

Increased vasohibin-1 expression is associated with metastasis and poor prognosis of renal cell carcinoma patients

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The microvascular density detected by markers of endothelial cells (ECs), such as CD31 and CD34, is considered to be a biomarker for angiogenesis, and it is generally associated with the malignant potential of solid tumors. However, there is a conflicting relationship between the microvascular density and prognosis in clear-cell renal cell carcinoma (ccRCC) patients. It may be explained by the suggestion that the microvascular density cannot fully reflect the angiogenic activity in ccRCC, as the markers of ECs are expressed by both quiescent and activated ECs. To investigate the real angiogenic activity, we examined vasohibin-1 (VASH1), a recently identified regulator of angiogenesis, which was demonstrated to be specifically expressed by ECs of newly formed blood vessels. Expression of VASH1 and CD34 were immunohistochemically examined in 116 primary untreated ccRCCs, 10 metastatic untreated ccRCCs, and 9 metastatic ccRCCs treated with sunitinib. ECs in the tumor microvessels were sporadically immunostained for VASH1, although no VASH1 staining was observed in the non-neoplastic renal tissues. CD34 was ubiquitously expressed by all ECs in both ccRCC and non-neoplastic renal tissues. Multivariate Cox analysis indicated that an elevated VASH1 density, but not microvascular density, was a significant and independent predictor of overall survival (odds ratio, 7.71; $P=0.003$). The microvascular density was significantly decreased in the sunitinib-treated metastases compared with untreated tumors ($P=0.001$). On the other hand, the VASH1 density was significantly higher in the metastatic ccRCCs treated with sunitinib compared with non-treated ones ($P=0.010$), indicating that VASH1 may be associated with the resistance of ECs to sunitinib treatment. Thus, VASH1 expression may reflect the actual activity of angiogenesis, and VASH1 can serve as a new prognostic and predictive biomarker in patients with ccRCC.

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Renal cell carcinoma (RCC) is the most common malignancy of the kidney,¹ and ~13% of patients who undergo curative surgery develop metastasis during follow-up.² As conventional therapies such as chemotherapy and radiation therapy are not effective in patients with clear-cell RCC (ccRCC), and only 10 to 20% of the patients benefit from immunotherapy,^{3–5} recent studies have focused on the development of angiogenesis inhibitor agents. Inhibitors of multitargeted tyrosine kinases in the receptors including vascular endothelial growth factor (VEGF) and platelet-derived growth factor, such as sunitinib and sorafenib, are now widely used as standard molecular target therapy for patients with advanced ccRCC.⁶

The formation of new blood vessel networks, that is, angiogenesis, has an important role in various pathological conditions, such as inflammatory diseases and neoplasms.^{7,8} Angiogenesis is regulated by the balance between angiogenic stimulators and inhibitors, and the microvascular density is evaluated by the expression of CD31, CD34, and von Willebrand factor, all of which are ubiquitously expressed by vascular endothelial cells (ECs).⁹ The microvascular density is generally associated with the malignant potential of many tumors, such as cancers of the esophagus, colon, breast, and lung.^{10,11} However, the relationship between the tumor vascularity and prognosis is controversial in ccRCC patients.¹² Previous studies have shown that a lower

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microvascular density correlates with longer patient survival,^{13,14} but another group reported the opposite finding.¹² In these studies, the microvascular density was determined by immunohistochemistry for CD31 or CD34.^{12–14} As these biomarkers are expressed by both quiescent and activated ECs, the immunohistochemical data may not reflect the real activity of tumor angiogenesis.

Vasohibin-1 (VASH1) was recently identified as one of the VEGF-induced genes in ECs using microarray analysis.^{8,15,16} Because of its antiangiogenic activity, VASH1 was originally thought to be a negative feedback regulator of angiogenesis.¹⁷ However, immunohistochemical analysis showed the preferential expression of VASH1 in ECs at sites of angiogenesis.¹⁶ Previous studies also revealed that VASH1 expression is restricted to ECs of blood vessels in the tumor stroma of endometrial adenocarcinoma, prostatic adenocarcinoma, RCC, and urothelial carcinoma of the upper urinary tract, but not in non-neoplastic tissues.^{18–21} The expression levels of VASH1 were reported to correlate with the microvascular density in neoplastic tissue and the malignant potential of breast, endometrial, upper urinary tract, and prostate cancers.^{20–23} In addition, recent studies provided evidence that VASH1 protects ECs from premature senescence and cell death when they are exposed to oxidative or serum starvation stress.²⁴ From these data, we hypothesized that VASH1 is expressed by ECs within tumor tissue vessels in ccRCCs, as proposed by experimental studies.^{16,17,24}

In the present study, we evaluated the number of VASH1-positive ECs as the VASH1 density, and showed that the VASH1 density is associated with distant metastasis and poor survival. The microvascular density, calculated by immunohistochemistry for CD34, was decreased in metastatic ccRCCs treated with sunitinib compared with untreated tumors, whereas the VASH1 density was higher in sunitinib-treated metastatic ccRCCs than in untreated ones. Our data suggest that VASH1 is a good marker of neovascularization and may be involved in the resistance of ECs to sunitinib treatment.

