Evaluation of Angiogenesis in Non-small Cell Lung Cancer: Comparison between Anti-CD34 Antibody and Anti-CD105 Antibody[1]

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ABSTRACT

Purpose: **Angiogenesis is an essential process in the progression of malignant tumors. Whereas pan-endothelial markers, such as CD34, are generally used in evaluation of angiogenesis, pan-endothelial antibodies react with not only "newly forming" vessels but also normal vessels just trapped within tumor tissues. It has been recently reported that anti-CD105 antibody preferentially reacts with "activated" endothelial cells in angiogenic tissues. Thus, the superiority of anti-CD105 monoclonal antibody (mAb) in evaluation of angiogenesis of non-small cell lung cancer (NSCLC) was assessed.**

Experimental Design: **A total of 236 patients with resected NSCLC were retrospectively reviewed. Intratumoral microvessel density (IMVD) was determined with an anti-CD34 mAb (CD34-IMVD) and with an anti-CD105 mAb (CD105-IMVD).**

Results: **The mean CD34-IMVD and CD105-IMVD were 179.9 and 41.6, respectively. Whereas CD34-IMVD was significantly correlated with the expression of vascular endothelial growth factor ($P = 0.003$), CD105-IMVD was more closely correlated with vascular endothelial growth factor expression ($P < 0.001$). The 5-year survival rate of the lower CD105-IMVD patients was 74.9%, significantly higher than that of the higher CD105-IMD patients (60.4%, $P = 0.018$). Whereas the 5-year survival rate of the lower CD34-IMVD patients seemed higher than that of the higher CD34-IMVD patients (63.7%), the difference did not reach a statistical significance ($P = 0.137$). Multivariate analysis confirmed that higher CD105-IMVD was a significant factor to predict poor prognosis ($P = 0.029$), whereas CD34-IMVD was not ($P = 0.070$).**

Conclusions: **Anti-CD105 mAb proved to be superior to anti-CD34 mAb in evaluation of angiogenesis in NSCLC.**

INTRODUCTION

Angiogenesis is an essential process in progression of malignant tumors because sold tumors cannot grow beyond 1–2 mm in diameter without angiogenesis (1). Many clinical studies on NSCLC[3] have revealed that IMVD, a measurement of tumor angiogenesis determined with antibodies against ECs, is closely correlated with tumor growth and postoperative prognosis (2–7). However, several studies have failed to find that IMVD is a significant prognostic factor (8–10). One of the most probable reasons for these conflicting results may be the reactivity of anti-EC antibodies used to highlight intratumoral microvessels (11, 12). Whereas antibodies against pan-ECs, such as anti-CD31 and anti-CD34 antibodies, have been used in evaluation of angiogenesis, these pan-EC antibodies can react with not only "newly forming" vessels but also normal vessels just trapped within tumor tissues. Thus, pan-EC antibodies may not be the ideal reagents to visualize tumor-associated blood vessels (12–14).

CD105 (endoglin) is a M_r 180,000 homodimeric membrane glycoprotein expressed on ECs that can bind transforming growth factor-β1 and transforming growth factor-β3 (11). It has been demonstrated that anti-CD105 antibodies have greater affinity for "activated" ECs in tissues participating in angiogenesis (11, 13, 15–17). In contrast to pan-EC antibodies, therefore, anti-CD105 antibodies may preferentially react with ECs of all angiogenic tissues, including tumors, but weakly or not at all with those of most normal tissues (13, 15). These experimental results may suggest the superiority of CD105 as a marker of angiogenesis in clinical studies. In fact, Kumar *et al.* (18) have recently reported that IMVD determined with an anti-CD105 mAb correlates with postoperative survival in breast carcinoma, whereas IMVD determined with an anti-CD34 mAb does not. In the present study, we assessed the validity of an anti-CD105 mAb in evaluation of angiogenesis of NSCLC.

PATIENTS AND METHODS

Patients and Tissue Preparation. A total of 237 consecutive patients with p-stage I–IIIa NSCLC, who underwent

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; EC, endothelial cell; p-stage, pathological stage; IMVD, intratumoral microvessel density; mAb, monoclonal antibody; Sq, squamous cell carcinoma; Ad, adenocarcinoma; IHS, immunohistochemical staining; VEGF, vascular endothelial growth factor; PS, performance status; La, large cell carcinoma.

