GENERAL THORACIC SURGERY

MICROMETASTATIC P53-POSITIVE CELLS IN THE LYMPH NODES OF NON-SMALL-CELL LUNG CANCER: PROGNOSTIC SIGNIFICANCE

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Objectives: Approximately one fourth of all patients with stage I non-small-cell lung cancer die of tumor recurrence, despite radical removal of their tumors. We thus tried to detect micrometastasis in the regional lymph nodes indicated to be tumor free by conventional histopathologic methods in patients with non-small-cell lung cancer by using immunohistochemical staining for p53 protein. We also investigated the relation between micrometastatic p53-positive cells in the lymph nodes and the prognosis of the patients. **Methods:** Samples from 480 regional lymph nodes were taken from 47 patients who underwent pulmonary resection for non-small-cell lung cancer and whose primary lesions were positive for p53 immunohistochemical staining. These samples were fixed in formalin and embedded in paraffin. We used p53 immunohistochemical staining to detect micrometastatic tumor cells in the lymph nodes. **Results:** Cells positive for p53 protein were found in 14 of 31 (45%) patients with a negative pathologic lymph node status and in 26 of 315 (8.3%) lymph nodes in these patients. The proportion of patients with micrometastasis who survived was also significantly lower than the proportion of patients without micrometastasis who survived ($p = 0.0001$). **Conclusions:** Immunohistochemical staining for the p53 protein offers a rapid, sensitive, and useful way of detecting for micrometastasis to the regional lymph nodes in patients with non-small-cell lung cancer that is positive for p53 staining. The patients with such micrometastases have a poor prognosis and thus need to be carefully followed up after the initial pulmonary resection. (J Thorac Cardiovasc Surg 1997;114:339-46)

Despite undergoing radical surgery, about one fourth of all patients with stage I non-small-cell lung cancer (NSCLC) die of disease recurrence.¹

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This poor prognosis suggests the occurrence of micrometastasis, which often takes place before the primary operation and therefore cannot be assessed by routine clinical examinations. As a result, it may be important to detect occult micrometastasis so that disease recurrence can be predicted more accurately.

Currently, micrometastasis can be detected by immunohistochemical (IHC) staining or genetic methods. Several investigators²⁻⁶ have recently used these techniques to detect micrometastasis of various malignant tumors in the lymph nodes, while also reporting their prognostic significance. In carcinoma of the breast, micrometastasis to the axillary lymph nodes was detected by using IHC staining with monoclonal antibodies against such epithelial antigens as cytokeratin and epithelial membrane antigen, and a significant correlation between the detection of micrometastasis and a poor prognosis was

Table I. Clinical characteristics of the patients and the distribution of positive p53 IHC staining in the resected NSCLC

Background factors of the patients	No. of patients	No. of patients with positive p53 IHC staining		p Value*
		No.	%	
Total	101	47	47	
Age (yr)				0.2701
>60	66	34	52	
≤60	35	14	40	
Sex				0.7024
Male	69	33	48	
Female	32	14	44	
Histology				0.1467
Adenocarcinoma	56	22	39	0.0431
Squamous cell carcinoma	38	23	61	
Large cell carcinoma	4	2	50	
Adenosquamous carcinoma	2	0	0	
Mucoepidermoid carcinoma	1	0	0	
Pathologic stage				0.5482
I	51	25	49	
II	6	1	17	
IIIA	31	15	48	
IIIB	7	4	57	
IV	6	2	33	
T status				0.5707
T1	29	12	41	
T2	52	23	44	
T3	11	7	64	
T4	9	5	56	
N status				0.2339
N0	60	31	52	
N1	12	3	25	
N2	29	13	45	
Curability				0.5319
Complete	88	42	48	
Incomplete	13	5	38	

* χ^2 test.

