Tumor Biol. (2016) 37:7267–7276
DOI 10.1007/s13277-015-4595-4

ORIGINAL ARTICLE

The effect of Vasohibin-1 expression and tumor-associated macrophages on the angiogenesis in vitro and in vivo

Zhanlong Shen[1] · Yichao Yan[1] · Chunxiang Ye[1] · Bo Wang[1] · Kewei Jiang[1] · Yingjiang Ye[1] · Harri Mustonen[2] · Pauli Puolakkainen[2] · Shan Wang[1]

Received: 15 September 2015 / Accepted: 6 December 2015 / Published online: 14 December 2015
© International Society of Oncology and BioMarkers (ISOBM) 2015

Abstract Vasohibin-1 is an intrinsic inhibitor of angiogenesis induced by VEGF-A. However, there little is known about the relationship between Vasohibin-1 expression, angiogenesis, and tumor-associated macrophages (TAMs). Vasohibin-1 expression, VEGF-A expression, microvessel density (MVD) marked with CD34, and density of cells marked with CD68 were measured in 111 paraffin-embedded tissues of gastric cancer by immunohistochemistry. The length of tube forming structures of endothelial cells and mobility rate of gastric cancer cells in Matrigel were tested by three-dimensional live cell imaging system. The effect of TAMs on the tumor growth, MVD, and Vasohibin-1 expression was measured by nude mice tumor genesis assay in vivo. We found that high Vasohibin-1 protein expression correlated significantly with worse TNM stage (P=0.002), metastatic lymph node (P=0.014), distant metastasis (P=0.022), overall survival (P<0.001), and progression-free survival (P<0.001) compared to those with low Vasohibin-1 expression. Vasohibin-1 protein expression had statistical correlation with the MVD (r=0.860, P<0.001), density of $CD68^+$ cells (r=0.882, P<0.001), and VEGF-A expression (r=0.719, P<0.001) in the gastric cancer tissues. Decreasing Vasohibin-1 expression with siRNA increased the length of tube forming structures of endothelial cells in co-culture with endothelial cells (EA-hy923), macrophages, and gastric cancers (Hs746T). Tumor volume (P=0.001), Vasohibin-1 (P<0.001), and VEGF-A (P<0.001) expression in mice inoculated with AGS and THP (10:1) was significantly higher than that with AGS alone (P=0.001). Vasohibin-1 protein expression had statistical correlation with VEGF expression (r=0.786, P<0.001) and MVD (r=0.496, P=0.014) in gastric xenografted tumor. Therefore, Vasohibin-1 might be a potential marker of worse prognosis and therapeutic target in gastric cancer. Vasohibin-1 might play an important role in the process of angiogenesis regulated by TAMs.

Keywords Vasohibin-1 · TAMs · Angiogenesis · Prognosis · Gastric cancer

Zhanlong Shen and Yichao Yan own equal first authorship; Shan Wang and Pauli Puolakkainen own equal last authorship.

Electronic supplementary material The online version of this article (doi:10.1007/s13277-015-4595-4) contains supplementary material, which is available to authorized users.

✉ Zhanlong Shen
shenlong1977@163.com

✉ Pauli Puolakkainen
Pauli.Puolakkainen@hus.fi

✉ Shan Wang
shanwang60@sina.com

[1] Department of Gastroenterological Surgery, Laboratory of Surgical Oncology, Peking University People's Hospital, Beijing 100044, People's Republic of China

[2] Department of Surgery, Helsinki University Central Hospital, University of Helsinki, Helsinki 00290, Finland

Introduction

Angiogenesis, also called neovascularization, is a fundamental process of blood vessel growth and a hallmark of cancer development [1]. The angiogenesis is the balance of activities of angiogenesis stimulators and inhibitors [2, 3]; Vasohibin-1 was originally identified as an endothelium-derived VEGF-inducible angiogenesis inhibitor that acts in a negative feedback manner [4]. The expression of Vasohibin-1 has been shown in endothelial cells in both physiological and pathological conditions including cancer associated with angiogenesis

[5–7]. Actually, Vasohibin-1 is not only expressed in endothelial cells but also other cell types, like hematopoietic cells [8] and cancer cells [9]. In 2012, we firstly reported the Vasohibin-1 could express in gastric cancer cells and could be regulated by tumor-associated macrophages (TAMs) [10]. TAMs has been shown to play a critical role in contributing to tumor angiogenesis by regulating the balance of proangiogenic and antiangiogenic factors [11–13]. However, there is little known about the relationship between Vasohibin-1 expression, angiogenesis, and TAMs by now, except of our previous report.

In this study, we investigated the Vasohibin-1, VEGF-A expression, microvessel density (MVD) labeled by CD34, and density of TAMs labeled by CD68 by immunohistochemistry in gastric cancer tissues. The relationship between the Vasohibin-1 expression, MVD, and density of TAMs was analyzed. The effect of Vasohibin-1 expression and TAMs on the angiogenesis was analyzed by measuring length of tube forming structures of endothelial cells in vitro under three-dimensional (3D) live cell imaging system and nude mouse tumorigenicity assay in vivo. We hope this study might throw more information about the regulation of Vasohibin-1 expression and angiogenesis induced by TAMs in gastric cancer.

Materials and methods

Samples

Samples of the gastric carcinoma tissue were obtained from 111 consecutive patients with gastric cancer who had undergone tumor resection at the Department of Gastroenterological Surgery at Peking University People's Hospital between January 2005 and July 2006. Informed consent was obtained from each patient who participated the study. The specimens were fixed in formalin in 5 min after surgical resection, and then embedded with paraffin until used. Clinicopathological data are shown in Table 1. Cancer staging relies on the 7th TNM system designed by the American Joint Committee on Cancer (Supplementary Table 1). The study obtained the approval of the Institutional Review Board and through the ethic committee in the locality.

