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Vasculogenic Mimicry: a New Prognostic Sign of Gastric Adenocarcinoma

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Abstract Vasculogenic mimicry (VM) has been generally recognized as a new pattern of tumor neovascularization. It presents in many human malignancies. Till now, there is no report about VM in gastric adenocarcinoma (GAC). In this study, we collected 173 paraffin-embedded human GAC samples, with detailed follow-up and clinicopathologic data. CD31/ periodic acid-Schiff (PAS) double staining, immunohistochemical staining of CK8 & 18 and laminin were performed to validate the existence of VM in GAC. Microvascular density (MVD) and vasulogenic mimicry density (VMD) were counted respectively. VM was observed in 40 of the 173 GAC patients, especially in poorly differentiated GAC (P=0.014). Patients with VM were prone to hematogenous metastasis and distant recurrence compared with patients without VM (P=0.020, 0.029). Higher VMD values was also associated with hematogenous metastasis (P=0.003). Immunohistochemical staining index (SI) of hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, and MMP-9 were compared between the VM and non-VM group. The SI of four factors were all higher in the VM group than those of non-VM group (P=0.000, 0.000, 0.004, 0.009, respectively). The Kaplan-Meier survival analysis showed that the VM group has shorter life span compared with non-VM group (P=0.022). Cox proportional hazards model indicated that the presence of VM and TNM stage were independent predictors of poor prognosis (P=0.039 and 0.004) for GAC. In conclusion, VM exists in GAC, especially in poorly differentiated GAC. Additionally, it is an unfavorable prognostic indictor for GAC. Hypoxia may play a role in VM formation in GAC.

Keywords Gastric adenocarcinoma · Hypoxia-inducible factor 1α · Microvascular density · Poorly differentiated · Tissue array · Vasculogenic mimicry

Abbreviations

ECM	extracellular matrix
EDV	endothelium dependent vessel
GAC	gastric adenocarcinoma
HIF-1α	hypoxia-inducible factor 1 α
MMP	matrix metalloproteinase
MV	mosaic vessel
MVD	microvascular density
PAS	periodic acid-Schiff
SI	staining index
RBCs	red blood cells
VEGF	vascular endothelial growth factor

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VM vasculogenic mimicry
VMD vasulogenic mimicry density

Introduction

Tumor cells acquire the capability of survival, invasion and metastasis via sufficient blood supply. From previous studies [1, 2], three patterns of blood supply have been generally accepted in the tumor neovascularization field, i.e. endothelium dependent vessel (EDV), vasculogenic mimicry (VM) and mosaic vessel (MV). VM, the new phenomenon different from traditional tumor angiogenesis and vasculogenesis, was first described as the fluid-conducting channels formed by highly aggressive and genetically dysregulated melanoma cells by Maniotis et al in 1999 [1]. This term was denominated owing to these de novo channels formed by pluripotent embryonic-like and high invasive tumor cells mimicking the function of vessels. The predominant characteristic of VM is that numerous periodic acid-Schiff (PAS) and/or laminin positive patterns are composed of extracellular matrix (ECM). Red blood cells (RBCs) can flow through these channels [3]. Several studies have identified that PAS-positive patterns of VM played a substantial role in the tumor blood supply [1, 4, 5].

VM has been observed in several human malignancies of distinct origin, including melanoma, soft tissue sarcomas and GIST, inflammatory breast cancer, prostate, ovarian and hepatocelluar carcinoma [1, 6–12]. Some studies have shown that patients with VM had a shorter 5-years survival rate and a higher blood-borne metastasis rate than non-VM patients [9, 10, 12–14]. It suggests that the presence of VM is a potential predictor of poor prognosis for many tumors.

Does VM exist in gastric adenocarcinoma (GAC)? This topic, never reported previously, attracts us with extreme interest because GAC remains the second leading cause of cancer mortality worldwide due to higher distant metastasis in the early stage [15]. Angiogenesis and associated factors of GAC are intimately correlated with hematogenous metastasis and poor prognosis [16–19].