Table 1 Patients' characteristics and IMVD determined with an anti-CD34 or an anti-CD105 antibody

	No. of patients (%)	IMVD			
		CD34 (mean ±SE)	*P*	CD105 (mean ±SE)	*P*
Total	236 (100)	179.7 ± 6.4		41.6 ± 3.2	
Age (mean ± SD, years)	62.4 ± 9.7				
Lower age (<64 years)	118 (50.0)	181.0 ± 9.4	0.869	42.2 ± 4.5	0.844
Higher age (≥64 years)	118 (50.0)	178.5 ± 8.8		41.1 ± 4.5	
Sex					
Male	170 (72.0)	175.4 ± 7.6	0.273	44.1 ± 4.0	0.167
Female	66 (28.0)	190.8 ± 11.7		35.3 ± 5.0	
PS					
0	206 (87.3)	181.9 ± 6.9		40.6 ± 3.3	
1	28 (11.9)	158.5 ± 18.0	0.600	51.1 ± 12.1	0.562
2	2 (0.8)	255.5 ± 63.5		15.0 ± 14.0	
Histologic type					
Sq	85 (36.0)	171.8 ± 10.7	0.292 (Sq *vs.* Ad)	52.2 ± 5.9	0.046 (Sq *vs.* Ad)
Ad	130 (55.1)	186.3 ± 8.5	0.557 (Sq *vs.* La)	38.0 ± 3.8	0.215 (Sq *vs.* La)
La	13 (5.5)	187.7 ± 26.4	0.960 (Ad *vs.* La)	29.0 ± 16.9	0.614 (Ad *vs.* La)
Others	8 (3.4)				
Tumor cell differentiation[a]					
Well differentiated	80 (35.1)	190.6 ± 12.2		37.5 ± 4.9	
Moderately differentiated	90 (39.5)	165.7 ± 9.0	0.754	43.5 ± 5.0	0.205
Poorly differentiated	58 (25.4)	188.6 ± 12.6		48.1 ± 7.7	
p-stage					
I	138 (58.5)	189.9 ± 8.6		42.6 ± 4.2	
II	44 (18.6)	145.8 ± 13.2	0.288	47.4 ± 7.1	0.927
IIIa	54 (22.9)	181.3 ± 13.2		45.2 ± 6.6	

[a] Other histologic types were excluded in the analysis.

complete tumor resection and mediastinal lymph node dissection without any preoperative therapy at Kyoto University Hospital between January 1, 1985, and December 31, 1990, was retrospectively reviewed. One patient was excluded from the study because of operation-related death, and a total of 236 patients was finally evaluated (Table 1). P-stage was reevaluated and determined by the current tumor-node-metastasis classification (19). Histological type and cell differentiation were determined using the current classification by WHO (20). For analysis according to the differentiation of cancer cells, well-differentiated Sq and Ad were classified as well-differentiated tumors, moderately differentiated Sq and Ad as moderately differentiated tumors. La carcinoma and poorly differentiated Sq and Ad were classified as poorly differentiated tumors; the other histological types were excluded in the analysis as described in an earlier article (21).

For all these patients, the inpatient medical records, chest X-ray films, whole-body computed tomography films, bone and gallium scanning data, and records of surgery were reviewed. Intraoperative therapy was not performed on any patient. 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 (22). Follow-up of the postoperative clinical course was conducted by outpatient medical records and by inquiries by telephone or letter. The follow-up survey was successfully completed for 100% of patients for 5 years after surgery. The day of thoracotomy was considered the starting day for counting postoperative survival days.

All primary tumor specimens were immediately fixed in 10% (volume for volume) formalin and then embedded in paraffin. Serial 4-μm sections were prepared from each sample and served for routine H&E staining and IHS. Results of IHS were evaluated by two authors independently (F. T. and Y. O.) without knowledge of clinical data.

