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Original Article

CXCL6 is upregulated in hepatocellular carcinoma and promotes invasion of liver cancer cells via targeting MMP9

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Abstract: Hepatocellular carcinoma (HCC) is a common primary liver neoplasm characterized by high morbidity and mortality. HCC also featured by high proliferation capacity and metastatic potentials. The prognosis of HCC is still poor partly due to few effective treatment measures. It is important to find new treatment targets for prevention of HCC proliferation and invasion. In our study, we investigated oncogene CXCL6, an important member of CXC chemokine, which was significantly upregulated in primary HCC tissues and related with tumor size and invasion. Besides, the HCC patients with high CXCL6 expression have poor prognosis compared with low expression patients. Furthermore, we revealed that the migration and invasion of HCC cells were inhibited when CXCL6 was knocked-down. The promotive effect of CXCL6 in HCC cells was carried out through activating MMP9. All the results above suggest that CXCL6 plays important roles in promoting HCC proliferation and invasion, and may act as a therapeutic target against HCC proliferation and metastasis.

Keywords: CXCL6, hepatocellular carcinoma, tumor invasion, metastasis

Introduction

Hepatocellular carcinoma (HCC) ranks the fifth most common cancer and is more common in East Asia due to high HBV infection rates in these countries [1]. As a rapid progress tumor, HCC featured by fast growth, easy to metastasis and short survival time. Until now, early diagnosis and timely surgery are the most curative measures for HCC patients [2]. Nevertheless, postoperative recurrence and metastasis are tricky problems that result in poor prognosis of postoperative HCC patients [3]. Therefore, it is urgent to find new treatment target against tumor progression for HCC.

Chemotactic cytokines (chemokines) were reported to play important roles in promoting metastasis of HCC [4]. Chemotaxis of cancer cells leading by tumor-derived chemokines is an important step during tumor metastasis. In other word, chemokines and their receptors play crucial role in tumor invasion and metastasis. CXC chemokines are divided into pro-angiogenic (ELR+) and anti-angiogenic (ELR-) according to whether with a Glu-Leu-Arg (ELR) N-terminal motif [5]. The granulocyte chemotactic protein 2 (GCP-2) which also known as CXCL6 belongs to ELR+ CXC chemokines. Similar to other CXC ELR+ chemokines, CXCL6 was demonstrated to facilitate tumor growth in animal experiment. Besides, CXCL6 has been illustrated as important mediators of progression in several solid tumors [6]. CXCL6 was found up-regulated in solid tumors, including small cell lung cancer, melanoma and colorectal cancer. Among common HCC cell lines, MHCC97H or HCCLM3 cells characterized with high metastatic capacity and HepG2 or SMMC7721 cells with low metastatic capacity [7].

Table 1. HCC patients' clinical and pathological characteristics and their correlation with CXCL6 expression

	CXCL6		
Variable	High expression	Low expression	*P* value
Age			
Age ≤50	31	92	
Age >50	48	86	0.059
Gender			
Female	10	24	
Male	71	152	0.064
Liver Cirrhosis			
Liver cirrhosis Yes	61	143	
Liver cirrhosis No	19	34	0.039
Tumor differentiation			
Grade I	5	8	
Grade II	35	72	
Grade III	40	96	0.037
Vascular Invasion			
Yes	51	52	
No	30	124	0.0027
Tumor Size			
≤5 cm	57	50	
>5 cm	37	113	0.0017
TNM stage			
I	49	110	
II	13	18	
III	18	49	0.0559

Levels of CXCL6 in MHCC97H or HCCLM3 were higher than those from HepG2 or SMMC7721 cells. This result suggests that CXCL6 may facilitate HCC cell invasion [8]. However, further studies about the role of CXCL6 on the invasion and growth of HCC cells remain insufficient. Moreover, the significance of CXCL6 in predicting prognosis of HCC patients remains unclear.

In the present study, we examined the expression of CXCL6 in paired HCC and non-tumor tissues, and found the positive expression rate of CXCL6 in HCC tissues was significantly higher than that in non-tumor liver tissues. Subsequently, we demonstrated the relevance of CXCL6 with HCC patients' prognosis and pathological features through tissue microarray assay. Our results showed that the overall survival and relapse-free survival of CXCL6 high expression HCC patients were lower than CXCL6 low expression patients. Furthermore, we performed CXCL6 knockdown assay in MHCC97H and HCCLM3 and found the proliferation and invasion of MHCC97H and HCCLM3 cells were suppressed in vitro. All these data suggest that CXCL may act as a prognostic factor and facilitate HCC progression.

Materials and methods

Clinical samples and tissue microarray construction

All paired HCC tissues and corresponding non-tumor liver tissues were obtained from surgical sample at Department of Liver Surgery, Shandong Provincial Hospital. Tissue microarrays were constructed by Xi'an Ailina Biotechnology (Ailina Biotechnology Co, Xi'an, China). 196 HCC samples of tissue microarrays were collected from 2005 to 2011 and follow-up until in December 2013. We obtained approval from the Regional Ethical Committees, Shandong Provincial Hospital, Jinan, China for the use of clinical HCC patients' tissues.

