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Vasohibin-1 Increases the Malignant Potential of Colorectal Cancer and Is a Biomarker of Poor Prognosis

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Abstract. *Background: Vasohibin-1 (VASH1) is related to angiogenesis and poor prognosis in several types of cancers. However, the biological function and clinical significance of VASH1 in colorectal cancer (CRC) are not fully known. Materials and Methods: The associations between VASH1 expression and clinicopathological features were investigated by immunohistochemistry in 429 CRC tissues. To evaluate the function of VASH1 in vitro, small-interfering VASH1-targeting RNA was transfected into human CRC cell lines. Results: We found that VASH1 was highly expressed in the cytoplasm of CRC tissues. High VASH1 expression in the cytoplasm was significantly associated with tumor progression, such as larger tumor size, advanced T-stage, lymph node metastasis, distant metastasis and poor prognosis. Moreover, a significant positive correlation was detected between VASH1 expression and microvessel density. VASH1 siRNA inhibited CRC cell proliferation, migration, and invasion, and promoted anoikis. Conclusion: Overexpression of VASH1 in CRC cells increased malignant potential and promoted metastasis.*

Colorectal cancer (CRC) is the third most common cancer worldwide and its incidence is rapidly increasing, especially in Eastern Asia (1). Despite recent advances in chemotherapeutic regimens with molecular-targeting drugs, the prognosis for patients with metastatic CRC remains poor (2, 3). In addition, the mechanism underlying the metastatic process in CRC is complex and not completely understood (4). Therefore, the identification of well-characterized molecular markers related to metastasis development is urgently needed to monitor metastatic progression in patients with CRC and thereby facilitate appropriate therapeutic intervention.

Angiogenesis, the growth of new blood vessels, is a major feature of various types of cancer. It is essential for tumor growth and metastasis (5). Indeed, increased vascularity through angiogenesis enhances growth of the primary tumor by supplying nutrients and oxygen, and provides an avenue for hematogenous metastasis (6-8). Microvessel density (MVD) assessment is the most commonly used technique to quantify intratumoral angiogenesis (9). Elevated MVD has been proposed to identify patients with CRC with poor prognosis (10-12).

Vasohibin-1 (VASH1) has recently been identified as a novel endothelium-derived inhibitor of angiogenesis that is induced by vascular endothelial growth factor (VEGF)-A (13-15). Up-regulation of VASH1 counteracts excessive angiogenic activity directed by VEGFA. It plays important roles in pathological angiogenesis, such as those in retinal disease (16) and diabetic nephropathy (17). In addition, VASH1 is exclusively expressed in endothelial cells (ECs) of several tumor types, including breast cancer, cervical cancer and prostate cancer, and influences the clinical course. Indeed, VASH1 expression in ECs is associated with tumor grade and histological type (18-21). VASH1 has also been shown to be expressed by cancer cells including those in hepatocellular carcinoma (HCC) (22) and CRC (23). VASH1 expression in tumor cells is closely correlated with tumor angiogenesis, as reflected by increased MVD as well as VEGFA. Moreover, it has been reported to be an independent prognostic factor for unfavorable disease-free survival and overall survival (OS) (22, 23). However, the function and clinical significance of VASH1 in CRC have not been fully-investigated.

In the present study, we first evaluated the relationship between VASH1 expression and clinicopathological features in more than 400 patients with CRC. Next, we assessed the relationship between VASH1 expression and MVD. Finally, we used RNA interference to investigate the functional role of VASH1 in CRC.

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Materials and Methods

Patients and samples. A total of 429 patients with CRC, who were treated between January 2002 and December 2011 at the Department of Gastrointestinal and Pediatric Surgery of Mie University Graduate Hospital Japan, were included in this retrospective study. Written informed consent of all patients was obtained according to the local ethics guidelines. The study was approved by the Institutional Review Board of our institution (No. 2216). Patients with incomplete clinical data, inadequate follow-up, or inadequate tissue sampling were excluded from this analysis. Among the patients, 255 had histologically confirmed carcinoma of the colon and 174 had that of the rectum. The patient cohort comprised 181 women and 248 men whose age range was 12-91 years (median=68 years). Tumor histology distinguished 383 tubular adenocarcinomas, four papillary adenocarcinomas, 20 poorly differentiated adenocarcinomas, 20 mucinous adenocarcinomas, and two signet cell adenocarcinomas. The distribution of T-stages according to the Union for International Cancer Control TNM classification (seventh edition) was as follows: Tis, 19 tumors (4.4%); T1, 48 tumors (11.1%); T2, 58 tumors (13.5%); T3, 206 tumors (48.0%); and T4, 98 tumors (22.8%). The N stages were: N0, 247 cases (57.6%); N1, 103 cases (24 %); N2, 63 cases (14.7%); and N3, 16 cases (3.7%). Thirty-three patients had peritoneal metastasis (7.7%), 57 patients had hepatic metastasis (13.3%) and 59 patients had distant metastasis (13.8%). The stages were distributed as follows: Stage 0, 19 cases (4.4%); stage 1, 76 cases (17.7%); stage 2, 133 cases (31.0%); stage 3, 109 cases (25.4%); and stage 4, 92 cases (21.5%). The follow-up period had a range of 0.13-118.4 months (median 24.1 months).

