Original Article
The effect of hepatocellular carcinoma-associated fibroblasts on hepatoma vasculogenic mimicry

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Abstract: Vasculogenic Mimicry (VM) is the main source of blood supply in the early stage of tumor growth. Carcinoma-associated fibroblasts (CAFs) are one of the most important host cells in the tumor microenvironment. Some studies have found that CAFs can promote tumor angiogenesis, but there are few reports on the relationship between CAFs and VM. Tissue samples were collected from 60 cases of hepatocellular carcinoma (HCC) and 10 persons with normal liver function. The relationship between VM expression and clinicopathologic features was analyzed. Furthermore, the relationship between VM expression and vimentin or α-SMA expression was analyzed. Primary culture of hepatocellular CAFs and the collection of conditioned media were carried out. The effects of hepatocellular CAF conditioned medium on the formation of VM and the levels of VM-related proteins and genes in MHCC-97H cells were studied. The positive rate of VM was 35.0% in HCC tissues. There was no VM expression in normal liver tissues. VM expression was related to tumor diameter, Edmondson grade, clinical stage, and liver cirrhosis. The expression of vimentin and α-SMA in VM-positive patients was higher than in VM-negative patients. Different concentrations of hepatocellular CAF conditioned medium could promote the formation of VM and increase the expression of VM-related genes and proteins (MMP2 and EphA2) in MHCC-97H cells. The results show that there was a significant correlation between VM formation and the expression of vimentin or α-SMA in HCC tissues. The conditioned medium of hepatocellular CAFs may promote VM formation and the expression of VM-related genes and proteins (MMP2 and EphA2) in hepatoma cell line MHCC-97H.

Keywords: Hepatocellular carcinoma, carcinoma-associated fibroblasts, vasculogenic mimicry

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of death due to malignant tumors worldwide [1], and has become a serious public health problem. Risk factors of HCC include chronic hepatitis B and C virus infection, alcohol consumption, smoking, aflatoxin contamination, obesity, and diabetes [2]. Presently, traditional surgical resection is recognized as the most effective treatment option of HCC. In addition, non-operative treatments such as transcatheter arterial chemoembolization, local radiofrequency ablation, radiotherapy, and emerging molecular-targeted therapy have also achieved certain results [3], but do not significantly improve the survival time of HCC patients. Therefore, exploring the molecular mechanism of invasion and metastasis is of

great significance for the molecular diagnosis, prevention, and treatment of HCC.

Vasculogenic Mimicry (VM) is a simulation model of the vascular wall structure in which malignant tumor cells imitate endothelial cells and interact with the extracellular matrix (ECM) through self-deformation, in order to reshape the tumor microcirculation. The microvasculature wall in VM is lined by tumor cells without an endothelial cell barrier. After the tumor cells release proteolytic enzymes to dissolve the ECM, they are directly exposed to the bloodstream, which significantly promotes the metastasis of tumor cells [4]. VM may be the main source of blood supply in the early stage of tumor growth. Therefore, anti-VM therapy has become a focus in liver cancer therapy research. Current studies have shown that EphA2, MMPs,

VE-cadherin [5], HIF-1α [6], VEGF [7], and PI3K [8] are all involved in VM formation. In addition, epithelial-mesenchymal transformation (EMT) and tumor stem cells (CSCs) are also related to VM formation. EMT-related nuclear transcription factors can inhibit E-cadherin promoter, reduce its transcriptional activity, and promote VM formation [9, 10]. Tumor stem cells enhance the plasticity of tumor cells, reshape ECM to form VM, and connect VM ducts to host blood vessels [11]. So far, the research on VM has mainly focused on the morphological structure of VM, the formation mechanism of VM, and the signal pathways involved. Among the various regulatory mechanisms of VM, a highly specific therapeutic target for anti-VM formation has not been found.

In 1993, Anderson et al. put forward the concept of "tumor microenvironment", which refers to the internal environment in which tumors occur and develop [12]. The tumor microenvironment consists of tumor cells, a variety of stromal cells, hypoxia, and ECM. Stromal cells mainly include CAFs, vascular-associated cells (such as endothelial cells and pericytes), immune cells (such as tumor-associated macrophages), and inflammatory cells [13]. Tumor-associated macrophages can promote the secretion of VEGF-C by tumor cells and the formation of tumor microlymphatic vessels, and also induce lymph node metastases and tumor invasion [14]. Gassmann et al. found that hepatic sinusoidal endothelial cells express chemokine ligand (CXCL12), tumor cells express chemokine receptor (CXCR4), and CXCL12 binds to CXCR4, which mediates intrahepatic metastasis of tumor cells [15]. In hypoxic conditions, the tumor promotes VM formation by inducing HIF-1α factor to regulate the changes of tumor VM-related genes, such as VEGF, VEGFR2, etc. [16]. It can be seen that the tumor microenvironment is closely related to the occurrence, development, invasion, and metastasis of different tumors. CAFs are one of the most important host cells in the tumor microenvironment [17]. Some studies have found that CAFs can activate signal pathways, promote tumor immunosuppression, promote tumor cells to undergo epithelial-mesenchymal transformation, promote tumor invasion and metastasis, and increase the malignant phenotype of tumor cells through direct cell-cell contact, cytokine secretion, and modification of the extracellular matrix components [18-21]. In

addition, there is evidence that CAFs can promote the formation of endothelium-dependent blood vessels in tumors [22, 23]. However, at present, there are few reports on the effect of CAFs on the formation of VM in HCC. Some scholars have found that the genome of CAFs is more stable than tumor cells, and CAFs are not easily resistant to drugs [24, 25]. If CAFs also have a definite effect on VM formation, then tumor-individualized therapies targeting CAFs will bring new opportunities for the treatment of malignant tumors.

