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Vascular Biology, Atherosclerosis and Endothelium Biology

Vasohibin-1 Expression in Endothelium of Tumor Blood Vessels Regulates Angiogenesis

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In this study, we characterized the significance of the vascular endothelial growth factor-inducible angiogenesis inhibitor vasohibin-1 to tumors. In pathological sections of non-small cell lung carcinoma, vasohibin-1 was present in the endothelial cells of blood vessels of the tumor stroma, but not in the lymphatics. In cancer cells, the presence of vasohibin-1 was associated with hypoxia-inducible factor 1α/vascular endothelial growth factor and fibroblast growth factor-2 expression. We then examined the function of vasohibin-1 in the mouse by subcutaneously inoculating with Lewis lung carcinoma cells. Resultant tumors in *vasohibin-1*$^{-/-}$ mice contained more immature blood vessels and fewer apoptotic tumor cells than tumors in wild-type mice. In wild-type mice that had been inoculated with Lewis lung carcinoma cells, tail vein injection of adenovirus containing the human *vasohibin-1* gene inhibited tumor growth and tumor angiogenesis. Moreover, the remaining tumor vessels in adenoviral human *vasohibin-1* gene-treated mice were small, round, and mature, surrounded by mural cells. The addition of adenoviral human *vasohibin-1* gene to cisplatin treatment improved cisplatin's antitumor activity in mice. These results suggest that endogenous vasohibin-1 is not only involved in tumor angiogenesis, but when sufficient exogenous vasohibin-1 is supplied, it blocks sprouting angiogenesis by tumors, matures the remaining vessels, and enhances the antitumor effect of conventional chemotherapy. ***(Am J Pathol 2009, 175:430–439; DOI: 10.2353/ajpath.2009.080788)***

Angiogenesis, also called neovascularization, is a fundamental process of blood vessel growth, and a hallmark of cancer development. Multiple studies show that tumor angiogenesis in non-small cell lung carcinoma (NSCLC) is associated with metastases and poor survival.[1] The importance of tumor angiogenesis is further emphasized by clinical studies of anti-angiogenic agents.[2] Indeed, anti-angiogenic therapy shows promise as an effective treatment for various cancers, including NSCLC.[3]

The local balance between angiogenesis stimulators and inhibitors regulates angiogenesis. The most important molecule that stimulates angiogenesis is vascular endothelial growth factor (VEGF).[4] The circulating level of VEGF before treatment predicts the survival of patients with NSCLC.[5–7] Hypoxia, one of the triggers of angiogenesis, induces the expression of various molecules including VEGF. Hypoxia frequently occurs in tumors due to the increased oxygen requirement of the proliferating cancer cells and the poor blood supply.[8] The induction of VEGF in the hypoxic condition is mediated by a transcription factor, hypoxia-inducible factor 1 (HIF1), a heterodimeric complex of HIF1α and HIF1β subunits. HIF1α is easily degraded under normoxic conditions, but becomes stable under hypoxic conditions, and makes a heterodimeric complex with HIF1β. The dimer binds to the hypoxia responsive element in the promoter of the VEGF gene.[9] A worse prognosis in patients with NSCLC is associated with increased expression of HIF1 in cancer cells.[10] Fibroblast growth factor (FGF)-2 is another growth factor that has potent angiogenic activity. The induction of FGF-2 in cancer cells is also associated with the angio-

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Table 1. Patient Profile

Number of patients	44
Sex (%)	
Male	28 (63.6)
Female	16 (36.4)
Age	
Mean (range)	66.2 (45–82)
Histology (%)	
Adenocarcinoma	35 (79.5)
Squamous cell carcinoma	9 (20.5)
Stage (%)	
IA	29 (65.9)
IB	5 (11.3)
IIA	0 (0)
IIB	1 (2.3)
IIIA	7 (15.9)
IIIB	1 (2.3)
IV	1 (2.3)

genic switch.[11] Indeed, FGF-2 is expressed in some NSCLC cells.[5–7]

The activities of angiogenesis stimulators are normally countered by angiogenesis inhibitors. A number of angiogenesis inhibitors have been identified to date.[12] Thrombospondin 1 is the best-characterized angiogenesis inhibitor in NSCLC. The expression of thrombospondin 1 is under the control of p53.[13] In cancer cells including NSCLC, alteration of the p53 gene is associated with decreased expression of thrombospondin 1.[14] The decreased expression of thrombospondin 1 in NSCLC is confirmed by others,[15–17] and correlates with poor prognosis.[15]

We have recently isolated a novel angiogenesis inhibitor, vasohibin (VASH), from endothelial cells (ECs).[18] Because a homologue of VASH was reported as VASH2,[19] the prototype VASH is now called VASH1. The characteristic feature of VASH1 is that it is induced in ECs by VEGF and FGF-2, two potent angiogenic factors.[18,20] Here we characterize the significance of VASH1 in tumors. We first verify the localization of VASH1 in relation to HIF1α, VEGF, or FGF-2 in the pathological sections of NSCLC. We then evaluate the role of VASH1 in tumor angiogenesis in animal models. Our analysis reveals that endogenous VASH1 is expressed in ECs of tumor blood vessels, and it is involved in the termination of tumor angiogenesis. When applied exogenously, VASH1 inhibits sprouting angiogenesis in tumors, matures the remaining vessels, and enhances the antitumor effect of chemotherapy.

Table 2. Relationship between VEGF and HIF-1α in NSCLCs

	VEGF Negative	VEGF Positive	Total
HIF-1α Negative	9	2	11
HIF-1α Positive	12	21	33
Total	21	23	44

Materials and Methods

Materials

We used the following materials: anti-human CD31 monoclonal antibody (mAb), anti-human α-smooth muscle actin (αSMA) mAb, and Rabbit/Mouse Ig Immunohistochemistry Kit (DAKO Cytomation, Glostrup, Denmark); anti-human HIF-1α goat antibody (Ab), and anti-human

