

Clinical implications of vasohibin-1 in esophageal carcinoma cells: Inhibition of cell growth and migration

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Abstract. As one of the first-established negative feedback regulators of angiogenesis, mesenchymal vasohibin-1 (VASH1) serves important roles in the progression and prognosis of various types of tumor. However, the clinical implications of VASH1 in esophageal carcinoma (EC) cells have not been reported and the direct effects of VASH1 on EC cells remain unknown. In the present study, the expression of VASH1 in EC cells was observed using immunohistochemistry and western blotting; a χ^2 test was used to analyze the correlation of VASH1 with clinical parameters, and it was observed that VASH1 was negatively-correlated with tumor size ($r=-0.399$; $P<0.01$) and invasion depth ($r=-0.318$; $P<0.01$). Survival analysis demonstrated that VASH1 was positively-correlated with increased overall survival ($P=0.039$) and disease free survival ($P=0.012$). The direct effects of VASH1 on EC cells were analyzed by altering VASH1 expression, and it was observed that downregulation of VASH1 increased proliferation, clone formation and the migratory ability of EC9706 cells, whereas upregulation of VASH1 inhibited proliferation, clone formation and the migratory ability of EC1 cells. The results of the present study demonstrated that VASH1 in EC cells was negatively-correlated with progression and poor prognosis of patients with EC. VASH1 was able to directly inhibit the growth and migration of EC cells.

Introduction

Esophageal carcinoma (EC) is among the most common types of malignancy worldwide, with the 8th highest incidence, and is the 6th leading cause of cancer-associated mortality (1-3). Despite the development of a combination of surgery, radiotherapy and chemotherapy, the prognosis for EC remains poor

due to the insidious symptomatology, late clinical presentation and rapid progression (4,5). Therefore, it is necessary to understand the pathophysiological mechanisms underlying the progression of EC and to elucidate novel targets which are able to inhibit the malignant processes, so as to provide an improved prognosis for patients with EC.

Vasohibin-1 (VASH1) is a member of the vasohibin family. Previous studies have reported that VASH1 is selectively expressed in endothelial cells, and is able to inhibit angiogenesis by regulating endothelial cell death and biological functions (6-8). Cumulative evidence has indicated that VASH1 in the tumor mesenchyme may function as a molecular marker for prognosis in numerous types of cancer, including breast cancer (9), renal carcinoma (10), hepatocellular cancer (11) and prostate cancer (12). Previous studies have demonstrated that VASH1 is not restricted to endothelial cells, and is additionally expressed in other types of cells, including tumor cells (13,14). However, the expression of VASH1 in EC cells and the relevance of this to clinical parameters and prognosis remains unknown. The direct effect of VASH1 on the biological behaviors of EC cells has not been reported.

The present study demonstrated the expression of VASH1 in EC cells using immunohistochemistry (IHC) and western blotting, and it was observed that increased VASH1 expression was negatively correlated with tumor size, invasion depth and poor prognosis. Following alteration of VASH1 expression in EC cells using transfection, it was observed that VASH1 markedly inhibited the proliferation, clone formation and migration of EC cells. The results of the present study demonstrated the suppressive effects of VASH1 in EC and provide evidence of a novel target to inhibit EC progression.

Materials and methods

Cell lines and culture. EC cell lines EC1 and EC9706 were purchased from the American Type Culture Collection (Manassas, VA, USA). EC1 and EC9706 cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

IHC. Approved by the Review Board and Ethics Committee of Yishui Central Hospital of Linyi (Linyi, China),

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100 primary EC specimens obtained between January 2006 and January 2009 were selected from the hospital. No chemotherapy, radiotherapy or immunotherapy had been performed prior to surgery. Patient characteristics are presented in Table I.