Immunohistochemistry

Envision method was used to perform immunohistochemical staining for Vasohibin-1, VEGF-A, CD34, and CD68 in gastric cancer tissues. Specimens had been fixed in 10 % formalin, embedded in paraffin, cut into 4 μm thick sections, and placed on the glue-coated glass slides. Sections were deparaffinized in xylene and hydrated with graded alcohols and distilled water. To retrieve the immunoreactivity, tissue sections were boiled in citrate buffer (pH 6.0) for CD34 and VEGF-A in 10 mM EDTA (pH 9.0) for Vasohibin-1 and CD68 for 20 min at above 95 °C. Mouse polyclonal Vasohibin-1 antibody, mouse monoclonal VEGF-A antibody, rabbit polyclonal CD34 antibody, and rabbit monoclonal CD68 antibody were added to adjacent tissue sections, respectively, and incubated overnight at 4 °C. The primary antibodies were used as follows: anti-human Vasohibin-1 polyclonal antibody (sc-365541, Santa Cruz Biotechnology, Europ) and anti-CD34 (ab27448, Abcam, Cambridge, UK) both diluted at 1:100, anti-CD68 (ab955, Abcam, Cambridge, UK) diluted at 1:200, anti-VEGF-A (ab105219, Abcam, Cambridge, UK) diluted at 1:50. Horseradish peroxidase conjugated second antibody was added to the sections and incubated at room temperature for 20 min. DAB was used for the color reaction according to the manufacturer's instructions, and then the slides were counterstained with hematoxylin. The tissue sections were washed with phosphate buffer solution (PBS) (0.01 M, pH 7.4) between each step. Positive and negative controls were simultaneously used to ensure the specificity and reliability of staining. As a positive control, tissue sections from endometrial carcinoma were used. Sections were processed with 0.01 M PBS instead of the primary antibody, which was used as negative control. The positive result showed yellow or brown coloration in cytoplasm and/or plasma membranes.

Immunohistochemical analysis

Two independent assessors unaware of the patient outcome carried out this semiquantitative analysis. The average number counted by the two investigators was used for subsequent analysis. The degree of Vasohibin-1 and VEGF-A staining was estimated by semiquantitative evaluation and categorized by the extent and intensity of staining as follows [14]: The extent of positive cells was estimated as 0=positive staining cells 5 %, 1=positive staining cells in 6~25 %, 2=positive staining cells in 26~50 %, 3=positive staining cells in 51~75 %, and 4=positive staining cells >75 %. The intensity of staining was scored as 0=achromatic, 1=light yellow, 2=yellow, and 3=brown. Combined staining score was used to evaluate the results of staining. The extent of positive cells was multiplied by the intensity of staining and scored as follows: (–)=0, (+)=1–4, (++)=5–8, and (+++)=9–12. Negative (–) and weak (+) were categorized as low expression, and moderate (++) and strong (+++) as high.

Microvessel density counting

Microvessels marked by CD34 were counted. The areas with the greatest number of distinctly highlighted microvessels were selected. Any cell clusters with CD34-positive signals were regarded as a single countable microvessel, regardless of whether the lumen was visible. Unstained lumina were considered artifacts, even if they contained blood or tumor cells.

Table 1 Correlation between Vasohibin-1 expression and clinicopathologic factors

	Expression of Vasohibin-1 [number of patients (row %)]			*P* value
	Number of patients (*n*=111)	Low expression (*n*=67)	High expression (*n*=44)	
Sex				0.357
Male	81	51 (63.0 %)	30 (37.0 %)	
Female	30	16 (53.3 %)	14 (46.7 %)	
Age				0.763
≤60 years	46	27 (58.7 %)	19 (41.3 %)	
>60 years	65	40 (61.5 %)	25 (38.5 %)	
Tumor differentiation				0.577
Poorly	57	35 (61.4 %)	22 (38.6 %)	
Moderately	43	24 (55.8 %)	19 (44.2 %)	
Well	11	8 (72.7 %)	3 (27.3 %)	
Tumor invasion depth				0.002*
T1-T2	34	28 (82.4 %)	6 (17.6 %)	
T3-T4	77	39 (50.6 %)	38 (49.4 %)	
Lymph node status				0.014*
Negative	35	27 (77.1 %)	8 (22.9 %)	
Positive	76	40 (52.6 %)	36 (47.4 %)	
Distant metastasis				0.022*
Negative	94	61 (64.9 %)	33 (35.1 %)	
Positive	17	6 (35.3 %)	11 (64.7 %)	
TNM stage				0.002*
I-II	39	31 (79.5 %)	8 (20.5 %)	
III-IV	72	36 (50 %)	36 (50 %)	

*Significant at the $p<0.05$ level. Pearson's Chi-Square test was used

The results of MVD were expressed as the highest number of capillaries and small venules identified within any single ×200 field (×20 objective and ×10 ocular, 0.785 mm^2 per field) in gastric cancer tissues [15] and ×400 (×40 objective and ×10 ocular, 0.785 mm^2 per field) field in the xenografts of nude mice, in accordance with an original method.

Density of $CD68^+$ cells

A single pathologist, who was blinded to the clinical assessments of each case, scored the cases by counting the number of $CD68^+$ cells (macrophage marker) in six independent fields under a ×200 magnification. $CD68^+$ cell counts were expressed as the mean±standard deviation.

Isolation of monocytes and macrophages

Mononuclear cells were isolated form healthy subjects blood with density gradient centrifugation (Ficol-Paque, Amershamn, Uppsala, Sweden). The cells were washed with PBS+10 % acid citrate dextrose (ACD) solution two times. The cells were counted and 1.4×10^6 cells were placed on Matrigel (Matrigel, BD Biosciences, San Jose, CA) covered cover slip (Nalge Nunc International Corporation, Naperville, German). The isolated cells were grown in serum-free medium designed for macrophages (Macrophage serum-free medium, Gibco, Paisley, UK) with granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/mL, Immuno Tools, Oldenburg, Germany), antibiotics, and 5 % CO_2 at 37 °C. Monocytes adhered to the Matrigel overnight and differentiated to macrophages due to GM-CSF. Monocytes were fully differentiated into macrophages after 6 days and then used for experiments. When co-cultured with cancer cells, macrophages developed into TAMs with special surface marker, CD 206, $CD14^+$ [16]. After co-culturing with gastric cancer cells, the portion of CD 206^+, $CD14^+$ positive macrophages reached more than 80 % measured with flow cytometry.

Cells culture

AGS cell line (CRL-1739) was purchased from ATCC (American Type Culture Collection). The cells were cultured in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 10 % fetal bovine serum (FBS),

and antibiotics (100 μg/mL penicillin and 100 U/mL streptomycin).

Hs 746T (HTB-135) cell line was purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L bicarbonate, 4.5 g/L glucose, 10 % FBS, and antibiotics (100 μg/mL penicillin and 100 U/mL streptomycin).