Immunohistochemistry stain

The tissue microarray glass slides were baked at 56°C for about one hour, and subsequently de-paraffinized gradually through pure xylene, 50% xylene, gradient ethanol until immersed in water. The TMA slides were immersed in 0.3% Hydrogen peroxide at 37°C for thirty minutes against endogenous peroxidase activity. Antigen retrieval was performed via microwave for about fifteen minutes. We then incubate the slide with CXCL6 antibody overnight at 4°C after the slide restored at room temperature. The TMA slides were washed with phosphate buffer solution (PBS) for three times in the next day. Subsequently, we incubated the slide with HRP-coupled anti-mouse secondary antibody (1:200 dilution, Dako, USA) for one hour at room temperature. Immunostaining was performed using diaminobenzidine substrate chromogen (Dako, USA) and chromogenic reaction was strictly controlled under microscope. Then, the slide was immersed into hematoxylin for nuclear staining for about 2 minutes. Next, the slide was dehydrated through gradient concentrations of ethanol, cleared using xylene, and coverslipped with balsam (Beyotime, China). The IHC staining results were judged by two experienced pathologists according to following criterion, 0-5% positive cells scored 0;

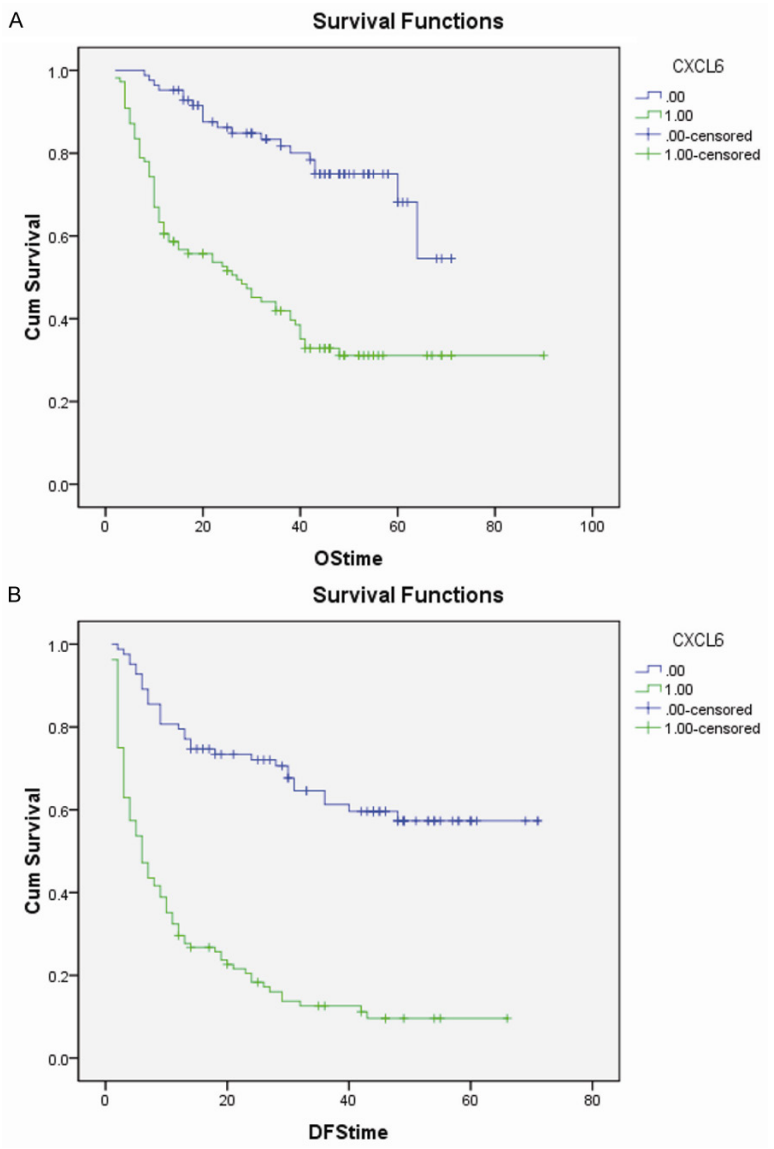

Figure 1. Kaplan-Meier survival analysis illustrated that the overall survive time and progression-free survival time in CXCL6 high expression HCC patients were shorter than low expression patients. The overall survive rate and progression-free survival rate in CXCL6 high expression HCC patients were lower than low expression patients.

6-35% positive cells scored 1; 36-70% positive cells scored 2; 70-100% positive cells scored 3; and scored 0 or 1 was identified low expression, scored 2 or 3 was identified as high expression.

Clinicopathological features correlation and prognosis analysis

A complete follow-up data including patients' age, gender, tumor size, liver cirrhosis, local invasion status, TNM stage, grade, tumor microsatellite, vascular invasion, thrombosis were provided by pathologists in Shandong Provincial Hospital. We use SPSS 16.0 software to analyze the correlation between CXCL6 expression and HCC patients' clinicopathological features. Besides, the correlation of CXCL6 with the overall survival and relapse-free survival of HCC patients were analyzed by Kaplan-Meier method using SPSS software.