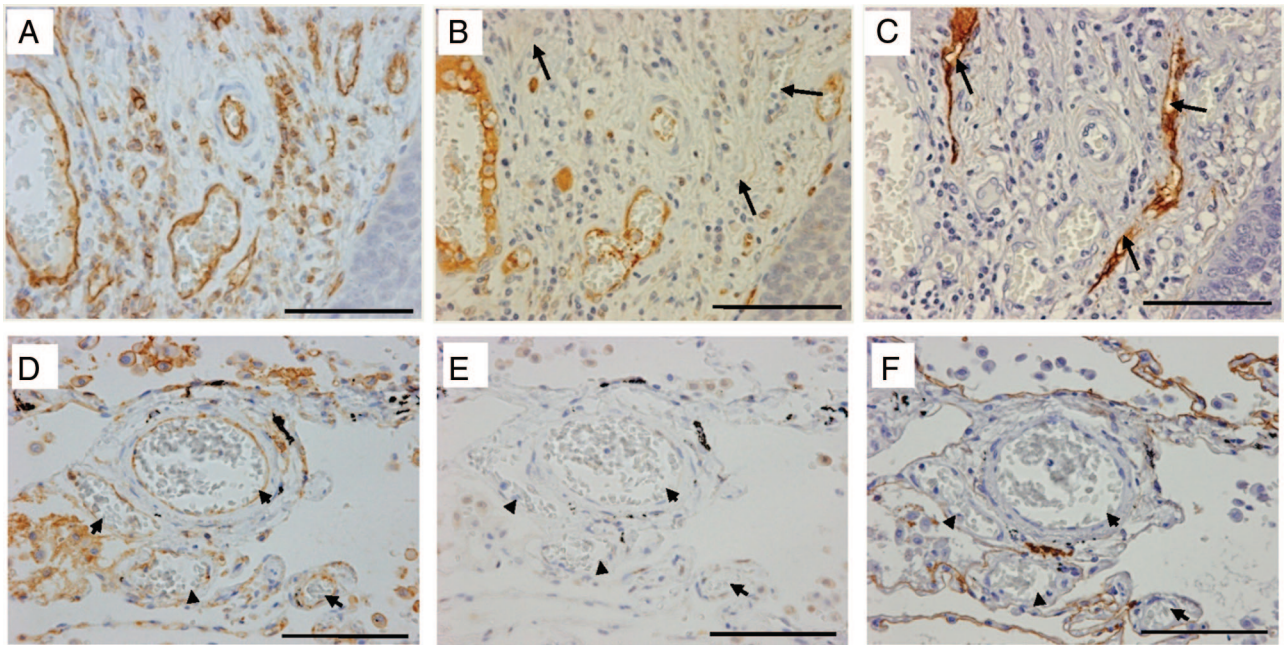

Figure 1. Presence of VASH1 in blood vessel ECs in the tumor stroma of NSCLCs. Pathological sections of NSCLC were immunostained for endothelial cell marker CD31 (**A** and **D**), VASH1 (**B** and **E**), and the lymphatic endothelial cell marker podoplanin (**C** and **F**). (**A**), (**B**) and (**C**) show tumor stroma, whereas (**D**), (**E**), and (**F**) show non-cancerous region in the same patient. Scale bar = 100 μm. VASH1 was present in blood vessel ECs in the tumor stroma of NSCLCs. **Arrows** indicate lymphatic vessels in tumor stroma and **arrowheads** indicate blood vessels in non-cancerons resion.

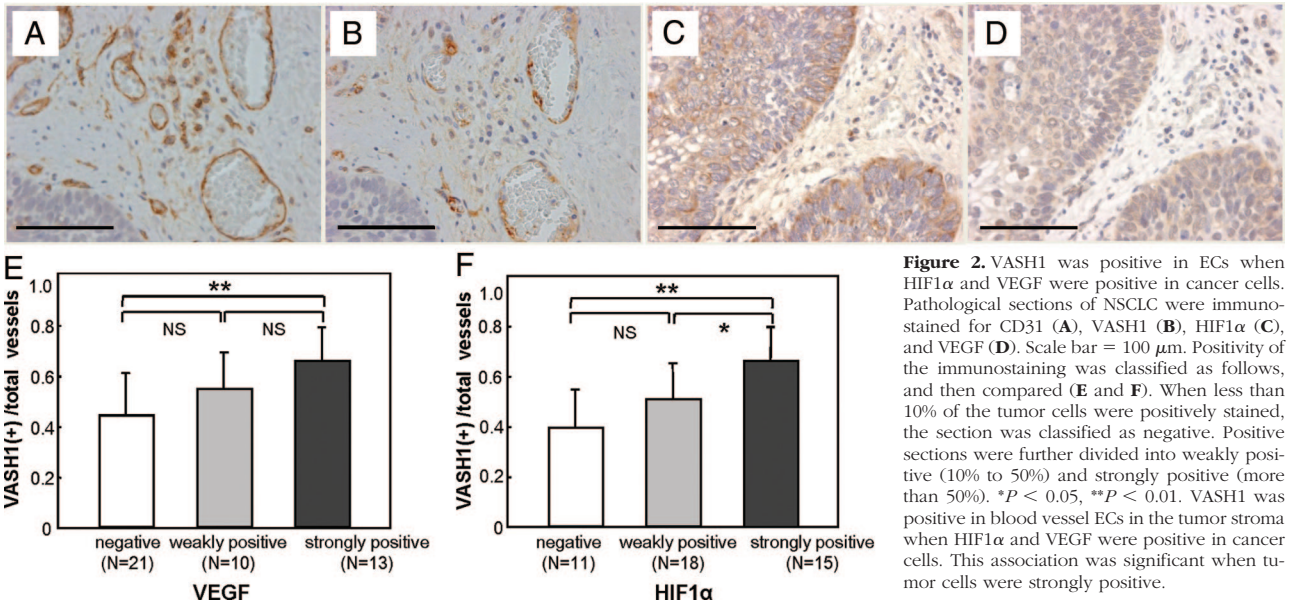

Figure 2. VASH1 was positive in ECs when HIF1α and VEGF were positive in cancer cells. Pathological sections of NSCLC were immunostained for CD31 (**A**), VASH1 (**B**), HIF1α (**C**), and VEGF (**D**). Scale bar = 100 μm. Positivity of the immunostaining was classified as follows, and then compared (**E** and **F**). When less than 10% of the tumor cells were positively stained, the section was classified as negative. Positive sections were further divided into weakly positive (10% to 50%) and strongly positive (more than 50%). $^{*}P < 0.05$, $^{**}P < 0.01$. VASH1 was positive in blood vessel ECs in the tumor stroma when HIF1α and VEGF were positive in cancer cells. This association was significant when tumor cells were strongly positive.

FGF-2 rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA); anti-human podoplanin mAb (AngioBio, Del Mar, CA); anti-human VEGF mAb (LAB VISION, Fremont, CA); anti-human β-actin mAb, anti-mouse αSMA mAb, horseradish peroxidase-conjugated anti-mouse IgG and *cis*-diammineplatinum dichloride (CDDP) (Sigma, St. Louis, MO); anti-mouse CD31 rat Ab (Fitzgerald Industries International, Concord, MA); anti-mouse platelet-derived growth factor receptor β goat Ab (R&D Systems, Minneapolis, MN); biotin-conjugated anti-mouse or anti-goat IgG, IgA, IgM Ab, and streptavidin-biotin peroxidase complex (Nichirei Biosciences, Tokyo, Japan); Alexa fluor 568 labeled goat anti-rat IgG; Alexa 488 labeled donkey anti-mouse IgG; Alexa 488 labeled donkey anti-goat IgG; TO-PRO-3 iodide (Molecular Probes, Eugene, OR); OCT compound (Sakura Finetechnical, Tokyo, Japan); Dulbecco's modified Eagle's Medium (Nissui Pharmaceutical Co., Tokyo, Japan); fetal bovine serum (Equitech-Bio, Kerrville, TX); and diaminobenzidine (Sigma). Anti-human vasohibin-1 (VASH1) mAb was described previously.[18]

Patients

The clinical study included 44 patients with NSCLC, who underwent partial resection of a lung (lobectomy or pneumonectomy) in Tohoku University Hospital between November 2003 and January 2007. Informed consents were obtained from all of the patients, and the study was approved by the Ethical Committee of Tohoku University.

