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The Relationship between Microvessel Count and the Expression of Vascular Endothelial Growth Factor, p53, and K-ras in Non-Small Cell Lung Cancer

Using immunohistochemical staining, we studied the relationship between the microvessel count (MC) and the expression of K-ras, mutant p53 protein, and vascular endothelial growth factor (VEGF) in 61 surgically resected non-small cell lung cancers (NSCLC) (42 squamous cell carcinoma, 14 adenocarcinoma, 2 large cell carcinoma, 2 adenosquamous carcinoma, and 1 mucoepidermoid carcinoma). MC of the tumors with lymph node (LN) metastasis was significantly higher than that of tumors without LN metastasis (66.1±23.1 vs. 33.8±13.1, $p<0.05$). VEGF was positive in 54 patients (88.5%). MC was 58.1±25.2 (mean±S.D.) in a ×200 field, and it was significantly higher in VEGF(+) tumors than in VEGF(-) tumors (61.4±23.7 vs. 32.9±23.8, $p<0.05$). VEGF expression was higher in K-ras-positive or mutant p53-positive tumors than in negative tumors ($p<0.05$). MC was significantly higher in K-ras(+) tumors than in K-ras(-) tumors, although it did not differ according to the level of mutant p53 protein expression. Survival did not differ with VEGF, mutant p53, or K-ras expression, or the level of MC. In conclusion, there is a flow of molecular alterations from K-ras and p53, to VEGF expression, leading to angiogenesis and ultimately lymph node metastasis. Correlations between variables in close approximation and the lack of prognostic significance of individual molecular alterations suggest that tumorigenesis and metastasis are multifactorial processes.

Key Words : *Vascular Endothelial Growth Factor; K-ras; Protein p53; Carcinoma, Non-Small-Cell Lung*

Yu-Ho Kang, Kyu-Sik Kim, Young-Kwon Yu, Sung-Chul Lim, Young-Chul Kim, Kyung-Ok Park

Department of Internal Medicine, Chonnam National University Medical School, Research Institute of Medical Science, Kwangju, Korea

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Address for correspondence
Young-Chul Kim, M.D.
Department of Internal Medicine, Chonnam National University Hospital, 8 Hak-dong, Dong-gu, Kwangju 501-757, Korea
Tel : +82.62-220-6573, Fax : +82.62-225-8578
E-mail : kyc0923@chonnam.ac.kr

INTRODUCTION

Angiogenesis is essential for tumor growth and metastasis. Tumors cannot exceed 1-2 μL in volume without developing new blood vessels (1). These new vessels may allow tumor dissemination by providing tumor cells with a portal of entry into the circulation (2), and decreased angiogenesis is associated with a decreased rate of metastasis (3).

Angiogenetic capacity, that is, the ability to induce neovascularization, is characteristic of most neoplastic cells. Among various angiogenic factors, vascular endothelial growth factor (VEGF) is known as a powerful endothelial cell-specific mitogen that is involved in tumor neovascularization (4). VEGF is also a highly potent direct mediator of microvascular permeability, and it is thought to be responsible for the characteristic leakiness of tumor blood vessels (5). VEGF expression has been demonstrated in a number of human cancer cell lines (6), as well as in clinical specimens of breast, brain, ovarian, esophageal, and colon cancer, suggesting a trophic role of VEGF in supporting tumor growth via host angiogenesis (7). In previous studies, VEGF expression was significantly associated with the degree of vascularization in non-small-cell lung cancer (NSCLC) (8, 9). Although hypoxia is a strong inducer of VEGF (10), mutationally activated *ras* or *p53* oncogenes act synergistically with hypoxia to induce VEGF expression (11, 12).

The wild-type (wt) *p53* oncogene has recently been shown to inhibit angiogenesis via regulation of thrombospondin-1, an inhibitor of angiogenesis (13), and to down-regulate VEGF-promoter activity (14). Mutant *ras* genes up-regulate the expression of a variety of other growth factors thought to have direct or indirect stimulating effects on angiogenesis, e.g., transforming growth factor (TGF)-β, TGF-α, and VEGF (15). Although experimental studies have shown that mutant *p53* and ras oncogenes contribute to angiogenesis (11-15), there are few reports on the relationship between these oncogenes and angiogenesis in NSCLC (16, 17).

