lung carcinomas [11, 27]. These point mutations are associated with p53 overexpression caused by the reduced breakdown of the mutant protein [15]. p53 overexpression is easily detectable by immunohistochemical staining [11, 12]. In non-small-cell lung carcinoma (NSCLC), p53 staining is positive in 45%–58% of tumors [3, 8, 9, 22, 28]. Either wild-type or mutant p53, if overexpressed in tumors, induces humoral and cellular immune responses. Winter et al. have reported that p53-specific autoantibodies in the sera of lung cancer patients appeared to be dependent on the type of p53 mutation, following investigations using established lung cancer cell lines, but most of the patients had small-cell lung carcinoma [29]. Another investigator has reported that analyses of p53 autoantibodies in the sera of patients with lung carcinoma defined immunodominant regions in the p53 protein [16, 26]. There have been few reports on the correlation between overexpression of p53, determined by immunohistochemical staining for p53, and serum anti-p53 autoantibodies in patients with NSCLC [17]. In this study, we describe the correlation between the presence of serum anti-p53 autoantibodies and immunohistochemical staining for p53 in primary resected NSCLC.

Materials and methods

Patients and controls

A group of 62 patients with primary resected NSCLC were included in the current study (43 men and 19 women ranging in age from 48 to 85 years, mean 65.7 years). There were 33 adenocarcinomas, 21 squamous cell carcinomas and 8 large-cell carcinomas. Pathological staging revealed 23 patients with stage I, 10 patients with stage II, 17 patients with stage IIIa, and 12 patients in stage IIIb disease. A total of 48 patients underwent complete resection and 14 patients incomplete resection. Histological type, degree of differentiation, pathological

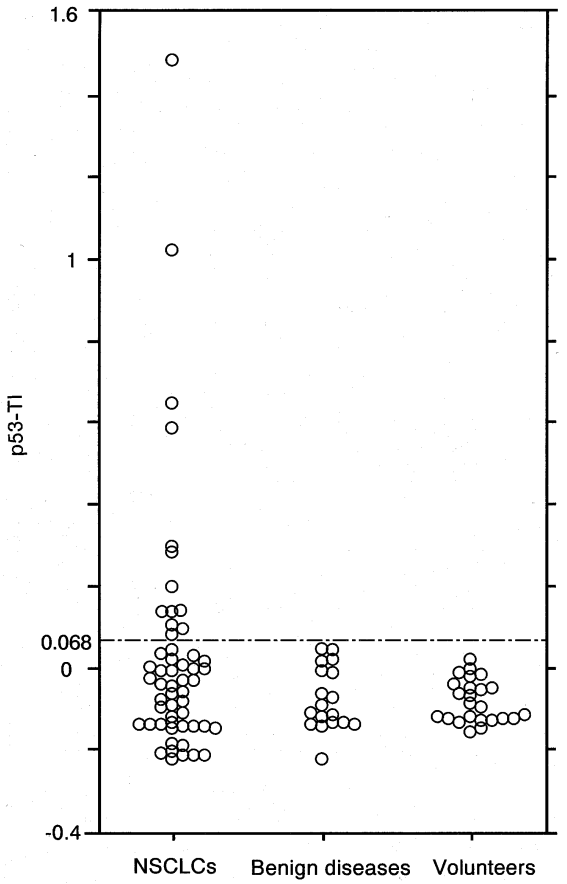

Fig. 1 Scatter diagram showing the titer index of serum anti-p53 antibodies in patients with non-small-cell lung carcinoma and benign diseases (including 12 benign tumors) and healthy volunteers. p53 titer index (*p53-TI*) = (absorption of patient sample – absorption of low control)/(absorption of high control – absorption of low control). The mean p53-TI value+3SD of healthy volunteers (0.068) was taken as the cut-off for normal values

stage, and resectability were classified according to the criteria of the American Joint Committee on Cancer [1] and the General Rules for Clinical and Pathological Recording of Lung Cancer described by the Japan Lung Cancer Society [13]. Cellular differentiation was graded as well-differentiated, moderate, or poor for those patients with non-large-cell carcinoma. Large-cell carcinoma was classified as undifferentiated [24].

Smoking history was scored by the parameter "pack-years" (the number of packs smoked per day multiplied by the number of years the subject smoked tobacco), because smoking is one of the major causes of the development of NSCLC; 36 patients scored 30 or more pack-years and 26 patients scored less than 30. No patient had received any treatment, including chemotherapy or radiotherapy, prior to their surgery. Nineteen patients diagnosed as having benign lung and mediastinal diseases, including 12 benign tumors, and 29 healthy volunteers were tested for p53 autoantibodies as control groups.