MATERIALS AND METHODS

Patients

Total or partial nephrectomy specimens were obtained from 116 patients, who were clinically and pathologically diagnosed with ccRCCs from 1989 to 2013 at the Keio University Hospital (Tokyo, Japan), and were used in the present study. Hematoxylin and eosin (HE)-stained ccRCC samples were reviewed by two certified pathologists. The International Union Against Cancer tumor node metastases system was used for tumor staging,²⁵ and nuclear grading was performed according to the WHO/International Society of Urological Pathology grading system.¹ Their clinicopathological parameters at the time of nephrectomy are summarized in Table 1. Statistical analysis of the tumors was carried out by dividing them into the following groups: groups of a low stage (pT1 and pT2) and high stage (pT3 and pT4) or groups of a low grade (grades 1 and 2) and high grade (grades 3 and 4).

Table 1 Relationship between clinicopathological parameters and VASH1 density or microvascular density in 116 ccRCCs

	VASH1 density		Microvascular density	
	Mean ± s.d.	P-value	Mean ± s.d.	P-value
<i>Gender</i>				
Male (n = 89)	292.1 ± 136.3		112.9 ± 58.5	
Female (n = 27)	246.3 ± 116.7	0.129	135.7 ± 67.8	0.113
<i>Age (years)</i>				
Less than median (<59) (n = 56)	280.8 ± 140.4		130.8 ± 67.9	
Median or over (≥59) (n = 60)	282.1 ± 126.7	0.590	116.7 ± 51.4	0.464
<i>Pathological tumor stage</i>				
pT1, 2 (n = 83)	273.3 ± 127.1		126.8 ± 61.4	
pT3, 4 (n = 33)	302.5 ± 146.7	0.143	96.6 ± 50.7	0.008
<i>Lymph node metastasis</i>				
pN0 (n = 111)	282.5 ± 132.5		119.4 ± 60.3	
pN1, 2 (n = 5)	261.6 ± 158.3	0.698	94.7 ± 51.3	0.399
<i>Distant metastasis</i>				
pM0 (n = 106)	271.7 ± 130.4		121.4 ± 62.4	
pM1 (n = 10)	385.0 ± 119.2	0.011	85.7 ± 45.8	0.023
<i>Histological grade</i>				
Low grade (G1, 2) (n = 77)	262.9 ± 107.5		137.1 ± 54.0	
High grade (G3, 4) (n = 39)	317.9 ± 168.8	0.132	92.1 ± 61.2	<0.001

Abbreviations: ccRCC, clear-cell renal cell carcinoma; VASH1, vasohibin-1.

During the follow-up period, 41 patients developed metastatic disease, and 22 patients died of the disease. Ten metastases were removed without treatment. Twenty-one patients were treated with sunitinib according to the protocol,⁴ and nine metastases were removed after the treatment. Among the nine metastases of the sunitinib-treated patients, seven metastases were removed because of their progression. Existing metastases of other two patients were stable, but new metastases were pointed out during the follow-up periods. New lesions were resected for the management of the disease. Ten other patients were treated with interferon- α or interleukin-2. This study was performed after approval by the Institutional Review Board of Keio University Hospital, and informed consent for the experimental use of the samples was obtained from the patients according to the hospital's ethical guidelines.

Immunohistochemistry

Paraffin sections of 116 primary ccRCCs, 10 untreated metastatic ccRCCs (3 bone, 4 lung, 2 pancreas, and 1 brain metastasis) and 9 sunitinib-treated metastatic ccRCCs (6 bone, 2 skin, and 1 lung metastasis) were immunohistologically investigated for the expression of VASH1 and CD34 in the present study. After deparaffinization through graded alcohols to distilled water, the slides were subjected to heat-induced epitope retrieval using a microwave oven in Target Retrieval Solution, pH 9.0 (Dako, Glostrup, Denmark), for VASH1 staining or 10 mM citrate buffer, pH 6.0, for CD34 staining, and they were cooled for 30 min at room temperature. The sections were incubated for 15 min in 0.3% H₂O₂ diluted in methanol to block endogenous peroxidase activity. The slides were incubated with 10% normal goat serum in PBS for 30 min to prevent nonspecific binding of the first antibodies. They were reacted with mouse anti-VASH1 monoclonal antibody (2 µg/ml),¹⁶ or mouse anti-CD34 monoclonal antibody (0.12 µg/ml; clone QBEnd 10; Dako) according to the previous methods.²⁶ The antibody against VASH1 was developed using a synthetic peptide corresponding to the 286–299 amino-acid sequence of VASH1,¹⁶ and the specificity has been characterized.^{16,20,21,23} After washing with PBS, they were incubated with anti-mouse immunoglobulin G (IgG) conjugated to peroxidase-labeled dextran polymer (no dilution: EnVision+Mouse; Dako) for 30 min, and the color was developed with 3,3'-diaminobenzamine tetrahydrochloride in 50 mM Tris-HCl, pH 7.5, containing 0.005% hydrogen peroxidase. The sections were counterstained with hematoxylin. The specificity of immunohistochemistry was checked using negative and positive controls. For negative controls, paraffin sections were incubated with non-immune mouse IgG (Sigma-Aldrich, St Louis, MO, USA) at the same concentration used for each antibody. Sections from upper urinary tract carcinoma with high-VASH1 and microvascular densities were used as positive controls for VASH1 and CD34.²¹

