RESEARCH ARTICLE

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VEGFR-1 activation-induced MMP-9-dependent invasion in hepatocellular carcinoma

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Aim: VEGFR-1 can promote invasion through epithelial–mesenchymal transition induction in hepatocellular carcinoma (HCC). This study aims to elucidate VEGFR-1 impact on proteolytic enzymes profile involved with invasion. **Materials & methods:** The effect on cell invasion was evaluated by invasive and migration assays with and without VEGFR-1 activation. The mechanism was investigated by real-time PCR, western blot and gelatin zymography using inhibitors for MMP-9. In total, 95 HCC patients were enrolled for its clinical value evaluation. **Results:** VEGFR-1 activation induced invasion in HCC cells with an increase in the expression and activity of MMP-9 and Snail. MMP-9 blockage effectively inhibited VEGFR-1-induced invasion. High coexpression of both in HCC predicted a worse clinical outcome. **Conclusion:** Data show a novel VEGFR-1 activation-to-MMP-9 mechanism promoting HCC invasion.

Hepatocellular carcinoma (HCC) is an aggressive disease with a poor prognosis due to the tumor invasiveness, intrahepatic spread and extrahepatic metastasis [1]. HCC metastasis is frequently associated with epithelial–mesenchymal transition (EMT) and extracellular matrix (ECM) degradation using proteases including matrix metalloproteinases (MMPs) [2,3]. Understanding of the molecular mechanisms regulating the metastatic and invasive behavior of this malignant tumor is essential for improving the treatments.

Activation of growth factor receptors is one of the most important mechanisms for survival and invasion of human cancers. VEGFR-1 is one of three typical membrane-bound tyrosine kinase receptors that specifically bind VEGF-B and PGF. VEGFR-1-mediated signaling promotes cancer metastasis via three major mechanisms: promotion of angiogenesis, activation of tumor cell proliferation and induction of tumor EMT, which endows tumor cell with a more invasive phenotype [4,5]. Activation of VEGFR-1 on the endothelial cells by its ligands results in the enhanced angiogenesis and metastasis. VEGFR-1 signaling facilitates malignant angiogenesis through the enhancement of endothelial migration and activity [6]. VEGFR-1 activation in breast cancer cells promotes tumor growth via activation of MAPK and PI3K/Akt signaling [7,8]. Moreover, activation of VEGFR-1 in cancer cells can also induce EMT, a critical mechanism for acquisition of invasive potential and promotion of cancer cell metastasis [4,9–10]. Furthermore, it is reported that activation/induction of proteolytic enzyme-mediated degradation of the ECM is also one of the essential steps in carcinoma invasion [1]. Many growth factors including TGF- β , EGF and IGF-1 have the ability to enhance the cancer cell invasiveness through activation of proteolytic enzymes [11–13]. While the mechanisms of angiogenesis and EMT are well established, the VEGFR-1 signaling pathways that regulate the activation of proteolytic enzymes are poorly understood.

KEYWORDS

- hepatocellular carcinoma
- invasion • MMP-9 • Snail
- VEGFR-1

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MMPs, a family of metalloendopeptidases that cleave the protein components of the ECM and endothelial cell basement membrane, play a pivotal role in tumor-associated angiogenesis and cancer metastasis [14–16]. MMP-9, a member of MMPs family, is particularly interesting, because it is correlated with the tumor recurrence and survival in patients with HCC. Quantitative real-time PCR studies have also shown that the expression of MMP-9 was significantly upregulated in the high-metastatic HCC cells lines [1]. Moreover, animal studies have confirmed that blocking MMP-9 can inhibit tumor cell invasion and metastasis. Importantly, it is hypothesized that MMP-9 enhances tumor angiogenesis through the VEGF–VEGFR signaling system [17]. Recent studies have shown that VEGFR-1 activation markedly promotes pulmonary metastasis through the induction of MMP-9 secretion in premetastatic lung endothelial cells and macrophages [18]. However, the mechanisms of MMP-9 regulation and its function of VEGFR-1 activation in HCC remain to be established.

In this study, we investigated one possible mechanism of MMP-9 regulation and its role in activation of VEGFR-1-mediated invasion. We showed that Snail, an EMT regulator induced by activation of VEGFR-1, was involved in the regulation of the expression of MMP-9. We also showed a strong correlation of high coexpression of VEGFR-1 and MMP-9 with the patient tumor invasion and demonstrated that high coexpression of VEGFR-1 and MMP-9 is a prognostic marker for HCC.

