CX3CR1 regulates hepatocellular carcinoma(HCC) metastasis via PI3K/AKT pathway

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Abstract

Background Fractalkine receptor CX3CR1 is differentially expressed in hepatocellular carcinoma and its function is unknown. As a special case of chemokine family, CX3CR1 only binds to its unique ligand CX3CL1, and CX3CL1 also only binds to its only receptor CX3CR1, the unique matching between ligands and receptors is not found in other chemokines, and this specificity is essential for diagnosis, prognosis and clinical target therapy. Methods CX3CR1 expression was analyzed by immunohistochemistry, migration and invasion abilities of SMMC-7721 cells were detected by wound-healing and transwell after overexpressing CX3CR1. In addition to overexpression experiments, interfering RNA siCX3CR1 was used for reverse validation in HepG2 cells. To further clarify the mechanism of CX3CR1 regulating HCC metastasis, the classical PI3K/AKT pathway were detected by Western blot. Results The CX3CR1 levels were positive correlated with pathological TNM stage, meanwhile a negative correlation between high expression of CX3CR1 and survival rate was found among patients at stagell-III. In our study, overexpression of CX3CR1 promoted migration and invasion ability of SMMC-7721 cells, whereas knockout of CX3CR1 inhibited these abilities of HepG2 cells. Mechanistically, CX3CR1 regulates the metastasis of HCC cells via PI3K/AKT pathway, and the PI3K inhibitor LY294002 could stop the promoting effect of CX3CR1 on SMMC-7721 cells. Conclusion CX3CR1 regulated HCC cell metastasis via PI3K/AKT signaling and this effect might be a new target for clinical diagnosis and treatment.

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world [1]. Although there are many methods to treat hepatocellular carcinoma, such as surgery, radiotherapy and chemotherapy [2-4], but many patients have lost the opportunity of surgical treatment because the early symptoms of primary hepatocellular carcinoma are not obvious [5]. The use of radiotherapy and chemotherapy is also limited because of its toxic and side effects. Therefore, it is particularly important to explore the mechanism of the occurrence and development of hepatocellular carcinoma and find new treatment methods.

Chemokines are 8-10KD proteins which can regulate leukocyte migration to infected sites by binding to their specific chemokine-receptors [6]. Chemokine receptor 1 (CX₃-C motif chemokine receptor 1, CX₃CR1) is the only known member of CX₃C family. It is mainly expressed on the surface of T cells, natural killer cells, monocytes and vascular endothelial cells [7-10]. More and more studies have shown that chemokines and their receptors play an important role in the growth, invasion and metastasis of tumors. It has been confirmed that CX₃CR1 is involved in regulating the occurrence and development of breast cancer [11], prostate cancer [12], pancreatic cancer [13], ovarian carcinoma [14] and other tumors[15], but there are few reports about its role and mechanism in hepatocellular carcinoma.

In this study, the effects of overexpression of CX₃CR1 on the migration and invasion of human hepatocellular carcinoma cells were explored in two aspects: overexpress CX₃CR1 in low-expression cell lines and inhibit CX₃CR1 in high-expression cell lines. The molecular mechanism of CX₃CR1 was preliminarily explored to provide a new basis for the treatment and prognosis of hepatocellular carcinoma.

Methods

Specimens and patients data

All 385 HCC patients data were used to analyze. The 20 HCC specimens were collected from the Pathology Archive of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between 2018-2019. This study was approved by the Medical Research Ethics Committee of Chongqing Medical University, and informed consent was obtained from all patients. The 365 mRNA expression profile was downloaded from The Human Protein Atlas, which patients data were collected from The Cancer Genome Atlas (TCGA) database.

Cell culture

Human normal hepatocyte LO2 cell line, human hepatocellular carcinoma SMMC-7721 and HepG2 cell line were cultured in DMEM medium (HyClone, California, USA) containing 10% FBS, 10U/ml penicillin-streptomycin (complete medium) at 37°C and 5% CO₂ incubator. When density of LO2, SMMC-7721 and HepG2 were reached 70%-80%, these cells were trypsinized by 0.5% trypsinase and carried out to new dishes.

