examined (Fig. 1). The average number of these positive vessels for each marker in control cases, ADC, and SCC is summarized in Table 2. CD31-positive vessels in ADC was significantly higher in inner ($P < .001$) and outer areas ($P < .001$) than extratumoral area in the tissue specimens examined, but no significant differences between inner and outer areas were found ($P = .7$). CD31-positive vessels in SCC were also higher in both inner ($P = .07$) and outer areas ($P = .02$) than in extratumoral areas and significantly higher in outer than inner areas of SCC ($P = .01$). MVD or the number of CD31-positive vessels in the inner area of the tumor was significantly higher in ADC than in SCC ($P = .002$; Fig. 2A). The average number of VASH-1–positive microvessels as a cytoplasmic staining per each field (×200) in ADC was significantly higher in both inner ($P < .001$) and outer areas ($P < .001$) than in extratumoral areas and also significantly higher in outer than inner areas in ADC ($P = .01$). The average number of VASH-1–positive microvessels per each field in SCC was significantly higher in inner ($P = .01$) and outer areas ($P = .006$) than extratumoral areas, but no significant differences between inner and outer areas were found ($P = .07$). The number of positive VASH-1 vessels in both inner and outer areas of the tumors was significantly higher in ADC than in SCC ($P = .03$ and $P = .02$, respectively; Fig. 2B). The average number of endoglin-positive vessels did not show any significant differences between ADC and SCC ($P = .72$ and $P = .68$; Fig. 2C).

The ratio of VASH-1– to CD31-positive vessels in the same areas [9] of ADC was significantly higher in both inner ($P < .001$) and outer areas ($P < .001$) than in extratumoral areas, but there was no difference between inner and outer areas ($P = .45$). In SCC, the ratio was significantly higher in inner ($P = .006$) than in extratumoral areas, but there were no significant differences between inner and outer areas ($P = .32$). The VASH-1/CD31-positive ratio in inner and outer areas did not show any significant differences between ADC and SCC ($P = .39$ and $P = .36$, respectively; Fig. 2E). There were no significant differences of endoglin/CD31-positive ratios between ADC and SCC ($P = .33$ and $P = .61$, respectively; Fig. 2F).

3.2. Correlation between the ratios of VASH-1 or endoglin/CD31-positive vessels with Ki-67 labeling in endothelial cells

Statistically significant correlation was detected between the ratio of Ki-67/CD31- and VASH-1/CD31-positive vessels in the cases examined ($P = .03$; Figs. 3A and 4A, B), whereas no significant differences were detected between the ratio of Ki-67/CD31 and endoglin/CD31 ($P = .17$; Fig. 3B). VASH-1/CD31 tended to be correlated with endoglin/CD31, but the correlation did not reach statistical significance ($P = .08$; Fig. 3C).

3.3. The status of pericytes and their coverage identified by nestin immunohistochemistry in the intratumoral vessels of NSCLC

Nestin immunoreactivity was demonstrated to be most consistent with histologically identifiable pericytes in this study (data not shown). Therefore, we used nestin as an immunohistochemical marker of pericytes in the following studies.

The mean of pericytes counted based on nestin immunoreactivity as a cytoplasmic staining (Fig. 1E, J) did not show any significant differences between inner and extratumoral areas ($P = .45$), whereas it was significantly higher in outer than in extratumoral areas ($P = .004$) of ADC cases. In SCC, the value was significantly higher in outer than in inner areas of the tumor ($P = .01$). Overall nestin immunoreactivity in ADC in inner and outer areas was significantly higher than that in SCC ($P = .004$ and $P = .02$; Fig. 2D).

3.4. CD31/nestin and VASH-1/nestin ratios evaluated by double immunohistochemistry in the vessels of NSCLC

The ratio of CD31/nestin was subsequently obtained in individual cases of NSCLC. The CD31/nestin-positive ratio in ADC was significantly higher in inner ($P = .008$) and outer areas of the tumors ($P > .001$) than in extratumoral area, but

Table 2 Mean expression of examined markers in control, ADC cases, and SCC cases

Marker	Mean expression of MVD						
	Control	ADC			SCC		
		Inner area	Outer area	Extratumoral area	Inner area	Outer area	Extratumoral area
CD31	11.12 ± 7.41	15.11 ± 1.15	16.54 ± 1.48	8.12 ± 1.33	9.63 ± 1.34	13.20 ± 1.52	7.03 ± 1.67
VASH-1	4.62 ± 4.65	7.19 ± 0.61	10.14 ± 0.94	3.05 ± 0.69	5.38 ± 0.75	7.06 ± 1.03	2.97 ± 0.72
VASH-1/CD31-positive ratio	0.31 ± 0.20	0.84 ± 0.26	1.00 ± 0.21	0.32 ± 0.16	1.02 ± 0.20	0.91 ± 0.24	0.56 ± 0.18
Nestin	3.62 ± 2.31	15.37 ± 1.28	19.73 ± 1.85	13.65 ± 1.82	9.89 ± 1.43	13.44 ± 2.06	12.58 ± 2.29
CD31/Nestin-positive ratio	0.75 ± 0.21	1.59 ± 0.31	1.39 ± 0.22	0.84 ± 0.15	1.68 ± 0.34	1.21 ± 0.24	0.65 ± 0.27
VASH-1/Nestin-positive ratio	0.25 ± 0.05	0.42 ± 0.13	0.64 ± 0.26	0.29 ± 0.05	0.86 ± 0.12	0.61 ± 0.26	0.37 ± 0.06

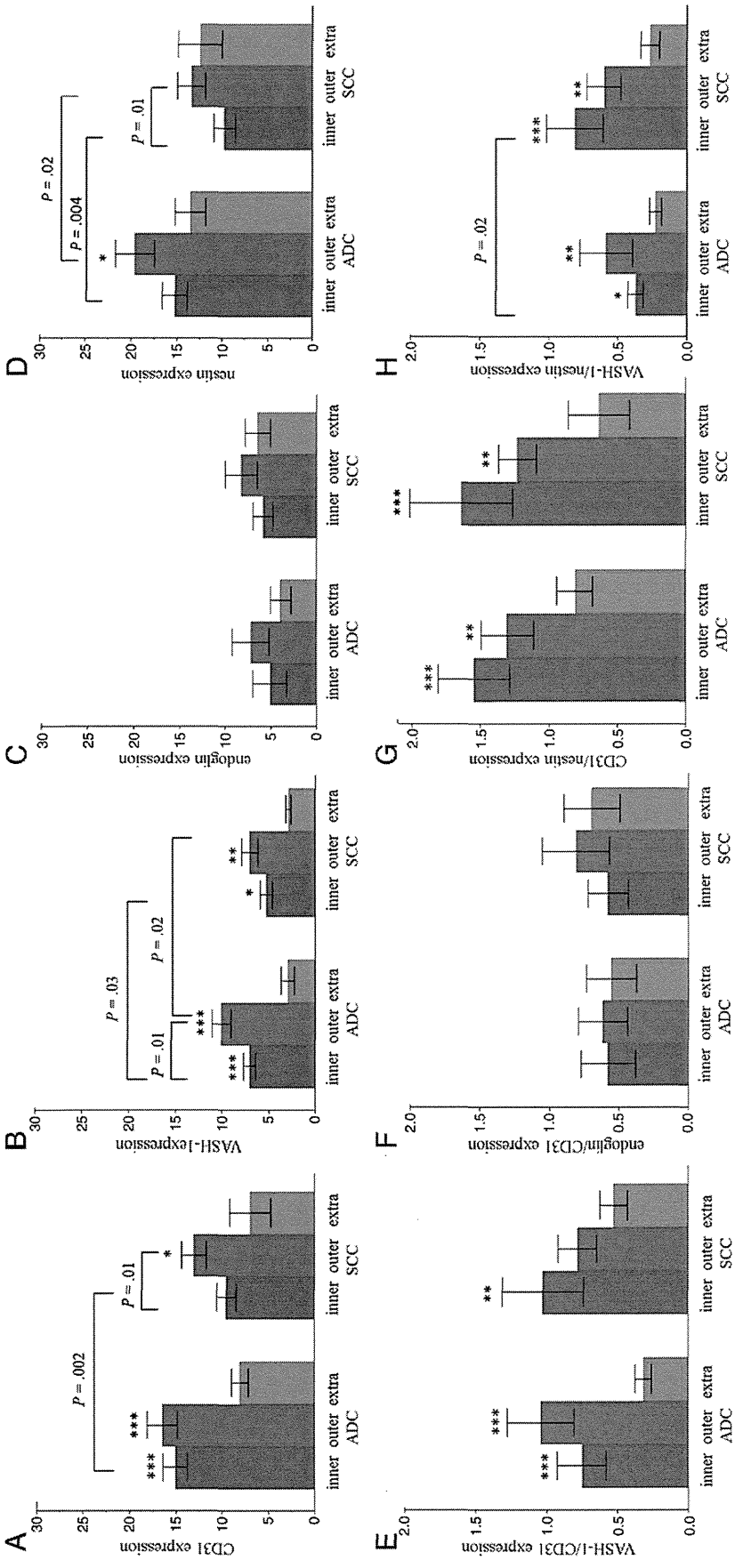

Fig. 2 Summary of the results of immunohistochemistry in ADC and SCC in inner, outer, and extratumoral areas. Mean expression of CD31 (A), VASH-1 (B), endoglin (C), nestin (D), VASH-1/CD31-positive ratio (E), endoglin/CD31 (F), CD31/nestin (G), and VASH-1/nestin (H). The significant difference of the inner and outer areas from the extratumoral area was defined by the following: $*P < .05$, $**P < .01$, and $***P < .001$. Standard error is defined for each graph. Extratumoral area is summarized as "extra" in graphs.

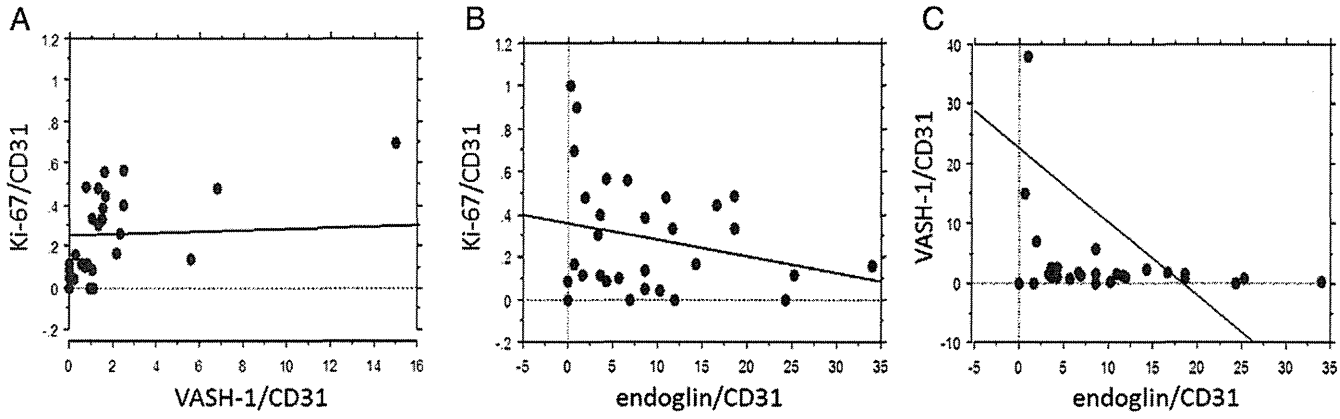

Fig. 3 The correlation between Ki-67 labeling index and VASH-1/CD31 (A) and endoglin/CD31-positive (B) ratios in 30 NSCLC cases examined in our present study (15 ADCs and 15 SCCs), which reveals the significant correlation between Ki-67/CD31 and VASH-1/CD31. C, The correlation between VASH-1/CD31- and endoglin/CD31-positive ratios.

there were no significant differences between inner and outer areas of the tumors examined ($P = .45$). In SCC, the CD31/nestin ratio was also significantly higher in inner ($P > .001$) and outer ($P = .009$) areas of the tumors than in extratumoral areas. The CD31/nestin ratio did not show any significant differences between SCC and ADC in both inner and outer areas ($P = .83$ and $P = .76$, respectively; Fig. 2G). The VASH-1/nestin-positive ratio was also significantly higher in both inner ($P = .03$) and outer ($P = .006$) areas than in extratumoral areas, but there were no significant differences between inner and outer areas in ADC cases ($P = .07$). In SCC cases, the value was also significantly higher in inner ($P > .001$) and outer ($P = .009$) areas of the tumor than in extratumoral area, but not between inner and outer areas ($P = .23$). There were significant differences in VASH-1/nestin ratios in the inner area between ADC and SCC ($P = .02$; Figs. 2H and 5).

3.5. Correlation between clinicopathologic variables and VASH-1/CD31, CD31/nestin, and VASH-1/nestin ratios in intratumoral vessels of NSCLC

The correlation between clinicopathologic variables examined and VASH-1/CD31, CD31/nestin, and VASH-1/nestin ratios in the inner area of the tumor was summarized in Table 3. We studied the correlation in inner areas of the tumor because of the difference in VASH-1/nestin ratios between ADC and SCC, and intratumoral necrosis was usually detected in the inner areas of NSCLC. The necrosis score (%) of the tumor area was significantly correlated with VASH-1/nestin ratio in ADC ($P = .01$).

