Correlation between Angiogenesis and p53 Expression in Lung Adenocarcinoma of Young Patients

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Lung cancer commonly occurs in individuals who are 60 years of age or older. Lung cancer in patients younger than 40 years of age is rare and is often advanced when discovered. However, the biological features of lung cancer in young adults have not yet been fully elucidated. This study was conducted to determine the role of p53 expression and neoangiogenesis in lung adenocarcinomas of young patients. Lung adenocarcinomas, which were surgically resected from 20 patients younger than 40 years of age between 1977 and 1996, were compared with lung adenocarcinomas selected with random sampling from 45 patients older than 60 years of age. The expression of p53, vascular endothelial growth factor (VEGF), CD34, a marker for vascular endothelial cells, and proliferating cell nuclear antigen (PCNA) were studied immunohistochemically in both young and elderly patient groups. Lung adenocarcinomas with p53-positive staining showed higher expression of VEGF protein than p53-negative tumors in both the young and the elderly groups. However, the intratumoral microvessel count was significantly higher in the p53-positive young group than in the elderly group. The percentage of VEGF-positive cells correlated significantly with intratumoral microvessel counts in the young group. The survival rate tended to be poorer in patients with a high VEGF labeling index and p53-positive staining than in other young patients. Lung adenocarcinoma occurring in young patients tends to have a poorer prognosis, and angiogenesis of lung adenocarcinoma in young patients is more closely correlated with p53 expression than in elderly patients. ———— lung adenocarcinoma; neoangiogenesis; p53; VEGF; young.

Lung cancer commonly occurs in individuals who are 60 years of age or older, and it has been reported that, among lung cancer patients younger than 40 years of age, females are becoming increasingly affected and adenocarcinoma is becoming more common (Pemberton et al. 1983; Antkowiak et al. 1989; Sugio et al. 1992; Shimono et al. 1994; Putnam et al. 1997; Schönfeld et al. 1999; Whooley et al. 2000; Maruyama et al. 2001; Skarin et al. 2001). Although several reports have shown no differences in the prognosis of surgically treated cases of lung cancer (Sugio et al. 1992; Shimono et al. 1994; Schönfeld et al. 1999; Maruyama et al. 2001; Skarin et al. 2001), lung cancer in young adults is reported to be associated with a poor prognosis (Putnam et al. 1997) and to be more aggressive (Antkowiak et al. 1989; Whooley et al. 2000). Furthermore, it has been reported that lung cancer in patients younger than 40 years of age is often more advanced at the time of diagnosis than in elderly patients (Pemberton et al. 1983;

Schönfeld et al. 1999; Maruyama et al. 2001; Skarin et al. 2001). However, the biological features of lung cancer in young adults have not yet been fully elucidated.

The p53 gene is one of the most important genes involved in the development and progression of various cancers. Several studies have shown that p53 expression correlates with tumor angiogenesis, and that up-regulation of vascular endothelial growth factor (VEGF) in lung cancer is associated with loss of wild-type p53 (Giatromanolaki et al. 1998) or mutation of the p53 gene (Fontanini et al. 1999; Niklińska et al. 2001; Yuan et al. 2002). VEGF is the most important cytokine associated with angiogenesis, and it is associated with proliferation, differentiation status (Shalaby et al. 1995), migration, and tube-like formation (Fong et al. 1995) of endothelial cells. Furthermore, VEGF secreted by tumor cells accelerates tumor angiogenesis in various cancers (Fontanini et al. 1997; Miyagami et al. 1998; Doi et al. 1999; Saito et al. 1999). CD34 is a protein

on the cell surface of bone marrow progenitor cells and blood vessel endothelial cells. Several reports have evaluated intratumoral microvessels using an antibody that recognizes CD34 antigen, and they demonstrated that such tumor microangiogenesis is correlated with axillary lymph node involvement in breast cancer (Hansen et al. 2000) and hematogenous metastases of gastric cancer (Tanigawa et al. 1996), colon cancer (Tanigawa et al. 1997), and non-small cell lung cancer (Matsuyama et al. 1998).