Plasmid transfection

The SMMC-7721 cells in logarithmic growth phase were inoculated into 96-well plate, 24-well plate, 6-well plate and culture dish respectively. When the cell density reached 60%, the complete DMEM medium was replaced with medium without FBS nor penicillin-streptomycin. The mixture of 1µg/ml CX₃CR1 over-expression plasmid, 1µl/ml Lipo2000 (Cat.No.11668019, Invitrogen, California, USA), 100ug/ml medium without FBS was incubated at room temperature for 30 minutes, then the mixture was added into the culture medium evenly. The medium without FBS was changed to complete medium after 4-6 hours. At the same time, the same amount of empty vector plasmid was added in the negative control group (NC group) to exclude the influence of the plasmid itself on the experiment. The overexpression and empty plasmids were purchased from Genechem Co.,Ltd. (shanghai, China). 10nmol/ml LY294002 (Cat.No.HY-10108, MCE, China) was added in the SMMC-7721 cells after transfecting CX₃CR1 over-expression plasmid.

Interfering RNA transfection

HepG2 cells in logarithmic growth phase were inoculated into 96-well plate, 24-well plate, 6-well plate and culture dish respectively. When the cell density reached 50%, the mixture of 60µl/ml Buffer, 2.5µl/ml CX₃CR1 interfering RNA and 6µl/ml transfection reagent was evenly divided into the medium. At the same time, the same amount of negative control interfering RNA was added to establish negative control group (NC group). The CX₃CR1 and negative control interfering RNA were purchased from RIBOBIO Co.,Ltd. (Guangzhou, China).

Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time PCR was performed in a CFX-connect fast real-time PCR system (Bio-RAD, California, USA). The sample mixture was composed of 5µl SYBR (TaKaRa, Japan) 0.8µl primers 1µl cDNA and 3.2µl ddH₂O. The following touch-down cycling conditions: 1) 95°C for 10min. 2) 95°C for 20s. 3) 56°C for 10s. 4) temperature was reduced 3°C/cycle and the step2-3 were repeated. 5) 95°C for 20. 6) 55°C for 20s.

The primer sequence is as follows, CX₃CR1: Forward 5'CAACAGCAAGAAG CCCAAGAG3' Reverse 5'TGAAGAAGAAGGCGGTAGTGAA3';β-actin: Forward 5'CCACGAAACTACCTTCAACTCC3' Reverse 5'GTAGTCTCCTTCTG CATCCTGT3' (TaKaRa, Japan).

Western blot

1) Extraction of total cell protein: Collecting cell precipitation and add protein lysate (Roche, Switzerland), after mixing the cell suspension, the supernatant was collected by centrifuging 13000rpm for 15min at 4°C and adding 5 x Buffer to the supernatant (Beyotime, Shanghai, China). In the end, heating proteins to denaturation. 2) SDS-PAGE electrophoresis: 10% separating gum and 5% concentrating gum were prepared in turn, Adding samples containing 20ug protein in each hole, after SDS-PAGE electrophoresis, the membrane were blocked in 5% BSA at 37°C for 2h. After incubating with primary antibodies (CX₃CR1 1:1000, sc-30030, SantaCruz; p-AKT 1:1000, #4060, Cell Signaling Technology; AKT 1:1000, #4685, Cell Signaling Technology; MMP-3 1:1000, YT4465, Immunoway; MMP-9 1:1000, WL01580, Wanleibio) overnight at 4°C, the membranes were washed by 0.1% TBST for 3 times, 5 minutes at a time. Then the membranes were detected by chemiluminescence after incubating with HRP-anti-mouse/rabbit IgG (1:3000, ZSGB-BIO, Beijing, China) at 37°C for 1h.

Wound-healing test

When the density of cells were reached to 80% in the 6-well plate after transfecting with plasmids or siRNA, the cells were scratched with a small pipette tip, and washed with PBS for 3 times, then replaced with the medium containing 2% serum. The cells were observed and photographed under the microscope at 0 hours, 24 hours and 48 hours. Then, the wound-healing width was recorded at the same observation point. The average wound-healing widthh of multiple observation points was calculated, and the scratch healing rate of each group was obtained.

Transwell

400µL culture medium with 2% serum containing 40000 SMMC-7721 cells or 60000 HepG2 cells was added into the upper chamber (matrix glue was used in invasion experiment, matrix glue and culture medium were 1:5 ratio, 50µL, Cat.No.356234, Corning, USA) and 700µL complete culture medium containing 10% serum was added into the lower chamber. Four parallel controls were set up in each group. After 24 hours, the cells on the lower side of chamber membrane were fixed for 20 minutes with 500µL 4% polyformaldehyde. The cells were stained with 500µL crystal violet for 30 minutes. After washing and drying, the cells were counted under a microscope. At least 10 visual fields were observed in each chamber, and the average values were obtained after counting.

