Vasohibin-1 Is a Poor Prognostic Factor of Ovarian Carcinoma

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Vasohibin-1 (VASH1) is an identified negative feedback inhibitor of angiogenesis induced by vascular endothelial growth factor (VEGF) in vascular endothelial cells (ECs). Expression of VASH1 has been reported not only in ECs of normal tissue, but also in ECs surrounding malignant tumors. In malignant tumors, VASH1 is also gaining attention as a prognosis prediction marker. The aim of this study is to investigate the correlation between VASH1 expression and vascular-related factors and various clinicopathological outcomes in clinical cases of ovarian carcinoma. We retrospectively analyzed clinical records of 58 patients with ovarian carcinoma. The expression patterns of VASH1 and other vascular-related factors (CD31 as markers of microvessel density (MVD), VEGF receptor type 2 (VEGFR2), D2-40 as markers of lymphovessel density), and Ki67 (as proliferation markers of cancer cells) were examined immunohistochemically. We studied the correlation between immunohistochemical expression and overall survival. VASH1 expression pattern significantly differed between Federation of Obstetrics and Gynecology (FIGO) Stages. Numbers of VASH1-positive vessels had a significant positive correlation with MVD (Speaman's correlation coefficient (ρ) was 0.51, p < 0.001), VEGFR2-positive vessels (ρ = 0.61, p < 0.001), and percentage of Ki67 (ρ = 0.28, p = 0.034). The Cox univariable analyses revealed that the group of high VASH1 expression (> 14.6 vessels per mm^2) at Stages I-III is a prognostic factor (HR = 3.3, 95%CI = 0.4-8.4; p = 0.013). Our results indicate that VASH1 expression in ovarian carcinoma is significantly associated with vascular-related factors and Ki67 expression. We propose that VASH1 is a prognostic marker in ovarian carcinoma.

Introduction

Ovarian carcinoma is one of the most frequent causes of cancer death in women, and the number of deaths is expected to increase. More than 50% of patients with ovarian carcinoma were diagnosed in an advanced stage (Heintz 1988; Heintz et al. 2006). A combination of debulking surgery and multidrug chemotherapy is standard therapy for advanced stage carcinomas (Winter et al. 2007, 2008; Bookman et al. 2009; Elattar et al. 2011). Recently, vascular endothelial growth factor (VEGF) humanized monoclonal antibody therapy was combined with conventional chemotherapy in ovarian carcinoma to target a specific molecular pathway. Angiogenesis is attracting much attention as a new treatment strategy of ovarian carcinoma (Burger et al. 2011; Perren et al. 2011; Aghajanian et al. 2012). Vasohibin-1 (VASH1) is a negative feedback regu-

lator of angiogenesis. It was identified in VEGF-stimulated vascular endothelial cells (ECs) (Sato 2012). VASH1 was subsequently demonstrated to be specifically expressed in ECs in response to angiogenetic stimulators such as VEGF and basic fibroblastic growth factor (bFGF) (Watanabe et al. 2004; Sonoda et al. 2006). Previous analysis of samples of various tissues revealed that vasohibin was expressed in the brain, heart and kidney (Watanabe et al. 2004). Moreover, vasohibin expression was observed in ECs of human placenta and various developing organs of the human embryo (Watanabe et al. 2004), but it is reduced in expression in the post-neonate (Shibuya et al. 2006). Another report showed by *in situ* hybridization that VASH1 mRNA is expressed in a wide range of tissues and organs in the chicken embryo and suggested that the expression of VASH1 might not be limited to ECs (Nimmagadda et al. 2007), although the significance of these expression is