Evaluation of Immunostaining

Because VASH1-positive and VASH1-negative ECs were observed at random even in the same microvessels, the number of VASH1-positive ECs, but not the number of VASH1-positive vessels, was counted. The VASH1 density was determined by two independent observers in five randomly selected high-power fields (x400), and the average count was determined as the VASH1 density for each case. Similarly, the microvascular density was determined in the five randomly selected areas of each section by observing at x200 magnification, as described previously, and the average count was determined as the microvascular density for each case.²⁷

Statistical Analysis

Mann–Whitney's *U*-test was used to analyze the associations between clinicopathological parameters and the VASH1 or microvascular density. Receiver operating characteristic

(ROC) curve analysis was performed to determine the area under the curve (AUC), and the optimal cutoff value was obtained at the farthest point from the diagonal line of the curve.²⁸ The cases in which the VASH1 or microvascular density was equal to or more than the cutoff values were defined as VASH1 or microvascular density-high cases, and those less than the cutoff values were defined as low cases. Progression-free and overall survival rates were estimated using the Kaplan–Meier method and compared by the log-rank test. Univariate and multivariate analyses were carried out according to Cox proportional hazard analysis. Differences among groups were regarded as significant when *P*-values were <0.05. These analyses were performed using IBM SPSS 23 Windows version (PASW Statistics for Windows; SPSS, Chicago, IL, USA).

RESULTS

Expression of VASH1 and CD34 in 116 Primary ccRCCs and Correlation of VASH1 and Microvascular Density with Clinicopathological Parameters

VASH1 staining was negative or negligible in tumor cells and non-neoplastic renal tissues remote from the tumor, whereas CD34 was ubiquitously expressed by all ECs in the blood vessels including glomerular capillaries in the non-neoplastic renal tissues (Figures 1a and b). In low-grade ccRCCs, VASH1 was immunostained by the ECs within the tumors, and the stained ECs were sporadically located in the vessels (arrows in Figure 1c). On the other hand, CD34 was localized to all ECs (Figure 1d). In high-grade tumors, VASH1-positive ECs appeared to increase in the tumor microvessels (arrows in Figure 1e), whereas CD34-positive microvessels seemed to be relatively low compared with the low-grade tumors (Figure 1f). These data strongly suggest that VASH1-positive ECs are specific to ECs within ccRCCs, and thus they can be referred to as tumor-associated ECs.

The VASH1 density within the ccRCCs varied from 42 to 667 cells per mm² (median, 254 cells per mm²), showing a positive correlation with distant metastasis (*P*=0.011) (Table 1). There was no correlation between the VASH1 density and other clinicopathological parameters. On the other hand, the microvascular density varied from 32 to 405 vessels per mm², and its median was 105 vessels per mm². In contrast to the VASH1 density, the microvascular density was inversely correlated with the pathological tumor stage (*P*=0.008), distant metastasis (*P*=0.023) and histological grade (*P*<0.001; Table 1).

Prognostic Significance of VASH1 Density and Microvascular Densities in Patients with ccRCC

ROC curve analysis was performed to determine reasonable cutoff points for the VASH1 and microvascular densities. VASH1 cutoff points for progression-free and overall survival were 268.8 (AUC=0.715, *P*<0.001; Figure 2a) and 268.8 (AUC=0.673, *P*=0.012; Figure 2b), respectively. Microvascular density cutoff points for progression-free and overall