The purpose of this study was to explore the effect of CAFs on the formation of VM in HCC, which could contribute to making CAFs a therapeutic target for liver cancer, and provide new ideas and theoretical bases for the treatment of HCC.

Material and methods

Patients and tissue samples

In this study, we included 60 patients with HCC who underwent surgery in the Affiliated Hospital of the Southwest Medical University from November 2015 to December 2016. All patients were being diagnosed for the first time and did not receive hepatic artery embolization, radiotherapy, or chemotherapy before operation. Ten hepatolithiasis patients who underwent peripheral liver tissue resection during this same period were randomly selected to form the control group. Pathology of the resected samples showed a normal liver structure. Tissue samples in both the case and control group were collected. This study was approved by the Ethics Committee of Affiliated Hospital of Southwest Medical University and all participants provided their informed consent to participate in this study.

Immunohistochemical staining of vimentin, α-SMA, and the results analysis

All the specimens were fixed with 10% neutral formaldehyde solution. The samples were dehydrated and embedded in paraffin, and then cut into 4 µm serial sections. Immunohistochemical staining was performed by EnVision two-step method. The sections were boiled in a pressure cooker with citric acid buffer (pH 6.0) for 5 minutes and incubated with a primary antibody at room temperature for 1 hour. The primary anti-

body included vimentin and α-SMA. The sections were finally displayed in diaminobenzidine (DAB) chromogenic solution. The semi-quantitative method was used to evaluate vimentin and α-SMA expression. Under high magnification, 10 non-overlapping tumor cell visual fields were randomly selected and evaluated according to the staining intensity and the percentage of positive cells (the number of stained cells/ the number of tumor cells). The staining of vimentin and α-SMA was located in the cytoplasm. The staining intensity score was 0 for non-staining, 1 for light brown, 2 for tan, and 3 for yellowish brown. The percentage of positive cells score was 0 for non-staining, 1 for <10%, 2 for 10%-30%, and 3 for >30%. A semi-quantitative evaluation was done according to the product of the above two indexes. A score of 0-2 was marked as low expression, and a score ≥3 was marked as high expression.

CD31/Periodic acid-Schiff (PAS) double staining and VM analysis

The immunohistochemical staining method of CD31 was like that for vimentin and α-SMA. After that, the sections were reduced in 0.5% periodate solution for 10 minutes, washed with water for 2 minutes, and then placed in Schiff solution for 15 minutes in a dark environment. Gastric mucinous adenocarcinoma was used as the positive control for PAS and the mucus was stained red. Phosphate-buffered saline (PBS) solution was used instead of a primary antibody as the negative control. The appearance of brown granules in the cell membrane or cytoplasm was recorded as CD31-positive. The lumen of endothelial blood vessels or hepatic sinusoids was surrounded by CD31-positive cells.

VM analysis: Vascular endothelial cells were absent in the lumen which was surrounded by tumor cells. Red blood cells could be seen occasionally in the lumen. The red blood cells and tumor cells were separated by continuous or discontinuous PAS-positive membranes, and there was no obvious necrosis or inflammatory cell infiltration around them [26]. Those with VM were positive and those without VM were negative.

Primary culture of hepatocellular CAFs and the collection of conditioned medium

The tissues used in the primary culture came from the resected liver cancer specimens which were pathologically diagnosed as poorly differentiated HCC. The cells were extracted using the enzyme digestion method and cultured in DMEM/F12 medium containing 20% fetal bovine serum (FBS), 1% double antibody, and 1% L-glutamine. According to the difference in the growth rate and adhesion ability between fibroblasts and tumor cells, a combination of the differential enzyme digestion method and the repeated adhesion method were used to purify cells. The purified CAFs could be obtained after 3 passages. Cell climbing slices were made to identify CAFs, and the immunohistochemical staining steps of vimentin, α-SMA and the analysis of results were the same as before. When the growth of the purified cells reached 70%, the old medium was gently sucked out with a straw, and the cells were washed 3 times with PBS buffer. Then, a 3 ml serum-free medium was added and sucked out after 24 hours of culture. The supernatant was collected after 2000 rpm centrifugation for 5 minutes and filtered using a 0.22 μm microporous membrane.

Cell lines and coculture system

The human normal hepatocyte cell line L02, human hepatoma cell line SMMC-7721 and MHCC-97H were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. L02 cells were cultured in RPMI-1640 medium containing 10% FBS. SMMC-7721 cells and MHCC-97H cells were cultured in DMEM high glucose medium containing 10% FBS at 37°C in humidified air with 5% CO_2.