EA.hy926 cell line was the human umbilical vein cell line and purchased from ATCC. Cells were cultured in DMEM with 10 % FBS and antibiotics (100 μg/mL penicillin and 100 U/mL streptomycin).

THP cell line derived from the monocytes from the human perineal blood and purchased from ATCC. Cells were cultured in RPMI-1640 medium, with 2-mercaptoethanol to a final concentration of 0.05 mM added, 10 % FBS and antibiotics (100 μg/mL penicillin and 100 U/mL streptomycin).

All cell lines were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

Invasion and migration assay (3D dynamic migration imaging system)

Cells were grown on Matrigel-covered cover slip wells with serum-free medium designed for macrophages. Gastric cancer cells were grown either alone or with differentiated macrophages and/or EA.hy926 cells on Matrigel under normal oxygenic condition. siRNA transfection kit (sc-45064, Santa Cruz Biotechnology, USA) was used to down regulate the Vasohibin-1 mRNA expression. Gastric cancer cells were stained with fluorescent dye (CellTracker green CMFDA, Invitrogen, Eugene, OR) before imaging. During the invasion phase, the cancer cells invaded in Matrigel were imaged by 3D dynamic migration imaging system (Olympus A×70 Research System microscope, Japan). The average migration speed was calculated from the cells which could be tracked at least for 6 hours in one *z*-plane (ImagePro Plus, Media Cybernetics, Bethesda, MD).

The length of tube forming structures of endothelial cells in vitro

The endothelial cells were cultured in the Matrigel alone or with FBS+EGF, or with macrophages, or with Hs 746T cells, or with macrophages+Hs 746T fetal bovine serum (5 %, FBS) and EGF (10 ng/mL) were used as a positive control to stimulate tube formation. siRNA technique was used to decrease Vasohibin-1 expression (Santa Cruz Biotechnology) in cancer cells and non-targeting (NT) siRNA was used as negative control. The endothelial cells were colored with fluorescent dye (CellTracker green CMFDA, Invitrogen, Eugene, OR) before imaging. The movement of cells in Matrigel was imaged by 3D dynamic migration imaging system. We have manually searched for cell structures extending two elongated cells in length and having two to three cells side by side or over each other in the other direction along the diameter of a tube to be formed. The length of the elongated cells was measured manually, thereby analyzing length of possible tube forming structures. The analysis was done with ImagePro 7.01.

In vivo tumor growth assay

BALB/c female nude mice (4 weeks old) were purchased from Beijing Vital River Laboratories (China). These mice were maintained in a specific pathogen-free environment in the Experimental Animal Center of Peking University People's Hospital. The animal experiments were approved and reviewed by the Animal Research Committee of the Peking University People's Hospital. Care and handling of the animals were in accordance with the guidelines for Institutional and Animal Care and Use Committees.

Mice were randomly divided into four groups (AGS, AGS+THP (5:1), AGS+THP (10:1), and AGS+THP (20:1)) with six mice in each group. The concentration of basic cell solution of AGS and THP was 2×10^7 cells/mL and 4×10^6 cells/mL, respectively. The cells were mixed according to the percentage above. Cell solution (0.2 mL) was injected subcutaneously into mice. Tumor volumes were measured every 7 days and calculated according to the formula $V=0.5\times L$ (length)$\times W^2$ (width). At 21 days after the cell inoculation, mice were sacrificed and tumors were excised to measure the volume and weight. The expression of Vasohibin-1, VEGF-A, and CD34 was detected by immunohistochemistry.

Definition of overall survival and progression-free survival

Overall survival (OS) was calculated from the time of surgery to last follow-up or date of death. For patients who recurred or metastasized, progression-free survival (PFS) was calculated as the time from surgery to time of first recurrence; for those that did not recur or metastasize, PFS was defined as the time from surgery to last follow-up/death.

Results

Vasohibin-1 expression in the gastric cancer tissues and its relationship with clinicopathological characteristics and prognosis

Sixty-seven and 44 gastric cancer tissues showed low and high expression of Vasohibin-1 protein, respectively. There was no significant correlation between Vasohibin-1 protein expressions and age, sex, and tumor differentiation. Tumors with deeper depth of invasion had significantly higher Vasohibin-1 protein expression than those with superficial invasion ($P=0.002$). High Vasohibin-1 expression was detected

more often in patients with metastatic lymph node and distant metastasis ($P=0.014$, $P=0.022$, respectively). High Vasohibin-1 protein expression correlated significantly with worse TNM stage ($P=0.002$) (Table 1, Fig. 1).

Follow-up rate of gastric cancer patients is 96.4 % (107/111). Patients with high Vasohibin-1 expression had significantly worse OS and PFS compared with those with low Vasohibin-1 expression (mean OS time: 34.6 vs. 63.5 month, $P<0.001$; median PFS time: 33.7 vs. 62.9 month, $P<0.001$) (Fig. 2).

The correlation between the Vasohibin-1 and VEGF-A protein expressions in the gastric cancer tissues

The tissues with VEGF-A low expression accounted for 85.1 % (57/67) and 29.5 % (13/44) in the gastric cancer tissues with Vasohibin-1 low and high expression, respectively ($P<0.001$) (Fig. 1). There was significant correlation between Vasohibin-1 and VEGF-A protein expressions in the gastric cancer tissues by the analysis of immunohistochemistry ($r=0.719$, $P<0.001$) (Fig. 3).

The correlation between the Vasohibin-1 expression and the MVD in the gastric cancer tissues

The mean MVD in the gastric cancer tissues with Vasohibin-1 low and high expression was 28.9±12.3 and 61.4±19.1, which had statistical difference ($P<0.001$), respectively. Immunohistochemistry showed that Vasohibin-1 protein expression had statistical correlation with the MVD in the gastric cancer tissues ($r=0.860$, $P<0.001$) (Figs. 1 and 3).