The purpose of the present study was to determine the role of p53 expression and neoangiogenesis in lung adenocarcinoma occurring in young adults. For this purpose, immunohistochemically determined nuclear p53 protein expression, cytoplasmic VEGF expression, and intratumoral microvessel counts based on CD34 and PCNA expression were compared in lung adenocarcinomas from 20 patients younger than 40 years of age (the adenocarcinomas were selected to avoid differences due to histological type) and unselected lung adenocarcinomas from 45 patients older than 60 years of age.

PATIENTS AND METHODS

Among 930 lung cancer patients who underwent surgical resections from 1977 to 1996, 20 (2.2%) patients with non-small cell lung cancer were younger than 40 years of age. The male to female ratio was 1.1. Tumor histology included adenocarcinoma ($n = 13$, 65%), squamous cell carcinoma ($n = 5$, 25%), large cell carcinoma ($n = 1$, 5%), and carcinoid tumor ($n = 1$, 5%). At the time of diagnosis, seven (35%) patients had stage I, two (10%) patients had stage II, seven (35%) patients had stage IIIA, two (10%) patients had stage IIIB, and two (10%) patients had stage IV. In this immunohistochemical study, lung adenocarcinomas, which were surgically resected from 20 patients younger than 40 years of age, were analyzed, including 13 specimens from this department and seven specimens resected at the Sasebo City General Hospital. The control group consisted of unselected lung adenocarcinomas of 45 patients older than 60 years of age, which were resected during the period between 1977 and 1996. Patients who underwent preoperative chemotherapy were excluded from the study. Tumor specimens were stained with hematoxylin-eosin. The histological diagnoses were established by two independent pathologists. Tumor staging was performed according to the TNM staging of the World Health Organization. The study protocol was approved by the Human Ethics Review Committee of the Nagasaki University School of Medicine.

Immunohistochemistry

Serially cut, 5-μm-thick paraffin sections were immunohistochemically stained using the avidin biotin complex method. For p53 staining, the sections were pretreated with 0.1 M citric acid in a microwave for 10 min. The primary mouse monoclonal antibody used for p53 protein (DO-7, Dako Corporation, Carpinteria, CA, USA) was diluted 1 : 50 and added, and then the preparation was further incubated overnight at 4°C. The expression of p53 protein was expressed as the percentage of immunopositive cells among 1,000 tumor cells. DO-7 antibody is considered to be a marker of both mutant and wild-type p53 protein. In the present study, the cutoff value for p53-positive staining was 20%, based on the correlation between p53 gene mutations and the percentage of p53-positive stain-

ing cells determined in a recent study (Salinas-Sánchez et al. 2007).

For VEGF staining, the sections were pretreated with 0.01 M Tris-HCl buffer in a microwave for 10 min. The primary mouse monoclonal antibody used for VEGF (JH121, Neomarkers, Fremont, CA, USA) was diluted 1 : 50 and added, and then the preparation was further incubated overnight at 4°C. The JH121 antibody recognizes the four isoforms of VEGF that contain 206-, 189-, 165-, and 121-amino acid residues. VEGF expression was expressed as the percentage of immunopositive cells among 1,000 tumor cells.

For CD34 staining, the sections were pretreated with 0.1% trypsin at room temperature for 30 min. The primary mouse monoclonal antibody used for CD34 (NCL-END, Novocastra Laboratories, Newcastle upon Tyne, UK) was diluted 1 : 50 and added, and then the preparation was further incubated overnight at 4°C. The assessment of intratumoral microvessels was performed near the edge of the central stromal region of the adenocarcinoma. After determining the area of highest vascularization at low power (40 × and 100 ×), the mean number of microvessels was determined on a 250 × field (10 × objective and 25 × subjective) in the five areas of highest vascularization. Two investigators performed the counting independently, and large vessels were excluded from the counting.