The VASH-1/nestin ratio in deceased patients was significantly higher than that in living patients in ADC cases ($P = .04$), but not in SCC, whereas a multivariate

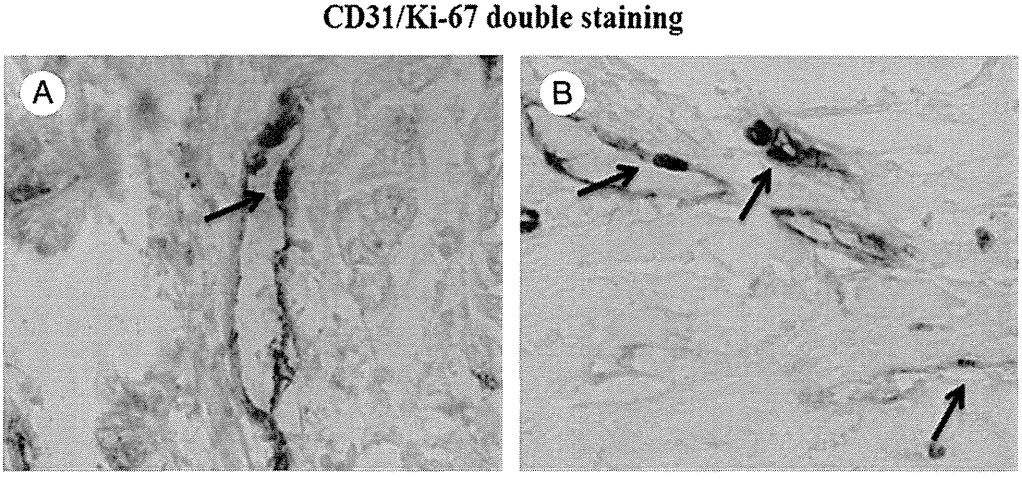

Fig. 4 Double immunostaining for determining proliferating endothelial cells in ADC (A) and SCC (B). Ki-67–positive vessels are illustrated by arrows. CD31 is represented in blue and Ki-67 in brown (×400).

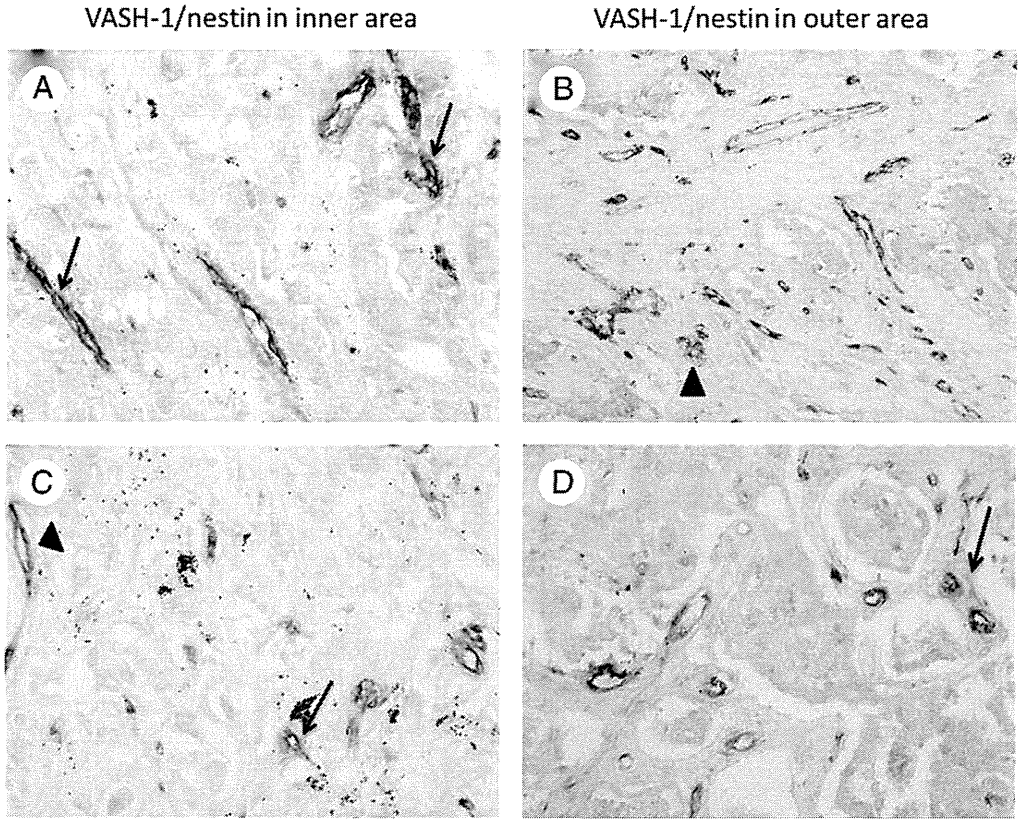

Fig. 5 Representative illustrations of double immunostaining of VASH-1/nestin (A) in the inner area and VASH-1/nestin (B) in the outer area in ADC. VASH-1/nestin (C) in the inner area and VASH-1/nestin (D) in the outer area in SCC. VASH-1 is represented in blue and nestin in brown. Double-positive vessels for VASH-1/nestin were detected both in ADC and in SCC (arrows), and the number of stained vessels only for VASH-1 is defined (arrowheads) (×200).

analysis revealed that histologic type (ADC, SCC; $P = .04$) and VASH-1/nestin ratio ($P = .42$) turned out to be independent prognostic factors. The VASH-1/nestin ratio in poorly differentiated ADC was also significantly higher than that in well- and moderately differentiated ADC cases ($P = .02$).

4. Discussion

In our present study, we evaluated the status of endothelial cells and pericytes in both ADC and SCC to understand the potential differences of the structures of intratumoral vessels between these 2 major histologic subtypes of NSCLC. VASH-1 and endoglin have been studied in some human malignancies but not in NSCLC. In addition, this is the first study to evaluate the status of nestin as an immunohistochemical marker of pericytes in human lung cancer.

The results of our present study did demonstrate significantly higher VASH-1 and CD31 immunoreactivity in both inner and outer areas of the tumor than in extratumoral areas in both ADC and SCC but not endoglin. These results demonstrated that not only high vascularity, as reported previously [16,17], but also neovascularization was more pronounced in tumor areas in the lung. MVD was reported to significantly increase from inner to outer areas in uterine cervical cancer, and a significantly positive correlation was also detected between CD34 level and lymphatic metastases in these patients, which suggested that an induction of angiogenesis could result in an increased potential of metastasis and subsequent adverse clinical outcome of the patients [22]. CD31, one of the most widely used immunohistochemical markers of endothelial cells, has been previously reported to be expressed in both proliferating and resting endothelial cells [23-25]. VASH-1 was, however, demonstrated to be expressed exclusively in proliferating endothelial cells [23]. Tamaki et al [9] proposed the use of the VASH-1/CD31-positive ratio determined by immunohistochemistry as an indicator of neovascularization in breast cancer. In our present study, the overall VASH-1/CD31-positive ratio in ADC was not significantly different from that in SCC but higher in outer than inner areas in both ADC and SCC. In NSCLC, MVD in ADC was also reported to be higher than that in SCC [8,26]. However, our results clearly demonstrated that the number of proliferating vessels was by no means higher in ADC than in SCC. Results of double immunostaining in this study further confirmed the significantly positive correlation between Ki-67–positive proliferating vascular endothelial

Table 3 Association between clinicopathologic variables and VASH-1/CD31, CD31/nestin, and VASH-1/nestin ratios in vessels of 93 NSCLCs

Clinical variables		ADC						SCC					
		VASH-1/CD31-positive ratio	*P*	CD31/nestin-positive ratio	*P*	VASH-1/nestin-positive ratio	*P*	VASH-1/CD31-positive ratio	*P*	CD31/nestin-positive ratio	*P*	VASH-1/nestin-positive ratio	*P*
Stage	I	0.78 ± 0.24		1.53 ± 0.31		0.36 ± 0.07		0.77 ± 0.41		2.13 ± 0.51		0.88 ± 0.30	
	II	0.53 ± 0.75		5.05 ± 2.09		0.38 ± 0.17		2.13 ± 0.76		0.94 ± 1.27		0.12 ± 0.49	
	III	0.96 ± 0.43		0.96 ± 0.69		0.37 ± 0.21		1.01 ± 0.59		1.10 ± 0.77		1.36 ± 0.38	
	IV	1.02 ± 0.21	.22	1.04 ± 0.23	.06	0.42 ± 0.28	.85	2.0 ± 1.87	.46	1.58 ± 0.48	.58	1.92 ± 0.21	.09
Differentiation	Well	0.48 ± 0.29		1.34 ± 0.38		0.25 ± 0.06		0.75 ± 0.42		2.08 ± 0.49		0.57 ± 0.38	
	Moderate	0.93 ± 0.26		3.08 ± 0.80		0.41 ± 0.07		1.40 ± 0.43		1.20 ± 0.70		1.05 ± 0.26	
	Poor	0.96 ± 0.50	.17	1.44 ± 0.41	.38	0.76 ± 0.16	.02*	0.57 ± 0.80	.54	0.86 ± 0.94	.31	0.12 ± 0.61	.08
pT	pT1	1.79 ± 0.27		1.77 ± 0.41		0.21 ± 0.07		0.93 ± 0.60		1.19 ± 0.84		0.86 ± 0.35	
	pT2	1.78 ± 0.34		1.77 ± 0.57		0.56 ± 0.09		1.06 ± 0.39		2.03 ± 0.52		0.5 ± 0.27	
	pT3	0.64 ± 1.32		2.00 ± 1.80		0.59 ± 0.25		1.25 ± 1.90		0.66 ± 2.23		0.92 ± 0.31	
	pT4	1.45 ± 1.32	.25	0.40 ± 1.80	.29	0.72 ± 0.24	.08	1.40 ± 0.95	.73	1.23 ± 1.11	.63	1.92 ± 0.45	.21
pN	pN0	0.73 ± 0.19		1.37 ± 0.27		0.35 ± 0.05		0.79 ± 0.38		2.07 ± 0.48		0.89 ± 0.29	
	pN1	0.53 ± 0.69		3.36 ± 0.90		0.73 ± 0.21		2.29 ± 0.63		0.82 ± 0.97		0.88 ± 0.42	
	pN2	0.99 ± 0.45		1.64 ± 0.90		0.57 ± 0.19		0.55 ± 0.68		1.33 ± 0.97		0.51 ± 0.67	
	pN3		.23		.69		.3	0.76 ± 1.80	.83	1.52 ± 0.54	.46	0.72 ± 0.21	.47
pM	pM0	0.77 ± 0.17		1.56 ± 0.26		0.38 ± 0.05		1.00 ± 0.29		1.64 ± 0.37		0.81 ± 0.20	
	pM1	0.10 ± 1.19	.2	0.95 ± 1.63	.89	0.10 ± 0.27	.32	2.00 ± 1.81	.17	1.25 ± 1.36	.41	1.23 ± 1.42	.52
Survival time	Alive	0.72 ± 0.23		1.58 ± 0.34		0.39 ± 0.22		0.48 ± 0.43		1.10 ± 0.51		0.63 ± 0.33	
	Dead	0.78 ± 0.27	.88	1.53 ± 0.44	.93	1.13 ± 0.28	.04*	1.50 ± 0.39	.09	2.36 ± 0.58	.11	1.41 ± 0.38	.13
Necrosis (%)			.90		.15		.01*		.20		.44		.1

NOTE. Statistical analysis was conducted by Fisher exact test, Wilcoxon rank sum test, and Pearson χ^2 test. The average of VASH-1/CD31, CD31/nestin, and VASH-1/nestin-positive ratio for each clinicopathologic variable is presented. *P* values less than .05 were considered significant and are indicated with an asterisk.

cells and VASH-1–positive endothelial cells, which also indicated that VASH-1 is considered a better marker of neovascularization compared with CD31 in NSCLC, as reported in breast cancer [10]. CD105 or endoglin was also proposed as a highly expressed marker of proliferative tumor vasculature [27], but no significant correlation was detected between endoglin/CD31 and Ki-67/CD31 ratios. Results of our present study were also consistent with those of Eleno et al [28], who reported in uterine paraganglioma that endoglin was exclusively detected in endothelial cells, but Ki-67 had no positive immunoreactivity in these endoglin-positive endothelial cells.