Three-dimensional culture

To each 24-well plate, 0.3 ml matrix collagen solution was added, and then the well plate was put into a 37°C incubator for 30 minutes. L02 cells in the logarithmic phase were digested, and the cell density was adjusted to 2×10^5/ ml. The cells were planted in the 24-well plate. Different volumes of conditioned medium were added, and the final concentration was 25%, 50%, and 75% based on which the low, medium, and high concentration groups were respectively constituted. Each concentration was set with three repeat holes. A blank control group was set. The three-dimensional culture model of SMMC-7721 and MHCC-97H were established using the same method. The arrangement and integrity of tubular structures
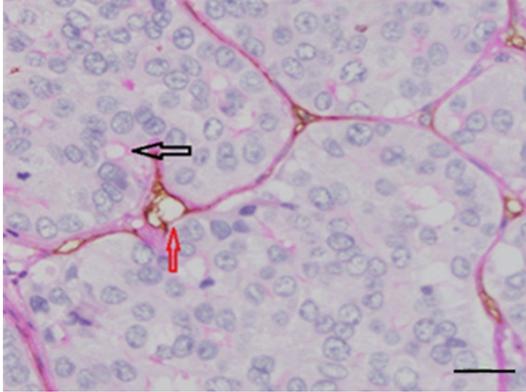
Figure 1. Immunohistochemical double staining of CD31 and PAS in HCC. The black arrow refers to VM. The red arrow refers to the vascular endothelium. Bar=50 µm.

were observed under an inverted microscope. Under a low magnification microscope, the upper, lower, left, right, and center visual fields were randomly selected to record and count the number of tubular structures. The culture was terminated after 5 days.

Real-time PCR steps

MHCC-97H cells were planted in a non-three-dimensional culture. Total RNA was extracted. The RNA concentration was determined and reverse transcription was performed. A pair of primers was designed according to the target genes MMP2 and EphA2, and β-actin was used as the internal reference gene. The real-time PCR system used was SYBR Premix Ex Taq II (Tli RNaseH Plus) (2×) 10 µl, PCR Forward Primer 0.8 µl, PCR Reverse Primer 0.8 µl, DNA template 2 µl, and sterilized water 6.4 µl.

Western blotting steps

The cells of each group were collected. The total protein was extracted, and the protein concentration was measured using the bicinchoninic acid assay method. The primary antibodies; MMP2, EphA2, and β-actin were added and incubated overnight after electrophoresis, membrane transfer, and sealing. The secondary antibody was added to incubate for 1 hour the next day. After rinsing, the PVDF film was immersed in the photoluminescence solution and photographed using the gel imaging system.

Statistical analyses

SPSS 17.0 software was used for statistical analysis of the data. The X^2 test was used to analyze the relationship between VM expression and clinicopathological features, and between VM expression and the expression of vimentin and α-SMA. The continuous variables were expressed as mean ± standard deviation (x ± s). One-way ANOVA was used to compare the mean among groups. The Student-Newman-Keuls (SNK) method was used for pairwise comparison. Levene's test was used to test the homogeneity of variance. When P<0.05, the difference was considered statistically significant.

Results

Patient characteristics

A total of 60 patients, 48 males and 12 females were included in the study. The clinicopathological data of the patients were intact. The median age of the patients was 46.5 (26-73) years. The tumor diameter was ≥5 cm in 33 cases and <5 cm in 27 cases. Thirty-two cases were HBsAg-positive. Patients with liver cirrhosis, Edmondson grade I-II, Edmondson grade III-IV, TNM stage I-II, and TNM stage III-IV were 25, 27, 33, 24, and 36, respectively.

VM expression in HCC

Among the 60 HCC cases, 21 cases were VM-positive. VM was a lumen-like structure surrounded by tumor cells. There were no CD31-positive endothelial cells on the wall. A PAS-positive ECM could be seen in the periphery of the lumen, and red blood cells could be seen in the lumen occasionally (**Figure 1**). Thirty-nine HCC cases were VM-negative. The positive expression rate of VM in the cases was 35.0%. All the control tissues were VM-negative. The difference in VM expression between both groups was statistically significant (X^2=5.862, *P*=0.021; **Table 1**).

The relationship between VM expression and clinicopathological features in HCC (Table 1)

There was no significant correlation between VM expression and sex, age, and HBsAg status in HCC (P>0.05). However, VM expression was related to tumor size, Edmondson grade, clini-

Table 1. The relationship between VM expression and clinicopathologic features in HCC patients

Clinicopathological features		N	VM		X^2	P
			positive	negative		
Organization category	HCC tissue	60	21 (35.0)	39 (65.0)		
	Control tissue	10	0 (0.0)	10 (100.0)	3.472	0.021
Gender	Men	48	16 (33.3)	32 (66.7)		
	Women	12	5 (41.7)	7 (58.3)	0.041	0.839
Age (years)	≥60	7	1 (14.3)	6 (85.7)		
	<60	53	20 (37.7)	33 (62.3)	0.642	0.423
Tumor size (cm)	≥5	33	16 (48.5)	17 (51.5)		
	<5	27	5 (18.5)	22 (81.5)	5.862	0.015
HBsAg (ng/ml)	+	32	12 (37.5)	20 (62.5)		
	-	28	9 (32.1)	19 (67.9)	0.188	0.664
Liver cirrhosis	yes	25	13 (52.0)	12 (48.0)		
	no	35	8 (22.9)	27 (77.1)	5.444	0.020
Edmondson classification	I+II	28	5 (17.9)	23 (82.1)		
	III+IV	32	16 (50.0)	16 (50.0)	6.782	0.009
TNM Staging	I+II	24	4 (16.7)	20 (83.3)		
	III+IV	36	17 (47.2)	19 (52.8)	5.910	0.015