Quantification of Angiogenesis (IMVD). IHS for CD34 and CD105 to highlight ECs was performed using a sensitive streptavidin-biotinylated horseradish peroxidase complex system (TSA-Indirect Kit; NEN Life Science Products, Boston, MA). All of the procedures were performed following the manufacture's protocol as described previously (23). Sections were incubated with an anti-CD34 mAb QBEnd10 (mouse IgG 1, κ, 50 μg/ml; Dako, Kyoto, Japan) diluted at 1/50 or an anti-CD105 mAb SN6h (mouse IgG 1, κ, 366 μg/ml; Dako) diluted at 1/100 for 1 h at room temperature. Normal mouse IgG was used as a substitute for the primary antibody for the negative controls. The 10 most vascular areas within a section were selected for quantitation of angiogenesis, and vessels labeled with the anti-CD34 mAb or the anti-CD105 mAb were counted under light microscopy with a 200-fold magnification. The average counts were recorded as the CD34-IMVD or the CD105-IMVD for each case.

Expression and Grade of VEGF. Expression of VEGF was evaluated using a standard streptavidin-biotinylated horseradish peroxidase complex method (LSAB kit; Dako) as described in an earlier article (21). After retrieval of the antigen with heating in a microwave oven for 5 min three times each, the sections were incubated with an anti-VEGF polyclonal antibody A-20 (200 μg/ml rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA). VEGF expression was evaluated according to a

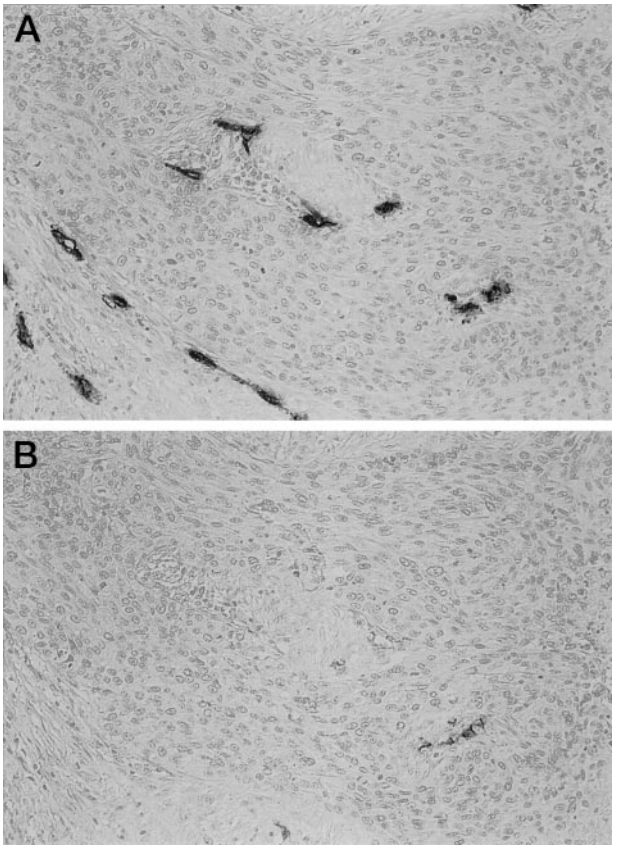

Fig. 1 Intratumoral microvessels highlighted with an anti-CD34 mAb (A) and with an anti-CD105 mAb (B). More vessels were recognized with the anti-CD34 mAb than with the anti-CD105 mAb.

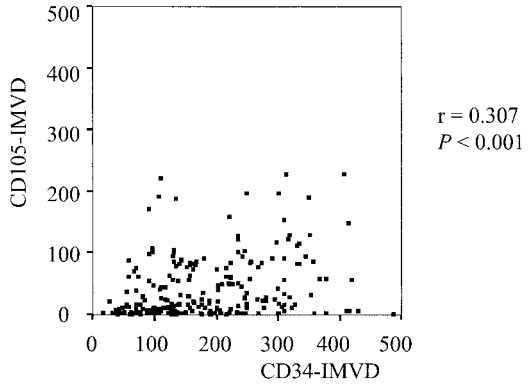

Fig. 2 Correlation between IMVD was determined with an anti-CD34 mAb (*CD34-IMVD*), and IMVD was determined with an anti-CD105 mAb (*CD105-IMVD*)

Table 2 Expression of VEGF and IMVD determined with an anti-CD34 or an anti-CD105 antibody[a]