Immunohistochemistry (IHC). Paraffin-embedded tissue sections (3 μm) were prepared from surgical specimens of patients with CRC. After deparaffinization and hydration, specimens were pretreated in an autoclave at 121˚C for 10 min in 10 mM citrate buffer (pH 6.0). Specimens were then blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated with mouse monoclonal antibody against VASH1 (1:2000; Abnova, Taipei, Taiwan) and Cluster of Differentiation 31 (CD31) (1:1000; Cell Signaling, Beverly, MA, USA) overnight at 4˚C. Antibody binding was detected by Envision HRP reagents kit (Dako Cytomation, Glostrup, Denmark). All sections were counterstained with Mayer's hematoxylin. The specificity of the immunoreaction was verified by staining for known positive and negative controls.

Each slide was observed by scanning the whole section at medium (×40) and high (×200) magnification. Two independent researchers, with no prior knowledge of clinical or pathological parameters of the samples, evaluated VASH1 immunoreactivity based on staining intensity and extent. Staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong) (Figure 1). The extent of VASH1 staining was scored according to the percentage of cancer cells with cytoplasmic VASH1 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% (Figure 2). Final IHC scores were calculated by multiplication of the intensity and extent scores, resulting in a scale with a minimum score of 0 and a maximum score of 12. Any discrepancies encountered were resolved using a multihead microscope until a consensus was reached. For further analysis, VASH1 expression of each section was dichotomized into either high-expression (score ≥6) or low-expression (score <6) groups according to the cutoff value determined by receiver operating characteristic curve analysis to predict OS.

Scanning of immunostained sections under low magnification (×40 and ×100) was used to examine microvessels, which were identified based on architecture, including a lumen lined by endothelial cells, which were distinguished by positivity to antibody to CD31. The areas with the greatest number of distinctly highlighted microvessels, within each section, were selected for counting at higher power (×200; 0.74 mm^2 per field). Using this measurement method, the intratumoral MVD of each individual tumor was determined as the absolute mean value of three counted fields by a single observer (24, 25).

Cell lines and culture conditions. Human colon cancer cells lines, Caco2, DLD1, HT29, LoVo, and SW480, were obtained from the Riken Cell Bank (Ibaraki, Japan). Cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRl, Tokyo, Japan), 2 mM glutamine, 100 IU/ml penicillin, and 100 ng/ml streptomycin at 37˚C in 5% CO_2.

Western blot analysis. Cells were homogenized in lysis buffer for 5 min on ice, then, the supernatants were collected after centrifugation at 12000 × *g* for 15 min at 4˚C and protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL, USA). Lysates containing 20 μg total protein were mixed with appropriate quantities of Lammeli's loading buffer containing β-mercaptoethanol and denatured by heating at 98˚C for 5 min. Samples were then separated by electrophoresis on 12.5% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate at 25 mA for 30 min, followed by semi-dry transfer to an Immune-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 12 V for 30 min. The membranes were then blocked for 2 h at room temperature with 1% skim milk in Tris Buffer Saline (TBS), pH 7.5, supplemented with 0.1% Tween 20 (TBS-T). Next, the blots were incubated overnight at 4˚ with mouse monoclonal antibody against VASH 1 (1:100; Abnova) and mouse monoclonal antibody to actin (clone C4) (1:400; ICN Biomedicals, Aurora, OH, USA). After three washes with TBS-T, blots were incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1000; Promega, Madison, WI, USA) diluted in TBS-T containing 1% skim milk. Following addition of the chemiluminescence detection solution, signals were visualized using a CS Analyzer and AE-6962 Light Capture (ATTO Corp., Tokyo, Japan).

VASH1 RNA interference. VASH1-specific siRNA (Silencer Validated siRNA; sense: GAACAGCCGCAGUGAAAGAtt; antisense: UCUUUCACUGCGGCUGUUCct) and control siRNA (Silencer Select Negative Control #1 siRNA) were purchased from Ambion (Austin, TX, USA). Reverse transfections were performed by mixing suspended cells with 50 nM siRNA oligonucleotides and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) diluted in Optimem I (Invitrogen) before plating. The final siRNA oligonucleotide concentration was 30 nM.

Proliferation assay. Cell proliferation was evaluated using the WST-8 colorimetric assay kit (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, 24 h after transfection with *VASH1* siRNA or control siRNA, DLD1 and HT29 cells were seeded at 5×10^3 cells per well in 96-well flat-bottomed microtiter plates in culture medium at a final volume of 100 μl. After 0-48 h culture, 10 μl Cell Counting Kit-8 reagent was added to each well, and the plates were incubated for a further 2 h, whereupon the absorbance of each well was

measured by SoftMax Pro (Molecular Devices Corp., Sunnyvale, CA, USA) at a wavelength of 450 nm. Each independent experiment was performed three times.

