Expression of the p53 Family in Lung Cancer

HIDETAKA URAMOTO[1], KENJI SUGIO[1], TSUNEHIRO OYAMA[2], SHOJI NAKATA[1], KENJI ONO[1], TADAHIRO NOZOE[1] and KOSEI YASUMOTO[1]

[1]Second Department of Surgery and [2]Department of Environmental Health, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan

Abstract. *Background: p53 is mutated in about 50% of various malignant diseases including lung cancer. The p53 family consists of p53, p73 and p63. Although transactivating protein isoforms display p53-like functions, the ΔNp73 or ΔNp63 isoforms act toward p53 in a dominantly negative way. The aim of this study was to detect p53, ΔNp73 and ΔNp63 expressions in lung cancer and to evaluate the relationship between the expression levels of the proteins and the prognosis of patients with resectable lung cancer. Materials and Methods: Immunohistochemistry was employed to analyze the protein expression of p53, ΔNp73 and ΔNp63 in paraffin-embedded tumor samples from 132 well-characterized lung cancer patients. The correlation among the expression levels of p53, ΔNp73 and ΔNp63, clinical variables and survival outcome was analyzed. Results: Positive expressions of p53, ΔNp73 and ΔNp63 were detected in the tumor cells in 52, 77 and 44 of the 132 patients, respectively (39.4%, 58.3% and 33.3%) with lung cancer. The incidence of p53 positive expression was 54.5% and 27.6% in patients with squamous cell carcinoma and adenocarcinoma, respectively (p=0.03). The incidence of a positive expression of ΔNp73 was 64.5% and 43.6% in male and female patients, respectively (p=0.03). The incidence of ΔNp63 positive expression was 68.2% and 15.8% in the patients with squamous cell carcinoma and adenocarcinoma, respectively (p<0.0001). The expressions of p53 and ΔNp63 were not found to significantly affect survival. However, lung cancer patients with a positive ΔNp73 expression had a poorer prognosis than those with a negative ΔNp73 expression. In addition, multivariate analysis indicated that a positive expression of ΔNp73 was a significantly independent factor for predicting a poor prognosis (p<0.0001, risk ratio=3.38). Conclusion: Clinical evidence that the p53 family is frequently overexpressed in lung cancer specimens, especially ΔNp63 in squamous cell carcinoma, was provided. The expression of ΔNp73 may be a useful marker for predicting a poor prognosis in resectable lung cancer. Understanding how groups of lung cancer cell genes are coordinately expressed in response to physiological, immunological and micro-environmental stimuli remains an important goal. A better understanding of the gene expression profiles of tumors may help to identify molecular targets, such as ΔNp73, for effective therapy.*

Lung cancer is an aggressive carcinoma with a poor outcome and an overall survival rate of about 11 to 14% (1). The TNM staging system for lung cancer (2) is widely used as a guide to predict prognosis. Despite investigation into various therapeutic modalities, the survival rate of lung cancer patients has improved little and the management of patients still remains far from satisfactory due to rapid and extensive metastasis (3). Therefore, it is important to evaluate the malignant potential of tumor cells to make a more precise evaluation of the prognosis of patients with lung cancer. Recent advances in molecular biology and genetics have created new diagnostic and therapeutic possibilities for clinical oncology (4).

The expression of *p53* is altered in a high proportion of human neoplasms and is mutated in about 50% of various malignant diseases including lung cancer (5). The transcription factor and tumor suppressor, p53 and its two homologs, p73 and p63, form a family of proteins. The transactivating domain (TA) p73 or TAp63 isotypes might behave in a similar manner to p53 because they have various transactivating p53 downstream targets, induce apoptosis, and mediate cell cycle control. However, the ΔNp73 or ΔNp63 isotypes have been shown to display opposing functions as oncoproteins (6-9).

This is the first report to describe the relationship between p53 family gene expression and the prognosis of patients with lung cancer. This study was a retrospective cohort study, in which the p53 family gene expression was

Abbreviations: TA, transactivating domain; IHC, immunohistochemical.