The rapid growth of the tumor is generally considered to result in the insufficient tumor blood supply compared with that required for sustaining the tumor cell proliferation [29]. Bevacizumab was reported to cause central cavity formation in the advanced patients with squamous histology, which was proposed to contribute to the development of intratumoral hemorrhage [29,30]. SCC is the most common lung cancer associated with this central cavity formation [29], but it is also true that the risks of intratumoral hemorrhage after bevacizumab treatment were by no means associated with the development of central cavity formation [29,30]. Formation of pericytes is generally considered as a pivotal step in the process of maturation in newly formed blood vessels [13]. Nestin is one of the most common immunohistochemical markers identifying pericytes [31,15] and has been also used in the analysis of pericytes in intratumoral vessels of pancreatic [32] and colorectal cancers [33]. Our results firstly indicated in lung cancer that the status of nestin immunoreactivity was significantly higher in ADC than in SCC. Results of double immunostaining further demonstrated that the vessels including those newly formed in the tumor microenvironment in ADC were more frequently covered by pericytes than those in SCC. VASH-1–positive vessels in inner areas of tumor in SCC were also demonstrated to be less covered by pericytes than in ADC. These findings all demonstrated that newly formed vessels in SCC, especially those in inner parts of the tumors, were less covered by pericytes. These differences in intratumoral vascular structures between SCC and ADC may also reflect the relative fragility of the vascular wall, which was considered to ultimately increase the risks of intratumoral hemorrhage [13]. Therefore, the relatively higher frequency of lack or absence of pericytes in intratumoral vessels of SCC, especially in newly formed ones, is considered one of the reasons for the increased incidence of intratumoral hemorrhage reported in the patients with SCC after bevacizumab treatment [4]. Results of our present study also demonstrated the significantly positive correlation between intratumoral

necrosis and VASH-1/nestin-positive ratio in ADC, which suggests that the number of newly formed vessels without the concomitant association of pericytes, increases in tumors with higher percentage of necrosis. Tilton et al [34] also reported that the inert basement membrane of the vessels remained intact, forming a tubular scaffold in which regenerating endothelial cells develop to form neovessels after necrosis. Therefore, in ADC, pericytes may also act as a tubular scaffold for neovascular formation after necrosis. In addition, the level of VASH-1/nestin-positive ratio in ADC was higher in deceased patients than in living ones. The number of cases studied was rather small in our present study, but the VASH-1/nestin ratio or the degree of maturation of newly formed intratumoral vessels may serve as a prognostic factor in ADC. However, results of multivariate analysis also demonstrated no significant correlation between VASH-1/nestin ratio and survival in ADC. In addition, pulmonary hemorrhage after the treatment of bevacizumab was mostly detected in patients with clinically advanced inoperable lung cancer [35]. In our present study, none of the patients had received bevacizumab in their clinical course, and further investigations including the correlation between the degrees of angiogenesis/maturation and therapeutic response to bevacizumab are required to establish the relevant targets of bevacizumab in surgical pathology materials of NSCLC cases.

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References

[1] Ryuge S, Sato Y, Wang GQ, et al. Prognostic significance of nestin expression in resected non–small cell lung cancer. Chest 2011;139:862-9.
[2] Yuan Y, Wang F, Liu XH, Gong DJ, Cheng HZ, Huang SD. Angiogenin is involved in lung adenocarcinoma cell proliferation and angiogenesis. Lung Cancer 2009;66:28-36.
[3] Wakelee H. Antibodies to vascular endothelial growth factor in non–small cell lung cancer. J Thorac Oncol 2008;3:113-8.
[4] Di Costanzo F, Mazzoni F, Micol Mela M, Antonuzzo L, Checcacci D, Saggese M. Bevacizumab in non–small cell lung cancer. Drugs 2008; 68:737-46.
[5] Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther 2006;28:1779-802.
[6] Manegold C. Bevacizumab for the treatment of advanced non–small-cell lung cancer. Expert Rev Anticancer Ther 2008;8:689-99.
[7] Herbst RS, Sandler A. Bevacizumab and erlotinib: a promising new approach to the treatment of advanced NSCLC. Oncologist 2008;13: 1166-76.
[8] Kojima H, Shijubo N, Abe S. Thymidine phosphorylase and vascular endothelial growth factor in patients with stage I lung adenocarcinoma. Cancer 2002;94:1083-93.
[9] Tamaki K, Moriya T, Sato Y, et al. Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. Cancer Sci 2009;100:88-94.
[10] Tamaki K, Sasano H, Maruo Y, et al. Vasohibin-1 as a potential predictor of aggressive behavior of ductal carcinoma in situ of the breast. Cancer Sci 2010;101:1051-8.
[11] de Andrade Santos PP, de Aquino AR, Oliveira Barreto A, de Almeida FR, Galvao HC, de Souza LB. Immunohistochemical expression of nuclear factor kappaB, matrix metalloproteinase 9, and endoglin (CD105) in odontogenic keratocysts, dentigerous cysts, and radicular cysts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112: 476-83.
[12] Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 2005;7:452-64.
[13] Lu C, Shahzad MM, Moreno-Smith M, et al. Targeting pericytes with a PDGF-B aptamer in human ovarian carcinoma models. Cancer Biol Ther 2010;9:176-82.
[14] Rivera LB, Brekken RA. SPARC promotes pericyte recruitment via inhibition of endoglin-dependent TGF-beta1 activity. J Cell Biol 2011; 193:1305-19.
[15] Nico B, Ennas MG, Crivellato E, et al. Desmin-positive pericytes in the chick embryo chorioallantoic membrane in response to fibroblast growth factor-2. Microvasc Res 2004;68:13-9.
[16] Giatromanolaki A, Koukourakis MI, Sivridis E, O'Byrne K, Gatter KC, Harris AL. 'Invading edge vs. inner' (edvin) patterns of vascularization: an interplay between angiogenic and vascular survival factors defines the clinical behaviour of non–small cell lung cancer. J Pathol 2000;192:140-9.
[17] Giatromanolaki A, Sivridis E, Minopoulos G, et al. Differential assessment of vascular survival ability and tumor angiogenic activity in colorectal cancer. Clin Cancer Res 2000;8:1185-91.
[18] Yatabe Y, Takahashi T, Mitsudomi T. Epidermal growth factor receptor gene amplification is acquired in association with tumor progression of EGFR-mutated lung cancer. Cancer Res 2008;68: 2106-11.
[19] Pyne J, Sapkota D, Wong JC. Aggressive basal cell carcinoma: dermatoscopy vascular features as clues to the diagnosis. Dermatology Practical and Conceptual 2012;2:3-11.
[20] Ito M, Moriya T, Ishida T, et al. Significance of pathological evaluation for lymphatic vessel invasion in invasive breast cancer. Breast Cancer 2007;14:381-7.
[21] Hu CC, Zhang C, Qian Q, Tatum NB. Reparative dentin formation in rat molars after direct pulp capping with growth factors. J Endod 1998; 24:744-51.
[22] Ancuta C, Ancuta E, Zugun-Eloae F, Carasevici E. Neoangiogenesis in cervical cancer: focus on CD34 assessment. Rom J Morphol Embryol 2010;51:289-94.
[23] Hosaka T, Kimura H, Heishi T, et al. Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. Am J Pathol 2009;175:430-9.
[24] El Gehani K, Al-Kikhia L, Mansuri N, Syrjanen K, Al-Fituri O, Elzagheid A. Angiogenesis in urinary bladder carcinoma as defined by microvessel density (MVD) after immunohistochemical staining for Factor VIII and CD31. Libyan J Med 2011:6.
[25] Morishita C, Jin E, Kikuchi M, et al. Angiogenic switching in the alveolar capillaries in primary lung adenocarcinoma and squamous cell carcinoma. J Nihon Med Sch 2007;74:344-54.
[26] Shibusa T, Shijubo N, Abe S. Tumor angiogenesis and vascular endothelial growth factor expression in stage I lung adenocarcinoma. Clin Cancer Res 1998;4:1483-7.
[27] Wood LM, Pan ZK, Guirnalda P, Tsai P, Seavey M, Paterson Y. Targeting tumor vasculature with novel *Listeria*-based vaccines directed against CD105. Cancer Immunol Immunother 2011;60: 931-42.
[28] Eleno N, Duwel A, Munoz A, Paz-Bouza J, Lopez-Novoa JM, Lozano F. Endoglin as a marker in cervical paragangliomas. Head Neck 2010; 32:737-43.

[29] Sandler AB, Schiller JH, Gray R, et al. Retrospective evaluation of the clinical and radiographic risk factors associated with severe pulmonary hemorrhage in first-line advanced, unresectable non–small-cell lung cancer treated with carboplatin and paclitaxel plus bevacizumab. J Clin Oncol 2009;27:1405-12.

[30] Marom EM, Martinez CH, Truong MT, et al. Tumor cavitation during therapy with antiangiogenesis agents in patients with lung cancer. J Thorac Oncol 2008;3:351-7.

[31] Alliot F, Rutin J, Leenen PJ, Pessac B. Pericytes and periendothelial cells of brain parenchyma vessels co-express aminopeptidase N, aminopeptidase A, and nestin. J Neurosci Res 1999; 58:367-78.

[32] Kawamoto M, Ishiwata T, Cho K, et al. Nestin expression correlates with nerve and retroperitoneal tissue invasion in pancreatic cancer. HUM PATHOL 2009;40:189-98.

[33] Teranishi N, Naito Z, Ishiwata T, et al. Identification of neovasculature using nestin in colorectal cancer. Int J Oncol 2007;30:593-603.

[34] Tilton RG, Hoffmann PL, Kilo C, Williamson JR. Pericyte degeneration and basement membrane thickening in skeletal muscle capillaries of human diabetics. Diabetes 1981;30:326-34.

[35] Crino L, Dansin E, Garrido P, et al. Safety and efficacy of first-line bevacizumab-based therapy in advanced non-squamous non–small-cell lung cancer (SAiL, MO19390): a phase 4 study. Lancet Oncol 2010;11:733-40.

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Vasohibin-1 is a new predictor of disease-free survival in operated patients with renal cell carcinoma

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ABSTRACT

Background Vasohibin-1 (VASH1) is an endothelium-produced angiogenesis inhibitor. Renal cell carcinoma is highly vascularised, but the significance of endogenous VASH1 in renal cell carcinoma has not been defined.

Aims To identify VASH1 expression and its possible relationship with various clinicopathological factors and prognosis in renal cell carcinoma.

Methods A retrospective analysis of 122 tumours obtained from 118 consecutive patients with renal cell carcinoma was performed. The expression patterns of VASH1, CD31, vascular endothelial growth factor (VEGF) and VEGF receptor type 2 (VEGFR2) were examined immunohistochemically and their relationships with clinicopathological factors were analysed.

Results Microvessel density, VASH1 and VEGFR2 expression were significantly higher in clear cell carcinoma than in other subtypes. The VEGF expression pattern differed significantly between clear cell carcinoma and other histological subtypes. VASH1, pT factor and TNM stage were significantly associated with disease-free survival ($p=0.030$, $p = 0.0012$ and $p = 0.0018$, respectively). Cox models of multivariable disease-free survival analyses indicated that VASH1 and stage are independent prognostic factors ($p=0.019$ and $p = 0.024$).

Conclusions VASH1 expression may be useful for estimating the prognosis of renal cell carcinoma. Further studies of the role of VASH1 in renal cell carcinoma involving larger sample sizes are warranted.

Renal cell carcinoma typically has high vascularity and high histological microvessel density (MVD).[1 2] Angiogenesis is intimately involved in renal cell carcinoma oncogenesis. In clear cell renal cell carcinoma, von Hippel–Lindau gene dysfunction evokes activation of hypoxia-inducible factor (HIF) leading to overexpression of vascular endothelial growth factor (VEGF), angiogenesis acceleration and tumour growth.[3–6] Angiogenesis in the tumour microenvironment is determined by a balance of various stimulatory and inhibitory factors. Angiogenetic factors such as VEGF,[5–10] basic fibroblast growth factor (bFGF),[11 12] thymidine phosphorylase[13 14] and platelet-derived growth factor[15–17] have been studied in renal cell carcinoma; however, the study of natural anti-angiogenic factors in renal cell carcinoma is limited.[18 19]

Vasohibin-1 (VASH1) is an endothelium-derived negative feedback regulator of angiogenesis[20] which regulates endothelial proliferation, migration and lymphangiogenesis.[21] VASH1 expression has been studied in breast cancer,[22 23] uterine cancer[24 25] and cancer of the upper urinary tract[26] but it has not been studied in renal cell carcinoma.

In this study we used immunohistochemistry to examine the expression of VASH1 in renal cell carcinoma and analysed possible relationships between MVD, expression of VEGF and VEGF receptor type 2 (VEGFR2), tumour histology, nuclear grade, vascular invasion, fat invasion and TNM stage. The relationships between VASH1 expression and tumour progression and survival in patients with renal cell carcinoma were also examined.

MATERIALS AND METHODS

A total of 122 tumours obtained from 118 consecutive patients with renal cell carcinoma were reviewed. The samples were obtained from surgical resections performed in the Department of Urology of Kawasaki Medical School, Kurashiki, Japan, from 1986 to 1999.

The paraffin blocks were extracted and thin sections of 5 μm were cut and placed on to Matsunami Adhesive Slide coated glass slides (Matsunami, Osaka, Japan). After deparaffinisation and hydration, hot-bath antigen retrieval was performed at 95°C for 40 min in Target Retrieval Solution, pH 9.0 (Dako, Glostrup, Denmark) for CD31, VASH1 and VEGF, or in citrate buffer, pH 6.0 for VEGFR2. The antibodies used are listed in table 1. The signal was visualised with EnVision Plus (Dako), the chromogen used was 3,3′-diaminobenzidine-tetrachloride and counterstained with haematoxylin for nuclear staining.

The MVD of the tumours was determined by CD31 immunohistochemistry. The densest area ('hot spot') of CD31 reaction was chosen by scanning power and CD31-positive vessels were then counted under a ×20 objective lens (0.785 mm^2, BH-2, Olympus, Tokyo, Japan). The presence of a visible blood vessel lumen was not required for the vessel to be defined as positive. VASH1 and VEGFR2-positive vessels were counted in the same way. The VEGF immunohistochemical pattern was classified as a 'membranous pattern' if the reaction was limited to the cell membrane without cytoplasmic staining or with cytoplasmic staining <10%; 'mixed pattern' if the cytoplasmic staining was 10–90%; and 'cytoplasmic' if the cytoplasmic staining was >90%. One of the authors (NK) evaluated the immunohistochemistry without any knowledge of the patient data.