Scratch wound migration assay. DLD1 (2×10^6 cells/well) and HT29 (2×10^6 cells/well) cells transfected with *VASH1* siRNA or negative control siRNA in media with 10% FBS were seeded into 6-well plates in triplicate and incubated for 12 h at 37˚C. Once a confluent monolayer was attained, wounds were generated using a sterile 200-μl pipette tip. The cells were grown for an additional 48 h then wound closure was assessed using an Olympus I×71 (OLYMPUS, Tokyo, Japan) microscope at ×10 magnification. Cell migration distance was calculated by Adobe Photoshop Elements 10.0 software (Adobe Systems Incorporated, San Jose, CA, USA) by comparison with baseline measurements. Each independent experiment was performed three times.

Invasion assay. Control or *VASH1* siRNA-transfected DLD (5×10^4 cells/well) and HT29 (5×10^4 cells/well) cells were seeded in serum-free media in 24-well Falcon™ cell culture inserts (8-μm pore size; BD Biosciences, Bedford, MA, USA) or 24-well Matrigel™ Invasion Chambers (8-μm pore size; BD Biosciences) for migration and invasion assays, respectively. Inserts were placed into Falcon companion plates containing 10% FBS. After incubation for 48 h at 37˚C, the media plus cells were removed from the top chamber using cotton swabs and phosphate-buffered saline and the number of cells migrating to or invading the membrane undersides was determined. To do this, membranes were fixed and stained with Diff-Quick stain™ (Sysmex, Kobe, Japan) and mounted on glass slides. The number of migrating or invading cells in five fields of view was counted with a light microscope at ×100 magnification. Each independent experiment was performed three times.

Anoikis assay. Anoikis assays were performed in 6-well Costar Ultra-Low Attachment microplates (Corning, NY, USA). Briefly, *VASH1* siRNA- or negative control siRNA-transfected DLD1 and HT29 cells were suspended in RPMI-1640 with 10% FBS at a density of 5×10^5 cells/ml. Cell suspensions (2 ml) were added to each well and incubated for 24 h. MTT assays were performed to confirm anoikis induction. To do this, floating cells from the anoikis assay were seeded at 5×10^3 per well in 96-well flat-bottom microtiter plates in a final volume of 100 μl culture medium. Spectrophotometric absorbance of the samples was measured as described for the proliferation assay. Each independent experiment was performed three times.

Statistical analysis. Results are expressed as median values (interquartile range) or as mean±standard deviation. The associations between clinicopathological parameters and VASH1 expression were validated by Chi-square tests. Linear regression was used to analyze the correlation between VASH1 and MVD. To calculate cut-off values, receiver operating characteristic curves were analyzed according to the most accurate value obtained to predict OS. An OS curve was generated by the Kaplan–Meier method, and comparisons were made using the log-rank test. The Cox proportional hazards model was used to evaluate independent prognostic factors by univariate and multivariate analysis of OS. The statistical significance of differences *in vitro* was calculated usingStudent's *t*-test. All statistical analyses were carried out using JMP 10 for windows software (SAS Institute, Cary, NC, USA). Two-sided *p*-values of less than 0.05 were considered statistically significant.

Table I. *Relationship between expression of Vasohibin-1 and clinicopathologicalfeatures on 429 patients with colorectal cancer.*

Feature	VASH1 expression			*p*-Value
	n	High	Low	
Gender				
Male	248	117	131	
Female	181	87	94	0.855
Age (years)				
<68 (median)	214	103	111	
≥68	215	101	114	0.810
Histrological grade				
Differentiated	387	183	204	
Undeffierentiated	42	21	21	0.738
Tumor size (cm)				
<4	191	79	112	
≥4	238	125	113	**0.021**
T-Stage				
T1,T2	125	42	83	
T3,T4	304	162	142	**0.0002**
Lymphatic invasion				
Negative	106	38	68	
Positive	323	166	157	**0.005**
Venous invasion				
Negative	213	83	130	
Positive	216	121	95	**0.0004**
Lymph node metastasis				
Negative	247	98	149	
Positive	182	106	76	**0.0001**
Hepatic metastasis				
Negative	372	168	204	
Positive	57	36	21	**0.011**
Peritoneal metastasis				
Negative	396	180	216	
Positive	33	24	9	**0.002**
Distant metastasis				
Negative	370	164	206	
Positive	59	40	19	**0.0007**
TNM stage				
0, 1, 2	228	85	143	
3, 4	201	119	82	**<0.0001**
MVD (n/mm^2)				
<39 (median)	214	75	139	
≥39	215	129	86	**<0.0001**

Categorical data were compared by chi-square test. Bold indicates statistically significant difference at $p<0.05$.

Results

Cytoplasmic VASH1 expression is associated with clinicopathological features in CRC. Specific immunostaining of VASH1 was identified in the cytoplasm of tumor cells in patient-derived CRC tissue (Figure 3A) at differing intensities, as evidenced by the various shades of brown interspersed in a granular or lamellar fashion. In contrast, VASH1 expression was low to absent in the cytoplasm of adjacent normal colonic

