IMMUNOHISTOCHEMICAL DETECTION OF P53 PROTEIN IS NOT ASSOCIATED WITH A POOR PROGNOSIS IN NON-SMALL-CELL LUNG CANCER

Immunohistochemical detection of the p53 gene product by monoclonal antibodies has been shown to be associated with a poor clinical outcome in carcinomas of the breast and stomach. Because the prognostic relevance of p53 immunostaining in lung cancer is still under debate, we studied the expression pattern and clinical significance of such staining in 73 patients with operable non-small-cell lung cancer. p53 expression was detected on frozen sections with the use of monoclonal antibody p1801, which recognizes both the wild-type and mutant gene product (alkaline phosphatase-anti-alkaline phosphatase method). A tumor was considered p53 positive if more than 1% of the tumor cells were stained. The p53 expression pattern was compared with clinicopathologic parameters, and analysis of follow-up, based on the data of 65 patients, was done by a log rank test (median observation time, 780 days). Nuclear p53 staining was detected in 33 of 73 non-small-cell lung cancers (45.2%). Comparison with clinicopathologic parameters demonstrated that the p53 protein was detected more frequently in younger patients (younger than 50 years, $p = 0.014$), whereas no correlation was found with sex, tumor differentiation, tumor histologic type, or TNM stage. Surprisingly, follow-up analysis revealed that p53 staining was associated with an increased rate of disease-free survival, especially in patients with early stage tumor disease ($p = 0.004$) and in male patients ($p = 0.023$). Counter to previous studies in other solid tumors, immunocytochemical detection of p53 expression does not predict a poor clinical outcome in non-small-cell lung cancer. In early-stage lung cancer it might be associated with an improved disease-free survival, which suggests that the majority of the detected protein inherits the wild-type tumor suppressor function. (J THORAC CARDIOVASC SURG 1995;109:1205-11)

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The prognosis of patients with non-small-cell lung cancer (NSCLC) is still poor. The disease in most patients is inoperable at the time of diagnosis and in patients with operable primary tumors metastatic relapse is a frequent event. In fact, about 40% to 50% of the patients thought to have limited tumor disease (tumor stage I or II) will have a relapse within 3 years after operation, which indicates that these patients cannot be cured by operation alone.¹

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To identify these patients at risk, additional prognostic parameters need to be established.

Mutations of the p53 gene play an important role in the carcinogenesis and progression of solid human tumors. The expression of nuclear p53 is regulated during the cell cycle and it has been suggested that one of its main functions is to block the transition from G₀/G₁ to the S phase of the cell cycle.² Recently, it has been demonstrated that by using the polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) assay, point mutations of the p53 gene occur in approximately 45% of resected NSCLCs, and patients in which this occurs indeed have a poorer prognosis.³ Surprisingly, this prognostic influence was only significant in patients with advanced-stage disease, whereas the relapse rate in patients at stages I and II were unaffected.

An indirect but simpler method to detect p53 mutations is based on the observation that in comparison with the wild-type gene product, the mutant

Table I. Frequency of p53 expression in NSCLC

Parameter	No. of patients	p53 ⁺ (%) [*]	p53 ⁻	p Value [†]
All patients	73	33 (45.2)	40	—
Age (yr)				
< 50	15	11 (73.3)	4	0.014
≥ 50	58	22 (37.9)	36	
Sex				
Female	16	8 (50.0)	8	0.662
Male	57	25 (37.3)	32	
Grade of differentiation [‡]				
G1-2	34	15 (44.1)	19	0.702
G3	37	18 (48.6)	19	
Tumor histologic type				
Adenocarcinoma	35	14 (40.0)	21	0.864
Squamous cell carcinoma	30	15 (50.0)	15	
Adenosquamous carcinoma	2	1 (50)	1	
Large-cell carcinoma	6	3 (50)	3	
Tumor stage [§]				
Stage I-II	38	20 (52.6)	18	0.139
Stage IIIa-IV	34	12 (35.3)	22	

^{*}Frequency of p53 expression was determined by immunohistochemistry using mAb p1801. A tumor was considered to be positive if more than 1% of tumor cells were stained.