HCC cell culture

HCC cell lines were cultured in vitro at 37°C, 100% humidity and 5% CO_2 condition in Dulbecco's modified Eagle medium (DMEM) supplemented with 5-10% fetal bovine serum. All the cell culture medium was added with penicillin and streptomycin.

Real time quantitative PCR reaction

Total RNA extraction was carried out using Trizol reagent (Takara, China) from HCC tissues and cells. Besides, reverse transcription reaction was performed using PrimeScript RT-PCR reagent (Takara, China) following the manufacturer's instructions. Subsequently, we performed real-time PCR reaction in SYBR Green method using SYBR Premix Ex Taq Kit and monitored fluorescence value in ABI7300 PCR instrument (Applied Biosystems Inc). The primers for CXCL6 are as follows: forward primer: 5'-CGC TGG TCC TGT CTC TGC-3', the reverse primer: 5'-GTT TTT CTT GTT TCC ACT GTC C-3'. The relative expression levels of CXCL6 were normalized to 18S RNA (forward primer: 5'-TGCGAGTACTCAACACCAACA-3'; reverse primer: 5'-GCATATCTTCGGCCCACA-3').

Western blot

The total proteins of HCC tissues and corresponding normal liver tissues were extracted

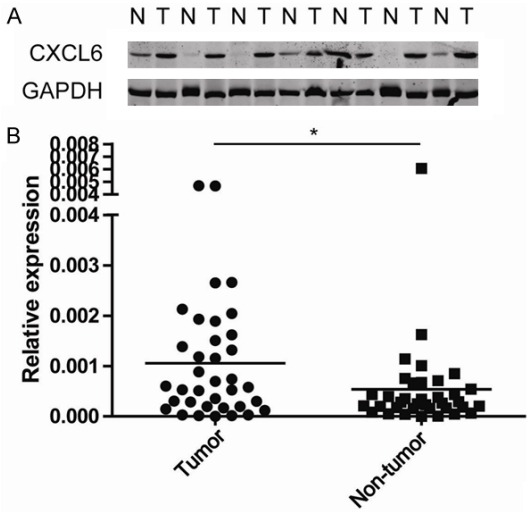

Figure 2. The CXCL6 expression level in HCC tumor tissue and non-tumor tissue were detected by western blot assay and qPCR assay. The CXCL6 expression level was higher in tumor tissue than non-tumor tissue in qPCR (B) and Western blot results (A).

using T-Per tissue protein extraction reagent (Thermo Scientific) following the manufacturer' instruction. Besides, proteins of HCC cells were extracted using IP lysis buffer (Beyotime Jiangsu China). Before Western blot assay, protein lysates were detected quantitatively by BCA method. For western blot assay, protein lysates were added into SDS-PAGE gel and electrophoresis in constant voltage condition. Afterwards, the proteins were transferred into nitrocellulose membrane (Millipore) in constant current condition. Next, the membrane was blocked in 5% fat-free milk (BD) for one hour at room temperature. After blocked with milk, the membrane was incubated with primary antibody for CXCL-6, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech Group, Chicago IL) at 4°C overnight. In the next day, the membrane was washed using TBS (Tris Buffer Solution) for three times and incubated with secondary antibodies IRDye 800 anti-rabbit (LI-COR, Lincoln, NE) or IRDye 680 anti-mouse (LI-COR, Lincoln, NE) for one hour at room temperature. Then the membrane was washed using TBS supplemented with Tween-20 for three times away from light. In the end, the fluorescence signal was detected using Odyssey infrared imaging system (LI-COR, Lincoln, NE) and grey value was quantitatively analyzed by Image J software.

Small interfering RNA transfection

Small interfering RNA (SiRNA) targeted CXCL6 were designed and synthesized by Sangon Biotech. The sequences are, SiRNA-1: forward, 5'-GACCACGCAAGGAGUUCAUTT-3', reverse, 5'-AUGAA CUCCUUGCGUGGUCTT-3'; SiRNA-2: forward, 5'-UCUGCAAGUGUUCGCCAUATT-3', reverse, 5'-UAUGGCGAACACUUGCAGAT-3'; SiRNA-3: forward, 5'-UGGAAACAAGGAAAACUGATT-3', reverse, 5'-UCAGUUUUCCUUGUUUCCATT-3'; SiRNA-NC: forward, 5'-UUC UCCGAACGUGUCACGUTT-3', reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. SiRNA transfection was carried out using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Cell proliferation assay

HCC cell proliferation was measured using the Cell Counting Kit-8 reagent (CCK-8, Dojindo, Japan). HCC cells were seeded into 96-well plate at density of 2000 per 100 μl and cultured at 5% CO_2, 100% humidity, 37°C condition. For detection step, 10 μl CCK-8 reagent was added into each well and the reaction time was one hour. The WST-8 was major ingredient of CCK-8 reagent which can be oxidized to produce a colorimetric dye and be detected at 450 nm using a microplate reader (BIO-TEK).