Table 2. The relationship between vimentin expression and VM (cases, %)

VM	n	Vimentin		X^2	P
		High expression	low expression		
Positive	21	18 (85.7)	3 (14.3)		
negative	39	21 (53.8)	18 (46.2)	6.093	0.014

Table 3. The relationship between α-SMA expression and VM (cases, %)

VM	n	α-SMA		X^2	P
		High expression	low expression		
Positive	21	17 (81.0)	4 (19.0)		
negative	39	20 (51.3)	19 (48.7)	5.038	0.024

cal stage, and liver cirrhosis (X^2=5.862, 6.782, 5.910, 5.444, respectively; P<0.05). The positive rates of VM in samples with tumor diameter ≥5 cm and <5 cm were 48.5% and 18.5%, respectively, and the difference was statistically significant (X^2=5.862, P=0.015). The positive rate of VM in patients with liver cirrhosis was 52.0%, and was significantly higher than in patients without liver cirrhosis (22.9%, X^2=5.444, P=0.020). The positive rate of VM in the Edmondson grade I-II group was 17.9%, and was 50% in the grade III-IV group. There was a significant difference between the two groups (X^2=6.782, P=0.009). The positive rate of VM in the TNM stage I-II group was 16.7%, and was 47.2% in the stage III-IV group. The difference was statistically significant (X^2=5.910, P=0.015).

The relationship between the expression of vimentin, α-SMA, and VM

Among the HCC cases, 39 cases had high vimentin expression and 21 cases had low vimentin expression. The high vimentin expression rate was 65.0%. The high vimentin expression rate in the VM-positive group was 85.7%, and was 53.8% in the VM-negative group. The difference was statistically significant (X^2=6.093, P=0.014; **Table 2**). Among the HCC patients, 37 cases (61.7%) had high α-SMA expression, and 23 cases had low α-SMA expression. The high α-SMA expression rate in the VM-positive group was 81.0%, and was 51.3% in the VM-negative group. The difference was statistically significant (X^2=5.038, P=0.024; **Table 3**).

Primary culture and identification of hepatocellular CAFs

After 3 days of culture, a large number of cells adhered to the wall and all kinds of cells were mixed (**Figure 2A**). The CAFs were fusiform or star-shaped, with multiple protuberances and round or oval nuclei. After purification, there

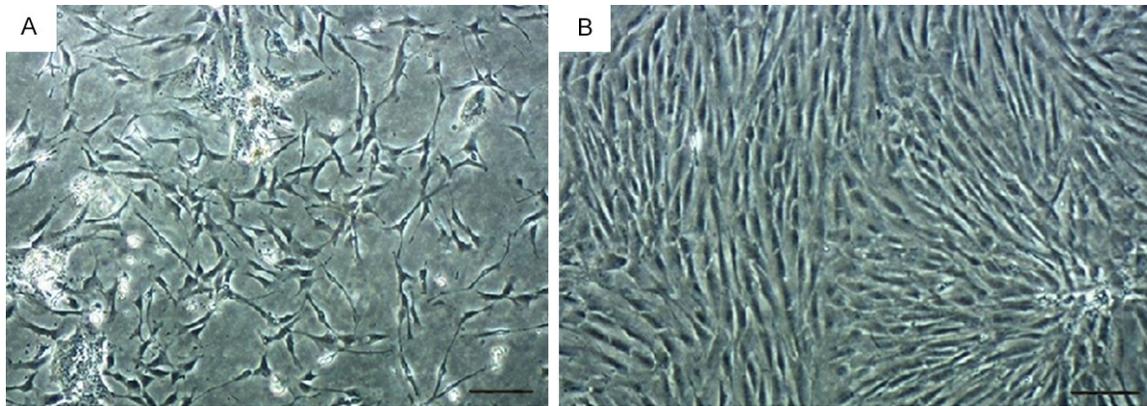**Figure 2.** The morphological changes of the primary cell. A. The morphology of CAFs on day 3. B. The highly purified CAFs. Bar=200 µm.
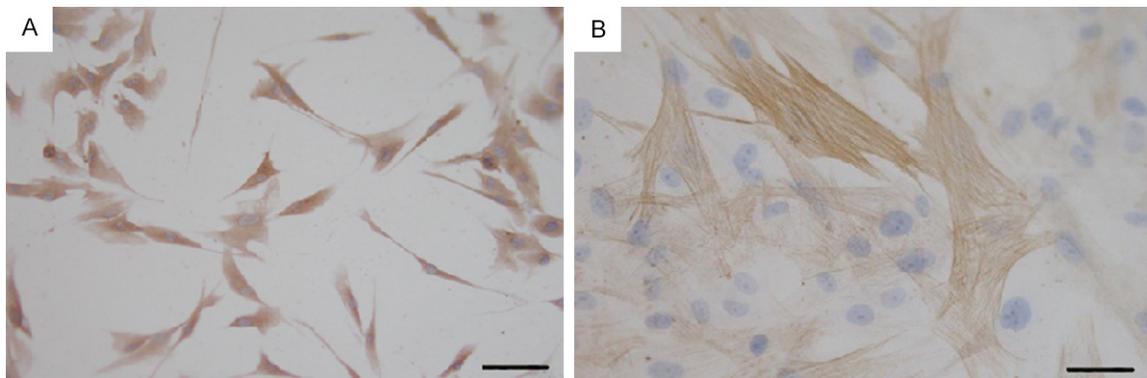