Materials & methods

• Reagents

Recombinant human VEGF-B¹⁶⁷ was purchased from R&D Systems, Inc. (MN, USA). Purified human immunoglobulin (Sigma, MO, USA), a nonspecific IgG, was used as a control. Both of them were added to cultures at a final concentration of 50 ng/ml. The broad spectrum MMPs inhibitor (GM6001) was purchased from Chemicon (CA, USA). Anti-MMP-9 monoclonal antibody (Ab-1) for functional blocking was purchased from Calbiochem (CA, USA). Monoclonal anti-VEGFR-1 and polyclonal anti-Snail were purchased from Abcam Cambridge (MA, USA). Polyclonal anti-MMP-9 and anti- β -actin were purchased from Santa Cruz Biotechnology (CA, USA). FITC-conjugated antirabbit IgG, horseradish peroxidase

(HRP)-conjugated antirabbit IgG and HRP-conjugated antigoat IgG were purchased from Zhongshan Biotechnology (Beijing, China).

• Cell cultures

All cell lines, except for those with particular notes, were obtained from Cell bank of Chinese Academy of Science. MHCC97H was obtained from the Liver Cancer Institute of Zhongshan Hospital, Fudan University, China. The human umbilical vein endothelial cells (which are used as positive control of VEGFR-1 expression) and NIH3T3 were kindly provided by Professor Ling Gao (Central Laboratory of Provincial Hospital Affiliated to Shandong University, China). HCC cell line and NIH3T3 was cultured as described previously [19]. SMMC7721 and HepG2 cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% FBS (Gibco, NY, USA) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin; Gibco), at 37°C in 5% CO₂ and 95% air. Results from all studies were confirmed in at least three independent experiments.

• Patients, follow up & tissue microarrays construction

A total of 95 HCC patients who underwent curative resection treatment at Provincial Hospital Affiliated to Shandong University during October 2006 to August 2007 were analyzed. The criteria for curative resection were described previously [20]. No patients received any preoperative anticancer treatment. All pathological features such as cirrhosis, tumor encapsulation, tumor size and tumor number were defined histologically. Tumor differentiation and stage were grade by Edmondson grading system and the 2002 International Union Against Cancer TNM classification system (6th edition), respectively. This study was approved by the Ethics Committee of Provincial Hospital Affiliated to Shandong University, and informed consent was obtained from all patients. Patients' clinical characteristics were summarized in [Table 1](#).

All of the patients received follow-up tests including liver function, tumor markers and ultrasonography with an interval of 4–7 months after curative resection. In case of suspicious tumor recurrence, computed tomography scanning was used immediately. Treatment modalities after relapse were administered depending on the individual situation, including radiofrequency ablation and selected transcatheter

Table 1. Characteristics and univariate survival analysis of 95 hepatocellular carcinoma patients.

Factor	n	Mean RFS (95% CI)	p-value	Mean OS (95% CI)	p-value
Age (≤50 years/>50 years)	41/54	31.6 (27.6–35.5)/28.4 (25.3–31.5)	0.236	44.2 (41.4–47.0)/38.9 (35.8–42.1)	0.087
Gender (male/female)	79/16	30.6 (28.0–33.3)/25.3 (19.3–31.3)	0.074	40.7 (38.3–43.2)/43.1 (38.2–47.9)	0.293
Liver cirrhosis (no/yes)	15/80	29.6 (23.7–35.5)/29.8 (27.1–32.5)	0.724	45.8 (42.6–49.1)/40.4 (37.9–42.9)	0.459
Tumor size (≤5 cm/>5 cm)	38/57	38.6 (35.3–41.8)/23.7 (21.3–26.1)	0.0001*	47.5 (45.4–49.6)/37.0 (34.0–39.9)	0.0001*
Tumor number (single/multiple)	50/45	35.5 (32.2–38.9)/23.3 (20.8–32.2)	0.0001*	47.1 (45.1–49.2)/34.8 (31.7–38.0)	0.0001*
Tumor tissue encapsulation (yes/no)	53/42	35.2 (32.2–38.2)/22.5 (19.8–25.3)	0.0001*	45.6 (43.1–48.1)/35.5 (32.3–38.6)	0.0001*
TNM stage (I–II/III–IV)	50/45	36.9 (34.0–39.7)/21.5 (19.1–23.9)	0.0001*	47.6 (45.7–49.4)/34.0 (30.9–37.0)	0.0001*
VEGFR-1 (low/high) [†]	46/43	33.2 (29.4–36.9)/25.1 (22.2–27.9)	0.0001*	44.5 (41.9–47.0)/37.2 (33.6–40.8)	0.023*
Snail (low/high) [†]	43/49	34.0 (30.4–37.6)/24.7 (22.0–27.4)	0.0001*	47.4 (45.1–49.8)/35.1 (32.2–37.9)	0.001*
MMP-9 (low/high) [†]	53/33	30.7 (27.3–34.1)/28.5 (24.6–32.5)	0.327	42.2 (39.0–45.4)/40.1 (36.9–43.4)	0.274
VEGFR-1/MMP-9 high coexpression (yes/no)	19/60	23.2 (19.7–26.6)/30.4 (27.3–33.6)	0.003*	35.6 (30.6–40.7)/42.5 (39.8–45.2)	0.02*