Here, we investigated the relationship and correlation between tumor angiogenesis and VEGF, mutant p53, and K-ras protein expression by immunohistochemistry. In addition, we evaluated the relationship between tumor angiogenesis and overall survival.

MATERIALS AND METHODS

Paraffin-embedded tumor specimens from 61 patients with NSCLC who had undergone surgery at the Chonnam National University Hospital were studied using immunohistochemistry. Pathologic and clinical data at the time of surgery were reviewed to provide accurate staging. The clinical follow-up of the patients (46 men and 15 women) ranged from 1.5 to 75.7 months (average 18.5 months). Tumors were staged and classified using the TNM staging system (18) and the World Health Organization Histological Classification (19) after a complete mediastinal lymph node dissection in all cases. The mean age of patients was 56.3 yr (range, 31 to 71 yr). The characteristics of the patients are listed in Table 1. All the patients had an Eastern Cooperative Oncology Group performance status of 0 or 1, and normal findings on abdominal computed tomograms and radionuclide bone scans. The histologic types included 42 squamous cell carcinoma, 14 adenocarcinoma, 2 large cell carcinoma, 2 adenosquamous carcinoma, and 1 mucoepidermoid carcinoma. TNM staging consisted of 1 Stage Ia, 8 Ib, 13 IIb, 30 IIIa, and 9 IIIb. Patients who had died within 1 month of the surgery were excluded.

Vascular endothelial growth factor (VEGF) expression

Immunohistochemical staining for VEGF was performed using a Microprobe immuno/DNA staining system (Fisher Scientific, Pittsburgh, PA, U.S.A.), which is based on capillary action (20). Three-micrometer sections were cut from paraffin blocks and mounted on Probe on Plus slides (Fisher Scientific). Dewaxed sections were incubated with Pepsin solution (Research Genetics, Huntsville, AL, U.S.A.) for 1 min at 45℃. After washing with Universal Buffer (Research Genetics), sections from each tumor were incubated with primary VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a 1:50 dilution for 2 hr at room temperature. The slides were washed with Universal Buffer and incubated in biotinylated anti-mouse IgG (Sigma, St. Louis, MO, U.S.A.) for 7 min at 45℃.

Endogenous peroxidase activity was blocked with Autoblocker (Research Genetics) for 5 min at 45℃. After washing with Universal Buffer, the sections were incubated with streptavidin horseradish peroxidase (STR-HRP) (Research Genetics) for 7 min at 45℃. The reaction products were again washed in Universal Buffer, and then developed with the chromogen 3-amino-9-ethylcarbazole (AEC) for 7 min at 45℃. Sections were washed in running tap water and lightly counterstained with hematoxylin. To evaluate VEGF expression, a score corresponding to the percentage of positive cells (0=0% positive cells, 1=≤10%, 2=10 to 25%, 3=26 to 50%, 4=≥50%) was established. Negative controls were stained similarly, but without the primary antibody. VEGF expression was considered positive if staining of the cytoplasm was seen in more than 25% of the tumor cells in the slide containing the largest section of the tumor.

Determination of microvessel count

Microvessels were identified by immunohistochemistry using the CD34 mouse monoclonal antibody (QBEnd/10, BioGenex, San Ramon, CA, U.S.A.) in a 1:50 dilution at 45℃ for 15 min. The microvessel count (MC) was assessed by light microscopy in the areas of the tumor containing the highest number of capillaries and small venules at the invasive edge, as described previously (21). The three highly vascular areas were identified by scanning tumor sections at low power ×40 and ×100). After the area of highest neovascularization was identified on a ×200 field ×20 objective and (10 ocular, 0.739 mm^2 per field), the highest value was taken as the MC.

p53 and K-ras protein expression

Three-micrometer sections were cut from paraffin blocks and mounted on Probe on Plus slides. Dewaxed sections, were immersed in citric acid buffer (0.01 M, pH 6.0), and then placed in a microwave oven at 100℃ for 10 min (for p53). Sections from each tumor were incubated with primary antibodies for p53 (B*P53*.12, Zymed, South San Francisco, CA, U.S.A.) and K-ras (F234, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 15 to 25 min. The slides were washed with Universal Buffer and incubated with biotinylated anti-mouse IgG for 7 min.