Although the precise molecular mechanisms of VM formation is still unknown, some researchers have reported the high level of matrix metalloproteinases (MMPs) and the effect of local tumor microenvironment, such as hypoxia were associated with the formation of VM channels [20–25]. Hypoxia is an indispensable process during the tumor growth that can promote tumor progression by stimulating angiogenesis [26, 27]. The hypoxia-inducible factor 1 α (HIF-1α) is a major transcript regulator under hypoxic and ischemic environments [26, 27]. It can up-regulate vascular endothelial growth factor (VEGF) which is one of its downstream genes [27]. Some data have shown that high expression of HIF-1α and VEGF were both associated with gastric cancer mortality [28–30].

So the objective of this study was designed to determine whether VM should occur in GAC or not and to evaluate its clinical significance if any. Moreover, we intended to detect the expression of HIF-1α, MMP-2, MMP-9 and VEGF in the VM and non-VM patients in order to elucidate their intrinsic association with VM formation.

Materials and Methods

Tissue Specimens

Paraffin-embedded GAC tissue samples (n=208) were obtained from the Tianjin Cancer Hospital from 1998 to 2003. Detailed pathological and clinical data were collected by reviewing medical charts and pathological records for all of the samples, including age and gender, tumor location and size, histological grade and TNM stage, blood-borne and lymph node metastasis, and survival duration. None of the patients had received treatment before operation. Clinical outcome was followed from the date of surgery to the date of death or until the end of 2008. Cases lost during follow-up were regarded as censored data for the survival analysis. 173 patients with enough follow-up were selected, 134 males and 39 females (mean age, 60.68±12.05 years). The survival durations ranged from 1 to 113 months (mean, 35.42± 27.32 months). Of the 173 patients, 21 had TNM stage I, 45 had TNM stage II, 62 had TNM stage III, and 45 had TNM stage IV. According to WHO classification, we categorized them as either well differentiated or poorly differentiated GAC. The patients information is summarized in Table 1. The use of these tissues in this study was approved by the institutional research committee.

Main Agents

The primary antibodies included the antibody against CD31 (mouse monoclonal, Z2136, ZETA, USA), the antibody against CK8 & 18 (mouse monoclonal, 18-0213, Invitrogen, USA), the antibody against laminin (rabbit polyclone, ready to use, RB-082-R7, Labvision Neomarkers, USA), the antibody against HIF-1α (mouse monoclonal, MS-1164-P0, Labvision Neomarkers, USA), the antibody against VEGF (rabbit monoclone, Z2092, ZETA, USA), the antibody against MMP-2 (rabbit polyclone, Z2089, ZETA, USA), the antibody against MMP-9 (rabbit polyclone, Z2090, ZETA, USA). The 0.5% periodic acid and Schiff solutions were made in the pathology department of Tianjin Cancer Hospital. The staining system was PV6001 or PV6002 (Zhongshan, Beijin, PR China).

Immunohistochemical Staining and CD31/PAS Double-Staining

We performed preliminary screening on H&E-stained slides. The presence of VM was recognized when the channels were lined by tumor cells, instead of endothelial cells, without necrosis, hemorrhage or inflammatory cells in the vicinity, and RBCs could be seen in the lumens.

After that, we performed the CD31/PAS double-staining to validate the existence of VM. First, immunohistochemical staining for CD31 was performed by using primary antibody against CD31 after microwave antigen retrieval according to the manufacturer's instructions, the procedure was completed as described by Zhang et al in previous report [9]. After CD31 immunohistochemical staining, the sections were then exposed to 0.5% periodic acid solution for 15 min, and rinsed with distilled water for 2–3 min. In a dark chamber, these sections were treated with Schiff solution for 15–30 min. After rinsing with distilled water, sections were counterstained with hematoxylin. Normal gastric mucosa was chosen as a positive control.

Furthermore, we performed the immunohistochemical staining of CK8 & 18 and laminin to verify that the external cells lined the channels were GAC cells, and whether PAS-positive patterns was coincidence with laminin positive staining. The slides stained for laminin were pretreated with proteinase K (ZLI-9016, Zhongshan, Beijin, PR, China). The diaminobenzidine method was used as a visualization system. Appropriate positive and negative controls were applied.