Correspondence to: Hidetaka Uramoto or Tsunehiro Oyama, Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. Tel: +81-93-691-7442, Fax: +81-93-692-4004, e-mail: hidetaka@med.uoeh-u.ac.jp

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detected in lung cancer patients using immunohistochemical (IHC) staining, in order to evaluate the relationship between the protein expression levels of tumors and patient prognosis.

Materials and Methods

Patients and follow-up. The study included 132 consecutive patients with stage I to III lung cancer, who had undergone surgical resection between April 1993 and July 1996 at the University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan. The clinicopathological data were obtained from a retrospective chart review. The tumor stage was classified according to revisions in the international system for staging lung cancer (2). There were 93 men and 39 women in the series, with a mean age of 66.2 years (range, 40 to 84). The pathological types included 76 adenocarcinomas (among them seven patients with bronchioloalveolar type), 44 squamous cell carcinomas, two adenosquamous cell carcinomas, three carcinoids, five large cell carcinomas and two small cell carcinomas. Institutional review board-approved informed consent was obtained from all the patients or their guardians for the use of tumor tissue specimens collected at tumor resection.

For the postoperative follow-up, the patients were examined every month within the first year and at approximately 2- to 4-month intervals thereafter. The evaluations included a physical examination, chest roentgenography, a blood chemistry analysis and measurements of such classic tumor markers as carcinoembryonic antigen. Chest, abdominal and brain computed tomographic scans and a bone scintiscan were performed every 6 months for the first 2 years and then annually thereafter. If any symptoms or signs suggestive of recurrence appeared, then additional evaluations to confirm the site of recurrence were performed. The survival data were updated in January 2005. A follow-up was available for all patients. The median follow-up was 52.3 months.

Immunohistochemical (IHC) staining. A formalin-fixed, paraffin-embedded, 3-μm section was obtained from each of the 132 primary lesion samples. All the specimens were stained with hematoxylin and eosin for histopathological diagnosis. IHC staining was performed by the streptavidin-biotin-peroxidase complex method (10). The sections were briefly immersed in citrate buffer (0.01 mol/liter citric acid: pH 6.0) and then were incubated for two 5-minute intervals at 100˚C in a microwave oven for antigen retrieval. They were then incubated with the p53 (DO-1, Oncogene, Boston, MA, USA), ΔNp73 (Sigma genosis, Cambridgeshire, UK), ΔNp63 (p40, Oncogene Research Products, San Diego, CA, USA) antibody diluted at 1:100, 1:1000 or 1:3000, respectively, overnight in a cold room using a Labeled Streptavidin Biotin kit (Dako Corp., Carpinteria, CA, USA), as previously described (11-13). The antibody was diluted in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). Negative controls were processed by immunostaining with a pre-immune serum and by the exclusion of the primary antibody.

Immunohistochemical evaluation. The slides were independently reviewed by two of the authors (H.U. and K.S.), who were blinded to the clinicopathological data. To evaluate any correlation with the clinicopathological characteristics, the p53 expression scores were regarded as positive when the proportion of nuclear staining was 10% or more (11). The cells were judged to be positive for ΔNp73 when the cytoplasm, or both the nuclei and cytoplasm were stained (12). Because the tumor samples showed various degrees of staining intensity and different numbers of positive cells, ΔNp63 immunoreactivity was semi-quantified using the combined intensity and percentages based on the positive scoring method (13, 14). Intense nuclear staining was scored as 2, weak as 1 and negative as 0. Normal epithelial cells from the basal layer of the bronchus showed intense nuclear staining, which was used as an internal positive control of intensity (13). The percentage of positive cells was calculated by counting more than 1000 cells in randomly-chosen fields (10 x 40). The ΔNp63 staining score was defined as the sum of the percentage of positive cells at each intensity level multiplied by the intensity score. The mean value of the ΔNp63 score was 30.2±4.2. The ΔNp63 expression scores were divided into two groups, positive or negative. The ΔNp63 expression scores were positive when the proportion of stained cells was 30% or more (13).