Endogenous peroxidase activity (for p53 and K-ras) was blocked with Autoblocker for 5 min. After washing with Universal Buffer, the sections were incubated with STR-HRP (for p53 and K-ras) for 7 min. The reaction products were again washed in Universal Buffer, then developed

Table 1. Clinical characteristics of subjects

	n=61
Mean age (yr)	56.3±9.3
Male : Female	46 : 15
Smoking (pack-years)	24.1±17.7
Histology	
Squamous cell carcinoma	42
Adenocarcinoma	14
Large cell carcinoma	2
Adenosquamous cell carcinoma	2
Mucoepidermoid carcinoma	1
N status	
N0	15
N1-3	46
TNM stage	
≤ IIB	22
≥ IIIA	39

using 3-amino-9-ethylcarbazole (AEC, for p53 and K-ras, 7 min) as a chromogen. All steps in the staining procedure were done at 45℃. Sections were washed in running tap water and lightly counterstained with hematoxylin. All procedures were performed using a Microprobe immuno/DNA staining system, which is based on capillary action (23). Positive controls consisted of colon cancer for p53 and gastric cancer for K-ras. For negative controls, the same positive control slides were stained without the primary antibody. The immunoreactivity of the slides was examined by two investigators using standard light microscopy, and scored in quartiles as the percentage of positive tumor cells: 0=0%, 1=≤10%, 2=10 to 50% and 3=≥50%. The intensity of the staining reaction was not quantified.

K-ras immunoreactivity was defined as a diffuse cytoplasmic stain in neoplastic cells. p53 immunoreactivity was defined as nuclear reactivity in neoplastic cells. The expression of p53 and K-ras was considered to be positive if respective staining of the cytoplasm or nuclei was seen in more than 10% of the tumor cells in the slide containing the largest section of the tumor.

Statistical analyses

The statistical analyses were performed with the SPSS for Windows program package. The survival time was recorded in months (m) from the day of surgery. Univariate and multivariate analyses of survival data were undertaken, using survival curves and applying the Kaplan and Meyer method with log rank analysis, and the Cox regression model. Data were expressed as the mean±standard deviation (S.D.). The significance of associations was determined by using the χ^2 test, Fisher's exact probability test, or a two-tailed Student's t-test. $p<0.05$ was considered significant.

RESULTS

Microvessel count in tumors

Blood vessels were heterogeneously distributed in the stroma of the tumors (Fig. 1A). The mean MC was 58.1±25.2 and the median was 58 (range, 14-124) in a ×200 field. MC according to the pathologic state, and VEGF,