Table 1 Clinicopathologic data of 173 patients with GAC

Factors	n
Gender	
Male	134
Female	39
Age	
≥60	98
<60	75
Tumor location	
Proximal	51
Distant	122
Tumor size	
≥10 cm	42
<10 cm	131
Histological grade	
Poorly-differentiated	148
Well-differentiated	25
Blood-borne metastasis	
Positive	43
Negative	130
Lymph node metastasis	
Positive	103
Negative	70
TNM stage	
I	21
II	45
III	62
IV	45

Tissue Array Methods and Immunohistochemical Staining

Original H&E slides from 173 selected patients were reviewed and representative tumor regions were marked by a pathologist under microscope. Tissue cylinders (2 mm in diameter) were punched from the corresponding paraffin-embedded gastric tumors (donor blocks) and arranged in a new recipient paraffin block by using a trephine apparatus. Two cores were chosen from each case for analysis. The four tissue array blocks were then completed. The blocks were heated at 42°C for 3 min, and then were routinely sectioned at 4 μm thickness. Those sections were used to perform HIF-1α, MMP-2, MMP-9 and VEGF immunohistochemical staining.

Quantization Methods

The positive expression of MMP-2, MMP-9 and VEGF was in the cytoplasm, that of HIF-1α was either in the nucleus or in the cytoplasm. The staining index (SI) was assessed by both the staining intensity and the percentage of positive cells. The average percentage of positively stained cells in 5 visual fields of each section was converted into a score as follows: 0 for <10%, 1 for <25%, 2 for <50%, and 3 for >50%. The score of the staining intensity of each section was completed as follows: 0 for no staining, 1 for light yellow, 2 for moderate yellow, 3 for brown. The SI equals the product of staining intensity and positive cell scores which were used to determine the final result for each section.

Microvessel was defined as any single CD31-stained cells or cluster of endothelial cells in low light microscopy examination. VM was determined as PAS-positive channels lined by tumor cells exclusively, not endothelial cells where RBCs were therein. Five fields of greatest number of neovascularization were chosen to assess the microvascular density (MVD) and the vasculogenic mimicry density (VMD). The average count of five fields under 400 magnification (0.28 mm^2 field area) was recorded as MVD and VMD. The values were defined as the mean count of microvessels or VM per 0.28 mm^2 field area.

Statistical Analysis

The statistical analysis was conducted using SPSS version 13.0 (SPSS, Chicago, IL, USA). P-value less than 0.05 was defined as significant level. Values are shown as mean ± SD or percentages. The χ^2 test, the Student's *t*-test, the Mann-

Whitney test and Spearman correlation analysis were used in our study. Kaplan-Meier survival analysis and log-rank test were performed to compare the survival time between two groups. Multivariate survival analysis was performed using the Cox proportional hazards model.

Results

Definition of VM in GAC

VM was identified by CD31/PAS double-staining. The cells lined the external wall of PAS-positive channels were tumor cells, RBCs were present in those channels. The cells surrounded VM were negative for CD31, but positive for CK8 & 18 staining, indicating that they were GAC cells, rather than endothelial cells. A great deal of PAS-positive patterns were observed in the VM-positive slides, so did laminin (Fig. 1a–f). There were 40 (23%) patients recognized to be VM positive.

Relation Between VM and Clinicopathologic Data

The detailed clinicopathologic data between the VM and non-VM groups are summarized in Table 2. The incidence of VM was obviously associated with differentiated degree and blood-borne metastasis of GAC. The frequency of VM was significantly higher in poorly differentiated GAC (39/

