

# C-C chemokine receptor type 2 promotes epithelial-to-mesenchymal transition by upregulating matrix metalloproteinase-2 in human liver cancer

HUIJIN LI<sup>1,2\*</sup>, HUI LI<sup>3\*</sup>, XIAO-PENG LI<sup>4</sup>, HAOJUN ZOU<sup>1</sup>, LITING LIU<sup>1</sup>, WANGQIAO LIU<sup>5</sup> and TAO DUAN<sup>1,6</sup>

<sup>1</sup>Department of General Surgery, The Second People's Hospital of Changzhi, Changzhi, Shanxi 046011;

<sup>2</sup>Shaanxi Key Laboratory of Ischemic Cardiovascular Disease, Institute of Basic and Translational Medicine, Xi'an Medical University, Xi'an, Shaanxi 710021; <sup>3</sup>Department of Dermatology, The Second People's Hospital of Changzhi, Changzhi, Shanxi 046011; <sup>4</sup>Shaanxi Normal University General Publishing House Co., Ltd., Xi'an, Shaanxi 710062;

<sup>5</sup>Department of Ophthalmology, The Second People's Hospital of Changzhi, Changzhi, Shanxi 046011;

<sup>6</sup>Department of Hepatobiliary Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China

Received January 29, 2018; Accepted July 30, 2018

DOI: 10.3892/or.2018.6660

**Abstract.** C-C chemokine receptor type 2 (CCR2) is aberrantly expressed in a variety of tumor cells, and participates in the regulation of tumor cell progression, metastasis and immune escape. However, the mechanism of action of CCR2 in liver cancer remains unclear. In the present study, the aim was to elucidate the molecular mechanism underlying the regulation of epithelial-to-mesenchymal transition (EMT) by CCR2 in liver cancer cells. Initially, CCR2 expression in liver cancer tissues was measured, and the survival time of patients was analyzed by Kaplan-Meier analysis. In liver cancer cells, the mRNA and protein expression levels of CCR2, matrix metalloproteinase-2 (MMP2), E-cadherin and vimentin were evaluated by reverse transcription-quantitative polymerase chain reaction and western blotting. Cell viability, migration and invasion were determined by Cell Counting Kit-8, wound healing and Transwell chamber assays, respectively. Additionally, the binding between CCR2 and MMP2 was identified by co-immunoprecipitation (Co-IP). It was observed that CCR2 was abnormally upregulated in liver cancer tissues and significantly associated with the tumor diameter, metastasis and stage. The survival of patients with high CCR2 expression was lower compared with that of patients

with low CCR2 expression. In addition, the number of cells that penetrated the transwell chamber membrane was significantly reduced following treatment with CCR2-small interfering RNA (siRNA). Furthermore, CCR2 was found to participate in MMP2-induced EMT, while CCR2-siRNA transfection reduced the expression and activity of MMP2, and confirmed the specific binding between CCR2 and MMP2. Co-IP also identified the independent interaction between endogenous proteins in HepG2 cells. These results revealed that CCR2 promotes EMT in liver cancer. Thus, CCR2 is an attractive novel target for inhibiting invasion and metastasis of liver cancer cells.

## Introduction

Liver cancer is the sixth most common cancer worldwide and the second major tumor type in China (1,2). Despite significant advances in the detection and treatment of liver cancer, the survival of liver cancer patients remains poor and the precise mechanisms underlying liver cancer remain unclear (3). Therefore, novel diagnostic markers are urgently needed to improve the survival rate of liver cancer patients (4).

Previous investigations have demonstrated that epithelial-to-mesenchymal transition (EMT) participates in the progression and metastasis of various types of tumor, including liver cancer (5,6). Since EMT is the initial step of tumor metastasis, further understanding on the mechanisms of EMT will shed new light on the use of targeted therapeutic strategies for liver cancer. However, the molecular mechanism of EMT is yet unknown. An increasing number of transcription factors that can promote EMT have been identified, including zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2 and Twist (7).

C-C chemokine receptor type 2 (CCR2) is the specific receptor of monocyte chemoattractant protein-1 (MCP-1), which is also known as C-C motif chemokine ligand 2 (CCL2), and is also a receptor for CCL7, CCL8, CCL11, CCL12 and CCL13. CCR2A and CCR2B are the two isoforms of CCR2, both of which are derived from the same gene, with the

*Correspondence to:* Dr Tao Duan, Department of General Surgery, The Second People's Hospital of Changzhi, 83 Peace West Road, Changzhi, Shanxi 046011, P.R. China  
E-mail: duantao2082425@163.com

Dr Huijin Li, Shaanxi Key Laboratory of Ischemic Cardiovascular Disease, Institute of Basic and Translational Medicine, Xi'an Medical University, No. 1 Xin Wang Road, Xi'an 710021, P.R. China  
E-mail: lihuijin017@126.com

\*Contributed equally

**Key words:** liver cancer, C-C chemokine receptor type 2, matrix metalloproteinase-2, epithelial-to-mesenchymal transition, metastasis

exception of a difference in the carboxy-terminus, and CCR2B is the major functional form. CCR2 is abnormally expressed in a variety of tumor cells, including prostate cancer, renal cell carcinoma, non-small cell lung cancer, myeloma and colorectal cancer (8-10). Furthermore, it is associated with tumor invasion and metastasis (11,12). However, the specific mechanism of CCR2 in the regulation of liver cancer remains to be clarified.