[†]Pearson's χ^2 test.

[‡]In tumors with mixed histologic types grade of differentiation was not determined ($n = 2$).

[§]In one patient tumor staging was not available.

p53 gene product is more stable and has a prolonged half-life.⁴ This accumulation of mutant p53 protein allows its immunohistochemical detection on frozen or paraffin-embedded sections. In contrast to the PCR-SSCP assay, this technique can easily be done and established in clinical pathologic examinations. Hence detection of p53 mutations by immunohistochemistry might be a practicable tool leading to an improved staging of the disease in individual patients.

We, therefore, performed a prospective study in 73 patients with operable NSCLC using immunohistochemistry with the monoclonal antibody (mAb) p1801, which recognizes both the wild-type and mutant gene product. Counter to our expectations, we observed that immunohistochemical detection of p53 expression predicts an improved disease-free survival, which raises doubt on the suitability of p53 immunostaining for indirect assessment of p53 gene mutations.

Patients and methods

Patients and follow-up. Tumor specimens from 73 patients with NSCLC were examined. Before operation all patients underwent the conventional staging procedures and were found to have operable primary tumors (TNM stage less than or equal to T3 N2 M0). The median age at the time of operation was 59 years, range 38 to 77. In

general lobectomies or, if necessary, pneumonectomies were done, followed by a radical lymphadenectomy. Patients whose tumors were classified pathologically as T3 N2 M0 or higher after operation had adjuvant radiation therapy.

Follow-up care was organized by the department of pulmonary medicine. Evaluation included physical examination, chest roentgenography, and blood examinations every 3 months and bronchoscopy, computed tomographic scan of the thorax, abdominal ultrasonography, and a bone scan every 6 months after operation. Complete information was obtained from a representative group of 65 patients with a median follow-up of 780 days (range 450 to 1170). Six patients with deaths unrelated to cancer have been excluded from the analyses ($n = 3$ in the p53-positive group and $n = 3$ in the p53-negative group). Two patients were lost to follow-up.

Tumor samples and immunohistochemistry. Representative tumor samples were obtained from the fresh surgical specimen and immediately snap-frozen in liquid nitrogen. The frozen tissue was stored at -80°C until use. Out of each frozen tumor sample 10 to 15 serial cryostat sections of 5 μm thickness were cut and transferred onto glass slides. After air drying for 24 hours, slides were stored at -20°C .

p53 protein was detected by mAb p1801 (immunoglobulin [Ig] G1, Dianova, Hamburg, Germany), which recognizes both the wild-type and mutant gene product.

For immunohistochemistry the slides were fixed in acetone for 10 minutes and air dried for 45 minutes. Then the cryostat sections were stained according to the alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining procedure described by Cordell and associates.⁵ After rehydration through incubation with Tris-buffered solution (pH 7.6) for 15 minutes, AB serum (pooled serum from donors with blood group AB, diluted 1:10 with phosphate-buffered solution; Biotest, Dreieich, Germany) was applied for 20 minutes to block unspecific binding. Subsequently, specimens were incubated for 45 minutes with the primary antibody diluted with Tris-buffered solution (1:50). After each incubation step, the slides were washed three times with Tris-buffered solution. The bridging antibody (Dako Corp., Carpinteria, Calif.) was applied for 30 minutes, followed by incubation with the APAAP complex (Dako) for 30 minutes. Staining was visualized by the Neufuchsin method. Counterstaining was done with Mayer's hemalum solution.

As a negative control for unspecific antibody binding, the primary antibody was replaced by an irrelevant mouse myeloma protein of identical isotype (MOPC 21, IgG1; Sigma, Deisenhofen, Germany).

Evaluation of the specimens. The slides were evaluated, in a double-blind fashion, by two observers with light microscopy. A tumor was considered p53 positive if more than 1% of all visible tumor cells were stained. Localization of the epithelial tumor cells within the specimen was confirmed by the anti-epithelial antibody EP4 (IgG1, Dako⁶), which stains more than 96% of all bronchogenic carcinomas (data not shown).