VEGF score	No. of patients (%)	IMVD	
		CD34 (mean ±SE)	CD105 (mean ±SE)
0	35 (14.8)	137.9 ± 19.5	10.1 ± 2.9
2	13 (5.5)	165.5 ± 14.6	18.5 ± 9.2
3	28 (11.9)	171.0 ± 16.3	22.1 ± 2.7
4	80 (33.9)	178.4 ± 10.1	39.8 ± 5.1
5	59 (25.0)	206.0 ± 14.0	64.7 ± 7.2
6	21 (8.9)	201.5 ± 19.4	83.5 ± 12.2

[a] *P* for correlation between VEGF score and IMVD; 0.003 (for CD34); <0.001 (for CD105).

scoring method reported by Mattern *et al.* (24). A percentage score was defined as follows: score 0, no VEGF-positive staining cell; score 1, the percentage of VEGF-positive staining cells ≤25%; score 2, the percentage ≤50%; score 3, the percentage >50%. A intensity score was defined as follows: score 0, no staining; score 1, weak staining intensity; score 2, moderate staining intensity; score 3, high staining intensity comparable with that of smooth muscle cells of either bronchial wall or blood vessels, which were served as internal positive control for VEGF staining (25). Grade of VEGF expression was represented as the sum of the percentage score and the intensity score (VEGF score).

Statistical Methods. The χ^2 was used to compare counts. Continuous data were compared using Student's *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 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 statistical manipulations were performed using the SPSS for Windows system (SPSS, Inc, Chicago, IL).

RESULTS

IMVD in NSCLC. As demonstrated in Fig. 1, A and B, more vessels were generally recognized by the anti-CD34 mAb than by the anti-CD105 mAb. The mean CD34-IMVD and CD105-IMVD, calculated for all 236 patients studied, were 179.7 and 41.6, respectively (Table 1); the median CD34-IMVD and CD105-IMVD were 157 and 19, respectively. There proved to be a significant correlation between the CD34-IMVD and the CD105-IMVD ($P < 0.001$, $r = 0.307$; Fig. 2). No significant correlation was observed between the IMVD (CD34-IMVD or CD105-IMVD) and age, sex, PS, differentiation of cancer cells, or p-stage (Table 1).

IMVD and VEGF Expression. The mean IMVDs stratified by the VEGF scores were shown in Table 2. Whereas both the CD34-IMVD and the CD105-IMVD were significantly correlated with the VEGF score, the CD105-IMVD was more closely correlated. To examine the correlation more clearly, VEGF expression was classified according to the following grading system reported by Mattern *et al.* (24): negative VEGF expression, the VEGF score 0–2; weak VEGF expression, the score 3–4; strong VEGF expression, the score 5–6. The CD105-IMVD markedly increased in response to enhanced VEGF expression ($P < 0.001$; Fig. 3B), whereas the CD34-IMVD also increased ($P = 0.003$; Fig. 3A).

Kumar *et al.* (11, 13, 15–18) and others have suggested that anti-CD105 antibodies preferentially react with activated