unclear. Furthermore, VASH1 is not only expressed in normal tissue, but VASH1 expression in vascular ECs around malignant tumors has also been documented in various carcinomas. In addition to expression in malignant tumors, the relationship to prognosis, tumor growth, metastasis, and more has been examined; VASH1 is gaining attention as a new target or biomarker for predicting prognosis (Li et al. 2010; Chan et al. 2012; Coch et al. 2014). For example, current studies report cases of poor prognosis having high expression of VASH1 in breast carcinoma and non-small cell lung carcinoma (NSCLC) (Hosaka et al. 2009; Tamaki et al. 2009). On the other hand, in renal cell carcinoma, VASH1 expression was associated with long progression-free survival (PFS) (Kanomata et al. 2013). These reports show the correlation of expression of VASH1 and different prognosis between organs. In ovarian carcinoma, VASH1 was identified to inhibit tumor vascularization and growth in *in vivo* and *in vitro* tumor cell line experiments (Takahashi et al. 2015, 2016). In addition, high VASH1 expression prolonged host survival in those studies.

The aim of our study is to examine the correlation of VASH1 expression and clinicopathological factor and prognosis in human ovarian carcinoma tissue. In addition, we investigated the correlation of VASH1 expression and another angiogenetic factor (VEGF receptor 2 (VEGFR2), microvessel density (MVD), and lymphatic vessel density (LVD)) and Ki67 as markers which are closely linked to cancer cell proliferation by immunohistochemistry.

Materials and Methods

Patients of this study were operated on between 1990 and 2016 at the Kawasaki Medical School Hospital (Okayama, Japan). A total of 58 tumors with available prognostic dates were obtained from surgical pathology files. Staging was based on final pathological findings and determined according to the 2014 Federation of Obstetrics and Gynecology (FIGO) classification system. All Japanese patients with FIGO stage I-IV tumors underwent primary cytoreductive surgery. In order to evaluate local invasion (pT factor), lymph node metastasis (pN factor) and distant metastasis (M factor), TNM classification (Union for International Cancer Control 7th edition) was employed.

This study was a retrospective study. An appropriate informed consent was obtained from each patient and approved by the Research Ethics Committee of Kawasaki Medical School and Hospital.

The paraffin blocks were extracted and thin 5 μm sections of the tumors were cut and placed on Matsunami Adhesive Slide coated glass slides (Matsunami, Osaka, Japan). After deparaffinization and hydration, hot-bath antigen retrieval was performed at 95℃ for 40 minutes in Target Retrieval solution pH 9.0 (Dako, Glostrup, Denmark) for CD31, Ki67, and vasohibin-1, and citrate buffer for D2-40 and VEGFR2. The antibodies used are listed in Table 1. The signal was visualized with EnVision Plus (Dako). The chromogen used was 3,3-diaminobenzidine-tetrachloride and counterstained with hematoxylin for nuclear staining.

Antihuman vasohibin-1 monoclonal antibody was raised against the synthetic fragment (Gly286-Arg299) of human vasohibin-1 as described by Watanabe et al. (2004). The VASH1-positive vessels of tumors were determined by vascular ECs, which distinctly stained. Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single vessel. The densest area ('hot spot') of antibody-positive vessels was chosen by scanning power, and staining vessels were counted under a 20× objective lens (0.785 mm², BH-2, Olympus, Tokyo, Japan). The presence of a visible blood vessel lumen was not required for the vessel to be defined as positive. We evaluated three areas. The average of the three areas of immunostaining vessels was evaluated. We regarded the number of VASH1-positive signals per mm² as 'VASH1 expression'.

The MVD of the tumors was determined by CD31 (as markers of vascular ECs) immunohistochemistry. The LVD was determined by D2-40 (as markers of lympovascular ECs). VEGFR2 was immunostained by Flk-1. These were counted in the same manner as VASH1 expression. Ki67 expression (as nuclear located protein during all active phases of the cell cycle) was counted as the percentage of immunoreactive nuclears per 1,000 cancer cells.

Numbers of median stained vessels in each VASH1, MVD, VEGFR2, D2-40 were divided into high expression and low expression groups. Ki67 was divided into 2 groups, a high expression group and a low expression group, according to the median percentage of stained cells. Furthermore, statistics were analyzed not only as a continuous variable, but the two groups were also analyzed as categorical data.

Statistical analyses were performed using JMP 7 (SAS Institute,

Table 1. Antibody list in current study.

