

Original Article

Erythropoietin and erythropoietin receptor in hepatocellular carcinoma: correlation with vasculogenic mimicry and poor prognosis

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Abstract: To evaluate erythropoietin (Epo) and erythropoietin receptor (EpoR) expression, its relationship with vasculogenic mimicry (VM) and its prognostic value in human hepatocellular carcinoma (HCC), we examined Epo/EpoR expression and VM formation using immunohistochemistry and CD31/PAS (periodic acid-Schiff) double staining on 92 HCC specimens. The correlation between Epo/EpoR expression and VM formation was analyzed using two-tailed Chi-square test and Spearman correlation analysis. Survival curves were generated using Kaplan-Meier method. Multivariate analysis was performed using Cox regression model to assess the prognostic values. Results showed positive correlation between Epo/EpoR expression and VM formation ($P < 0.05$). Patients with Epo or EpoR expression exhibited poorer overall survival (OS) than Epo-negative or EpoR-negative patients ($P < 0.05$). Epo-positive/VM-positive and EpoR-positive/VM-positive patients had the worst OS ($P < 0.05$). In multivariate survival analysis, age, Epo and EpoR were independent prognostic factors related to OS. These results will provide evidence for further research on HCC microcirculation patterns and also will provide new possible targets for HCC diagnosis and treatment.

Keywords: Erythropoietin, erythropoietin receptor, vasculogenic mimicry, prognosis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), the fifth most prevalent primary malignancies in worldwide, is the second leading cause of cancer death in China [1]. Both the incidence and mortality rates of HCC have been steadily increasing in recent years [2]. As an aggressive solid tumor, its prognosis remains unsatisfactory despite significant advances in surgical techniques and medical treatment [3]. High incidence of recurrence and metastasis contributed to the poor prognosis of HCC [4, 5]. Adequate blood supply is the main cause of recurrence and metastasis. Unfortunately, because of complicated blood supply pattern, therapeutic effect of anti-angiogenesis on HCC patients was unsatisfactory. Besides angiogenesis, vasculogenic mimicry (VM) and mosaic vessels which served as alternative pathways should be considered [6]. VM was first reported by Maniotis et al. in 1999,

which describes the ability of highly aggressive uveal melanoma to form highly patterned vascular channels in vivo composed of a basement membrane stained positive with periodic acid-Schiff (PAS) in the absence of endothelial cells [7]. Up to now, many studies were performed aiming at clarifying the underlying molecular pathways of VM.

Findings indicated erythropoietin (Epo) is a low-molecular-weight glycoprotein hormone stimulator of erythropoiesis produced in the fetal liver and subsequently in the adult kidney [8]. Epo exerts its action through specific binding to its cognate receptor (EpoR), a member of the cytokine receptor superfamily, which is mainly expressed on erythroid colony-forming units [9]. Epo can triggers a chain of intracellular signaling events, such as activation of the receptor-associated tyrosine kinase JAK2, phosphorylation and nuclear translocation of STAT5, thus

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promoting progenitor cell survival, proliferation and differentiation [10]. Many studies indicated that the functions of Epo and EpoR are not strictly limited to erythroid or hematopoietic lineages. For instance, EpoR expression has been detected in umbilical cord and placental endothelial cell lines, and Epo was found to be capable of stimulating endothelial cell proliferation in vitro [11, 12]. In addition, many kinds of cancers, such as breast cancer, renal cancer, gastric cancer, hepatocellular carcinoma and central nervous system tumors have shown Epo/EpoR expression [13-17].

There is increasing evidence suggesting a wider biological role for Epo/EpoR related to malignant biological behavior of tumor, including tumor angiogenesis [15]. Epo induces endothelial cell proliferation and migration [11, 18]. Tumor cells lining the VM networks express multiple endothelial markers, and resemble endothelial cell functions [19]. The expression of EpoR in tumor cell and vascular endothelium maybe imply that Epo/EpoR may affect the tumor microenvironment, perhaps by stimulating tumor angiogenesis and VM formation.

Epo is a hypoxia responsive cytokine. Low oxygen tension increases activity of hypoxia-inducible factor (HIF) that binds to cis-acting DNA hypoxia response elements (HREs) to activate EPO transcription [20]. Ribatti and others described erythropoietin as a pro-angiogenic factor comparable to the classical pro-angiogenesis factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [21]. Our previous studies indicated that hypoxia condition, HIF and VEGF are particularly relevant in VM formation of tumors [22, 23]. So we hypothesize Epo/EpoR expression may associated with VM formation.