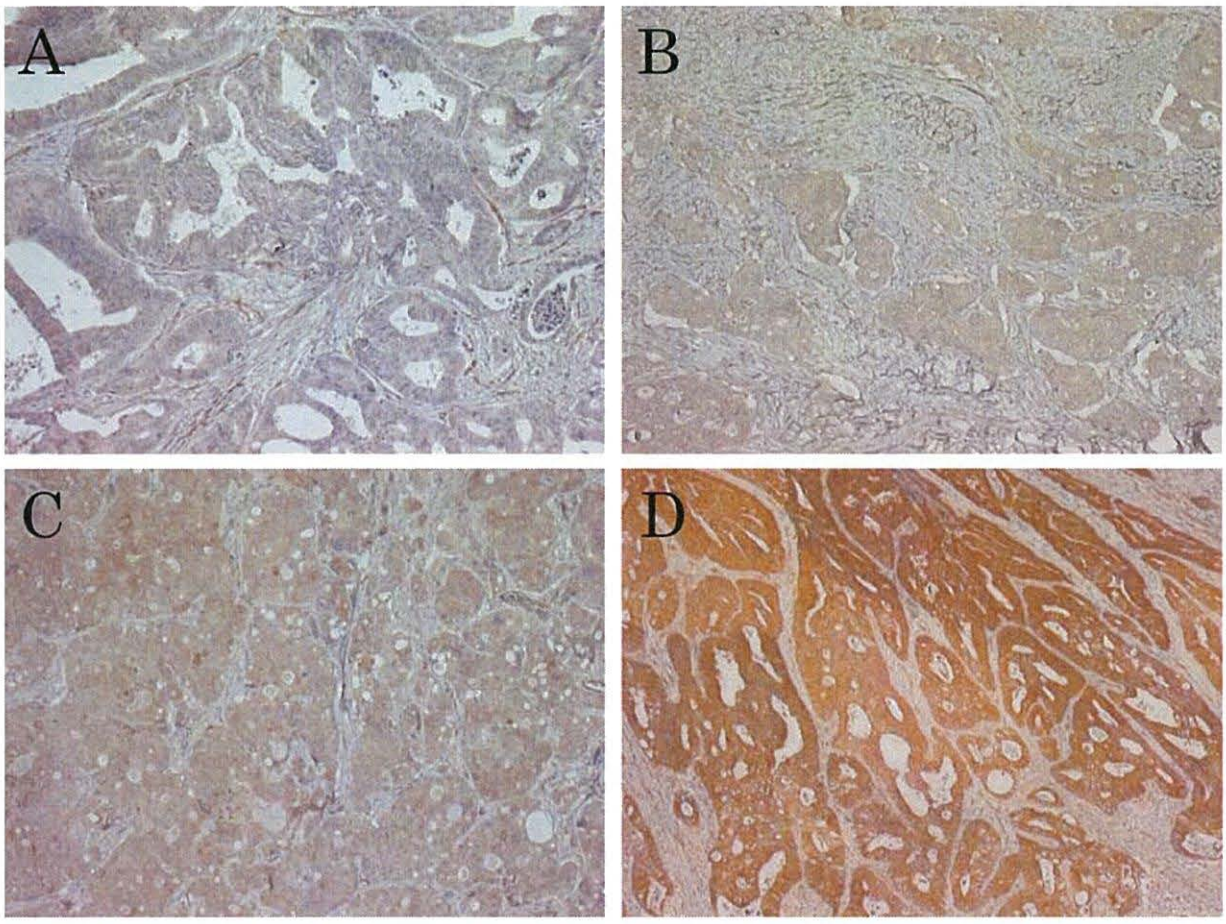

Figure 1. *Staining intensity of Vasohibin-1 (VASH1) expression in colorectal cancer (CRC). The staining intensity of cytoplasmically-expressed VASH1 protein in CRC tissues was scored as negative=0 (A), weak=1 (B), medium=2 (C), and strong=3 (D). Representative images are shown. All images were captured at ×200 magnification.*

mucosa (Figure 3B). Quantification of VASH1 immunostaining in CRC showed that its expression was high in 47.5% (204/429) of cases and low in 52.4% (225/429) of cases. As outlined in Table I, analysis of the relationships between VASH1 scoring and clinicopathological features revealed that VASH1 was significantly associated with tumor size (p=0.021), T-stage (p=0.0002), lymphatic invasion (p=0.005), venous invasion (p=0.0004), lymph node metastasis (p=0.0001), hepatic metastasis (p=0.011), peritoneal metastasis (p=0.002), distant metastasis (p=0.0007), and TNM stage (p<0.0001). Moreover, immunostaining of CD31 was identified in endothelial cells of CRC tissues (Figure 3C) and used to calculate tumor MVD, which was found to be a median of 39/mm^2 (range=5.63-102.3/mm^2). A significant positive correlation was found between VASH1 expression and MVD (r=0.356, p<0.001) (Figure 4).

Prognostic impact of VASH1 expression in CRC. According to the survival analysis, the 1-, 3-, and 5-year OS rates were 92.1, 81.4, and 72.5%, respectively. Patients with high VASH1 expression (n=204) had significantly worse OS than those with low VASH1 expression (log-rank test, p=0.03) (Figure 5). Furthermore, by univariate analysis, VASH1 expression was found to significantly influence OS, along with tumor size, histological grade, lymphatic invasion, venous invasion, and TNM stage. In contrast, MVD did not affect OS (p=0.095). However, multivariate analysis revealed that VASH1 was not an independent prognostic factor for OS (HR=1.05, p=0.819) (Table II).