Paraffin-embedded tissue samples were cut into 4- μ m slices. Then, the slides were kept at 60°C for 30 min, de-paraffinized in xylene at room temperature and rehydrated in graded ethanol. Following antigen unmasking, the sections were immersed in 3% H₂O₂ for 10 min to inhibit the endogenous peroxidase and in goat serum blocking solution (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) at room temperature for 15 min to block non-specific antigens. Following incubation at room temperature for 2 h with primary antibody against VASH1 (1:600; Abnova, Taipei, Taiwan), the sections were incubated in horseradish peroxidase goat anti-rabbit/mouse immunoglobulin G polymer (cat. no. KIT-7710; Fuzhou Maixin Biotech Co., Ltd.) at room temperature for 30 min. The slides were stained with 3,3'-diaminobenzidine for 5-10 sec and counterstained with hematoxylin for 20 sec at room temperature. The staining assessment was performed by two independent pathologists simultaneously at x400 magnification using an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan). The proportion score represented the estimated fraction of positive staining tumor cells (0, \leq 25%; 1, 26-50%; 2, 51-75%; 3, >75%). The intensity score represented the estimated average staining intensity of positive tumor cells (0, negative; 1, weak; 2, moderate; 3, strong). The expression level of VASH1 was evaluated using the product of the proportion score and intensity score at five fields and the mean value was obtained (low expression, \leq 4; high expression, >4). The overall survival (OS) period was defined as the period of time from surgery to cell death induced by different factors. Disease-free survival (DFS) was defined as the period of time from surgery to the recurrence or progression of disease.

Transfection. Plasmid pEZ-M61/VASH1 (Shanghai Gene Pharma Co., Ltd, Shanghai, China) was transfected into EC1 cells to upregulate VASH1 expression; p-GPU6/VASH1-sh1/2 (Shanghai GenePharma Co., Ltd) was transfected into EC9706 cells to downregulate VASH1 expression. All procedures were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and empty vectors were used as a control. Cells were harvested 48 h subsequently and transfection efficiency was determined using reverse transcription-quantitative polymerase chain reaction and western blotting. The two shRNA sequences used for silencing VASH1 were as follows: shRNA1, 5'-AGCGCTACATCAGAGAGCTGCAGTA-3'; shRNA 2, 5'-CCTACTTCTCAGGGAAGTACT-3'.

RT-qPCR. Cells were collected and total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized as a template using PrimeScript RT-PCR kit (Takara Biotechnology Co. Ltd., Dalian, China), according to the manufacturer's protocol. The qPCR was performed using specific primers and Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) with a CFX96 Touch Deep Well™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following thermocycling conditions: VASH1; 4°C for 5 min, then 40 cycles of 94°C for

30 sec, 57°C for 30 sec and 72°C for 30 sec, followed by 72°C for 5 min; the same thermocycling conditions were applied for GAPDH, however, 36 cycles were performed instead of 40. mRNA levels were normalized to GAPDH. The primers were as follows: VASH1: forward, 5'-CAAGGACCGGAAGAA GGAUGUUUCU-3' and reverse, 5'-CAACCAAGGAGAGGA GUAUUGGUCU-3'; and GAPDH: forward, 5'-AGAAGGCTG GGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCACAG TCTTC-3'. The 2^{- $\Delta\Delta$ Cq} method was used for quantification (15). The experiment was performed \geq 3 times.

Western blot. Protein was extracted from cells using radioimmunoprecipitation lysis buffer containing 1% PMSF (Beyotime Institute of Biotechnology, Haimen, China) and the concentration was detected using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 300 mg/well protein was loaded into a 5% SDS-PAGE gel and then separated by a 10% separating gel and transferred to polyvinylidene difluoride membrane. Following blocking in TBS with Tween-20 containing 5% non-fat dried milk for 1 h at room temperature, the membrane was incubated in primary antibodies of VASH1 (1:1,000) and GAPDH (cat. no. 10494-1-AP; 1:5,000, Proteintech Group, Inc., Wuhan, China) at 4°C overnight. Following incubation with horseradish peroxidase-conjugated secondary antibody (cat. no. SA00001-2; 1:5,000, Proteintech Group, Inc.) at room temperature for 1 h, the protein on the membrane was detected using a chemiluminescence solution (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions, and measured using Image-Pro software (version 5.1; Media Cybernetics, Inc., Rockville, MA, USA).