Statistical analysis. Statistical significance was evaluated using the Pearson's Chi-square test. Survival curves were plotted according to the Kaplan-Meier method (15) and differences between the curves were analyzed by the log rank test (16). The Cox proportional hazards model was applied to a multivariate survival analysis (17). These results did not change when the follow-up was up to 5 years. The statistical difference was considered to be significant when the *p*-value was less than 0.05. All the data were analyzed using Survival Tools for StatView (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Immunohistochemical detection of p53 expression in lung cancer. The relationship between p53 expression and various clinicopathological characteristics of the patients is summarized in Table I. Among the 132 specimens, 52 (39.4%) stained positive for p53 in the nuclei of the tumor cells. The incidence of a positive expression of p53 was 27.6% and 54.5% in patients with adenocarcinoma and squamous cell carcinoma, respectively ($p=0.03$). No significant difference was observed between the p53 expression and gender, age at operation, pathological stage, pathological T status, pathological N status, ΔNp73 or ΔNp63 expression.

Immunohistochemical detection of ΔNp73 expression in lung cancer. The relationship between the ΔNp73 expression and various clinicopathological characteristics of the patients is summarized in Table II. Seventy-seven samples (58.3%) stained positively for ΔNp73, mainly in the cytoplasm of the tumor cells and, in six (4.5%) cases, where a positive expression of ΔNp73 was found both in the nuclei and cytoplasm. The incidence of positive ΔNp73 expression was 64.5% and 43.6% in male and female patients, respectively ($p=0.03$). Further, a positive expression for ΔNp73 was determined in 52.2%, 50.0% and 70.2% of patients at stages I, II and III, respectively ($p=0.04$, I-II *vs.* III). No significant

Table I. *Relationships between the level of p53 expression and the clinicopathological characteristics in 132 lung cancer patients.*

		p53			
Characteristics	Total	Positive	(%)	Negative	*P*
All cases	132	52	(39.4)	80	
Gender					
Male	93	40	(43.1)	53	
Female	39	12	(30.8)	27	0.19
Age (y)					
<65	46	19	(41.3)	27	
>=65	86	52	(38.3)	53	0.34
Histological type					
adenocarcinoma	76	21	(27.6)	55	
squamous cell carcinoma	44	24	(54.5)	20	0.03[a]
adenosquamous cell carcinoma	2	1	(50.0)	1	
carcinoid	3	2	(66.7)	1	
large cell carcinoma	5	3	(60.0)	2	
small cell carcinoma	2	2	(100.0)	0	
Pathological stage					
I	67	23	(34.3)	44	
II	18	8	(44.4)	10	
III	47	21	(44.7)	26	0.35[b]
pT					
T1	35	11	(31.4)	24	
T2	66	29	(43.9)	37	
T3	18	8	(44.4)	10	
T4	13	4	(30.8)	9	0.92[c]
pN					
N0	80	29	(36.3)	51	
N1	11	4	(36.4)	7	
N2	38	19	(50.0)	19	
N3	3	0	(0)	3	0.36[d]
ΔNp73					
Positive expression	77	29	(37.7)	48	
Negative expression	55	23	(41.8)	32	0.63
ΔNp63					
Positive expression	44	21	(47.7)	23	
Negative expression	88	31	(35.2)	57	0.17

The *p* value was calculated between adenocarcinoma and squamous cell carcinoma [a], between pathological stages I-II and III [b], between T1-2 and T3-4 carcinomas [c] and between N0 and N1-3 [d] status.

Table II. *Relationships between the level of ΔNp73 expression and the clinicopathological characteristics in 132 lung cancer patients.*

		ΔNp73			
Characteristics	Total	Positive	(%)	Negative	*P*
All cases	132	77	(58.3)	55	
Gender					
Male	93	60	(64.5)	33	
Female	39	17	(43.6)	22	0.03
Age (y)					
<65	46	25	(54.3)	21	
>=65	86	52	(60.5)	34	0.50
Histological type					
adenocarcinoma	76	46	(60.5)	30	
squamous cell carcinoma	44	23	(52.3)	21	0.38[a]
adenosquamous cell carcinoma	2	1	(50.0)	1	
carcinoid	3	2	(66.7)	1	
large cell carcinoma	5	3	(60.0)	2	
small cell carcinoma	2	2	(100.0)	0	
Pathological stage					
I	67	35	(52.2)	32	
II	18	9	(50.0)	9	
III	47	33	(70.2)	14	0.04[b]
pT					
T1	35	17	(48.6)	18	
T2	66	38	(57.6)	28	
T3	18	12	(66.7)	6	
T4	13	10	(76.9)	3	0.10[c]
pN					
N0	80	43	(53.7)	37	
N1	11	6	(54.5)	5	
N2	38	25	(65.8)	13	
N3	3	3	(100.0)	0	0.19[d]
ΔNp63					
Positive expression	44	24	(54.5)	20	
Negative expression	88	53	(60.2)	35	0.53