In vitro cell migration and invasiveness assays

Cell migration and invasion assays were carried out using Transwell chambers (Millipore). For Transwell invasion assays, Matrigel (BD Biosciences, Franklin Lakes, NJ) was added to the base of the top chamber of Transwell (with 8 μm filter membrane, Corning Costar Corp, Corning, NY) for 45 mins at 37°C. Before Transwell assay, the HCC cells were serum and growth factor starved for 24 hours. The HCC cells were added to the top chambers at a density of 1×10^5 per well and the bottom chambers were added DMEM medium supplemented with 10% fetal bovine serum. Cultures were maintained for about 48 hours and cells resided in upper surface of the membrane were cleared using cotton swab carefully. Invading cells located in lower surface of the membrane were fixed using 4% paraformaldehyde for fifteen mins and stained with 0.1% crystal violet for about thirty mins at room temperature and counted or photographed in six fields under

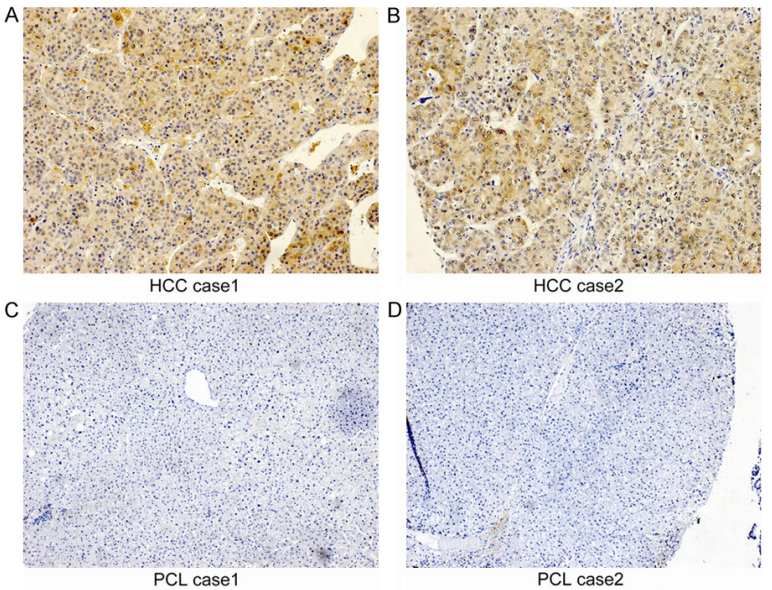

Figure 3. Immunohistochemical analysis of CXCL6 expression in normal liver tissue (down panels), Hepatocellular carcinoma tissue (up panels). Original magnification, 200×.

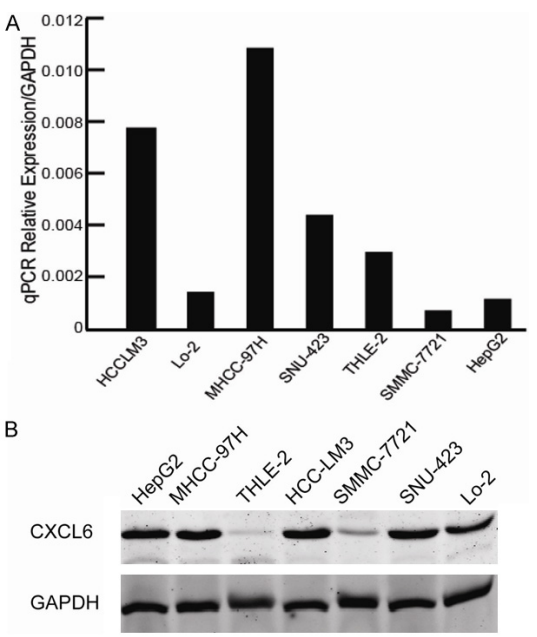

Figure 4. Expression of CXCL6 in Hepatocellular carcinoma cell lines. Lysates from HCCLM3, Lo-2, MHCC-97H, SNU-423, THLE-2, SMMC-7721, HepG2 cells were subjected to SDS-PAGE, followed by Western Blotting for CXCL6. GAPDH was chosen as loading control. The mRNA levels of CXCL6 in these cell lines were detected by quantitative real-time PCR, which were normalized to GAPDH.

100× microscope. Assays were carried out in triplicate wells and repeated three times. In vitro migration assay was performed followed the invasion assay steps without Matrigel added and HCC cells were added to the top chambers at a density of 5×10^4 per well. The migration assay time was maintained for about 24 hours.

Statistical analysis

We used SPSS 16.0 software to analyze the statistical significance of differences in our study. Statistic differences were calculated using two-tailed Student's t-test. Kaplan-Meier method was used to evaluate the correlation between CXCL6 and prognosis of HCC patients. $P<0.05$ was considered statistically significant and $P<0.01$ was considered very statistically significant.

Results

CXCL6 expression is closely related to HCC tumor size, invasion and patients' prognosis

In order to further investigate the clinical significance of CXCL6 in HCC, we detected CXCL6 expression in tissue microarray which included 212 HCC samples using immunohistochemistry assay. By analyzing 178 available paired tissues, we found that CXCL6 was elevated in 71.91% (128/178) of HCC patients, but was down-regulated in 14.04% (25/178) of HCC patients (**Table 1**).