The correlation between the Vasohibin-1 expression and the density of $CD68^+$ cells in gastric cancer tissues

There was a significant difference between the density of $CD68^+$ cells (macrophage marker) in the gastric cancer tissues with Vasohibin-1 low and high expression (32.7±18.3 and 73.7±19.8, $P<0.001$). Significantly positive correlations were found between Vasohibin-1 expression and the density of $CD68^+$ cells in the gastric tissues ($r=0.882$, $P<0.001$) (Figs. 1 and 3)

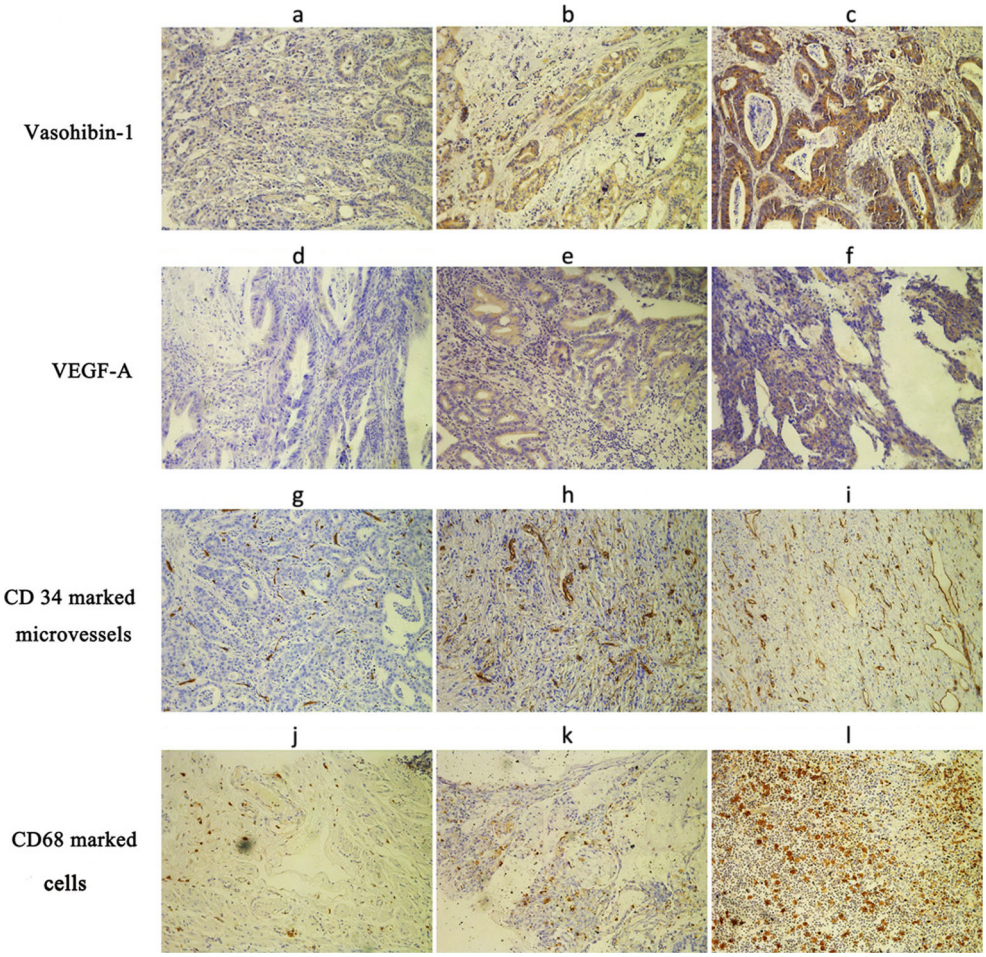

Fig. 1 Vasohibin-1 expression detected by immunohistochemistry in gastric cancer tissues. Vasohibin-1 expression: (*a*) Week (*light yellow*), (*b*) positive (*yellow*), (*c*) strong positive (*brown*). ×200; VEGF expression: (*d*) week (*light yellow*), (*e*) positive (*yellow*), (f) strong positive (*brown*). ×200; microvessels marked with CD34 (*g*, *h*, *i* ×200); $CD68^+$ cells (*j*, *k*, *l* ×200)

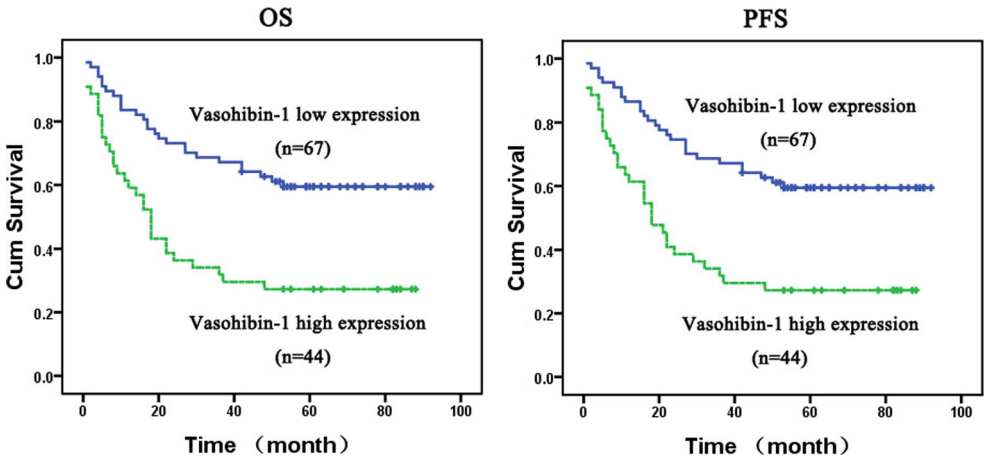

Fig. 2 Gastric cancer patients with high Vasohibin-1 expression in tumors show poor overall survival (OS) and progression-free survival (PFS). Vasohibin-1 expression was measured by immunohistochemistry in tumors of gastric cancer patients

The effect of TAMs and Vasohibin-1 on the length of tube forming structures of endothelial cells in Matrigel

The length of tube forming structures in Matrigel of the group of EA.hy923 cells alone, EA.hy923+FBS+EGF, EA.hy923+macrophages, EA.hy923+Hs746T cells, EA.hy923+macrophages+Hs746T, EA.hy923+macropahges+Hs746TVasohibin-1 siRNA, and EA.hy923+macrophages+Hs746T NT were 40.7±7.5, 65.5±8.6, 52.1±6.3, 42.0±4.9, 38.1±2.3, 61.4±3.1, and 38.6±3.0 μm, respectively. There was statistical difference of tube length between the EA.hy923 alone group and EA.hy923+macrophages group ($P<0.001$). The tube length of EA.hy923+macrophages+Hs 746TVasohibin-1 siRNA group was significantly longer than EA.hy923+macrophages+Hs 746T group ($P<0.001$) and NT group ($P<0.001$) (Fig. 4).