VASH1 promotes proliferation of CRC cells. Among the five CRC cell lines, Caco2, DLD1, HT29, LoVo, and SW480, VASH1 protein expression was highly detected in

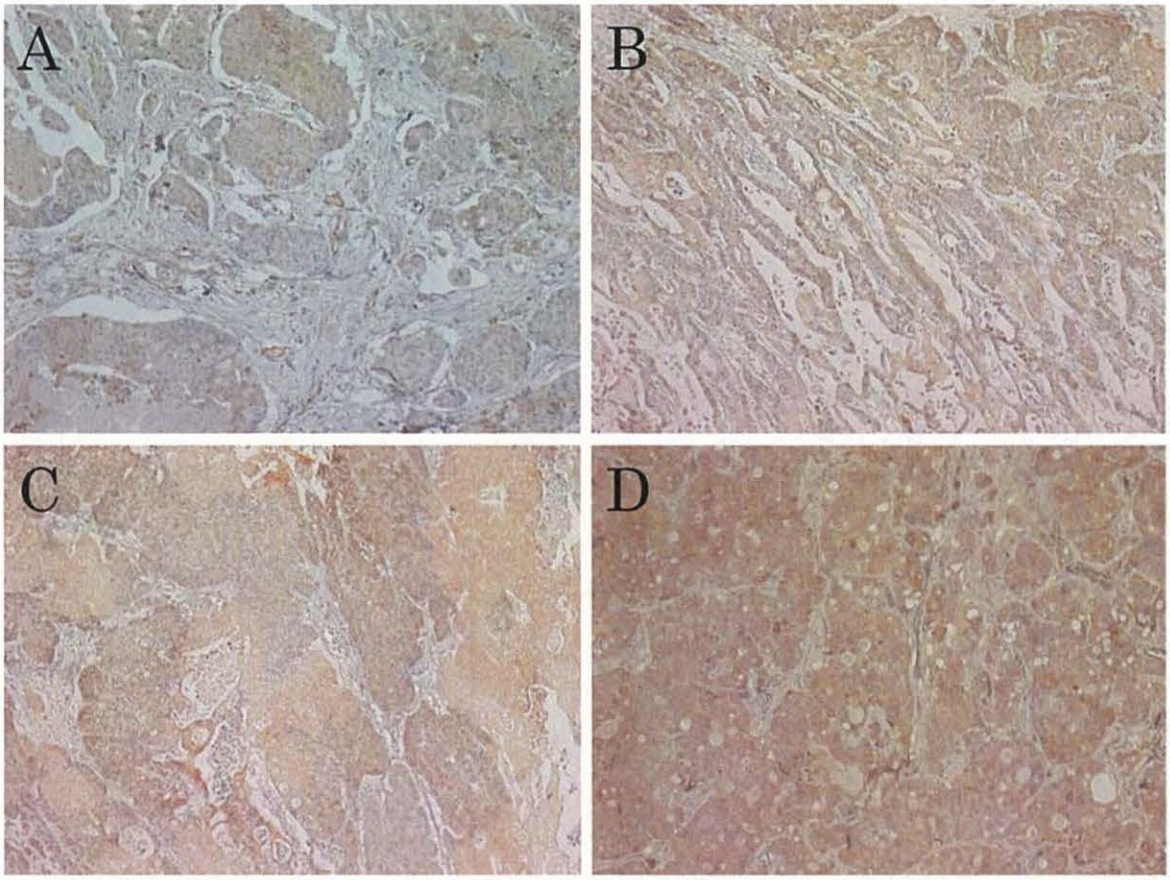

Figure 2. *Extent of Vasohibin-1 (VASH1) immunostaining in colorectal cancer (CRC). The extent of VASH1 staining in patient-derived CRC tissues was scored according to the percentage of cancer cells with cytoplasmic VASH1 staining, where 0=0%, 1=1-25% (A), 2=25-50% (B), 3=51-75% (C), and 4=76-100% (D). Representative images are shown. All images were captured at ×200 magnification.*

DLD1 and HT29 cells (Figure 6A). Therefore, DLD1 and HT29 cells were used to further analyze the function of VASH1 in CRC using *VASH1* siRNA. We first examined whether down-regulation of *VASH1* (Figure 6B-C) influenced the growth of DLD1 and HT29 cells. As a result, the proliferation of both cell lines transfected with *VASH1* siRNA was significantly inhibited when compared to that of control siRNA-transfected counterparts ($p<0.05$) (Figure 6D-E).

VASH1 increases the migratory capacity of CRC cells. Next, to examine the effect of VASH1 on tumor cell motility, wound-healing assays were performed and the migratory potential of CRC cells with and without *VASH1* knockdown were compared. Twenty-four hours after *VASH1* siRNA transfection, both cell lines showed significantly decreased migratory capacity ($p=0.004$) compared with that of control siRNA-transfected cells (Figure 6F-I).

VASH1 promotes the invasive ability of CRC cells. Furthermore, to examine the role of VASH1 on tumor cell invasion, we performed *in vitro* invasion assays. We found that the capacity of *VASH1* siRNA-transfected DLD1 and HT29 cells to invade through a Matrigel-coated membrane was significantly reduced compared with that of control siRNA-transfected cells ($p<0.05$) (Figure 6J–M).