Statistical analyses were performed using StatView V.5.0 (SAS Institute, Cary, North Carolina, USA) and SPSS Statistics V.19 (IBM, Armonk, New York, USA). The χ^2 test, Fisher exact test and Mann–Whitney test were used to identify

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Table 1 Antibody list

Antigen	Clone	Supplier	Dilution	Incubation
CD31	JC70A	Dako, Glostrup, Denmark	1 : 50	30 min at room temperature
VASH1	(raised against the synthetic fragment Gly286–Arg299 of human VASH1)[20]		2 μg/ml	30 min at room temperature
VEGF	JH121	Lab Vision Co. Fremont, California, USA	1 : 100	Overnight at 4°C
VEGFR2 (Flk-1)	sc-6251	Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA	1 : 100	30 min at room temperature

VASH1, vasohibin-1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor type 2.

significant differences in the frequencies of the clinicopathological factors and immunohistochemical results. Survival curves were drawn using the Kaplan–Meier method and the differences were assessed by the log-rank test. We also used univariate and multivariable analyses with the Cox proportional hazard regression model to analyse the predictive power of VASH1 expression and other clinicopathological factors in tumour disease-free survival. A p value <0.05 was considered significant.

RESULTS

Characteristic of patients

The patients comprised 79 men and 39 women whose ages ranged from 37 to 85 years (median 56 years). Of the total 122 tumours, 110 tumours were unilateral single tumours and 12 were bilateral and/or multiple tumours. The tumour histology was as follows: 90 clear cell carcinoma (73.8%), 17 chromophobe carcinoma (13.9%), 6 papillary carcinoma (4.9%), 4 acquired cystic disease-associated renal cell carcinoma (3.3%), 1 mucinous tubular and spindle cell carcinoma (0.8%), 1 tubulocystic carcinoma (0.8%) and 3 unclassified carcinoma (2.5%) (figure 1A,B). Fuhrman's nuclear grades were as follows: G1, 2 tumours (1.6%); G2, 34 tumours (27.9%); G3, 72 tumours (59.0%); G4, 14 tumours (11.5%).

The distribution of pT factors according to the Union for International Cancer Control 7th edition were as follows: pT1a, 44 tumours (36.1%); pT1b, 28 tumours (23.0%); pT2a, 17 tumours (13.9%); pT2b, 3 tumours (2.5%); pT3a, 25 tumours (20.5%); pT3b, 4 tumours (3.3%); pT3c, 1 tumour (0.8%). The pN factors were: pN0 or cN0/pNx, 112 tumours (91.8%); pN1, 7 tumours (5.7%); pN2, 3 tumours (2.5%). Nine cases (7.3%) had had distant metastasis at the time of operation. The stages were: stage I, 70 cases (57.4%); stage II, 17 cases (13.9%); stage III, 25 cases (20.5%); stage IV, 10 cases (8.2%).

The treatment comprised radical nephrectomy in 106 tumours (86.9%) and nephron-sparing surgery in 16 tumours (13.1%). The prognostic data were available for 89 of the 110 unilateral single tumours. The follow-up period was 20–7834 days (median 3026). Tumour recurrence occurred in 24 patients. Eighteen patients died from renal cancer and three died of other diseases.

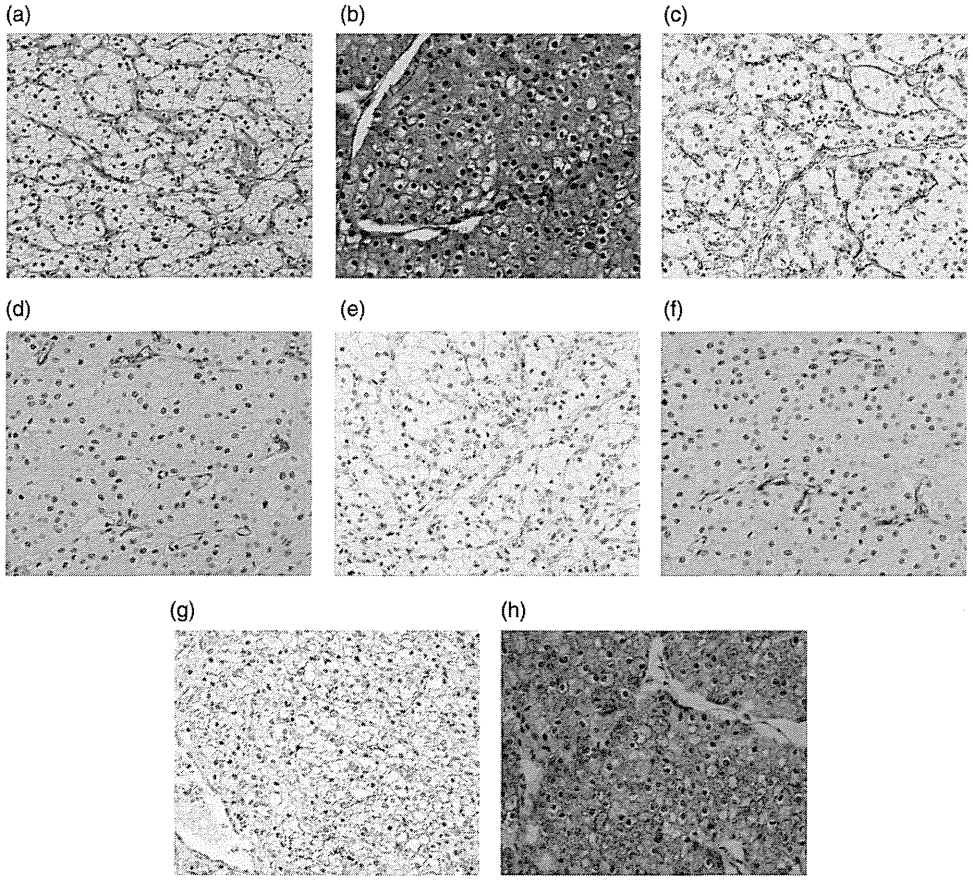

Figure 1 (A, C, E and G) Clear cell renal cell carcinoma; (B, D, F and H) chromophobe renal cell carcinoma. (A and B) H&E staining. (C and D) Immunohistochemical staining of CD31. (E and F) Immunohistochemical staining of vasohibin-1. (G and H) Immunohistochemical staining of vascular endothelial growth factor; membranous pattern (G) and cytoplasmic pattern (H).

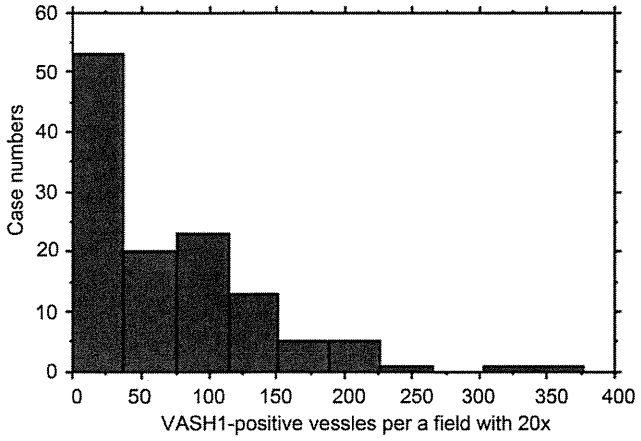

Figure 2 Vasohibin-1 (VASH1) expression of renal cell carcinoma showing a bimodal distribution.

Immunohistochemistry

The tumour MVD was 17–484 per field (median 177), corresponding to 21.7–616.6/mm^2 (median 225.5; figure 1C,D). The number of VASH1-positive vessels ranged from 0 to 378 per field (median 52), corresponding to 0–481.5/mm^2 (median 66.2; figures 1E,F and 2). The number of VEGFR2-positive vessels ranged from 0 to 116 per field (median 6), corresponding to 0–147.8/mm^2 (median 7.6). VEGF immunostaining was classified as membranous in 64 tumours, of mixed pattern in 7 tumours and cytoplasmic in 51 tumours (figure 1G,H).

The comparisons of VASH1 expression and various clinicopathological factors are shown in table 2. The number of VASH1-positive vessels showed a vague bimodal distribution (figure 2), and the cut-off value was set at 95 per field for χ^2 test and Fisher exact test analyses ($t=14.42$, $p<0.0001$). VASH1 expression was significantly associated with tumour histology ($p<0.0001$), MVD (CD31 staining; $p<0.0001$) and VEGFR2 ($p=0.0006$). By contrast, VASH1 was not associated with age ($p=0.5334$), sex ($p=0.3886$), pT factor ($p=0.3714$), pN factor ($p=0.4319$), distant metastasis ($p=0.7076$), vascular invasion (macroscopically and/or microscopically; $p=0.5081$), fat invasion (extrarenal and/or renal sinus; $p=0.0558$) or Fuhrman's nuclear grade ($p>0.999$) at the cut-off level of 95 per field for VASH1. VASH1 expression was significantly lower in tumours with fat invasion than in those without fat invasion (Mann–Whitney test, $p=0.0074$).

The MVD ($p<0.0001$) and expression levels of VASH1 ($p<0.0001$), VEGFR2 ($p<0.0001$), VASH1/CD31 ($p=0.0079$) and VEGFR2/CD31 ($p<0.0001$) were significantly higher in clear cell renal cell carcinoma than in other subtypes. The expression pattern of VEGF differed significantly between clear cell carcinoma and other subtypes ($p=0.0001$; table 3).

Among 122 tumours, overall survival could be analysed for 89 of the 110 patients with a unilateral single tumour. By Kaplan–Meier analysis, overall survival was significantly related

Table 2 Comparison of VASH1 and clinicopathological factors

	Low VASH1	High VASH1	p Value
Sex			
Men	52	21	0.3886
Women	23	14	
pT factor			
T1 or T2	62	30	0.3714
T3	23	7	
pN factor			
N0	68	34	0.4319
N1 or N2	7	1	
Distant metastasis			
M0	70	32	0.7076
M1	5	3	
TNM stage			
I or II	52	26	0.6575
III or IV	23	9	
Tumour histology			
Clear cell RCC	54	36	<0.0001*
Others	31	1	
Vascular invasion (macro and/or micro)			
Absent	61	29	0.5081
Present	24	8	
Fat invasion			
Absent	68	35	0.0558
Present	17	2	
	Median (average±SD)	Median (average±SD)	
Age	61 (60.71±11.08)	63 (62.51±10.51)	0.5334
Fuhrman's nuclear grade	3 (2.85±0.627)	3 (2.81±0.569)	>0.999
Microvessel density (CD31)	139 (163.74±94.66)	273 (268.89±65.75)	<0.0001*
VEGFR2	4 (11.41±22.49)	13 (15.86±16.55)	0.0006*

*p<0.05.
RCC, renal cell carcinoma; VASH1, vasohibin-1.

Table 3 Histology and immunohistochemistry results

	Clear cell RCC	Other subtypes	p Value
Number of tumours	90	32	
MVD (CD31)	219 (226.4±87.3)	98.5 (110.3±80.6)	<0.0001*
VASH1	76 (85.9±72.5)	15 (25.5±30.9)	<0.0001*
VEGFR2	8.5 (16.7±23.2)	0 (1.84±2.64)	<0.0001*
VEGF			
Membranous or mixed	62	9	0.0001*
Cytoplasmic	28	23	
VASH1/CD31	0.342 (0.372±0.270)	0.172 (0.235±0.210)	0.0079*
VEGFR2/CD31	0.045 (0.0710±0.0924)	0 (0.0176±0.0260)	<0.0001*

*p<0.05.
MVD, microvessel density; RCC, renal cell carcinoma; VASH1, vasohibin-1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor type 2.

to pT factor (p=0.0253), pN factor (p=0.0075), distant metastasis (p<0.0001) and TNM stage (p<0.0001). Overall survival was not significantly related to age (p=0.1597), histology (p=0.8587), Fuhrman's nuclear grading (p=0.1482), MVD (p=0.7859), expression of VASH1 (p=0.4720) or VEGFR2 (p=0.8854; figure 3).