By statistical analysis, we found that the expression of CXCL6 was closely related with tumor size and vascular invasion which are important clinicopathological parameters of HCC progression (**Table 1**). This result suggested that CXCL6 may be related with HCC progression. Furthermore, we found that higher CXCL6 expression was related with poor overall survival rate (OS) as well as relapse-free survival rate (RFS). The HCC patients with low CXCL6 expression had better prognosis than high expression patients (**Figure 1**). The result suggested that CXCL6 may serve as a candidate prognostic marker of HCC.

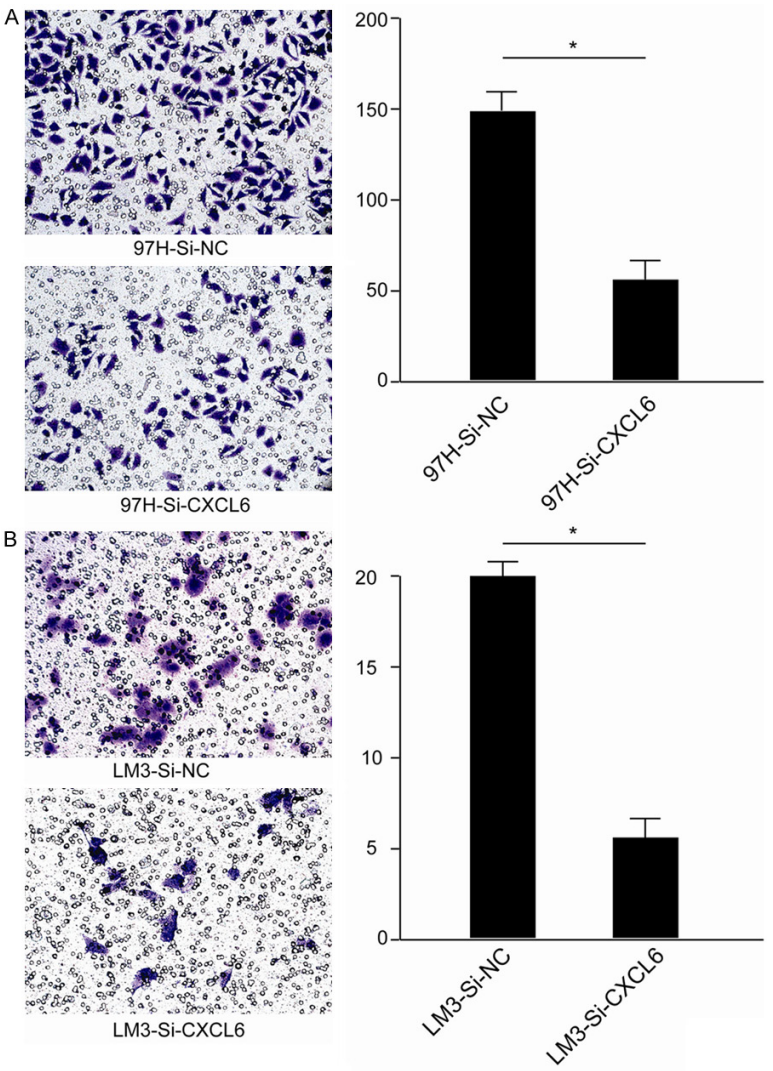

Figure 5. Silencing of CXCL6 inhibits Hepatocellular carcinoma cells invasion in vitro. LM3 and 97H cells were seeded into transwell which contained Matrigel. The cells invaded through the Matrigel and onto the lower surface of transwell membrane were stained with methyl violet. There were fewer invaded cells in the CXCL6 siRNA groups than in the negative control siRNA group.

CXCL6 expression is elevated in HCC tissues

We detected the expression level of CXCL6 in HCC and Corresponding Normal Liver (CNL) tissues using quantitative real time polymerase chain reaction (PCR) and western blot method. Our results showed that the CXCL6 mRNA and protein expression level in HCC tissues were significantly higher than that in CNL tissues. Our results illustrated that CXCL6 is up-regulated in HCC tissues compared with CNL tissues (**Figures 2** and **3**).

CXCL6 is up-regulated in HCC cells with high metastatic capacity

Compared with HepG2 and SMMC7721 cells, MHCC97H and HCCLM3 cells have higher metastatic capacity. We detected the CXCL6 protein expression in eight HCC cell lines and non-HCC cell line (Lo2, immortal liver cell) and found CXCL6 was highly expressed in MHCC97L, MHCC97H and HCCLM3 cells, but down-regulated in HepG2 and SMMC7721 cells (**Figure 4**). Moreover, the mRNA expression level of CXCL6 in HCC cell lines was generally consistent with protein expression status. These results demonstrated that CXCL6 may play role in facilitating motility of HCC cells.

Furthermore, we assessed the role of CXCL6 by transfecting small interfering RNA (siRNA) into MHCC97H and HCCLM3 cells using Lipofectamine 2000 reagent. The knockdown effect of siRNA in HCC cells was detected using qPCR and western blot assay. The results showed that CXCL6 expression levels were remarkably silenced by siRNA at 48 hours after transfection (Supplementary Figure 1).