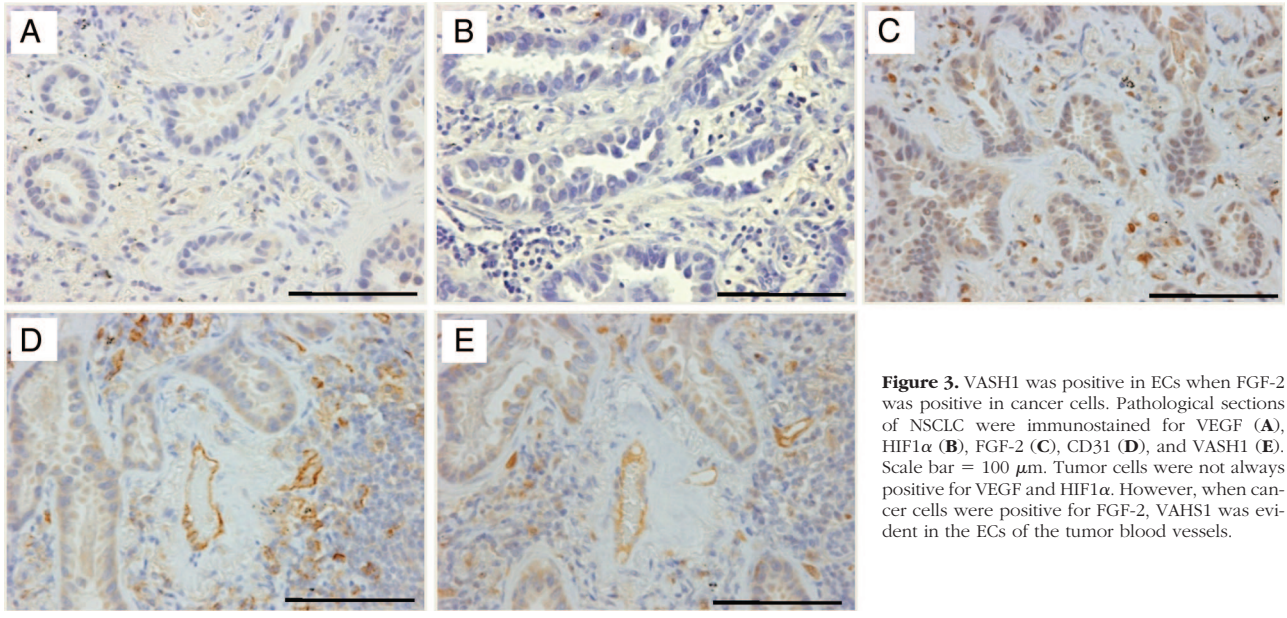

Figure 3. VASH1 was positive in ECs when FGF-2 was positive in cancer cells. Pathological sections of NSCLC were immunostained for VEGF (**A**), HIF1α (**B**), FGF-2 (**C**), CD31 (**D**), and VASH1 (**E**). Scale bar = 100 μm. Tumor cells were not always positive for VEGF and HIF1α. However, when cancer cells were positive for FGF-2, VAHS1 was evident in the ECs of the tumor blood vessels.

Table 3. Relationship between VEGF and FGF-2 in NSCLCs

	VEGF Negative	VEGF Positive	Total
FGF-2 Negative	15	3	18
FGF-2 Positive	6	20	26
Total	21	23	44

Immunohistochemical Analysis of Human Lung Specimens

Human lung specimens were fixed in 10% formalin, embedded in paraffin, and cut into 3-μm thick sections. Sections were dewaxed in xylene, rehydrated in a graded ethanol series (100%, 90%, 80%, and 70%), and incubated in 10% H_2O_2/methanol to block endogenous peroxidase activity. Sections were then incubated in citrated buffer (pH 6.0) for 5 minutes at 121°C in a microwave oven for VASH1, CD31, and FGF-2 staining, in citrated buffer (pH 6.0); in the same conditions for 15 minutes for VEGF staining; and in 0.1% Trypsin/0.05 mol/L Tris buffer (pH 7.6) for 30 minutes at 37°C for αSMA staining. Thereafter, sections were incubated for 10 minutes at room temperature in a blocking solution of 10% rabbit or goat serum (Nichirei Biosciences). Primary antibody reactions were performed overnight at 4°C at a dilution of 1:400 for anti-human VASH1 mAb, 1:40 for anti-human CD31 mAb, 1:800 for anti-human αSMA mAb, 1:100 for anti-human HIF1α goat Ab, 1:200 for anti-human podoplanin mAb, 1:50 for anti-human VEGF mAb, and 1:200 for anti-FGF-2 rabbit Ab. A secondary antibody reaction was performed with biotin-conjugated anti-mouse or anti-goat IgG, IgA, and IgM Ab for 30 minutes at room temperature. Streptavidin-biotin peroxidase complex formation was performed for 30 minutes at room temperature. Sections were visualized using diaminobenzidine/H_2O_2 and sodium azide in 0.05mol/L Tris buffer, (pH 7.6). Nuclei were counterstained with hematoxylin.

To localize VASH1, mirror sections were prepared. One section was stained for CD31 and αSMA, and the other was stained for VASH1. For CD31 and αSMA, primary antibody reactions were performed overnight at 4°C; for anti-human CD31 mAb reactions were performed at day one. On day 2, sections were visualized using diaminobenzidine, 0.1 M/L glycine buffer (pH 2.2) for 30 minutes at room temperature. After treatment in 0.1% Trypsin, 0.05 mol/L Tris buffer, pH7.6, for 30 minutes at 37°C and washing with PBS three times, the sections underwent the primary antibody reaction for anti-human αSMA mAb overnight at 4°C. On day three, sections were visualized using 4 chloro-1naphtol and ethanol, 0.05 mol/L Tris buffer (pH 7.6)/H_2O_2, for 30 minutes at room temperature. After being washed with PBS three times, the sections were covered with aqueous mounting medium.