Statistical analysis

SPSS 16.0 statistical software was used to analyze the experimental results. Each experiment was repeated three times. The differences between protein and mRNA expression were compared by t-test or Bonferroni-corrected t-test. The Log-rank(Mantel-Cox) test or Gehan-Breslow-Wilcoxon test was used to analyze difference between survival curves. The difference was statistically significant with $*P < 0.05$ $**P < 0.005$ $***P < 0.001$.

Results

CX₃CR1 expression in HCC tissues is related to clinical stages and is a marker of poor prognosis

In order to clarify the relationship between CX₃CR1 and the progression of HCC, we detected the expression level of CX₃CR1 in 20 cases of hepatocellular carcinoma by immunohistochemistry between high-differentiation and low-differentiation groups, finding that the expression of CX₃CR1 was uniform and higher in the low-differentiated group, but it was not uniform in the high-differentiated group, which had low-expression and high-expression regions. (Fig. 1a). Then we analyzed the mRNA expression data of CX₃CR1 in 365 HCC tissues which were downloaded from The Human Protein Atlas. These results showed that the expression level of CX₃CR1 was higher at stageⅢ than that at stageⅡ (Fig. 1b). The correlation between CX₃CR1 expression and prognosis of HCC patients at stageⅡ-Ⅲ was verified by Kaplan-Meier survival analysis, finding the patients with high expression of CX₃CR1 had lower short-term survival rate (Gehan-Breslow-Wilcoxon test) in total-population (Fig. 1c) and male-population (Fig. 1d), the long-term survival rate (Log-rank(Mantel-Cox) test) was also lower in male-population but no difference in total-population. On the contrary, both short-term survival rate and long-term survival rate was no difference in female-population (Fig. 1e). In conclusion, there was a negative correlation between high expression of CX₃CR₁ and survival rate was found among patients at stageⅡ-Ⅲ.

CX₃CR1 is expressed at low levels in SMMC-7721 cells and at high levels in HepG2 cells

Western blot analyses were used to detect the mRNA expression and protein expression levels of CX₃CR1 in different HCC cells compared with human normal hepatocytes LO2, finding that CX₃CR1 expression levels were higher in HepG2 and lower in SMMC-7721 cells (Fig. 2a). Therefore, we choose HepG2 cell lines to verify interference experiments, and SMMC-7721 cells lines to verify overexpression experiments. we downregulated CX₃CR1 in HepG2 cells using siRNA and upregulated CX₃CR1 in SMMC-7721 cells by transfecting CX₃CR1-plasmid, the Q-PCR (Fig. 2b c and d) and western blot analyses showed these treatment were effective (Fig. 2e and f).

CX₃CR1 regulates the migration and invasion ability of HCC cells

To explore the role of CX₃CR1 in metastasis, Wound-healing test showed that the migration ability was promoted in SMMC-7721 cells after overexpressing CX₃CR1 (Fig. 4a), and migration ability of HepG2 was inhibited by knockout of CX₃CR1 (Fig. 4b). Transwell assays revealed that CX₃CR1 overexpression promoted the migration and invasion of SMMC-7721 cells (Fig. 3a and b), whereas knocked of CX₃CR1 inhibited the migration and invasion of HepG2 cells as compared with control cells (Fig. 3c and d). Consistent with these results, the protein levels of matrix metalloproteinase [16-17] MMP-3 and MMP-9 were up-regulated in SMMC-7721 cells by overexpressing CX₃CR1 (Fig. 4c), and the level of MMP-3 and MMP-9 were down-regulated in HepG2 by knocking out CX₃CR1 (Fig. 4d). These findings demonstrated that CX₃CR1 regulated the migration and invasion of HCC cells.

CX₃CR1 regulates the migration and invasion of HCC cells via PI3K/AKT pathway

PI3K/AKT pathway has been proved to play an important role in the process of chemokine-axis regulating tumor [18-20]. Western blot test was used to verify whether PI3K/AKT signaling pathway was involved in regulating metastasis of hepatocellular carcinoma. The results revealed the phosphorylation of AKT(p-AKT) was up-regulated in SMMC-7721 cells by transfecting CX₃CR1-plasmid compared with control group (Fig. 5a) , on the contrary, the phosphorylation-AKT level was down-regulated in the HepG2 cells after knocking out CX₃CR1 (Fig. 5b). To investigate whether CX₃CR1 was mediated through the PI3K/AKT pathway, we added the PI3K/AKT pathway inhibitor LY294002. Wound-healing test showed that LY294002 inhibited the migration ability of SMMC-7721 cells after overexpressing CX₃CR1 (Fig. 5c and d),

and the migration and invasion-related protein MMP-9 also reduced by using LY294002 compared with over-expression CX₃CR1 groups (Fig. 5e f and g), these results showed that CX₃CR1 regulated the metastasis of HCC cells via PI3K/AKT pathway.

Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. The death toll of HCC patients is as high as 745,000 [21]. The occurrence and development of HCC is a complex process involving multiple factors. Because of its initial symptoms, most patients have lost the best opportunity for surgical treatment [22]. With the continuous exploration and research, the chemokine axis is becoming increasingly important. More and more attention has been paid to the role of CXCR4 [23-24], CCL20 [25], CCL2-CCR2 [26], CCR4 [27] and other chemokine axes in the occurrence and development of hepatocellular carcinoma [28-30]. As a chemokine receptor, CX₃CR1 has also been proved to be involved in regulating the biological effects of various tumors. In addition, tumor microenvironment, which consists of stromal cells, vascular endothelial cells, immune cells and extracellular matrix, is also an indispensable part of the process of tumorigenesis and development [31-34]. Chemokines and their receptors have been proved to play a very important role in tumor microenvironment. Studies have confirmed that chemokine axis CXCL9/10-CXCR3, CXCL12-CXCR4, CCL20-CCR6, CCL5-CCR5, CXCL5-CXCR2 and so on can regulate the functions of various immune cells in tumor microenvironment to further promote immune escape of tumor cells and angiogenesis of tumors to help metastasis and progress of tumors [35-39]. CX₃CR1 has also been shown to play an important role in regulating tumor and tumor microenvironment [40]. Although there are many studies on CX₃CR1 in tumors, the relationship between CX₃CR1 and hepatocellular carcinoma is still unclear.

In this study, we found that CX₃CR1 was differentially expressed in different stages of HCC and there was a significant negative correlation between higher-level CX₃CR1 and prognosis in male-population. At the same time, CX₃CR1 also showed differential expression in different hepatocellular carcinoma cell lines. In order to explore the direct relationship between CX₃CR1 and biological characteristics of hepatocellular carcinoma cells, the CX₃CR1 overexpression plasmid was transfected with SMMC-7721 cells, which expressed the CX₃CR1 at low-level, on the contrary, the CX₃CR1 interference RNA was transfected with HepG2 cells, which expressed the CX₃CR1 at high-level. The migration and invasion ability of SMMC-7721 cells overexpressing CX₃CR1 was significantly enhanced. At the same time, the levels of MMP-3 MMP-9 and p-AKT were significantly increased, and the migration and invasion ability of HepG2 cells inhibiting CX₃CR1 was significantly weakened. At the same time, the levels of MMP-3 MMP-9 and p-AKT were significantly decreased. In conclusion, while overexpression of CX₃CR1 in SMMC-7721 cells can promote its migration and invasion ability, interfering with CX₃CR1 can significantly inhibit the migration and invasion of HepG2 cells. Its mechanism may be related to activation of PI3K/AKT

signaling pathways. Thus, CX₃CR1 may become a potential new target for the treatment of hepatocellular carcinoma.

Conclusions

Our study reveal for the first time the relationship between CX₃CR1 and HCC metastasis. Here, we find that CX₃CR1 is significantly correlate with the survival of patients with hepatocellular carcinoma, and this phenomenon is more evident in male patients. CX₃CR1 regulates migration and invasion ability of SMMC-7721 and HepG2 cells via PI3K/AKT pathway, and the PI3K inhibitor LY294002 reverse the promoting effect of CX₃CR1 on SMMC-7721 cells. Thereby CX₃CR1 regulates HCC cell metastasis via PI3K/AKT signaling and this effect might be a new target for clinical diagnosis and treatment.

Abbreviations

AKT	Serin/theronine kinase
BSA	Bovine serum albumin
ddH ₂ O	Double distilled water
EP	Eppendorf
FBS	Fetal bovine serum
h	Hour
Min	Minute
s	Second
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3kinase
P/S	Penicillin/Streptomycin
PVDF	Polyvinylidene fluoride
RT	Reverse transcription
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
TBST	Tris buffered saline

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Declarations

Ethics approval and consent to participate

This study was approved by the Medical Research Ethics Committee of Chongqing Medical University, and informed consent was obtained from all patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

Q.S. supervision, conceived of the study. M.T.F. obtained most part of the data. Y.G.W. was responsible for collating experimental data and writing manuscript. X.L. provided HCC specimens. Y.J.J. provided the methods of experiments. X.W.W. provided the methods of experiments. M.J.B. was responsible for analysing data. L.Q.A. was responsible for analysing data. All authors interpreted the data and contributed to the final version of this report. All authors approved the final manuscript.

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Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5