

# Expression of Angiopoietins and Its Clinical Significance in Non-Small Cell Lung Cancer<sup>1</sup>

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## ABSTRACT

Angiopoietin (Ang)-1 and -2 have been recently identified as potent angiogenic factors which function in concert with vascular endothelial growth factor (VEGF), but no detailed clinical study on Ang expression has been reported. To assess the clinical significance of Ang expression in non-small cell lung cancer (NSCLC), a total of 236 patients with pathological stage-I-IIIa disease were retrospectively reviewed. Expression of Ang-1, Ang-2, or VEGF was examined immunohistochemically; intratumoral microvessel density (IMVD) was examined with immunohistochemical staining against CD34, a marker of pan-endothelial cells (CD34-IMVD), and that against CD105, a marker of proliferative endothelial cells (CD105-IMVD). Positive expression of Ang-1 and that of Ang-2 were seen in 101 (42.8%) and 40 patients (16.9%), respectively. There was no significant correlation between Ang-1 expression and CD34-IMVD or CD105-IMVD. In contrast, the average CD105-IMVD for Ang-2-positive tumor was significantly higher than that for Ang-2-negative tumor (56.7 versus 38.5;  $P = 0.032$ ). More interestingly, such an angiogenic effect of Ang-2 was seen only when VEGF expression was high; when VEGF expression was high, the average CD105-IMVD for Ang-2-positive tumor was significantly higher than that for Ang-2-negative tumor (89.1 versus 63.6;  $P = 0.045$ ); when VEGF expression was low, the average CD105-IMVD for Ang-2-positive tumor and that for Ang-2-negative tumor were almost the same (27.4 and 27.1, respectively). Moreover, positive expression of Ang-2, not Ang-1, was a significant factor to predict a poor postoperative survival (5-year survival rates for Ang-2-positive patients and -negative patients were 53.5 and 70.3%, respectively;  $P = 0.027$ ), which was confirmed by a multivariate analysis. The influence of Ang-2 status on postoperative survival was enhanced when VEGF expression was high. That said, the 5-year survival of Ang-2-positive and VEGF-high patients was extremely low (41.4%) as compared with that for Ang-2-negative and VEGF-low patients (66.6%), as compared with that for Ang-2-positive and VEGF-low patients (63.6%), and as compared with that for Ang-2-negative and VEGF-low patients (71.8%). In conclusion, positive Ang-2 expression was significantly correlated with a poor prognosis, as well as with aggressive angiogenesis in resected NSCLC that was enhanced in the presence of high VEGF expression.

## INTRODUCTION

Angiogenesis is an essential process in the development and progression of malignant tumors including NSCLC<sup>3</sup> (1, 2). VEGF is the most potent angiogenic factor, and many clinical studies have demonstrated that increased expression of VEGF is associated with increased IMVD (a measurement of angiogenesis) and with a poor survival in NSCLC (3).

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<sup>3</sup> The abbreviations used are: NSCLC, non-small cell lung cancer; Ang, angiopoietin; VEGF, vascular endothelial growth factor; IMVD, intratumoral microvessel density; EC, endothelial cell; Sq, squamous cell carcinoma; Ad, adenocarcinoma; IHC, immunohistochemical staining; mAb, monoclonal antibody.

Ang-1 (4, 5) and Ang-2 (6) have been identified as ligands for Tie2, which is a receptor tyrosine kinase specifically expressed on ECs, and Angs play critical roles in angiogenesis in concert with VEGF (2). Ang-1 binds to Tie2, and maintains and stabilizes mature vessels by promoting interaction between ECs and surrounding extracellular matrix. Ang-2 competitively binds to Tie2, and antagonizes the stabilizing action of Ang-1, which results in destabilization of vessels. These destabilized vessels may undergo regression in the absence of angiogenic factors such as VEGF; in the presence of VEGF, however, these destabilized vessels may undergo angiogenic changes. Thus, angiogenesis is controlled by a dynamic balance between vessel regression and growth that are mediated by VEGF, Ang-1, and Ang-2 (2), which has been proved in many experimental studies (7–9). However, little has been reported on the correlation between the status of Angs and angiogenesis in clinical materials (10), although some clinical studies on Angs expression have been conducted in a variety of malignant tumors, including glioma (10), glioblastoma (11), neuroblastoma (12), colon carcinoma (13), gastric carcinoma (14), hepatocellular carcinoma (15), and NSCLC (16, 17, 18). In addition, only a few clinical studies have documented a correlation between Ang expression and clinical features or prognosis (12, 14). We have previously conducted a clinical study on angiogenesis in NSCLC in which IMVD was determined with an antibody against CD34, a pan-endothelial marker (CD34-IMVD), or with an antibody against CD105, a specific marker of activated ECs (CD105-IMVD), and have demonstrated that CD105-IMVD, not CD34-IMVD, was significantly correlated with postoperative survival (19). In the present study, therefore, we assessed expression of Angs in correlation with angiogenesis and clinical outcome in NSCLC.