[†]Cores of six (VEGFR-1), three (Snail) and nine (MMP-9) patients were detached from tissues microarrays during immunostaining.

**p* < 0.05 was considered statistically significant.

OS: Overall survival; RFS: Recurrence-free survival.

arterial chemoembolization. The median follow-up period was 38 months. Overall survival (OS) and recurrence-free survival (RFS) were defined as the interval between curative resection and death or recurrence.

A total of 95 pathology-proven HCC tissues and 33 peritumoral tissues (only corresponding to HCC tissues of moderate differentiation) were collected. After examining HE-stained slides for the location and differentiation of cancerous tissues and peritumoral tissues, tissue microarrays (TMAs; collaborated with Shanghai Biochip Co, Ltd, Shanghai, China) were constructed.

• Immunofluorescent microscopy

Paraformaldehyde-fixed, permeabilized SMMC7721 cells cultured on glass slide for 48h were incubated with primary antibodies overnight at 4°C followed by incubation with secondary fluorescent antibodies. Nuclei were stained with DAPI (Vector Laboratories, CA, USA), and the resultant immunofluorescence was observed under a fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Negative controls were treated identically but with the primary antibodies omitted.

• Cell migration assay

Cell migration was examined utilizing a wound healing assay as described previously [21]. Briefly, SMMC7721 cells were digested and plated in a six-well plate until 100% confluence. Three parallel wounds with a size of approximately 400 μm were made in the cell monolayer using a sterile pipette tip. The monolayer was washed twice with PBS followed by incubation with

VEGF-B¹⁶⁷ (R&D Systems, Inc., MN, USA) or nonspecific human IgG (Sigma) in DMEM + 1% FBS for 24 h. Relative cell migration was calculated as the percentage of the remaining cell-free area among the initial wounded areas. Closure of the wounded area was monitored using an inverted microscope (Leica Microsystems, Germany) attached with a digital camera. Cell migration distance was determined by measuring the width of the wound and calculated using the following equation: cell migration distance = initial half-width of the wound - the width of the wound after migration/2. Results are expressed as mean ± standard deviation of three independent experiments.

• Matrigel invasive assay

Invasion assay was performed using 24-well Transwell chamber with a pore size of 8 μm (Costar, NY, USA) and the inserts were coated with Matrigel (BD, Bioscience, MA, USA). Cells were digested and diluted to 1 × 10⁶ cells/ml in DMEM + 1% FBS. A total of 100 μl of cells was then added on the upper chamber of the insert. Consequently, DMEM + 10% with or without VEGF-B¹⁶⁷ was added to the upper chamber. After 48 h of incubation, cells on the upper surface of the filters were removed with a cotton tip. The migrant cells were fixed with 4% paraformaldehyde and stained with hematoxylin. Numbers of cells migrating to the membrane were counted in five randomly chosen fields under a light microscope (×200). The average number of migrated cell per microscopic field was analyzed. Results are expressed as mean ± standard deviation of three independent experiments.

• Western blot analysis

Cells were grown to 70–80% confluence in DMEM + 10%FBS and were then serum-deprived overnight in DMEM + 1% FBS. Subsequently, cells were treated with VEGF-B¹⁶⁷ or nonspecific IgG in DMEM + 1% FBS. Cells in both groups were collected by scraping, and the whole cell lysate was prepared using lysis buffer (1 × PBS, 0.5% sodium dextrocholate, 1 mM Na₃VO₄, 1% NP-40, 0.1% SDS, 5 mM EDTA, 1 mM PMSF). After centrifugation, 40 µg of total protein from each group were loaded onto 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, which was blocked, incubated with appropriate antibodies and developed with chemiluminescence as described previously [19]. The fluorescence of the protein bands was detected and quantified with LAS-3000 system (Fuji Systems, Tokyo, Japan). β-actin was used as a loading control.

• Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on ABI 7500 Real-time PCR system (Applied Biosystems, CA, USA) using the SYBR Premix Ex Taq (TaKaRa Biotechnology [Dalian] Co., Ltd, Dalian, China) according to the manufacturer's instructions. Primer pairs were as follows: Snail: forward 5'-TAGGCCCTGGCTGCTACAAG-3', reverse 5'-GAGAAGGTCCGAGCACACG-3'; β-actin: forward 5'-TGACGTGGACATCCGCAAAG-3', reverse 5'-CTGGAAGGTGGACAGCGAGG-3'; MMP-9: forward 5'-GTGCTGGGCTGCTTTGCTG-3', reverse 5'-GTGCGCCCTCAAAGGTTTGGAAT-3'. The data were quantified with the comparative C_t method for relative gene expression [22]. β-actin was used as the reference gene.

• Gelatin zymography

Cells at 70–80% confluence were treated with VEGF-B¹⁶⁷ or nonspecific IgG after overnight serum deprivation in DMEM + 1% FBS. These conditioned media were centrifuged to remove cell debris and concentrated 15–20-fold using Spin-x UF concentrators (Costar, NY, USA) before being utilized as gelatinase. Volumes for each group were adjusted to 15 µg protein as determined by BCA. Gelatin zymography was performed as described previously [23]. Briefly, samples were mixed with SDS sample buffer without reducing agent and separated on 10%

SDS-PAGE gels containing 0.1% gelatin. Gels were incubated at 37°C in digestion buffer after SDS was removed by washing with 2.5% Triton X-100-containing buffer. Gels were stained with Coomassie Brilliant Blue R250 and the gelatinolytic activities were detected as clear bands against a blue background.

• Immunohistochemistry analysis

The immunohistochemistry protocols were described previously [24]. Negative controls were treated identically but with the primary antibodies omitted. Immunoreactivity was evaluated independently by two pathologists. Immunohistochemical expression of VEGFR-1 and Snail were quantified by determining the percentage of positive tumor cells at each intensity scores: 1+ (absent), 2+ (weak), 3+ (moderate) and 4+ (strong). The sum of these scores gave a final score for every core: 1, 1.01–2; 2.01–3; 3.01–4, as reported elsewhere [12]. Positive expression on the endothelial cells was not counted. Staining of MMP-9 was quantified by log-transformed integrated optical density as reported previously [2].

• Statistical analysis

For VEGFR-1, Snail and MMP-9, the cutoff for definition of subgroups was the median value of 2.3, 1.9 and 5.2, respectively [3]. The normality test indicated that the raw data has normal distribution and data were analyzed using SPSS15.0 and expressed as mean ± standard deviation. Differences between means were compared using either a standard two-sided independent t-test for two-group comparisons or one-way analysis of variance for multiple comparisons. The association was assessed using Pearson correlation coefficient. Survival curves were generated using Kaplan–Meier method, and Log-rank test was used to compare patients' survival between subgroups. *p* < 0.05 was considered as statistically significant.

Results

• VEGFR-1 expression in HCC cell lines & surgical specimens

Western blot demonstrated that VEGFR-1 (180 kDa) was expressed in four HCC cell lines including Hep3B, HepG2, SMMC7721 and MHCC97H (Figure 1A). human umbilical vein endothelial cells was used as a positive control [25]. Immunofluorescence analysis showed

Figure 1. VEGFR-1 protein expression in hepatocellular carcinoma. (A) Western blot shows expression of VEGFR-1 protein in all four cell lines. HUVECs were used as a positives control. (B) Immunoreactivity for the VEGFR-1 was clearly observed (arrow, upper panel). The negative control was performed similarly without the primary antibody (lower panel). Scale bar: 50 μ m. (C) Immunohistochemical staining of VEGFR-1 was localized in hepatocytes of peritumoral tissues (i), the cell membrane in the parenchyma (ii) and the vascular endothelial cell (upper arrow) and bile duct epithelial cells (lower arrow) in stroma of hepatocellular carcinoma tissues (iii). Scale bar: 50 μ m. HUVEC: Human umbilical vein endothelial cell.

that VEGFR-1 was localized in the cytomembrane of SMMC7721 cells (Figure 1B, upper panel). By contrast, the negative control without addition of primary anti-VEGFR-1 antibody did not show specific staining (Figure 1B, lower panel). Immunohistochemical staining was performed to determine the expression of VEGFR-1 in the HCC tumor and peritumoral

tissues of surgical specimens. VEGFR-1 was barely detectable in the hepatocyte of peritumoral tissues (Figure 1Ci). However, VEGFR-1 expression was distinctly observed on the cell membrane in the parenchyma (Figure 1Cii) and also localized at the vascular endothelial cells and bile duct epithelial cells in the stroma of HCC tissues (Figure 1Ciii).