Antigen	Clone	Supplier	Dilution	Incubation
VASH1	(raised against the synthetic fragment Gly286-Arg299 of human VASH1) (Watanabe et al. 2004)		2µg/ml	4℃ overnight
CD31	JC70-A	Dako, Glostrup, Denmark	1 : 50	4℃ overnight
VEGFR2 (Flk-1)	A-3	Santa Cruz, Calfornia, USA	1 : 100	30 min at room temperature
D2-40	D2-40	Dako, Glostrup, Denmark	1 : 50	30 min at room temperature
Ki67	MIB-1	Dako, Glostrup, Denmark	1 : 50	30 min at room temperature

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

Cary, North Carolina, USA). The χ^2 test, Mann-Whitney test, and Kruskal Wallis test were used to identify significant differences in the frequencies of clinicopathological factors and immunohistochemical results. Spearman's product-moment correlation coefficient was used to study the relationship between multivariables. 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 logistic regression analyses or Cox proportional hazard regression model to analyze the clinicopathological factors and overall survival (OS).

A p value < 0.05 was considered significant.

Results

Of the 58 tumors, 28 were high-grade serous carcinoma, 14 clear cell carcinoma, 6 mucinous carcinoma, 7 endometrioid carcinoma, 2 mixed carcinoma, and 1 squamous cell carcinoma. The follow-up period was 0 to 143 months (median 37 months). Ages ranged from 21 to 77 years (median 51 years). FIGO Stages were as follows: Stage I, 19; Stage II, 7; Stage III, 25; Stage IV, 7. T factor were as follows: pT1, 21; pT2, 8; pT3, 29. Ten cases had lymph node metastasis (pN1). Seven cases had distant metastasis (M1) at the time of operation. Twenty-seven patients were positive for lymphatic vessel invasion. Fifty-three patients had postoperative chemotherapy, 2 patients had postoperative chemotherapy and interval debulking surgery, and 3 patients had no additional postoperative therapy.

The median of VASH1-positive vessels was 14.6 per mm^2 (interquartile range: 4.9 to 21.7), MVD was 19.1 (11.9 to 29.5), LVD was 2.5 (0.4 to 10.0), and VEGFR2 was 4.6 (0.4 to 10.6). The Ki67 positive ratio was 42.7% (32.4 to 59.5). We showed images of VASH1 which classified into high and low expression group according to the median of immunostained vessels (Fig. 1).

VASH1 expression was significantly associated with pT factor (p = 0.031) and FIGO Stage (p = 0.036). It was not associated with age, pN factor, M factor, or lymphatic invasion (Table 2).

VASH1 expression showed a significant positive correlation with MVD (Speaman's correlation coefficient (ρ) was 0.51, p < 0.001), expression of VEGFR2 (ρ = 0.61, p < 0.001) and Ki67 (ρ = 0.28, p = 0.034). Correlation was not observed with LVD.

Patients were tentatively classified into two groups according to the median of VASH1 positive vessels (14.6 vessels per mm^2). We compared immunohistochemical results in two density groups (Table 3). High VASH1 expression was significantly associated with MVD (p < 0.001), expression of VEGFR2 (p = 0.0018) and Ki67 (p = 0.012).