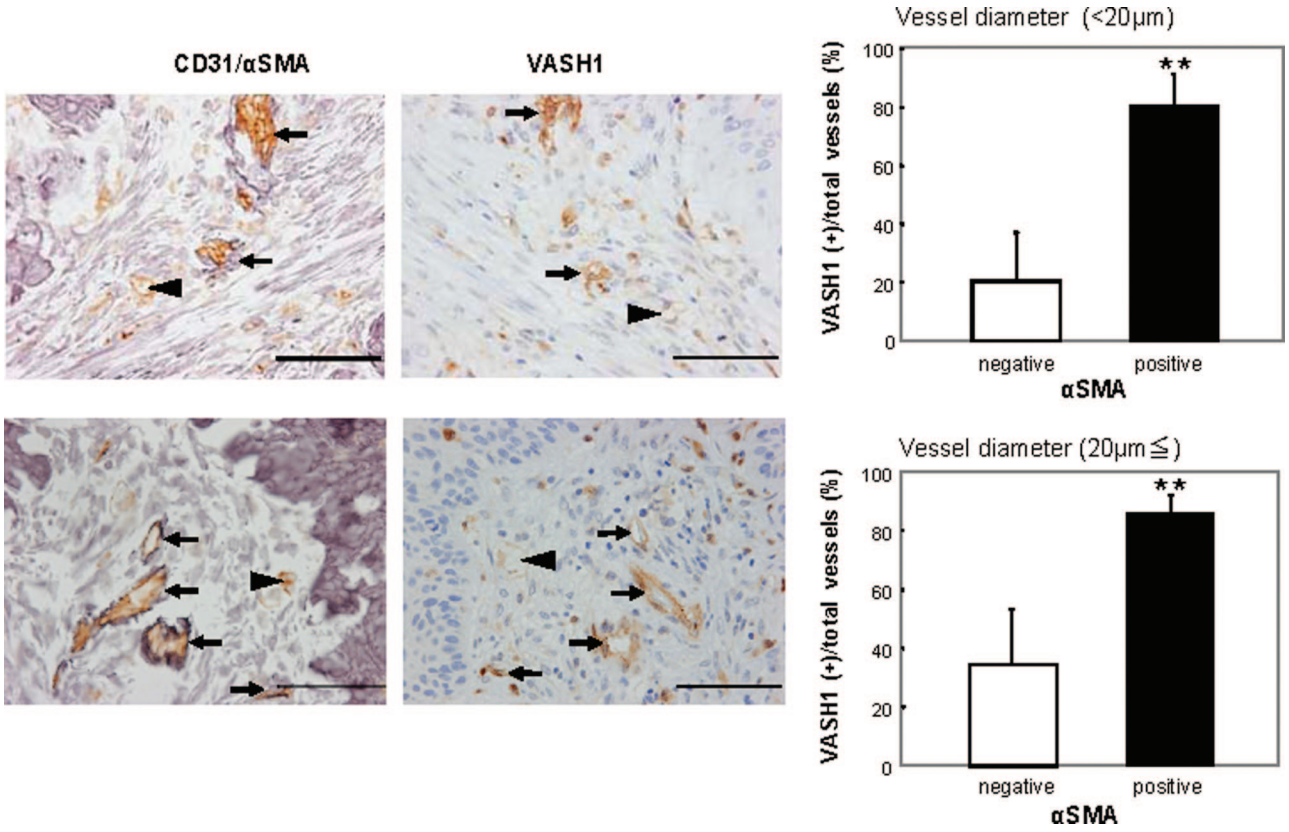

Figure 4. VASH1 was positive in ECs of tumor vessels when they were associated with mural cells. Mirror sections of NSCLC were immunostained for CD31 (brown) and αSMA (purple) on the left, and VASH1 (brown) on the right. Scale bar = 100 μm. VASH1 was positive when the vessels were associated with mural cell (**arrows**). VASH1 was negative when the vessel was not associated with mural cell (**arrowheads**). Quantification revealed that the positivity of VASH1 with mural cell association was evident regardless of the size of blood vessels. $^{**}P < 0.01$.

Interpretation of the Immunohistochemical Staining

Two pathologists evaluated all of the slides independently. To analyze CD31 and VASH1 staining, slides were scanned at low magnification (×100), and then vessels were counted in four random intratumoral areas of 1 mm^2, and in four random peritumoral areas of 1 mm^2. To determine HIF-1α, VEGF, and FGF-2 staining, slides were scanned at low magnification (×100). When less than 10% of the tumor cells were positively stained, the section was classified as negative; when more than 10% were positively stained, the section was classified as positive. In some cases, positive sections were further divided in to weakly positive (10% to 50%) and strongly positive (more than 50%).

Cells

Lewis lung carcinoma (LLC) cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 4 mmol/L L-glutamine.[21]

Tumor Growth in Mice

Male C57 BJ/6 J mice, 6 to 8-week-old (Charles River, Japan) or *VASH1*$^{-/-}$ mice of C57 BJ/6 background[22]

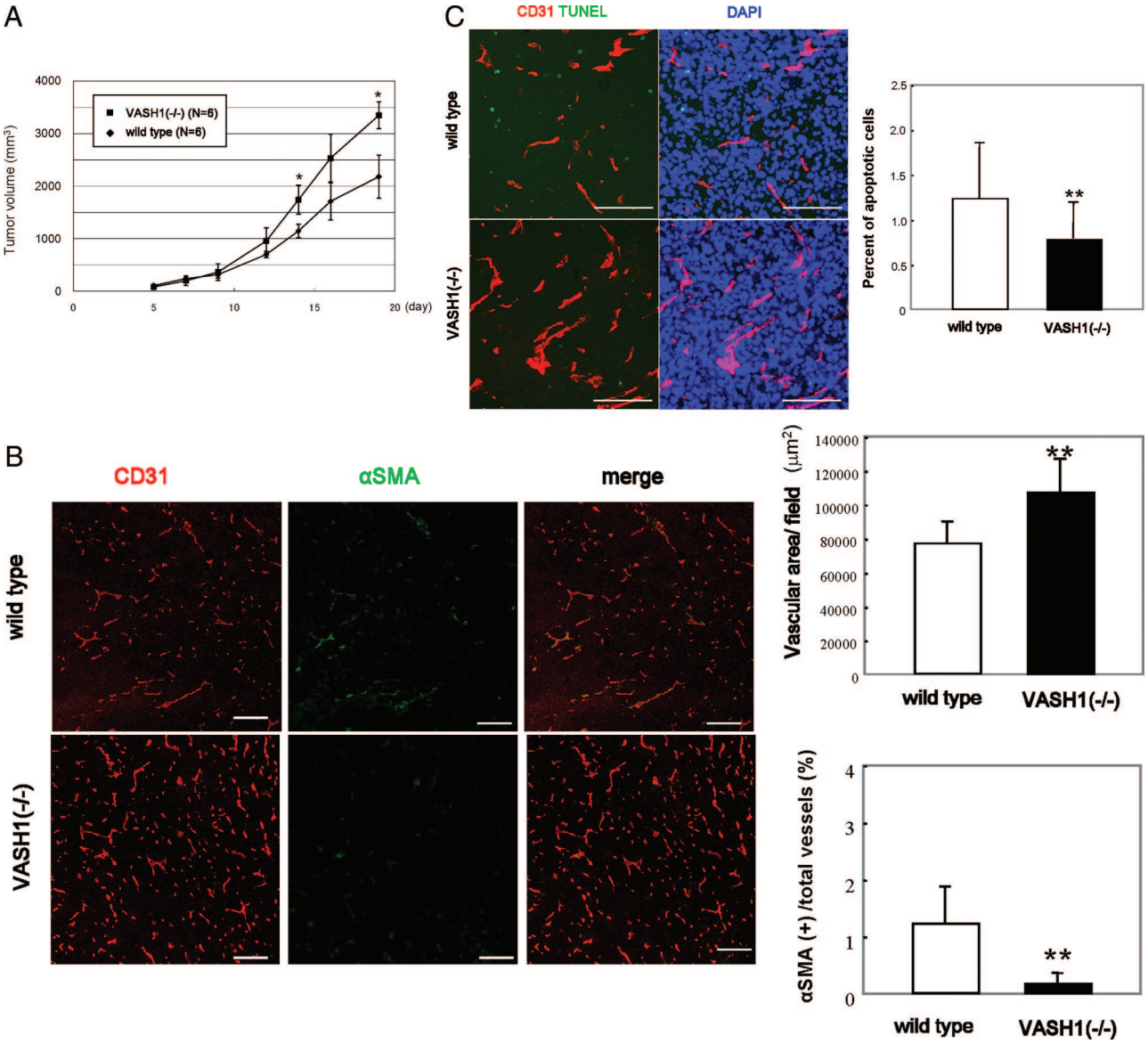

Figure 5. Tumor growth and tumor angiogenesis in *VASH1*$^{-/-}$ mice. **A:** LLC cells (5×10^6) were inoculated in wild-type and *VASH1*$^{-/-}$ mice, and tumor growth was evaluated. Data are expressed as the means and SDs. Tumors in *VASH1*$^{-/-}$ mice tended to grow bigger. $^*P < 0.05$. **B:** Tumor sections were co-immunostained for CD31 and αSMA. Tumors in *VASH1*$^{-/-}$ mice contained numerous small vessels. Scale bar = 100 μm. For this analysis, we counted 221 to 444 vessels per field (357 ± 66 per field) in wild-type and 328 to 780 vessels per field (472 ± 129 per field) in knockout mice. Quantification revealed that the vascularized tumor area in *VASH1*$^{-/-}$ mice was significantly increased, and vessels in *VASH1*$^{-/-}$ mice were more immature. $^{**}P < 0.01$. **C:** Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling. Scale bar = 100 μm. Tumors in *VASH1*$^{-/-}$ contained fewer apoptotic tumor cells. $^{**}P < 0.01$.