Figure 2. VEGFR-1 activation enhances SMMC7721 cell migration and invasion (see facing page). (A) Wound healing assay was performed to analyze the cell migration. Bars indicated standard deviation; * $p < 0.05$ versus non-specific IgG group; ** $p = 0.01$ versus untreated group. (B) Photographs of cell migration at the point of 12 h and 24 h post-scratch. Scale bar: 100 μm . (C) Matrigel invasive assay was performed to analyze the cell invasion. Data shown are representative of three independent experiments. Bars indicated standard deviation; * $p < 0.0001$ versus nonspecific IgG group. Scale bar: 100 μm .

• VEGFR-1 activation promotes cell migration & invasion of SMMC7721 cell line

Having determined that VEGFR-1 is present in HCC cell lines and tissues, we determined its role in the tumor progression. We first tested if activation of VEGFR-1 by VEGF-B¹⁶⁷ can enhance the migration and invasion of SMMC7721 by wound healing and cell invasion assays. At the point of 12 h and 24 h postscratch, the relative cell migration was 60.3 and 12.3%, respectively, for the untreated group, 60.5 and 8.4%, respectively, for the nonspecific IgG group, but 58.6 and 1.4%, respectively, for the VEGF-B¹⁶⁷-treated group. There was no significant increase in the migration among these three groups in initial 12 h. However, the motility of the VEGF-B¹⁶⁷-treated cells was significantly increased compared with the nonspecific IgG-treated cells after 24 h (Figure 2A & B).

Because the activation of VEGFR-1 appeared to promote the migration of HCC cells, we next assessed the effect of VEGFR-1 on invasion using Matrigel Invasive assay. Cells were treated as described in Material and methods and invasion was determined at 48 h. As shown in Figure 2C, invasion of cells treated with VEGF-B¹⁶⁷ were significantly increased compared with that of the cell treated with nonspecific IgG. These data suggest that the activation of VEGFR-1 by VEGF-B¹⁶⁷ promotes the migration and invasion of SMMC7721 cells.

• VEGFR-1 activation induces MMP-9 expression in HCC cell lines

MMP-9 and MMP-2 are the major proteinases for the proteolytic degradation of the ECM components, leading to the cancer cell invasion [2]. In HCC metastasis, MMP-9 possesses greater biological activity than MMP-2 [20,26]. Therefore, we wondered whether VEGFR-1 activation-induced cell invasion was due to the elevated expression of MMP-9. As shown in Figure 3A, the level of MMP-9 mRNA expression in the cell treated with VEGF-B¹⁶⁷ for 32 h was increased by 4.3-fold and 2.6-fold in SMMC7721 and HepG2 cells, respectively. The expression level was declined at 48 h

post-treatment. Gelatin zymography assay showed that MMP-9 gelatinolytic activity in the culture medium from VEGF-B¹⁶⁷-treated SMMC7721 cells was significantly higher than that in the non-specific IgG group or untreated cells (Figure 3B, left panel). However, MMP-2 activity was barely detectable (Figure 3B, left panel). In contrast, in the VEGF-B¹⁶⁷-treated HepG2 cells, the activity of both MMP-9 and MMP-2 were increased in comparison with the controls (Figure 3B, right panel). The predominant band was detected at 92 kDa and 72 kDa corresponding to the pro-active form that becomes active during zymography. Western blot indicated that the level of MMP-9 protein, detected as two bands at 92 kDa and 82 kDa, was increased in these representative two cell lines (Figure 3B). Immunofluorescence detection was performed to confirm the changes of MMP-9. As shown in Figure 3C, the immunoreactivity for MMP-9 in the cytoplasm of both SMMC7721 and HepG2 cells was significantly elevated in the VEGF-B¹⁶⁷-treated group. However, there were not significant differences in the immunoreactivity for MMP-2 between two groups of HepG2 cells (data not shown). Taken together, these data indicated that MMP-9 expression and activity were increased in HCC cells upon activation of VEGFR-1.