## PATIENTS AND METHODS

**Patients and Tissue Preparation.** A total of 237 patients with pathological stage-I-IIIa NSCLC, who underwent complete resection without any preoperative therapy at Kyoto University Hospital from January 1, 1985, through December 31, 1990, were retrospectively reviewed. One patient was excluded from the study because of operation-related death, and a final total of 236 patients were evaluated (Table 1; Refs. 19–21). Pathological stage was re-evaluated and determined with the present tumor-node-metastasis classification as revised in 1997 (22). Histological type and cell differentiation were determined using the current classification by WHO as revised in 2000 (23). For analyses according to the differentiation of cancer cells, well-differentiated Sq and Ad were classified as well-differentiated tumors and moderately differentiated Sq and Ad as moderately differentiated tumors. Large cell carcinoma and poorly differentiated Sq and Ad were classified as poorly differentiated tumors, and the other histological types were excluded in the analyses (20).

For all of these patients, the inpatient medical records, chest X-ray films, whole-body computed tomography films, bone scanning data, and records of surgery were reviewed. Intraoperative therapy was not performed in any patient. As postoperative adjuvant therapy, cisplatin-based chemotherapy, radiation, and oral administration of tegafur (a fluorouracil-derivative drug) were prescribed for 55, 35, and 58 patients, respectively (21). Follow-up of the postoperative clinical course was conducted by outpatient medical records and by inquiries by telephone or letter.

All of the primary tumor specimens were immediately fixed in 10% (v/v) formalin, and then embedded in paraffin. Serial 4- $\mu$ m sections were prepared from each sample and served for H&E staining and IHS. Results of IHS were evaluated by two authors (F. T. and S. I.) independently without knowledge of any clinical data.

**Expression of Ang-1, Ang-2, and VEGF.** Expression of Ang-1, Ang-2, or VEGF was evaluated immunohistochemically using a standard streptavidin-biotinylated horseradish-peroxidase complex method (LSAB-2 kit; DAKO, Kyoto, Japan). After retrieval of the antigen by heating the sections in a microwave oven three times for 5 min each, the sections were incubated with an anti-Ang-1 polyclonal antibody (goat IgG, 200  $\mu$ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1/50, an anti-Ang-2 polyclonal antibody (goat IgG, 200  $\mu$ g/ml; Santa Cruz Biotechnology) diluted at 1/50, or an anti-VEGF polyclonal antibody A-20 (rabbit IgG, 200  $\mu$ g/ml, Santa Cruz Biotechnology) diluted at 1/50. The anti-VEGF antibody recognizes the 165-, 189-, and 121-amino splicing variants of VEGF. As a chromogen, 3,3'-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO) was used, and the sections were counterstained with hematoxylin. For the negative controls, normal goat IgG was used as a substitute for the primary antibody against Ang-1 or Ang-2, and normal rabbit IgG was used for that against VEGF.

Ang-1 or Ang-2 expression was judged to be positive, when the percentage of cancer cells with positive staining exceeded 5%. VEGF expression was classified according to the following grading system as described previously (19). Briefly, a percentage score was defined as follows: score 0 if no VEGF-positive staining cell was documented; score 1 if the percentage of VEGF-positive staining cells was  $\leq$ 25%; score 2 if the percentage was  $>$ 25% and  $\leq$ 50%; and score 3 if the percentage was  $>$ 50%; an intensity score was defined as follows: score 0 if no staining was documented; score 1 if the staining intensity was weak; score 2 if the intensity was moderate; and score 3 if the intensity was high. The staining intensity of tumor cells was judged as

high (score 3) when the staining intensity was comparable with that of smooth muscle cells of either bronchial wall or blood vessels. Grade of VEGF expression was represented as the sum of the percentage score and the intensity score (VEGF score), and VEGF expression was finally defined as follows: weak expression when the VEGF score is  $\leq$ 4; and strong expression when the VEGF score is 5 or 6.

**Quantification of Angiogenesis (IMVD).** IHS for CD34 and CD 105 to highlight ECs was performed using a sensitive streptavidin-biotinylated horseradish-peroxidase complex system (TSA-Indirect kit; NEN Life Science Products, Boston, MA) as described previously (19). Briefly, dewaxed sections were incubated with an anti-CD34 mAb QBEnd10 (mouse IgG1 $\kappa$ , 50  $\mu$ g/ml; DAKO) diluted at 1/50 or an anti-CD105 mAb SN6 h (mouse IgG1 $\kappa$ ; 366  $\mu$ g/ml; DAKO Japan) diluted at 1/100. The 10 most vascular areas within a section were selected for evaluation of angiogenesis, and vessels, labeled with the anti-CD34 mAb or the anti-CD105 mAb, were counted under light microscopy at  $\times$ 200. The average counts were recorded as the CD34-IMVD or CD105-IMVD for each case.

