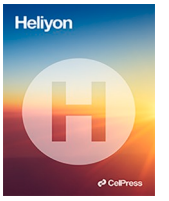
Heliyon 10 (2024) e30782

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

The effect of CXCL12 on survival outcomes of patients with viral hepatitis-associated hepatocellular carcinoma after hepatectomy

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ARTICLE INFO

Keywords:
Hepatocellular carcinoma
Chemokine
CXCL12
CXCR4
CXCR7

ABSTRACT

Background: The CXCL12-CXCR4/CXCR7 axis is garnering growing attention. But the comprehension of its function in the progression of HCC remains controversial. The purpose of this study was to investigate the effects of CXCL12 and its receptor on the prognosis of patients with viral hepatitis-associated HCC after hepatectomy.

Methods: A total of 86 patients had been enrolled who had undergone hepatectomy for HCC and followed up to July 31, 2019, and their clinicopathological and follow-up data were recorded. Tumor and peritumoral tissues were obtained to detect the expression of CXCL12, CXCR4, and CXCR7 using immunohistochemistry. Real-time polymerase chain reaction was utilized to detect hepatitis B or C virus loads, while survival analysis was performed using the Kaplan–Meier method. Furthermore, the Cox proportional hazards regression model was employed to analyze the factors affecting the prognosis.

Results: The results revealed that the CXCL12, CXCR4, and CXCR7 expression in tumor tissues was lower than in the corresponding non-tumor tissues in 20.93 %, 22.09 %, and 23.26 % of the patients, respectively, and that only CXCL12 was found to be related to the extrahepatic invasion of HCC. The survival analysis and Cox regression showed that only CXCL12 was associated with the postoperative survival of patients with HCC, and that it was an independent prognostic risk factor in the CXCL12-CXCR4/CXCR7 axis. The $CXCL12_{low}$ group represented shorter progression-free survival and lower overall survival rates. However, the subgroup analysis displayed that the survival difference associated with CXCL12 was only manifested in patients with higher expression of CXCR4 or CXCR7 in HCC, as compared to the surrounding tissues.

Conclusions: Our findings suggest that, when assessing the prognostic significance of CXCL12 in HCC, it is essential to consider the expression level of its receptor. Nevertheless, CXCL12 can potentially serve as a promising prognostic marker for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, ranking fifth in terms of malignancies and second among

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https://doi.org/10.1016/j.heliyon.2024.e30782
Received 6 October 2023; Received in revised form 5 May 2024; Accepted 6 May 2024
Available online 7 May 2024

males in terms of cancer death [1]. In China, HCC is the second leading malignant tumor in mortalities; 81 % of HCC is caused by liver disease related to the hepatitis B virus (HBV), and approximately 10%–15 % of HCC is caused by the hepatitis C virus (HCV) [2,3]. While the postoperative five-year survival rate of HCC was enhanced between 1970 and the late 1990s, it did not improve for more than ten years after 2000 [2]. The primary cause is that tumor recurrence and metastasis remain challenging to overcome [3]. The most prevalent form of HCC is intrahepatic metastasis. Moreover, patients with high-risk recurrence factors, such as tumor thrombus in portal veins or bile ducts, multiple lesions, and satellite lesions, are at a high risk of experiencing recurrences or metastases within one year after surgery [4,5].