Clinical follow-up information was available for 87 cases of curatively operated tumours not accompanied by distant metastasis at the time of the operation. Sixteen of 62 tumours (25.8%) with low VASH1 expression recurred compared with only 2 of 25 tumours (8.0%) with high VASH1 expression. VASH1 expression was significantly associated with disease-free survival (p=0.030). pT factor and TNM stage were also significant prognostic factors (p=0.0012 and p = 0.0018, respectively). pN factor, age, tumour histology, Fuhrman's nuclear grading, MVD and VEGFR2 did not have significant prognostic

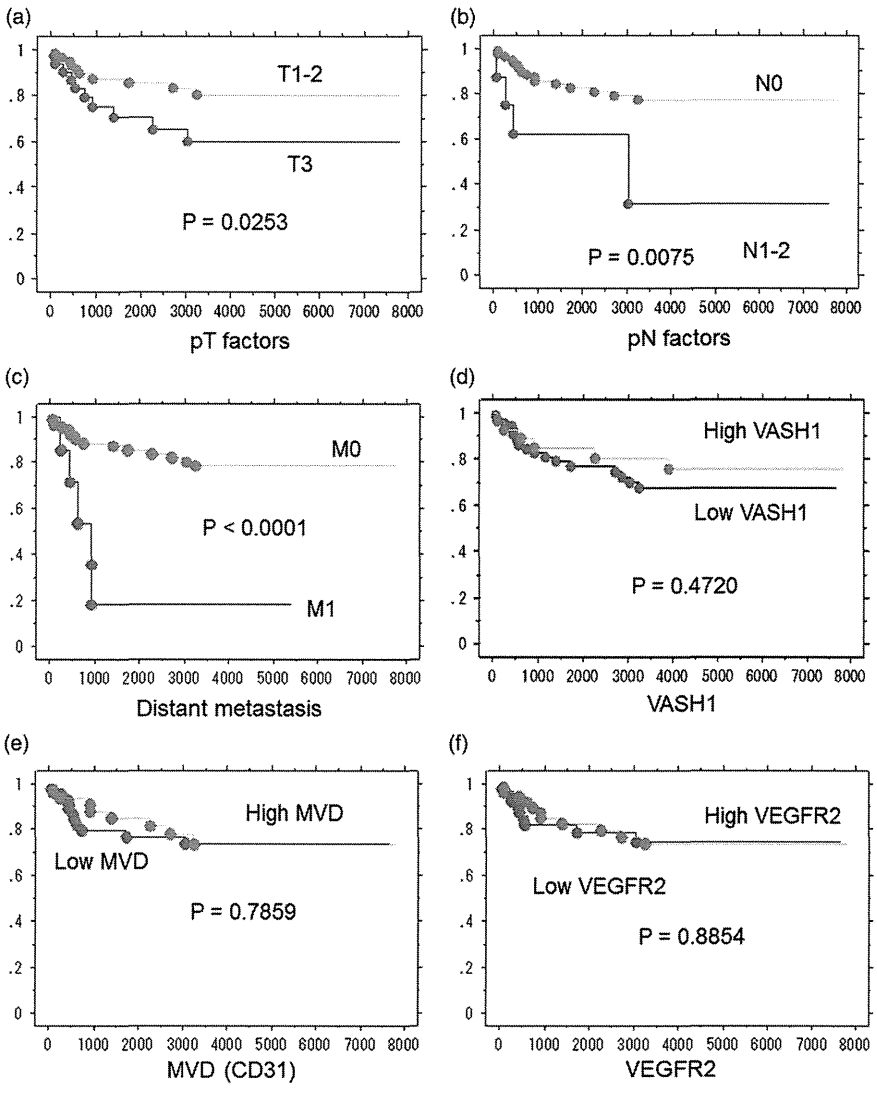

Figure 3 Kaplan–Meier analyses of overall survival according to pT factor (A), pN factor (B), distant metastasis (C), vasohibin-1 (VASH1) expression (D), microvessel density (MVD) (E) and vascular endothelial growth factor receptor type 2 (VEGFR2) expression (F).

correlation (p=0.0586, p = 0.3987, p = 0.9466, p = 0.4274, p = 0.4540 and p = 0.3280, respectively; figure 4). The Cox model of multivariable disease-free survival that included VASH1 (at the cut-off level of 95 per field), pT factor and stage indicated that VASH1 and stage were independent prognostic factors (p=0.019; HR 0.486, and p=0.024; HR 4.053; table 4).

DISCUSSION

VASH1 is an endothelium-derived negative feedback regulator of angiogenesis.[20] Two isoforms of human VASH1 are known: full-length VASH1A and its splicing variant, VASH1B. Human VASH1A protein is composed of 365 amino acid residues and human VASH1B protein is composed of 204 amino acid residues. Both forms of VASH have anti-angiogenic activity.[27]

VASH1 correlates positively with tumour grade of endometrial cancer.[24] Its expression is higher in uterine cervical cancer tissue than in normal uterine cervical tissue.[25] In breast cancers, VASH1 expression correlates with VEGF, bFGF and VEGFR2,[23] and high VASH1 expression indicates a poor prognosis for breast cancer.[23] Miyazaki *et al*[26] reported recently that VASH1 is an independent prognostic factor of upper urinary tract urothelial carcinoma. The expression of VASH1 in renal cell carcinoma has not been well studied with only one report being available for VASH1 expression in renal cell carcinoma.[28] The expression level of VASH1 in renal cell carcinoma tissue was significantly lower than that in non-tumorous renal tissues. The possible prognostic impact of VASH1 has not been investigated. The aim of this study was to elucidate VASH1 expression and to identify possible relationships between VASH1 expression and various clinicopathological factors including the prognosis for renal cell carcinoma.

We found that high VASH1 expression correlated with better disease-free survival of curatively operated patients with renal cell carcinoma whereas MVD did not correlate with disease-free survival or overall survival. The significance of MVD on the prognosis of kidney cancer is controversial. High MVD has been reported to be an indicator of better prognosis,[29–36] worse prognosis,[12 37–39] or to have no prognostic value.[40 41] It is interesting that only VASH1 expression had prognostic significance in our study, although VASH1 and MVD correlated positively. Renal cell carcinomas seem to have some different mechanisms of VASH1-related angiogenetic feedback mechanism. Renal tumours where the vessels are less covered by VASH1 seem to have aggressive behaviour and tumours with an abnormal/broken VASH1-related vascular mechanism are likely to be aggressive.

Our series included three patients who died of other diseases (pneumonia, liver cirrhosis and sepsis). This might account for the finding that VASH1 was associated with disease-free survival but was not associated with overall survival.

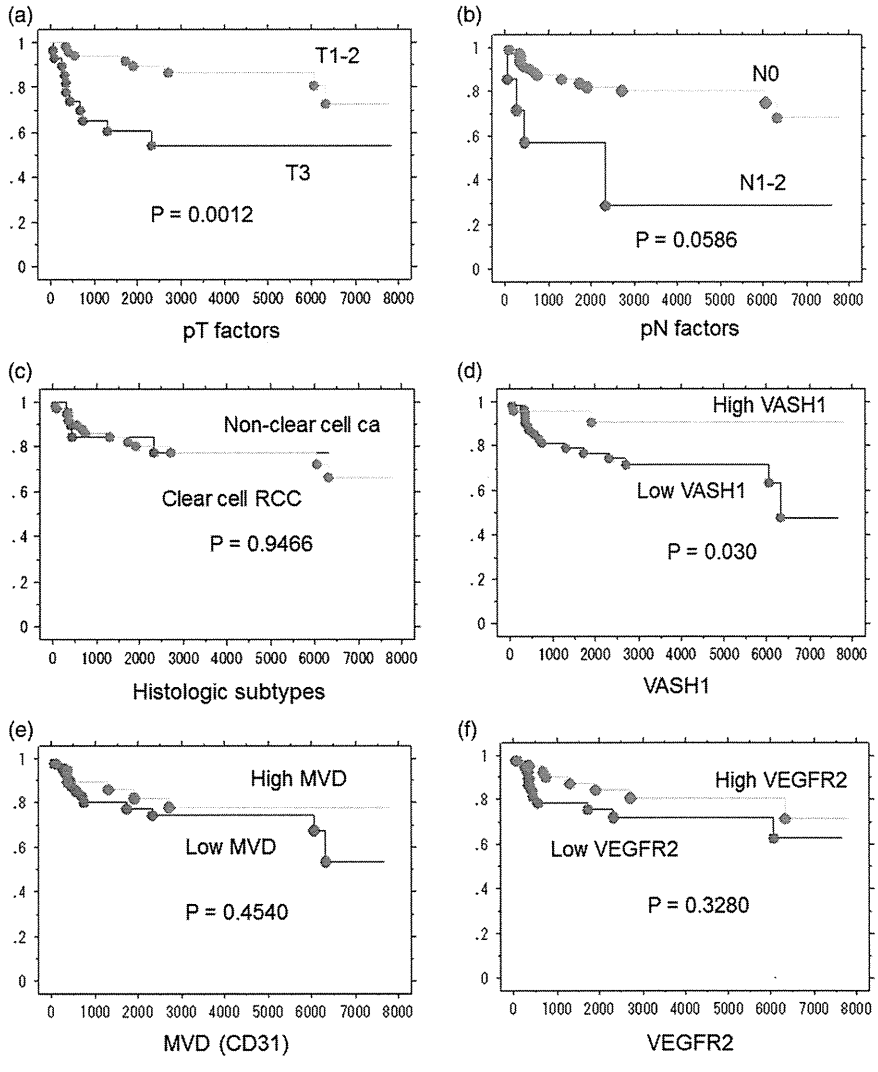

Figure 4 Kaplan–Meier analyses of disease-free survival according to pT factor (A), pN factor (B), histology (C), vasohibin-1 (VASH1) expression (D), microvessel density (MVD) (E) and vascular endothelial growth factor receptor type 2 (VEGFR2) expression (F).

Table 4 Cox proportional hazards model for cancer-specific survival

		Univariate		Multivariable	
Parameter	Criteria	p Value	HR (95% CI)	p Value	HR (95% CI)
pT	1/2 vs 3	0.0028*	4.168 (1.636 to 10.620)	0.065	3.420 (0.927 to 12.609)
pN	0 vs ½	0.0729	3.122 (0.899 to 10.839)		
pStage	1/2 vs ¾	0.0038*	3.992 (1.563 to 10.194)	0.024*	4.035 (1.197 to 13.600)
Histology	Clear cell vs others	0.9466	0.963 (0.315 to 2.940)		
Fuhrman's grade	1/2 vs ¾	0.4312	0.640 (0.210 to 1.946)		
MVD	<177 vs ≥177	0.4564	0.697 (0.269 to 1.805)		
VASH1	<95 vs ≥95	0.0461*	0.218 (0.049 to 0.974)	0.019*	0.486 (0.280 to 0.890)
VASH1	(continuum)	0.2815	0.996 (0.988 to 1.004)		
VEGFR2	<6 vs ≥6	0.3322	0.630 (0.247 to 1.605)		

* p<0.05.
MVD, microvessel density; VASH1, vasohibin-1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor type 2.

We found that the VASH1 density histogram was bimodal, although there was an overlap (figure 2). The VASH1 density histogram of clear cell carcinoma was also bimodal (data not shown). According to Brannon and coworkers, unsupervised consensus clustering of DNA microarray can divide clear cell carcinoma into two categories.[42] Clear cell carcinomas designated type A (ccA) have markedly improved disease-specific survival compared with type B (ccB) tumours. The pathway analysis of the angiogenesis gene set showed that ccA and ccB are highly dissimilar. VASH1 expression might differ between ccA and ccB.

We showed that high levels of VASH1 expression were associated with a good prognosis for renal cell carcinoma but, in breast cancer, a high level of VASH1 expression was associated with a poor prognosis.[23] Almost all the vessels in renal cell carcinoma are sandwiched by the tumour cells without any interventional tissue. However, in breast cancers the 'tumour vessels' do not always touch the tumour cells as desmoplastic stromal cells are usually present between the vessels and the breast tumour cells. Renal cell carcinoma typically shows apparent hypervascularity by angiographic study while breast cancer shows lower vascularity. These differences might be important for the VASH1-related vascular mechanism and the different impact of VASH1 on the prognosis of kidney cancer and breast cancer.

We also found that VASH1 expression was significantly lower in tumours with fat invasion than in those without fat invasion. Lower VASH1 expression by itself or an imbalance between VASH1 level and the levels of other angiogenic/anti-angiogenic factors might influence fat invasion in renal cell carcinoma. VASH1 may play an important role in the cancer–stroma interaction in the cancer microenvironment. Cancer-associated stromal cells are important for cancer invasion, especially through their production of matrix metalloproteinases.[43] VASH1 is produced in the bone marrow,[44] and bone marrow-derived myofibroblasts have been reported to contribute to the cancer-induced stromal reaction.[45] The relationship between VASH1 and cancer-associated stromal fibroblast-type cells is an attractive topic for further investigation.

We identified VASH1 expression in the endothelium of the kidney cancer blood vessels. VASH1 was higher in clear cell carcinoma than other subtypes such as MVD and VEGFR2. The VEGF expression pattern differed between clear cell carcinoma and non-clear cell carcinoma. Sandlund *et al*[32] reported that MVD is higher in clear cell renal cell carcinoma than in papillary renal cell carcinoma. These results suggest that the angiogenic mechanism differs between clear cell carcinoma and non-clear cell carcinoma. Clear cell renal cell carcinoma is known to overexpress VEGF and HIF-1 because of von Hippel-Lindau gene dysfunction,[3–6] whereas other subtypes of renal cell carcinoma do not have this abnormality. The tumour histology might be important to choose anti-angiogenic therapy.

VASH1 is naturally present in the human body and the administration of VASH1 to patients seems to be possible. VASH1 might be able to control the local invasion or distant metastasis of renal cell carcinoma. VASH1 has a different anti-angiogenic mechanism from that of sorafenib, sunitinib and bevacizumab. Combined therapy with these agents may also be promising. The development of VASH1 therapy for renal cell carcinoma through in vivo models, for example, is encouraged.

Take-home messages

- Microvessel density, vasohibin-1 (VASH1) and vascular endothelial growth factor receptor type 2 (VEGFR2) expression were significantly higher in clear cell renal carcinoma than in other subtypes.
- The pattern of VEGF expression differed significantly between clear cell renal carcinoma and other histological subtypes.
- VASH1 was significantly associated with disease-free survival in curatively operated patients with renal cell carcinoma.

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Competing interests None.

Patient consent Obtained.