**Statistical Methods.** The  $\chi^2$  was used to compare counts. Continuous data were compared using the Student *t* test if the distribution of samples was normal, or the Mann-Whitney *U* test if the sample distribution was asymmetrical. The postoperative survival rate was analyzed by the Kaplan-Meier method, and the differences in survival rates were assessed by the log-rank test. Multivariate analysis of prognostic factors was performed using Cox's regression model. Differences were considered significant when  $P < 0.05$ . All of the statistical manipulation was performed using the SPSS for Windows software system (SPSS Inc., Chicago, IL).

## RESULTS

**Expression of Ang-1 and Expression of Ang-2 in NSCLC.** Positive expression of Ang-1 and that of Ang-2 were mainly seen in the

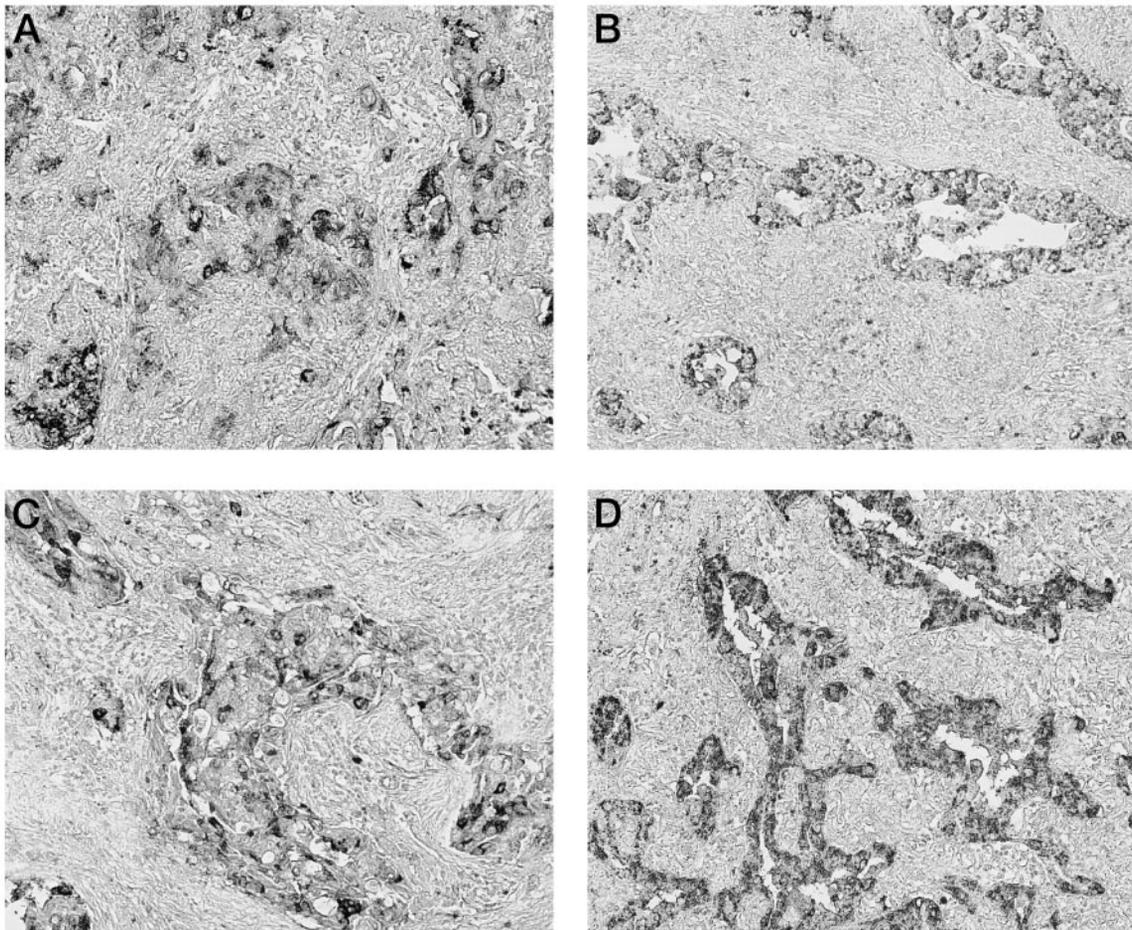


Fig. 1. IHS for Ang-1 or Ang-2 in NSCLC. Positive staining of cytoplasm of tumor cells for Ang-1 was documented in a case of Ad (A) and in another case of Ad (B). Positive staining for Ang-2 was documented in each case (C and D).