Table 4 shows the immunohistochemistry analysis according to histological subtypes. There is a tendency to have higher expression of VASH1 in serous carcinoma but we could not find a significant difference between histological types. Expression of VEGFR2 (p = 0.020), and Ki67 (p
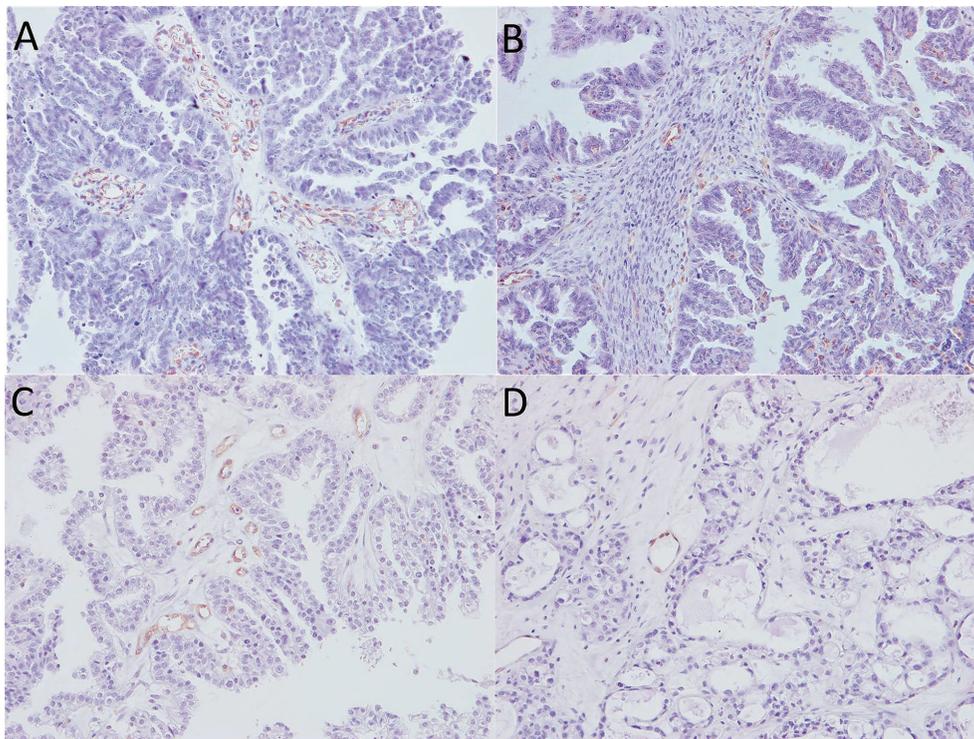

Fig. 1. Immunohistochemistry of VASH1.
Shown are representative images of serous carcinoma (A, B) and clear cell carcinoma (C, D). The cancer specimens shown in A and C are categorized as high expression group (> 14.6 vessels per mm^2), and those in B and D represent low expression group (≤ 14.6 vessels per mm^2). Original magnification of each photo is 200×.

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= 0.024) is significantly associated with histology.

In the Kaplan-Meier analysis, comparisons of patients with low versus high group of VASH1 expression demon-strated that prognosis of the high VASH1 group tended to be poorer than the low VASH1 group but there was no significant difference (p = 0.071) at Stage I-IV. At Stage I-III the high VASH1 group showed significantly poor prognosis (p = 0.015, Fig. 2). Similar to the Kaplan-Meier analysis, the Cox model of overall survival indicated an insignificant correlation at Stage I-IV. At Stage I-III, the high VASH1 group showed significantly poor prognosis in a univariable analysis (HR = 3.3, p = 0.013, Table 5). There was no significant difference in multivariable analysis.

Discussion

This study is a retrospective analysis of the correlation between VASH1 expression and clinicopathological factors and prognosis in patients with ovarian carcinoma. We confirmed that VASH1 expression in human ovarian carcinoma was significantly associated with MVD, VEGFR2 and Ki67. We also found that VASH1 is a prognostic factor of ovarian carcinoma.

Potential roles of angiogenesis have been identified in tumor growth and metastasis (Ueda et al. 2005; Spannuth et al. 2008; Gomez-Raposo et al. 2009; Jayson et al. 2016), suggesting that VASH1 can also be a prognostic factor. In ovarian carcinoma, correlations have been reported between MVD and/or VEGF expression and prognosis (Weidner 1995; Hartenbach et al. 1997; Paley et al. 1997; Nishida et al. 2004), and it is hypothesized that their expression may influence cell sensitivity to chemotherapy (Pyaskovskaya et al. 2007). In this situation, VASH1 was discovered as an endothelium derived anti-angiogenic factor (Sato 2012). Additionally, VASH1 expression not only in ECs of normal tissue but also ECs surrounding malignant tissue has been reported. Until the current time, VASH1 expression has been reported in carcinomas in the mammary gland (Tamaki et al. 2009), large intestine (Liu et al. 2015), lung (Zhang et al. 2014), uterine corpus (Yoshinaga et al. 2008), uterine cervix (Yoshinaga et al. 2011), upper urinary tract (Miyazaki et al. 2012), prostate (Kosaka et al. 2013), and renal cell carcinoma (Kanomata et al. 2013) in humans.