Given the importance of CCR2 in tumor progression, the aim of the present investigation was to elucidate the possible mechanism of CCR2 in liver cancer cell invasion. Kaplan-Meier survival analysis demonstrated that the survival time of patients with high expression of CCR2 was lower in comparison with that of patients with low expression of CCR2. In addition, wound healing and the Transwell chamber invasion assays revealed that the number of cells transfected with CCR2-small interfering RNA (siRNA) was significantly reduced. Matrix metalloproteinase 2 (MMP2) expression and activity were also significantly decreased following CCR2-siRNA transfection. Furthermore, there was a positive correlation between CCR2 and MMP2 in liver cancer tissues, and CCR2 interacted with MMP2 in HepG2 cells. Taken together, the present study results revealed that CCR2 promotes EMT through MMP2 in liver cancer, and that CCR2 is an attractive, novel target for inhibiting the invasion and metastasis of liver cancer cells.

## Materials and methods

**Clinical samples.** Liver cancer tissues (n=39) and paired normal tissues were obtained from the First Affiliated Hospital of the Fourth Military Medical University (Xi'an, 710032, China) between June 2004 and June 2007. All tissues were obtained by resection surgery, and none of the patients had previously received preoperative radiotherapy, chemotherapy or biotherapy. The present study was approved by the First Affiliated Hospital of the Fourth Military Medical University (approval no. KY20163226-1) and written informed consent was obtained from all patients. The clinicopathological data of the included cases are shown in Table I. Tumor and paired tissues were snap-frozen in liquid nitrogen and preserved at -80°C.

**Immunohistochemical staining.** Liver cancer tissues samples were fixed in 10% neutral formaldehyde for 24 h, dehydrated and embedded in paraffin. Next, 5- $\mu$ m sections were cut from each tissue. Subsequent to dewaxing and blocking the endogenous peroxidase activity, specimens were incubated with antibodies against CCR2 (dilution 1:100; cat. no. LBP60766; Abcam, Cambridge, MA, USA) at 4°C overnight. Next, specimens were incubated with the Biotin-labeled goat anti-mouse (1:1,000; ZSGB-BIO, Beijing, China) for 60 min at room temperature. Following treatment with horseradish peroxidase-labeled streptavidin solution (ZSGB-BIO) for 30 min at room temperature, the samples were stained with 3,3'-diaminobenzidine. Finally, the sections were counterstained with 0.02% hematoxylin and visualized under a microscope.

**Cell culture and treatment.** Three human liver cancer cell lines (HepG2, SMMC-7721 and MHCC97-H) and a normal

liver cell line (HL-7702) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose supplemented with 10% fetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in humidified chamber with 5% CO<sub>2</sub>. Cells were transfected with CCR2-siRNA and negative control siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) were transfected using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.** Total RNA was extracted from the cells or tissues with Fast 200 kit (Fastagen, Shanghai, China), RNA concentration was measured by NanoDrop. Next, 1-2  $\mu$ g RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) and qPCR was performed on the CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The conditions for the qPCR reaction were as follows: Initial denaturation at 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 55°C for 15 sec and 72°C for 20 sec. The primers used in PCR are listed in Table II.  $\Delta$ Cq values were normalized to GAPDH, serving as the internal control, and comparative quantification was performed with the  $2^{-\Delta\Delta Cq}$  method (13).

**Western blot assay and antibodies.** Cells were lysed in radioimmunoprecipitation assay buffer containing 1 mM phenylmethane sulfonyl fluoride and cocktail protease inhibitor (Roche Diagnostics, Nutley, NJ, USA). Protein concentration was measured by BCA protein assay kit, then separated by 12% SDS-PAGE gel and transferred to nitrocellulose membranes. The nitrocellulose membrane was then blocked with 5% non-fat milk in 0.01 M PBS buffer for 1 h at room temperature, followed by incubation with primary antibodies against E-cadherin (1:1,000; cat. no. ab76055; Abcam, Hong Kong, China), vimentin (1:500; cat. no. 5741; Cell Signaling Technology, Danvers, MA, USA), CCR2 (1:1,000; cat. no. LBP60766; Abcam), MMP2 (1:1,000; cat. no. 40994; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 2118; Cell Signaling Technology, Inc.). After washing with PBST, the blots was reacted with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2,000; cat. nos. 7076 or 7074; Cell Signaling Technology, Inc.). The immunoreactive bands were subsequently detected with an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.), and the signals were analyzed by ChemiDoc MP Imaging System and (Bio-Rad Laboratories, Inc.).

**Cell viability assay.** Cells were seeded in 96-well plates (3x10<sup>3</sup>/well) and incubated for 24, 48 or 72 h. Subsequently, cell growth was tested by Cell Counting Kit-8 (CCK-8; 7Sea, Shanghai, China), according to the protocol provided by the manufacturer. At 2 h after addition of CCK-8, cell growth viability was detected by measuring the optical density at 450 nm on an EnSpire plate reader (PerkinElmer, Inc., Waltham, MA, USA).

Table I. Association of CCR2 expression with the clinicopathological characteristics of liver cancer patients.

Clinical characteristics	Case no.	CCR2 expression		P-value
		Low	High	
Total	39	18	21	0.573
Sex				
Male	25	11	14	
Female	14	7	7	0.720
Age (years)				
≤60	18	10	8	
>60	21	9	12	0.0221 <sup>a</sup>
Tumor size (cm)				
<5	21	14	7	
≥5	18	4	14	0.215
Histologic grade (differentiation)				
Good/moderate	20	10	10	
Poor	19	8	11	0.0187 <sup>a</sup>
N status				
N0	15	10	5	
N1/2	24	8	16	0.0158 <sup>a</sup>
Clinical stage				
I-II	17	10	7	
III-IV	22	8	14	

<sup>a</sup>P<0.05. CCR2, C-C chemokine receptor type 2.