Table 1 Patients' characteristics and expression of Ang-1 and -2

|   | All patients<br>n <sup>a</sup> (%) | Expression of Ang-1 and Ang-2 |                   |                              |                   |
|---|------------------------------------|-------------------------------|-------------------|------------------------------|-------------------|
|   |                                    | Positive expression of Ang-1  |                   | Positive expression of Ang-2 |                   |
|   |                                    | n (%)                         | P                 | n (%)                        | P                 |
| Total                                   | 236 (100%)                         | 101 (42.8%)                   |                   | 40 (16.9%)                   |                   |
| Age <sup>b</sup>                        |                                    |                               |                   |                              |                   |
| Lower age (<64 yr)                      | 116 (49.2)                         | 49 (42.2%)                    | 0.896             | 17 (14.7%)                   | 0.389             |
| Higher age (≥64 yr)                     | 120 (50.8)                         | 52 (43.3%)                    |                   | 23 (19.2%)                   |                   |
| Sex                                     |                                    |                               |                   |                              |                   |
| Male                                    | 170 (72.0)                         | 67 (39.4%)                    | 0.107             | 28 (16.5%)                   | 0.847             |
| Female                                  | 66 (28.0)                          | 34 (51.5%)                    |                   | 12 (18.2%)                   |                   |
| Performance status (PS)                 |                                    |                               |                   |                              |                   |
| 0                                       | 206 (87.3)                         | 88 (42.7%)                    |                   | 36 (17.5%)                   |                   |
| 1                                       | 28 (11.9)                          | 12 (42.9%)                    | 0.979             | 3 (10.7%)                    | 0.306             |
| 2                                       | 2 (0.8)                            | 1 (50.0%)                     |                   | 1 (50.0%)                    |                   |
| Histological type                       |                                    |                               |                   |                              |                   |
| Sq                                      | 85 (36.0)                          | 35 (41.2%)                    | 0.486 (Sq vs. Ad) | 16 (18.8%)                   | 0.304 (Sq vs. Ad) |
| Ad                                      | 130 (55.1)                         | 60 (46.2%)                    |                   | 20 (15.4%)                   |                   |
| Large cell                              | 13 (5.5)                           | 1 (7.7%)                      |                   | 1 (7.7%)                     |                   |
| Others                                  | 8 (3.4)                            | 5 (62.5%)                     |                   | 3 (37.5%)                    |                   |
| Tumor cell differentiation <sup>c</sup> |                                    |                               |                   |                              |                   |
| Well differentiated                     | 81 (35.2)                          | 32 (39.5%)                    |                   | 7 (8.6%)                     |                   |
| Moderately differentiated               | 91 (39.6)                          | 45 (49.5%)                    | 0.219             | 21 (23.1%)                   | 0.039             |
| Poorly differentiated                   | 58 (25.2)                          | 21 (36.2%)                    |                   | 10 (17.2%)                   |                   |
| Pathological stage                      |                                    |                               |                   |                              |                   |
| I                                       | 138 (58.5)                         | 59 (42.8%)                    |                   | 19 (13.8%)                   |                   |
| II                                      | 44 (18.6)                          | 12 (46.2%)                    | 0.924             | 5 (19.2%)                    | 0.285             |
| IIIa                                    | 54 (22.9)                          | 30 (41.7%)                    |                   | 16 (22.2%)                   |                   |

<sup>a</sup> n, number of patients.

<sup>b</sup> Age (mean ± SD), 62.4 ± 9.7 years.

<sup>c</sup> Other histological types were excluded in the analysis.

edge of tumor tissues and were seen not only in the cytoplasm of tumor cells but also in ECs (Fig. 1). However, the degree and the extent of Angs expression in ECs were extremely heterogeneous, and the staining intensity in ECs was generally not so strong as to be clearly distinguished from negative expression. For these reasons, in the present study, we have focused on Ang expression in tumor cells, and Ang-1 or Ang-2 expression was judged based on the percentage of positive-staining tumor cells as described in the previous section. The staining intensity of IHS for Ang-1 or Ang-2 was heterogeneous even in the same tissue, and we could not evaluate semiquantitatively the degree of Ang-1 or Ang-2 expression.

Expression of Ang-1 was judged to be positive in 101 (42.8%) of all of the patients. No significant correlation between Ang-1 expression and any patient characteristic was documented (Table 1). Expression of Ang-2 was positive only in 40 patients (16.9%), and positive Ang-2 expression was significantly less in well-differentiated tumor (Table 1).

Both Ang-1 expression and Ang-2 expression were positive [Ang-1(+)/Ang-2(+)] in 27 patients (11.4%), and both were negative [Ang-1(-)/Ang-2(-)] in 122 patients (51.7%); the numbers of Ang-1(-)/Ang-2(+) and Ang-1(+)/Ang-2(-) patients were 13 (5.5%) and 74 (31.4%), respectively.

**Angs Expression and IMVD.** The average CD34-IMVD for Ang-1-negative tumor and that for Ang-1-positive tumor were 179.3 and 180.3, respectively, showing no correlation between Ang-1 status and CD34-IMVD. There was no significant correlation between Ang-1 status and CD105-IMVD, and the average CD105-IMVD for Ang-1-positive tumor was somewhat higher than that for Ang-1-negative tumor (48.0 versus 36.9;  $P = 0.093$ ). The average CD105-IMVD for Ang-2-positive tumor was significantly higher than that for Ang-2-negative tumor (56.7 versus 38.5;  $P = 0.032$ ), suggesting that Ang-2 plays an important role in angiogenesis. The average CD34-IMVD for Ang-2-positive tumor was somewhat higher than that for Ang-2-negative tumor (199.3 versus 177.0), but the difference was not statistically significant ( $P = 0.347$ ). When angiogenesis was analyzed in combination with Ang-1 status and Ang-2 status, Ang-1-negative and Ang-2-positive tumor or Ang-1-positive and Ang-2-positive tumor showed higher IMVD, suggesting that Ang-2, not Ang-1, played major roles in tumor angiogenesis (Table 2).