Although Epo/EpoR involved in angiogenesis in HCC has been reported on previous study [16], the relationship between Epo/EpoR expression and VM in HCC and the relevance of their co-existence within clinical parameters remain unclear. In the current study, expression patterns of Epo/EpoR and VM were examined by immunohistochemistry (IHC) on 92 samples of human HCC samples. The correlation of Epo/EpoR expression and VM and its relevance to clinicopathologic parameters were explored. Prognostic roles of Epo/EpoR expression and VM in human HCC were also evaluated using Cox regression and Kaplan-Meier analysis. To

our knowledge, this study is the first to report the correlation of Epo/EpoR expression and VM and its clinical significance for HCC.

Materials and methods

Patients

Tissue specimens were obtained from 92 patients who had undergone hepatectomy for HCC in Tianjin Medical University Cancer Institute and Hospital from September 2000 to December 2004. The diagnoses of these samples were independently verified by two pathologists. The data of clinicopathological parameters were harvested from the patients' clinical records and pathological reports. We collected paraffin-embedded tumor tissue samples from patients who had not undergone therapy prior to tumor surgical operation. This study was approved by the Ethical Committee of Tianjin Medical University prior to its initiation.

Immunohistochemistry and CD31/periodic acid Schiff (PAS) double staining

Tissue sections (4 μ m) were deparaffinized and hydrated following standard procedures. After immersing in 3% H₂O₂ for 30 min to eliminate endogenous peroxidase, the sections were microwaved for antigen retrieval in 0.01 M sodium citrate for 15 min. After blocking with 10% goat serum for 30 min at room temperature, the slides were incubated with a primary antibody overnight at 4°C and a homologous secondary antibody for 1 h at room temperature in a humidified box. Then the sections were stained with freshly dispensed diaminobenzidine solution (DAB) for observation under a microscope. In the process, the slides were all rinsed three times in phosphate-buffered saline (PBS) (pH 7.2) before each step, except for the procedure of serum blocking to incubation with the primary antibody. After counterstaining with hematoxylin or PAS, the slides were ready for microscopic examination.

In the current study, the primary antibodies to Epo and EpoR were purchased from Santa Cruz Biotechnology (Epo 1:50; EpoR 1:200, Santa Cruz, CA, USA) and CD31 (Zhongshan Golden Bridge Biotechnology Limited Company (Beijing, China). Positive control and negative control were performed for each batch. For the negative control, PBS was used instead of the primary antibody. For the positive control, a fore-