For PCNA staining, the sections were pretreated with 0.1 M citric acid in a microwave for 10 min. The primary mouse monoclonal antibody used for PCNA protein (PC-10, DAKO) was diluted 1 : 50 and added, and then the preparation was further incubated overnight at 4°C. For the evaluation of PCNA, the PCNA labeling index, which represents the number of immunopositive cells among a total of 1,000 tumor cells, was used. The peroxidase reaction was developed using diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. Normal mouse IgG (at a concentration similar to that of the primary antibody) was substituted for the primary antibody in each staining, and then the preparation was used as a negative control.

Statistical analysis

The data are expressed as means ± S.D. Differences in the clinicopathological factors between the elderly group and the young group were evaluated using the chi-squared test. The mean values of immunohistochemical variables were compared between the two age groups using the unpaired, two-tailed *t*-test. The mean values of the immunohistochemical variables related to p53 status were compared using Mann-Whitney's U test, because these groups were small and not normally distributed. A linear regression analysis was performed to assess the correlation between the percentages of VEGF-positive cells and the number of intratumoral microvessels. A *P* value < 0.05 was considered statistically significant.

RESULTS

Clinicopathological differences

Table 1 shows the age-based comparison of the clinicopathological characteristics of patients with lung adenocarcinoma. The male-to-female ratio in the young group (0.82) was not significantly different from that in the elderly group (1.81). With regard to tumor differentiation, 20% ($n = 4$) were well differentiated, 45% ($n = 9$) were moderately differentiated, and 35% ($n = 7$) were poorly differentiated in the young group, while the proportions in the elderly group were 53.3% ($n = 24$), 35.6% ($n = 16$), and 11.1% ($n = 5$),

respectively. There was a significant difference between the two age groups with respect to tumor differentiation ($p = 0.017$). The clinical stage at the time of diagnosis in the young group was stage I in 40% ($n = 8$), stage II in 10% ($n = 2$), stage IIIA in 30% ($n = 6$), stage IIIB in 15% ($n = 3$), and stage IV in 5% ($n = 1$), while the proportions in the elderly group were 46.7% ($n = 21$), 4.4% ($n = 2$), 31.1% ($n = 14$), 13.3% ($n = 6$), and 4.4% ($n = 2$), respectively. There was no significant difference in clinical stage between the two age groups.

TABLE 1. Age-based comparison of clinicopathological characteristics of patients with lung adeno-carcinoma.

	Young group ($n = 20$)	Elderly group ($n = 45$)
Sex (male : female)	9 : 11	29 : 16
Age (years)*	36.1 ± 4.7	64.6 ± 2.8
Differentiation (well / moderate / poorly)	4 / 9 / 7	24 / 16 / 5
Stage at diagnosis		
Stage I	8	21
Stage II	2	2
Stage IIIA+IIIB	9	20
Stage IV	1	2

*Data are mean ± S.D.

Differences in immunohistochemical staining for p53, VEGF, CD34, and PCNA

Nuclear expression of p53 protein was detected in the adenocarcinomas of both the young and the elderly groups (Fig. 1A). Table 2 shows that the percentage of p53-positive cases was significantly lower in the young group than in the elderly group ($p = 0.0256$). In the young group, for p53-positive staining adenocarcinomas, the clinical stage was stage I in 33.3% ($n = 2$), stage II in 0% ($n = 0$), stage IIIA in 33.3% ($n = 2$), stage IIIB in 33.3% ($n = 2$), and stage IV in 0% ($n = 0$), while for the p53-negative staining adenocarcinomas, the proportions for each clinical stage were 42.9% ($n = 6$), 14.3% ($n = 2$), 28.6% ($n = 4$), 7.1% ($n = 1$), and 7.1% ($n = 1$), respectively. In the elderly group, for the p53-positive staining adenocarcinomas, the clinical stage was stage I in 48.1% ($n = 13$), stage II in 7.2% ($n = 2$), stage IIIA in 29.6% ($n = 8$), stage IIIB in 7.2% ($n = 2$), and stage IV in 7.2% ($n = 2$), while for the p53-negative staining ade-

TABLE 2. Age-based comparison of immunohistochemical features of lung adenocarcinoma.