Enzyme-linked immunosorbent assays (ELISA) and criteria for positive titers of p53 autoantibodies

The detection of p53 autoantibodies in the patients' sera was performed with a commercially available ELISA (p53-autoantibodies ELISA, lot. no. 1795; Dianova, Hamburg, Germany). The assay was performed in duplicate-well assays according to the manufacturer's instructions. Briefly, 100 µl patient serum diluted 1:100 was added to microtiter wells coated with human recombinant p53 protein for 60 min at 37 °C in a humid chamber. After five washes with phosphate-buffered saline, goat anti-(human IgG) antibody conjugated with peroxidase was added for 30 min at 37 °C. Immediately after washing, the substrate 3,5,3,5-tetramethylbenzidine was added for 30 min at room temperature in the dark. The enzymatic process was stopped by adding 2 M hydrogen chloride, and light absorption was measured at 450 nm in a spectrophotometer (ImmunoReader NJ-2000; InterMed Co., Tokyo, Japan). Human serum (negative control) and three different concentrated anti-p53 antibody standards (low, medium, and high controls) were provided by the manufacturer. The controls had been standardized for autoimmunoreactivity by Western blotting. The anti-p53 autoantibody titer index (p53-TI) was defined as follows:

p53-TI = (absorption of patient sample – absorption of low control)/(absorption of high control – absorption of low control).

The mean p53-TI value+3SD of 29 healthy volunteers was considered a cut-off value. In addition, the data of the 101 control samples for the p53-TI provided by the manufacturer were noted separately. We measured samples for p53-TI at least twice to determine the reproducibility.

Table 1 Serum anti-p53 autoantibody in primary resected non-small-cell carcinoma. *P* calculated from the χ^2-text. *ca.* carcinoma

Clinical features	Anti-p53 antibody		*P*
	Positive	Negative	
Age			
70 years or more	5	20	
Less than 70 years	8	29	0.878
Sex			
Male	11	32	
Female	2	17	0.179
Smoking history			
30 pack years or more	9	27	
Less than 30 pack years	4	22	0.358
Histology			
Adenocarcinoma	6	27	
Squamous cell ca.	3	18	
Large-cell ca.	4	4	0.031[a]
Differentiation			
Well, moderately	6	33	
Poorly, undifferentiated	7	16	0.160
Stage			
I, II	8	25	
IIIa, IIIb	5	24	0.499
T classification			
T1, T2	9	33	
T3, T4	4	16	0.897
Tumor size			
30 mm or less	6	25	
More than 30 mm	7	24	0.755
N classification			
N0	9	27	
N1, N2, N3	4	22	0.358

[a] Large-cell carcinoma versus squamous cell carcinoma and adenocarcinoma

Immunohistochemistry

Immunohistochemical staining for p53 was performed by the streptavidin-biotin-peroxidase method with a Histofine SAB-PO(M) kit (Nichirei, Tokyo, Japan). The anti-p53 antibody DO-7 (Dako Japan Co. Ltd, Kyoto, Japan) was used. All slides were evaluated independently by two pathologists (H.O., K.H.) and positive tumors were those in which more that 10% of tumor cell nuclei were stained with DO-7 antibody.