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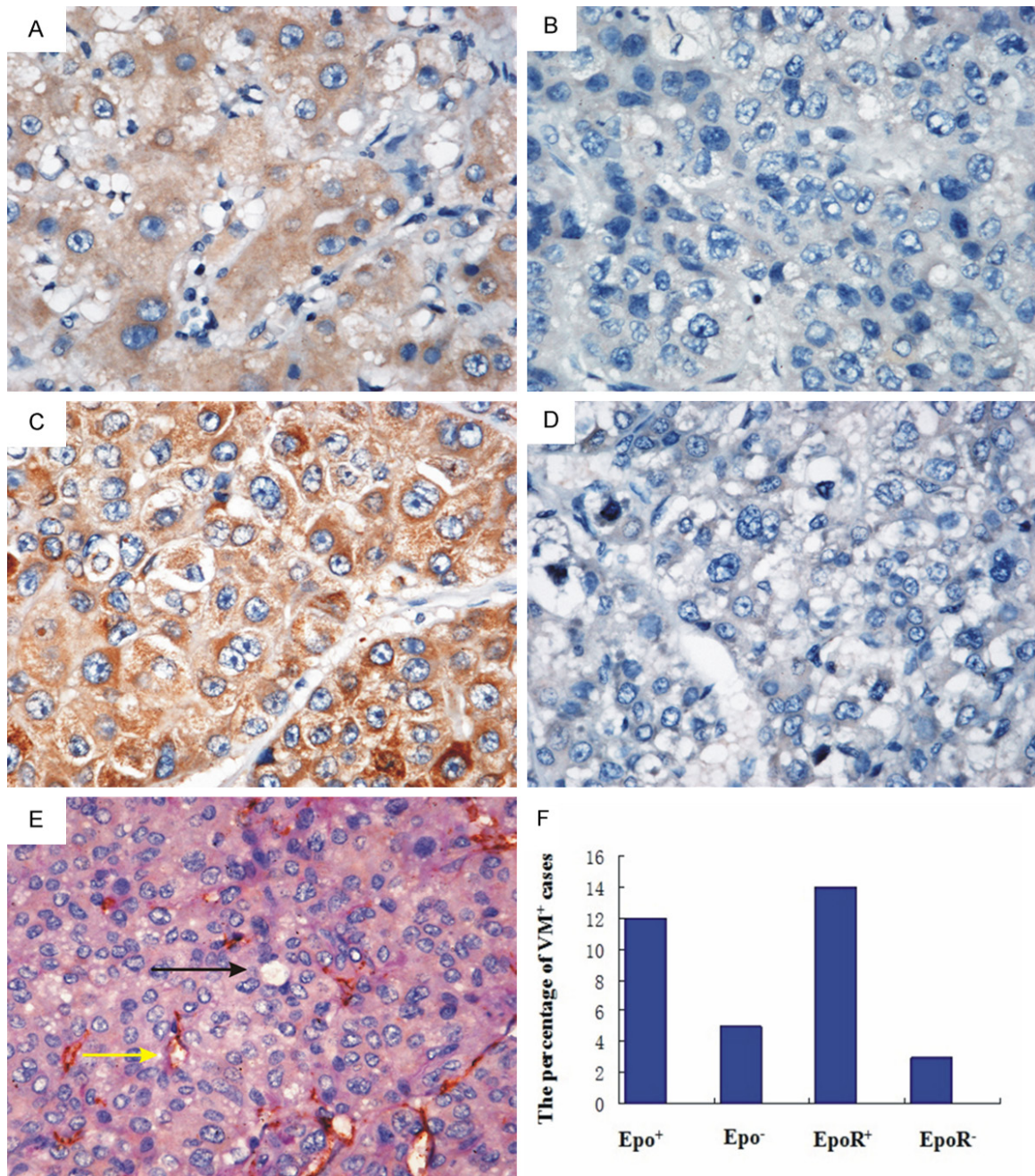


Figure 1. Epo/EpoR expression and VM formation in HCC specimens. A. Positive expression of Epo was a weak and diffuse cytoplasmic staining in the tumor cells. B. Negative expression of Epo in HCC. C. Positive expression of EpoR was brown granules staining in the cytoplasm/membrane of the tumor cells. D. Negative expression of EpoR in HCC. E. VM formation in HCC tissue (black arrows indicate VM channels formed by tumor cells; yellow arrows indicate typical blood vessels with brown CD31⁺ staining). F. Epo/EpoR-positive expression directly correlated with VM formation in HCC samples.

gone positively expressed tissue section was used. The results were evaluated following the method described by Bittner et al [24]. The percentage and the intensity of the positive cells were both measured. The percentage was stratified as follows: 0 for less than 5% positive

cells, 1 for less than 30% positive cells, 2 for less than 60% positive cells, and 3 for more than 60% positive cells. The intensity was also classified as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The sum of positive cell and staining intensity scores, which was

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Table 1. Relationship between Epo/EpoR and clinicopathologic characteristics/VM formation of patients with HCC

Variables	Epo		χ^2	P	EpoR		χ^2	P
	Negative	Positive			Negative	Positive		
Ages (year)								
< 50	11	11	1.434	0.231	10	12	0.262	0.609
≥ 50	45	25			26	44		
Sex								
Male	48	31	0.003	0.957	29	50	1.376	0.241
Female	8	5			7	6		
Tumor size (cm)								
> 5	18	22	7.483	0.006*	14	26	0.507	0.467
≤ 5	38	14			22	30		
Histological differentiation								
I/II	34	12	6.571	0.010*	23	23	4.563	0.033*
III/IV	22	24			13	33		
Stage								
I/II	19	24	9.435	0.002*	23	20	6.988	0.008*
III/IV	37	12			13	36		
Metastasis								
Yes	11	18	9.335	0.002*	9	20	1.165	0.280
No	45	18			27	36		
VM								
Positive	5	12	8.664	0.003*	3	14	4.041	0.044*
Negative	51	24			33	42		

*Significantly different.

more than 3 for the final result, was considered as the positive sample for each slide.