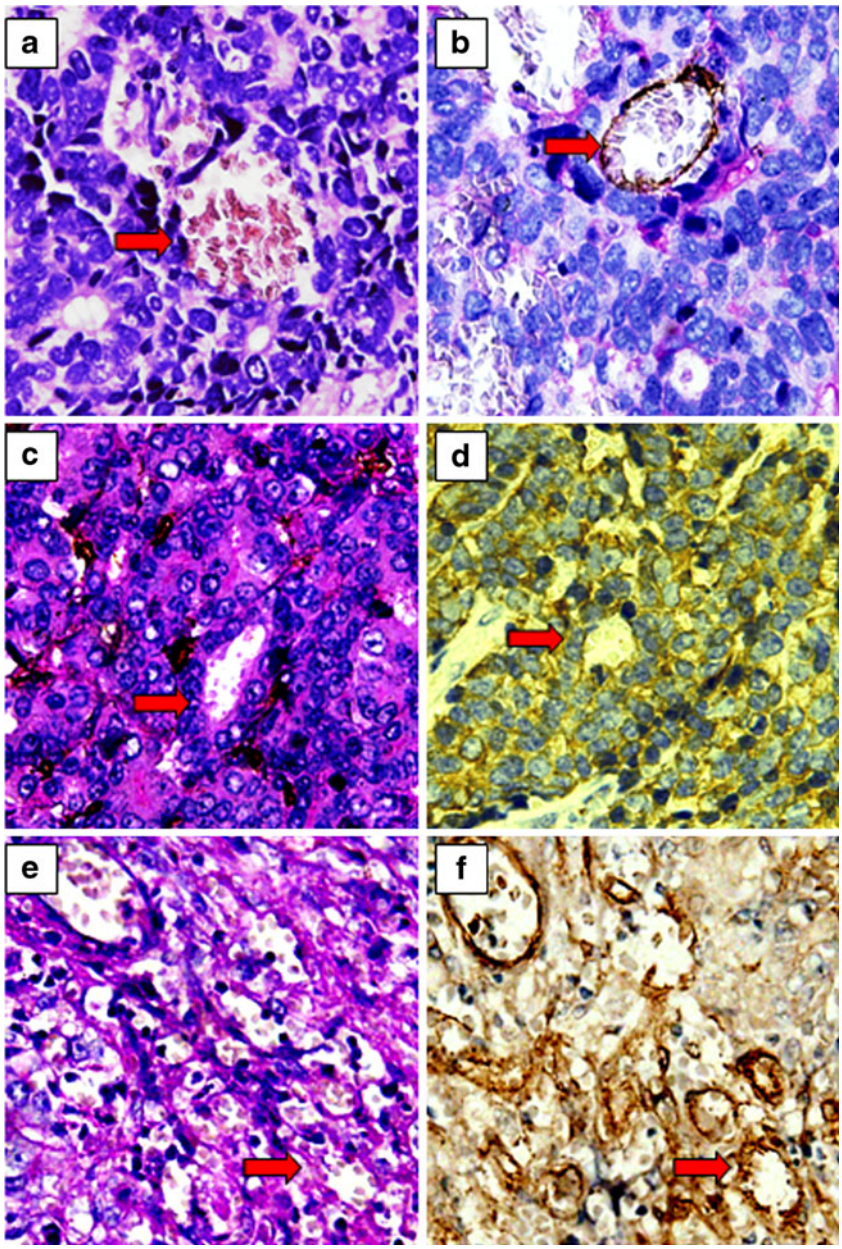

Fig. 1 Evidence of vasculogenic mimicry (VM) in gastric adenocarcinoma (GAC). **a** Morphologic appearance of VM with H&E staining. The VM channel (*arrow*) lined only by tumor cells, RBCs therein. There was absent of hemorrhage and necrosis cells in the vicinity. H&E, ×400. **b** The presence of CD31/ periodic acid-Schiff (PAS) double-staining of the endothelium dependent vessel (EDV). The endothelial cells lining the vessel were positive for CD31. x400 **c** The presence of CD31/PAS double-staining of the VM (*arrow*). The wall of the VM channel was positive for PAS staining, while tumor cells lining the external wall were negative for CD31 staining. ×400. **d** The cells lining the VM channel (*arrow*) were positive for CK8 & 18, which indicated that they were GAC cells. ×400. **e**, **f** Serial section from the VM-positive slides showed that extracellular matrix (ECM) was composed of PAS-positive patterns (*arrow*, **e**), parallelled with laminin positive-staining (*arrow*, **f**). RBCs can be seen in those channels. ×400

Table 2 Relation of VM with clinicopathologic data of GAC

Characteristic	Total	VM		*P*
		Positive	Negative	
Gender				
Male	134	34	100	
Female	39	6	33	NS
Age				
≥60 y	98	19	79	
<60 y	75	21	54	NS
Tumor location				
Proximal	51	16	35	
Distant	122	24	98	NS
Tumor size (cm)				
≥10	42	10	32	
<10	131	30	101	NS
Histological grade				
Poorly differentiated	148	39	109	
Well differentiated	25	1	24	0.014
Blood-borne metastasis				
Positive	43	16	27	
Negative	130	24	106	0.020
Lymph node metastasis				
Positive	103	29	74	
Negative	70	11	59	NS
TNM stage				
I	21	3	18	
II	45	10	35	
III	62	20	42	
IV	45	7	38	NS