thus reported.² In colorectal cancer, a genetic diagnosis of micrometastasis to the regional lymph nodes using mutant-allele-specific amplification (MASA) to detect mutations in *K-ras* or *p53* was examined, and the lymph node micrometastasis identified by this genetic method was found to be significantly correlated with disease recurrence.³ In addition, in esophageal adenocarcinoma, p53-positive findings on IHC staining of the primary tumor and the regional lymph nodes appeared to be associated with a reduced overall survival.⁷ In NSCLC, an IHC analysis using antibodies against epithelial antigens including anticytokeratin and Ber-EP4 for

detecting disseminated cancer cells in the regional lymph nodes was performed, and the frequency and prognostic significance of micrometastasis was thus reported.^{4,5} In these reports, micrometastasis in the regional lymph nodes was identified in 63% and 16% of patients with pN0 disease by using anticytokeratin and Ber-EP4, respectively. Nodal micrometastasis was also associated with a reduced survival in the study in which Ber-EP4 was used. The detection of micrometastasis in the regional lymph nodes of NSCLC using genetic procedures with reverse transcriptase-polymerase chain reaction for surfactant protein was examined in pulmonary adenocarcinoma and micrometastatic nodal involvement was thus identified in about half of the lymph nodes that had been judged to be tumor free according to a routine pathologic examination.⁶

The identification of micrometastasis in bone marrow by means of an IHC assay with anticytokeratin antibody was done on patients with gastric cancer, and micrometastasis in the bone marrow was detected in about one third of the patients at the time of the primary operation.⁸ The disseminated tumor cells were detected with the use of anticytokeratin in the bone marrow of patients with esophageal carcinoma, and the patients with tumor cells thus found in their bone marrow had a significantly shorter survival than the corresponding patients without any tumor cells in their bone marrow.⁹ In NSCLC, a similar IHC assay was performed with anticytokeratin antibody to detect micrometastasis in the bone marrow, and the micrometastases detected by this method closely correlated with a poor prognosis in such patients.¹⁰ The detection of micrometastasis not only in the lymph nodes but also in the bone marrow is important for predicting the prognosis of patients with various malignant diseases.

Alteration of the p53 tumor suppressor gene is one of the most frequent genetic changes in NSCLC, and it is mutated in about half of all patients with NSCLC.¹¹⁻¹⁴ An overexpression of p53 protein is specifically detected in tumor cells containing a p53 mutation but is not detected in either tumor cells without a p53 mutation or nontumor cells.¹⁵ Its immunoreactivity is found at a rate similar to that of the p53 gene mutations,^{12,16,17} and these characteristics are also preserved in the metastatic regional lymph nodes.³ For these reasons, we applied IHC staining of p53 protein in patients with positive p53 IHC staining for their primary NSCLC to detect micrometastatic tumor cells in the regional lymph nodes and also evaluated their prognostic significance.

Fig. 1. Section of the regional lymph node in a case of primary lung cancer. Three p53 IHC-positive tumor cells (arrows) are seen in a background of benign lymphatic cells. One of them, which is located at the center of this picture, has the most characteristic deep brown stained nucleus.

Patients and methods

Patients. We studied 101 consecutive patients with NSCLC who had undergone a surgical resection at the Department of Respiratory Surgery in National Oita Hospital during the 4-year period from 1987 to 1990. Tumor stage, TNM classification, and the curability of the operation were classified according to the fourth edition of the TNM classification of the International Union Against Cancer (1987). The patient characteristics are shown in Table I. According to the preoperative clinical staging, 57 patients had stage I disease, 4 had stage II, 32 had stage IIIA, 3 had stage IIIB, and 5 had stage IV disease. The 13 patients with an incomplete resection included 6 with unresected distant metastasis and 7 with a pleural dissemination of the tumor.

We retrospectively investigated the postoperative records of all 101 patients. The patients were examined every month within the first year after operation and thereafter at 2- to 4-month intervals as a rule. The evaluations included a physical examination, chest roentgenography, analysis of blood chemistry, and carcinoembryonic antigen assay. If any symptoms or signs of recurrence appeared in these examinations, further evaluations including bronchoscopy, computed tomography, abdominal ultrasonography, and a bone scan were performed. Only one patient died of respiratory failure within 1 month after the operation.

IHC staining. Samples including regional lymph nodes or a primary tumor were fixed in formaldehyde (formalin) and embedded in paraffin. At first, two slices of 4 μ m sections were obtained from samples of the primary lesions. The samples were all obtained from the widest transected areas of the primary tumors. One was for staining with anti-p53 protein antibody. Another

was for staining with hematoxylin and eosin. After paraffin was cleaned from the sections, they were placed in a citrate buffer and incubated five times for 5 minutes each in a microwave oven for antigen retrieval. They were then stained with mouse monoclonal antibody DO-1 (Oncogene Science Inc., Cambridge, Mass.) by means of the labeled streptavidin-biotin method (DAKO LSAB Kit, CA930 13, Dako Corp., Carpinteria, Calif.).