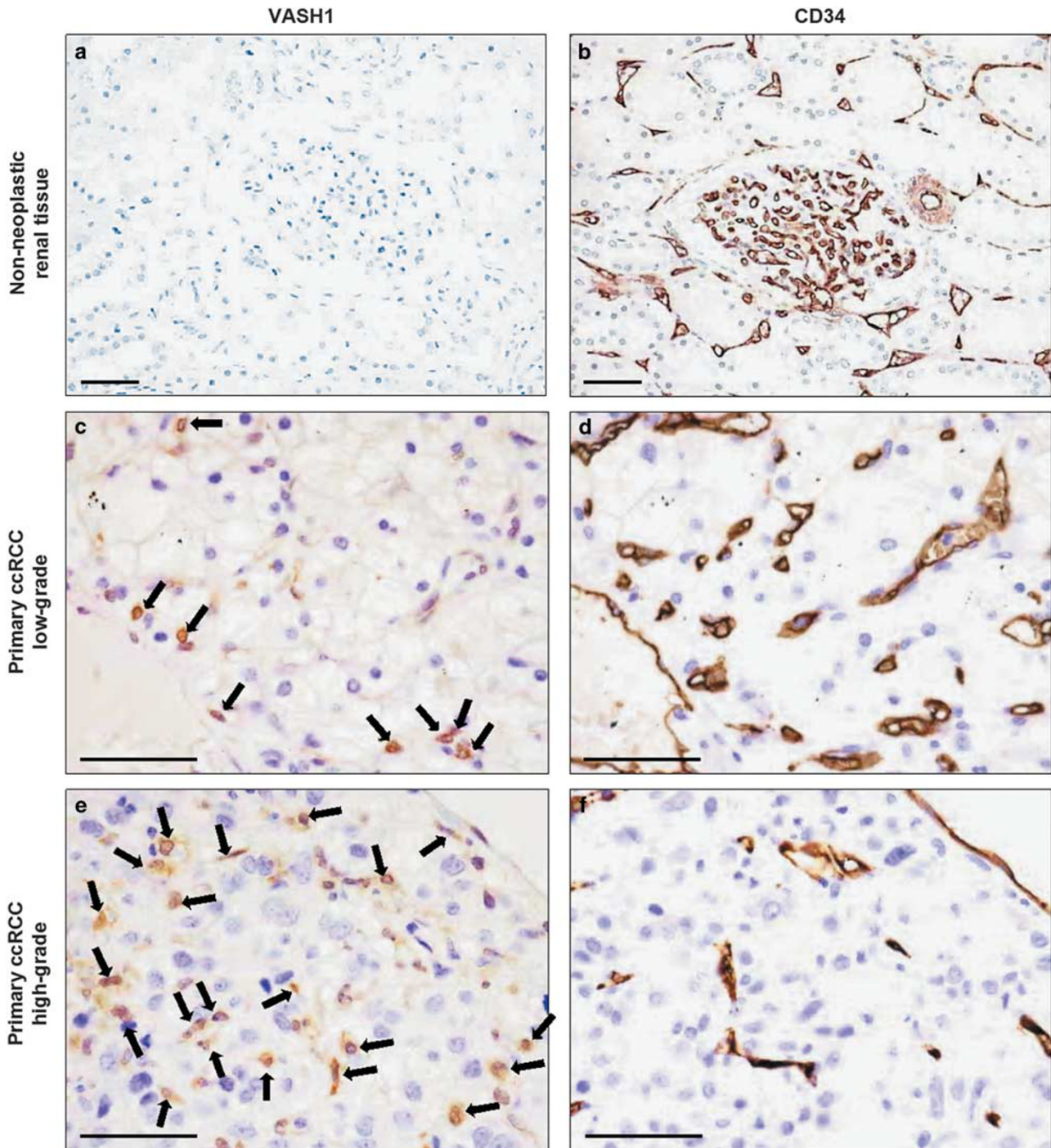


Figure 1 Immunohistochemical expression of vasohibin-1 (VASH1) and CD34. (a and b) Non-neoplastic renal tissues, (c and d) low-grade clear-cell renal cell carcinoma (ccRCC) tissues, and (e and f) high-grade ccRCCs tissues. Serial sections were immunostained with anti-VASH1 and anti-CD34 antibodies. Arrows in (c and e) indicate VASH1-positive endothelial cells (ECs). Note that there are VASH1-positive and -negative ECs in the same microvessels of low-grade ccRCC (c), whereas many ECs in high-grade tumors were positive for VASH1 (e). Bars, 50 μ m.

survival were 91.6 (AUC = 0.599, $P = 0.078$; Figure 2c) and 93.2 (AUC = 0.611, $P = 0.105$; Figure 2d), respectively. Patients with low-VASH1 density tumors had significantly higher progression-free and overall survival rates than those with high-VASH1 density tumors ($P < 0.001$ (Figure 2e) and

$P = 0.002$ (Figure 2f)). In contrast, patients with low-microvascular density tumors had significantly lower progression-free and overall survival rate than those with high-microvascular density tumors ($P = 0.002$ (Figure 2g) and $P = 0.002$ (Figure 2h)).