Viability assay. An MTT assay was used to detect viability. Cells were counted and plated in 96-well plates in triplicate at a starting number of 3x10³ cells/well. The absorbance of each sample was measured at 490 nm for 5 days using MTT (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. The experiment was repeated 3 times.

Clone formation assay. Cells were plated in 6-well plates at 1,000 cells/well. After 2 weeks, cell colonies were stained using giemsa at room temperature for 15 min (Beijing Solarbio Science and Technology Co., Ltd.) and counted under a light microscope (magnification, x200; >50 cells as one clone; XDS-200; Olympus Corporation). The experiment was repeated three times.

Migration assay. A total of 1x10⁵ cells were plated in the upper chambers of Transwell plates in RPMI-1640 without FBS. RPMI 1640 supplemented by 20% FBS was plated in the lower chamber. Following incubation for 24 h, migrated cells were fixed using 100% methanol at room temperature for 30 min, stained with 0.1% crystal violet at room temperature for 25 min and counted in five random fields at x200 magnification.

Statistical analysis. All data are expressed as the mean \pm standard error of the mean and SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. The differences between two groups were analyzed

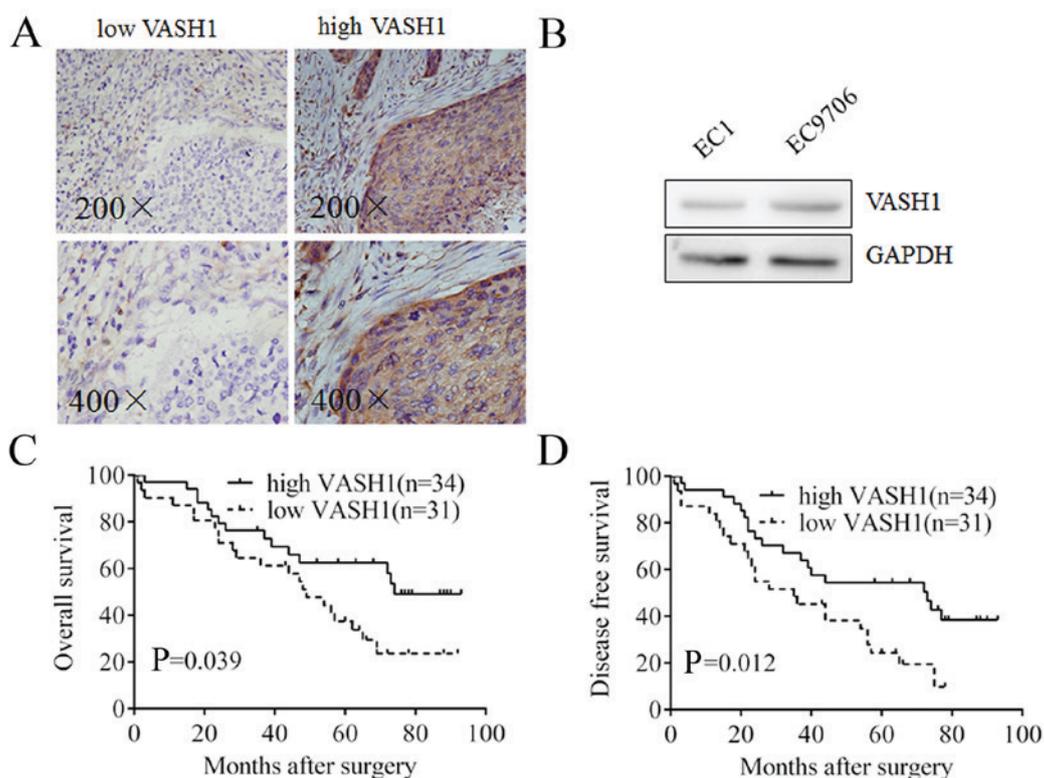


Figure 1. Expression of VASH1 in EC cells. (A) VASH1 expression in EC tissues demonstrated by immunohistochemical staining. (B) VASH1 expression in EC cells demonstrated by western blot analysis. (C) The overall survival period of patients with high VASH1 expression was increased compared with patients with low VASH1 expression. (D) The disease-free survival period of patients with high VASH1 expression was increased compared with patients with low VASH1 expression. VASH1, vasohibin 1; EC, esophageal carcinoma.