were inoculated in the subcutaneous tissue of the right abdominal wall with 5×10^6 LLC cells. Every 2 days after the inoculation, perpendicular tumor diameters were measured by digital calipers, and the tumor volume was calculated as $0.5 \times \text{length} \times \text{width}^2$. At indicated periods after the inoculation, mice were sacrificed, and tumors were collected.

To evaluate the effect of VASH1, we used a replication-defective adenovirus vector encoding the human *VASH1* gene (AdVASH1). A replication-defective adenovirus vector encoding the *β-galactosidase* gene (AdLacZ) was used for the control.[18,23] A total of 100 μl AdVASH1 or AdLacZ, containing 1×10^9 plaque-forming units were injected into the mouse tail vein on day 7 after the inoculation. For the combination with CDDP, AdVASH1 or AdLacZ was injected in the tail vein at day 6 after the inoculation. The mice were then given an intraperitoneal injection of CDDP (2.5 mg/kg) on days 10, 14, and 18.[24,25]

Tumor tissues were embedded in OCT compound to make frozen tissue specimens, and sectioned at 7 μm. Sections were fixed with methanol for 20 minutes at 20°C, blocked with Protein Block Serum Free (DAKO Cytomation) for 10 minutes at room temperature, and stained with anti-mouse CD31 rat Ab (1:200), anti-mouse α-SMA mAb (1:200) or anti-mouse platelet-derived growth factor receptor (PDGFR) β goat Ab (1:10) overnight, followed by staining with Alexa fluor 568 labeled goat anti-Rat IgG (1:400), Alexa 488 labeled donkey anti-mouse IgG (1:400), Alexa 488 labeled donkey anti-goat IgG (1:400), and TO-PRO-3 iodide (1:1000) for 60 minutes at room temperature. After being washed with PBS three times, the sections were covered with fluorescent mounting medium. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining used Fluorescein FragEL DNA Fragmentation Detection Kit (EMD Chemicals Inc., Darmstadt, Germany) following manufacturer protocols. Stained samples were visualized using an Olympus FluoView FV1000 confocal microscope (Tokyo, Japan). The vascular lumen was traced and the luminal area was analyzed with NIH ImageJ software.

Statistical Analysis

Data are expressed as mean ± SD The statistical significance of differences was evaluated using the

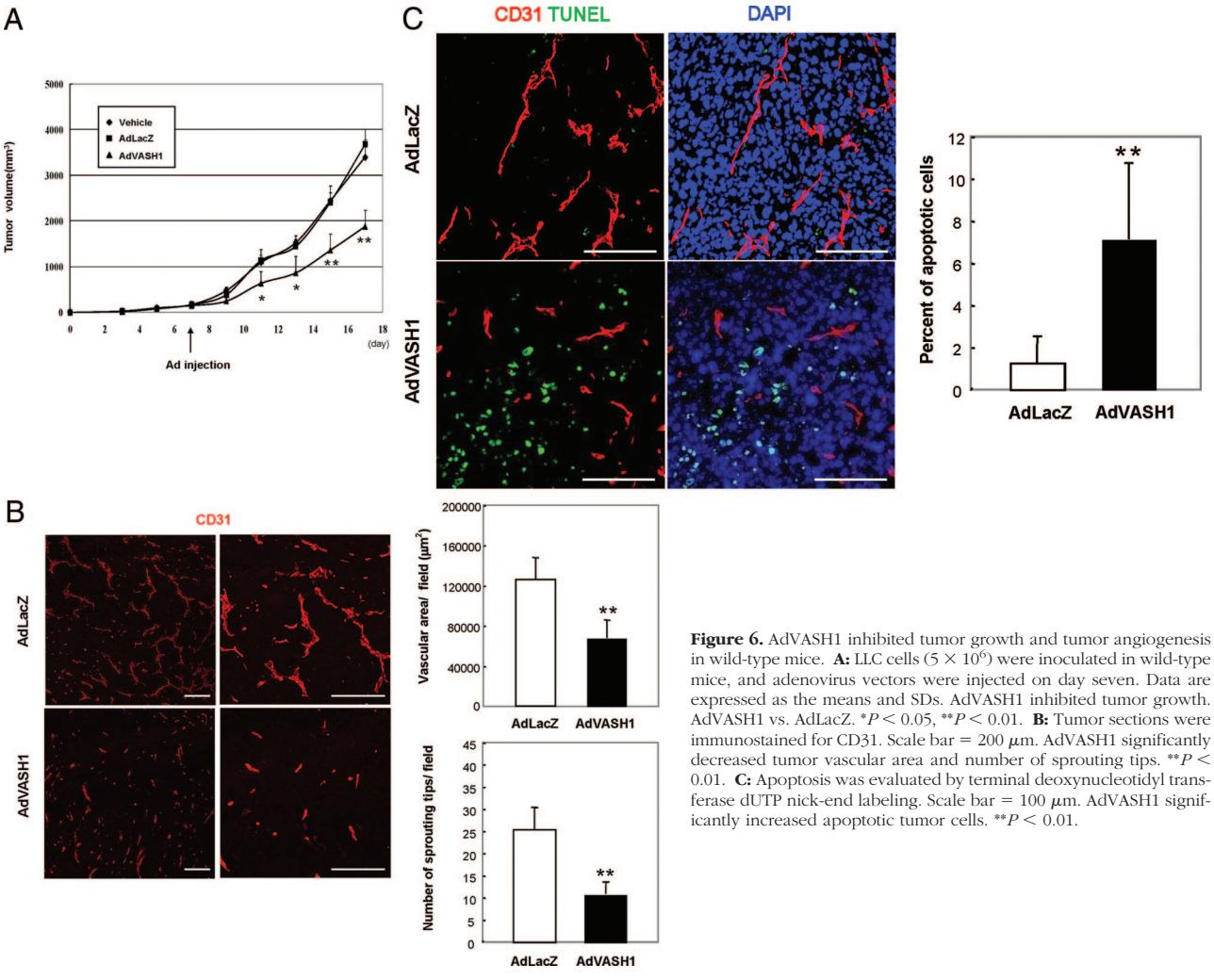

Figure 6. AdVASH1 inhibited tumor growth and tumor angiogenesis in wild-type mice. **A:** LLC cells (5×10^6) were inoculated in wild-type mice, and adenovirus vectors were injected on day seven. Data are expressed as the means and SDs. AdVASH1 inhibited tumor growth. AdVASH1 vs. AdLacZ. $^*P < 0.05$, $^{**}P < 0.01$. **B:** Tumor sections were immunostained for CD31. Scale bar = 200 μm. AdVASH1 significantly decreased tumor vascular area and number of sprouting tips. $^{**}P < 0.01$. **C:** Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling. Scale bar = 100 μm. AdVASH1 significantly increased apoptotic tumor cells. $^{**}P < 0.01$.