Table 2. Correlation of clinicopathological parameters and VASH1 expression.

	VASH1 median (IQR)	p Value
Age		
< 55	14.0 (4.3-25.1)	0.93
≥ 55	15.7 (6.4-21.2)	
T factor[†]		
1-2	11.5 (3.4-17.2)	0.031[*]
3	17.4 (6.8-25.5)	
N factor[†]		
0	14.4 (4.5-20.0)	0.66
1	15.3 (3.8-30.3)	
M factor[†]		
0	14.9 (5.1-22.9)	0.77
1	6.4 (3.8-21.2)	
Stage[‡]		
I-II	11.7 (2.8-17.1)	0.036[*]
III-IV	17.0 (6.5-25.7)	
Ly[†]		
negative	15.7 (4.2-20.4)	0.58
positive	11.0 (3.8-17.4)	

IQR, interquartile range; Ly, lymphatic invasion; VASH1, vasohibin-1; *p < 0.05; [†], TNM classification (Union for International Cancer Control 7th edition); [‡], The 2014 Federation of Obstetrics and Gynecology (FIGO) classification.

Table 3. Immunohistological results according to the level of the VASH1 expression.

	Low VASH1 n = 29	High VASH1 n = 29	p Value
MVD	14 (6.2-21.4)	28 (17.2-38.6)	< 0.001[*]
VEGFR2	2.1 (0-5.7)	9.3 (3.4-14.6)	0.0018[*]
LVD	2.1 (0-8.5)	3.0 (0.4-11.0)	0.41
Ki67	35.7 (19.1-51.1)	50.2 (34.9-63.2)	0.012[*]

Results other than Ki67 are expressed as median (interquartile range) of each antibody-positive vessels. Ki67 results are the percentage of immunoreactive cells per 1,000 cancer cells.
LVD, lymphatic vessel density; MVD, microvessel density; VASH1, vasohibin-1; VEGFR2, vascular endothelial growth factor receptor 2; *p < 0.05.

Table 4. Immunohistological results according to histological type.

Variable	Overall	Serous	Clear	Mucinous	Endometrioid	Others	p Value
	n = 58	n = 28	n = 14	n = 6	n = 7	n = 3	
VASH1	14.6 (4.9-21.7)	16.7 (8.1-24.1)	11.3 (3.4-22.3)	11.3 (0-18.9)	6.8 (2.1-22.9)	18.7 (17.0-25.9)	0.28
MVD	19.1 (11.9-29.5)	21.7 (13.9-37.7)	17.6 (4.6-28.2)	13.8 (6.5-35.6)	14.0 (9.8-28.0)	18.2 (11.9-38.6)	0.47
LVD	2.5 (0.4-10.0)	2.5 (0.4-9.3)	1.3 (0.4-9.9)	10.0 (4.1-25.3)	2.5 (0-5.5)	3.0 (0.4-19.1)	0.38
VEGFR2	4.6 (0.4-10.6)	7.4 (3.2-14.9)	3.6 (0.6-7.1)	0.8 (0-3.1)	0.4 (0-8.5)	7.2 (0-10.6)	0.020*
Ki67	42.7 (32.4-59.5)	50.1 (34.1-66.2)	40.8 (13.8-49.6)	30.6(24.4-34.0)	50.3(43.1-71.1)	65.0 (35.5-68.9)	0.024*

Results other than Ki67 are expressed as median (interquartile range) of each antibody-positive vessels. Ki67 results are the percentage of immunoreactive cells per 1,000 cancer cells.