NS non-significant

148, 26.4%) than in well differentiated GAC (1/25, 4%) (χ^2= 6.011, *P*=0.014). There was a total 43 of GAC patients with blood-borne metastasis. In the VM group, the rate of blood-borne metastasis was higher (16/40, 40%) than that of non-VM group (27/133, 20.3%) (χ^2=6.389, *P*=0.020). The difference of MVD between the two groups was of no statistical significance, but there was slight higher in the VM group (29.39±10.43/0.28 mm^2) than that of non-VM group (27.71±13.95/0.28 mm^2) (*F*=1.596, *P*=0.482). In addition, we compared the presence of VM with age and gender, tumor size and location, lymph node metastasis, and the TNM stage, yet, no statistical significance was detected between the two groups.

VM Associated with Poor Prognosis and Short Survival

Among 173 GAC patients, there were 117 patients who accepted both surgery and chemotherapy. The VM group (26/117) showed poor response to anti-cancer therapy, as there was higher distant recurrence (12/26, 46.15%) than that of non-VM group (22/91, 14.18%) (χ^2=4.748, *P*=0.029). A *t*-test was used to analyze the different values of VMD between the hematogenous metastatic group and non-hematogenous metastatic group. The prior group had more VM channels (16.19±5.88/0.28 mm^2) than that of later group (10.58±5.29/0.28 mm^2) (*t*=3.140, *P*=0.003). The survival rates of the VM group were shorter than that of non-VM group with a Kaplan-Meier survival analysis (*P*= 0.022) (Fig. 2). The means and medians for survival time of the VM group were 34.78 and 24.13 months, compared with 51.73 and 32.22 months for non-VM group. The five-year survival rate was 26.5% and 36.2% in the VM and non-VM group respectively. Cox proportional hazards model analysis was performed and showed that the presence of VM and the TNM stage were independent indicators of poor prognosis (*P*=0.039 and 0.004).

The Results of Immunohistochemical Staining of HIF-1α, MMP-2, MMP-9 and VEGF Between the VM and Non-VM Group

We performed immunohistochemical staining for HIF-1α, MMP-2, MMP-9 and VEGF in the VM and non-VM group to further identify their association with VM formation. HIF-1α was expressed either in the nucleus or in the cytoplasm of GAC cells, MMP-2, MMP-9 and VEGF were all located in cytoplasm (Fig. 3a–h). The SI of HIF-1α, MMP-2, MMP-9 and VEGF were all higher in the VM group than that of non-VM group (*P*=0.000, 0.004, 0.009, 0.000) (Table 3). The expression of HIF-1α was positively

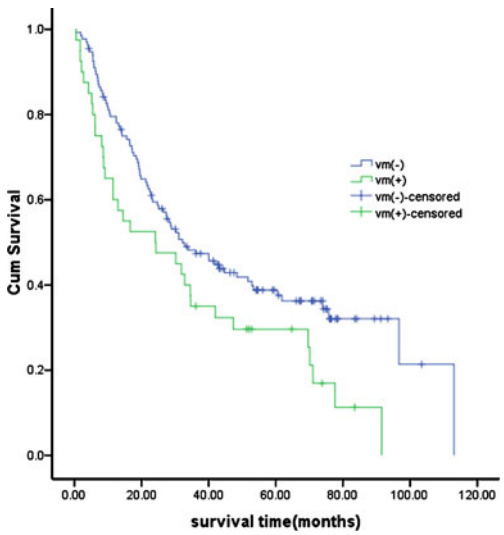

Fig. 2 Kaplan-Meier survival analysis showing the VM group has a shorter survival time than that of non-VM groups in gastric adenocarcinoma