Statistical analysis

All data in the study were evaluated with SPSS17.0 software (SPSS, Chicago, IL, USA). The correlation of Epo/EpoR expression and VM formation and clinicopathologic parameters was analyzed using two-tailed Chi-square test and Spearman correlation analysis. Survival curves were estimated using Kaplan-Meier method and compared by log rank test. Univariate or multivariate analysis of prognostic factors was tested for Cox proportional-hazard regression models. All *P* values were two-sided, and *P* < 0.05 was considered statistically significant.

Results

Epo/EpoR expression and VM formation in HCC specimens

To investigate Epo/EpoR expression in HCC, IHC staining was performed on 92 HCC tissue

sections. In most cases, Epo showed a weak and diffuse cytoplasmic pattern in HCC cells (**Figure 1A**). By contrast, negative Epo expression is shown in **Figure 1B**. EpoR positive expression appeared as brown granules staining in the cytoplasm/membrane of the tumor cells (**Figure 1C**). Negative EpoR expression is shown in **Figure 1D**. Among 92 HCC specimens, Epo was detected in 36 cases (39.13%) as well as EpoR was detected in 56 cases (60.86%). According to CD31/periodic acid Schiff double staining, VM was found in 17 out of 92 HCC samples (18%) (**Figure 1E**). Hepatocellular carcinoma cells mimic endothelial cells to form extracellular matrix-rich channels (PAS-positive) without necrosis and inflammatory cells infiltrating around the channels (black arrows indicate VM, yellow arrows indicate typical blood vessels with brown CD31⁺ staining).

Of the 92 cases analyzed, 12 (13.04%) were positive for both Epo and VM formation, 51 (55.43%) were both negative, 24 were Epo positive only, and 5 were VM positive only. 14 (15.21%) were positive for both EpoR and VM formation, 33 (35.86%) were both negative, 42

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Table 2. Correlation between expression of Epo/EpoR and clinicopathologic characteristics/VM formation of patients with HCC

Variables	Epo		<i>r</i>	<i>P</i>	EpoR		<i>r</i>	<i>P</i>
	Negative	Positive			Negative	Positive		
Ages (year)								
< 50	11	11	-0.024	0.823	10	12	0.004	0.970
≥ 50	45	25			26	44		
Sex								
Male	48	31	-0.078	0.459	29	50	-0.022	0.836
Female	8	5			7	6		
Tumor size (cm)								
> 5	18	22	0.075	0.476	14	26	0.101	0.340
≤ 5	38	14			22	30		
Histological differentiation								
I/II	34	12	0.040	0.709	23	23	0.072	0.493
III/IV	22	24			13	33		
Stage								
III	19	24	0.289	0.005*	23	20	0.287	0.006*
III IV	37	12			13	36		
Metastasis								
Yes	11	18	0.084	0.425	9	20	-0.048	0.646
No	45	18			27	36		
VM								
Positive	5	12	0.352	0.001*	3	14	0.181	0.084
Negative	51	24			33	42		

*Significantly different.

were EpoR positive only, and 3 were VM positive only.

Relationship between Epo/EpoR and VM formation as well as clinicopathological features in HCC

According to Epo/EpoR presence, all samples were divided into two groups respectively: Epo-positive group (n=36)/Epo-negative group (n=56) and EpoR-positive group (n=56)/EpoR-negative group (n=36). Then, the relationship between Epo/EpoR and VM formation as well as clinicopathological features was analyzed separately. Statistical data in **Table 1** showed that Epo/EpoR was significantly associated with VM formation ($P=0.003$ and 0.044 , resp.). In addition, Epo was significantly associated with tumor size, histological differentiation, stage and metastasis ($P=0.006$, 0.010 , 0.002 and 0.002 , resp.). EpoR was significantly associated with histological differentiation and stage ($P=0.033$ and 0.008 , resp.). Interestingly, correlation analysis revealed that only Epo-

positive expression is directly correlated with VM formation in these samples ($r=0.352$, $P=0.001$) (**Figure 1F**). Both Epo and EpoR are correlated with stage ($r=0.289$, 0.287 and $P=0.005$, 0.006 resp.). However, no significant correlation existed in Epo/EpoR with other clinicopathological features (**Table 2**).