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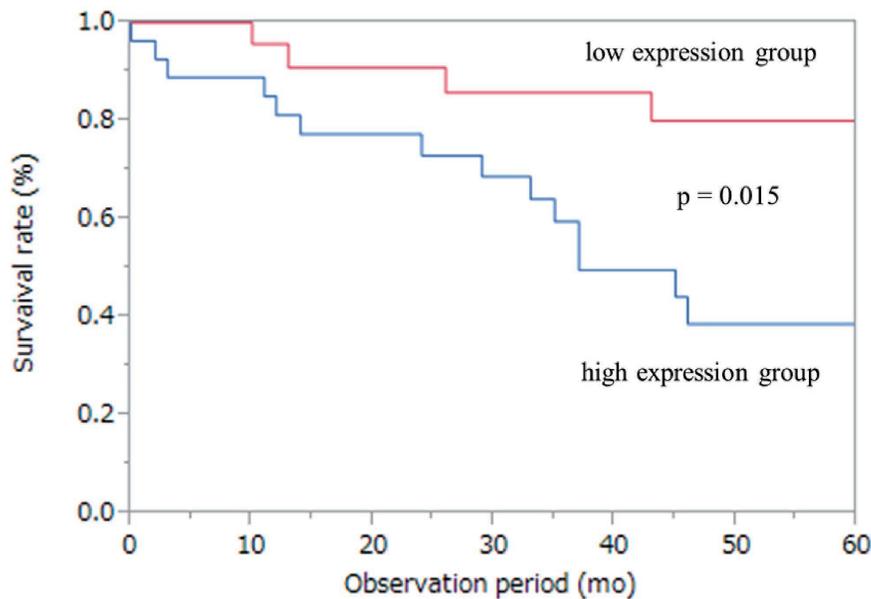

Fig. 2. Kaplan-Meier analyses of overall survival at Stages I-III according to the level of the VASH1 expression.
There are 27 cases in the high expression group and 24 cases in the low expression group. A p value was assessed by the log-rank test.

These reports suggest that the expression level of VASH1 differs between cancer invasion of the same organ depending on degree and histological type. Furthermore, the correlation between VASH1 expression and prognosis is different for each organ. We focused on VASH1 expression in ovarian carcinoma, on which few reports are available to date.

Regarding the relationship between VASH1 expression and prognosis, VASH1 expression was previously reported to have no effect on OS and PFS in colon carcinoma (Liu et al. 2015), while high VASH1 expression was shown to be associated with poor OS in breast carcinoma (Tamaki et al. 2009). Other studies reported a similar association in upper urinary tract carcinoma and prostate carcinoma, identifying VASH1 as a recurrence and prognostic marker (Miyazaki et al. 2012; Kosaka et al. 2013). In NSCLC, association of high VASH1 expression with short PFS and OS was reported (Heishi et al. 2010). In uterine corpus carcinoma, the VASH1 expression was reported to be higher as the differentiation is poorer, but its correlation has not been analyzed yet (Yoshinaga et al. 2008). In uterine cervix carcinoma, higher VASH1 expression was reported in infiltrating carcinoma than in in-situ carcinoma, and in adenocarcinoma than in squamous cell carcinoma, without regard to the correlation with prognosis (Yoshinaga et al. 2011). In renal cell carcinoma, a finding different from the above was reported where the high VASH1 expression group demonstrated a better PFS, but correlation with OS was not proven (Kanomata et al. 2013). In addition, mice grafted with VASH1-expressing tumor cells showed the microvessel area in the tumor was significantly lower, downsized tumors, and prolonged host survival compared to mice that

Table 5. Univariate and multivariate analysis of overall survival for ovarian carcinoma at Stage I-III.