The effect of TAMs and Vasohibin-1 on the invasion of gastric cancer cells in the Matrigel

The mobility rate of Hs746T with macrophages (5.1±0.0 μm vs. 3.1±0.0 μm/h (without macrophages), $P<0.001$), with EA.hy923 (8.4±0.1 μm vs. 3.1±0.0 μm/h, $P<0.001$) and with macrophages+EA.hy923 (8.5±0.1 μm vs. 3.1±0.0 μm/h, $P<0.001$) was significantly faster than that of Hs746T alone in Matrigel.

Vasohibin-1 siRNA significantly increased the mobility of Hs746T cells in above all groups (Hs 746T Vasohibin-1 siRNA vs. Hs746T: 3.6±0.1 μm vs. 3.1±0.0 μm/h, $P=0.001$; Hs 746T Vasohibin-1 siRNA+macrophages vs. Hs746T+macrophages: 6.2±0.1 μm vs. 5.1±0.0 μm/h, $P<0.001$; Hs 746T Vasohibin-1 siRNA+EA.hy923 vs. Hs746T+EA.hy923: 8.7±0.1 μm vs. 8.4±0.1 μm/h, $P=$

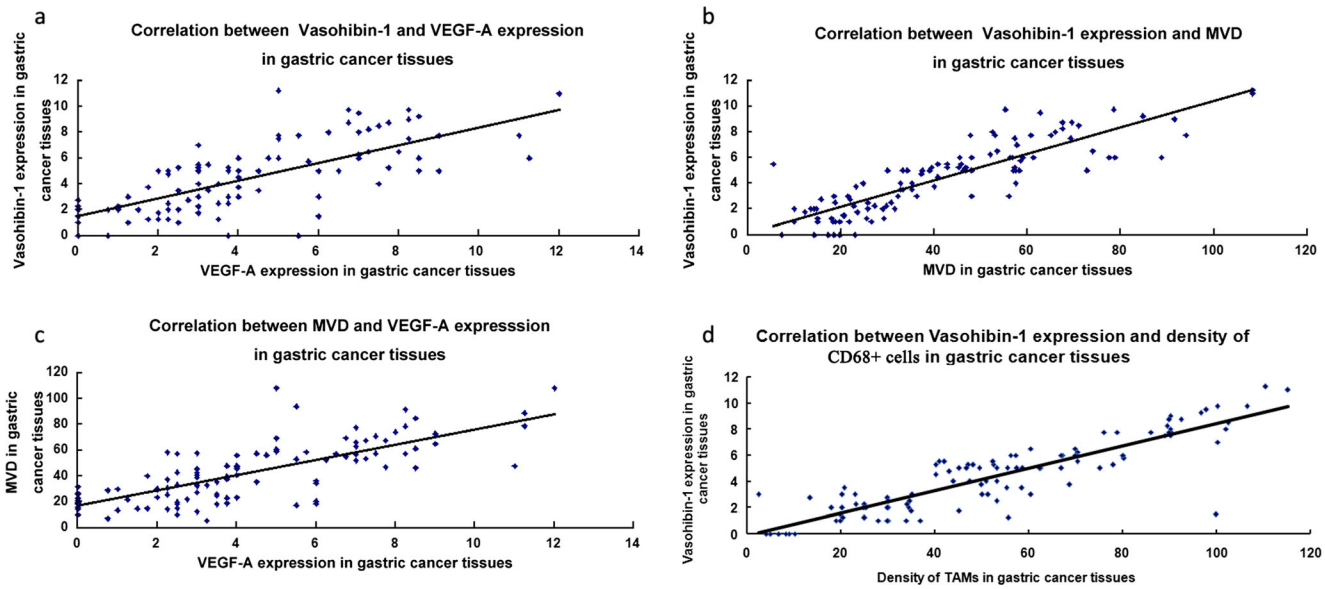

Fig. 3 Correlation within Vasohibin-1, VEGF-A expression, microvessel density (MVD), and density of $CD68^+$ cells in gastric cancer tissues analyzed by immunohistochemistry

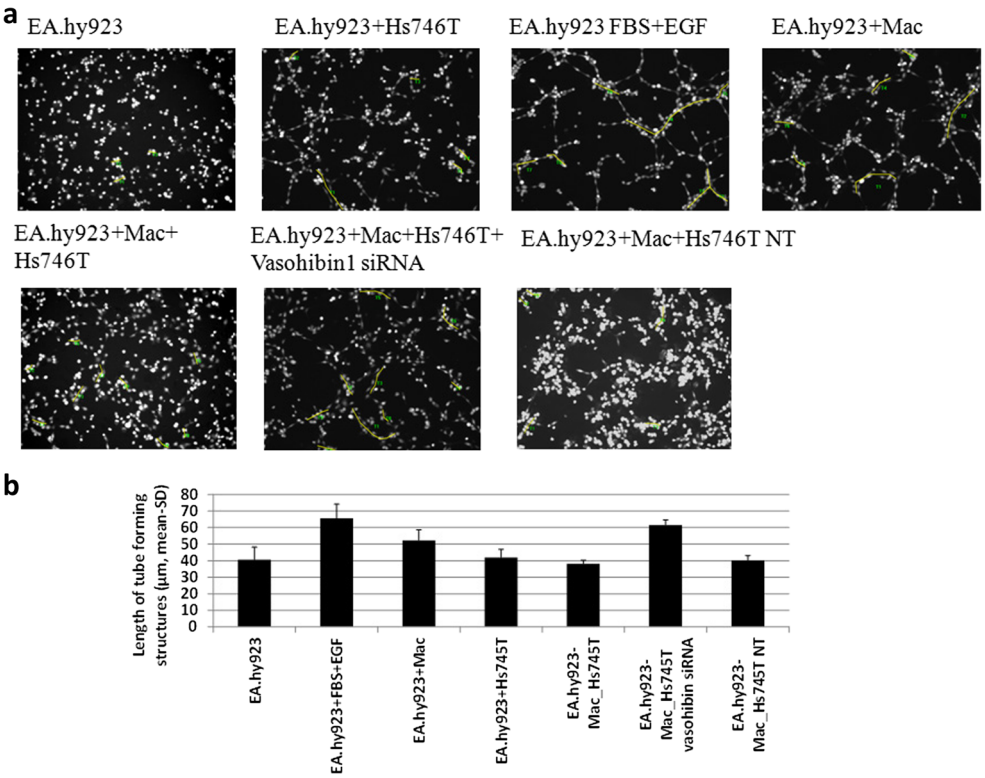

Fig. 4 **a** The effect of Vasohibin-1 on the length of tube forming structures in co-culture of endothelial cells, macrophages, and gastric cancer cells. Endothelial cells (EA.hy923) were cultured alone or with gastric cancer cells and/or with macrophages. FBS (5 %) and EGF (10 ng/mL) were used as positive controls. Vasohibin-1 expression was decreased in cancer cells with siRNA technique. Non-targeting siRNA was used as a control. **b** Decreasing Vasohibin expression increases the length of tube forming structures

0.018; Hs 746T Vasohibin-1 siRNA+macrophages+EA.hy923 vs. Hs746T+macrophages+EA.hy923: 11.3±0.1 μm vs. 8.5±0.0 μm/h, $P<0.001$).