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REFERENCES

1 Lang EK. Arteriographic assessment and staging of renal-cell carcinoma. Analysis of a series of 120 patients. *Radiology* 1971;101:17–27.
2 Mattern J, Volm M. Microvessel density and vascular endothelial growth factor expression in human tumors of different localization. *Oncol Rep* 1996;3:465–8.
3 Kondo K, Kaelin WG Jr. The von Hippel-Lindau tumor suppressor gene. *Exp Cell Res* 2001;264:117–25.
4 Brown LF, Berse B, Tognazzi K, *et al*. Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int* 1992;42:1457–61.
5 Takahashi A, Sasaki H, Kim SJ, *et al*. Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 1994;54:4233–7.
6 Nicol D, Hii SI, Walsh M, *et al*. Vascular endothelial growth factor expression is increased in renal cell carcinoma. *J Urol* 1997;157:1482–6.
7 Chang SG, Jeon SH, Lee SJ, *et al*. Clinical significance of urinary vascular endothelial growth factor and microvessel density in patients with renal cell carcinoma. *Urology* 2001;58:904–8.
8 Hemmerlein B, Kugler A, Ozisik R, *et al*. Vascular endothelial growth factor expression, angiogenesis, and necrosis in renal cell carcinomas. *Virchows Arch* 2001;439:645–52.
9 Minardi D, Lucarini G, Filosa A, *et al*. Prognostic role of tumor necrosis, microvessel density, vascular endothelial growth factor and hypoxia inducible factor-1alpha in patients with clear cell renal carcinoma after radical nephrectomy in a long term follow-up. *Int J Immunopathol Pharmacol* 2008;21:447–55.
10 Verheul HM, van Erp K, Homs MY, *et al*. The relationship of vascular endothelial growth factor and coagulation factor (fibrin and fibrinogen) expression in clear cell renal cell carcinoma. *Urology* 2010;75:608–14.
11 Slaton JW, Inoue K, Perrotte P, *et al*. Expression levels of genes that regulate metastasis and angiogenesis correlate with advanced pathological stage of renal cell carcinoma. *Am J Pathol* 2001;158:735–43.
12 Fukata S, Inoue K, Kamada M, *et al*. Levels of angiogenesis and expression of angiogenesis-related genes are prognostic for organ-specific metastasis of renal cell carcinoma. *Cancer* 2005;103:931–42.
13 Kawata N, Yagasaki H, Yuge H, *et al*. Histopathologic analysis of angiogenic factors in localized renal cell carcinoma: the influence of neoadjuvant treatment. *Int J Urol* 2001;8:275–81.
14 Suzuki K, Morita T, Hashimoto S, *et al*. Thymidine phosphorylase/platelet-derived endothelial cell growth factor (PD-ECGF) associated with prognosis in renal cell carcinoma. *Urol Res* 2001;29:7–12.
15 Tawfik OW, Kramer B, Shideler B, *et al*. Prognostic significance of CD44, platelet-derived growth factor receptor alpha, and cyclooxygenase 2 expression in renal cell carcinoma. *Arch Pathol Lab Med* 2007;131:261–7.
16 Xu L, Tong R, Cochran DM, *et al*. Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model. *Cancer Res* 2005;65:5711–19.
17 Sulzbacher I, Birner P, Traxler M, *et al*. Expression of platelet-derived growth factor-alpha alpha receptor is associated with tumor progression in clear cell renal cell carcinoma. *Am J Clin Pathol* 2003;120:107–12.
18 Izutsu T, Konda R, Sugimura J, *et al*. Brain-specific angiogenesis inhibitor 1 is a putative factor for inhibition of neovascular formation in renal cell carcinoma. *J Urol* 2011;185:2353–8.
19 Baltaci S, Orhan D, Gogus C, *et al*. Thrombospondin-1, vascular endothelial growth factor expression and microvessel density in renal cell carcinoma and their relationship with multifocality. *Eur Urol* 2003;44:76–81.
20 Watanabe K, Hasegawa Y, Yamashita H, *et al*. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J Clin Invest* 2004;114:898–907.
21 Sato Y, Sonoda H. The vasohibin family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arterioscler Thromb Vasc Biol* 2007;27:37–41.
22 Tamaki K, Sasano H, Maruo Y, *et al*. Vasohibin-1 as a potential predictor of aggressive behavior of ductal carcinoma in situ of the breast. *Cancer Sci* 2010;101:1051–8.
23 Tamaki K, Moriya T, Sato Y, *et al*. Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. *Cancer Sci* 2009;100:88–94.
24 Yoshinaga K, Ito K, Moriya T, *et al*. Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Sci* 2008;99:914–19.
25 Yoshinaga K, Ito K, Moriya T, *et al*. Roles of intrinsic angiogenesis inhibitor, vasohibin, in cervical carcinomas. *Cancer Sci* 2011;102:446–51.
26 Miyazaki Y, Kosaka T, Mikami S, *et al*. The prognostic significance of vasohibin-1 expression in patients with upper urinary tract urothelial carcinoma. *Clin Cancer Res* 2012;18:4145–53.
27 Sato Y. The vasohibin family: novel regulators of angiogenesis. *Vascul Pharmacol* 2012;56:262–6.
28 Zhao G, Yang Y, Tang Y, *et al*. Reduced expression of vasohibin-1 is associated with clinicopathological features in renal cell carcinoma. *Med Oncol* 2012;29:3325–34.
29 Rioux-Leclercq N, Epstein JI, Bansard JY, *et al*. Clinical significance of cell proliferation, microvessel density, and CD44 adhesion molecule expression in renal cell carcinoma. *Hum Pathol* 2001;32:1209–15.
30 Schraml P, Struckmann K, Hatz F, *et al*. VHL mutations and their correlation with tumour cell proliferation, microvessel density, and patient prognosis in clear cell renal cell carcinoma. *J Pathol* 2002;196:186–93.
31 Yildiz E, Ayan S, Goze F, *et al*. Relation of microvessel density with microvascular invasion, metastasis and prognosis in renal cell carcinoma. *BJU Int* 2008;101:758–64.
32 Sandlund J, Hedberg Y, Bergh A, *et al*. Evaluation of CD31 (PECAM-1) expression using tissue microarray in patients with renal cell carcinoma. *Tumour Biol* 2007;28:158–64.
33 Sabo E, Boltenko A, Sova Y, *et al*. Microscopic analysis and significance of vascular architectural complexity in renal cell carcinoma. *Clin Cancer Res* 2001;7:533–7.
34 Toge H, Inagaki T, Kojimoto Y, *et al*. Angiogenesis in renal cell carcinoma: the role of tumor-associated macrophages. *Int J Urol* 2009;16:801–7.
35 Mertz KD, Demichelis F, Kim R, *et al*. Automated immunofluorescence analysis defines microvessel area as a prognostic parameter in clear cell renal cell cancer. *Hum Pathol* 2007;38:1454–62.
36 Imao T, Egawa M, Takashima H, *et al*. Inverse correlation of microvessel density with metastasis and prognosis in renal cell carcinoma. *Int J Urol* 2004;11:948–53
37 Yoshino S, Kato M, Okada K. Prognostic significance of microvessel count in low stage renal cell carcinoma. *Int J Urol* 1995;2:156–60.
38 Joo HJ, Oh DK, Kim YS, *et al*. Increased expression of caveolin-1 and microvessel density correlates with metastasis and poor prognosis in clear cell renal cell carcinoma. *BJU Int* 2004;93:291–6.
39 Miyata Y, Koga S, Kanda S, *et al*. Expression of cyclooxygenase-2 in renal cell carcinoma: correlation with tumor cell proliferation, apoptosis, angiogenesis, expression of matrix metalloproteinase-2, and survival. *Clin Cancer Res* 2003;9:1741–9.
40 MacLennan GT, Bostwick DG. Microvessel density in renal cell carcinoma: lack of prognostic significance. *Urology* 1995;46:27–30.
41 Minardi D, Lucarini G, Mazzucchelli R, *et al*. Prognostic role of Fuhrman grade and vascular endothelial growth factor in pT1a clear cell carcinoma in partial nephrectomy specimens. *J Urol* 2005;174:1208–12.
42 Brannon AR, Reddy A, Seiler M, *et al*. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. *Genes Cancer* 2010;1:152–63.
43 Kanomata N, Nakahara R, Oda T, *et al*. Expression and localization of mRNAs for matrix metalloproteinases and their inhibitors in mixed bronchioloalveolar carcinomas with invasive components. *Mod Pathol* 2005;18:828–37.
44 Kimura H, Miyashita H, Suzuki Y, *et al*. Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. *Blood* 2009;113:4810–18.
45 Ishii G, Sangai T, Oda T, *et al*. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem Biophys Res Commun* 2003;309:232–40.

FULL PAPER

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The prognostic significance of vasohibin-1 expression in patients with prostate cancer

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Background: We recently isolated vasohibin-1 (VASH1), a novel angiogenic molecule that is specifically expressed in activated vascular endothelial cells (ECs), and the status of VASH1 expression has been documented in various cancer angiogenesis. The aim of this study was to assess the prognostic value of VASH1 expression in prostate cancer (PCa).

Methods: In this study, we retrospectively analysed the clinical records and evaluated the VASH1 expression of tumour microvessels in 167 patients with PCa who underwent radical prostatectomy. We immunohistochemically examined the microvessels positive for anti-CD34 as microvessel density (MVD) and the microvessels with activated ECs positive for VASH1 density.

Results: We found that the VASH1 expression was restricted to ECs in the tumour stroma. VASH1 density was significantly associated with pathological T stage, Gleason score and MVD. The 5-year PSA recurrence-free survival rate was 58.8% in patients with higher VASH1 density (≧12 per mm^2) and 89.1% in patients with lower VASH1 density (<12 per mm^2), respectively ($P<0.001$). Microvessel density was not an independent predictor of PSA recurrence. Multivariate analysis revealed that high VASH1 density was an independent prognostic indicator of PSA recurrence ($P=0.007$, HR = 2.950).

Conclusion: VASH1 density represents a clinically relevant predictor of patient prognosis and can be a new biomarker that would provide additional prognostic information in PCa.

Prostate cancer (PCa) is one of the most commonly diagnosed malignant tumours in men and the second leading cause of cancer-related deaths in the United States (Jemal *et al*, 2010). Nowadays, there are still few effective therapeutic options for advanced PCa (Scher and Sawyers, 2005; Chen *et al*, 2008). One of the most troublesome aspects of PCa is that androgen-dependent PCa inevitably progresses to highly aggressive and life-threatening castration-resistant prostate cancer after androgen ablation therapy. The key molecular events associated with PCa progression remains to be elucidated.

Angiogenesis, which is the formation of new blood vessel networks, not only plays a role in human normal development but also in pathophysiological conditions such as inflammation and cancer (Folkman, 1971; Sato, 2003). The development and establishment of blood supply has an important role in the development and growth of cancer cells by providing oxygen, nutrients and growth factors. In addition to its traditional role, angiogenesis is one of the potential pathways that contribute to solid tumour progression and metastasis by allowing cancer cells direct access to vessels. In general, angiogenesis is regulated by a balance between stimulatory and inhibitory factors (Sato and Sonoda, 2007, Sato, 2012). Molecules such as CD34, von Willebrand factor and vascular endothelial-cadherin, which are specifically expressed in vascular endothelial cells (ECs), could serve as biomarkers of angiogenesis (Weidner *et al*, 1991). One of the parameters of angiogenesis in neoplasms is microvessel density

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(MVD). In PCa, several studies indicated that MVD served as a predictor of poorly differentiated tumours and biochemical failure after treatment (Weidner *et al*, 1993; Bettencourt *et al*, 1998; Borre *et al*, 1998; Rubin *et al*, 1999; Krupski *et al*, 2000; Josefsson *et al*, 2005; Concato *et al*, 2007, 2009; Kosaka *et al*, 2007). However, to date the prognostic role of MVD in PCa has not been fully characterised. This is because those angiogenic molecules are expressed in quiescent ECs as well as in activated ECs, and thus cannot reflect 'angiogenic activity' alone.

We recently isolated vasohibin-1 (VASH1), a novel angiogenic molecule, that is specifically expressed in ECs and upregulated by vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) (Watanabe *et al*, 2004; Kimura *et al*, 2009). Previous studies found that the expression of VASH1 was restricted to ECs of blood vessels in the tumour stroma and correlated with the expression of VEGF and FGF-2 in tumour cells (Hosaka *et al*, 2009). No one has ever characterised the expression of VASH1 in relation to tumour angiogenesis in PCa. In this study, we will evaluate whether the expression of VASH1 can serve as a more accurate biomarker of tumour angiogenesis compared with MVD.

We will examine the expression of VASH1 and MVD in PCa specimens acquired by primary surgery and investigate whether VASH1 expression reflects angiogenic activity in PCa and if this is related to the clinical outcome.

MATERIALS AND METHODS

Patients selection. Samples of archival paraffin-embedded tissue sections and clinicopathological features were obtained from 167 patients with PCa diagnosed and operated on at Keio University hospital. One hundred and sixty-seven patients underwent curative surgery that included radical prostatectomy for localised PCa between January 2000 and December 2003. None of the patients had received hormonal treatment before the operation. The characteristics of these patients are shown in Table 1. After radical prostatectomy, patients were followed by serum PSA level and imaging studies. Prostate-specific antigen relapse was defined by an elevation of serum PSA level at three consecutive measurements. Histology of the specimens was evaluated by two independent pathologists using haematoxylin- and eosin-stained tissue preparations.