Table II. Primer and siRNA list.

mRNA	Sequence (5'-3')	Experimental use
CCR2	F: GAGCGGTGAAGAAGTCACCA R: CAGAAGCAAACACAGCCACC	qPCR
GAPDH	F: AAATCCCATCACCATCTTC R: TCACACCCATGACGAACA	qPCR
MMP2	F: GCATCCAGACTTCCTCAGGC R: CCATTAGCGCCTCCATCGTAG	qPCR
E-cadherin	F: GCTGCTCTTGCTGTTTCTTCG R: CCGCCTCCTTCTTCATCATAG	qPCR
Vimentin	F: AAGTTTGCTGACCTCTCTGAGGCT R: CTTCCATTTACGCATCTGGCGTT	qPCR
CCR2-siRNA	F: AAGCCAGGACGGTCACCTT R: AAGGTGACCGTCCTGGCTT	RNA interference
Control-siRNA	F: TTTTCGCATCGAGTCACGTCT R: AGACGTGACTCGATGCGAAAA	RNA interference

CCR2, C-C chemokine receptor type 2; MMP2, matrix metalloproteinase-2; siRNA, small interfering RNA; F, forward; R, reverse; qPCR, quantitative polymerase chain reaction.

*Migration and invasion assays.* The migration assay was conducted using a 24-well Transwell chamber, while the invasion assay was performed in a similar fashion using Matrigel-coated chambers. Briefly, cells (5x10<sup>5</sup>/ml in

serum-free medium) were seeded in 100  $\mu$ l in the top chamber. The bottom wells were filled with complete medium containing 20% FBS. After culturing for 24 h for the migration assay and 48 h for the invasion assay, cells on the upper surface was wiped off with cotton swabs. Cells in the lower membrane surface were fixed with methanol and stained using 0.1% crystal violet. Finally, cells were detected, counted and averaged in five random fields under a phase contrast microscope (original magnification, x100).

**Co-immunoprecipitation (Co-IP).** The Co-IP experiments were conducted utilizing the Pierce Crosslink IP kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The Co-IP protocol was performed on ice, unless otherwise indicated. Briefly, HepG2 cells (150 cm<sup>2</sup> plate, 10<sup>7</sup> cell/plate) were treated with 1 ml extraction buffer, and the binding of the anti-CCR2 or anti-MMP2 to Protein A/G Agarose was performed according to the procedure described in the kit. Subsequently, the Protein A/G Agarose was incubated with anti-CCR2 or anti-MMP2 antibody on a mixer at 25°C for 1 h. The immunoprecipitated products were eluted with Laemmli buffer. The eluting mixture was finally tested by western blot assay.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean, and were analyzed by one-way analysis of variance using the SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) statistical software. The patient survival rate was analyzed by the Kaplan-Meier method, and the log-rank test was performed to evaluate the presence of significant differences. Differences were considered to be statistically significant at  $P < 0.05$ .

## Results

**CCR2 is upregulated in liver cancer tissues and associated with liver cancer progression.** In order to compare the expression of CCR2 in different liver cancer tissues, the mRNA levels of CCR2 were detected in 39 liver cancer tissues and paired adjacent normal liver tissues using RT-qPCR analysis. The results demonstrated that the CCR2 level in liver cancer tissues was higher compared with that in adjacent normal tissues ( $P = 0.0014$ ; Fig. 1A). To further investigate the correlation of CCR2 expression with the clinicopathological characteristics, the relative CCR2 expression in the liver cancer tissues obtained from 39 patients was classified into two groups, including patients with low ( $n = 18$ ; CCR2 expression ratio  $<$  median ratio) and high ( $n = 21$ ; CCR2 expression ratio  $\geq$  median ratio) relative levels of CCR2 mRNA ( $P = 0.0003$ ; Fig. 1B). The results of the clinicopathological analysis revealed that CCR2 was significantly correlated with tumor size ( $P = 0.0221$ ; Fig. 1C), metastasis ( $P = 0.0187$ ; Fig. 1D) and clinical stage ( $P = 0.0158$ ; Fig. 1E). However, no statistically significant correlations were obtained between CCR2 expression and other clinicopathological characteristics, including gender, age and histologic grade ( $P > 0.05$ ; Table I). Kaplan-Meier analysis and log-rank test were used to evaluate the association between CCR2 expression in liver cancer and patient survival. The median 5-year survival time was 27 months in the low CCR2

expression group, whereas it was 17 months in the high CCR2 expression group ( $P = 0.0135$ ; Fig. 1F). Taken together, these results suggest that overexpression of CCR2 may be involved in liver cancer development, progression and metastasis.