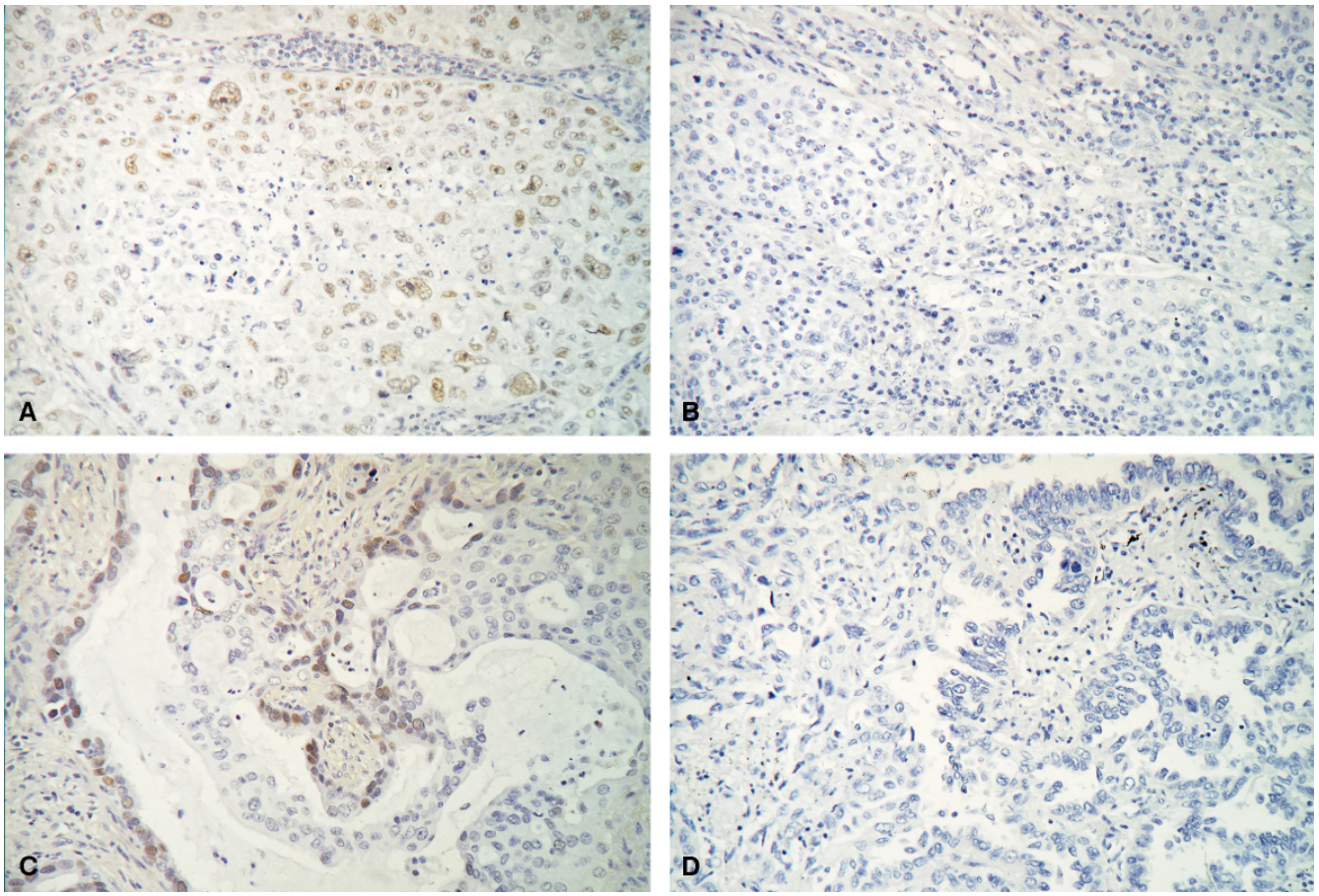

Fig. 2A–D Representative immunohistochemical stains of non-small-cell carcinoma with an anti-p53 antibody (DO-7). There is nuclear staining of a large number of tumor cells in a case of squamous cell carcinoma (**A**) and in a case of adenocarcinoma (**C**), whereas staining is negative in a case of squamous cell carcinoma (**B**) and in a case of adenocarcinoma (**D**)

Statistical analyses

Differences between each clinical feature were estimated by the χ^2-test. Correlations between the titer of serum anti-p53 autoantibodies and immunohistochemical staining for p53 were estimated by the χ^2-test. Differences were regarded as statistically significant at levels of $P < 0.05$.

Results

Anti-p53 autoantibodies and clinical features in 62 primary resected NSCLC

The mean value ± SD (standard deviation) of p53-TI was 0.028 ± 0.284 in 62 patients of NSCLC, –0.066 ± 0.077 in 19 patients with benign lung and mediastinal diseases, and –0.076 ± 0.048 in 29 healthy volunteers. The cut-off value of p53-TI adopted was 0.068, the mean index+3SD in the volunteers. The mean index and standard deviation of 101 healthy volunteers provided by the company were –0.077 and 0.032 respectively. Sera from all patients with NSCLC or benign diseases and all volunteers were tested twice to confirm the results.

Out of 62 patients, 49 (79.0%) had a p53-TI at the cut-off value (0.068) or less, regarded as indicating the absence of anti-p53 autoantibodies (Fig. 1). Thirteen patients (21.0%) demonstrated a p53-TI of more than 0.068, otherwise all 19 benign lung and mediastinal diseases were within the normal range. There was a statistically significant difference between p53-TI values of patients with large-cell carcinoma and those the other two groups (P = 0.031). However, the other clinical features showed no significant correlations (Table 1).

Correlation between serum anti-p53 autoantibodies and immunohistochemical staining for p53

Out of 62 patients, 25 (40.3%) had positive immunohistochemical staining for p53 (Fig. 2). None of the clinical features demonstrated a significant correlation with immunohistochemical staining for p53 (Table 2).

Ten of 25 patients (40%) with positive immunohistochemical staining for p53 had a positive serum p53-TI (Table 3). The serum p53-TI was positive in 3 of 37 patients (8%) with negative immunohistochemical staining. There was a statistically significant difference between these two groups (P = 0.0025).