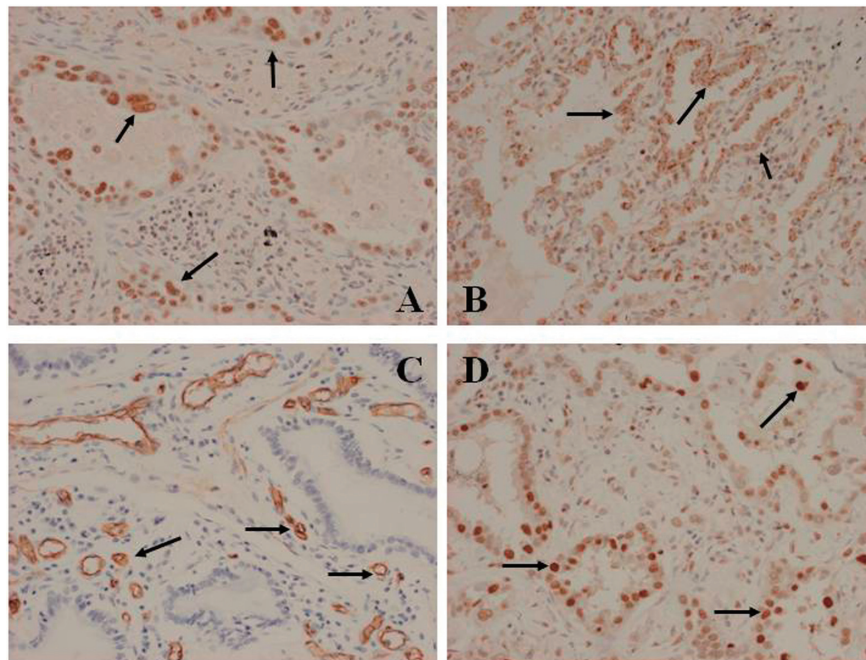|	Young group	Elderly group	P value
p53-positivity	6(30%)	27(60%)	0.026
VEGF-positivity*	64.0 ± 20.7	68.3 ± 15.8	NS
Intratumor microvessel counts*	91.3 ± 19.9	77.2 ± 18.8	0.008
PCNA labeling index*	42.0 ± 16.7	37.2 ± 11.6	NS

*Data are mean ± S.D.

Fig. 1. Immunohistochemical findings in representative adenocarcinomas of young adult patients. A) Nuclear expression of p53 protein (arrows). B) VEGF expression in the cytoplasmic and perinuclear regions (arrows). C) CD34 expression in large and small vascular endothelial cells (arrows). D) Nuclear expression of PCNA protein (arrows). Magnification, × 250.

A) Young group

r = 0.446、p = 0.0481

(Intratumor microvessel counts vs VEGF labeling index)

B) Elderly group

r = 0.01、p = 0.9474
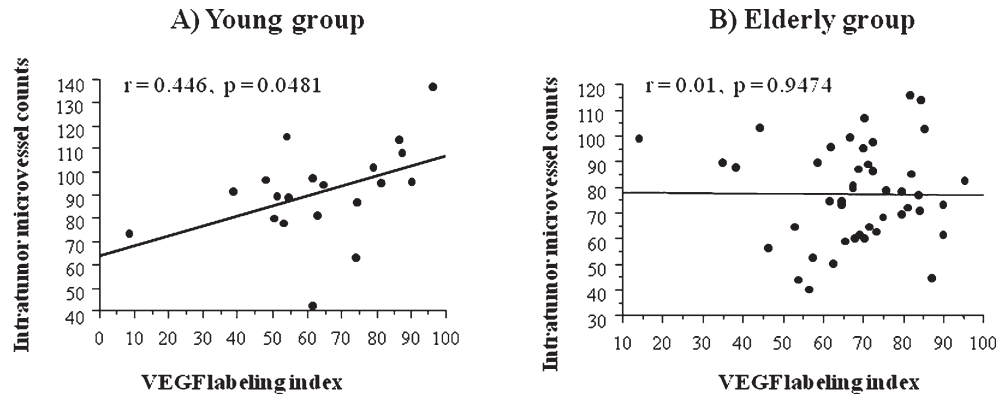
(Intratumor microvessel counts vs VEGF labeling index)

Fig. 2. Linear regression analysis of percentage of VEGF-positive cells and intratumoral microvessel counts in the two age groups.