The C-X-C chemokine ligand 12 (CXCL12), a member of the CXC chemokine subfamily, has been attracting growing attention, along with its receptors CXCR4/CXCR7. The active CXCL12-CXCR4/CXCR7 pathway is accepted as being involved in hepatocarcinogenesis. In brief, CXCL12 interacts with its receptors CXCR4 and CXCR7 to form coupling molecular pairs, thereby triggering downstream signaling pathways and playing a crucial role in the differentiation and proliferation [6], angiogenesis [7,8], invasion and metastasis, immune escape [9], and tumor microenvironment of HCC [10]. For many years, CXCR4 was the sole receptor known to bind CXCL12. However, in recent years, researchers have discovered that CXCR7 can also bind CXCL12, and its binding force is even stronger than that of CXCR4 [11–13]. While CXCR7 differs from CXCR4 in its binding mechanism, it relies on G-coupled proteins. CXCR7 internalizes CXCL12 through intracellular pathways induced by β-arrestin, including Akt, MAPK, and JAK/STAT3 pathways [10], leading to the degradation of CXCL12 [14] and modulation of CXCR4's effects [15]. When CXCR4 and CXCR7 are co-expressed, they can form homodimers or heterodimers to regulate CXCR4 downstream signaling [16]. Several studies have also explored the effect of CXCL12-CXCR4/CXCR7 expression on aggressive tumor behavior in HCC tissue, metastasis development, and poor prognosis. However, the outcome of these studies remains controversial, with some findings suggesting that CXCR4, but not CXCR7, influences the prognosis of HCC, while others suggest that decreased CXCL12 expression in HCC is linked to a poor prognosis [17–19]. In addition, it is widely recognized that the liver possesses a high concentration of CXCL12, however, prior research pertaining to the prognostic significance of the CXCL12-CXCR4/CXCR7 axis in HCC patients has seldom discussed the impact of CXCL12-CXCR4/CXCR7 in the surrounding environment of HCC. Hence, in the present study, we analyzed the correlation between CXCL12-CXCR4/CXCR7 expression and the clinical pathological characteristics of HCC, and assessed its prognostic significance in the postoperative survival of HCC patients by determining the relative expression concentration of CXCL12-CXCR4/CXCR7 in HCC compared to the surrounding tissue.

2. Materials and methods

2.1. Patients

A total of 86 paraffin-embedded HCC samples were obtained from March 2008 to July 2014 at the Department of General Surgery of the Shengjing Hospital of China Medical University. Tissues collected more than 2 cm away from the tumor were utilized as paired non-tumor tissues. All diagnoses of HCC are confirmed by pathological examination. The tumor histopathological grade was defined according to the Edmondson grading system. All clinical and pathological records, including age, gender, tumor size, serum α-fetoprotein (AFP) level, serum HBV DNA level, recurrence-related factors (portal vein or bile duct tumor thrombus, multiple satellite lesions, and invasion of surrounding organs and tissues), histologic grade, and TNM stage, were carefully documented. Tumor staging was defined according to the AJCC 8th Edition Cancer Staging Manual. No patient received any preoperative anticancer treatments. The study protocol was approved by the Ethics Review Board of Shengjing Hospital, China Medical University, and waived the need for informed consent from patients. All procedures were carried out in accordance with the Declaration of Helsinki and the pertinent policies in China.

2.2. Immunohistochemistry

The subcellular localization and expression patterns of CXCL12 (1:100 dilution; Santa Cruz Biotechnology), CXCR4 (1:100 dilution; Santa Cruz Biotechnology), and CXCR7 (1:100 dilution; ABGENT, USA) were detected using immunohistochemistry (IHC) methods as follows. The 5-μm-thick sections were first mounted onto glass slides, then dewaxed in xylene. Subsequently, the slides were rehydrated and washed with tris-buffered saline (TBS). The endogenous peroxidase activity was extinguished by incubating in a mixture of 3 % hydrogen peroxide solution for 5 min. After boiling in citrate buffer pH 6.0 for 15 min, the sections were sealed and maintained at room temperature in 10 % non-immune goat serum in TBS (pH 7.5) for 20 min. Subsequently, they were incubated with a primary antibody at 4 °C overnight, followed by a secondary antibody labeled with horseradish peroxidase (Envision™ Detection Kit; Gene Tech, Shanghai, China) at room temperature for 30 min. After washing with TBS, the slides were treated with diaminobenzidine for 5–10 min and counterstained with Mayers hematoxylin.

All the specimens were observed and photographed. Under a high-power view, images of four representative fields were captured using identical image system settings with Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions, Cambridge, UK). Additionally, the integrated optical density (IOD) pixels were measured using NIS-Elements Br3.0. software.

2.3. Hepatitis B virus DNA load quantified by real-time polymerase chain reaction (RT-PCR)

The DNA was extracted from cell lysates and amplified using **RT-PCR**. The amplification was performed in strict accordance with the manufacturer's protocol (Qiagen, Hilden, Germany) using a Rotor-Gene Q cycler at 37 °C for 5 min, 94 °C for 1 min, 95 °C for 5 s,

and then 60 °C for 30 s, for a total of 40 cycles. Values under or over the detection range were recorded.