The effect of TAMs on the tumor growth and angiogenesis and the relationship with Vasohibin-1 expression in vivo

After 21 days, the tumor volume of mice inoculated with AGS cells alone was 1551.1±213.8 mm^3, whereas that injected with AGS+THP macrophages by the ratio of 5:1, 10:1, and 20:1 was 1881.9±128.2 mm^3 ($P=0.334$), 2781.9±353.2 mm^3 ($P=0.001$), and 2333.2±189.9 mm^3 ($P=0.030$), respectively (Fig. 5).

Compared to the AGS group (0.87±0.18 g), the tumor weight was reduced by 6.9 % in the AGS+THP (5:1) group (0.77±0.10 g, $P=0.568$), but that was increased by 8.0 and 23.0 % in the AGS+THP (10:1) group (1.14±0.10 g, $P=0.123$) and AGS+THP (20:1) group (1.36±0.10 g, $P=0.010$) (Fig. 5).

Both of Vasohibin-1 and VEGF-A expression scores in AGS+THP (5:1) group ($P<0.001$, $P=0.022$), AGS+THP (10:1) group ($P<0.001$, $P<0.001$), and AGS+THP (20:1) group ($P<0.001$, $P=0.001$) were significantly higher than that of AGS alone group. The Vasohibin-1 and VEGF-A expression scores in AGS+THP (10:1) group were significantly higher than both of AGS+THP (5:1) group ($P<0.001$, $P=0.007$) and AGS+THP (20:1) group ($P<0.001$, $P=0.001$) (Fig. 5).

The mean MVD in the AGS+THP (10:1) group was significantly higher than that of AGS alone group ($P=0.030$), AGS+THP (5:1) group ($P=0.001$), but not AGS+THP (20:1) group ($P=0.111$). The mean MVD in AGS+THP (5:1) group was statistically lower than that of AGS alone group ($P=0.043$) (Fig. 5).

Vasohibin-1 protein expression detected by immunohistochemistry had statistical correlation with VEGF expression ($r=0.786$, $P<0.001$) and MVD in the gastric xenografted tumor ($r=0.496$, $P=0.014$) (Fig. 5).

Discussion

We firstly reported that Vasohibin-1 could be expressed in gastric cancer cell lines except in the endothelium cells of vessels, and found that Vasohibin-1 expression in cancer cells could be regulated by TAMs [10]. However, there is little information about the Vasohibin-1 expression in gastric cancer tissues and the relationship with the survivals before our research. In this study, we found that Vasohibin-1 high expression was associated with tumor deeper depth of invasion, lymph node metastasis, distant metastasis, and worse OS and PFS. Kitajima and colleagues [17] found that overexpression of Vasohibin-1 in colon cancer cells increased malignant potential and promoted metastasis. There were also reports about high Vasohibin-1 expression presented worse tumor clinicopathological characteristics in lung cancer [18], renal cancer [19], hepatocellular cancer [20], and prostate cancer [21]. Therefore, Vasohibin-1 might be a molecular marker of