Knockdown of CXCL6 inhibits HCC cell invasion in vitro

Our preliminary analysis showed that CXCL6 high expression was closely related with vascular invasion in HCC. This result indicated that CXCL6 may play role in facilitating HCC cell motility. Therefore, we designed in vitro invasion assays to validate this speculation. Through in vitro invasion assays, we concluded that silencing of CXCL6 significantly down-regulated the invasion capacities of MHCC97H and HCCLM3 cells (**Figure 5**).

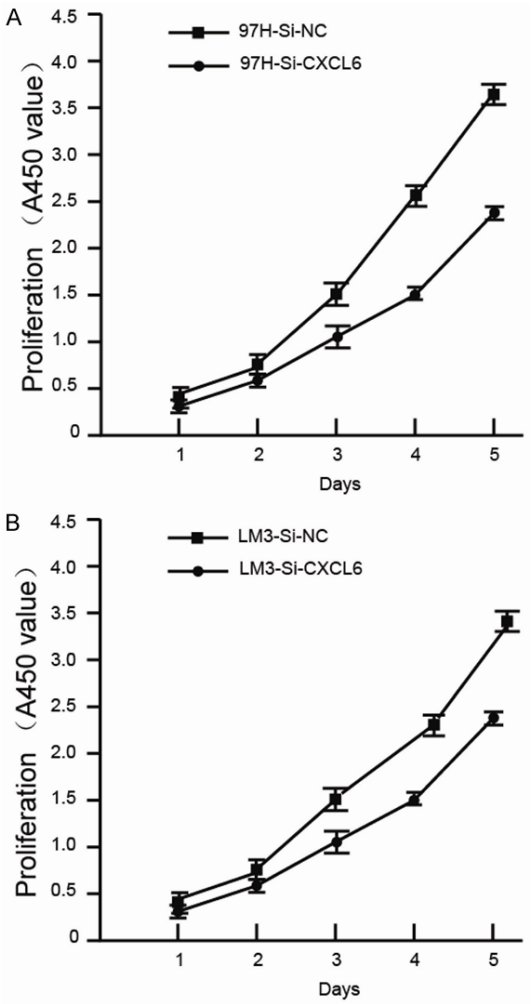

Figure 6. Effect of CXCL6 knockdown on Hepatocellular carcinoma cells proliferation (97H and LM3 cell lines). Equal amount of cells were seeded initially and the OD450nm value were detected by CCK-8 assay for up to five days.

Silencing of CXCL6 suppresses HCC cell proliferation in vitro

Our IHC assay suggested that CXCL6 expression was related with HCC tumor size. Given this, we surmise that CXCL6 may play roles in modulating HCC cell proliferation. In order to verify our surmise, we carried out in vitro cell proliferation assay in MHCC97H and HCCLM3 cells which were transfected with siRNA targeted CXCL6. The CCK-8 assay showed that silencing of CXCL6 significantly inhibited HCC cell proliferation in vitro (**Figure 6**).

Overexpression of CXCL6 promotes HCC cell migration and invasion in vitro

In order to further investigate the role of CXCL6 in HCC cell, we carried out overexpression assay through lentivirus transfection method. The results showed that the SMMC-7721 which was transfected with lenti-CXCL6 vector had higher invasion and migration abilities than control (**Figure 7**).

CXCL6 promotes HCC cell invasion via MMP9 pathway

Subsequently, we explored how CXCL6 promotes HCC cell invasion in vitro. The MMP9 was knockdown using SiRNA manner in HCC cells transfected with lenti-vector or lenti-CXCL6. The results showed that the pro-invasion effect of CXCL6 in SMMC-7721 cell was inhibited by knockdown of MMP9. Thus, the pro-invasion effect of CXCL6 in HCC cell may through activate or interact with MMP9 (**Figure 8**).

Discussion

Hepatocellular carcinoma is characterized by high metastatic potential and high mortality [9]. High malignancy of HCC cells and tumor microenvironment are main cause of poor prognosis in HCC patients [10]. Exploring and identifying the functional role of genes which differentially expressed between HCC tissues and corresponding non-tumor tissues is an effective method for us to find new therapeutic target against HCC progression [11]. Moreover, these differentially expressed genes may act as predictor for HCC patients' prognosis [12].

In this study we verified that the expression of CXCL6 was significantly elevated in HCC tissues compared with that in corresponding non-tumor liver tissues [13]. In addition, our results indicate that high expression of CXCL6 relates to invasion and metastasis of HCC, and CXCL6 high expression predicts poor prognosis of HCC patients [14-17]. Although CXCL6 has been found to contribute to proliferation, migration, invasion and angiogenesis, the exact role of CXCL6 in HCC progression has not been systematic studied yet [18]. Our study showed that expression of CXCL6 was positively related to the malignant potential of HCC.