**Knockdown of CCR2 inhibits tumor development in liver cancer cells.** The mRNA and protein expression levels of CCR2 were also analyzed in three liver cancer cell lines (HepG2, SMMC-7721 and MHCC97-H) and the HL-7702 normal liver cell line. As shown in Fig. 2A, the results indicated that the CCR2 mRNA level in HepG2, SMMC-7721 and MHCC97-H cells was markedly higher compared with that in the HL-7702 normal liver cell line. Furthermore, the protein expression of CCR2 in cell lines HepG2, SMMC-7721 and MHCC97-H was also higher than in HL-7702 cell line (Fig. 2B). Due to the protein level of CCR2 in SMMC-7721 and MHCC97-H cells which was roughly similar, HepG2 and MHCC97-H cells were selected in subsequent experiments. Subsequently, CCR2 expression in liver cancer cells was manipulated to analyze its association with tumor progression. The expression level of CCR2 was silenced by siRNA transfection, and CCR2 expression level and liver cancer cell viability were detected following CCR2 inhibition. Knockdown of CCR2 was observed to successfully inhibit the CCR2 expression level (Fig. 2C and D) and viability (Fig. 2E and F) in liver cancer cells. Taken together, these results suggest that CCR2 is required for tumor development in liver cancer cells.

**CCR2 regulates liver cancer cell migration and invasion.** In order to explore the role of CCR2 in the regulation of liver cancer metastasis, the migration and invasion of liver cancer cells (HepG2 and MHCC97H) were detected following treatment with CCR2-siRNA. As shown in Fig. 3A and B, the silencing of CCR2 in HepG2 and MHCC97H cells decreased cell mobility compared with that in the control cells. In addition, downregulation of CCR2 significantly suppressed cell invasion subsequent to the treatment of cells with CCR2-siRNA, in contrast to the control cells (Fig. 3C and D). Therefore, these data suggest that downregulation of CCR2 blocked EMT in liver cancer cells.

**CCR2 enhances EMT by MMP2 in liver cancer cells.** To determine whether CCR2 regulates the EMT in liver cancer cells, the mRNA and protein levels of E-cadherin, vimentin and MMP2 were initially evaluated in HepG2 and MHCC97H cells transfected with CCR2-siRNA. As reported earlier, CCR2-siRNA decreased CCR2 expression in liver cancer cells. This silencing also enhanced the EMT in HepG2 and MHCC97H cells, as shown by the decreased expression of E-cadherin and increased expression of vimentin detected by RT-qPCR (Fig. 4A and B) and western blotting (Fig. 4C and D). In order to further explore the molecular mechanisms of CCR2 in liver cancer cells, the expression of MMP2 following the treatment of cells with CCR2-siRNA was also analyzed. The results demonstrated that the mRNA and protein expression levels of MMP2 were suppressed after silencing of CCR2 compared with the control cells (Fig. 4A-D). Furthermore, the activity of MMP2 was tested, and the results revealed that MMP2 activity was weakened by downregulation of CCR2 (Fig. 4E and F). The association between CCR2 and

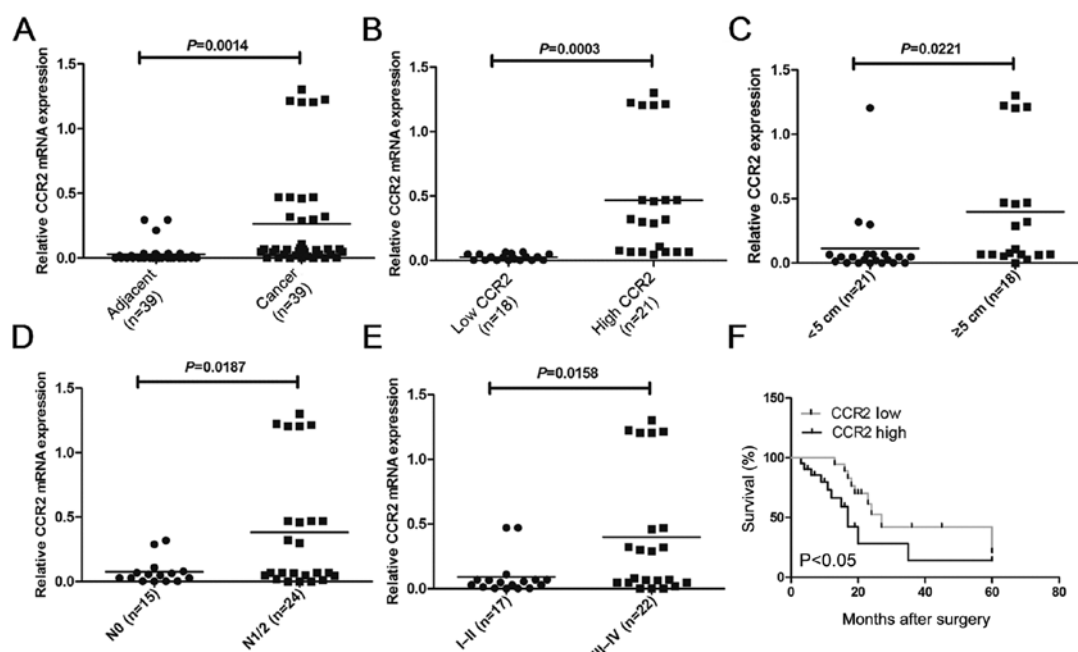


Figure 1. CCR2 is upregulated in liver cancer tissues and associated with liver cancer progression. (A) mRNA level of CCR2 in liver cancer tissues is higher than that in adjacent cancer tissues ( $P=0.0014$ ). (B) CCR2 mRNA expression was classified into two groups, including the low and high CCR2 expression groups ( $P=0.0003$ ). CCR2 mRNA expression is positively correlated with (C) tumor size ( $P=0.0221$ ), (D) metastasis ( $P=0.0187$ ), and (E) clinical stage ( $P=0.0158$ ). (F) Kaplan-Meier survival curves and log-rank tests were used to evaluate recurrence rates and survival of all liver cancer patients. CCR2, C-C chemokine receptor type 2.