The sections were then examined for nuclear staining under a light microscope. We evaluated p53 immunoreactivity according to the proportion of the tumor cells in which the nuclei were stained. The sections from the primary lesions were scored positive when the proportion of stained cells was 10% or more. This criterion was based on the findings of another report,¹⁸ and the concordance rate between the DNA analysis and IHC staining was relatively constant when this cutoff level was used.

Next, the lymph node samples from all patients whose primary lesions were scored positive with p53 IHC staining were sectioned into slices 4 μ m thick. Every five slices from ten sections were then attached to glass slides and p53 IHC staining was done in the same manner. The sections from the lymph nodes were scored positive when the nuclei of one or more cells within the whole body section of the lymph nodes were stained.

The specimens were examined and checked by three of the authors (K.D., K.S., and T.O.) who had no knowledge of the patients' characteristics including the routine histopathologic lymph node status (N status).

Statistical analysis. Differences in several proportions were then compared by a χ^2 test. Survival curves were estimated by the Kaplan-Meier method, and any differences were analyzed by the log-rank test. The Cox proportional hazards model was used for a multivariate

Table II. The frequency of lymph node micrometastasis based on p53 IHC staining according to the pathological N status

Pathologic N status	No. of patients	No. of lymph nodes	No. of positive lymph nodes by pathologic study	Negative lymph nodes by pathologic study				
				Patients with p53-positive nodes		No. of lymph nodes examined	Lymph nodes with p53-positive cells	
				No.	%		No.	%
pN0	31	315	0	14	45	315	26	8.3
pN1	3	26	3	1	33	23	1	4.3
pN2	13	139	38	4	31	101	12	11.9
Total	47	480	41	19	40	439	39	8.9

Table III. Discrepancy in the nodal status between the pathologic study and p53 IHC staining

Nodal status	No. defined by pathology	No. defined by IHC staining		
N0	31	17		
N1	3	7	{	N0 5
				N1 2
N2	13	23	{	N0 9
				N1 1
				N2 13

*Forty-seven patients whose primary lesions were positive for p53 IHC staining were evaluated.

analysis. The data were considered to be significant when the *p* value was less than 0.05.

Results

Overexpression of the p53 protein in the primary lesions. Positivities of the IHC stain for p53 protein in the primary tumor are shown in Table I according to the background factors of the patients. The positive rate was 47% in the 101 patients. The rate of p53 protein overexpression was not associated with age, sex, stage, T status, N status, or curability of treatment. The positive rate was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma. In addition, the p53 protein overexpression was not correlated with patient survival.

Application of p53 IHC staining for the detection of micrometastasis in the lymph node. We examined p53 protein overexpression for the detection of micrometastatic tumor cells in the regional lymph nodes obtained from all 47 patients who had p53-positive primary lesions. A small number of tumor cells having nuclei that stained for p53 were seen in either the subcapsular or medullary sinuses of lymph node specimens, as shown in Fig. 1.

Table IV. A univariate analysis of various prognostic factors of 47 patients in Table III

Prognostic factors	No. of patients	Five-year survival (%)	<i>p</i> Value
Age (yr)			0.9861
>60	34	47	
≤60	13	46	
Sex			0.4234
Male	33	42	
Female	14	57	
Histology			0.4148
Squamous cell carcinoma	23	38	
Non-squamous cell carcinoma	24	54	
T status			0.2531
T1-2	35	51	
T3-4	12	33	
Pathologic N status			0.0132
N0	31	58	
N1-2	16	25	
M status			0.0050
M0	45	49	
M1	2	0	
Curability			0.0012
Complete	42	52	
Incomplete	5	0	
p53 immunohistochemical N status			0.0001
N0	17	88	
N1-2	30	23	

Prevalence of micrometastatic p53-positive cells in the regional lymph nodes. We examined 480 lymph nodes from the 47 patients described herein. A formal systematic dissection of mediastinal lymph nodes was performed in 35 patients, and samples of the mediastinal lymph nodes were taken in 12. In 19 (40%) of these 47 patients, p53-positive cells were detected in the pathologically negative lymph nodes. Of 439 lymph nodes that were negative by conventional pathologic examination, 39 (8.9%) contained

Fig. 2. The survival curves of the patients with pathologic N0 disease with and without micrometastasis in the regional lymph nodes detected by p53 IHC staining.

p53-positive cells. Of 315 lymph nodes from the patients with pN0 disease, 26 (8.3%) were positive for p53 on IHC staining. In the lymph nodes judged negative by pathologic examination, the positive rates of p53 overexpression were 8.3%, 4.3%, and 11.9%, respectively, in pN0, pN1, and pN2 disease (Table II).