The *p* value was calculated between adenocarcinoma and squamous cell carcinoma [a], between pathological stages I-II and III [b], between T1-2 and T3-4 carcinomas [c] and between N0 and N1-3 [d] status.

difference was observed between the ΔNp73 expression and the age at operation, histological type, pathological T status, pathological N status or ΔNp63 expression.

Immunohistochemical detection of ΔNp63 expression in lung cancer. The relationship between the ΔNp63 expression and various clinicopathological characteristics of the patients is summarized in Table III. Forty-four (33.3%) samples were ΔNp63-positive in the nuclei. The incidence of a positive expression of ΔNp63 was 15.8% and 68.2% in patients with adenocarcinoma and squamous cell carcinoma, respectively ($p<0.0001$). No significant differences were observed between the ΔNp63 expression and gender, age at operation, pathological stage, pathological T or pathological N status.

Influence of clinicopathological factors on survival. Univariate or multivariate analyses were performed to detect prognostic factors. Five variables (gender, pathological stage, pathological T status, pathological N status and expression of ΔNp73) were found, by univariate analysis, to significantly affect the survival of patients (Table IV). The expressions of p53 and ΔNp63 were not found to significantly affect the survival of any patient, employing univariate or multivariate analyses (Tables IV and V). Differences in survival remained insignificant even after stratification according to stage or histological type. Furthermore, a multivariate analysis demonstrated three variables (pathological T status,

Table III. *Relationships between the level of ΔNp63 expression and the clinicopathological characteristics in 132 lung cancer patients.*

		ΔNp63			
Characteristics	Total	Positive	(%)	Negative	*P*
All cases	132	44	(33.3)	88	
Gender					
Male	93	32	(34.4)	61	
Female	39	12	(31.0)	27	0.69
Age (y)					
<65	46	12	(26.1)	34	
>=65	86	32	(37.2)	54	0.20
Histological type					
adenocarcinoma	76	12	(15.8)	64	
squamous cell carcinoma	44	30	(68.2)	14	<0.0001[a]
adenosquamous cell carcinoma	2	0	(0)	2	
carcinoid	3	1	(33.3)	2	
large cell carcinoma	5	0	(0)	5	
small cell carcinoma	2	1	(50.0)	1	
Pathological stage					
I	67	23	(30.3)	44	
II	18	7	(38.9)	11	
III	47	14	(30.8)	33	0.52[b]
pT					
T1	35	10	(28.6)	25	
T2	66	21	(31.8)	45	
T3	18	9	(50.0)	9	
T4	13	4	(30.8)	9	0.25[c]
pN					
N0	80	28	(35.0)	52	
N1	11	5	(45.5)	6	
N2	38	9	(23.7)	29	
N3	3	2	(66.7)	1	0.61[d]

The *p* value was calculated between adenocarcinoma and squamous cell carcinoma [a], between pathological stages I-II and III [b], between T1-2 and T3-4 carcinomas [c] and between N0 and N1-3 [d] status.

Table IV. *Univariate analysis using a proportional hazard model for the survival of 132 lung cancer patients.*

		Univariate analysis		
Variable	n	95% CI	HR	*P*
Gender				
Female	39		1	
Male	93	1.07-3.26	1.87	0.03
Age (y)				
<65	46		1	
>=65	86	0.73-1.96	1.20	0.47
Pathological stage				
I-II	85		1	
IIIA	47	1.95-5.00	3.11	<0.0001
pT				
T1-2	101		1	
T3	31	1.99-5.35	3.27	<0.0001
pN				
N0	80		1	
N1-2	52	1.62-4.15	2.60	<0.0001
p53 expression				
Positive	52	065-1.60	1.03	
Negative	80		1	0.923
ΔNp73 expression				
Positive	77	2.11-6.45	3.69	
Negative	55		1	<0.0001
ΔNp63 expression				
Positive	77	0.51-1.42	0.85	
Negative	88		1	0.625