Prognostic significance of EPO/EPOR expression and VM formation in HCC

To evaluate the prognostic significance of EPO/EPOR expression, Kaplan-Meier survival analysis was performed. Survival analysis revealed that Epo-positive and EpoR-positive patients showed lower overall survival (OS) than those with Epo-negative and EpoR-negative expression ($P=0.002$ log-rank test, resp.) (**Figure 2A, 2B**). Notably, patients with Epo-positive/VM formation or EpoR-positive/VM formation exhibited the worst survival. By contrast, patients with both negative Epo and VM or EpoR and VM demonstrated the highest survival (**Figure 2C, 2D**).

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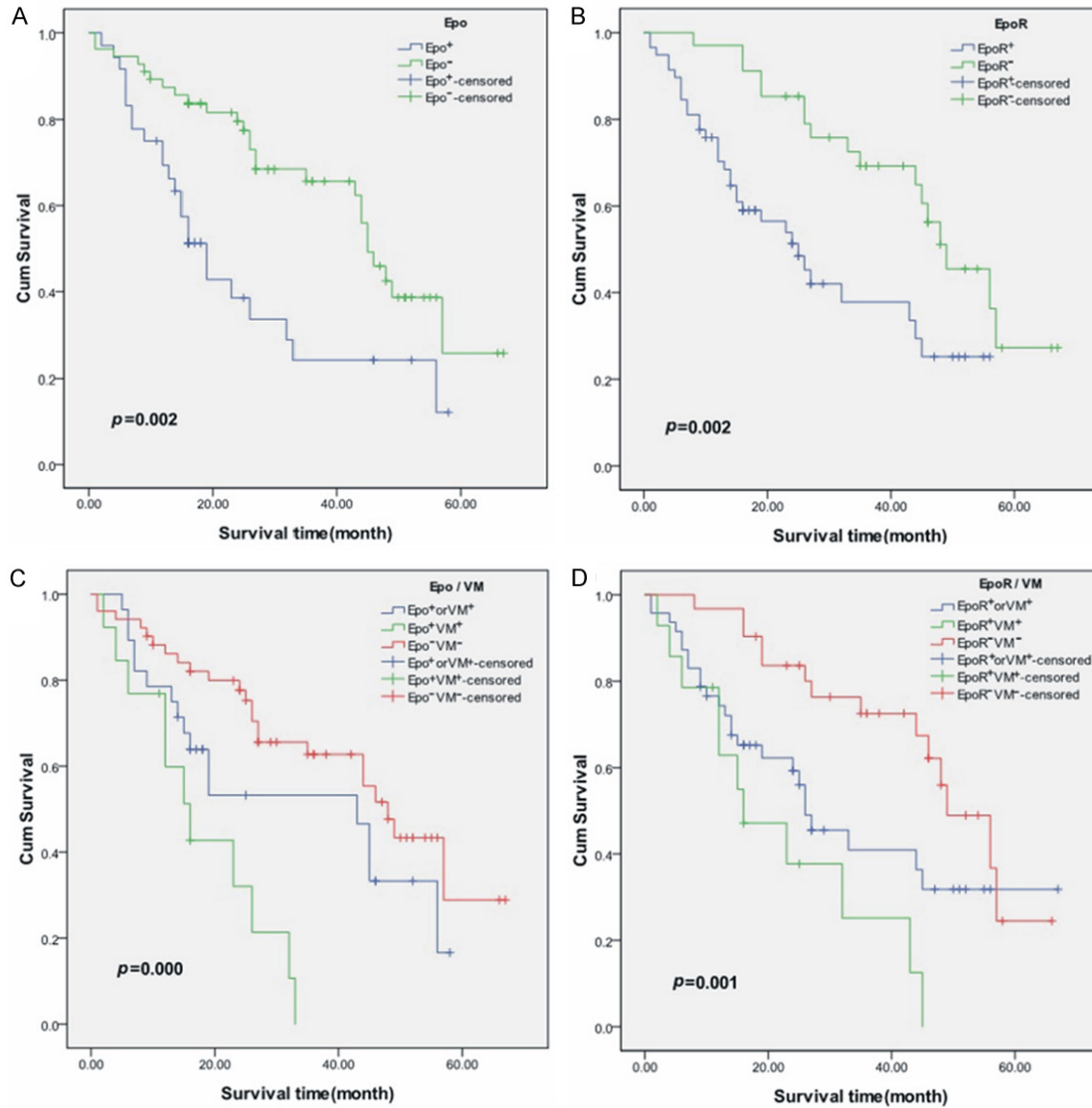