Of the 31 patients whose regional lymph nodes were negative according to a conventional pathologic study, 14 (45%) patients had p53-positive cells in their lymph nodes (Table II). Five patients were upgraded to N1 status and nine were upgraded to N2 status. The details of the differences between the pathologic study and p53 IHC staining with regard to the N status are shown in Table III.

Influence of micrometastatic p53-positive cells in the lymph node on prognosis. The median survival time and 5-year survival of the patients with pN0 disease and micrometastasis were only 22 months and 21%, respectively. On the other hand, those of the patients with pN0 disease without micrometastasis were more than 60 months and 87%, respectively. Fig. 2 shows the significant difference of survival ($p = 0.0001$) between the patients with pN0 disease with and without micrometastasis.

A similar difference was also observed in patients with stage I disease. The 5-year survivals of patients with and without micrometastasis were 86% and 30%, respectively, and the difference was statistically significant ($p = 0.0013$) (Fig. 3). The disease-

free survival curve of the patients who had pN0 disease without micrometastasis was significantly better than that of the patients with micrometastasis ($p = 0.0018$). In addition, in stage I, the disease-free survival curve was significantly better in patients without micrometastasis than in those with micrometastasis ($p = 0.0343$).

A multivariate analysis was performed to determine whether the N status according to p53 IHC staining is an independent prognostic factor for survival. At first, the effect of several potential prognostic factors was analyzed by a univariate analysis (Table IV). The pathologic N status, distant metastasis, completeness of surgical resection, and N status according to p53 IHC staining were all statistically significant factors indicative of a poor prognosis. Thereafter, a multivariate analysis with a Cox regression model was done to determine which of these four factors has a predominant impact on survival. As shown in Table V, the N status on p53 IHC staining was found to be an independent and predominant prognostic factor ($p = 0.0016$, relative risk 11.4).

Discussion

The p53 tumor-suppressor gene mutation is one of the most frequent genetic changes for cancer.^{11,12} It is mutated in about half of the various known types of malignant diseases including NSCLC.^{12, 16, 17} The mutation of the p53 gene in tumor cells produces stabili-

Fig. 3. Differences in the survival curves between the patients with stage I disease with and without micrometastasis in the regional lymph nodes detected by p53 IHC staining.

Table V. A multivariate prognostic factor analysis of 47 patients in Table III

Prognostic factors	Estimated coefficient	Standard error	Relative risk	95% CI	p Value
Complete vs incomplete resection	0.74	0.65	2.09	0.58-7.49	0.259
M0 vs M1	0.84	0.94	2.32	0.37-14.7	0.373
Pathologic N0 vs N1-2	-0.22	0.45	0.80	0.33-1.95	0.622
p53 IHC N0 vs N1-2	2.44	0.77	11.4	2.51-52.0	0.002

CI, Confidence interval.

zation and accumulation of the protein in the nuclei, which reaches overexpressed levels detectable by IHC staining.

We used the antibody against p53 protein to detect micrometastasis in the regional lymph nodes of patients with NSCLC, because it was specifically overexpressed in the tumor cells and was undetectable in normal cells because of its short half-life and its low amounts.¹⁵ In this study, normal lung tissue specimens around the tumors were negative for p53 IHC staining.