There proved to be a marked difference in CD34-IMVD or CD105-IMVD between VEGF-negative tumor and VEGF-positive tumor, demonstrating VEGF was a most potent angiogenic factor; the average CD34-IMVD for VEGF-low tumor and that for VEGF-high tumor were 166.9 and 204.8, respectively ( $P = 0.006$ ), and the average

Table 2 IMVD and postoperative survival according to the status of combination of Ang-1 and Ang-2 expression

|                         | Ang-1(-)/Ang-2(-)<br>(n = 122) | Ang-1(-)/Ang-2(+)<br>(n = 13) | Ang-1(+)/Ang-2(-)<br>(n = 74) | Ang-1(+)/Ang-2(+)<br>(n = 27) |
|-------------------------|--------------------------------|-------------------------------|-------------------------------|-------------------------------|
| IMVD (mean ± SE)        |                                |                               |                               |                               |
| CD34-IMVD <sup>a</sup>  | 176.7 ± 9.1                    | 203.7 ± 25.2                  | 177.5 ± 11.3                  | 188.0 ± 19.2                  |
| CD105-IMVD <sup>b</sup> | 35.3 ± 4.0 <sup>c</sup>        | 51.9 ± 22.6                   | 43.9 ± 12.1 <sup>d</sup>      | 59.0 ± 10.4                   |
| 5-yr survival rate      | 69.2%                          | 66.7%                         | 72.0%                         | 45.9% <sup>e</sup>            |

<sup>a</sup> IMVD determined with an anti-CD34 mAb.

<sup>b</sup> IMVD determined with an anti-CD105 mAb.

<sup>c</sup>  $P < 0.05$  for Ang-1(-)/Ang-2(-) versus Ang-1(-)/Ang-2(+), versus Ang-1(+)/Ang-2(+).

<sup>d</sup>  $P < 0.05$  for Ang-1(+)/Ang-2(-) versus Ang-1(+)/Ang-2(+).

<sup>e</sup>  $P < 0.05$  for Ang-1(+)/Ang-2(+) versus any other group.

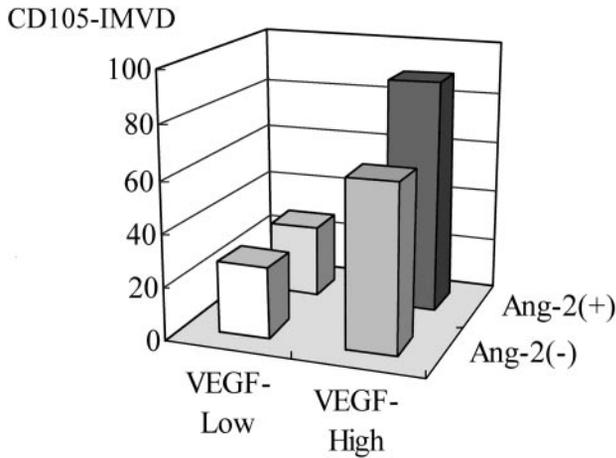


Fig. 2. Correlation between IMVD determined with an anti-CD105 mAb (CD105-IMVD) and expression of Ang-2 in combination with expression of VEGF. When VEGF expression in tumor was high, the average CD105-IMVD for Ang-2-positive tumor was significantly higher than that for Ang-2-negative tumor (89.1 versus 63.6;  $P = 0.045$ ). In contrast, when VEGF expression was low, the average CD105-IMVD for Ang-2-positive was almost the same as that for Ang-2-negative tumor (27.4 versus 27.1;  $P = 0.982$ ). Regardless of Ang-2 status, VEGF status was a significant factor to increase CD105-IMVD ( $P < 0.001$ ).

CD105-IMVD for VEGF-low tumor and that for VEGF-high tumor were 27.2 and 69.7, respectively ( $P < 0.001$ ).