Figure 2. Correlation of Epo/EpoR expression and VM with overall survival (OS). A. Epo-positive patients showed poorer prognosis for OS than Epo-negative patients. B. EpoR-positive patients showed poorer prognosis for OS than EpoR-negative patients. C, D. Patients with both Epo/EpoR-positive expression and VM formation exhibited the worst survival. By contrast, patients with both negative Epo/EpoR and VM demonstrated the highest survival for OS.

To analyze the relevance of Epo/EpoR expression, VM formation, and clinicopathological features with OS, univariate Cox regression analysis was performed with factors including age, sex, tumor size, histological differentiation, stage, metastasis, VM formation, Epo expression and Epo expression. Combined Epo-positive and VM formation, EpoR-positive and VM formation were also analyzed (Table 3). Statistical analysis indicated that VM formation, Epo positive expression and EpoR positive expression were significantly associated with

poor OS ($P=0.004$, 0.002 , 0.003 resp). More importantly, the combined Epo positive and VM formation, EpoR positive and VM formation were significantly associated with poor OS ($P=0.000$, 0.004 resp.).

In multivariate analysis, age ($P=0.012$; HR=0.449; CI 0.240-0.841), Epo ($P=0.001$; HR=2.711; CI 1.495-4.918), and EpoR ($P=0.001$; HR=3.088; CI 1.603-5.939) were independent prognostic factors related to OS (Table 3). However, VM formation, the combined Epo

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Table 3. Cox proportional-hazard regression model analysis for overall survival

Variables	Univariate Analysis			Multivariate Analysis		
	HR**	95% CI	p	HR**	95% CI	p
Ages (year) < 50, ≥ 50	0.495	0.266-0.922	0.027*	0.449	0.240-0.841	0.012*
Sex Male, Female	1.284	0.575-2.870	0.542			
Tumor size (cm) ≤ 5, > 5	0.990	0.567-1.728	0.971			
Histological differentiation I/II,III/IV	0.700	0.381-1.285	0.250			
Stage I/II, III/IV	0.475	0.264-0.855	0.013*			
Metastasis Yes, No	0.628	0.354-1.113	0.111			
VM Positive, Negative	2.528	1.340-4.769	0.004*			
Epo Positive, Negative	2.395	1.360-4.217	0.002*	2.711	1.495-4.918	0.001*
EpoR Positive, Negative	2.509	1.356-4.641	0.003*	3.086	1.603-5.939	0.001*
Epo/VM Epo ⁺ /VM ⁺ , Non Epo ⁺ /VM ⁺	3.573	1.764-7.237	0.000*			
EpoR/VM EpoR ⁺ /VM ⁺ , Non EpoR ⁺ /VM ⁺	2.744	1.379-5.462	0.004*			

*Significantly different. HR**: Hazard ratio.

positive and VM formation as well as EpoR positive and VM formation did not show independent prognostic significance for OS in the multivariate analysis.

Discussion

Three patterns of microcirculation including endothelium-dependent vessels, mosaic vessels (MV) and vasculogenic mimicry (VM) were reported to participate in tumor blood supply [7, 25-27]. Our previous study reported the number of vasculogenic mimicry decreased and the number of endothelial-dependent vessels increased during tumor growth in a mouse melanoma xenograph [6]. Mosaic vessels might be the interim state between vasculogenic mimicry and endothelium-dependent vessels. But how to transform between three patterns, in other words, how tumor epithelial cells integrated into the malignant tumor vasculature is unclear. As a form of angiogenesis, VM was described as characteristically had tumor cells, but not endothelial cells, expressed multiple endothelial markers and resemble endo-

thelial cell functions [7]. VM positive tumors are usually related to more aggressive tumor biology and poor clinical outcomes [28]. A considerable number of researches have shown that tumors exhibiting VM are associated with high tumor grade, invasion and metastasis, and short survival [29-32].