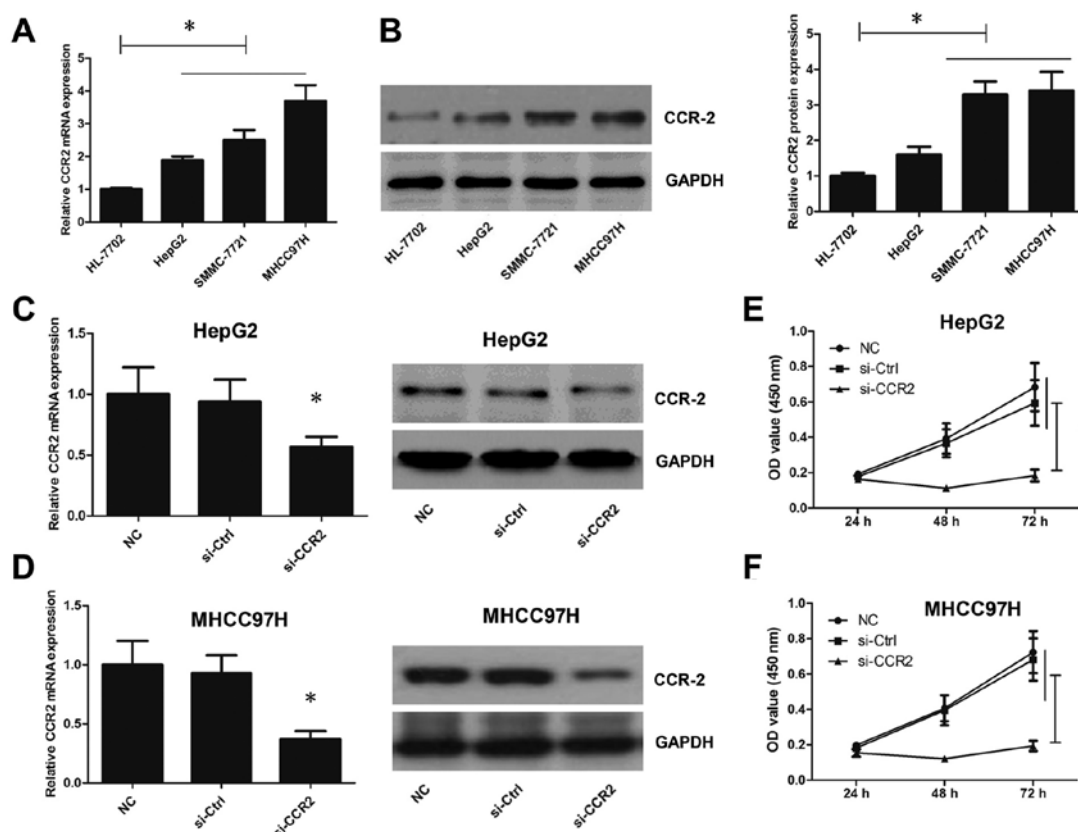


Figure 2. Knockdown of CCR2 inhibited the tumor development in liver cancer cells. (A) mRNA and (B) protein levels of CCR2 in liver cancer cell lines (HepG2, SMMC-7721 and MHCC97H) were higher compared with the levels in HL-7702 cells, as determined using RT-qPCR and western blotting, respectively. The results were normalized to GAPDH expression. (C) HepG2 and (D) MHCC97H cells transfected with si-Ctrl or si-CCR2 were examined using RT-qPCR and western blotting to determine the relative expression levels of CCR2 mRNA and protein. (E) HepG2 and (F) MHCC97H cells were subjected to CCK-8 assay to determine the cell viability and proliferation following transfection with si-Ctrl or si-CCR2.  $^*P<0.05$  vs. corresponding control groups. CCR2, C-C chemokine receptor type 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, normal control; si, small interfering RNA; Ctrl, control.