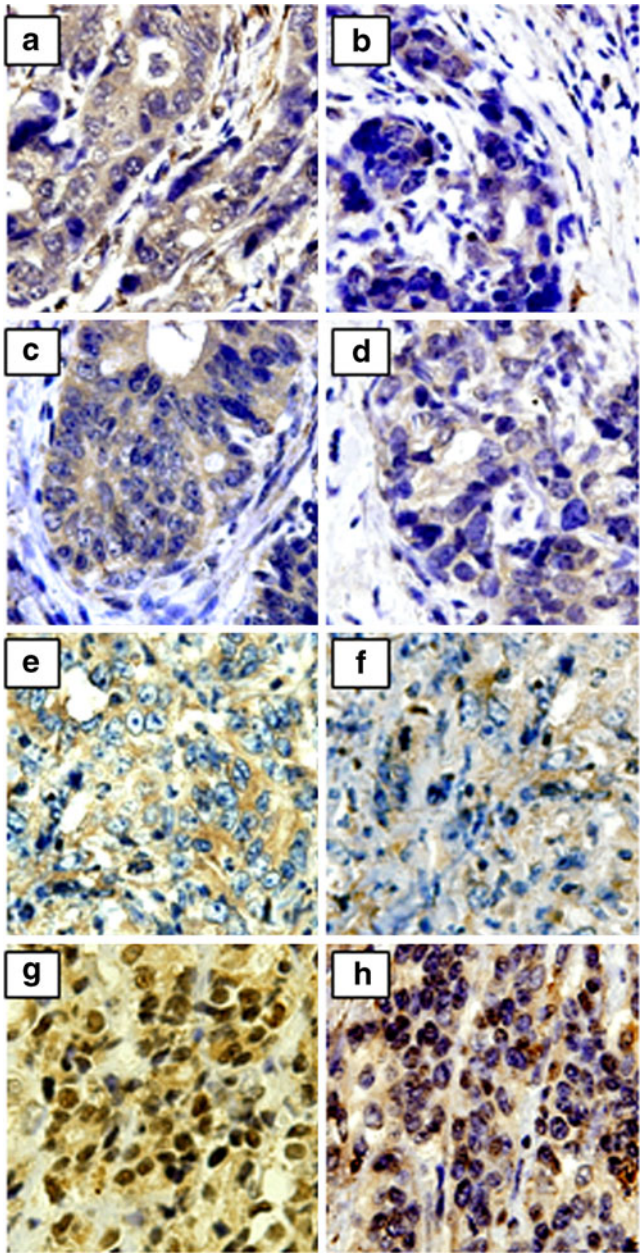

Fig. 3 Immunohistochemical staining expression of HIF-1α, VEGF, MMP-2 and MMP-9 in the VM and non-VM group of GAC. **a**, **b** Expression of MMP-2 in the VM and non-VM group, ×400 **c**, **d** Expression of MMP-9 in the VM and non-VM group, ×400 (**e**, **f**) Expression of VEGF in the VM and non-VM group, ×400 (**g**, **h**) Expression of HIF-1α in the VM and non-VM group, ×400

correlated with that of VEGF in the VM group (r_s=0.344, P=0.046).

Discussion

It is crucial for tumor cells to acquire sufficient blood supply for their survival, proliferation and metastasis. Angiogenesis is the process of formation of new vessels from pre-existing vasculature. Tumor angiogenesis is always the "hot spot" which has gained considerable attention. Since 1999, this prevailing assumption that tumor blood supply only depends on the angiogenesis has been challenged by a new discovery, termed as "vasculogenic mimicry". It was the novel vascular channels generated by aggressive tumor cells, instead of endothelial cells [1]. Another conspicuous feature was that there was abundance of PAS-positive patterns in the ECM. Several researches have demonstrated that those patterns could communicate with the tumor microcirculation [1, 4, 5]. Either RBCs or plasma could appear in those patterns [4]. It has been the typical evidence to be used for VM judgement in previous studies [1, 6–12, 14].

In our study, the CD31/PAS double-staining combined with CK8 & 18 immunohistochemical staining were performed for the first time to validate the existence of VM in GAC. The appearance of VM was that the wall was composed of PAS-positive patterns, GAC cells lined externally, and RBCs were therein. Moreover, we performed immunohistochemical staining of laminin, the outcome revealed the PAS-positive patterns were rich in laminin, that was similar to the previous report [3]. VM were frequently found in the periphery of tumors, that might provide the sufficient blood supply for tumor cells to invade adjacent normal tissues. Resently Zhang et al [31] have reported that SGC7901, a gastric adenocarcinoma cell line, can form the vascular-like networks in vitro. This finding happens to provide the evidence that GAC cells have the possible ability to form VM.