2.4. Adjuvant therapy and follow-up

Before resuming their diet, all patients were administered prophylactic antibiotic therapy, liver medication, and the necessary fluid replacement. Patients who had already started antiviral treatment preoperatively should continue to receive it after surgery. The diagnosis of recurrence is made by the typical imaging signs of liver lesions, which are acquired through computed tomography (CT), magnetic resonance imaging (MRI), or contrast-enhanced ultrasound, combined with elevated levels of AFP. Patients who experienced recurrence were treated with surgical resection, ablation, transhepatic arterial chemotherapy, and transarterial chemoembolization embolization, as well as targeted therapies, such as sorafenib, lenvatinib treatment, or supportive care. The choice of treatment was determined by the recurrence pattern and the patient's hepatic functional reserves. As of July 31, 2019, we had recorded comprehensive follow-up data for all 86 patients.

2.5. Statistical analysis

All statistical analyses were conducted using SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). Differences between the groups were analyzed using a Student's *t*-test, a paired *t*-test, an X^2 test, or a Wilcoxon test, as appropriate. The overall survival (OS) and progression-free survival (PFS) time after surgery was analyzed using the Kaplan–Meier method, while the estimation of the differences in survival time was carried out using the log-rank test. Prognostic factors were examined using the Cox proportional

Table 1
Relationship between CXCR4 expression and clinicopathologic parameters of HCC patients.

Characteristics	Number of cases	CXCR4 expression		*p* value[b]
		High (n = 67)	Low (n = 19)	
Age (years)	86	54.4 ± 8.41	59.3 ± 8.51	0.027
Gender				1.000
Male	70	55	15	
Female	16	12	4	
Tumor size				0.545
<5 cm	40	30	10	
≥5 cm	46	37	9	
Tumor number				0.676
Single	72	55	17	
≥2	14	12	2	
HBV load				0.107
$<10^3$	33	29	4	
$\geq 10^3$	32	23	9	
HBSAg				0.329
Negative	22	15	7	
Positive	64	52	12	
AFP level				0.138
<200 ng/mL	49	41	8	
≥200 ng/mL	37	26	11	
AJCC stage[a]				0.099
I	54	39	15	
II, III	32	28	4	
Embolus				0.238
No	66	49	17	
Yes	20	18	2	
Satellite lesions				0.262
No	72	54	18	
Yes	14	13	1	
Extrahepatic invasion				0.351[c]
No	83	64	19	
Yes	3	3	0	
Pseudo-capsule(69)				0.054
Yes	48	33	15	
No	21	19	2	
Histologic grade				0.254
Well	27	19	8	
Mid + Poorly	59	48	11	
Portal hypertension				0.738
Yes	18	13	5	
No	68	54	14	

[a] Tumor stage was obtained according to AJCC TNM criteria(8th).
[b] all statistical tests were two sided, significance level: $p < 0.05$.
[c] Mann-Whitney Test.

hazards regression model. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics

Out of the 86 cases of HCC patients, 70 were males and 16 were females, with an average age of 55.5 years (range = 27–76 years). The demographic characteristics of the patients are presented in Table 1. Preoperative examinations using enhanced CTs, enhanced MRIs, or postoperative pathology confirmed early recurrence-related factors in 27 patients, such as portal vein or bile duct tumor thrombus, multiple lesions, and multiple satellite lesion [4,5]. In total, 69 cases of tumor capsule and 18 cases of portal hypertension were documented. Fluorouracil implants (Simcere Pharmaceutical Group) were applied in 37 cases of surface omentum liver wounds. Notably, no recurrences or progressions occurred in 24 cases during the follow-up period.

3.2. Viral DNA load and antiviral therapy in HCC patients

Among the 75 patients with HBV infection, 65 completed the viral load test. There were 32 patients with an HBV DNA level of $>1.0 \times 10^3$ copy/mL and 33 patients with an HBV DNA level of $<1.0 \times 10^3$ copy/mL. All patients with HBV DNA of $>10^3$ were given antiviral therapy before operation. In addition, among the 11 patients with HCV infection, seven completed the viral load test, and for HCV RNA positive patients, antiviral therapy was also administered after surgery.