Figure 3. Immunohistochemical staining of CAFs. (A) Positive expression of Vimentin. (B) Positive expression of α-SMA. Bar=50 µm (A), 20 µm (B).

were almost no epithelioid cells in the fourth generation, and the CAFs were arranged in a mat pattern (**Figure 2B**). The positive expression of vimentin and α-SMA in CAFs resulted in a stained cytoplasm and showed brown granules (**Figure 3**).

VM formation of three cell lines in three-dimensional culture

For 5 consecutive days, the formation of VM in three kinds of cell lines was observed using an inverted phase contrast microscope. Normal hepatocyte cell line L02 and low metastatic hepatoma cell line SMMC-7721 did not form VM tubular structures, and some cells gathered into cell colonies. Only highly metastatic hepatoma cell line MHCC-97H formed VM tubular structures in the three-dimensional culture (**Figure 4**). It was also observed that MHCC-97H cells began to protrude slender protuberances

between the 6th to 8th hour, and connected to each other into a grid-like or tubular structure. The peak was attained at the 24th hour, and the tubular structures began to loosen after 48 hours.

The effect of hepatocellular CAF conditioned medium on VM of MHCC-97H cells in three-dimensional culture

After 24 hours of culture, the tubular structures of MHCC-97H cells cultured in different concentrations of CAF conditioned medium were photographed and counted (**Table 4**). The results showed that the three concentrations of the CAF conditioned medium could promote MHCC-97H cells to form VM, and the tubular structures were smaller than those of the control group. The number of VM formed in each experimental group was significantly different from that in the control group ($F=10.889$, $P<0.01$),
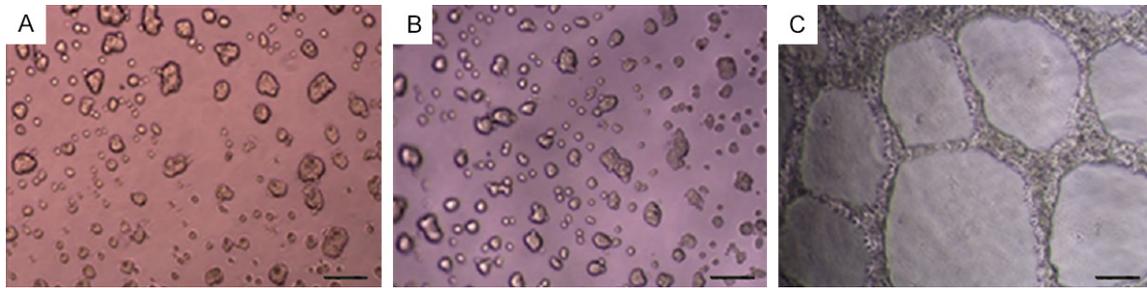
Figure 4. Observing the ability of vasculogenic mimicry in different cell lines in three-dimensional culture. A. L02; B. SMMC-7721; C. MHCC-97H. Bar=100 μm.

Table 4. The effect of different concentrations of the CAF conditioned medium on the formation of VM in MHCC-97H ($\bar{x} \pm s$)

Group	Number of VM (24 h)
Control	14.67±1.528
Low concentration	24.33±2.517
Medium concentration	25.33±4.041
High concentration	27.00±3.000
F	10.889
P	0.003

but there was no significant difference in the three experimental groups (P>0.05, **Figures 5**, **6**).

Detection of the mRNA level expression of VM-related genes MMP2 and EphA2 by real-time PCR

The mRNA levels of VM-related genes, MMP2 and EphA2 in MHCC-97H cells were detected by real-time PCR. The results showed that the mRNA level expression of MMP2 in the three experimental groups of CAF conditioned medium was significantly higher than in the control group (**Figure 7A**), and there was a statistical significance (F=22.524, P<0.01; **Table 5**). The mRNA level expression of MMP2 in the medium concentration group was higher than in the high and low concentration groups, but there was no significant difference among the three groups (P>0.05). Compared with the control group, the mRNA level expression of EphA2 in the experimental groups was significantly higher (**Figure 7B**; **Table 5**; F=18.784, P<0.01). The expression in the low concentration group was higher than in the medium and high concentration groups, but there was no significant difference among the three groups (P>0.05). The

results showed that the CAF conditioned medium could increase the mRNA level expression of MMP2 and EphA2 in MHCC-97H cells, but there was no significant difference in the different concentrations.

Detection of the protein level expression of MMP2 and EphA2 by Western blot

The expression of VM-related proteins MMP2 and EphA2 in MHCC-97H cells were detected by Western Blotting. The results showed that, the expression of MMP2 and EphA2 increased in the experimental groups when compared with the control group (**Figure 8**). The protein increase of MMP2 in the low concentration group was more obvious than in the medium and high concentration groups. The protein increase of EphA2 in the medium concentration group was more obvious than in the low and high concentration groups. There was no significant difference among the three experimental groups (P>0.05; **Figure 9**). The results showed that the CAF conditioned medium could increase the protein expression of MMP2 and EphA2 in MHCC-97H cells, but there was no significant difference in different concentrations.