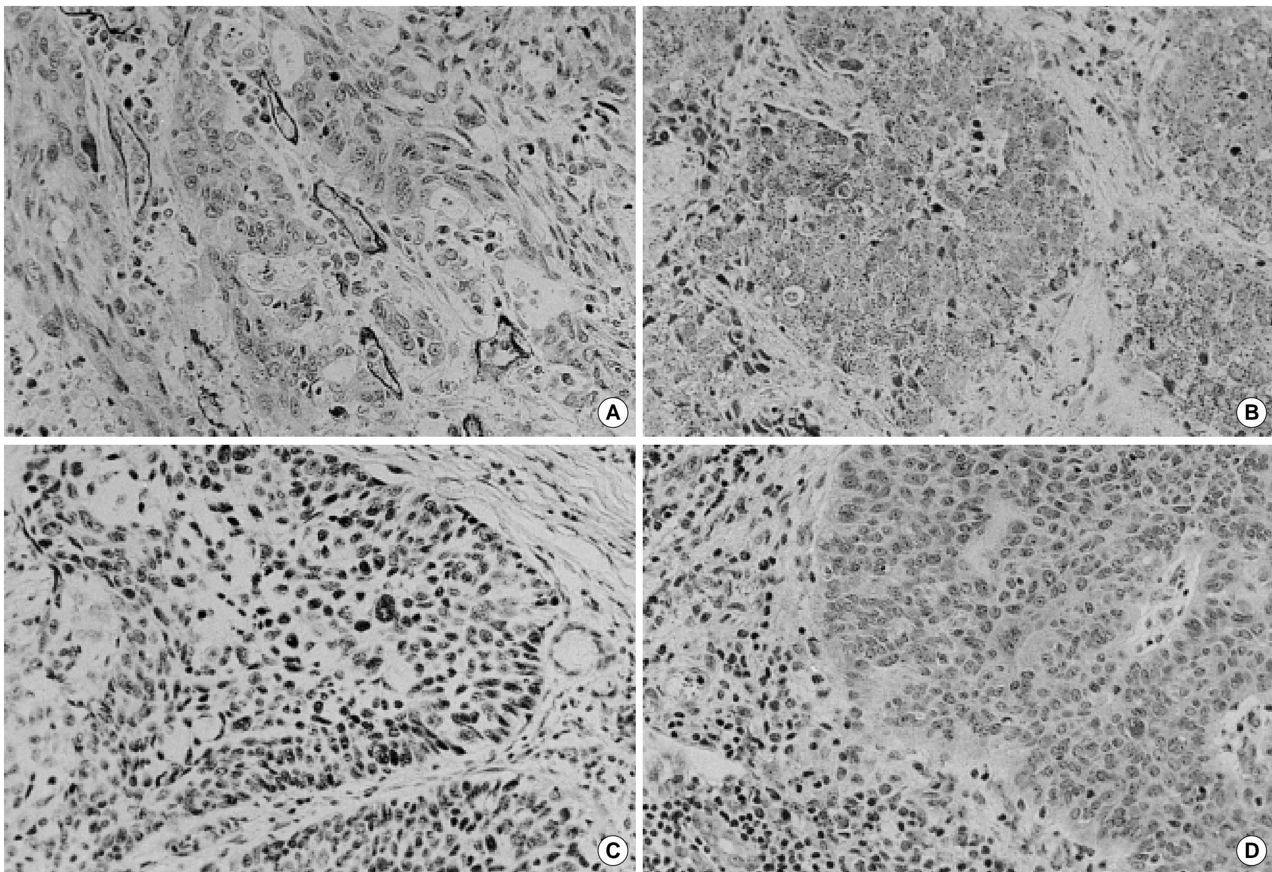


Fig. 1. A: Tumor area with high microvessel count in squamous cell carcinoma (ABC method, ×200). B: cytoplasmic VEGF expression in squamous cell carcinoma (×200). C: p53 protein accumulation in the nuclei of neoplastic cells in squamous cell carcinoma (×200). D: Cytoplasmic K-ras expression in squamous cell carcinoma (×200).

mutant p53, and K-ras protein expression are shown in Table 2. There was no significant difference in MC among the different histological subtypes of NSCLC. In addition, MC was not significantly different between low stage (≤ Stage IIb) and advanced stage (≥ Stage IIIa). The patients with lymph node metastasis had a higher MC than those without metastasis (66.1±23.1 vs. 33.8±13.1, p<0.001).

VEGF expression and microvessel count

VEGF expression was mainly identified in the cytoplasm of tumor cells (Fig. 1B). Positive staining for VEGF was seen in 54 out of 61 cases (88.5%). MC of VEGF-positive tumors was significantly higher than that of VEGF-negative tumors (61.4±23.7 vs. 32.9±23.8, p=0.004) (Table 2). VEGF expression did not differ according to lymph node metastasis or stage (low stage vs. advanced stage) (Table 3).

p53 staining, microvessel count, and VEGF

Mutant p53 was stained in the nuclei of neoplastic cells (Fig. 1C). Of 61 specimens, 34 (55.7%) stained positive for mutant p53 protein. p53 expression did not differ according to lymph node metastasis or stage. MC did not differ between p53-positive and p53-negative tumors (61.7±30.8 vs. 55.3 ±19.7, p=0.353) (Table 2). However, VEGF expression in p53-positive tumors (33/34, 97.1%) was greater than that in p53-negative tumors (21/27, 77.8%) (p=0.037) (Table 3).

K-ras staining, microvessel count, and VEGF

K-ras was stained in the cytoplasm of neoplastic cells (Fig. 1D). Forty-seven specimens stained positive for K-ras protein (77%). K-ras expression did not differ according to lymph node metastasis or stage (data not shown). MC in K-ras-positive tumors was significantly higher than that in K-ras-negative tumors (62.0±25.6 vs. 45.2±19.6, p= 0.028) (Table 2). VEGF expression in K-ras-positive tumors (45/47, 95.7%) was greater than that in K-ras-negative tumors (9/14, 64.3%) (p=0.005) (Table 3).