Table 2 Immunohistochemical staining for p53 in primary resected non-small-cell carcinoma. *P* calculated from the χ^2-text. *ca.* carcinoma

Clinical features	p53 immunohistochemical staining		*P*
	Positive	Negative	
Age			
70 years or more	8	17	
Less than 70 years	17	20	0.155
Sex			
Male	19	24	
Female	6	13	0.351
Smoking history			
30 pack years or more	15	21	
Less than 30 pack years	10	16	0.800
Histology			
Adenocarcinoma	14	19	
Squamous cell ca.	8	13	
Large-cell ca.	3	5	0.861[a]
Differentiation			
Well, moderately	17	22	
Poorly, undifferentiated	8	15	0.495
Stage			
I, II	16	17	
IIIa, IIIb	9	20	0.162
T classification			
T1, T2	16	26	
T3, T4	9	11	0.604
Tumor size			
30 mm or less	12	21	
More than 30 mm	13	16	0.498
N classification			
N0	15	21	
N1, N2, N3	10	16	0.800

[a] Large-cell carcinoma versus squamous cell carcinoma and adenocarcinoma

Table 3 Correlation between immunohistochemical staining for p53 and serum anti-p53 autoantibodies in primary resected non-small-cell carcinoma. *P* calculated by the χ^2-test

Serum anti-p53 autoantibodies	Immunohistochemical staining for p53		P
	Positive	Negative	
Positive	10	3	0.0025
Negative	15	34	
Total	25	37	

Discussion

The clinical significance of serum anti-p53 autoantibodies is not known. In this study, we confirmed that sera from 21.0% of NSCLC patients contained autoantibodies against p53. A positive titer for these autoantibodies was significantly correlated with positive immunohistochemical staining for p53. The prevalence of p53 autoantibodies in sera has been reported to be 8.4% [10], 11.8% [31], and 24% [26] in lung cancer, 15% [25] in breast cancer, 25% [4] in esophageal cancer, and 24% [2] in ovarian cancer. However, *p53* gene mutations have been found in 43%–75% of NSCLC [6, 18, 20, 21], and mutated p53 protein has been immunohistochemically detected in 46%–75% of cases as well [5, 11, 18, 19]. We also detected p53 protein in 40% of our cases. The prevalence of p53 autoantibodies in sera has been much lower than the frequency of *p53* gene mutations and mutated p53 protein quoted in most reports, including our study.

No autoimmune response should occur against wild-type p53 protein if it is not overexpressed in tumors, because the antigen is recognized as self. Wild-type p53 protein is short-lived. However, mutated p53 proteins accumulate in the presence of *p53* gene mutations as a result of stabilization [15]. Labrecque and Schlichtholz have reported that autoantibodies for p53 did not recognize epitopes in the mutated portion. They suggested that the immunogenicity of mutated p53 protein was not high, although p53 autoantibodies were produced with accumulation of mutated p53 proteins [14, 25].

Another researcher has suggested that a 70-kDa heat-shock protein that complexes with accumulated p53 protein as a chaperone is responsible for the conformation of p53 autoantibodies [7]. We could not clarify the presence of these complexes in NSCLC immunohistochemically (data not shown). Our results suggest that the generation of p53 autoantibodies in the sera of NSCLC patients not only reflects accumulation of p53 and stabilization of mutant p53, but also partly results from differences in the immune response of each host.

The serum anti-p53 autoantibodies were not associated statistically with any clinical feature except for histological type. No correlation was demonstrated between immunohistochemical staining for p53 and any clinical feature. Schlichtholz and Peyrat have demonstrated a correlation between serum p53 autoantibodies and histological grade and hormone receptor status in breast cancer [23, 25]. This is the first report demonstrating a correlation between anti-p53 autoantibodies and histological types in lung cancer.

We could not find any symptom related to the autoimmune response in those patients who had detectable titers of p53 autoantibodies. However, the autoimmune response to overexpressed p53 proteins has to occur in each host. Our clinical interest in this phenomenon lies in the alterations in the malignant characteristics and prognosis of NSCLC. Some studies have reported on prognosis and p53 autoantibodies in other malignancies [2, 23, 30].

In conclusion, the mechanism accounting for the presence of serum anti-p53 autoantibodies remains obscure, but appears to depend on differences in the immune response of each host to the overexpression of p53 proteins in tumor cells. As a test for p53 autoantibodies in serum, for use in diagnostics or the monitoring of tumor burden, the assay used in this study would have a sensitivity of 40% and a specificity of 92%. At this level of performance, the method may be useful for the detection of p53 autoantibodies as surrogate clinical markers for the presence of *p53* mutations and p53 overexpression.

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