VASH1 inhibits anoikis of CRC cells. Anoikis is a form of detachment-induced apoptosis. In the *in vitro* experimental setting, it is induced when adherent tumor cells are forced to undergo non-adherent growth. Anoikis resistance was evaluated by the proportion of viable tumor cells that proliferated non-adherently in low-attachment culture plates. Transfection with *VASH1* siRNA significantly reduced the proportions of anoikis-resistant tumor cells (floating viable tumor cells with non-adherent growth) in both cell lines compared with those of cells receiving control siRNA ($p<0.05$) (Figure 6N–O).

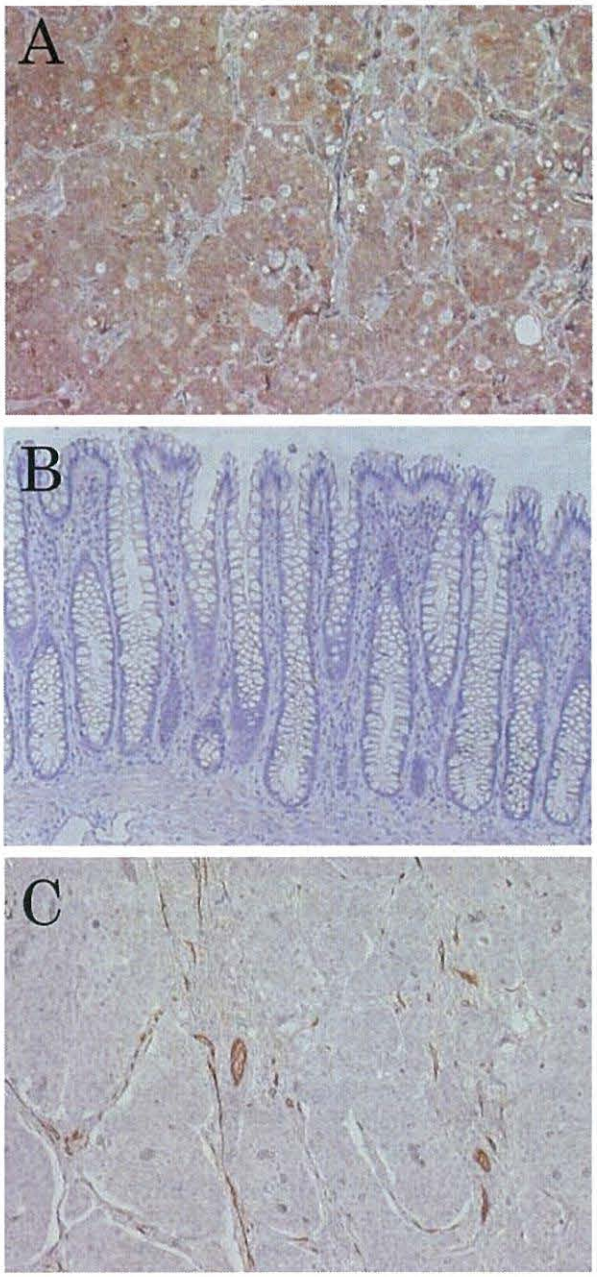

Figure 3. *Expression of Vasohibin-1(VAS1) in colorectal cancer (CRC). Immunohistochemistry was used to examine VASH1 expression in patient-derived CRC tissue (A) and normal mucosa (B). Cluster of Differentiation 31 (CD31) expression was also examined (C). All images were captured at ×200 magnification.*

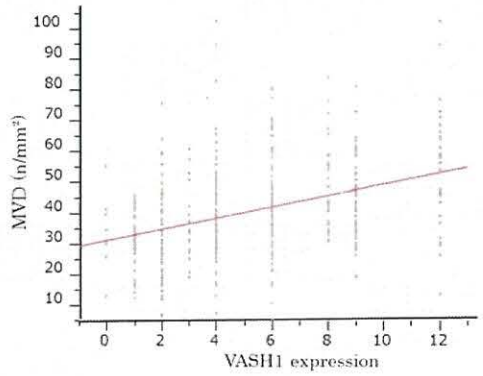

Figure 4. *Analysis of the correlation between Vasohibin-1 and microvessel density in colorectal cancer (r=0.356, p<0.001).*

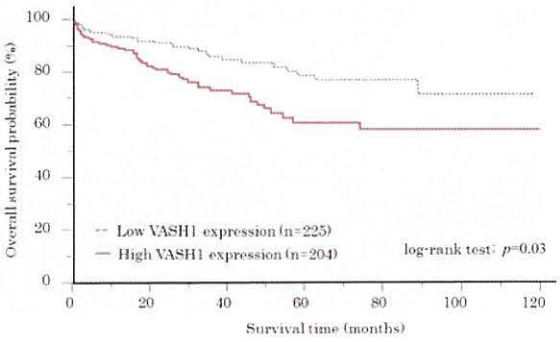

Figure 5. *Vasohibin-1 (VASH1) expression in colorectal cancer (CRC) correlates with reduced overall survival. Kaplan–Meier survival curves were generated to examine overall survival of patients with CRC with either high or low VASH1 expression; p=0.03, log-rank test.*

Discussion

In this study, we examined the biological function and clinical significance of VASH1 in CRC. We found that CRC cells with elevated VASH1 exhibited greater metastatic potential through acquisition of enhanced proliferation, invasion, and motility, and reduced anoikis. By IHC analysis of clinical samples, we found that the expression of VASH1 protein in CRC tissues was significantly greater than that in adjacent normal colonic mucosa. In addition, we observed that high VASH1 expression in the cytoplasm was associated with more aggressive clinical behavior, including high T-stage (T3/T4), lymph node metastasis, and distant metastasis of CRC. Because VASH1 was found to be significantly related to the promotion and establishment of metastasis in CRC, these results indicate that it could be a promising therapeutic target.