• VEGFR-1 activation promotes MMP-9-dependent Matrigel invasion

Our data have indicated that VEGFR-1 activation enhances the invasiveness accompanied by an increase in expression of MMP-9 in SMMC7721 cell line. To determine the specific contribution of MMPs, especially MMP-9 in this cellular invasion, a broad-spectrum pharmacologic MMP inhibitor (GM6001), and a functional blocking anti-MMP-9 antibody (Ab-1) were used to block the proteinase activity. VEGFR-1 activation-induced Matrigel invasion was completely blocked after addition of GM6001, indicating that metalloproteinase activity is required for the cellular invasion (Figure 4A). To examine if MMP-9 is involved in the cellular invasion, functional blocking

Figure 3. VEGFR-1 activation upregulates the expression and activity of MMP-9 in hepatocellular carcinoma cell lines (see facing page). (A) Relative expression of *MMP-9* and β -actin was determined by quantitative real-time PCR, normalized to non-specific IgG group, and arbitrarily set at 1.0. Bars indicate standard deviation; * $p < 0.001$ for both versus the zero time point, and the similar results were obtained in three independent experiments. (B) The expression and activity of MMP-9 in SMMC7721 and HepG2 cells was analyzed by western blot and gelatin zymography. Data shown are representative of three independent experiments. (C) Immunofluorescence detection of MMP-9 in SMMC7721 (upper panel) and HepG2 cells (lower panel). Scale bar: 50 μ m.

antibody against MMP-9 was used and the results showed that abrogation of MMP-9 activity significantly reduced cell invasion by approximately 84% when compared with SMMC7721 cells treated with VEGF-B¹⁶⁷ alone (Figure 4B). These results support a key role for MMP-9 in VEGFR-1 activation-induced invasion.

• VEGFR-1 activation induces Snail expression in HCC cell lines

Recent reports have shown that Snail promotes HCC invasion by inducing EMT and/or upregulating MMPs expression [2,3]. Moreover, Snail is activated downstream of several reporters and bio-active compounds including VEGFRs [12,27–29]. Accompanied with activation of VEGFR-1, Snail expression is significantly elevated in pancreas cancer cells [4]. Thus, we examined the specific role of Snail in VEGFR-1 mediated MMP-9 expression in HCC cells. The results showed that mRNA level of *Snail* in SMMC7721 and HepG2 were increased by 8.5-fold and 2.2-fold after 28 h and 24 h of VEGF-B¹⁶⁷ treatment, respectively (Figure 5A). Western blot analysis also indicated the Snail protein level was increased after VEGF-B¹⁶⁷ treatment (Figure 5B).

• High expression of both VEGFR-1 & MMP-9 correlated with tumor metastasis

The results that activation of VEGFR-1 promotes MMP-9 expression of in HCC cell lines suggest that VEGFR-1 and MMP-9 might be involved in the tumor invasion and metastasis. To test this hypothesis, we investigated the clinical significance of VEGFR-1 in HCC and its relationship to MMP-9 and Snail expression by immunohistochemistry (Table 1). The representative examples of VEGFR-1, MMP-9 and Snail staining in HCC tissues and corresponding peritumoral tissues were shown in Figure 6A. As expected, VEGFR-1 was localized on the cell membrane; Snail was localized in the nucleus, whereas the MMP-9 was present in both cytoplasmic and extracellular compartment. High expression of VEGFR-1, Snail and MMP-9 was confirmed in

48.3, 53.3 and 45.3%, respectively, of all the cases. High expression of VEGFR-1 was also associated with a worse prognosis of HCC cases ($p < 0.0001$ for RFS and $p = 0.023$ for OS, respectively), and there was also a trend toward a worse outcome in patients with high expression of MMP-9 ($p = 0.327$ for RFS and $p = 0.274$ for OS, respectively; Table 1). HCC tissues with high expression of VEGFR-1 also showed increased MMP-9 and Snail expression with a Pearson correlation coefficient of $r = 0.232$ ($p = 0.036$) and $r = 0.418$, respectively ($p < 0.001$) (Figure 6B). To demonstrate the prognostic significance of expression pattern of VEGFR-1 and/or MMP-9 in HCC, we divided the patients in four groups, I: high expression of both VEGFR-1 and MMP-9, II: low VEGFR-1 but high MMP-9 expression, III: high VEGFR-1 but low MMP-9 expression, IV: low expression of both VEGFR-1 and MMP-9, and performed a Kaplan–Meier survival analysis. The results showed that patients with high expression of both VEGFR-1 and MMP-9 had the worst prognosis when compared with other groups ($p = 0.008$ for RFS and $p = 0.128$ for OS, respectively) (Figure 6C). Therefore, we divided patients into groups I with high expression of both VEGFR-1 and MMP-9 and group II without high expression of both VEGFR-1 and MMP-9. Our results showed that patients with high expression of both VEGFR-1 and MMP-9 had a significantly worse RFS and OS than the patients without high expression of both VEGFR-1 and MMP-9 ($p = 0.003$ for RFS and $p = 0.02$ for OS, respectively) (Figure 6C). Collectively, these results strongly support that induction of MMP-9 by VEGFR-1 activation contributes to HCC invasion and metastasis, and it can be speculated a new model for cancer invasion (Figure 6D).