Through in vitro assays, we found that CXCL6 was down-regulated in HCC cell with low metastatic potential. To further explore whether CXCL6 has functional roles in HCC progression, we knockdown CXCL6 expression using SiRNA method. Our results demonstrated that both proliferation and invasion of HCC cells were

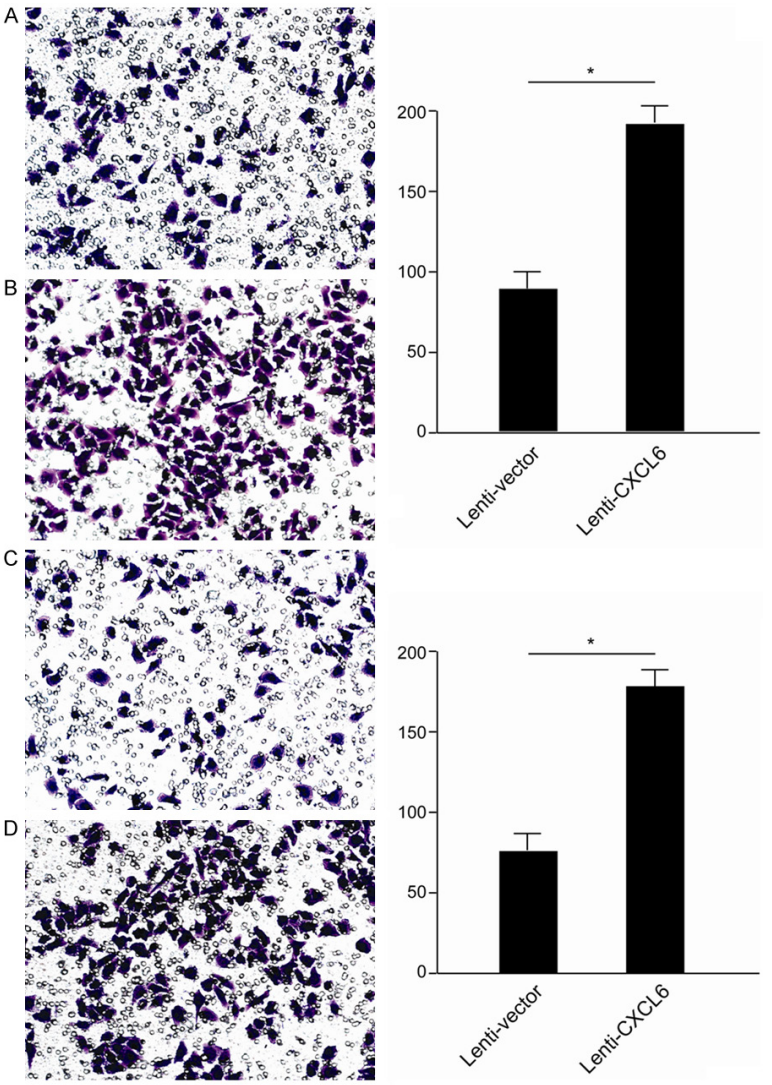

Figure 7. Representative figure of cell migration and invasion detection by transwell assay. The overexpression of CXCL6 (lenti-CXCL6) HCC cells obtained higher migration and invasion capacities than negative control cell group.

remarkably inhibited by SiRNA targeted CXCL6, indicating important roles of CXCL6 in HCC progression.

CXCL6 (also known as granulocyte chemotactic protein-2, GCP2) is a member of the CXC chemokine family which can be divided into ELR (Glu-Leu-Arg) + and ELR- chemokines [19-22]. The CXC chemokine family is characterized by Cys-X-Cys (CXC) motif forming by four cysteine residues near the amino terminus. CXCL6 belongs to ELR+ chemokines with neutrophil chemotactic and angiogenic properties [23-27]. The function of CXCL6 in neutrophil is similar to interleukin-8 [28, 29]. Besides, CXCL6 has ability to attract granulocytes (includes neutrophil) and stimulate secretion of matrix-metalloproteinase-9 and so on [30-33]. Neutrophils, firstly identified as effector cells participate in defense against bacterial invasion, account for 50-70% of leukocytes [34-36]. In addition to defense function, neutrophils can facilitate tumor cell motility, invasion by secreting hepatocyte growth factor, β2-integrins [37], MMP-9 [38], or neutrophil elastase [39-41]. It is reported that neutrophils enhance the invasion capacity of HepG2 cell line in vitro. We also know that neutrophils could promote the implantation of circulating cancer cells and formation of metastases via potentiating HCC cell adhesion to hepatic sinusoids. Therefore, CXCL6 may promote HCC progression through recruiting neutrophils [42-44] to HCC tumor microenvironment and enhancing expression of MMP-9 [45-49] which facilitate tumor invasion via degrading extracellular matrix [50-53]. CXCL6 binds to G-protein coupled receptors CXCR1 and CXCR2, which mediates its chemotactic and angiogenic capacities [54-56]. Besides, CXCL6 has been implicated in the progression of several solid tumors including osteosarcoma [57], breast cancer [58], gastrointestinal tumors [59] and prostate cancer [60-62]. However, the exact mechanism of how CXCL6 promote HCC progression remains unclear and need further research.