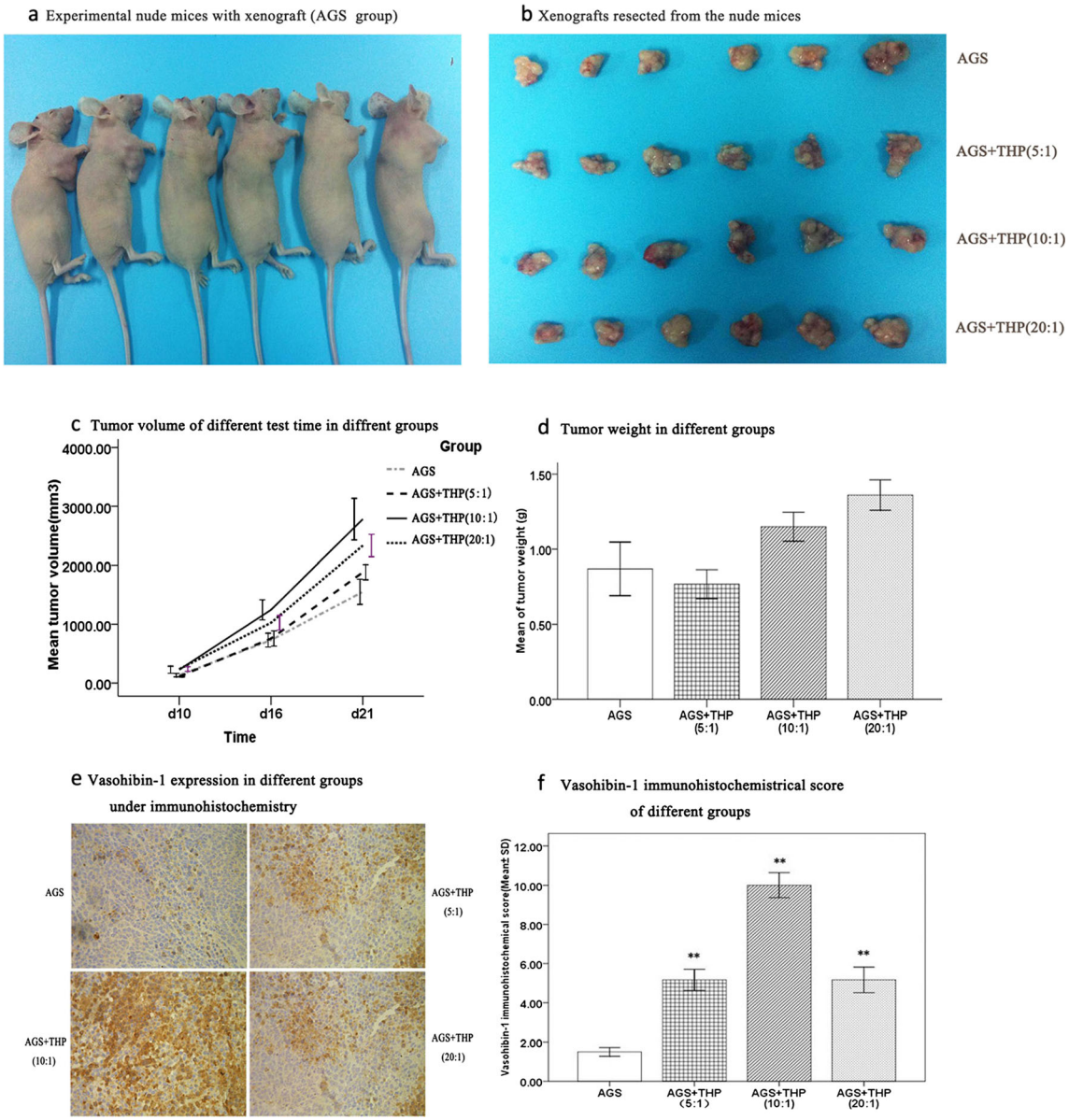

Fig. 5 Nude mice tumor genesis assay. **a** Experimental nude mice with xenograft (AGS group); **b** xenografts resected from the nude mice. **c** Tumor volume of different test time in different groups (groups: AGS alone and mixture of AGS and THP cells (5:1, 10:1, and 20:1)). **d** Tumor weight in different groups. **e** Vasohibin-1 expression in different groups under immunohistochemistry. **f** Vasohibin-1 immunohistochemistrical score of different groups. **g** VEGF-A expression in different groups under immunohistochemistry. **h** VEGF-A immunohistochemistrical score of different groups. **i** MVD marked by CD34 in different groups under immunohistochemistry. **j** Mean of MVD in xenografted tumor of different groups. **k** Correlation between Vasohibin-1 and VEGF-A expression in gastric xenografted tumor of nude mice. **l** Correlation between Vasohibin-1 expression and MVD in gastric xenografted tumor of nude mice

worse tumor biological behavior of cancer, even if Vasohibin-1 is an angiogenesis inhibitor. Moreover, we also found that the expression of Vasohibin-1 expression is parallel with the VEGF-A expression in gastric cancer tissue, which is in accordance with our previous study in the gastric cancer cells lines. Therefore, we speculated that the anti-angiogenesis of Vasohibin-1 might keep the balance with the pro-angiogenesis with VEGF-A expression in the human body, which could be explained by the results from Kimura and colleagues that the function of endogenous Vasohibin-1 was to terminate angiogenesis rather than to inhibit it in the sprouting front [22]. Our study also showed that Vasohibin-1 siRNA significantly increased the mobility of gastric cancer cell and endothelium cells vessel formation in Matrigel. Hence, Vasohibin-1 might become a new anti-angiogenesis target of gastric cancer in future expect for bevacizumab, which is a humanized monoclonal antibody against angiogenesis induced by VEGF.

We firstly described the regulation of TAMs for Vasohibin-1 expression in gastric cancer [10] and pancreatic cancer [12]. Nude mice tumorigenicity assay showed gastric cancer cells