TABLE 3. Comparison of the two age groups by p53 protein status in lung adenocarcinoma.

	Young group			Elderly group		
	p53(−)	p53(+)	P value	p53(−)	p53(+)	P value
n	14	6		18	27	
VEGF labeling index	58.9 ± 19.4	75.9 ± 20.1	NS	59.1 ± 18.3	74.4 ± 10.4	0.004
Intratumor microvessel counts	83.8 ± 16.9	108.8 ± 15.3	0.003	73.9 ± 15.5	79.4 ± 20.7	NS
PCNA labeling index	35.6 ± 14.6	56.9 ± 11.4	0.004	34.3 ± 10.8	39.2 ± 11.9	NS

*Data are mean ± S.D.

nocarcinomas, the proportions were 44.4% ($n = 8$), 0% ($n = 0$), 33.3% ($n = 6$), 22.2% ($n = 4$), and 0% ($n = 0$), respectively. There were no significant differences between the two p53 status groups with respect to the clinical stage in either the young or the elderly group. VEGF was expressed in the cytoplasmic and perinuclear regions in sections of both the young and elderly groups (Fig. 1B). The percentage of VEGF-positive cells was not different between the two age groups ($p = 0.3625$). The mean number of intratumoral microvessels was significantly higher in the young group (Fig. 1C) than in the elderly group ($p = 0.0079$). The PCNA labeling index was not significantly different between the young group (Fig. 1D) and the elderly group ($p = 0.1916$). A linear regression analysis showed that the percentage of VEGF-positive cells was significantly correlated with the intratumoral microvessel counts in the young group ($p = 0.0481$, $r = 0.446$, Fig. 2A), but not in the elderly group ($p = 0.9474$, $r = 0.01$, Fig. 2B).

Differences in immunohistochemical staining for VEGF, CD34, and PCNA according to p53 status

The mean percentages of VEGF-positive cells and the intratumoral microvessel counts were also compared in the p53-negative and p53-positive tumors in each of the two age groups (Table 3). In the young group, the VEGF labeling index tended to be higher in the p53-positive than in the p53-negative tumors, though the difference was not significant ($p = 0.1171$). In the elderly group, the VEGF labeling index was significantly higher in the p53-positive cases than in the p53-negative cases ($p = 0.0036$). On the other hand, the intratumoral microvessel count was significantly higher in the p53-positive young group than in the p53-negative cases of the same age group ($p = 0.003$), while the microvessel count in the elderly group was not affected by p53 expression ($p = 0.3421$). The PCNA labeling index was significantly higher in the p53-positive than in the p53-negative tumors of the young group ($p = 0.0039$), but no such difference was noted in the elderly group ($p = 0.1509$). Linear regression analysis demonstrated that the intratumoral microvessel count was significantly correlated with the PCNA labeling index in the young ($p = 0.0122$, $r = 0.543$, Fig. 3A) and elderly ($p < 0.0001$, $r = 0.648$, Fig. 3B) groups.