Overall survival analysis

The three-year survival rate (YSR) was higher in low stages (≤IIb) than in advanced stages (≥IIIa) (3YSR: 79.8% vs. 28.8%, p=0.0043) (Fig. 2). The patients were divided into two groups: those with low MC (<58) or high MC (≥58: median of MC). The overall survival did not differ between the low and high MC groups (Fig. 3A). In addition, the overall survival did not differ according to VEGF, p53, or K-ras expression (Fig. 3B, 4). The univariate analysis using a Cox regression model (shown in Table 4) underlines the strong prognostic relevance of TNM stage (p=0.0098).

Table 2. Correlation between the microvessel counts and VEGF expression, mutant p53 protein, K-ras protein expression in tumor tissue

Factors	Microvessel counts	p value
Total	58.1±25.2	
Histologic types		
Squamous cell carcinoma (n=42)	57.5±24.2	
Non-squamous cell carcinoma (n=19)	59.6±27.9	0.766
Lymph nodal status		
N1-N3 (n=46)	66.1±23.1	
N0 (n=15)	33.8±13.1	0.000
TNM stage		
≤ IIB (n=22)	50.6±28.9	
≥ IIIA (n=39)	62.4±22.2	0.081
VEGF expression		
Positive (n=54)	61.4±23.7	
Negative (n=7)	32.9±23.8	0.004
P53 expression		
Positive (n=34)	55.3±19.7	
Negative (n=27)	61.7±30.8	0.353
K-ras expression		
Positive (n=47)	62.0±25.6	
Negative (n=14)	45.2±19.6	0.028

Table 3. VEGF expression according to the expression of mutant p53, K-ras protein, lymph nodal status, and TNM stage

Factors	VEGF expression		p-value
	Negative (n=7)	Positive (n=54)	
Lymph node status			
Negative	4	11	
Positive	3	43	0.055
TNM stage			
≤ IIB	4	18	
≥ IIIA	3	36	0.240
Mutant p53			
Negative	6	21	
Positive	1	33	0.037
K-ras			
Negative	5	9	
Positive	2	45	0.005

Table 4. Results of Cox regression analysis in 61 patients with NSCLC; univariate analysis of the various prognostic factors

Factors	Hazard ratio	95% Confidence interval	p value
Age (yr)	0.987	0.450-2.167	0.97
Sex	2.267	0.775-6.636	0.14
Lymph node status	2.617	0.781-8.769	0.12
TNM stage	4.938	1.471-16.581	0.0098
Microvessel counts	1.124	0.512-2.469	0.77
VEGF	1.083	0.324-3.626	0.90
Mutant p53	2.233	0.930-5.365	0.07
K-ras	0.924	0.385-2.218	0.86

Age; 0: <58, 1: ≥58, Sex: 0: Female, 1: Male. Lymph node status; 0: Negative, 1: Positive. TNM stage; 0: ≤IIB, 1: ≥IIIA. Microvessel count; 0: less than median, 1: equal or greater than median. Other factors; 0: negative, 1: positive.

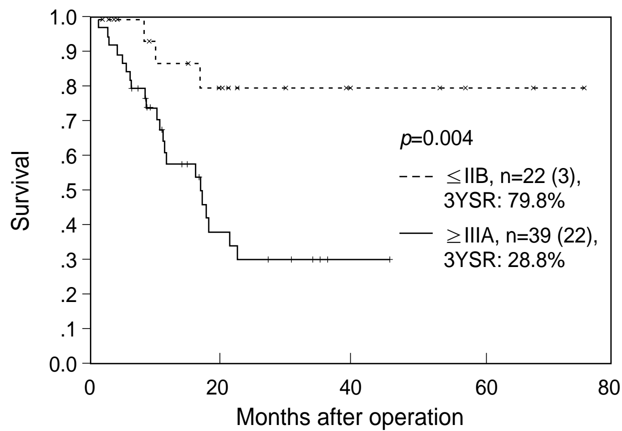
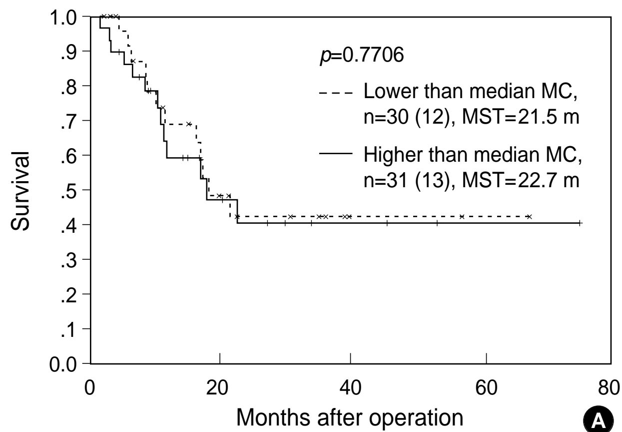


Fig. 2. Suvival difference according to the TNM stage. n represents the number of subjects with the number of deaths in parentheses. 3YSR, three year survival rate.