Discussion

In comparison with the well-established mechanisms of EMT and angiogenesis, the impact of VEGFR-1 mediated signaling on the expression profile of proteolytic enzymes involved in invasiveness is not well understood. In this study, we

Figure 4. VEGFR-1 activation promotes MMP-9-dependent Matrigel invasion by SMMC7721 cells. (A) Matrigel invasion by SMMC7721 cells was examined in the absence or presence of a broad-spectrum MMP inhibitor GM6001 (12.5 $\mu\text{mol/l}$), bars indicate standard deviation; * $p < 0.005$, versus nonspecific IgG group. (B) Matrigel invasion by SMMC7721 cells was examined in the presence or absence of function-blocking MMP-9 antibody Ab-1 (15 $\mu\text{g/ml}$). Bars indicated standard deviation; ** $p < 0.05$ versus VEGF-B¹⁶⁷-treated group. The similar results were obtained in three independent experiments.

confirmed that VEGFR-1 activation in HCC cell lines can regulate the expression of MMP-9, which might be a novel mechanism for HCC invasion.

HCC usually expresses high level of VEGF/VEGFR compared with the peritumoral tissue [16,30–31]. Among VEGFRs, VEGFR-1, expressed in cancer cells, is particularly interesting due to its direct tumor activation via an autocrine stimulatory pathway [4,7,32]. Previously studies have shown that overexpression of VEGF-B (a sole ligand for VEGFR-1) in cancerous tissues is associated with tumor invasion and poor prognosis in HCC. In addition, isoform VEGF-B¹⁶⁷ appears to be the clinically dominant isoform of VEGF-B [33]. Using VEGF-B¹⁶⁷ as the ligand to activate VEGFR-1, we found that VEGFR-1 activation can significantly promote cancer cell migratory and invasive ability *in vitro*. These results provided experimental evidence for the clinical significance of VEGF-B in HCC prognosis. In addition, these results are also supported by the *in vivo* results showing that high level of VEGFR-1 was associated with poor prognosis of HCC patients, which was consistent with the finding in renal cell cancer [34]. However, the clinical value of VEGFR-1 warrants further evaluation in larger clinical trials.

Recently, a monoclonal VEGFR-1 blocking antibody IMC-18F1 was used to block the function of VEGFR-1 to confirm that the changes in invasiveness were mediated through VEGFR-1, but not the aberrant expression of another VEGF tyrosine kinase receptor or due to the activation of neuropilin-1 in several studies [31,35]. However, VEGFR-B¹⁶⁷ is thought to be the sole ligand for VEGFR-1. There are no significant effects of the nonspecific IgG on the changes of invasiveness. These two aspects confirmed that the elevation in migration and invasion of HCC cells was caused by VEGFR-1 activation rather than unspecific stimulation.

MMP-9, which is expressed in many types of human carcinomas including HCC, has been closely associated with tumor invasion and poor prognosis [20,36–37]. Recent studies reported that MMP-9 was significantly upregulated by many growth factors, leading to the invasion a variety of solid cancers *in vitro* [11–13]. In present study, VEGFR-1 activation-to-MMP-9 signaling pathway was investigated and confirmed as a novel mechanism of HCC invasion *in vitro* and *in vivo*. Additional, these findings were reinforced by the results of immunohistochemistry on TMA showing that VEGFR-1 is positively correlated with the MMP-9 expression in cancerous tissues. Moreover, the results that

patients with high coexpression of VEGFR-1 and MMP-9 exhibited the worst clinical outcome also suggest that overexpression of both VEGFR-1 and MMP-9 may play a synergistic role in the progression of HCC and can serve as a prognostic marker for HCC.