Relationship between overall survival and immunohistochemical staining according to p53 status

The predictive cutoff values of the VEGF labeling index and the intratumor microvessel count in p53-positive patients were set at 72.4% and 86.4% (median value), respectively, in the present study. The survival rate in patients with a high VEGF labeling index in p53-positive tumors tended to be poorer than among other patients in the young group ($p = 0.0937$, Fig. 4A), but not in the elderly group ($p = 0.4523$, Fig. 4). The differences in the survival rates between patients with a high intratumor microvessel count in p53-positive tumors and other patients were not
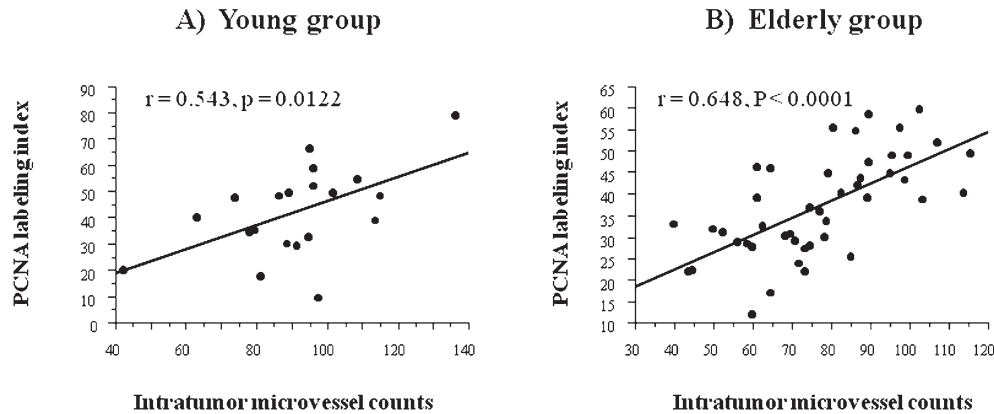
A) Young group   B) Elderly group

Fig. 3. Linear regression analysis of intratumoral microvessel counts and the percentage of PCNA-positive cells in the two
  age groups.
  *Data are mean ± s.d.

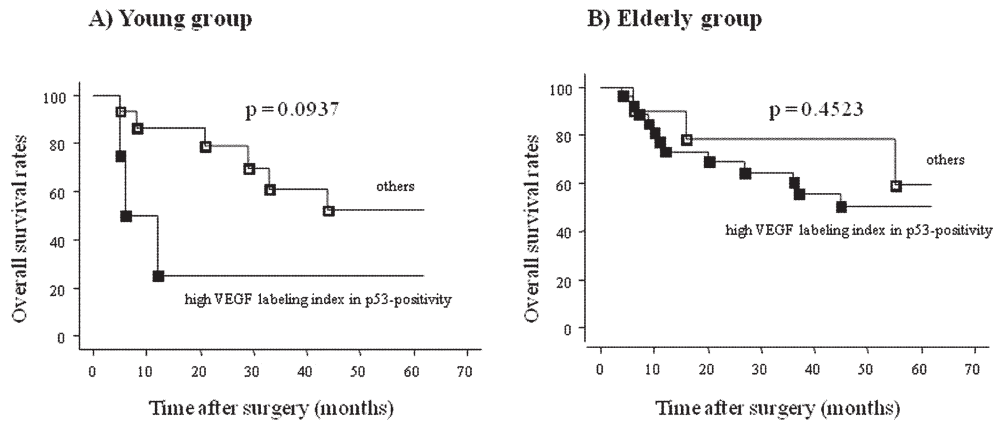## A) Young group   B) Elderly group

Fig. 4. Relationship between overall survival and VEGF labeling index (more and less than 72.4% [median value]) according
  to p53 status.

significant in both the young ($p = 0.3398$) and the elderly ($p = 0.3291$) groups.

DISCUSSION

No previous reports have provided detailed descriptions of the characteristic features of lung cancer in young adults. In the present study, the immunohistochemical features of 20 lung adenocarcinomas occurring in young patients that were less than 40 years of age were compared to those of tumors occurring in elderly patients.

P53 is one of the most important genes for the development and progression of lung cancer. However, Kashii et al. (1995) suggested that p53 gene alteration did not play an important role in lung carcinogenesis in their young patients who were ≤ 45 years old. The present immunohistochemical study narrowed the scope of the argument to lung adenocarcinoma, and the results showed that the p53-positive rate, which reflects the mutation status, was lower in the young group than in the elderly group. Considering that most lung cancers develop in patients older than 60, the low rate of p53-positive staining in the young group could reflect a low frequency of p53 mutation in this age group

because of short-term exposure to carcinogens. The current results suggest that the positive expression of p53 protein in young patients does not usually affect carcinogenesis but instead reflects the progression of lung adenocarcinoma.