Our approach enabled us to detect specifically micrometastatic tumor cells in the lymph nodes. We also examined p53 IHC staining in the lymph nodes obtained from seven patients with p53-negative primary lesions (five in pN0 and two in pN2). No positively stained cells were found in the lymph nodes of the five patients with pN0 disease, and the metastatic lesions in the lymph nodes of the two patients with pN2 disease were not stained at all. Therefore this method can be used for patients with

positive p53 staining in the primary tumor, which thus includes about half of all patients with NSCLC. In our investigation, the overexpression of p53 protein was preserved during metastasis to the regional lymph nodes. Their reactivity of the anti-p53 protein antibody was positive in all 41 lymph nodes with a pN1 and pN2 status. When the hematoxylin and eosin sections were reviewed after a p53 IHC examination, only two sections could be diagnosed as positive for micrometastasis among the 39 p53-positive lymph nodes diagnosed to be negative by pathologic study.

Currently, several antibodies against antigens specific for epithelial cells, including cytokeratins, are used for the detection of micrometastases.^{2, 4, 5, 8, 10} Chen and associates⁴ reported that lymph node micrometastasis was detected by means of an anti-cytokeratin antibody in 63% of patients with pN0 disease who had NSCLC. However, the antibodies against cytokeratins are specific for all epithelial cells but not for tumor cells, and thus they might

react positively with nontumor cells.^{4,5} Passlick and coworkers⁵ used a monoclonal antibody named Ber-EP4 to stain against two glycopolypeptides, both on the surface and in the cytoplasm of epithelial cells. This antibody was thus suggested to be more specific against cancer cells, and it also demonstrated nodal micrometastasis in 16% of the patients with pN0 disease and in 6.2% of the lymph nodes that were negative by routine histopathologic examination. Such IHC staining using anticytokeratin and anti-epithelial differentiation protein antibodies may thus be useful especially in patients with p53-negative tumors.

In our current study, the 5-year survival of the patients with pN0 disease and micrometastasis was 21%. However, the 5-year survival of those without micrometastasis was 87% and the difference was statistically significant. These results suggested that patients with micrometastasis should be followed up carefully regardless of pathologic N status.

Martini and colleagues¹ investigated the pattern of recurrences in resected stage I lung cancer and proved that about three fourths of the recurrences were hematogenous distant metastases. Among our 25 patients with stage I disease and p53-positive IHC staining in the primary tumor, four patients had a recurrence. All four recurrences were distant metastases. Furthermore, among our 31 patients with pN0 disease and p53-positive IHC staining in the primary tumor, all seven patients with recurrence had distant metastases, although five of them did concurrently have local recurrences (data not shown). Pantel and associates¹⁰ reported that micrometastasis in the bone marrow in patients with pN0 disease and NSCLC was an indicator for poor prognosis.¹⁰ Distant metastases are likely to occur frequently in patients with micrometastasis in the bone marrow. The reason that the presence of micrometastasis in the regional lymph nodes indicates the prevalence of distant metastasis remains to be clarified. Our preliminary analysis using anticytokeratin antibody and anti-p53 antibody regarding the relationship between p53 overexpression in the primary tumor and the detection of micrometastasis in the bone marrow suggested that micrometastatic cancer cells were more prevalent in the bone marrow of patients with a tumor having p53 protein overexpression than in those with a tumor having no p53 overexpression.

Various genetic approaches are expected to be used in the future for the detection of micrometastasis in the lymph nodes. Micrometastasis in the

lymph nodes of pulmonary adenocarcinoma were detected by means of reverse transcriptase-polymerase chain reaction with primers specific for surfactant protein.⁶ A genetic diagnosis of lymphatic micrometastasis in colorectal cancer using mutant-allele-specific amplification (MASA) for p53 gene mutation has also been tried.³ Such genetic diagnoses may also be useful in predicting the prognosis of patients. Both IHC and genetic DNA detection of p53 mutation seem to have the same ability to detect micrometastasis in the lymph nodes. In this study, we did not perform a molecular analysis. Harris and Hollstein¹¹ reported that more than 90% of the tumors with p53 missense mutations were positive in the test for abnormal accumulation of p53, whereas fewer than 20% in the tumors with p53 mutations are undetectable by p53 IHC staining owing to nonsense mutations. They also suggested that although the concordance between p53 gene mutation and the accumulation of p53 protein cannot therefore be perfect, immunoreactivity does appear to be an approximate indicator of the tumors with an altered p53 function.¹¹

In conclusion, IHC staining of p53 is considered to be a rapid and sensitive method to detect metastatic tumor cells in the lymph nodes. Micrometastasis in the lymph nodes detected by this procedure is an important indicator for prognosis after an initial pulmonary resection.

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