using the Student's two-tailed t-test. The associations between the expression of VASH1 and clinical parameters were analyzed using Pearson's χ^2 test. Survival curves were drawn using the Kaplan-Meier estimator method and compared by means of the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Increased VASH1 expression in EC cells is negatively correlated with tumor size, invasion depth and poor prognosis. In order to detect VASH1 expression in EC tissues, IHC was performed and it was observed that VASH1 was expressed in the cytoplasm of EC cells with different staining intensities; sporadic staining was observed in the mesenchyme (Fig. 1A). Different expression levels of VASH1 were observed in EC1 cells and EC9706 cells using western blotting (Fig. 1B). Due to the important roles of mesenchymal VASH1 in tumor progression, the correlation between VASH1 in EC cells and clinical parameters was analyzed. As presented in Table I, in the VASH1 high expression group, 12/42 cases exhibited a tumor size of ≥ 5 cm; in the VASH1 low expression group, 40/58 cases exhibited a tumor size of ≥ 5 cm ($P < 0.001$). In the VASH1 high expression group, 14/42 cases were at T3/4 stage; in the VASH1 low expression group, 38/58 cases were at T3/4 stage ($P = 0.001$). Survival analysis demonstrated that the OS period of VASH1 high expression group was increased compared with the VASH1 low expression group ($P = 0.039$; Fig. 1C); additionally, the DFS period of the VASH1 high

Table I. Correlation of VASH1 with clinical parameters of patients with esophageal carcinoma.

| Characteristic | VASH1 | | r | χ^2 | P-value |
|----------------|-------|------|--------|----------|--------------------|
| | Low | High | | | |
| Sex | | | | | |
| Male | 28 | 27 | -0.159 | 2.523 | 0.112 |
| Female | 30 | 15 | | | |
| Age, years | | | | | |
| <60 | 35 | 26 | -0.016 | 0.025 | 0.875 |
| ≥ 60 | 23 | 16 | | | |
| Tumor size, cm | | | | | |
| <5 | 18 | 30 | -0.399 | 15.925 | <0.01 ^a |
| ≥ 5 | 40 | 12 | | | |
| Invasion depth | | | | | |
| Tis, T1, T2 | 20 | 28 | -0.318 | 10.109 | <0.01 ^a |
| T3+T4 | 38 | 14 | | | |
| Metastasis | | | | | |
| Absent | 32 | 22 | 0.028 | 0.076 | 0.782 |
| Present | 26 | 20 | | | |
| Clinical stage | | | | | |
| I-IIa | 27 | 18 | 0.037 | 0.134 | 0.714 |
| IIb-IV | 31 | 24 | | | |

^a $P < 0.01$. VASH1, vasohibin 1.