Many factors were involved in the mechanism of VM formation. Hypoxia as a common phenomenon in solid tumors is an important microenvironment factor in evolution of malignant tumors. Recent research reported hypoxia and subsequent hypoxia-inducible factor-1 α (HIF-1 α) contributed to VM formation [22]. As a major HIF-1 target gene, Epo is closely related to malignant tumor growth, differentiation and angiogenesis [33]. EpoR is not regulated by HIF-1 though is also induced by hypoxia [34, 35]. In hypoxic tumor cell lines, Epo and EpoR were highly expressed [36]. Geza Acs et al. found hypoxic tumor regions displayed the highest levels of Epo and EpoR expression in specimens of breast carcinoma [34]. It is generally believed that EpoR expressed in tumor tissue

could stimulate tumor angiogenesis, thus increasing the oxygen content of tumor and contributing to tumor growth and development [37]. Several studies have demonstrated that Epo is secreted by tumor cells and it affects vascular endothelial cells and promotes angiogenesis via its receptor [15, 38, 39].

MMPs expression and activation and then degradation of extracellular matrix can be seen as prerequisite of VM formation. A previous study indicated increased expressions of matrix metalloproteinase 2 (MMP2) and MMP-14 are required for VM formation [40]. Additionally, MMP-2 and MMP-9 were present in all the VM cases but were found less frequently in non-VM cases [41]. Epo is a pleiotropic survival and growth factor. Recombinant human Epo (rh-EPO)-activated endothelial cells significantly increased secretion of MMP2 and MMP9 and then enhance neural progenitor cell migration [42]. These findings may imply Epo involved in VM formation by up-regulating MMPs expression.

In this study, we examined Epo/EpoR expression and VM formation in 92 human HCC specimens using IHC. The relationship between the clinicopathological factors and Epo/EpoR expression was analyzed. This retrospective study of 92 HCC patients showed that Epo/EpoR positive expression had strong correlation with histological differentiation and stage. A previous study also reported that high expression of Epo/EpoR is correlated with histological differentiation in HCC [16]. We also found that Epo positive expression is also correlated with tumor metastasis, which was consistent with a previous study on head and neck squamous cell carcinoma [43]. Our previous research reported that patients with VM had a higher metastasis rate than did those without VM in HCC [41]. Other studies also indicated VM contributes to distant metastasis in breast carcinomas and lymph node metastasis of laryngeal squamous cell carcinoma [44, 45]. Collectively, these findings may suggest that the increased Epo/EpoR expression in HCC promotes VM formation, thereby facilitating tumor cell migration and metastasis into the blood and lymphatic vessels and promoting HCC aggressiveness.

We found that patients with Epo/EpoR-positive expression showed poorer survival than Epo/EpoR-negative patients. This result is consis-

tent with earlier observations in tongue squamous cell carcinoma [46] and cervical cancers [47]. Our previous study found that patients in the VM group had a significantly shorter survival duration than did those in the non-VM group [41]. In the current study, Kaplan-Meier analyses revealed that Epo/EpoR-positive expression combined with VM formation significantly correlated with the worst OS in HCC. Patients with Epo/VM positive expression or EpoR/VM positive expression showed the worst survival, whereas those with double negative of Epo/VM or EpoR/VM exhibited the highest survival. Consistent with previous reports, our Cox multivariate analysis demonstrated that Epo/EpoR was an independent prognostic factor for OS in HCC patients. These results indicated that coexistence of VM and Epo/EpoR expression predicted worst survival and may serve as the key molecular prognostic indicator for HCC survival.

To our knowledge, this study is the first to present clinical evidence indicating that Epo/EpoR expression and VM formation are positively correlated in human HCC. Vascular endothelial growth factor A (VEGFA) is one of the major regulators of angiogenesis, which has been proposed to be involved in tumor VM formation. Our previous study by *in vitro* assays and clinical immunohistochemical analyses indicated that VEGF-a participated in the process of VM formation and appeared to play an important role in the formation of VM [23]. Others also reported VEGF and the phosphoinositide 3-kinase/AKT pathway exert a positive feedback regulation in the process of VM formation [48]. Jaquet et al. described Epo exhibits the same angiogenic potential on endothelial cells as VEGF [49]. Moreover, recent study indicated EPOR could be up-regulated and activated by VEGF in human retinal microvascular endothelial cells [50]. Thereby the correlation of Epo/EpoR-positive expression and VM formation suggested that Epo/EpoR could exert a promoting role in VM formation.

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Disclosure of conflict of interest

None.

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