Angiogenesis is the formation of new blood vessel networks. It plays a role in normal human development and also in pathophysiological conditions such as inflammation and cancer (26). The activities of angiogenesis stimulators are normally countered by angiogenesis inhibitors. The balance between angiogenesis stimulators and inhibitors decides whether

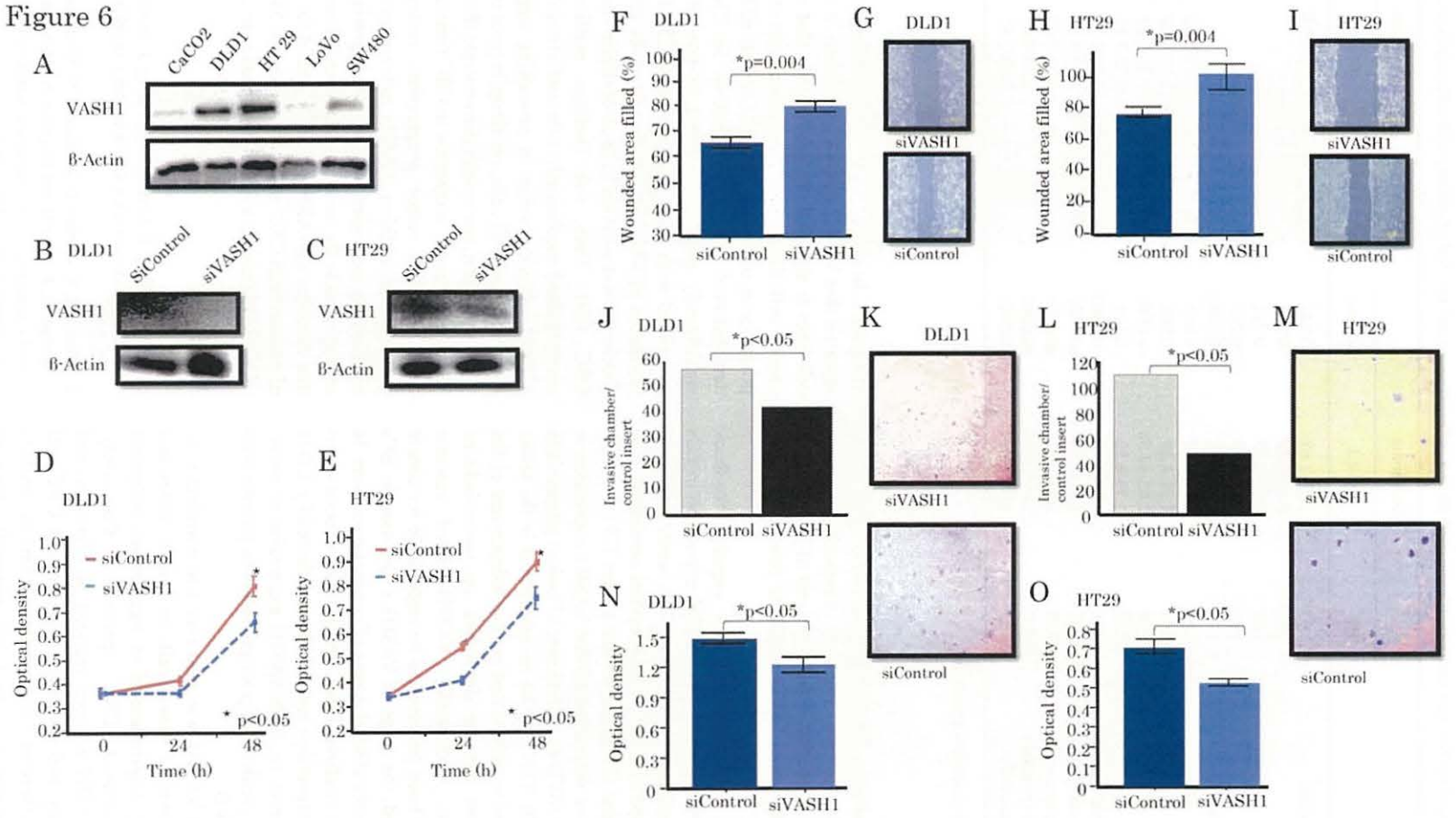

Figure 6. *Effect of Vasohibin-1 (VASH1) silencing on colorectal cancer (CRC) in vitro. A: Western blot analysis was used to determine VASH1 protein expression in five CRC cell lines, as indicated. Western blot analysis was used to validate knockdown of VASH1 protein expression 24 h after transfection with VASH1 siRNA (siVASH1) or control siRNA (siControl) in DLD1 (B) and HT29 (C) cell lines. Proliferation assays were performed using control siRNA-, and VASH1 siRNA-transfected DLD1 (D) and HT29 (E) cell lines ($p<0.05$). Wound healing assays were performed to examine the migratory behavior of control siRNA-, and VASH1 siRNA-transfected DLD1 (F, G) and HT29 (H, I) cells ($p=0.004$). Invasion assays were also performed in identically transfected DLD1 (J, K) and HT29 (L, M) cells ($p<0.05$). The anoikis assay was performed after anoikis induction for 24 h in low-attachment plates, while the MTT assay was performed to calculate the proportion of viable floating DLD1 (N) and HT29 (O) cells ($p<0.05$).*

Table II. *Univariate and multivariate analysis of prognostic factors influencing overall survival of patients with colorectal cancer.*

	Univariate			Multivariate		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
High VASH1 expression	1.65	1.03-2.67	0.034	1.05	0.64-1.75	0.819
MVD (≥39/mm²)	1.48	0.93-2.39	0.095			
Gender (male)	1.06	0.66-1.69	0.782			
Age (≥68 years)	1.31	0.82-2.10	0.243			
Tumor location (rectum)	1.27	0.79-2.02	0.308			
Tumor size (≥4 cm)	1.81	1.12-2.99	0.013	1.24	0.71-2.10	0.435
Histrological grade	2.46	1.31-4.28	0.006	2.26	1.17-4.10	0.016
T-Stage (T3,T4)	5.63	2.65-14.58	<0.0001	2.97	1.12-9.05	0.027
Lymphatic invasion (positive)	2.88	1.46-6.52	0.001	1.44	0.52-3.62	0.462
Venous invasion (positive)	2.54	1.54-4.36	0.0002	1.23	0.69-2.33	0.490
Lymph node metastasis (positive)	4.41	2.69-7.48	<0.0001	1.96	1.07-3.65	0.026
Hepatic metastasis (positive)	9.98	6.06-16.22	<0.0001	4.16	2.24-7.63	<0.0001
Peritoneal metastasis (positive)	12.15	7.00-20.42	<0.0001	4.54	2.26-9.07	<0.0001
Distant metastasis (positive)	6.22	3.69-10.24	<0.0001	1.36	0.69-2.61	0.365

HR, Hazard ratio; CI, confidence interval;

angiogenesis is induced. The histological MVD technique is the current gold-standard used to characterize tumor angiogenesis (27). Recently, a meta-analysis of all-published studies showed that high MVD predicted poor survival in patients with CRC (24).