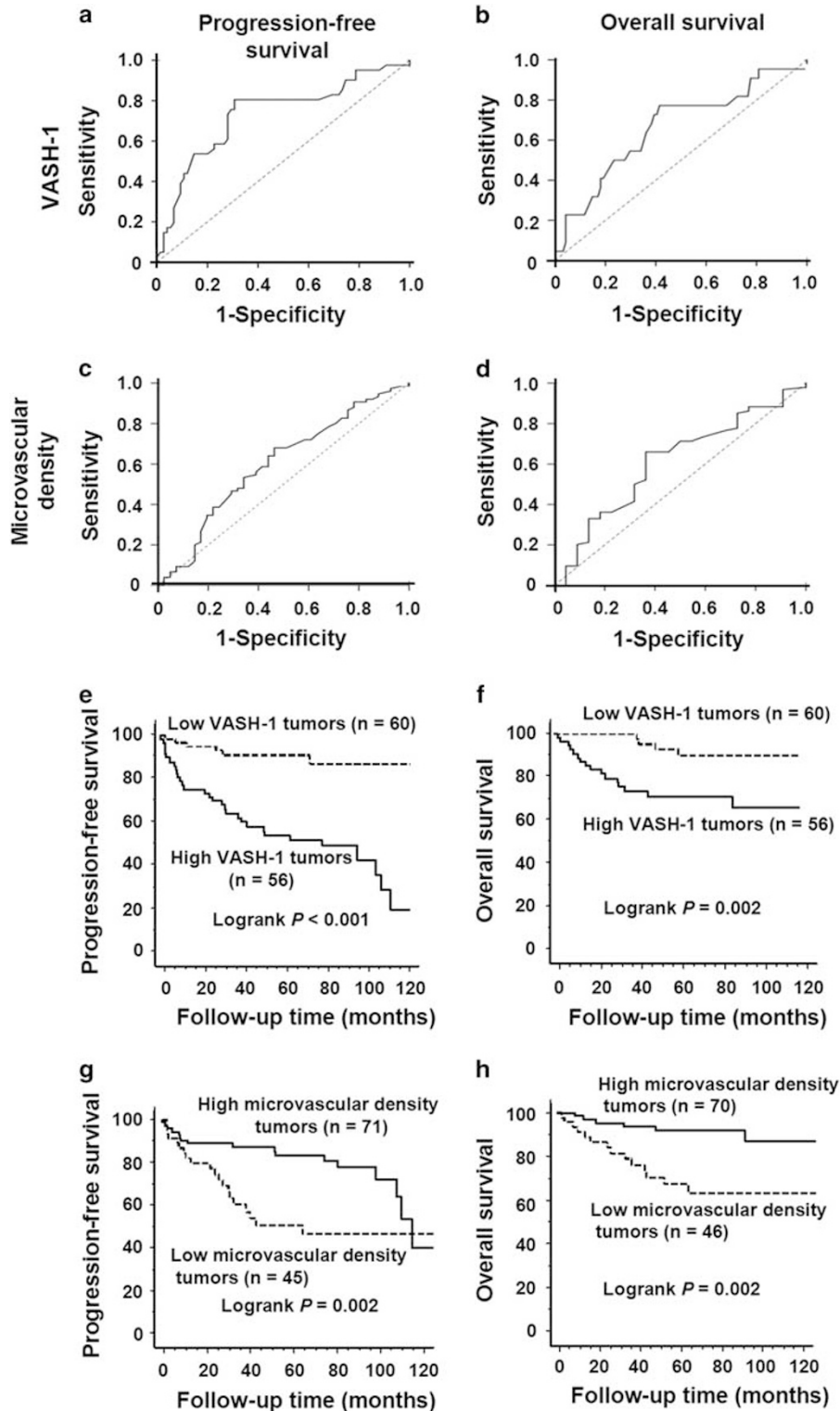


Figure 2 Receiver operating characteristic (ROC) curve analysis of vasohibin-1 (VASH1) and microvascular density cutoff scores, and Kaplan–Meier curves of progression-free and overall survival according to VASH1 and microvascular density in 116 cases of ccRCC. (a and b) ROC curves of progression-free and overall survival according to VASH1, and (c and d) ROC curves of progression-free and overall survival according to microvascular density. At each density, the sensitivity and specificity for the outcome being studied was plotted. The cutoff points of the VASH1 density for progression-free and overall survivals were 268.8 and 268.8, respectively. Those of the microvascular density were 91.6 and 93.2, respectively. High VASH1 tumors led to significantly poorer progression-free and overall survival compared with low VASH1 tumors (e and f). In contrast, tumors with a high-microvascular density were associated with better progression-free and overall survival compared with low-microvascular density tumors (g and h).