Next, correlation between IMVD and Angs status in combination with VEGF status was examined, because many experimental studies showed that angiogenesis was promoted by Angs in concert with VEGF. The average CD34-IMVD for Ang-1-negative and VEGF-low tumor and that for Ang-1-positive and VEGF-low tumor were 166.7 and 168.8, respectively ( $P = 0.843$ ); the average CD34-IMVD for Ang-1-negative and VEGF-high tumor and that for Ang-1-positive and VEGF-high tumor were 214.1 and 196.4, respectively ( $P = 0.446$ ). In addition, the average CD105-IMVD for Ang-1-negative and VEGF-low tumor and that for Ang-1-positive and VEGF-low tumor were 25.1 and 29.1, respectively ( $P = 0.543$ ); the average CD105-IMVD for Ang-1-negative and VEGF-high tumor and that for Ang-1-positive and VEGF-high tumor were 65.3 and 73.6, respectively ( $P = 0.513$ ). These results showed that Ang-1 did not affect angiogenesis induced by VEGF. In contrast, Ang-2 status affected CD105-IMVD when VEGF expression was high as follows: when VEGF expression was high, the average CD105-IMVD for Ang-2-positive tumor was significantly higher than that for Ang-2-negative tumor (89.1 versus 63.6;  $P = 0.045$ ); when VEGF expression was low, the average CD105-IMVD for Ang-2-positive tumor and that for Ang-2-negative tumor were almost the same (27.4 and 27.1, respectively;  $P = 0.982$ ; Fig. 2). When VEGF expression was high, the average CD34-IMVD for Ang-2-positive tumor was somewhat higher than that for Ang-2-negative tumor (216.7 versus 201.1), but the difference was not statistically significant ( $P = 0.166$ ). When VEGF expression was low, there was no difference between CD34-IMVD for Ang-2-positive tumor and that for Ang-2-negative tumor (171.8 and 166.1, respectively;  $P = 0.797$ ).

**Angs Expression and Postoperative Survival.** The 5-year survival rate for Ang-2-negative patients was 70.3%, significantly higher than that for Ang-2-positive patients (53.5%;  $P = 0.027$ ; Fig. 3B and Table 3). In contrast, there was no difference in the survival between Ang-1-positive patients and Ang-1-negative patients (Fig. 3A; Table 3). When analyzed in combination with Ang-1 status and Ang-2 status, Ang-1-positive and Ang-2-positive tumor showed the poorest prognosis (Table 2).

Next, analyses of postoperative survival stratified by pathological

stage were performed (Table 3). For pathological stage-I disease, the 5-year survival rate for Ang-2-positive patients was significantly lower than that for Ang-2-negative patients (61.8% versus 81.1%;  $P = 0.022$ ; Table 3). A multivariate analysis confirmed that positive Ang-2 expression was a significant factor to predict a poor prognosis (Table 4).

A univariate analysis failed to demonstrate that VEGF expression was a significant prognostic factor (Fig. 3C), whereas VEGF expression was the most potent angiogenic factor. Because angiogenesis was highly promoted by Ang-2 in the presence of high VEGF expression

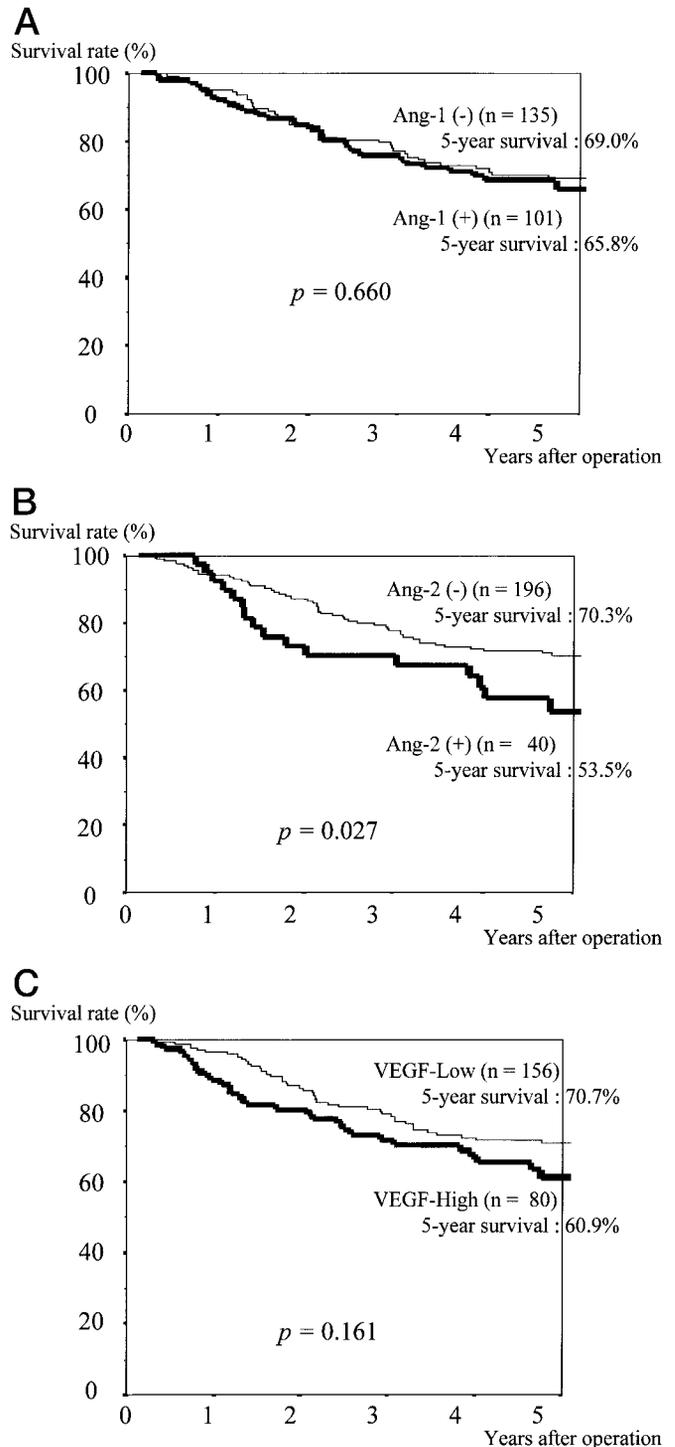


Fig. 3. Postoperative survival of pathological stage-I-IIIa NSCLC. Comparison according to the status of expression of Ang-1 (A), Ang-2 (B), or VEGF (C).