Adenocarcinoma, the commonest malignancy happened in stomach, accounts for approximately 90% of detected cases [32]. So it is representative and persuasive to select GAC as our research object. Histological grade according to morphological manifestation categorizes them as two groups: well differentiated and poorly differentiated GAC by the existence of glandular structure [33]. It is commonly recognized that poorly differentiated gastric cancer also have worse prognosis [34]. In our retrospective study of the 173 GAC patients, we have found that VM was correlated

Table 3 Differential expression of HIF-1α, MMP-2, MMP-9 and VEGF between the VM and non-VM group

Stain	VM		Z	*P*
	Positive	Negative		
HIF-1α	5.14±2.22	2.36±2.17	−5.532	0.000
MMP-2	3.63±2.03	2.29±2.16	−2.813	0.004
MMP-9	3.91±1.86	2.97±1.68	−2.622	0.009
VEGF	5.44±2.03	3.05±2.17	−5.349	0.000

with tumor differentiated degree, blood-borne metastasis and short survival time. This outcome showed that VM was an potential indicator of poor prognosis for GAC. VM has a more frequent occurrence in the poorly differentiated GAC. As we known, poorly differentiated cancer cells often have stronger aggressive and metastatic ability [35]. Sood AK et al [36] found that the molecular profile of aggressive cutaneous and uveal melanoma cells in the presence of VM was similar to that of pluripotent, embryonic-like stem cells. That gives us a hint that tumor cells composed of VM channels are generally poorly differentiated and of high plasticity, so that they can secret some proteins to be beneficial for penetration and metastasis. Owing to special structure of VM [1], i.e. there is no barrier formed by endothelial cells to block tumor cells from accessing to the microcirculation. Thus tumor cells should leak out and migrate into blood flow easily. So it is reasonable that GAC patients with VM and high VMD are favorable of hematogenous metastasis. Metastases are the cause of 90% of cancer deaths [37], so it can explain why VM is associated with shorter survival in many malignances. Moreover another valuable finding from this study is that, VM could be used as a predictor of lower response to clinical therapy. The possible reason is that some chemotherapies may only target the traditional angiogenesis associated factors, hence would not be effective for VM. Therefore, looking for the molecular marker of VM might provide better approach for cancer therapeutic strategies.

It is known to all, hypoxia may be the most principal switch for tumor angiogenesis [27]. There was a hypothesis that the formation of VM might be a complementary means for tumor cells to acquire an adequate supply of oxygen and metabolites in hypoxic environment [25]. Our co-workers validated this by animal-models [24]. They found melanoma cells in a hypoxic microenvironment could increase HIF-1α expression and enhance the VM formation. The result proves that VM formation is associated with hypoxia. HIF-1 is the key regulator in hypoxic condition, that is a heterodimer consisting of α and β subunits, HIF-1α is the active component expressed commonly in human cancers [26, 27]. Under hypoxic conditions, HIF-1α is expressed stably and dimerized with HIF-1β to target downstream gene, such as VEGF to promote tumor angiogenesis [26, 27]. In our study, the expression of HIF-1α and VEGF were both higher in the VM group than non-VM group. Although MVD count is not obviously different between two groups, it was slight higher in prior one. We presume that in the ischemic microenvironment, the EDV cannot provide an adequate blood supply for tumor growth. The poorly differentiated and aggressive tumor cells could transform themselves to shape "new vessels", on the other hand they secrete some angiogenesis-promoting genes, such as VEGF so as to enhance the blood supply. As a result, co-existing VM with EDV should distribute more blood and oxygen for tumor cells survival. Hypoxia also activates MMP-2 and MMP-9 which are two important enzymes to degrade ECM [38, 39]. Some experiments concerning of biological characteristic of VM have revealed that the VM-constitutive cells could express high levels of MMP-1, 2, 9, and 14 and the 5γ2 chain of laminin [20–23]. It can facilitate to remodel the ECM which is one of the prerequisite steps for the VM formation [20, 21]. In our study, we found the abundant expression of MMP-2 and MMP-9 in the VM group of GAC too.

In a word, VM exists in GAC, especially in poorly differentiated GAC. And it is associated with distant metastasis and short survival duration. Thus, VM is a predictor of poor prognosis for GAC. Hypoxia maybe play a role in the VM formation. Further research concerning molecular mechanisms of VM would shed light on new therapeutic strategies for GAC.

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