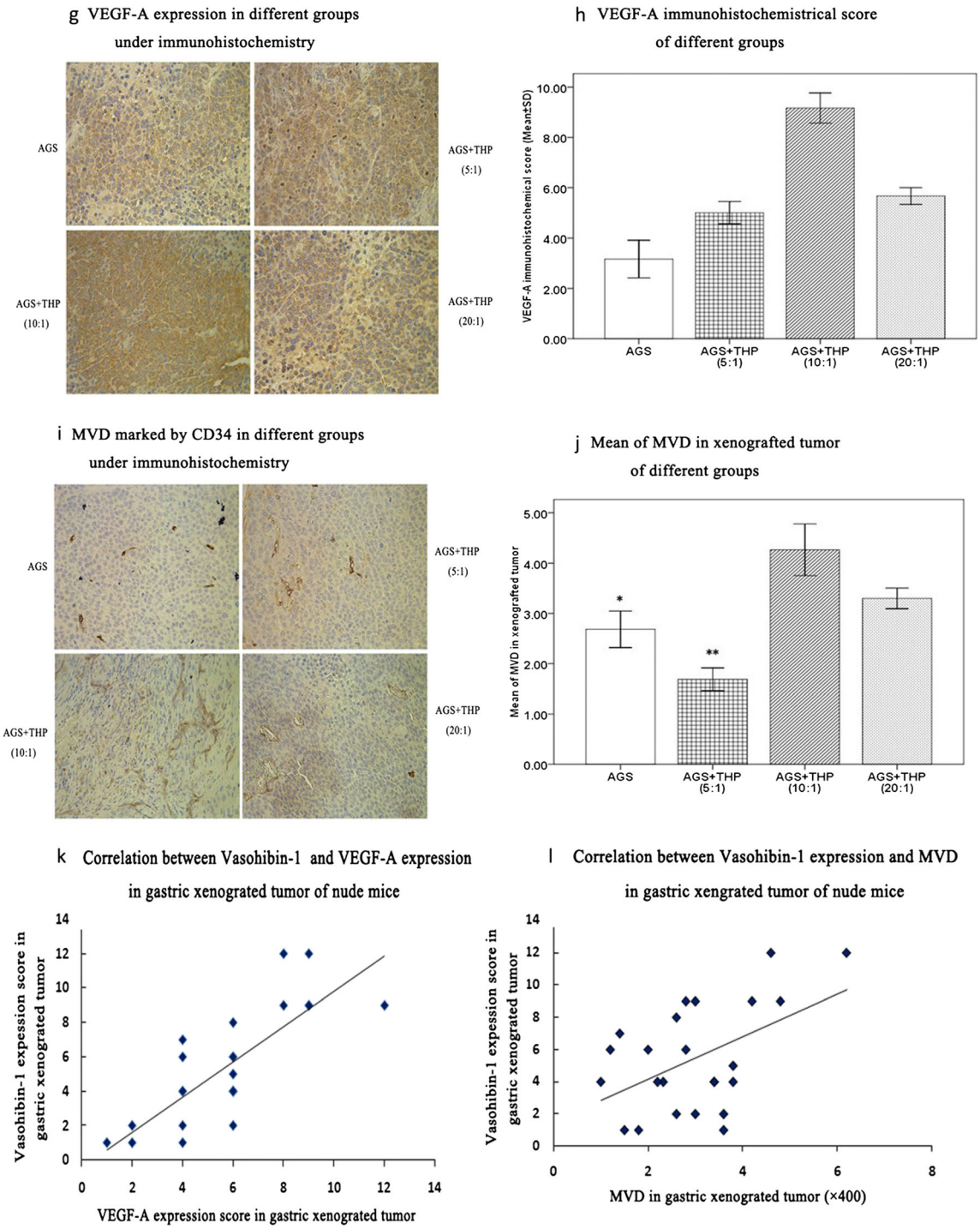

Fig. 5 (continued)

and macrophages mixed group, especially for the ratio of 10:1, significantly had bigger volume, weight, as well as higher Vasohibin-1, VEGF-A expression, and MVD compared to the gastric cancer cells alone group. It indicated that TAMs could promote the tumor growth and metastasis related to angiogenesis through cytokines including anti- and pro-angiogenesis factors. It synchronized our previous results from cell lines that TAMs could increase the mobility of gastric cancer cells in Matrigel and Vasohibin-1 expression in gastric cancer cells [10].

Moreover, in this study, the length of tube forming structures of endothelial cells in vitro showed that Vasohibin-1 expression in gastric cancer cells significantly decreased the ability of vessel formation of endothelium cells, which was up-regulated by TAMs. Other studies had shown TAMs could present angiostatic effects and angiogenic effects in tumors

through cytokines including IL12, MMP12, VEGF, and so on. Our series of studies indicated that TAMs might regulate the angiogenesis through the Vasohibin-1, inhibition of Vasohibin-1 could increase the effect of TAMs on the angiogenesis. Therefore, we speculated Vasohibin-1 might not only function as an intrinsic inhibitor of angiogenesis in the endothelium cells but also play an import role in the process of regulation by TAMs.

Acknowledgments This study was supported by grants from National Science Foundation of China (81372290, 81372291).

Compliance with ethical standards

Conflicts of interest None

References

1. Hosaka T, Kimura H, Heishi T, Suzuki Y, Miyashita H, Ohta H, et al. Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. Am J Pathol. 2009;175(1):430–9.
2. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature. 2000;407:242–8.
3. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. Semin Cancer Biol. 1999;9:211–20.
4. Sato Y. Is vasohibin-1 for more than angiogenesis inhibition? J Biochem. 2011;149:229–30.
5. Watanabe K, Hasegawa Y, Yamashita H, Shimizu K, Ding Y, Abe M, et al. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. J Clin Invest. 2004;114:898–907.
6. Yoshinaga K, Ito K, Moriya T, Nagase S, Takano T, Niikura H, et al. Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. Cancer Sci. 2008;99:914–9.
7. Tamaki K, Sasano H, Maruo Y, Takahashi Y, Miyashita M, Moriya T, et al. Vasohibin-1 as a potential predictor of aggressive behavior of ductal carcinoma in situ of the breast. Cancer Sci. 2010;101:1051–8.
8. Naito H, Kidoya H, Sato Y, Takakura N. Induction and expression of anti-angiogenic vasohibins in the hematopoietic stem/progenitor cell population. J Biochem. 2009;145:653–9.
9. Yan Y, Shen Z, Ye Y, Jiang K, Zhang H, Shen C, et al. A novel molecular marker of prognosis in colorectal cancer: Vasohibin-1. Med Oncol. 2014;31:816.
10. Shen Z, Kauttu T, Seppänen H, Vainionpää S, Ye Y, Wang S, et al. Vasohibin-1 and vasohibin-2 expression in gastric cancer cells and TAMs. Med Oncol. 2012;29:2718–26.
11. Shen Z, Seppänen H, Vainionpää S, Ye Y, Wang S, Mustonen H, et al. IL10, IL11, IL18 are differently expressed in CD14+ TAMs and play different role in regulating the invasion of gastric cancer cells under hypoxia. Cytokine. 2012;59:352–7.
12. Shen Z, Seppänen H, Kauttu T, Vainionpää S, Ye Y, Wang S, et al. Vasohibin-1 expression is regulated by transforming growth factor-β/bone morphogenic protein signaling pathway between tumor-associated macrophages and pancreatic cancer cells. J Interferon Cytokine Res. 2013;33(8):428–33.
13. Chittezhath M, Dhillon MK, Lim JY, Laoui D, Shalova IN, Teo YL, et al. Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. Immunity. 2014;41:815–29.
14. Wang SN, Yeh YT, Yang SF, Chai CY, Lee KT. Potential role of leptin expression in hepatocellular carcinoma. J Clin Pathol. 2006;59:930–4.
15. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N Engl J Med. 1991;324:1–8.
16. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549–55.
17. Kitajima T, Toiyama Y, Tanaka K, Saigusa S, Kobayashi M, Inoue Y, et al. Vasohibin-1 increases the malignant potential of colorectal cancer and is a biomarker of poor prognosis. Anticancer Res. 2014;34:5321–9.
18. Zhang T, Yu TT, Zhang DM, Hou XM, Liu XJ, Zhao D, et al. Vasohibin-1 expression detected by immunohistochemistry correlates with prognosis in non-small cell lung cancer. Med Oncol. 2014;31:963.
19. Kanomata N, Sato Y, Miyaji Y, Nagai A, Moriya T. Vasohibin-1 is a new predictor of disease-free survival in operated patients with renal cell carcinoma. J Clin Pathol. 2013;66:613–9.
20. Murakami K, Kasajima A, Kawagishi N, Sekiguchi S, Fujishima F, Watanabe M, et al. The prognostic significance of vasohibin 1-associated angiogenesis in patients with hepatocellular carcinoma. Hum Pathol. 2014;45:589–97.
21. Kosaka T, Miyazaki Y, Miyajima A, Mikami S, Hayashi Y, Tanaka N, et al. The prognostic significance of vasohibin-1 expression in patients with prostate cancer. Br J Cancer. 2013;108:2123–9.
22. Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, et al. Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. Blood. 2009;13:810–8.