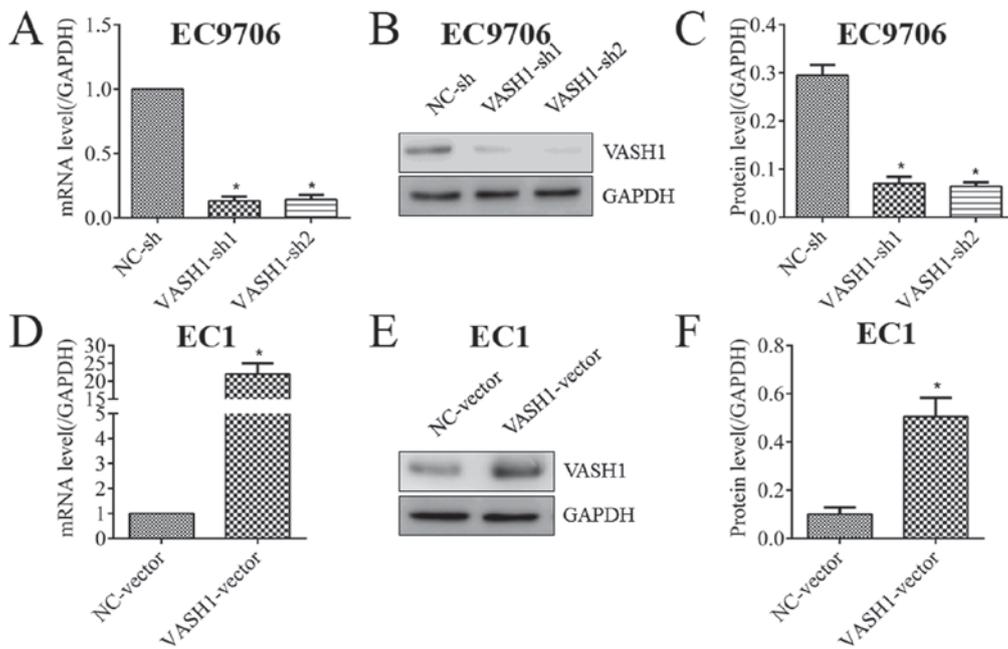


Figure 2. VASH1 expression level following transfection. (A) The VASH1 mRNA level was downregulated by pEZ-M61/VASH1 in EC9706 cells. (B) The VASH1 protein level was downregulated by pEZ-M61/VASH1 in EC9706 cells. (C) Histogram of the results of the downregulation of VASH1 protein expression by pEZ-M61/VASH1. (D) The VASH1 mRNA level was upregulated by p-GPU6/VASH1 in EC1 cells. (E) The VASH1 protein level was upregulated by p-GPU6/VASH1 in EC1 cells. (F) Histogram of the results of the upregulation of VASH1 protein expression by p-GPU6/VASH1. Error bars represent the standard error of three independent experiments. *P<0.05 vs. control. NC, normal control; sh, short hairpin; VASH1, vasohibin 1.