In approximately 85% of the cases both observers obtained the same results; the remaining slides were reevaluated and a consensus decision was made.

Fig. 1. Immunostaining of p53 protein (APAAP method) with mAb p1801 of squamous cell carcinoma of lung (tumor stage I) demonstrating intense nuclear staining of about 60% of tumor cells. Surrounding peritumoral tissue remains free of stain.

Statistical analysis. For comparison of clinicopathologic parameters contingency tables were tested by Pearson's χ^2 test. For analysis of follow-up data, Kaplan-Meier curves were calculated and a log rank test was done with the SPSS software package (SPSS Inc., Chicago, Ill.).

Results

Expression of p53 in NSCLC. By immunohistochemistry and the mAb p1801, p53 nuclear staining was detected in 33 (45.2%) of 73 patients with operable NSCLC (Table I, Fig. 1). The staining was limited to the nucleus in 29 cases, but some additional, weak cytoplasmatic staining was observed in four tumors. With a semiquantitative score less than 30% of the tumor cells were stained in 21.2% (7 of 33) of the tumors, in 45.5% (15 of 33) between 30% and 60% of the tumor cells were positive, and 33.3% (11 of 33) of the neoplasms displayed more than 60% positive cells. Normal lung tissue was always negative for p53 staining.

Comparison with clinicopathologic parameters demonstrated that the p53 protein was detected more frequently in younger patients (younger than 50 years; $p = 0.014$), whereas no correlation was found with sex, tumor grading, tumor histologic type, or TNM stage (Table I).

Prognostic significance of p53 expression. After a median observation time of 780 days, follow-up analysis revealed that p53 expression tended to be

associated with an increased rate of disease-free survival ($p = 0.134$, Fig. 2, A, Table II). This difference was significant in male patients ($p = 0.023$) and in patients with early-stage tumor disease (stage I or II, $p = 0.004$, Fig. 2, B). p53 expression was not of prognostic significance in patients with advanced tumor disease (Fig. 2, C). Interestingly, four out of the eight female patients with p53-positive tumors had a relapse within the observation period, in contrast to none of the female patients with p53-negative tumors, which suggests that in women p53 expression might be associated with a poorer prognosis.

Discussion

Mutations of the p53 gene have been described in different types of cancer and they play an important role in tumorigenesis and progression of certain solid tumors. The wild-type protein has a half-life time of only 5 to 10 minutes, which can be prolonged up to 30-fold in transformed cells with mutant forms of p53 protein resulting in its accumulation in the nucleus.⁴ Therefore, instead of demonstrating the gene mutation directly by deoxyribonucleic acid (DNA) sequencing or PCR-SSCP technology, immunohistochemical methods were used in the present study to indirectly assess p53 mutations. Nevertheless it is noteworthy that p53 protein can

Fig. 2. Influence of p53 immunostaining on disease-free survival in patients with NSCLC. **A**, All patients. **B**, Patients with tumor stages I or II. **C**, Patients with tumor stages IIIa through IV. Tumors were scored as positive if more than 1% of tumor cells were stained with mAb p1801 (*p* values by log rank test).

also be detected by immunohistochemistry despite the absence of a mutant p53 gene.⁷

In total, detectable p53 protein was found in 45.2% of the patients, which corresponds to findings in the respective work on NSCLC reported in the literature, including the incidence of mutations derived from DNA sequencing and PCR-SSCP analysis (Table III). Unspecific staining of cells was excluded by the use of an unrelated control antibody of the same immunoglobulin isotype as the anti-p53 mAb p1801. The majority of specimens displayed a nuclear staining of tumor cells. The biologic meaning of the cytoplasmatic staining observed in four samples is unclear. One explanation is that p53 protein can be stabilized in the cytoplasm through conjugation with other components such as heat shock proteins or mdm2 (murine double minute-2), a gene encoding for a nuclear phosphoprotein that may interact with both the mutant and wild-type p53 protein.

The definition of cutoff values in the analysis of immunohistochemical stainings is an obvious problem.⁸ Because the most invasive or metastatic clone of a primary tumor might account for only a small fraction of the entire tumor mass we decided to chose a low cutoff value. However, it should be noted that only seven of 33 tumors contained less than 30% p53-positive tumor cells.