unpaired analysis of variance (analysis of variance), and *P* values were calculated using the unpaired Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

Results

Presence of VASH1 in Blood Vessel ECs in the Tumor Stroma of NSCLCs

We first evaluated the presence of VASH1 protein in human NSCLC specimens. The study included 44 patients with NSCLC (28 males and 16 females). Their average age was 66.2 years (range, 45 to 82 years). Based on the World Health Organization's criteria for tumor types, 35 patients had adenocarcinomas, and nine had squamous cell carcinomas. Twenty-nine patients had stage IA cancers, five patients had stage IB, one had stage II, eight had stage III, and one had stage IV cancers (Table 1).

Pathological sections of NSCLC were analyzed as follows. VASH1 was present in CD31 positive ECs, which were negative for lymphatic EC marker podoplanin (Figure 1A, C, D, and F). Thus, VASH1 is preferentially expressed in ECs of blood vessels. VASH1 was evident only in the tumor stroma (Figure 1B), and not in the non-cancerous region of the surgically resected tissue of the same patient (Figure 1E). This distinction indicates that the expression of this protein is closely associated with tumor blood vessels.

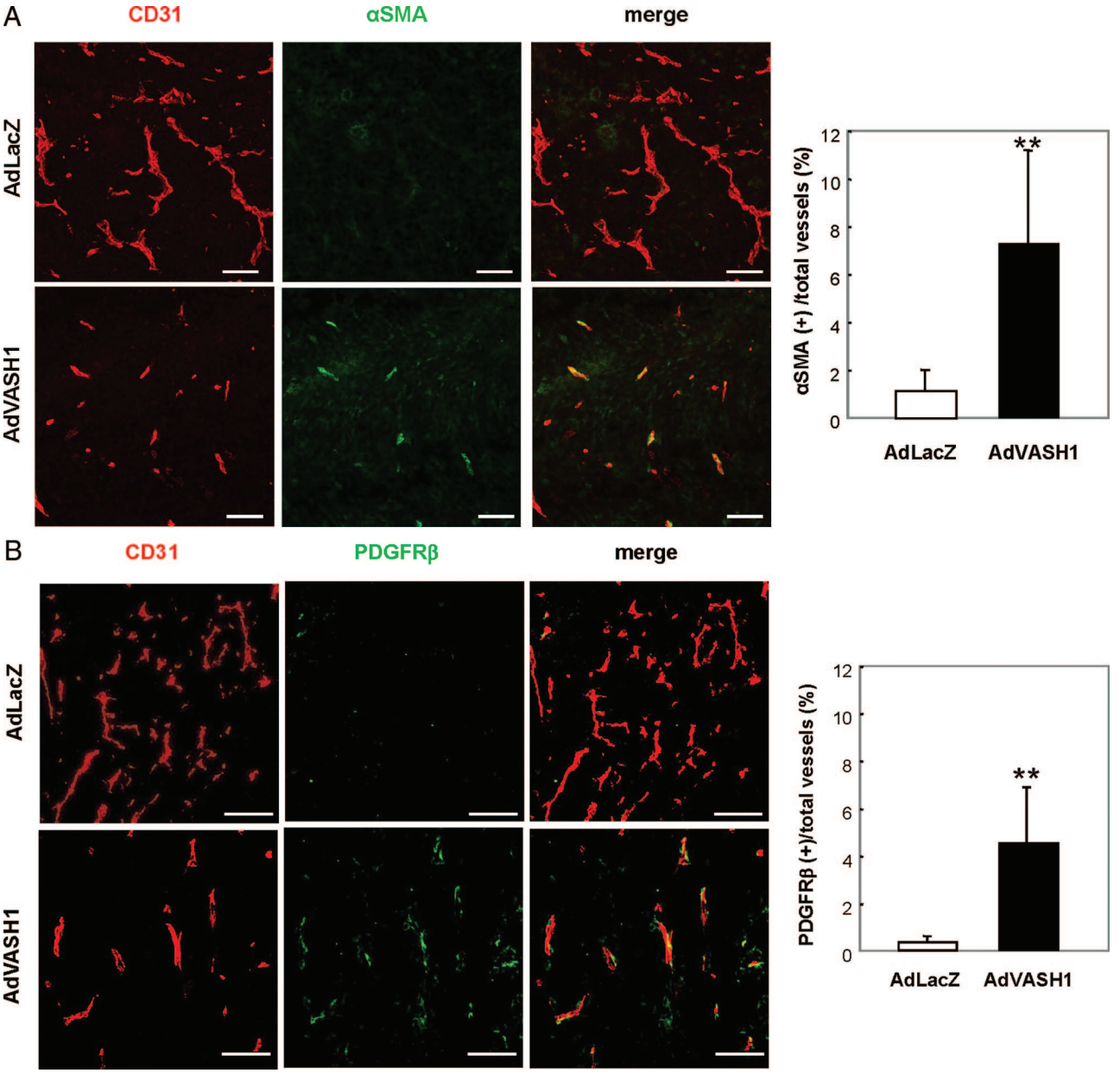

Figure 7. AdVASH1 matured tumor vessels in wild-type mice. Tumor sections were co-immunostained for (**A**) CD31 and αSMA or CD31 and (**B**) PDGFR β, an additional marker of mural cells. Scale bar = 100 μm. Tumor vessels in AdVASH1 injected mice were more frequently associated with mural cells. $^{**}P < 0.01$.

Relationship between VASH1 in ECs and VEGF or FGF-2 in Cancer Cells

Because VASH1 was inducible by two representative angiogenic factors, VEGF and FGF-2, in ECs,[18,20] we evaluated the relationships between VASH1 and VEGF or FGF-2. Because VEGF is induced in hypoxic conditions, the presence of VEGF was associated with the presence of HIF1α in cancer cells (Table 2).[26] Immunohistochemical analysis revealed that VASH1 was present in ECs in tumor blood vessels (Figure 2, A and B) when NSCLC cells were positive for VEGF and HIF-1α (Figure 2, C and D). Quantitative analysis confirmed that the presence of VASH1 in ECs was significantly higher in both VEGF positive and HIF1α positive tumors (Figure 2, E and F).

FGF-2 is another potent angiogenic factor. NSCLC cells were not always positive for VEGF. However, when cancer cells were positive for FGF-2, VASH1 was present in the ECs of tumor blood vessels (Figure 3A–E). The relationship between VEGF and FGF-2 is shown in Table 3.

Association of VASH1-Positive Tumor Vessels with Mural Cells

As we noticed that not all of the tumor blood vessels were positive for VASH1, we determined the characteristics of VASH1-positive tumor blood vessels. Immunostaining of αSMA in the mirror sections of NSCLC revealed that VASH1 was preferentially expressed in ECs when tumor blood vessels were associated with mural cells (Figure 4). Quantitative analysis further confirmed this association, independent of the sizes of the blood vessels (Figure 4).