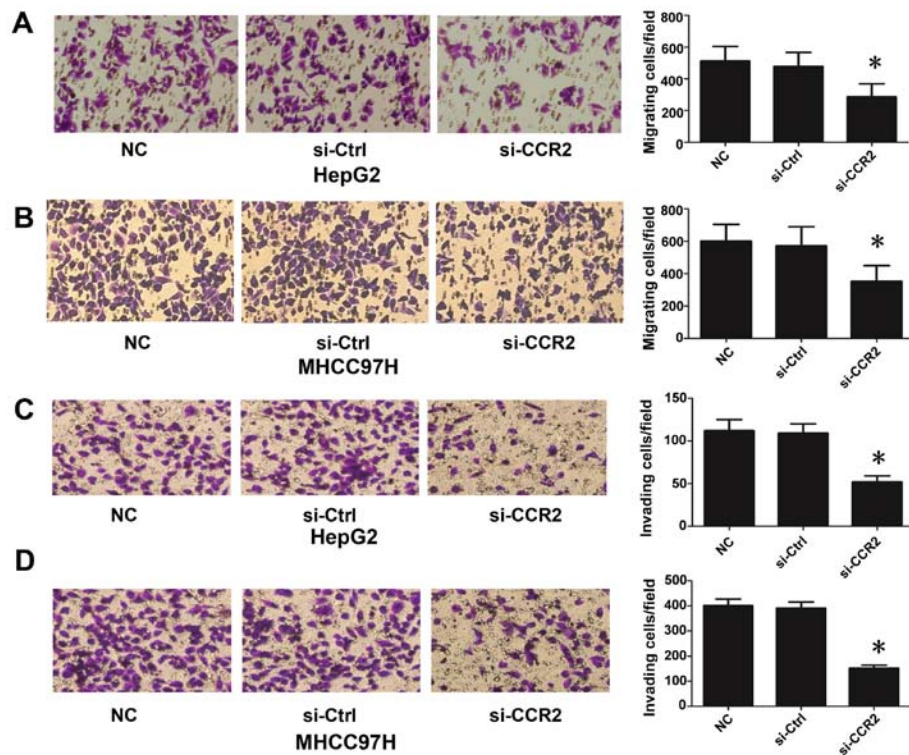


Figure 3. CCR2 regulates liver cancer cell migration and invasion. (A) HepG2 and (B) MHCC97H liver cancer cell migration following treatment with CCR2-siRNA. (C) HepG2 and (D) MHCC97H cell invasion following treatment with CCR2-siRNA, as compared with the control cells. \* $P < 0.05$  vs. control groups. CCR2, C-C chemokine receptor type 2; NC, normal control; si, small interfering RNA; Ctrl, control.

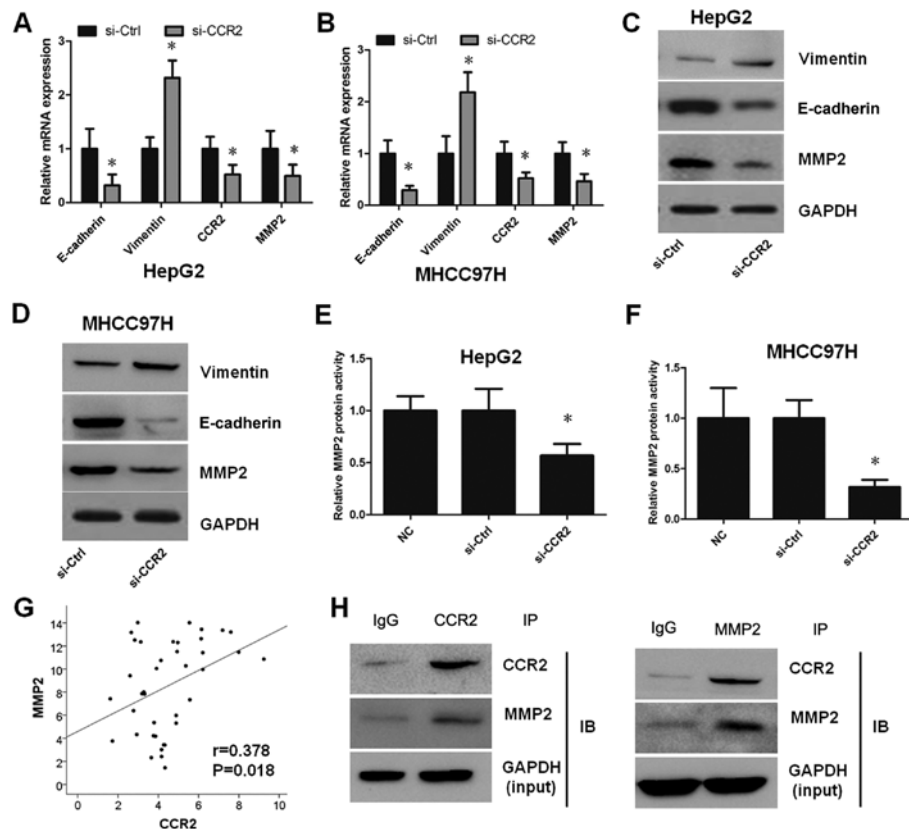


Figure 4. CCR2 enhanced epithelial-to-mesenchymal transition by MMP2 in liver cancer cells. mRNA expression levels of E-cadherin, vimentin, CCR2 and MMP2 in (A) HepG2 and (B) MHCC97H cells transfected with si-Ctrl or si-CCR2. Protein expression level of E-cadherin, vimentin, CCR2 and MMP2 in (C) HepG2 and (D) MHCC97H cells transfected with si-Ctrl or si-CCR2. Relative MMP2 protein activity in (E) HepG2 and (F) MHCC97H cells transfected with si-Ctrl or si-CCR2. (G) Correlation between CCR2 and MMP2 mRNA expression in liver cancer tissues ( $r = 0.378$ ,  $P = 0.018$ ). (H) Interaction between endogenous CCR2 and MMP2 in HepG2 cell lines by Co-IP. \* $P < 0.05$  vs. control group. CCR2, C-C chemokine receptor type 2; MMP2, matrix metalloproteinase-2; IP, immunoprecipitation; NC, normal control; si, small interfering RNA; Ctrl, control.

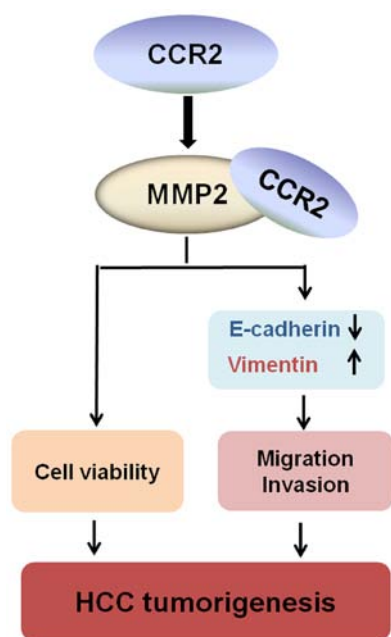


Figure 5. Schematic representation of the proposed mechanism of CCR2-mediated liver cancer epithelial-to-mesenchymal transition and progression. CCR2, C-C chemokine receptor type 2; MMP2, matrix metalloproteinase-2.