Table 2 Cox regression analysis for progression-free survival

Risk factors	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
<i>Pathologic tumor stage</i>				
pT3, 4	6.57 (3.34–12.95)	<0.001	2.61 (1.22–5.57)	0.013
<i>Lymph node metastasis</i>				
pN1, 2	4.82 (1.38–16.82)	0.014	0.92 (0.24–3.51)	0.899
<i>Distant metastasis</i>				
pM1	3.78 (1.69–8.42)	0.001	1.38 (0.54–3.56)	0.504
<i>Histological grade</i>				
High grade (G3, 4)	9.63 (4.68–19.84)	<0.001	7.24 (3.12–16.81)	<0.001
<i>VASH1 density</i>				
High	4.34 (1.99–9.46)	<0.001	3.73 (1.57–8.85)	0.003
<i>Microvascular density</i>				
Low	2.75 (1.44–5.26)	0.002	1.71 (0.80–3.66)	0.166

Abbreviations: CI, confidence interval; OR, odds ratio; VASH1, vasohibin-1.

The pathologic tumor stage, lymph node metastasis, distant metastasis, histological grade, VASH1 density, and microvascular density were prognostic factors for progression-free survival in univariate analysis (Table 2). Multivariate analysis revealed that the pathological stage, histological grade, and VASH1 density were independent prognostic factors for disease-free survival (Table 2). Similarly, univariate analysis indicated that the pathologic tumor stage, lymph node metastasis, distant metastasis, histological grade, VASH1 density, and microvascular density were prognostic factors for overall survival (Table 3). By multivariate analysis, the pathological stage, lymph node metastasis, histological grade, and VASH1 density were independent prognostic factors for overall survival (Table 3).

Expression of VASH1 and CD34 in Metastatic ccRCCs Obtained from the 19 Patients Treated or Untreated with Sunitinib

In the untreated metastatic ccRCCs, tumor-associated ECs in the tumor tissues were sporadically positive for VASH1 staining, although CD34 was expressed by all ECs (Figures 3a and b). On the other hand, VASH1 staining in tumor-associated ECs appeared to increase in sunitinib-treated ccRCCs, whereas CD34-positive microvessels decreased (Figures 3c and d).

Table 3 Cox regression analysis for overall survival

Risk factors	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
<i>Pathologic tumor stage</i>				
pT3, 4	8.60 (3.33–22.24)	<0.001	3.40 (1.20–9.67)	0.021
<i>Lymph node metastasis</i>				
pN1, 2	13.59 (4.31–42.89)	<0.001	5.33 (1.34–21.19)	0.018
<i>Distant metastasis</i>				
pM1	3.27 (1.09–9.84)	0.035	0.34 (0.09–1.35)	0.125
<i>Histological grade</i>				
High grade (G3, 4)	18.48 (5.39–63.36)	<0.001	11.0 (2.76–43.94)	<0.001
<i>VASH1 density</i>				
High	4.71 (1.58–14.0)	0.005	7.71 (1.96–30.27)	0.003
<i>Microvascular density</i>				
Low	3.94 (1.58–9.88)	0.003	2.50 (0.83–7.49)	0.102

Abbreviations: CI, confidence interval; OR, odds ratio; VASH1, vasohibin-1.

When the VASH1 and microvascular densities were calculated, the VASH1 density was significantly higher in the sunitinib-treated metastatic ccRCCs than in the untreated primary and metastatic tumors (** $P=0.001$ and * $P=0.01$; Figure 3e). In contrast, the microvascular density was markedly lower in the sunitinib-treated metastases than in the primary and metastatic tumors without treatment (** $P<0.001$, *** $P=0.001$; Figure 3f).

DISCUSSION

In the present study, we retrospectively evaluated the impact of VASH1 expression in 116 ccRCCs by immunohistochemistry, and obtained evidence that VASH1 is expressed almost selectively in tumor-associated ECs with correlations with distant metastasis. These data demonstrate, to the best of our knowledge, for the first time that the overexpression of VASH1 in tumor-associated ECs is related to the tumor aggressiveness of ccRCCs. Elevated VASH1 expression was identified as an independent prognostic factor for poor progression-free and overall survival rates in ccRCC patients. Similar findings have been reported involving cancers of the upper urinary tract, prostate, breast, and endometrium.^{20–23} Importantly, we have also shown that VASH1 expression is upregulated by tumor-associated ECs in sunitinib-treated metastatic tumors. Because VASH1 is a critical factor that improves the stress tolerance of