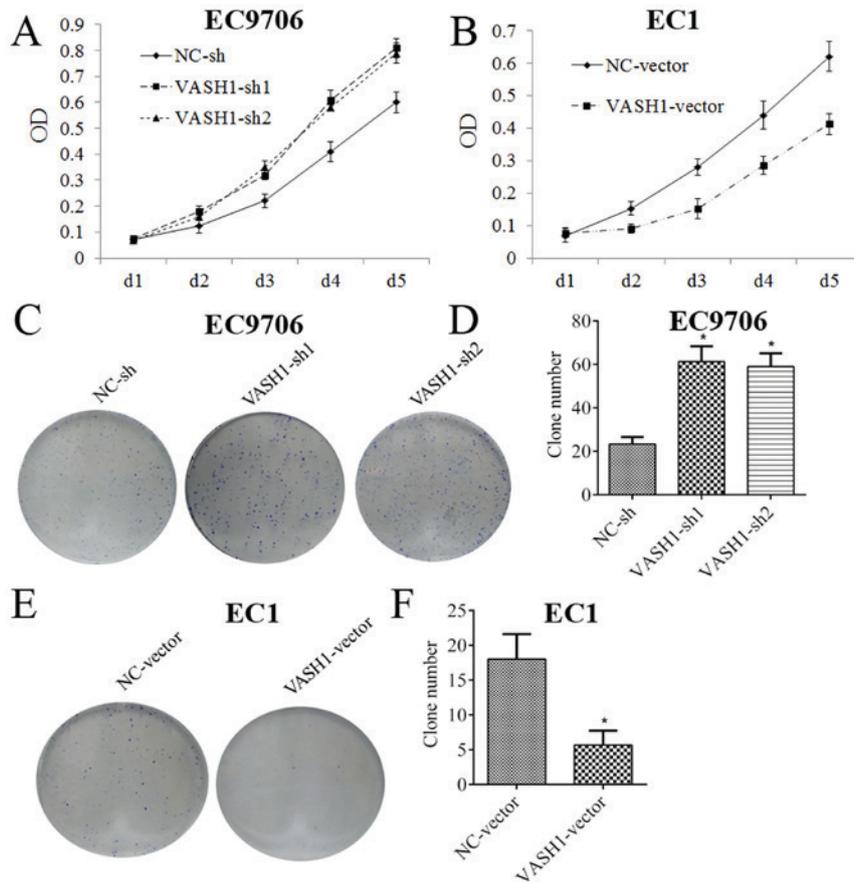


Figure 3. VASH1 inhibits the proliferation of esophageal carcinoma cells. (A) Growth rate was increased in EC9706 cells following downregulation of VASH1. (B) Growth rate was decreased in EC1 cells following upregulation of VASH1. (C) Clone formation ability was increased in EC9706 cells following downregulation of VASH1. (D) Histogram of the results of the clone formation assay in EC9706 cells. (E) Clone formation ability was decreased in EC1 cells following upregulation of VASH1. (F) Histogram of the results of the clone formation assay in EC1 cells. Error bars represent the standard error of three independent experiments. *P<0.05 vs. control. VASH1, vasohibin 1; NC, normal control; sh, short hairpin.