DISCUSSION

Various growth factors stimulate angiogenesis, including acidic fibroblast growth factor (aFGF), basic fibroblastic growth factor (bFGF), TGF-α, TGF-β, platelet-derived endothelial cell growth factor, hepatocyte growth factor, and VEGF (1, 7). In this study we focused on the role of VEGF.

As a result of alternative splicing of messenger RNA, VEGF exists as four different homodimeric molecular species; the monomers have 121, 165, 189, or 206 amino acids (VEGF121, VEGF165, VEGF189, VEGF206, respectively) (4). VEGF121 and VEGF165 are soluble proteins, whereas VEGF189 and VEGF206 are bound to heparin-containing proteoglycans on the cell surface or in the basement membrane (22). Experiments utilizing the gene transfection method (23, 24) have established strong evidence for a contribution of VEGF to the progressive growth



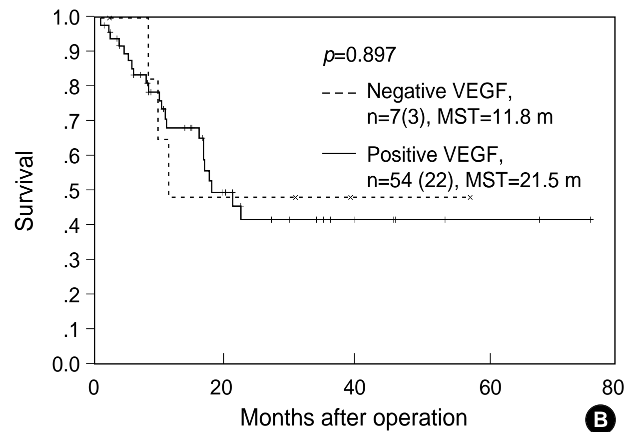
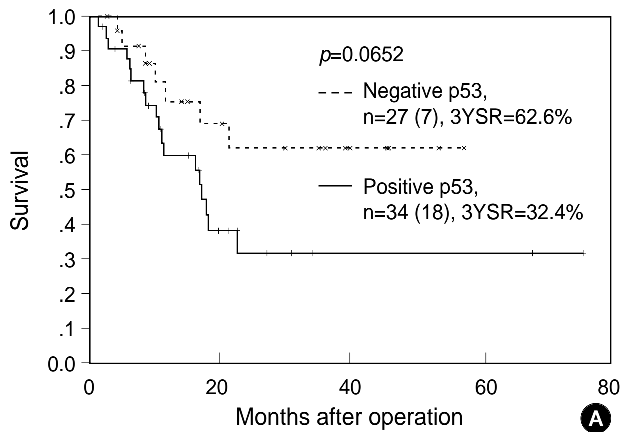


Fig. 3. Survival difference according to the microvessel count (A) and VEGF expression (B). n represents the number of subjects with the number of deaths in parentheses. MC, microvessel count; MST, median survival time (month).



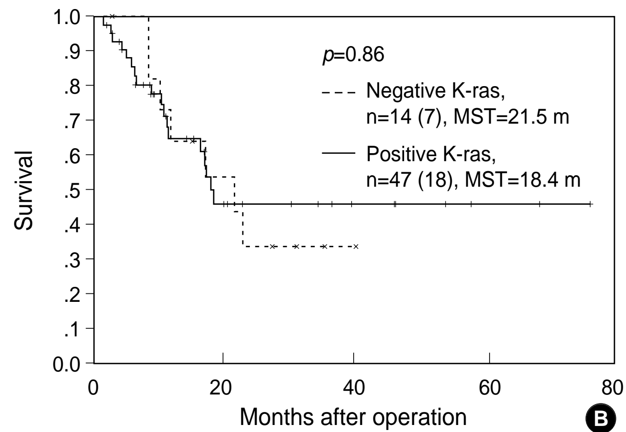


Fig. 4. Survival difference according to the p53 protein (A) and K-ras protein expression (B). n represents the number of subjects with the number of deaths in parentheses. 3YSR, three year survival rate; MST, median survival time (month).

of solid tumors through its effects in promoting tumor angiogenesis.