In conclusion, we showed that CXCL6 is upregulated in HCC and strong correlated with HCC patients' prognosis. Furthermore, knockdown of CXCL6 suppressed HCC proliferation and invasion in vitro. All the results above suggested that CXCL6 may serve as a potential thera-

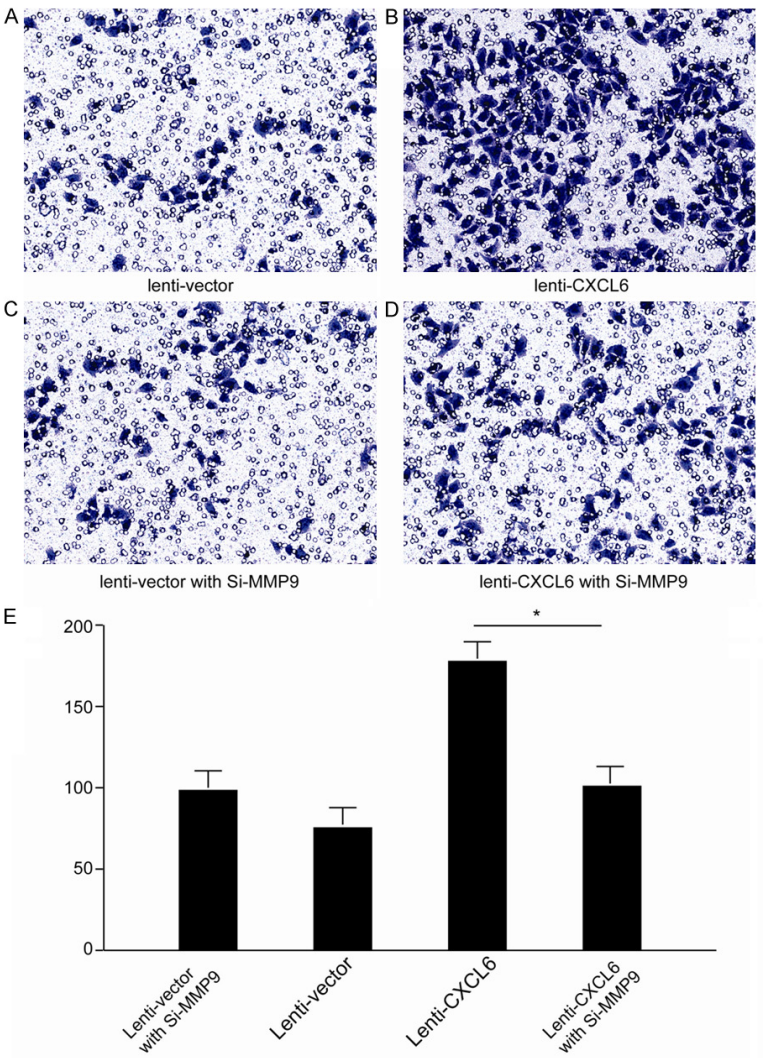

Figure 8. The lenti-CXCL6 HCC cells and lenti-vector HCC cells were transfected with MMP9 siRNA respectively and seeded into transwell 48 hours after siRNA transfection. The results showed that silencing of MMP9 can inhibited the promotion effect of CXCL6 in HCC cell invasion.

peutic target and predictor of prognosis in HCC patients.

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Disclosure of conflict of interest

None.

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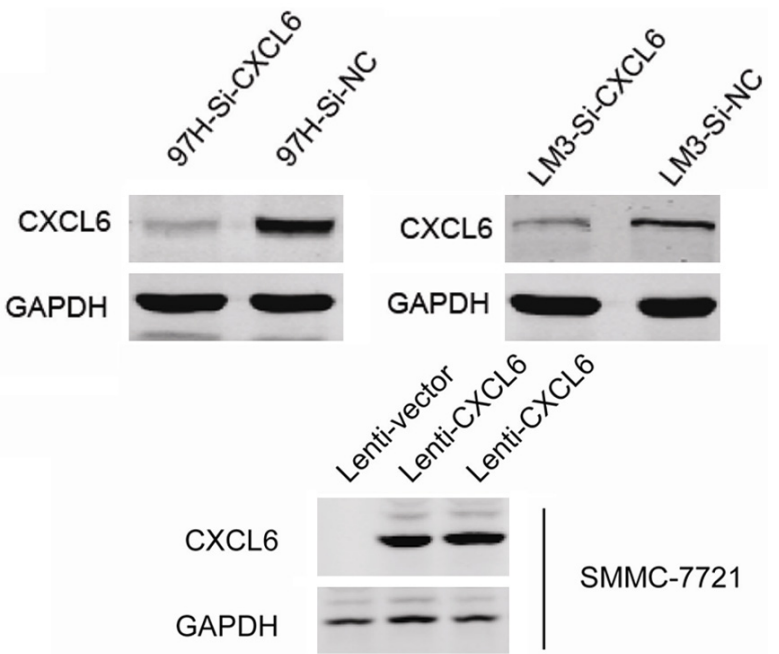

Supplementary Figure 1. Silencing of CXCL6 in LM3 and 97H cell lines were detected by western blot assay. Over-expression of CXCL6 in SMMC-7721 cells was detected by western blotting assay.