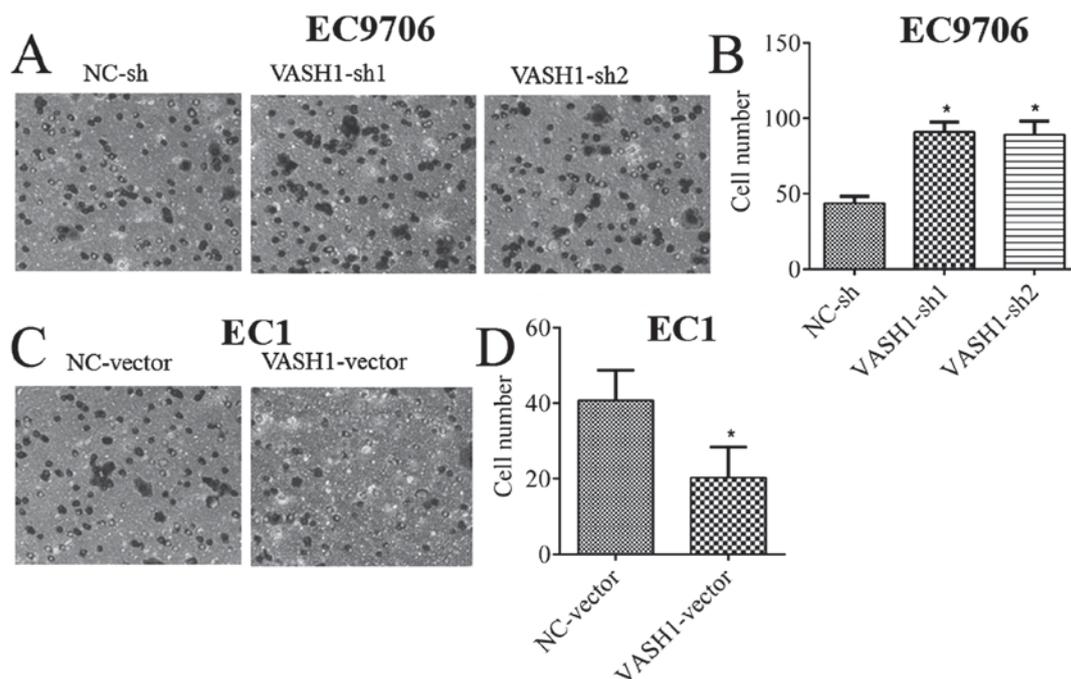


Figure 4. VASH1 inhibits the migration of esophageal carcinoma cells (A) Migratory ability was increased in EC9706 cells following downregulation of VASH1. (B) Histogram of the results of the migration assay in EC9706 cells. (C) Migratory ability was decreased in EC1 cells following upregulation of VASH1. (D) Histogram of the results of the migration assay in EC1 cells. Error bars represent the standard error of three independent experiments. * $P < 0.05$ vs. control. VASH1, vasohibin 1; NC, normal control; sh, short hairpin.

expression group was increased compared with the VASH1 low expression group ($P = 0.012$, Fig. 1D). The results of the present study confirmed that VASH1 in EC cells exhibited a negative correlation with progression and poor prognosis for patients with EC.

VASH1 inhibits the proliferative ability of EC cells. In order to study the direct effect of VASH1 on EC cells, the expression of VASH1 in EC cells was altered using transfection. As presented in Fig. 2, VASH1 in EC9706 cells was successfully downregulated at the RNA (Fig. 2A) and protein (Fig. 2B and C) levels; VASH1 in EC1 cells was successfully upregulated at the RNA (Fig. 2D) and protein (Fig. 2E and F) levels. MTT and clone formation assays are presented in Fig. 3. Following downregulation of VASH1 in EC9706 cells, the MTT assay demonstrated that the growth rate increased markedly (Fig. 3A); the clone formation assay demonstrated that clone number increased from 23.33 ± 3.21 to 61.33 ± 7.02 or 59.00 ± 6.08 ($P < 0.05$; Fig. 3C and D). Following upregulation of VASH1 in EC1 cells, the MTT assay demonstrated that growth rate decreased markedly (Fig. 3B); the clone formation assay demonstrated that clone number decreased from 18.00 ± 3.60 to 5.66 ± 2.08 ($P < 0.05$; Fig. 3E and F). The results of the present study demonstrated that VASH1 was able to directly inhibit the growth of EC cells.

VASH1 inhibits the migration of EC cells. Migratory ability is an important biological behavior of tumor cells and serves important roles in cancer invasion and metastasis (16,17). In order to detect the direct effect of VASH1 on the migratory ability of EC cells, a migration assay was performed; it was demonstrated that, following downregulation of VASH1 in

EC9706 cells, the number of migratory cells increased from 43.66 ± 4.51 to 91.00 ± 6.55 or 89 ± 9.16 ($P < 0.05$; Fig. 4A and B). Following upregulation of VASH1 in EC1 cells, the number of migratory cells decreased from 40.66 ± 8.08 to 20.33 ± 8.02 ($P < 0.05$; Fig. 4C and D). The results of the present study demonstrated the negative regulatory effect of VASH1 on the migratory ability of EC cells.

Discussion

The human VASH1 gene is located on chromosome 14q24.3, including 8 exons and 7 introns. Human VASH1 protein is composed of 365 amino acids with no glycosylation sites (18,19). As an acknowledged negative feedback regulator of angiogenesis, the anti-tumor function of mesenchymal VASH1 has been widely reported in multiple types of cancer (7,20,21). Kosaka *et al* (12) observed that VASH1 expression was restricted in the mesenchyme and negatively correlated with advanced clinical stage and poor recurrence-free survival of prostate cancer patients. Tamaki *et al* (9) reported that VASH1 was detected in human breast cancer endothelial cells and positively correlated with poor OS and DFS of patients. VASH1 expression has additionally been detected in parenchymal cells of several types of tumor. However, the roles of parenchymal VASH1 in cancer remains controversial. Zhao *et al* (10) reported that VASH1 was expressed primarily in the cytoplasm and on the membrane of renal cell carcinoma cells, and exhibited a negative correlation with tumor malignancy. In hepatocellular carcinoma, Wang *et al* (11) demonstrated that increased expression of VASH1 in the cytoplasm was positively-correlated with poor prognosis. In the present study, the expression of VASH1 in EC cells was