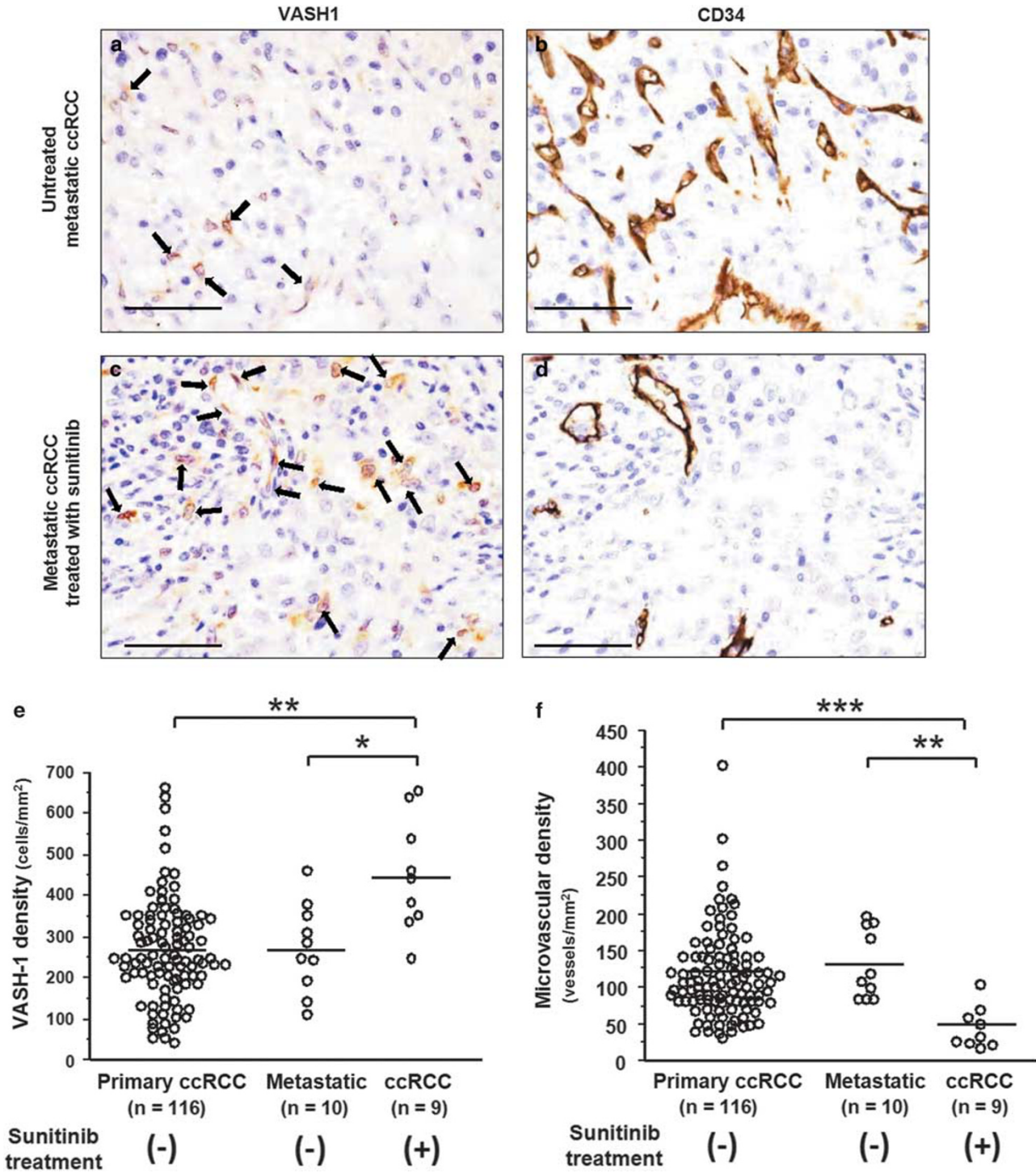


Figure 3 Immunohistochemical expression of vasohibin-1 (VASH1) and CD34 in metastatic clear-cell renal cell carcinomas (ccRCCs) treated without or with sunitinib, and statistical analyses of the difference in VASH1 density. (a–d) Expression of VASH1 and CD34. Serial sections were immunostained with anti-VASH1 and anti-CD34 antibodies. Bars, 50 μ m. (e and f) Statistical analysis of the difference in VASH1 density and microvascular density. Bars in (e and f) show mean values. * $P=0.01$, ** $P=0.001$, and *** $P<0.001$.

ECs,²⁴ VASH1 may be involved in the resistance of tumor-associated ECs to sunitinib treatment.

An increase in the microvascular density is commonly associated with early progression in several neoplasms,

including carcinomas of the breast or prostate and hematological malignancies.¹¹ However, an inverse correlation of the microvascular density with metastasis and the prognosis has been reported in RCC patients,¹² suggesting that such an

association may depend on the types of malignancy. Although ccRCCs are generally hypervascular, their vascular density has been suggested to decrease in high-grade ccRCCs compared with low-grade tumors because of the overwhelmed proliferation of tumor cells in high-grade ccRCCs.²⁹ The tumor microvascular density has usually been evaluated by the calculation of blood vessels positively immunostained with antibodies against CD31 or CD34, both of which are expressed by activated and quiescent ECs.^{20,21} In contrast, as shown in the present study, as quiescent ECs are almost negative for VASH1 expression, and VASH1 is inducible in response to angiogenic stimuli such as VEGF in an autocrine and/or paracrine manner,^{16,30} it is likely that VASH1 expression reflects the actual activity of angiogenesis. In fact, previous studies on prostate, upper urinary tract, breast, or endometrial cancer suggested that VASH1 has an important role in the regulation of tumor angiogenesis and is associated with the neovascularization, malignant potential, and unfavorable prognosis of patients.^{20–23}