VASH1 is an endothelium-derived negative feedback regulator of angiogenesis (13, 14). VASH1 expression in ECs has been significantly correlated with MVD, tumor grade, and the histological type of carcinoma in cervical carcinoma (21), renal cell carcinoma (28), and prostate cancer (29). Notably, several reports have demonstrated that VASH1 expression in ECs is superior to MVD as an indicator of patient prognosis in cancer (21, 28, 29). This may be because MVD in the tumor environment includes vessels that are not independent of the tumor environment but are dependent on tumor-induced neovascularization. Additionally, VASH1-related vascular mechanisms have been proposed to be responsible for cancer aggression. Indeed, the level of VASH1 expression in ECs effectively reflects the clinical course of cancer progression. In our study, we also confirmed that a positive correlation exists between VASH1 expression and MVD, as reflected by CD31 expression. Furthermore, high VASH1 expression in tumor cells was a better predictor of poor prognosis in patients with CRC than was MVD.

Tumor-derived VASH1 was also found to correlate with increased metastasis of tumor cells to lymph nodes and distant organs by acquisition of an aggressive, malignant phenotype in clinical CRC specimens. Conversely, knockdown of *VASH1 in vitro* reduced proliferation, and impaired migratory and invasive capabilities of CRC cell lines. These data, therefore, suggest that VASH1 is involved in driving the malignancy in CRC, especially through metastasis. In agreement with our findings, Shen *et al.* have reported that *VASH1* mRNA expression in metastatic cancer cell lines is significantly greater than that in non-metastatic cancer cell lines, and that it has important roles in the progression of gastric cancer (30). In addition, Yan *et al.* demonstrated that *VASH1* mRNA in CRC cell lines is significantly greater than that in normal tissues, and is associated with malignant potential (23). Furthermore, up-regulation of *VASH1* expression in tumor cells was related to shorter patient survival in hepatocellular carcinoma (22) and CRC (23). Thus, our findings confirm a previously unrecognized mechanistic role and clinical significance of VASH1 overexpression in promoting aggressiveness and metastases of CRC cells, resulting in poor prognosis.

In conclusion, our results demonstrate that up-regulation of VASH1 expression is common in CRC tissues and significantly correlates with tumor progression, metastasis, and poor prognosis. In addition, VASH1 expression in tumor cells is significantly associated with MVD, suggesting that it increases angiogenesis in the tumor environment. Therefore, we conclude that detection of VASH1 could be useful for the identification of patients with CRC with poor prognosis. Moreover, targeting of VASH1 could be a useful therapeutic strategy in CRC.

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