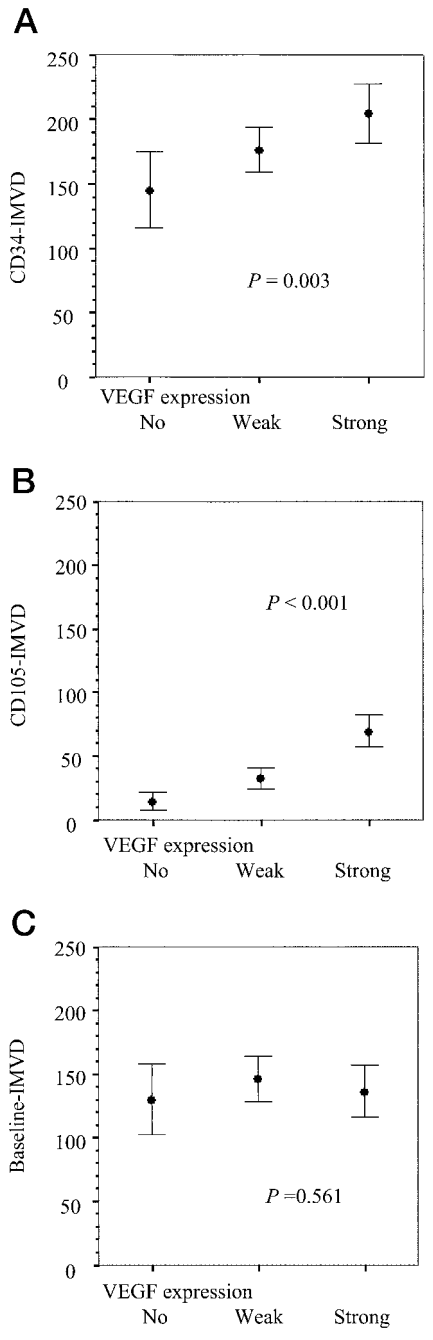

Fig. 3 (A), correlation between VEGF expression (*No, Weak,* and *Strong*) and IMVD determined with an anti-CD34 mAb (*CD34-IMVD*). (B), correlation between VEGF expression (*No, Weak,* and *Strong*) and IMVD determined with an anti-CD105 mAb (*CD105-IMVD*). (C), correlation between VEGF expression (*No, Weak,* and *Strong*) and the baseline IMVD that is defined as (CD34-IMVD–CD105-IMVD). It should be noted that there was no correlation between VEGF expression and the baseline IMVD.

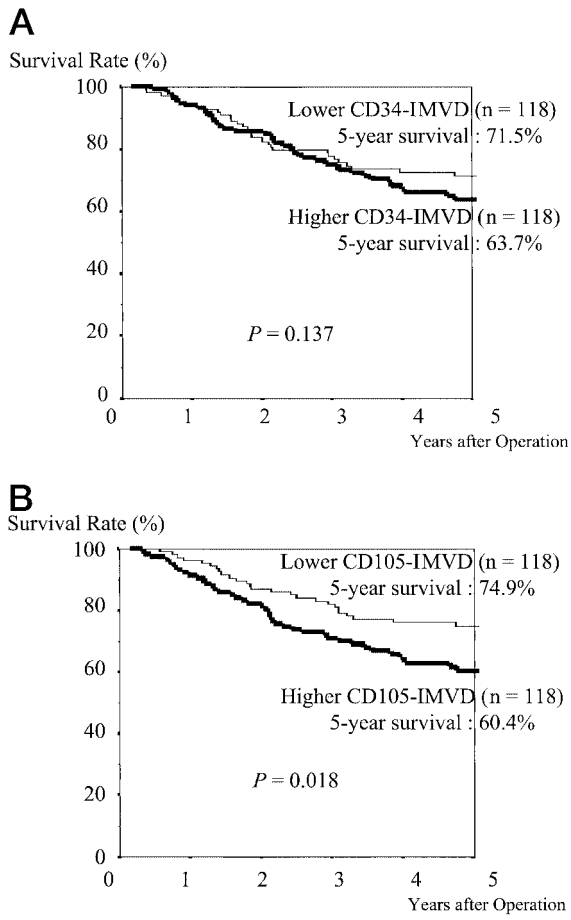

Fig. 4 Postoperative survival of completely resected p-stage I–IIIA NSCLC. Comparison according to IMVD determined with an anti-CD34 (A) or an anti-CD105 (B) mAb.

ECs. If it is true, vessels that are positively stained with pan-EC antibodies and are not positively stained with anti-CD105 antibodies should represent normal vessels just entrapped within tumor tissues. When we tried to define CD34-IMVD minus CD105-IMVD as the "baseline" IMVD, there proved to be no correlation between VEGF expression and the baseline IMVD (Fig. 3C).

IMVD and Postoperative Prognosis. For all patients, the 5-year survival rate of patients with tumors showing the lower CD105-IMVD (CD105-IMVD $<$ 19, the median value) was 74.9%, significantly higher than that of the higher CD105-IMD patients (60.4%, $P = 0.018$; Fig. 4B). Whereas the 5-year survival rate of the lower CD34-IMVD patients (CD34-IMVD $<$ 157, 71.5%) also seemed to be higher than that of the higher CD34-IMVD patients (63.7%), the difference did not reach a statistical significance ($P = 0.137$; Fig. 4A).

Analyses only for p-stage I patients revealed a significant difference between the lower and the higher CD34-IMVD patients (5-year survival rates, 86.4 and 72.2%, respectively; $P = 0.023$), as well as between the lower and the higher CD105-IMVD patients (5-year survival rates, 91.8 and 66.7%, respectively; $P = 0.002$). However, it should be noted that the difference was bigger when the CD105-IMVD was used as a marker of angiogenesis (Table 3).