Tissue samples and immunohistochemistry. All the tissue samples were fixed in 10% formalin, embedded in paraffin and cut into 4-μm-thick sections. All pathological specimens were reviewed again by genitourinary pathologists to unify the reproducibility of the diagnosis. As for the pathologic stage, all neoplasms were classified according to the 2006 TNM staging system. All study participants provided their informed consent, and the study design was approved by the ethics review board of Keio University. We carried out immunohistochemical staining for VASH1 and CD34 (as markers of vascular ECs). Tissue sections were deparaffinised in xylene, and hydrated by immersion in graded alcohols and finally in distilled water. After antigen retrieval was performed, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxidase for 20 min at room temperature. The tissue sections were then incubated with a blocking solution of 5% dry milk in PBS. They were stained for 60 min at room temperature with primary antibodies, followed by staining for 30 min at room temperature with secondary antibodies. The primary antibodies were all mouse monoclonal antibodies (mAbs): anti-human VASH1 mAb diluted at 1:400 and anti-CD34 (Nichirei Biosciences, Tokyo, Japan) diluted at 1:200. We previously described a mouse mAb against a synthetic peptide corresponding to the 286–299 amino-acid sequence of VASH1 (Watanabe *et al*, 2004). The specificity of this antibody, which detects VASH1, has been documented in our previous studies (Watanabe *et al*, 2004; Yoshinaga *et al*, 2008, 2011; Hosaka *et al*, 2009; Kimura *et al*, 2009; Tamaki *et al*, 2009, 2010; Miyashita *et al*, 2012; Miyazaki *et al*, 2012). After washing with PBS, the tissue sections were incubated with secondary antibodies (Histofine Simple Stain MAX PO (M); Nichirei Biosciences). Colour was developed with 3, 3′-diaminobenzamine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.005% hydrogen peroxide. The sections were counterstained with haematoxylin. The positive control slide CD34 antigen was prepared from paraffin-fixed bladder cancer tissue with high MVD. The appropriate negative controls slides for CD34 antigen and VASH1 were prepared by substituting the primary antibody with the immune globulin fraction of nonimmune mouse serum at the same concentration in each staining run.

Table 1. Correlation of clinicopathological parameters and MVD or VASH1 expression in the 167 study patients

Characteristic	No. of patients (%)	MVD (mean ± s.d.)	*P*-value	VASH1 density (mean ± s.d.)	*P*-value
Age (years)					
<65	99 (59.3)	133.1 ± 58.3	0.201	9.3 ± 7.4	0.101
≧65	68 (40.7)	115.6 ± 41.2		10.8 ± 7.2	
PSA					
<15	138 (82.6)	120.5 ± 47.5	0.588	9.4 ± 6.8	0.139
≧15	29 (17.4)	133.7 ± 67.9		12.3 ± 8.9	
Gleason score					
≦6	69 (41.3)	105.8 ± 42.2	<0.001	7.5 ± 5.6	<0.001
≧7	98 (58.7)	134.6 ± 54.4		11.6 ± 7.9	
Pathological Tstage					
≦pT2	113 (67.7)	111.2 ± 46.3	<0.001	8.6 ± 6.5	<0.001
≧pT3	54 (32.3)	146.8 ± 54.3		12.6 ± 8.2	

Abbreviations: MVD = microvessel density; PSA = prostate-specific antigen; VASH1 = vasohibin-1.

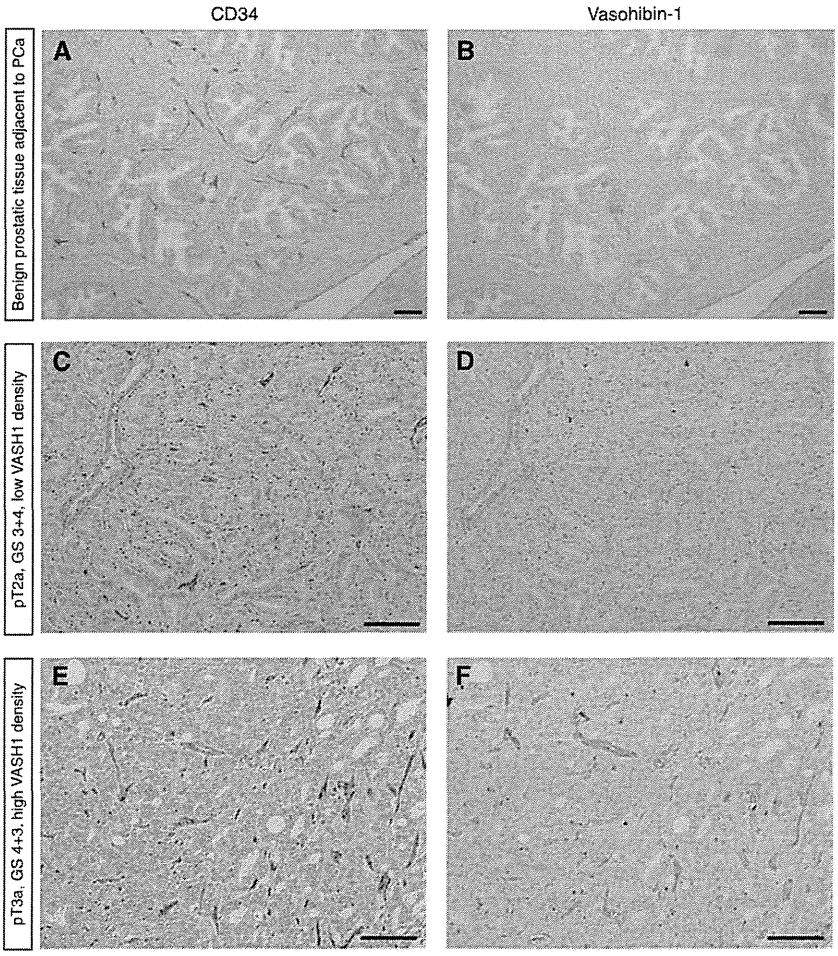

Figure 1. Immunostaining for CD34 (**A**, **C** and **E**) and VASH1 (**B**, **D** and **F**) in PCa. CD34 staining (**A**) and VASH1 staining (**B**) of vascular ECs in benign prostatic tissue adjacent to cancerous tissue. VASH1 staining of vascular ECs was negative or negligible. Low pT stage and PCa with low VASH1 density (**C** and **D**) or high pT stage with high VASH1 density (**E** and **F**). Bar = 0.1 mm.

Evaluation of immunostaining. Two authors independently evaluated immunoreactivity. They were blinded to the clinical course of the patients and the average of the numbers counted by the two investigators was used for subsequent analyses. Olympus IX71 (Olympus, Tokyo, Japan) was used for the analysis. The microvessels within the tumour were counted. Microvessels were identified based on their architecture, lumen lined by ECs and complemented by positivity of the ECs for anti-CD34 after scanning the immunostained section at low magnification (× 40 and × 100). The areas with the highest number of distinctly highlighted microvessels were selected, and these were counted at high magnification (× 200). We evaluated at least six areas in high-power fields and selected one area with the highest number of vessels. Any immunostained EC or cluster separated from adjacent vessels was counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the 'hot spot' as shown previously (Mikami *et al*, 2006; Kosaka *et al*, 2007; Shirotake *et al*, 2011; Yoshinaga *et al*, 2011). The highest number of microvessels in the hot spot was counted for MVD. Vasohibin-1-positive signals were counted in the 'hot spot' in which the highest number of vessels positive for anti-CD34 was identified. We regarded the number of VASH1-positive signals per mm^2 as 'VASH1 density' (Tamaki *et al*, 2009, 2010; Yoshinaga *et al*, 2011). The median values of MVD and VASH1 density were 119.5 and 9.9 per mm^2, respectively. We used a median MVD of ≧120 per mm^2 and a VASH1 density of ≧12 per mm^2 as the cutoff levels.

Statistical analysis. The associations between each clinicopathological parameter and VASH1 density of the tumour were analysed. These associations were validated using χ^2 test or Mann–Whitney *U*-test. Biochemical recurrence-free survival was estimated using the Kaplan–Meier method and was compared by using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model. Differences among groups were regarded as significant when $P<0.05$. These analyses were performed with the SPSS version 18.0 statistical software package (IBM corporation, New York, NY, USA).

RESULTS

Patient characteristics and VASH1 expression in PCa. Table 1 shows the clinicopathological characteristics of the patients and their association with MVD or VASH1 density in our study population. The median age of the patients was 66.6 years (range 46–75 years). Pathological T (pT) stage was ≦pT2 in 113 cases (67.7%) and ≧pT3 in 54 cases (32.3%). Gleason score (GS) was ≦6 in 69 cases (41.3%) and ≧7 in 98 cases (58.7%). During a median follow-up of 4.9 years, 48 patients (28.7%) experienced PSA recurrence. To elucidate the biological significance of VASH1 in PCa, we examined the expression of VASH1 by immunohistochemical staining (Figure 1). Although CD34 staining of vascular ECs in benign prostatic tissue adjacent to cancerous tissue was

Table 2. Univariate and multivariate analysis for PSA recurrence-free survival in 167 PCa patients

	Recurrence-free survival		
	Univariate	Multivariate	
Characteristic	*P*-value	HR (95%CI)	*P*-value
Age (years) <65 ≧65	0.525		
PSA	<0.001		
<15 ≧15			
Gleason score	0.073		
≦6 ≧7			
Pathological T stage	<0.001		<0.001
≦pT2 ≧pT3		4.667 (2.358-9.234)	
MVD	<0.001		
<120 per mm^2 ≧120 per mm^2			
VASH1 density	<0.001		0.007
<12 per mm^2 ≧12 per mm^2		2.950 (1.349–6.449)	

Abbreviations: CI = confidence interval; HR = hazard ratio; MVD = microvessel density; PCa = prostate cancer; PSA = prostate-specific antigen; VASH1 = vasohibin-1.

Table 3. Clinicopathological parameters in 167 patients according to the level of the VASH1 density

	No. of patients (%)		
Characteristic	Patients with VASH1 density <12 per mm^2	Patients with VASH1 density ≧12 per mm^2	*P*-value
No. of patients	83	84	
Age (years)			
<65 ≧65	53 (63.9) 30 (36.1)	46 (54.8) 38 (45.2)	0.271
PSA			
<15 ≧15	72 (86.7) 11 (13.3)	66 (78.6) 18 (21.4)	0.220
Gleason score			
≦6 ≧7	48 (57.8) 35 (42.2)	21 (25.0) 63 (75.0)	<0.001
Pathological T stage			
≦pT2 ≧pT3	65 (78.3) 18 (21.7)	48 (57.1) 36 (42.9)	0.005
MVD			
<120 per mm^2 ≧120 per mm^2	62 (74.7) 21 (25.3)	21 (25.0) 63 (75.0)	<0.001

Abbreviations: MVD = microvessel density; PSA = prostate-specific antigen; VASH1 = vasohibin-1.

positive as shown previously (Figure 1A), VASH1 staining of vascular ECs was negative or negligible (Figure 1B). Vasohibin-1 staining of vascular ECs was negative or negligible in low pT stage and low GS PCa (Figure 1D). On the other hand, in high pT stage PCa, strong VASH1 staining of vascular ECs was detected in many cases (Figure 1F). Strong VASH1 staining was observed in ECs of microvessels in the tumour lesion of high pT stage and high GS specimens. VASH1 staining of vascular ECs was negative or negligible in large-size vessels of the tumour detected by CD34.

The average MVD and VASH1 density (counts per mm^2) were 123 ± 51.6 and 9.9 ± 7.3 in 167 patients, respectively (Table 1). Patients with high GS tumours ($P<0.001$) or ≧pT3 ($P<0.001$) had significantly higher levels of both MVD and VASH1 density. As it has been reported that VASH1 associates with CD34, we also investigated the relationship between VASH1 and CD34 expression. Using Spearman's correlation coefficient test, we detected a significant positive correlation between MVD and VASH1 density in microvessels in the tumour ($\rho = 0.504$, $P<0.001$).

Prognostic significance of VASH1 expression in PCa patients. We performed univariate and multivariate analysis to determine the indicators for subsequent PSA recurrence following surgery (Table 2). Univariate analysis revealed that high PSA concentration ($P<0.001$), high pT stage ($P<0.001$), high MVD ($P<0.001$) and high VASH1 density (≧12 per mm^2) ($P<0.001$) were significant predictors of tumour recurrence. Multivariate analysis showed that high pT stage ($P<0.001$, HR = 4.667) and high VASH1 density ($P = 0.007$, HR = 2.950) were also independent predictors of PSA recurrence. A high level of MVD was not an independent predictor of PSA recurrence. Table 3 shows the association between the level of VASH1 density and clinic–pathological characteristics in 167 patients. High VASH1 density was significantly associated with GS ($P<0.001$), pT stage ($P = 0.005$) and MVD ($P<0.001$). The 5-year Kaplan–Meier PSA recurrence-free survival rate was 58.8% in patients with high VASH1 density compared with 89.1% ($P<0.001$) in their counterparts (Figure 2A).