Snail, one of the key regulators of EMT, is involved in the process of VEGFR-mediated cancer cell invasion [4,29]. Correlative studies have shown Snail also can induce MMP-9 expression at the level of mRNA and protein *in vitro* [12,23,38]. In this study, we found that a distinct increase of Snail expression was detected in the cells with activation of VEGFR-1. Additionally, a strong positive correlation of them was also observed in HCC tissue. These results indicated that Snail may participate in the regulation of VEGFR-1 activation-to-MMP-9 signaling pathway. VEGFR-1 activation led to

the enhanced expression of EMT regulators including Snail, Slug and Twist in pancreatic cancer cells. Among them, Twist appears to be regulated by VEGFR-1 activation [4]. However, the disadvantage of the present study was that only one regulator was detected. Further studies are required to analyze the other regulators expression in responding to the activation of VEGFR-1 and elucidate the exact role in this pathway.

Conclusion

In conclusion, we provide evidence for a new, stepwise signaling pathway from activation of VEGFR-1 to MMP-9 and demonstrate the critical role of MMP-9 in VEGFR-1-mediated HCC cell invasion. Based on the findings of this study, VEGFR-1 is present and functional in HCC cells and its activation significantly enhances the

Figure 5. VEGFR-1 activation promotes Snail expression in hepatocellular carcinoma cell lines. (A) Relative expression of *Snail* and β -actin was determined by quantitative real-time PCR, normalized to nonspecific IgG group, and arbitrarily set at 1.0. Bars indicated standard deviation; * $p < 0.001$ for both versus the zero time point, and the similar results were obtained in three independent experiments. (B) The expression of Snail was detected by western blot. Data shown are representative of three independent experiments.

Figure 6. High coexpression of VEGFR-1/MMP-9 in hepatocellular carcinoma patients indicates the worst outcome and a proposed model of VEGFR-1 activation promoting invasion via induction of MMP-9 (see facing page). (A) Immunohistochemistry staining of VEGFR-1, MMP-9 and Snail in two representative patients with high coexpression of three proteins (Case83) and low for three proteins (Case88). The black arrows indicated the expression of VEGFR-1; the blue arrows indicated the expression of MMP-9; the red arrows indicated the expression of Snail. Scale bars, 50 μ m. (B) Comparison of the recurrence-free survival (upper left panel) and overall survival (lower left panel) of patients categorized by VEGFR-1/MMP-9 immunohistochemistry result. Difference of recurrence-free survival (upper right panel) and overall survival (low right panel) in hepatocellular carcinoma patients with or without high coexpression VEGFR-1/MMP-9. (C) Pearson correlation test was performed to analyze the relationship between the VEGFR-1 and Snail (upper panel) and between VEGFR-1 and MMP-9 (lower panel). (D) A proposed model of VEGFR-1 activation induced invasion via MMP-9.

invasiveness of cell lines through its regulation of MMP-9. This study provided a novel pathway associated with the progression of HCC induced by VEGFR-1 activation.

Future perspective

It is significant to further confirm the role of Snail in the novel pathway associated with VEGFR-1 activation in the future. It is necessary to verify whether VEGFR-1 could be a target for the treatment of HCC invasion and metastasis.

Author contributions

T Li and C Qin conceived and designed the study, conducted most of experiments and drafted the manuscript. Y Zhu and L Han conducted some of the experiments, supervised the data collection and analysis, interpreted data and assisted in writing the manuscript. W Ren and H Liu conducted some of the experiments and interpreted the data.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

- Epithelial–mesenchymal transition and extracellular matrix degradation are critical for the initiation and progression of tumor invasion.
- VEGFR-1 activation can induce EMT by decreasing the expression of E-cadherin in hepatocellular carcinoma (HCC). However, the impact of VEGFR-1 activation on the expression profile of proteolytic enzymes involved with HCC invasion is unknown.
- In this study, we found that activation of VEGFR-1 in HCC cell lines can significantly increase the expression and activity of MMP-9, and then promoted the cell invasion. Functional blockage of MMP-9 inhibited tumor cell invasion, suggesting that VEGFR-1-induced cell invasion is dependent on the function of MMP-9.
- Snail, an epithelial–mesenchymal transition regulator induced by VEGFR-1, was increased by VEGFR-1 activation, indicating that Snail is involved in the regulation of the MMP-9 expression and cell invasion.
- High coexpression of VEGFR-1 and MMP-9 in HCC, which is associated with the patient tumor invasion and metastasis, is predictive of a worse clinical outcome.
- These data showed a novel VEGFR-1 activation-to-MMP-9 signaling pathway that promotes HCC invasion.

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