Numerous cytokines and growth factors produced by tumor and normal cells affect VEGF expression. It has been reported that VEGF expression is enhanced by epidermal growth factor, keratinocyte growth factor, TGF-α, TGF-β, insulin-like growth factor I, PGE2, IL-1β, IL-1α and IL-6 (7). In addition, oncogenes, such as *p53* and *ras,* affect VEGF expression. VEGF overexpression was correlated with nuclear accumulation of p53 in human lung and colon cancer (12, 16). Likewise, vessel counts in p53-positive tumors were significantly higher than in p53-negative tumors (15). Besides mutant *p53,* mutation of the *ras* gene also has been shown to up-regulate VEGF expression (11, 15).

As in other reports (8, 16, 25, 26), our results showed that lymph node (LN) metastasis was associated with the microvessel count. In addition, our data are in agreement with previous studies, in that VEGF expression was significantly associated with the degree of vascularization in NSCLC (8, 9). However, there were no direct correlations between LN metastasis and VEGF expression. These findings suggest that there are many other factors controlling the degree of LN metastasis that cannot be explained by VEGF expression alone.

In this study, p53 and K-ras were shown to have no direct effect on lymph node metastasis. However, both oncoproteins were correlated with VEGF expression. VEGF expression is correlated with the onset of mutations in both *ras* (11) and *p53* (12), and these mutations act synergistically with hypoxia to induce VEGF expression. Our data also showed that K-ras expression was correlated with MC, while p53 expression was not. These results suggest that K-ras induces angiogenesis via VEGF expression.

If we postulate a sequential pathway of angiogenesis, oncogenes (*p53* and *K-ras*), VEGF expression, microvessel count (angiogenesis), and finally degree of metastasis can be lined up. We observed a direct relationship between the closely approximated variables; however, direct relationships between distant variables are hard to prove. These findings suggest that there are many other factors controlling the dependent variable, since it cannot be explained with only one or two independent variables.

Although the role of VEGF in tumor metastasis has been demonstrated by several experiments that showed that VEGF induces neovascularization (1), the association of VEGF expression with the prognosis of NSCLC is controversial. Imoto et al. reported poor survival in VEGF-immunopositive NSCLC (27). However, Decaussin et al. (28) argued against this finding by demonstrating that positive staining for VEGF did not indicate a poor prognosis. Our data showed that survival did not differ according to VEGF expression.

Some studies have found that patients with p53-positive tumors have a significantly poorer prognosis in lung cancers (29), whereas others have found no differences in survival between p53-positive and p53-negative lung cancers (30). Moreover, a recent study by Lee et al. (31) showed that p53 expression implied a favorable prognosis in a series of 156 resected primary NSCLC. According to our data, p53 expression did not affect patient survival.

VEGF expression can be induced by the mutant *ras* oncogene (15), and mutant *K-ras* knockout sublines were incapable of forming tumors in nude mice (11). Furthermore, in serum-starved NIH 3T3 cells, a *ras*-transformed cell line expresses enhanced levels of VEGF transcripts (32). Although the *K-ras* mutation is known to be a poor prognostic factor in pulmonary adenocarcinoma (33), our data did not show any survival difference according to K-ras expression.

The prognostic significance of angiogenesis in NSCLC has not been established. Giatromanolaki et al. (25) and Harpole et al. (34) reported that vascularity was a significant factor in predicting poor survival. However, this study and others (10, 35) have found no differences in survival according to MC in lung cancer.

In conclusion, there is a flow of molecular alterations from K-ras and p53, and VEGF expression, that lead to angiogenesis and finally to lymph node metastasis. Correlations between variables in close approximation and the lack of prognostic significance of individual molecular alterations suggest that tumorigenesis and metastasis are multifactorial processes.

As shown in the report by Kim et al. (36), however, anti-VEGF monoclonal antibody inhibits the growth of rhabdomyosarcoma (A673) and leiomyosarcoma (SK-LMS-1) cell lines, and the magnitude of the response is greater in rhabdomyosarcoma, which proliferates more rapidly and is therefore more angiogenesis-dependent. The important role of VEGF expression in angiogenesis in NSCLC suggests a potential therapeutic use of anti-VEGF monoclonal antibody for blocking VEGF action in this type of tumor.

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