Table 3 Postoperative survival according to expression of Ang-1 or Ang-2

|                    | 5-yr survival rate (%) Ang-1 expression |          |       | 5-yr survival rate (%) Ang-2 expression |          |       |
|--------------------|---|----------|-------|---|----------|-------|
|                    | Negative                                | Positive | P     | Negative                                | Positive | P     |
| All patients       | 69.0%                                   | 65.8%    | 0.660 | 70.3%                                   | 53.5%    | 0.027 |
| Pathological stage |   |          |       |   |          |       |
| I                  | 79.1%                                   | 78.0%    | 0.782 | 81.1%                                   | 61.8%    | 0.022 |
| II                 | 63.5%                                   | 70.0%    | 0.956 | 63.5%                                   | 80.0%    | 0.895 |
| IIIa               | 48.0%                                   | 36.9%    | 0.779 | 45.6%                                   | 37.5%    | 0.943 |

Table 4 Multivariate analysis of prognostic factors in NSCLC

| Prognostic factors                    | $\beta$ | P      | Relative hazard (95% confidence interval) |
|---------------------------------------|---------|--------|---|
| Age                                   | 0.028   | 0.051  | 1.028 (0.990–1.056)                       |
| Sex (male/female)                     | -0.453  | 0.125  | 0.636 (0.357–1.134)                       |
| Performance status (0/1/2)            | 0.247   | 0.399  | 1.280 (0.722–2.271)                       |
| Histological type                     | -0.042  | 0.189  | 0.959 (0.900–1.021)                       |
| Pathological stage (I, II, IIIa)      | 0.621   | <0.001 | 1.860 (1.449–2.388)                       |
| Ang-1 (negative/positive)             | -0.006  | 0.981  | 0.994 (0.607–1.628)                       |
| Ang-2 (negative/positive)             | 0.377   | 0.041  | 1.458 (1.091–2.086)                       |
| VEGF (low-expression/high-expression) | 0.322   | 0.260  | 1.380 (0.788–2.417)                       |

(Fig. 2), postoperative survival was analyzed according to the status of Ang-2 expression in combination with the status of VEGF expression (Fig. 4). The 5-year survival of Ang-2-positive and VEGF-high patients was extremely low, suggesting that aggressive angiogenesis caused by Ang-2 in concert with VEGF might bring the poorest postoperative survival.

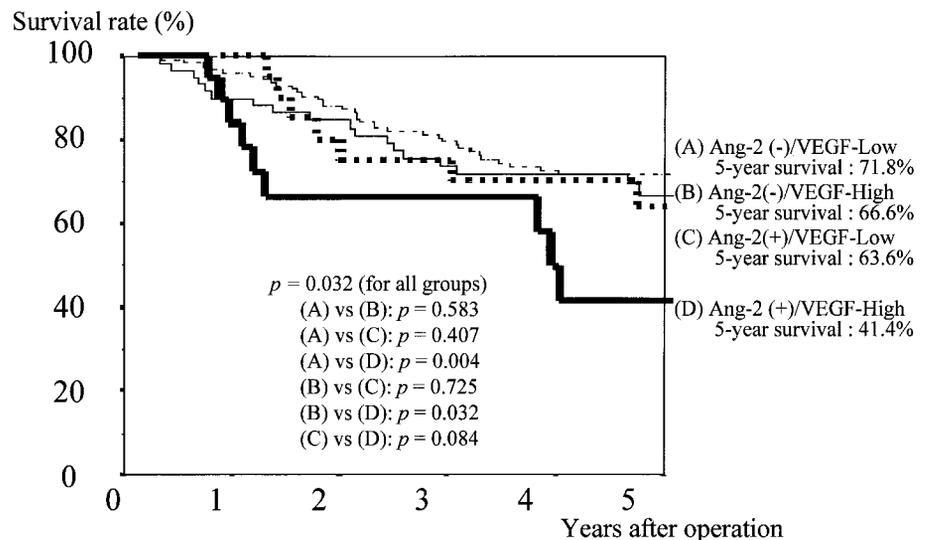
## DISCUSSION

In the present study, we clearly demonstrated that increased expression of Ang-2, not Ang-1, in tumor cells was significantly correlated with aggressive angiogenesis and a poor prognosis in NSCLC. Only two clinical studies have been reported on Ang-2 status in resected NSCLC (17, 18). In both studies, Ang-2 gene expression was assessed using reverse transcription-PCR and/or *in situ* hybridization, and no significant correlation between Ang-2 gene expression and angiogenesis or clinical outcome was documented in either study (17, 18). With respect to the correlation between Angs expression and angiogenesis, only one clinical study in glioma has been reported (10). In this study, Koga *et al.* (10) demonstrated that Ang-2 gene expres-