Discussion

Targeted therapy for CAFs can not only inhibit fibrosis, but also inhibit the progression of liver cancer [27]; therefore, CAFs are expected to become a new target for liver cancer. CAFs play an important role in the tumor microenvironment and have become a research hotspot. The premise of research is to obtain purified CAFs. Therefore, the primary culture, purification, and identification of CAFs are necessary. CAFs are activated fibroblasts in the tumor stro-
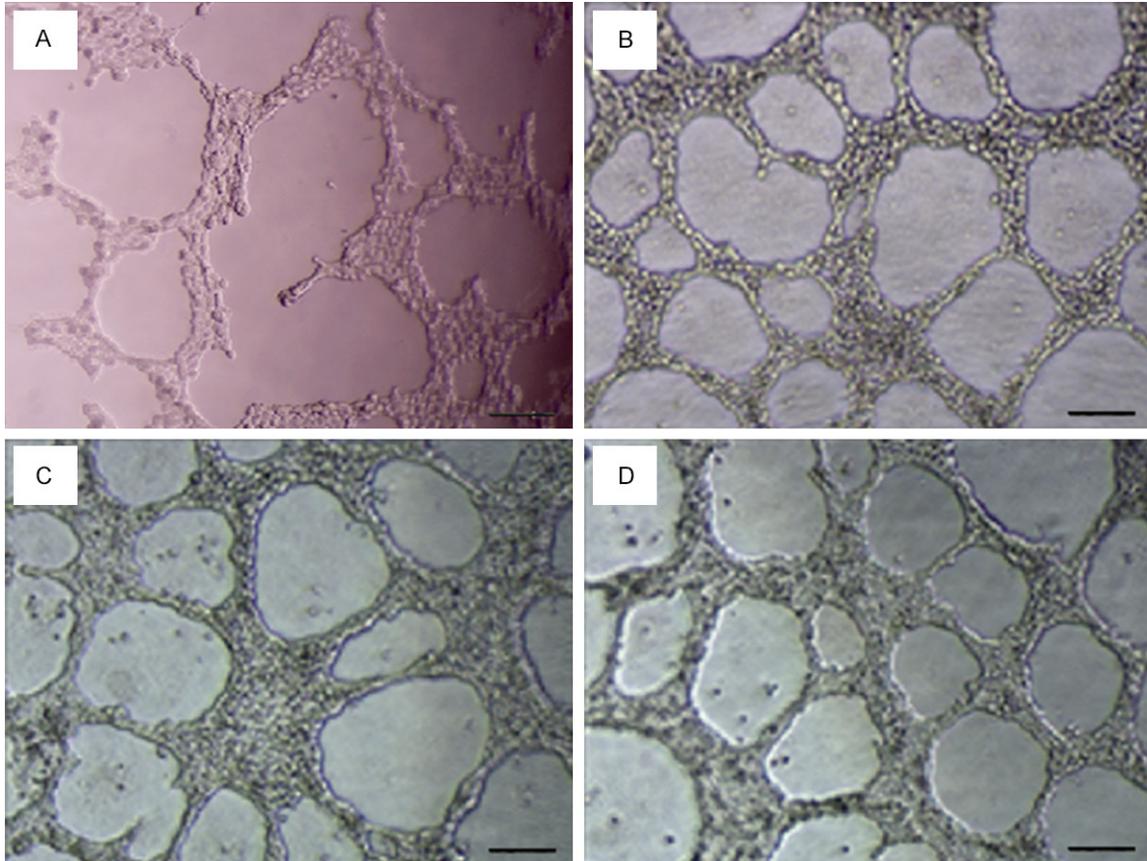
Figure 5. The effect of different concentrations of CAF conditioned medium on the formation of VM in MHCC-97H. A. The control group. B. Low concentration group. C. Medium concentration group. D. High concentration group. Bar=100 μm.
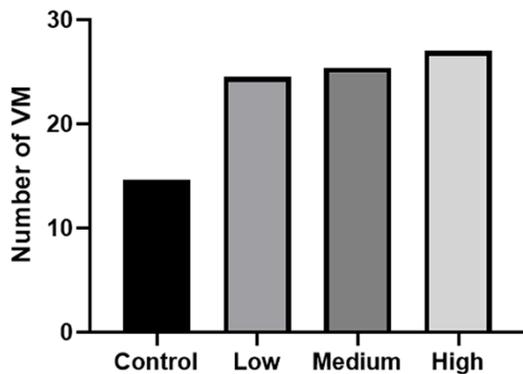

Figure 6. The effect of different concentrations of the CAF conditioned medium on the formation of VM in MHCC-97H.

ma, which have the dual characteristics of smooth muscle cells and fibroblasts in morphology and biological behavior. Vimentin is a marker of mesenchymal cells and mesoderm-derived cells, suggesting that CAFs may be derived from fibroblasts. The CAFs in our study expressed their own specific protein, α-SMA and the expression results were consistent with those reported in previous literature [28, 29]. This confirmed that the cells we established were CAFs with high purity. In this study, primary hepatocellular CAFs were successfully cultured and purified using the enzyme digestion method. The experiment was repeatable, and the cells with high vitality and high purity were obtained, which provided a good cell model for the follow-up study.