MMP2 in 39 liver cancer tissues was also detected, and the results suggest that the mRNA expression levels of CCR2 were positively correlated with MMP2 ( $r=0.378$ ,  $P=0.018$ ; Fig. 4G). To further confirm the specific binding between CCR2 and MMP2, the study also identified the interaction between endogenous proteins independently in HepG2 cell lines by Co-IP. The results indicated that endogenous CCR2 co-precipitated with MMP2 in HepG2 cells (Fig. 4F). Taken together, these findings reveal that CCR2 is highly expressed in liver cancer tissues and cells. In addition, CCR2 promoted EMT in liver cancer cells through the regulation of MMP2 (Fig. 5).

## Discussion

Mounting evidence has demonstrated that liver cancer is one of the deadliest types of cancer due to its complexity, heterogeneity, metastasis and recurrence (14). Tumor metastasis is one of the main reasons for the poor prognosis of liver cancer patients. EMT, the initial step of tumor metastasis that results in the loss of the epithelial phenotype and leads to mesenchymal characteristics, has become an important field in cancer research (15-18). Further exploration of the molecular mechanisms of EMT will shed new light on the development of liver cancer diagnostic and therapeutic strategies (19-20).

Previous studies have demonstrated that CCR2 serves an important role in tumor metastasis in breast, bladder, ovarian and prostate cancer (21-25). CCR2 is the specific receptor of MCP-1/CCL2. Hu *et al* (21) reported that CCR2 acts as a competing endogenous RNA by inhibiting the STARD13-RhoA-ROCK1-MLC-F-actin pathway in the regulation of ovarian metastasis. In addition, Izumi *et al* (25) demonstrated that knockdown of the androgen receptor in prostate cancer promoted PCa cell migration and invasion through the CCL2/CCR2-STAT3 axis and EMT pathways. Rao *et al* (23) also revealed that upregulation of estrogen

receptor  $\beta$  in mast cells and bladder cancer cells resulted in an enhanced CCL2/CCR2/EMT/MMP9 axis. Thus, exploration of the mechanisms of CCR2 in liver cancer may be useful for developing novel diagnostic markers and therapeutic drugs for liver cancer patients.

In the present study, the hypothesis that CCR2 serves an important role in liver cancer progression through the regulation of EMT was proposed. To that end, CCR2 was detected in 39 liver cancer and paired adjacent tissues. The results revealed that CCR2 levels in liver cancer tissues were higher when compared with those in adjacent normal tissues. Further investigation into the correlation of CCR2 expression with clinicopathological characteristics indicated that CCR2 was significantly correlated with tumor size, metastasis and clinical stage. The median 5-year survival time was evaluated, and the results indicated that this was higher in the low CCR2 expression group in comparison with that in the high CCR2 expression group. These results suggest that the overexpression of CCR2 may be involved in liver cancer development, progression and metastasis. To further dissect the role of CCR2 in the regulation of liver cancer EMT, CCR2 was silenced in liver cancer cells by siRNA transfection. The results indicated that knockdown of CCR2 inhibited liver cancer cell viability, migration and invasion. Furthermore, the molecular mechanism of CCR2 in the regulation of EMT in liver cancer cells was evaluated, and the results revealed that CCR2-siRNA inhibited the expression levels of E-cadherin and MMP2, while it increased the expression of vimentin. In addition, the activity of MMP2 was weakened by downregulation of CCR2. The mRNA expression levels of CCR2 were also found to be positively correlated with MMP2 in liver cancer tissues, while endogenous CCR2 co-precipitated with MMP2 in HepG2 cells.

In conclusion, CCR2 was aberrantly increased in liver cancer tissues and significantly associated with the tumor diameter, metastasis and stage. The survival of patients with high CCR2 expression was lower than that of patients with low CCR2 expression. CCR2 can promote cell viability, cell migration and invasion in liver cancer cells. Moreover, CCR2 was found to participate in the regulation of EMT by measuring RNA and protein expression levels of vimentin and E-cadherin, downregulating of CCR2, which reduced the expression of E-cadherin and MMP2, activity of MMP2, and enhanced the expression of vimentin. In addition, the specific binding between CCR2 and MMP2 was confirmed by Co-IP in HepG2 cells. These results revealed that CCR2 promotes EMT in liver cancer. Thus, CCR2 is an attractive novel target for inhibiting invasion and metastasis of liver cancer cells (Fig. 5).

## Acknowledgements

The authors would like to thank Dr Haimin Li from the First Affiliated Hospital of the Fourth Military Medical University for his assistance with the design and technical support.

## Funding

The present study was supported by the Nature Science Basic Research Program of Shanxi Province (grant



no. 201701D221177) and the Youth Tuoju Project of Shaanxi Association for Science and Technology (grant no. 20170408).