sion, as assessed with reverse transcription-PCR, was inversely correlated with vessel maturation but failed to demonstrate a significant correlation between Ang-2 gene expression and IMVD. In the present study, increased Ang-2 expression was significantly correlated with higher CD105-IMVD in NSCLC, suggesting that Ang-2 plays important roles in tumor angiogenesis. Tumor with positive Ang-2 expression also showed somewhat higher CD34-IMVD as compared with tumor with negative Ang-2 expression, but the difference did not reach a statistical significance, which may be attributable to the validity in the reactivity of the anti-CD34 antibody used in evaluation of angiogenesis. Thus, antibodies against pan-endothelial antigen such as CD34 may react with not only newly forming vessels but also stable vessels just trapped in tumors, whereas antibodies against CD105, a proliferation-related endothelial antigen, can preferentially react with active ECs of angiogenic tissue (24). In fact, Kumar *et al.* (25) and we (19) have demonstrated that CD105-IMVD is a significant prognostic factor, and CD34-IMVD is not, in breast carcinoma and NSCLC, respectively. These results along with the results documented in the present study may suggest that CD105 is superior to pan-endothelial markers such as CD34 in evaluation of angiogenesis in clinical studies. The present study also demonstrated that CD105-IMVD for Ang-2-positive tumor remained low in the absence of high VEGF expression whereas CD105-IMVD for Ang-2-positive tumor was elevated to the highest value in the presence of high VEGF expression. These results are in accordance with experimental results that angiogenesis is promoted by Ang-2 in concert with VEGF (2, 7). In the present study, we used only IMVD as a parameter of angiogenesis and showed that the status of Ang-1 did not affect angiogenesis. In future studies, other angiogenesis parameters, such as vessel maturity, length, and area, should be examined, because Ang-1 might affect other angiogenesis parameters.

With respect to a correlation between Angs expression and tumor progression or prognosis, only two clinical studies have been reported (12, 14). Eggert *et al.* (12) reported that increased Ang-2 gene expression, not Ang-1 gene expression, was significantly correlated with advanced tumor stages in neuroblastoma, but they failed to demonstrate a significant correlation between Ang-2 gene expression and postoperative survival. Etoh *et al.* (14) documented that increased Ang-2 gene expression was significantly correlated not only with an advanced tumor-stage but also with a poor prognosis in gastric carcinoma; this was the only clinical study that showed a significant correlation between Angs expression and a prognosis. Thus, the

Fig. 4. Postoperative survival of pathological stage-I-IIIa NSCLC. Comparison according to status of Ang-2 expression in combination with the status of VEGF expression.



present study added clinical evidence that Ang-2 played important roles in tumor progression through tumor angiogenesis and that Ang-2 could be a new prognostic marker. In addition, we demonstrated that the postoperative survival of patients with Ang-2-positive and VEGF-high tumor was extremely poor, which might be attributable to active angiogenesis in tumor tissues. Although Ang-2 significantly affected angiogenesis only when VEGF expression was high, a multivariate analysis of prognostic factors showed that Ang-2 proved to be an independent factor to predict a poor postoperative survival. The reason why Ang-2 status was an independent prognostic factor regardless of the VEGF status should be examined in a future study for a larger number of patients with more homogeneous characteristics.

In the present study, Angs expression was judged based on Angs expression in tumor cells, not in ECs, whereas Angs were expressed in ECs as well as in tumor cells. Although it had been initially reported that Angs were expressed in ECs, many experimental and clinical studies have revealed that Angs were expressed not only in ECs but also in tumor cells (9–14, 18, 26). In the present study, we did not assess Angs expression in ECs, not only because the degree and the extent of Angs expression in ECs were very heterogeneous, but also because the intensity of Angs expression was not so strong as to be clearly distinguished from negative expression. In fact, Ang-2 expression levels in whole tumor tissues were evaluated in most clinical studies, and detailed analyses of Ang-2 expression in tumor cells and that in ECs have not been reported. Only one clinical study has been reported on the quantitative evaluation of Ang-2 expression in ECs; that study used *in situ* hybridization to evaluate Ang-2 gene expression and demonstrated only that a correlation existed between the number of Ang-2-expressing vessels and VEGF expression (17). These results suggest that it may be difficult to assess Ang-2 expression quantitatively in ECs in clinical materials.

In conclusion, Ang-2 expression in NSCLC was significantly correlated with active angiogenesis, and can be a significant prognostic factor. In future studies, expression of Tie2 along with expression of the Ang ligands should be studied.

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## Expression of Angiopoietins and Its Clinical Significance in Non-Small Cell Lung Cancer

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