	Hazard ratio (univariate)	95CI	p value	Hazard ratio (multivariate)	95CI	p value
VASH1						
High / Low	3.3	1.3-10.2	0.013[*]	1.8	0.4-8.4	0.44
MVD						
High / Low	1.6	0.7-4.2	0.29			
VEGFR2						
High / Low	4.1	1.6-11.8	0.0025[*]	1.9	0.4-8.9	0.41
pT factor[†]						
3 / 1-2	3	1.2-8.1	0.015[*]	3.8	1.1-13.8	0.031[*]
pN factor[†]						
positive / negative	4.8	1.7-13.5	0.0048[*]	0.9	0.2-3.3	0.84
Ki67						
High / Low	0.9	0.4-2.2	0.80			

MVD, microvessel density; VASH1, vasohibin-1; VEGFR2, vascular endothelial growth factor receptor 2; *p < 0.05; [†], TNM classification (Union for International Cancer Control 7th edition)

did not express any VASH1 (Takahashi et al. 2015, 2016).

In our investigation of ovarian carcinoma in clinical cases, higher VASH1 expression as well as increased MVD was found in advanced stages compared to early stages. The high VASH1 expression group demonstrated poorer prognosis than that of the lower expression groups. Additionally, this study showed that VASH1 was a prognostic factor for Stage I-III ovarian carcinoma, suggesting that VASH1 is a good biomarker in cases without distant metastasis.

MVD did not show a statistical significance for survival, although MVD had a significantly positive correlation with VASH1. MVD simply indicates the number of blood vessels and does not represent actual blood flow. The actual blood flow is affected by various angiogenetic factors and inhibitors. VASH1, a negative feedback regulator of angiogenesis, might be a good marker of the actual blood flow in ovarian cancer.

Intravenous infusion of a viral vector encoding the human VASH1 gene was reported to have inhibited lymph node metastasis in a xenograft model of mice with cancer cells (Heishi et al. 2010). In the present study, however, no significant difference was found in VASH1 expression between cases with and without lymph node metastasis.

VASH1 is a VEGF-related factor and its relationship with cancer cells and apotheosis has been demonstrated (Hosaka et al. 2009; Affara et al. 2013). Its correlations with angiogenesis and other markers are also drawing attention. We focused on Ki67, which was previously reported

to show higher expression in advanced cancer and recognized as a prognostic marker (Aune et al. 2011; Marinas et al. 2012). Ki67 is a nuclear protein that is closely linked to the proliferative potential of tumor. Recently, Ki67 was identified as an important prognostic factor for many tumor entities with respect to chemosensitivity and disease recurrence/death (Falato et al. 2014; Li et al. 2016; Rowe et al. 2016). Its prognostic value in ovarian carcinoma remains controversial (Liu et al. 2012; Masoumi-Moghaddam et al. 2015; Feng et al. 2016; Green et al. 2016) and its correlation with VASH1 is not well known. The present study demonstrated that the high Ki67 expression group had a good prognosis but that there were no significant differences. However, Ki67 has a significant positive correlation with VASH1 expression, and the expression of a high level of VASH1 was significantly associated with high Ki67 expression, which matches the results of a breast cancer study showing the same correlation (Tamaki et al. 2009). In human ovarian carcinoma, as in many other carcinomas, VASH1 expression strongly correlated with both of VEGFR2 expression and MVD was recognized. Additionally, VASH1 was recognized to have a significant correlation similar to that of Ki67. These findings suggest a possible relationship of VASH1 with cancer cell proliferation in addition to angiogenesis.

This study is limited, as a retrospective study does not allow sufficient bias adjustment regarding stages and other characteristics to provide appropriate comparison among tissue types. In ovarian carcinoma, varied VEGF expres-

sion volume was reported depending on the tissue type (Mabuchi et al. 2010); therefore, VASH1 expression may similarly vary. In addition, clinical cases of ovarian carcinoma are often detected in advanced stages, when the tumor angiogenesis environment may be different from those in other organs where cancer is detected in an early stage. When investigating ovarian carcinoma focusing on angiogenesis, expression characteristics should be analyzed by tissue type and stage. Similar studies enrolling a considerable number of cases are warranted in the future.

VASH1 expression in human ovarian carcinoma was significantly associated with angiogenetic factors and Ki67 expression. Furthermore, in patients of ovarian carcinoma, VASH1 expression is a potential prognostic factor.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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