### Availability of data and materials

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

### Authors' contributions

HJL and HL conceived and designed the study. HJZ, LTL, HJL, XPL, WQL and TD performed the experiments. HJL and XPL wrote the paper. HJL, HL, HJZ and TD reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The present study was approved by the First Affiliated Hospital of the Fourth Military Medical University (approval no. KY20163226-1; Xi'an, China), and written informed consent has been provided by all patients.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 66: 7-30, 2016.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in china, 2015. *CA Cancer J Clin* 66: 115-132, 2016.
3. Jayachandran A, Dhungel B and Steel JC: Epithelial-to-mesenchymal plasticity of cancer stem cells: Therapeutic targets in hepatocellular carcinoma. *J Hematol Oncol* 9: 74, 2016.
4. Ozer Etik D, Suna N and Boyacioglu AS: Management of hepatocellular carcinoma: Prevention, surveillance, diagnosis, and staging. *Exp Clin Transplant* 15: 31-35, 2017.
5. Giannelli G, Koudelkova P, Dituri F and Mikulits W: Role of epithelial to mesenchymal transition in hepatocellular carcinoma. *J Hepatol* 65: 798-808, 2016.
6. van Zijl F, Zulehner G, Petz M, Schneller D, Kornauth C, Hau M, Machat G, Grubinger M, Huber H and Mikulits W: Epithelial-mesenchymal transition in hepatocellular carcinoma. *Future Oncol* 5: 1169-1179, 2009.
7. Bottoni P, Isgro MA and Scatena R: The epithelial-mesenchymal transition in cancer: A potential critical topic for translational proteomic research. *Expert Rev Proteomics* 13: 115-133, 2016.
8. Yang X, Lu P, Ishida Y, Kuziel WA, Fujii C and Mukaida N: Attenuated liver tumor formation in the absence of CCR2 with a concomitant reduction in the accumulation of hepatic stellate cells, macrophages and neovascularization. *Int J Cancer* 118: 335-345, 2006.
9. Zhang XW, Qin X, Qin CY, Yin YL, Chen Y and Zhu HL: Expression of monocyte chemoattractant protein-1 and CC chemokine receptor 2 in non-small cell lung cancer and its significance. *Cancer Immunol Immunother* 62: 563-570, 2013.
10. Hiratsuka S, Ishibashi S, Tomita T, Watanabe A, Akashi-Takamura S, Murakami M, Kijima H, Miyake K, Aburatani H and Maru Y: Primary tumours modulate innate immune signalling to create pre-metastatic vascular hyperpermeability foci. *Nat Commun* 4: 1853, 2013.
11. Zhuang H, Cao G, Kou C and Liu T: CCL2/CCR2 axis induces hepatocellular carcinoma invasion and epithelial-mesenchymal transition *in vitro* through activation of the hedgehog pathway. *Oncol Rep* 39: 21-30, 2018.
12. Lim SY, Yuzhalin AE, Gordon-Weeks AN and Muschel RJ: Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* 7: 28697-28710, 2016.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta C_T$  method. *Methods* 25: 402-408, 2001.
14. Dutta R and Mahato RI: Recent advances in hepatocellular carcinoma therapy. *Pharmacol Ther* 173: 106-117, 2017.
15. Wong SHM, Fang CM, Chuah LH, Leong CO and Ngai SC: E-cadherin: Its dysregulation in carcinogenesis and clinical implications. *Crit Rev Oncol Hematol* 121: 11-22, 2018.
16. Vu T and Datta PK: Regulation of EMT in colorectal cancer: A Culprit in metastasis. *Cancers* 9: E171, 2017.
17. Garg M: Epithelial plasticity and cancer stem cells: Major mechanisms of cancer pathogenesis and therapy resistance. *World J Stem Cells* 9: 118-126, 2017.
18. Gaianigo N, Melisi D and Carbone C: EMT and treatment resistance in pancreatic cancer. *Cancers* 9: E122, 2017.
19. Qiu L, Tang Q, Li G and Chen K: Long non-coding RNAs as biomarkers and therapeutic targets recent insights into hepatocellular carcinoma. *Life Sci* 191: 273-282, 2017.
20. Giannelli G, Koudelkova P, Dituri F and Mikulits W: Role of epithelial to mesenchymal transition in hepatocellular carcinoma. *J Hepatol* 65: 798-808, 2016.
21. Hu J, Li X, Guo X, Guo Q, Xiang C, Zhang Z, Xing Y, Xi T and Zheng L: The CCR2 3'UTR functions as a competing endogenous RNA to inhibit breast cancer metastasis. *J Cell Sci* 130: 3399-3413, 2017.
22. Cai J, Wang M, Zhu M, Zhang Q, Zhang X, Yan Y, Qian H and Xu W: N-methyl-N-nitro-N'-nitrosoguanidine induces the expression of CCR2 in human gastric epithelial cells promoting CCL2-mediated migration. *Mol Med Rep* 13: 1083-1090, 2016.
23. Rao Q, Chen Y, Yeh CR, Ding J, Li L, Chang C and Yeh S: Recruited mast cells in the tumor microenvironment enhance bladder cancer metastasis via modulation of ER $\beta$ /CCL2/CCR2 EMT/MMP9 signals. *Oncotarget* 7: 7842-7855, 2016.
24. Moisan F, Francisco EB, Brozovic A, Duran GE, Wang YC, Chaturvedi S, Seetharam S, Snyder LA, Doshi P and Sikic BI: Enhancement of paclitaxel and carboplatin therapies by CCL2 blockade in ovarian cancers. *Mol Oncol* 8: 1231-1239, 2014.
25. Izumi K, Fang LY, Mizokami A, Namiki M, Li L, Lin WJ and Chang C: Targeting the androgen receptor with siRNA promotes prostate cancer metastasis through enhanced macrophage recruitment via CCL2/CCR2-induced STAT3 activation. *EMBO Mol Med* 5: 1383-1401, 2013.