pathological N status and expression of ΔNp73) to be independently associated with the survival of all patients (Table V). The overall 5-year survival rates for ΔNp73-positive and ΔNp73-negative patients were 32.1% and 71.4%, respectively ($p<0.0001$). Among stage I patients, the 5-year survival rates for patients with positive and negative ΔNp73 expressions were 56.6% and 74.5%, respectively ($p=0.067$). For stage II patients, the 5-year survival rates with positive and negative ΔNp73 expressions were 22.2% and 88.9%, respectively ($p=0.001$), while for stage III patients the corresponding results were 9.1% and 52.2% ($p<0.001$). The positive expression of ΔNp73 was associated with an increased risk of death by a factor of 3.38 as seen by a multivariate analysis ($p<0.0001$). Among adenocarcinoma patients, the 5-year survival rates for ΔNp73-positive and -negative cases were 37.0% and 72.7%, respectively ($p<0.01$), while in squamous cell carcinoma patients, the corresponding results were 26.1% and 64.2% ($p<0.01$).

Discussion

The p53 gene, a tumor suppressor gene, is often rendered non-functional in human cancer by point mutations (18). As a result, wild-type p53 gene therapy represents a potentially valuable tool for the treatment of various types of cancer, including lung cancer (19). However, the induction of apoptosis by the p53 protein has not been detected in all cases (20). To overcome these restrictions, the genes that promote apoptosis by p53-independent mechanisms are particularly useful (21). The recently identified p53 family member represents such a molecule. p53 and p73 or p63 share significant homology in their structural organization as characterized by an NH2-terminal transactivation domain, a central DNA-binding domain and a COOH-terminal oligomerization domain (6-9). In addition, both p73 and p63 can block the cell cycle or induce cell death in response to DNA damage (22-25). However, despite a strong functional homology, the biological functions of p73 and p63 are different. Unlike p53-deficient mice, spontaneous tumors did not develop in p73 or p63 knockout mice, although such mice exhibited hippocampal dysgenesis (26) or showed defects in epidermal development (27).

Table V. *Multivariate analyses of various prognostic factors.*

Variable	Characteristics		Risk ratio	95% CI	*P* value
	Unfavorable	Favorable			
Gender	Male	Female	1.16	0.64-2.10	0.62
T status	Pathological T3-4	Pathological T1-2	2.96	1.76-5.00	<0.0001
N status	Pathological N1-3	Pathological N0	2.96	1.44-3.85	0.006
p53 expression	Positive	Negative	0.89	0.55-1.44	0.63
ΔNp73 expression	Positive	Negative	3.38	1.91-6.00	<0.0001
ΔNp63 expression	Positive	Negative	0.89	0.53-1.50	0.66

We previously reported that a positive expression of ΔNp73 in lung cancer was a significant independent factor for predicting poor prognosis (12). However, ΔNp63 was not a prognostic indicator in squamous cell carcinoma of the lung (13). These results were consistent with previous findings (28-30). In the present study, the frequency of p53 family expression was investigated and the prognostic significance of the biological markers, as detected by immunohistochemistry using specific antibodies in 132 cases with lung cancers, were determined. Clinical evidence was provided that the p53 family is frequently overexpressed in lung cancer specimens. Moreover, univariate and multivariate analyses demonstrated that, among the clinicopathological T and N factors, a positive expression of ΔNp73 was a significantly independent factor for predicting a poor prognosis. As a result, ΔNp73 expression may be a marker of the malignant potential of lung cancer. In our studies, no significant relationships were observed between the p53 expression and ΔNp73 or ΔNp63 expressions. Further study might be needed to clarify how these complex families, including the different N- and C-terminal variants, interact to help or hinder the function of an individual member (7).

We also previously reported the utility of biomarkers, such as VEGF (31), YB-1 (32, 33), CK (34-36), c-erbB-2 (37) 3p (38), k-ras (39, 40), telomerase activity (41) and Grp78 (42) in determining the accurate staging of diseases and the selection of candidates for adjuvant therapy. Notably, these aims are assisted by the proper interpretation of the gene profiles of individual tumors.

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