One of the novel findings in the current study is that VASH1-positive or -negative ECs were located in a random manner in the same microvessels of the ccRCC tissues. Kanomata *et al*,¹⁹ showed a similar immunohistochemical staining pattern of VASH1 in RCCs, albeit it was not mentioned, in their paper.¹⁹ The authors classified microvessels into VASH1-positive or -negative ones, and suggested that high-VASH1-positive vessels are associated with a longer disease-free survival.¹⁹ On the other hand, our data on the VASH1 density, which was determined by calculating VASH1-positive ECs, but not VASH1-positive microvessels, revealed positive correlations with the distant metastasis and poor prognosis of patients with ccRCC. As an inverse correlation between the microvascular density and prognosis or metastasis was obtained in the present study, we consider that VASH1 is a reasonable marker for the angiogenic activity and prognostic analysis of ccRCCs.

Sunitinib, which contributes to the inhibition of angiogenesis by blocking VEGF receptor kinase, is now widely used for patients with metastatic ccRCC.³¹ Apoptosis in tumor cells and ECs is induced in ccRCCs treated with sunitinib, and the microvascular density has been reported to decrease in primary ccRCCs of patients who received sunitinib treatment compared with an untreated group.²⁷ In the present study, we showed that the microvascular density is lower in sunitinib-treated metastatic ccRCCs than in the untreated metastatic tumors, confirming that targeting therapy on tyrosine kinase pathways by sunitinib treatment exhibits clinical effects mainly through the inhibition of angiogenesis, as suggested by a previous paper.⁶ In contrast, however, our study showed that the VASH1 density is significantly higher in sunitinib-treated metastatic ccRCCs than in untreated metastatic tumors. The elevated VASH1 expression in tumor-associated ECs can be a feedback upregulation of the VEGFR inhibitor. However, an *in vitro* study showed that VEGFR inhibitor had no effect on basal expression of VASH1 in

human umbilical endothelial cells (HUVECs) and that it induced cell death of HUVECs.²⁴ In addition, VASH1 enhances the maintenance of ECs by strengthening their resistance to oxidative or serum starvation stress, and overexpression of VASH1 reduces the death of ECs treated with VEGFR inhibitor.²⁴ These suggest that sunitinib induces apoptosis of tumor-associated ECs with low VASH1 expression, and tumor-associated ECs with high-VASH1 expression may survive during sunitinib treatment of ccRCCs, leading to the finding that many ECs within sunitinib-treated metastatic ccRCCs are positive for VASH1. Therefore, our findings of VASH1 overexpression by tumor-associated ECs in sunitinib-treated ccRCCs may help clarify the resistance of the ECs to sunitinib treatment, although further experimental studies are necessary to support our hypothesis.

A previous study reported the expression of VASH1 in both cancer cells and ECs, and showed that VASH1 expression is lower in RCC tissues than in the adjacent non-neoplastic renal tissues.³² However, our present and another group's studies indicated that VASH1 staining is almost specific to tumor-associated ECs in RCCs.¹⁹ Although the reason for the different staining patterns is not clear, it may be due to the different antibodies used for VASH1 immunostaining. As our antibody is monospecific to VASH1, as reported by our previous studies,^{15,16,18–21,23} the present study strongly suggests that VASH1 is predominantly expressed by tumor-associated ECs and at a negligible level by ccRCC cells, if any.

The limitation of this study is that relatively small sets of metastatic ccRCC with or without treatment were investigated. Therefore, the findings of the potential value of VASH1 for metastasis and/or prognosis of the ccRCC patients need further validation. RNA-based detection and/or liquid biopsy using urine samples would be easier to quantify the expression level of VASH1 compared with immunohistochemical analysis. In fact, urinary and plasma levels of VASH1 were reported to be useful for prediction of renal functional deterioration in patients with chronic renal disease.³³ Because VASH1 was associated with progression and resistance to sunitinib treatment of ccRCCs, quantification of VASH1 expression by RNA-based detection and/or liquid biopsy specimen using urine samples would be an easier method to predict the disease progression and useful for the choice of molecular targeted therapy in the future.

In summary, we have demonstrated the expression of VASH1 by tumor-associated ECs in ccRCC tissues, showing positive correlations with distant metastasis. The VASH1 density was also an independent prognostic predictor of progression-free and overall survival of patients with ccRCC. The finding of an increase in VASH1-positive tumor-associated ECs in the sunitinib-treated metastatic ccRCCs suggests that VASH1 is related to the resistance of ECs to the therapy. Our data also suggest that VASH1 can serve as a new prognostic and predictive biomarker for patients with ccRCC.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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