Tumor Vessels in VASH1$^{-/-}$ Mice

The presence of endogenous VASH1 in tumor vessels promoted us to evaluate the function of this molecule in the animal model. When LLC cells were inoculated in *VASH1*$^{-/-}$ mice, tumors in *VASH1*$^{-/-}$ mice tended to grow bigger than in wild-type mice (Figure 5A). We then evaluated tumor vessels in *VASH1*$^{-/-}$ mice. The vascular area was increased and tumor vessels were more immature lacking mural cells in *VASH1*$^{-/-}$ mice (Figure 5B). Although the number of apoptotic cancer cells in wild-type mice was at a rather low level, it was further decreased in *VASH1*$^{-/-}$ mice (Figure 5C). These results indicate that endogenous VASH1 does function as an angiogenesis inhibitor in tumors.

Effect of Exogenous VASH1 on Tumor Growth and Tumor Angiogenesis

We next examined the effect of exogenous VASH1. We inoculated LLC cells into wild-type mice, and injected AdVASH1 in the tail vein 7 days later. This procedure supplies sufficient VASH1 protein to regulate angiogenesis as described previously.[23] AdLacZ was used as a negative control.[23] AdVASH1 injection significantly inhibited the growth of tumor in mice (Figure 6A). The immunohistochemical analysis revealed that the tumor vascular area was decreased and tumor cell apoptosis was augmented in the AdVASH1-treated mice (Figure 6, B and C). Moreover, the remaining tumor vessels in the AdVASH1-treated mice were small, round, and mature, associating with mural cells; whereas tumor vessels in the control AdLacZ-injected mice were dilated, erratic, and immature, containing sprouting endothelial cells with few mural cells (Figure 7, A and B).

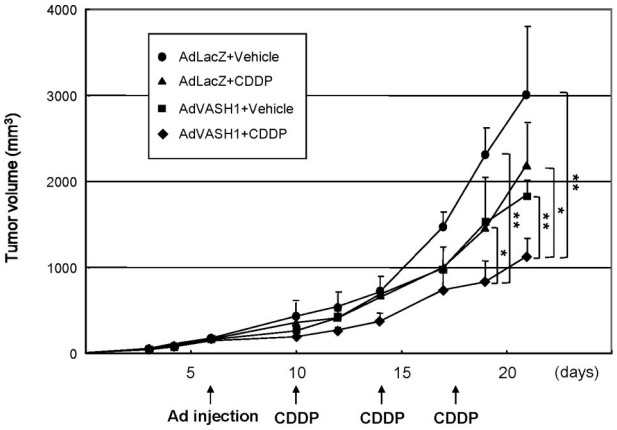

Figure 8. Anti-tumor activity of CDDP was enhanced when combined with AdVASH1. LLC cells (5×10^6) were inoculated in wild-type mice. Adenovirus vectors were injected on day seven, and CDDP (2.5 mg/kg) was injected on days 10, 14, and 18. Data are expressed as the means and SDs. AdVASH1 injection improved the anti-tumor effect of CDDP. $^*P < 0.05$, $^{**}P < 0.01$.

Because the VASH1 treatment caused the remaining tumor vessels to mature, we anticipated that those vessels would deliver anti-cancer drugs efficiently. We therefore tested the efficacy of the combination of VASH1 with CDDP. As expected, AdVASH1 injection improved the anti-tumor effect of CDDP (Figure 8).

Discussion

The search for molecular biomarkers of angiogenesis has been intensively pursued. Molecules that are specifically expressed in ECs can be candidates for such biomarkers. CD31, von Willebrand factor, and vascular endothelial-cadherin are used for histological identification of ECs.[27] However, those molecules are expressed in quiescent ECs as well, and thus cannot be specific for active angiogenesis. Endoglin and aminopeptidase N are expressed preferentially in ECs during angiogenesis.[28,29] Therefore, these two molecules are more suitable markers of angiogenesis. Additionally, several attempts have been made to isolate molecules that are expressed in cancer-specific ECs.[30–32] Nevertheless, such molecules had not been characterized in the clinical setting. In this report, we characterized the significance of VASH1, a VEGF-inducible angiogenesis inhibitor, in NSCLCs. Our data reveal that the expression of VASH1 was restricted to ECs of blood vessels in the tumor stroma, and was correlated with the expression of HIF-1α and VEGF, or FGF-2 in tumor cells. We have previously showed the presence of VASH1 in tumor vessels of human endome-

trial cancers as well.[33] We therefore propose that VASH1 should be further tested as a candidate of tumor angiogenesis biomarker.

We have previously determined the role of VASH1 in the mouse subcutaneous angiogenesis model.[22] Angiogenesis is normally synchronized and transient, as hypoxia-mediated angiogenic stimuli withdraw when blood starts to flow in the newly formed vessels. Our previous analysis in the mouse subcutaneous angiogenesis model has revealed that endogenous VASH1 is present in newly formed blood vessels behind the sprouting front where angiogenesis terminates (termination zone), and those VASH1-positive vessels are mature associated with mural cells.[22] As cancers contain complex lesions where angiogenesis is not synchronized and sprouting occurs randomly, it is difficult to dissect the spatio-temporal expression pattern of VASH1 in cancers. Here we show that VASH1 is prevalent in tumor blood vessels of NSCLC when they are associated with mural cells (Figure 4). This result suggests that the spatio-temporal expression profile of VASH1 is maintained even in tumor vessels.

Increased expression of angiogenesis stimulators, together with decreased expression of angiogenesis inhibitors, is proposed to occur in various cancers.[34] The presence of VASH1 in tumor vessels thus raises the question whether endogenous VASH1 acts as an angiogenesis inhibitor in tumors. We have previously demonstrated in the mouse subcutaneous angiogenesis model that the function of endogenous VASH1 is to terminate angiogenesis, but is not to inhibit angiogenesis in the sprouting front.[22] Here we compared tumors in wild-type and *VASH1*$^{-/-}$ mice. Tumors growth in wild-type mice was slower than in *VASH1*$^{-/-}$ mice. Histological analysis further demonstrated the distinction that tumors in *VASH1*$^{-/-}$ mice contained more immature vessels and fewer apoptotic tumor cells. This observation indicates that endogenous VASH1 does participate in the inhibition of tumor angiogenesis.

The persistence of sprouting ECs in tumor vessels in wild-type mice implies that endogenous VASH1 is ineffective in blocking sprouting angiogenesis in tumors. Importantly, when sufficient VASH1 is supplied exogenously, it can block angiogenesis in the sprouting front.[22] Here we show that sprouting ECs disappear from tumor vessels by the injection of AdVASH1. Tumor vessels in the control AdLacZ-injected mice were dilated, erratic and immature, containing fewer mural cells, whereas those in the AdVASH1-treated mice were small, round, and mature, associated with mural cells. These morphological differences imply that exogenous VASH1 has two modalities affecting tumor vessels. One is to inhibit sprouting angiogenesis, and the other is to participate in the termination of angiogenesis.

Abnormal tumor vessels bring about deficient blood flow within the tumor, which should impair the delivery of drugs to the tumor.[35] Maturation of tumor vasculature enhances the efficacy of cytotoxic anti-cancer therapies, as it increases the delivery of anti-cancer drugs to the tumor cells.[35] We therefore anticipated that exogenous VASH1 should enhance the efficacy of cytotoxic anti-cancer therapies. Indeed, the combination of AdVASH1 with CDDP did improve its efficacy.