With the exception of a preferential expression of p53 protein in younger patients (Table I), our present data confirm the results of several previous studies that demonstrated no correlation between p53 expression and conventional risk factors. In the only two reports that describe an association between p53 expression and an advanced tumor stage, these correlations were either relatively weak⁹ or based on the combination of different detection methods.⁷ Thus the prognostic significance of p53 alterations in NSCLC is still a point of contention.

In the present study, we demonstrate for the first time that p53 protein detected by immunohistochemistry is not associated with an unfavorable prognosis in patients with early-stage NSCLC. This is in contrast to the results reported by Quinlan and coworkers,¹⁰ who used the same anti-p53 mAb but described a poorer prognosis in patients with positive p53 staining. However, paraffin sections were used in this retrospective study, which might have hampered immunostaining. Furthermore the value of statistical analysis done in that study remains unclear, because of the lack of information on the length of the observation period and the use of χ^2

Table II. Prognostic significance of p53 expression in NSCLC

Parameter	No. of patients at risk	No. of tumor recurrences (%)	p Value*
All patients†			
p53 ⁺	30	14 (46.7)	0.134
p53 ⁻	35	20 (57.1)	
Age (yr)			
<50			
p53 ⁺	11	4 (36.4)	0.599
p53 ⁻	4	2 (50.0)	
≥50			
p53 ⁺	19	10 (54.5)	0.230
p53 ⁻	31	18 (58.1)	
Sex			
Female			
p53 ⁺	8	4 (50.0)	0.057
p53 ⁻	6	0	
Male			
p53 ⁺	22	10 (45.5)	0.023
p53 ⁻	29	20 (69.0)	
Grade of differentiation‡			
G1-2			
p53 ⁺	13	6 (46.2)	0.654
p53 ⁻	16	8 (50.0)	
G3			
p53 ⁺	17	8 (47.1)	0.106
p53 ⁻	18	12 (66.7)	
Tumor histologic type§			
Adenocarcinoma			
p53 ⁺	12	5 (41.7)	0.124
p53 ⁻	17	10 (58.8)	
Squamous			
p53 ⁺	14	8 (57.1)	0.442
p53 ⁻	14	8 (57.1)	
Tumor stage			
Stage I-II			
p53 ⁺	18	3 (16.7)	0.004
p53 ⁻	16	8 (50.0)	
Stage IIIa-IV			
p53 ⁺	11	10 (90.0)	0.465
p53 ⁻	19	12 (63.2)	

*p Value by log-rank test.

†Follow-up data were available from 65 patients.

‡Tumors with mixed histologic types were not analyzed (n = 1).

§Because of the small numbers tumors with mixed histologic types and large-cell carcinomas were not included.

||In one patient tumor staging was not available.

analysis of Kaplan-Meier curves. Although the follow-up period of our study was relatively short, most tumor recurrences in patients with NSCLC occur within the first 3 postoperative years. The presented data might therefore be meaningful. By stratifying the patients for tumor stage the relative numbers in each subgroup become small, which

Table III. *p53* in NSCLC

<i>Author</i>	<i>No. of patients</i>	<i>Method</i>	<i>mAb</i>	<i>Percentage of p53-positive tumors</i>	<i>Correlation to clinicopathologic parameters</i>	<i>Correlation to prognosis</i>
Fontanini (1993) ⁹	100	Immunocytochemistry (frozen sections, ABC method)	p240	35.9	Higher expression in N1-3	Not determined
Marchetti (1993) ⁷	53	Immunocytochemistry (frozen sections, ABC method)	p1801	68.0	No correlation	Not determined
		PCR-SSCP	p240	58.0		
		Immunocytochemistry + PCR-SSCP	p1801	47.0	No correlation	Not determined
				75.0	Higher expression in N1-3 and stage II-III	Not determined
Horio (1993) ¹¹	70	PCR-SSCP	—	49.0	No correlation	Poorer prognosis
Quinlan (1992) ¹⁰	114	Immunocytochemistry (paraffin sections, ABC method)	p1801	42.9	No correlation	Poorer prognosis
Iggo (1990) ¹⁶	40	Immunocytochemistry (frozen sections, APAAP method)	p240, JG8	55.3	Not determined	Not determined
Hiyoshi (1992) ¹⁷	88	Immunocytochemistry (paraffin sections, ABC method)	p1801	49.0	No correlation	Not determined
Chiba (1990) ¹⁸	51	cDNA sequencing	—	45.0	More frequent in squamous carcinomas	Not determined
Mitsudomi (1993) ³	120	PCR-SSCP	—	43.0	More frequent in squamous carcinomas	Poorer prognosis