Risk stratification for PCa according to pT stage and VASH1 density. We distributed the patients into three different groups according to pT stage and VASH1 density, which were the two statistically significant variables found by the multivariate Cox regression analysis (Figure 2C). The relative risk of death was calculated with the formula, exp (1.540 × pT stage + 1.082 × VASH1 density) for PSA recurrence-free survival. In this equation, the pT stage equaled 1 if the pT stage was pT3 or more, and it equaled 0 if the pT stage was pT2 or less. VASH1 density equaled 1 if VASH1 density was ≧12 per mm^2 and 0 if <12 per mm^2. On the basis of the relative risk of death, patients with PCa were divided into three risk groups: low (relative risk of PSA recurrence = 1), intermediate (8.02–12.7 for PSA recurrence-free survival) and high (13.8 for PSA recurrence-free survival). According to the risk stratification for PCa based on prognostic factors, 65 patients (38.9%) were in the low-risk group (low pT stage and low VASH1 density), 66 patients (39.5%) were in the high-risk group (high pT stage and high VASH1 density) and 36 patients (21.6%) were in the intermediate-risk group (all others). The 5-year PSA recurrence-free survival was 98.1% in the low-risk group, 69.7% in the intermediate-risk group and 33.7% in the high-risk group, respectively. The differences among the groups were significant ($P<0.001$ in PSA recurrence-free survival and for low- *vs* intermediate-risk group, $P<0.001$ for low- *vs* high-risk group

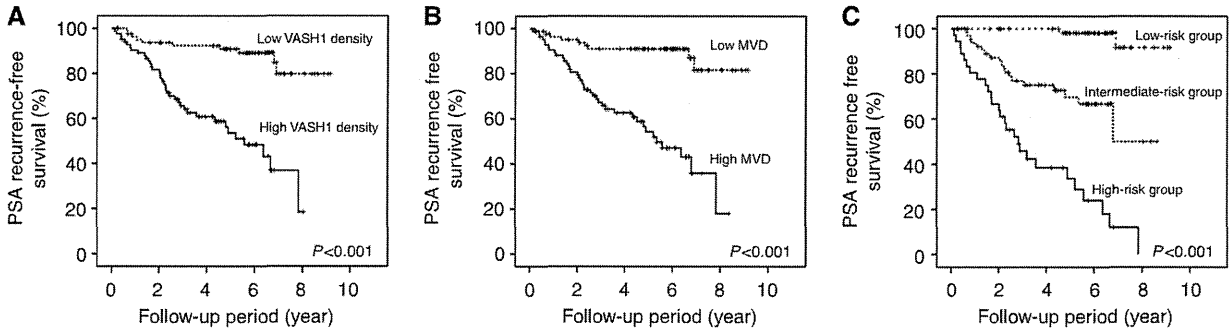

Figure 2. Kaplan–Meier curves of PSA recurrence-free survival of the patients after surgery for PCa according to VASH1 density (**A**) or MVD (**B**). Kaplan–Meier curves of PSA recurrence-free survival (**C**) of the patients after surgery for PCa according to pT stage and VASH1 density stratified according to three risk groups. Low-risk group consisted of patients with ≤pT2 and low VASH1 density. High-risk group consisted of those with ≥pT3 and high VASH1 density. All others were included in the intermediate-risk group.

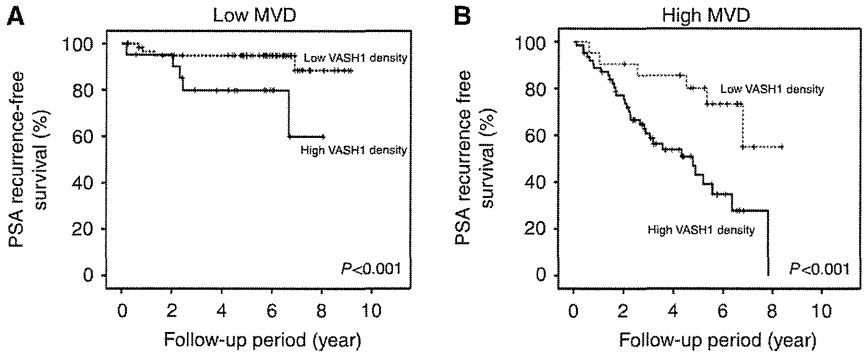

Figure 3. Kaplan–Meier curves of PSA recurrence-free survival of the patients among low (**A**) or high (**B**) MVD groups according to VASH1 density. The patients with high MVD and high VASH1 density had a significantly poorer prognosis than the counterpart group ($P<0.001$).

and $P<0.001$ for intermediate- *vs* high-risk group in PSA recurrence-free survival).

Microvessel density could be divided into two subclasses for PSA recurrence according to VASH1 density in PCa patients. Univariate analysis revealed that high MVD was one of the significant predictors of PSA recurrence (Figure 2B), while multivariate analysis showed that high MVD was not an independent predictor of PSA recurrence. Then, we re-evaluated univariate analysis of MVD according to high or low VASH1 density (Figure 3). In the low MVD group, 21 patients (25.3%) had high VASH1 density and 62 patients (74.7%) had low VASH1 density (Figure 3A). The 5-year PSA recurrence-free survival rate in the low MVD group was 79.9% for those with high VASH1 density and 94.9% for those with low VASH1 density. The difference between the groups was statistically significant ($P<0.001$). In the high MVD group, 63 patients (75.0%) had high VASH1 density and 21 patients (25.0%) had low VASH1 density (Figure 3B). The 5-year PSA recurrence-free survival rate in the high MVD group was 43.1% for those with high VASH1 density and 80.1% for those with low VASH1 density. The differences were significant ($P<0.001$). These results indicated that MVD in PCa could be subdivided into two subclasses for PSA recurrence according to VASH1 density.

DISCUSSION

In this study, we retrospectively evaluated the impact of VASH1 expression by immunohistochemical staining in a series of patients with PCa treated in a single centre. Our results suggested that VASH1 expression was a prognostic indicator in addition to other standard factors such as pT stage. High VASH1 density was related to shorter patient PSA recurrence-free survival. To the best of our knowledge, this is the first study evaluating the prognostic value of VASH1 expression in patients with prostate cancer.

Angiogenesis has a critical role in tumour growth and metastasis (Folkman, 1971; Chung *et al*, 2010). Recent studies revealed significant roles for angiogenesis in the prediction of survival in patients with different malignancies (Sato, 2003, 2012; Chung *et al*, 2010). One of the biomarkers that could reflect angiogenic aggressiveness was MVD (Weidner *et al*, 1991; Bochner *et al*, 1995; Kosaka *et al*, 2007; Shirotake *et al*, 2011). Several studies on PCa indicated that the status of MVD was associated with clinical features, such as GS and pathological stage, and could be an independent prognostic factor of patient survival (Weidner *et al*, 1993; Bettencourt *et al*, 1998; Borre *et al*, 1998; Rubin *et al*, 1999; Krupski *et al*, 2000; Josefsson *et al*, 2005; Concato *et al*, 2007, 2009; Kosaka *et al*, 2007). However, to date evidence of the prognostic role of MVD in PCa is contradictory, suggesting that the prognostic impact of MVD might be controversial. In this study, univariate analysis reveled that there was significant association between MVD and PSA recurrence. However, multivariate analysis including VASH1 density showed that MVD did not have a prognostic significance in PCa progression. One of the reasons might be because MVD corresponds to the number of accomplished vessels and includes vessels without the potential of neovascularisation in PCa. Our previous reports showed that VASH1 has been isolated from VEGF-inducible genes in ECs present in newly formed blood vessels behind the sprouting front where angiogenesis terminates (Watanabe *et al*, 2004; Sato and Sonoda, 2007; Hosaka *et al*, 2009). Recently, we have reported that histologic evidence of VASH1 expression has been found in

samples from patients with endometrial carcinoma (Yoshinaga *et al*, 2008), breast cancer (Tamaki *et al*, 2009, 2010) and cervical carcinoma (Yoshinaga *et al*, 2011). Our reports indicated that VASH1 expression was associated with tumour grade and histological type of carcinomas. Moreover, it was reported that VASH1 expression tended to be concordant with MVD, although partial dissociation was observed in some patients with breast carcinoma. VASH1 expression was significantly higher in invasive breast carcinoma, although no significant difference was observed in the level of MVD between patients with invasive disease or not (Tamaki *et al*, 2010). These studies suggested that an evaluation of the number of VASH1-positive vessels may become one of the prognostic biomarkers for metastasis and prognosis. These results indicate that VASH1 could become a new molecular biomarker of the angiogenic heterogeneity of tumours. In this study, we demonstrated that the prognostic value of MVD depends on the level of VASH1 density.

Our study demonstrated that PCa with a higher number of VASH1-positive vessels tended to have a poor prognosis. We found a significant correlation among VASH1 density, GS and pT stage. Multivariate analysis showed that high VASH1 density was an independent prognostic factor, suggesting that the status of VASH1 density could serve as a biomarker of the malignant potential of tumour angiogenesis. These results suggest that the level of VASH1 expression may influence the clinical course of prostate cancer progression. Moreover, VASH1 may become a molecular target in PCa patients.

Using VASH1 density and other independent indicators, we established a prognostic risk stratification for PCa. Patients were stratified into three groups according to statistical modelling based on the relative risk associated with the prognostic indicators derived from multivariate analysis. As shown in Figure 2C among patients with PCa, the prognosis of patients with higher pT stage and high VASH1 density was worse than that of the other groups ($P<0.001$). This stratification made it possible to predict PSA recurrence more accurately, suggesting more appropriate follow-up including PSA follow-up interval.

In conclusion, these results indicated that VASH1 can serve as a new biomarker for predicting PCa progression and could become a molecular target in PCa, especially for targeting tumour angiogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Bettencourt MC, Bauer JJ, Sesterhenn IA, Connelly RR, Moul JW (1998) CD34 immunohistochemical assessment of angiogenesis as a prognostic marker for prostate cancer recurrence after radical prostatectomy. *J Urol* **160**: 459–465.

Bochner BH, Cote RJ, Weidner N, Groshen S, Chen SC, Skinner DG, Nichols PW (1995) Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. *J Natl Cancer Inst* **87**: 1603–1612.

Borre M, Offersen BV, Nerstrom B, Overgaard J (1998) Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting. *Br J Cancer* **78**: 940–944.

Chen Y, Sawyers CL, Scher HI (2008) Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol* **8**: 440–448.

Chung AS, Lee J, Ferrara N (2010) Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer* **10**: 505–514.

Concato J, Jain D, Li WW, Risch HA, Uchio EM, Wells CK (2007) Molecular markers and mortality in prostate cancer. *BJU Int* **100**: 1259–1263.

Concato J, Jain D, Uchio E, Risch H, Li WW, Wells CK (2009) Molecular markers and death from prostate cancer. *Ann Intern Med* **150**: 595–603.

Folkman J (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**: 1182–1186.

Hosaka T, Kimura H, Heishi T, Suzuki Y, Miyashita H, Ohta H, Sonoda H, Moriya T, Suzuki S, Kondo T, Sato Y (2009) Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. *Am J Pathol* **175**: 430–439.

Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. *CA Cancer J Clin* **60**: 277–300.

Josefsson A, Wikstrom P, Granfors T, Egevad L, Karlberg L, Stattin P, Bergh A (2005) Tumor size, vascular density and proliferation as prognostic markers in GS 6 and GS 7 prostate tumors in patients with long follow-up and non-curative treatment. *Eur Urol* **48**: 577–583.

Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T, Sato Y (2009) Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. *Blood* **113**: 4810–4818.

Kosaka T, Miyajima A, Takayama E, Kikuchi E, Nakashima J, Ohigashi T, Asano T, Sakamoto M, Okita H, Murai M, Hayakawa M (2007) Angiotensin II type 1 receptor antagonist as an angiogenic inhibitor in prostate cancer. *Prostate* **67**: 41–49.

Krupski T, Petroni GR, Frierson Jr. HF, Theodorescu JU (2000) Microvessel density, p53, retinoblastoma, and chromogranin A immunohistochemistry as predictors of disease-specific survival following radical prostatectomy for carcinoma of the prostate. *Urology* **55**: 743–749.

Mikami S, Oya M, Mizuno R, Murai M, Mukai M, Okada Y (2006) Expression of Ets-1 in human clear cell renal cell carcinomas: implications for angiogenesis. *Cancer Sci* **97**: 875–882.

Miyashita H, Watanabe T, Hayashi H, Suzuki Y, Nakamura T, Ito S, Ono M, Hoshikawa Y, Okada Y, Kondo T, Sato Y (2012) Angiogenesis inhibitor vasohibin-1 enhances stress resistance of endothelial cells via induction of SOD2 and SIRT1. *PLoS One* **7**: e46459.

Miyazaki Y, Kosaka T, Mikami S, Kikuchi E, Tanaka N, Maeda T, Ishida M, Miyajima A, Nakagawa K, Okada Y, Sato Y, Oya M (2012) The prognostic significance of vasohibin-1 expression in patients with upper urinary tract urothelial carcinoma. *Clin Cancer Res* **18**: 4145–4153.

Rubin MA, Buyyounouski M, Bagiella E, Sharir S, Neugut A, Benson M, de la Taille A, Katz AE, Olsson CA, Ennis RD (1999) Microvessel density in prostate cancer: lack of correlation with tumor grade, pathologic stage, and clinical outcome. *Urology* **53**: 542–547.

Sato Y (2003) Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. *Int J Clin Oncol* **8**: 200–206.

Sato Y, Sonoda H (2007) The vasohibin family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arterioscler Thromb Vasc Biol* **27**: 37–41.

Sato Y (2012) The vasohibin family: Novel regulators of angiogenesis. *Vascul Pharmacol* **56**: 262–266.

Scher HI, Sawyers CL (2005) Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol* **23**: 8253–8261.

Shirotake S, Miyajima A, Kosaka T, Tanaka N, Maeda T, Kikuchi E, Oya M (2011) Angiotensin II type 1 receptor expression and microvessel density in human bladder cancer. *Urology* **77**: 1009, e19–e25.

Tamaki K, Moriya T, Sato Y, Ishida T, Maruo Y, Yoshinaga K, Ohuchi N, Sasano H (2009) Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. *Cancer Sci* **100**: 88–94.