In summary, VASH1 is selectively expressed in the ECs of tumor blood vessels. The expression is related to expression of angiogenic factors such as VEGF and FGF-2, and thus VASH1 can be further evaluated as an angiogenesis biomarker. Expression of endogenous VASH1 may participate in the inhibition of tumor angiogenesis, although it is not enough to block sprouting. However, exogenous VASH1 effectively inhibits sprouting angiogenesis, matures tumor vessels, and enhances antitumor efficacy when combined with conventional chemotherapy. We propose that VASH1 should be further tested in cancer diagnosis and therapy.

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References

1. Yano S, Matsumori Y, Ikuta K, Ogino H, Doljinsuren T, Sone S: Current status and perspective of angiogenesis and antivascular therapeutic strategy: non-small cell lung cancer. Int J Clin Oncol 2006, 11:73–81
2. Jain RK: Lessons from multidisciplinary translational trials on anti-angiogenic therapy of cancer. Nat Rev Cancer 2008, 8:309–316
3. Stinchcombe TE, Socinski MA: Bevacizumab in the treatment of non-small-cell lung cancer. Oncogene 2007, 26:3691–3698
4. Ferrara N, Kerbel RS: Angiogenesis as a therapeutic target. Nature 2005, 438:967–974
5. Bremnes RM, Camps C, Sirera R: Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. Lung Cancer 2006, 51:143–158
6. Mattern J, Koomagi R, Volm M: Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. Br J Cancer 1996, 73:931–934
7. Takanami I, Imamura T, Hashizume T, Kikuchi K, Yamamoto Y, Yamamoto T, Kodaira S: Immunohistochemical detection of basic fibroblast growth factor as a prognostic indicator in pulmonary adenocarcinoma. Jpn J Clin Oncol 1996, 26:293–297
8. Carmeliet P: VEGF as a key mediator of angiogenesis in cancer. Oncology 69 Suppl 2005, 3:4–10
9. Liao D, Johnson RS: Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev 2007, 26:281–290
10. Swinson DE, O'Byrne KJ: Interactions between hypoxia and epidermal growth factor receptor in non-small-cell lung cancer. Clin Lung Cancer 2006, 7:250–256
11. Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D: Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 1991, 66:1095–1104
12. Sato Y: Update on endogenous inhibitors of angiogenesis. Endothelium 2006, 13:147–155
13. Dameron KM, Volpert OV, Tainsky MA, Bouck N: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 1994, 265:1582–1584
14. Fontanini G, Boldrini L, Calcinai A, Chine S, Lucchi M, Mussi A, Angeletti CA, Basolo F, Bevilacqua G: Thrombospondins I and II messenger RNA expression in lung carcinoma: relationship with p53 alterations, angiogenic growth factors, and vascular density. Clin Cancer Res 1999, 5:155–161
15. Yamaguchi M, Sugio K, Ondo K, Yano T, Sugimachi K: Reduced expression of thrombospondin-1 correlates with a poor prognosis in

patients with non-small cell lung cancer. Lung Cancer 2002, 36:143–150
16. Mascaux C, Martin B, Paesmans M, Verdebout JM, Verhest A, Vermylen P, Bosschaerts T, Ninane V, Sculier JP: Expression of thrombospondin in non-small cell lung cancer. Anticancer Res 2002, 22:1273–1277
17. Dudek AZ, Mahaseth H: Circulating angiogenic cytokines in patients with advanced non-small cell lung cancer: correlation with treatment response and survival. Cancer Invest 2005, 23:193–200
18. Watanabe K, Hasegawa Y, Yamashita H, Shimizu K, Ding Y, Abe M, Ohta H, Imagawa K, Hojo K, Maki H, Sonoda H, Sato Y: Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. J Clin Invest 2004, 114:898–907
19. Shibuya T, Watanabe K, Yamashita H, Shimizu K, Miyashita H, Abe M, Moriya T, Ohta H, Sonoda H, Shimosegawa T, Tabayashi K, Sato Y: Isolation and characterization of vasohibin-2 as a homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin. Arterioscler Thromb Vasc Biol 2006, 26:1051–1057
20. Shimizu K, Watanabe K, Yamashita H, Abe M, Yoshimatsu H, Ohta H, Sonoda H, Sato Y: Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. Biochem Biophys Res Commun 2005, 327:700–706
21. Koizumi T, Abe M, Yamakuni T, Ohizumi Y, Hitotsuyanagi Y, Takeya K, Sato Y: Metronomic scheduling of a cyclic hexapeptide Ra-VII for anti-angiogenesis, tumor vessel maturation and anti-tumor activity. Cancer Sci 2006, 97:665–674
22. Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T, Sato Y: Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. Blood 2009, 113:4810-4818
23. Yamashita H, Abe M, Watanabe K, Shimizu K, Moriya T, Sato A, Satomi S, Ohta H, Sonoda H, Sato Y: Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. Biochem Biophys Res Commun 2006, 345:919–925
24. Li G, Tian L, Hou JM, Ding ZY, He QM, Feng P, Wen YJ, Xiao F, Yao B, Zhang R, Peng F, Jiang Y, Luo F, Zhao X, Zhang L, Zhou Q, Wei YQ: Improved therapeutic effectiveness by combining recombinant CXC chemokine ligand 10 with Cisplatin in solid tumors. Clin Cancer Res 2005, 11:4217–4224
25. Bardella C, Dettori D, Olivero M, Coltella N, Mazzone M, Di Renzo MF: The therapeutic potential of hepatocyte growth factor to sensitize ovarian cancer cells to cisplatin and paclitaxel in vivo. Clin Cancer Res 2007, 13:2191–2198
26. Kim SJ, Rabbani ZN, Dewhirst MW, Vujaskovic Z, Vollmer RT, Schreiber EG, Oosterwijk E, Kelley MJ: Expression of HIF-1α. CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer 2005, 49:325–335
27. McDonald DM, Foss AJ: Endothelial cells of tumor vessels: abnormal but not absent. Cancer Metastasis Rev 2000, 19:109–120
28. Bodey B, Bodey B Jr, Siegel SE, Kaiser HE: Immunocytochemical detection of endoglin is indicative of angiogenesis in malignant melanoma. Anticancer Res 1998, 18:2701–2710
29. Petrovic N, Bhagwat SV, Ratzan WJ, Ostrowski MC, Shapiro LH: CD13/APN transcription is induced by RAS/MAPK-mediated phosphorylation of Ets-2 in activated endothelial cells. J Biol Chem 2003, 278:49358–49368
30. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW: Genes expressed in human tumor endothelium. Science 2000, 289:1197–1202
31. van Beijnum JR, Dings RP, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH, Griffioen AW: Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. Blood 2006, 108:2339–2348
32. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-Cherry C, St Croix B: Genes that distinguish physiological and pathological angiogenesis. Cancer Cell 2007, 11:539–554
33. Yoshinaga K, Ito K, Moriya T, Nagase S, Takano T, Niikura H, Yaegashi N, Sato Y: Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. Cancer Sci 2008, 99:914–919
34. Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996, 86:353–364
35. Jain RK: Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005, 307:58–62