Multivariate analysis confirmed that higher CD105-IMVD was a significant and independent factor to predict poor prog-

Table 3 Postoperative survival and IMVD determined with an anti-CD34 or an anti-CD105 antibody

	5-year survival rate (%) IMVD (CD34)			5-year survival rate (%) IMVD (CD105)		
	Lower	Higher	*P*	Lower	Higher	*P*
All patients	71.5%	63.7%	0.137	74.9%	60.4%	0.018
p-stage						
I	86.4%	72.2%	0.023	91.8%	66.7%	0.002
II	65.5%	67.5%	0.740	72.1%	54.9%	0.104
IIIa	37.4%	36.3%	0.994	31.1%	46.7%	0.157

Table 4 Multivariate analysis of prognostic factors in NSCLC

Prognostic factors	β	*P*	Relative hazard (95% confidence interval)
a. Age	0.025	0.071	1.025 (0.998–1.052)
Sex (male/female)	−0.587	0.087	0.570 (0.301–1.036)
PS (0/1/2)	0.281	0.327	1.324 (0.756–2.319)
Histologic type (nonadenocarcinoma/ adenocarcinoma)	−0.038	0.238	0.963 (0.905–1.025)
p-stage (I, II, IIIa)	0.727	<0.001	2.068 (1.594–2.682)
CD34-IMVD (lower/ higher)	0.434	0.070	1.543 (0.965–2.466)
b. Age	0.024	0.077	1.024 (0.998–1.052)
Sex (male/female)	−0.446	0.127	0.640 (0.361–1.136)
PS (0/1/2)	0.279	0.330	1.321 (0.755–2.313)
Histologic type (nonadenocarcinoma/ adenocarcinoma)	−0.042	0.192	0.959 (0.900–1.021)
p-stage (I, II, IIIa)	0.628	<0.001	1.874 (1.464–2.399)
CD105-IMVD (lower/ higher)	0.520	0.029	1.662 (1.056–2.679)

nosis ($P = 0.029$; Table 4B). When the CD34-IMVD was used instead of CD105-IMVD in the regression model, IMVD failed to have a significant prognostic value ($P = 0.070$; Table 4A).

DISCUSSION

The present study demonstrated the validity and superiority of CD105 as a marker of angiogenesis in NSCLC; the CD105-IMVD was more closely correlated with the expression of VEGF than the CD34-IMVD. Kumar *et al.* (11, 13, 15–18) and others have demonstrated that anti-CD105 antibodies preferentially react with activated ECs in tissues participating in angiogenesis, such as tumor tissues, and that antibodies against pan-ECs, such as anti-CD34 antibodies, react with normal vessels, as well as activated vessels. According to the hypothesis, we tried to define the CD34-IMVD–CD105-IMVD as the baseline IMVD. As a result, the baseline IMVD proved not at all to be correlated with VEGF expression, suggesting the baseline IMVD was not a measurement of angiogenesis but a measurement of vessels just trapped within tumor tissues. Of course, it should be noted that angiogenesis is not influenced only by VEGF but also other angiogenic factors and antiangiogenic factors, such as angiostatin. Comparative studies on evaluation of angiogenesis using a pan-EC antibody and an anti-CD105 antibody in correlation with other factors influencing on angiogenesis should be conducted in the future.

The present study also demonstrated that the CD105-IMVD was an independent prognostic factor, whereas the CD34-IMVD was not, which was consistent with results of a retrospective study conducted by Kumar *et al.* (18). They evaluated angiogenesis in breast carcinoma using an anti-CD34 mAb and an anti-CD105 mAb and reported that the CD105-IMVD showed a statistical correlation with postoperative survival, whereas the CD34-IMVD did not. These results demonstrating the superiority of the CD105-IMVD over the CD34-IMVD as a prognostic factor also support the validity of CD105-IMVD in evaluation of angiogenesis. To confirm the prognostic significance of the CD105-IMVD, prospective studies should be conducted in the future.

In conclusion, CD105 proved to be superior to CD34 as a marker in evaluation of angiogenesis of NSCLC, not only because the CD105-IMVD was more closely correlated with VEGF expression but also because the CD105-IMVD, not the CD34-IMVD, was an independent prognostic factor.

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