observed using IHC and western blotting, and the results of the present study are consistent with the distribution of VASH1 in renal cell carcinoma and hepatocellular carcinoma reported by Zhao *et al* (10) and Wang *et al* (11). Further analysis demonstrated that VASH1 in EC cells was negatively correlated with tumor size, invasion depth and poor prognosis (OS and DFS); this is in contrast with the study of Wang *et al* and suggests that VASH1 exhibits anti-tumor effects in EC cells. Only 100 primary EC specimens were used in the present study; due to this limitation, the results of the present study may not completely reflect the clinical implications of VASH1 in EC.

Initial studies hypothesized that the anti-tumor effects of VASH1 were primarily mediated by inhibition of angiogenesis. The potential direct effects of VASH1 on tumor cells remain to be elucidated. Increased growth and migratory abilities are classical biological behaviors of malignant tumors, and are crucial for tumor occurrence, invasion, metastasis and prognosis (22-25). Conflicting reports about the direct effects of VASH1 on biologic behaviors of tumor cells have been published. Watanabe *et al* (7) reported that, although overexpression of VASH1 in lung cancer cells successfully inhibited angiogenesis *in vivo*, no effects on the proliferative ability of cancer cells were observed *in vitro*. By contrast, Liu *et al* (26) demonstrated that overexpression of VASH1 in colorectal cancer cells was able to inhibit growth and migration. Due to the results of the IHC in the present study, demonstrating that VASH1 was negatively correlated with tumor size and invasion depth, it was hypothesized that VASH1 may affect the growth and migration of EC cells directly. In order to examine this hypothesis, VASH1 expression in EC cells was altered using transfection and it was observed that overexpression of VASH1 inhibited proliferation, clone formation and the migratory ability of EC1 cells; however, silencing VASH1 enhanced proliferation, clone formation and the migratory ability of EC9706 cells. The results of the present study demonstrated the direct inhibitory effects of VASH1 on the growth and migration of EC cells, consistent with the inhibition of VASH1 on colorectal cancer cells reported by Liu *et al* (26). The results of the present study suggest that the negative correlation of VASH1 with EC progression and prognosis may be partially attributed to a direct effect on EC cells.

However, tumor growth is a complex process and may be regulated by various events, including proliferation, cell cycle progression, apoptosis and angiogenesis (27,28). Apart from proliferation and angiogenesis, Liu *et al* (26) has also observed that VASH1 was able to effectively promote apoptosis and senescence in CRC cells. However, apoptosis and senescence of EC cells were not investigated in the present study. Tumor invasion is a process which may be affected by the motility of tumor cells and degradation of the extracellular matrix by proteases produced in tumor cells (29,30). Although the results of the present study demonstrated the inhibitory effect of VASH1 on EC cell migration, this is insufficient to explain the negative correlation of VASH1 with EC invasion depth. It is possible that VASH1 may additionally affect the production of certain types of protease, including matrix metalloproteinase or collagenase, in EC cells; further research is required to investigate this hypothesis.

In conclusion, VASH1 in EC cells was negatively correlated with tumor size, invasion depth and poor prognosis.

VASH1 is able to prevent EC progression by anti-angiogenesis, and additionally through direct inhibition of the growth and migration of EC cells. The results of the present study increase the understanding of VASH1 in the context of tumors and contribute to the search for improved treatments for EC.

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