ABC, Avidin-biotin-peroxidase complex technique.

results in a certain insecurity with respect to the conclusions on the proposed beneficial impact of p53-positivity in patients with node-negative disease. The apparent evidence for a beneficial impact of p53 staining on prognosis in patients with node-negative disease (Fig. 2) is therefore only suggestive and needs to be corroborated in more extensive clinical investigations. Thus far, evaluation of overall survival has not revealed any significant changes between patients with p53-positive and p53-negative tumors (data not shown). However, it appears to be sufficient to present data on disease-free survival, because in patients with NSCLC the detection of a tumor recurrence within the first 3 years after operation is closely associated with a cancer-related death in the following years. To support this assumption we analyzed the follow-up data from 220 patients with NSCLC operated on in our clinic between March 1989 and December 1991. A total of 114 of 220 patients had a tumor recurrence within the first 3 years after operation and 95 (83.8%) of these 114 patients died of their disease within the subsequent 3 years (data not shown).

There are two reports in which the PCR-SSCP technique is used, and both describe a poorer prognosis for patients with NSCLC with p53 mutations.^{3, 11} Although Mitsudomi and colleagues³ found a significant prediction only in advanced-stage tumors, Horio and coworkers¹¹ described a short-

ened survival in all patients bearing p53 mutations independent from tumor stage. These findings are not in contrast to our results, because immunostaining for p53 with mAb p1801 might also include the detection of wild-type protein rather than the specific staining of the mutant gene product. This view is supported by the recent observation that in more than 30% of NSCLCs p53 overexpression without p53 mutation occurred.⁷ Antibodies to specific conformations of the mutant p53 protein are also available. However, the use of these antibodies in immunohistochemical analysis is greatly hampered by the fact that the specificity can be abolished on air-dried and fixed specimens, leading to unspecific staining (data not shown).

A better prognosis for patients with p53-positive tumors has also been described for patients with squamous cell carcinoma of the tongue,¹² whereas in patients with carcinoma of the esophagus the prognosis was independent from p53 immunostaining.¹³ Thus the prognostic significance of p53 immunostaining might depend on the particular type of carcinoma and might be influenced by the continuous exposure to specific carcinogens such as tobacco smoke. In this context it is noteworthy that overexpression of wild-type p53 protein leads to a block in the G₀ phase of the cell cycle, which allows DNA repair to be executed.^{14, 15} Thus lung tumor cells with high amounts of wild-type p53 might be able to

protect themselves more effectively against exogenous DNA-damaging agents. This could explain the prognostic influence observed in our study and that in squamous cell carcinoma of the tongue. This interpretation of why positive immunostaining might be related to improved disease-free survival is, however, speculative. Although immunostaining in general detects a greater frequency of p53 abnormalities than SSCP analysis for mutation, it is currently thought that the additional abnormalities probably represent accumulation of wild-type p53 protein by virtue of its having been complexed (for example with mdm2). This does not mean that accumulation of the wild-type p53 has a tumor-suppressor effect. In fact, just the opposite may be true.

In conclusion, the present findings provide suggestive evidence that immunocytochemical detection of p53 protein in patients with NSCLC predicts a better prognosis in tumor stages I and II. Although this conclusion needs to be confirmed in a larger clinical study, one might speculate that the approach presented here may be used to determine the risk of relapse in patients with early-stage NSCLC with potential consequences for adjuvant therapy.

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