CAFs are activated fibroblasts which secrete collagen, the main matrix component of many kinds of malignant tumors [30]. More and more literatures have reported that CAFs play a vital role in the development of tumors and may be a potential therapeutic target for tumors. Sukowati et al. [17] co-cultured hepatocellular CAFs and non-tumorous fibroblasts with hepatoma cell lines respectively, and found that CAFs upregulated the gene expression of TGF-β1 and FAP in hepatoma cell lines, while non-
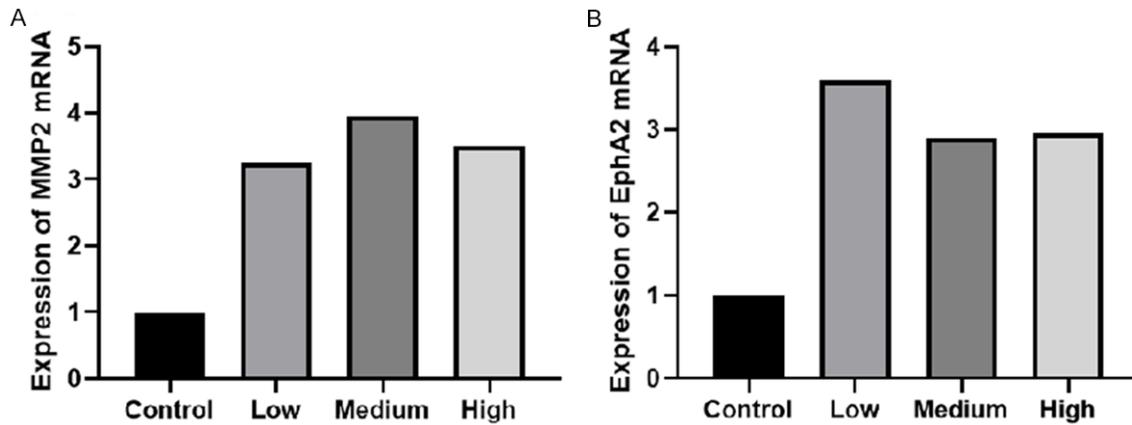
Figure 7. PCR detected the mRNA level expression of MMP2 (A) and EphA2 (B) in MHCC-97H.

Table 5. PCR detected the mRNA level expression of MMP2 and EphA2 in MHCC-97H (x ± s)

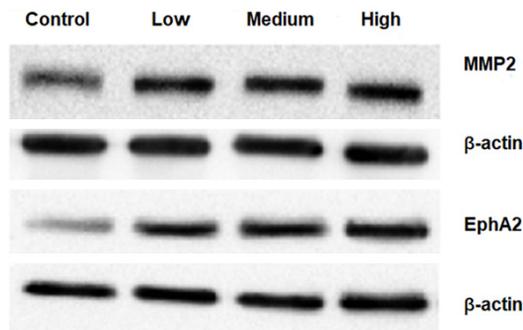| Group	The mRNA level expression of MMP2	The mRNA level expression of EphA2
Control	1±0.0000	1±0.0000
Low concentration	3.250±0.672	3.619±0.838
Medium concentration	3.950±0.432	2.922±0.281
High concentration	3.484±0.530	2.961±0.188
F	22.524	18.784
P	0.000	0.001

Figure 8. Western Blotting detected the protein level expression of MMP2 and EphA2 in MHCC-97H.

tumorous fibroblasts did not induce the expression of the two genes. Oral squamous cell CAFs can promote lymphangiogenesis through the c-Met/PI3K/AKT signaling pathway in vitro [31]. When CAFs were co-cultured with breast cancer cells, CAFs produced higher levels of chemokines such as CCL11 and CXCL14 which promoted the growth, drug resistance, and metastasis of breast cancer cells [32]. Wu et al. [33] also proposed that CAFs can directly provide tumor cells with essential nutrients and energy-rich metabolites, including lactic acid, ketone bodies, fatty acids, glutamine, etc. which can adapt to the metabolic needs of tumor cells in a dynamic way. More importantly, Jine Yang et al. found that co-implantation of CAFs and tumor cells significantly promoted the formation of VM in mouse xenografts. However, when the TGF-β or SDF1 signal is abrogated, the formation of VM promoted by the conditioned medium of cancer-associated fibroblast is attenuated. Recombinant TGF-β1 and SDF1 can induce VM formation. Their findings suggest that the miR-101-TGF-β/SDF1-VE-cadherin/MMP2/LAMC2 network may regulate the formation of VM and is a potential target for tumor therapy [18].