From the viewpoint of cellular senescence due to aging, in the elderly group, the p53-positive rate was high, and blocking of the p53 – p21 pathway may have been involved in carcinogenesis. To explain the low PCNA labeling index, the activated p16 – RB pathway may have been involved in suppressing cell growth (Tsuji et al. 2006; Kim and Sharpless 2006). Jie et al. (2007) reported a study of patients with colon cancer in which the expression of p16 and demethylation of the promoter regions were high in areas infiltrated by tumors and low in central areas. In addition, Milyavsky et al. (2007) reported that, in human fibroblasts, the p16 – RB pathway induces myocardin, thereby inducing differentiation via TGF-β. The tumor morphology in the elderly group had a high degree of differentiation, particularly in marginal regions, and it cannot be ruled out that the activated p16 – RB pathway may have been involved in suppressing the growth potential and keeping the cellular morphology in a well-differentiated state. In

other words, this may mean that differences in the p53 expression rate and in the degree of differentiation between the two groups reflect differences in the blocking of the p53 – p21 pathway and the p16 – RB pathway.

There is a correlation between p53 and angiogenesis in lung cancer. Giatromanolaki et al. (1998) reported that VEGF switch-on is dependent on the loss of wild-type p53. Furthermore, Fontanini et al. (1999) reported that p53 mutation is significantly correlated with VEGF protein overexpression. The present results confirmed that lung adenocarcinoma cells expressed VEGF and p53-positive tumors showed higher expression of VEGF protein than p53-negative tumors, both in the young and the elderly groups; however, the p53-positive patients with a high VEGF labeling index in the young group tended to have a poorer prognosis than the other patients. These results suggest that the control of VEGF expression by p53 protein expression affects prognosis in young patients.

Lung adenocarcinomas contained more intratumoral microvessels in the young group than in the elderly group, which was confirmed by a monoclonal antibody that recognizes CD34 antigen. Moreover, p53-positive tumors in the young group that expressed high levels of VEGF protein showed higher microvessel density than p53-negative tumors. In comparison, there was no significant difference in the microvessel density based on differences in p53 status in the elderly group. Thus, in lung adenocarcinomas, the young group and the elderly group showed no difference in the way that VEGF protein expression increases as p53 protein is overexpressed, but the capacity of the body to newly form intratumoral microvessels in response to increased expression of VEGF was more active in young patients. In other words, tumor neoangiogenesis was more sensitive to VEGF expression in the young group than in the elderly group. Folkman and Klagsbrun (1987) reported that formation of microvessels occurs through proliferation and migration of endothelial cells from large blood vessels in the neighboring area. Furthermore, Asahara et al. (1997) identified endothelial progenitor cells in human peripheral blood. These cells, which originate from the bone marrow, contribute to the formation of microvessels in areas with severe ischemia (Takahashi et al. 1999; Asahara et al. 1999). Therefore, the decline of either the reactivity of endothelial cells of the large blood vessels or the generation of endothelial progenitor cells in the bone marrow could explain the difference in intratumoral microvessel density between the young and elderly groups noted in the present study. In the present study, the difference in the proliferative activity of lung adenocarcinoma based on the p53 status between the two age groups was probably due, at least in part, to the difference in intratumoral microvessel count, which in turn was caused by differences in the sensitivity of microvessel formation to tumoral VEGF expression.

Based on the results of the present study, one can conclude that lung adenocarcinoma occurring in young patients tends to have a poorer prognosis, and angiogenesis of lung adenocarcinoma in young patients is more closely correlated with p53 expression than in elderly patients. Therefore, the present results suggest that future therapies designed to inhibit tumor angiogenesis could potentially be more effective against lung adenocarcinoma in young patients than in elderly patients.

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