In our study of HCC tissues, we found that the expression of CAF markers, α-SMA and vimentin in the VM-positive group was higher than in the VM-negative group. Through the three-dimensional culture of hepatoma cell lines, we found that only the highly invasive hepatoma cell line MHCC-97H could form VM, while the normal hepatocyte LO2 and the low invasive hepatoma cell line SMMC-7721 could not form VM. Therefore, we chose the highly invasive hepatoma cell line MHCC-97H as the main cell
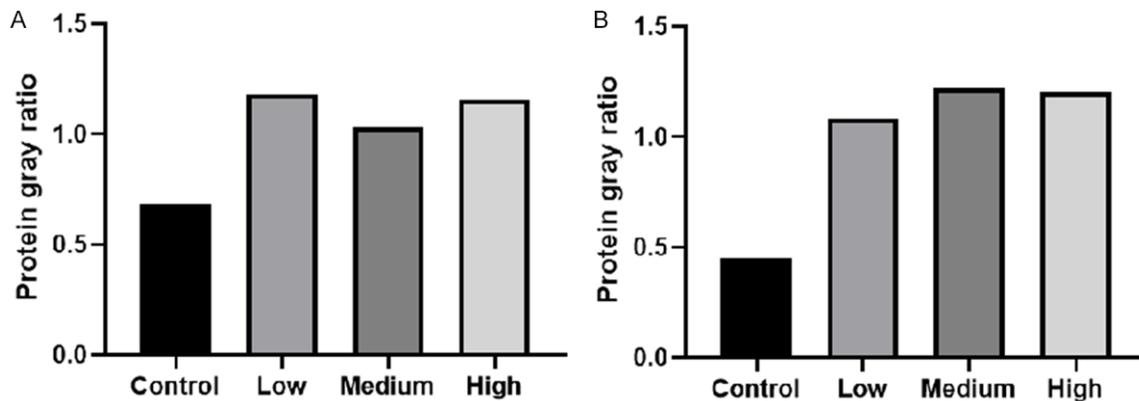
Figure 9. Western Blotting detected the protein level expression of MMP2 (A) and EphA2 (B) in MHCC-97H.

line for the follow-up study. The results showed that the hepatocellular CAF conditioned medium could increase the number of VM and up-regulate the mRNA and protein expression of MMP2 and EphA2 in MHCC-97H cells. The effect of the CAF conditioned medium on the formation of VM was not concentration-dependent. MMP2 is an extracellular proteolytic enzyme, which can degrade the tumor-mediated extracellular matrix and is closely related to VM formation and tumor metastasis. A lot of evidence shows that MMP2 activation can promote VM formation [34]. EphA2 is a member of the receptor tyrosine kinase family and is one of the important factors in VM formation. Wang et al. [35] have shown that, VM formation can be prevented by inhibiting the expression of EphA2 in pancreatic cancer. In hepatocellular carcinoma cell lines, Ji-Gang Zhang et al. found that ROCK affects the changes of VM-related factors such as EphA2 and MMP2. RhoC/ROCK2 may have an important effect on VM of hepatocellular carcinoma through ERK/MMPs signal pathway and may become a potential target for liver cancer therapy [36, 37]. Research on the molecular mechanism of VM formation is relatively mature, and many researchers have drawn a complete network diagram of the detailed regulation mechanism, though it is not completely unified. Among many signal pathways, most scholars suggest that the key pathway for VM formation may be the VEGF-EphA2-MMPs-VM pathway. The results of this study showed that the transcriptional and protein levels of EphA2 and MMP2 in the experimental groups were higher than in the control group, and the number of VM in the experimental groups was also higher than in the control group.

VM is only a temporary blood supply mode in the process of tumor development, which will be gradually replaced by endothelium-dependent blood vessels. When the tumor is treated by inhibiting the formation of endothelium-dependent blood vessels, the tumor microenvironment will show reactive changes such as aggravation of tumor tissue hypoxia, increase in the expression of angiogenic factors, increase in the coverage of vascular pericytes, promotion of the formation of tumor stem cells, and increase in tumor cell autophagy. In order to get more nutrition to meet the unlimited growth, tumor cells will continue to form VM and increase the ability of tumor cells to invade and metastasize. Therefore, in addition to the traditional anti-angiogenic therapy, the inhibition of VM should be taken into account. The genome of CAFs is relatively stable compared with tumor cells, and CAFs are not easily resistant to drugs. More importantly, it can up-regulate the protein expression of EphA2 and MMP2 in the hepatoma cell line, which is an important target to block the signal pathway in the process of VM formation. However, the current studies tend to focus on the promotion effect of CAFs on endothelium-dependent angiogenesis. There are few studies on the effect of CAFs on VM. This study shows that CAFs can also promote VM formation, which will be a major breakthrough and complement in the mechanism of tumor invasion and metastasis. The mechanism of CAFs promoting VM formation is not clear, and further research is needed.

Recent studies have shown that there are different subsets of CAFs, each of which has its own specific phenotype and function. The emergence of these subsets hinders the appli-

cation of CAFs in tumor diagnosis and targeted therapy. In various tumors, different CAF subsets secrete different cytokine profiles [38]. Su et al. [39] pointed out that although many literatures have suggested that CAFs play an important role in tumor prognosis, there are two results that have opposite effects on prognosis in different studies. This indicates that different subsets of CAFs may play an opposite role in the occurrence and development of tumors. Therefore, it is necessary to further explore the markers and functions of CAF subsets to accurately target the part of CAF subsets that promote tumor progression.

In this study, only three representative cell lines were selected. In order to obtain more accurate experimental results, we can increase the types of hepatoma cell lines. In addition, we can detect the species and proportion of various substances in the hepatocellular CAF conditioned medium by gene chip technology, and analyze the relationship between these substances and the formation of VM.

Acknowledgements

Disclosure of conflict of interest

None.

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