3.3. Expression of CXCL12, CXCR4, and CXCR7 in HCC tissues and surrounding tissues

The expression levels of CXCL12, CXCR4, and CXCR7 were analyzed using IHC in 86 paraffin-embedded HCC and corresponding non-tumor tissue samples. CXCR4 and CXCR7 were found to be positively expressed in the cytoplasm, while CXCL12 was detected in both the cytoplasm and nucleus. Representative IHC CXCR4, CXCR7 and CXCL12 staining images of cancer and paired non-tumor samples are respectively presented in Fig. 1(A–D), Fig. 2(A - D), and Fig. 3(A - D). The IOD values of CXCR4, CXCR7 and CXCL12 expression in tumor tissues and non-tumor tissues were 46.92 ± 85.71 and 38.85 ± 68.42, 73.67 ± 127.79 and 98.91 ± 379.01, 65.62 ± 106.05 and 39.90 ± 73.01, respectively, with no significant difference observed ($p > 0.05$). Furthermore, for CXCL12, no negative cytoplasmic staining was detected in the adjacent normal tissues. The expression of CXCR4 in tumor tissues was higher than or equal to that in non-tumor tissues in 77.91 % (67/86) of the patients, while it was lower than that in non-tumor tissues in the remaining 22.09 % (19/86) of patients. Similarly, the expression of CXCR7 in tumor tissues was higher than or equal to that in non-tumor tissues in 76.74 % (66/86) of the patients, while it was lower in the remaining 23.26 % (20/86) of patients. The CXCL12 expression in the tumor tissues was higher than or equal to that in the non-tumor tissues in 79.07 % (68/86) of the patients. Conversely, in the remaining 20.93 % (18/86) of patients, the CXCL12 expression in the tumor tissues was lower than that in the non-tumor tissues. In the subsequent study, we used the relative expression of CXCL12, CXCR4, or CXCR7 in HCC to pericancerous non-tumor tissues for grouping statistical analysis.

3.4. Correlations between CXCL12, CXCR4 and CXCR7 expression and clinicopathological characteristics

According to the results of the IHC, patients were classified into the following groups: low CXCR4 ($CXCR4_{low}$), CXCR7 ($CXCR7_{low}$), and ($tumor_{IOD} <$ non-$tumor_{IOD}$); high CXCR4 ($CXCR4_{high}$), CXCR7 ($CXCR7_{high}$), and ($tumor_{IOD} \geqq$ non-$tumor_{IOD}$); low CXCL12 ($CXCL12_{low}$) and ($tumor_{IOD} <$ non-$tumor_{IOD}$); and high CXCL12 ($CXCL12_{high}$) and ($tumor_{IOD} \geqq$ non-$tumor_{IOD}$). The average age of the $CXCR4_{high}$ group was lower than that of the $CXCR4_{low}$ group (Table 1). Based on Tables 1–3, no significant associations were observed between CXCR4, CXCR7, or CXCL12 expression and gender, HBV DNA load, AFP level, tumor size and number, tumor pseudo-capsule, or histologic grade, or portal hypertension. However, as shown in Table 3, the overexpression of CXCL12 in pericancerous tissues is associated with extrahepatic invasion of HCC.

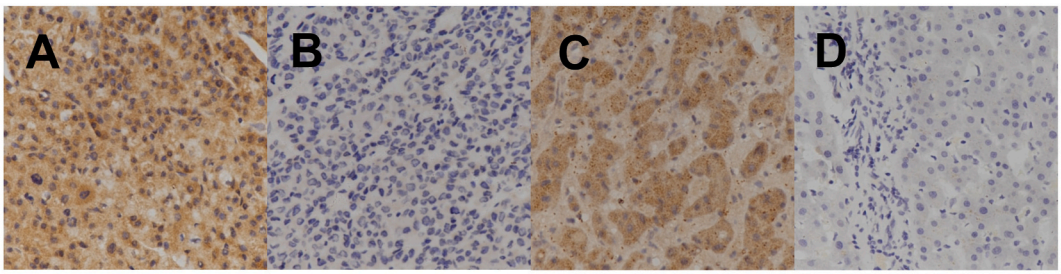

Fig. 1. CXCR4 expression was observed in the cell cytoplasm in HCC and paired non-tumor tissue (brown staining); A) high levels of CXCR4 expression in HCC; B) low levels of CXCR4 expression in HCC; C) high levels of CXCR4 expression in non-tumor tissue; D) low levels of CXCR4 expression in non-tumor tissue (IHC, 400 ×).

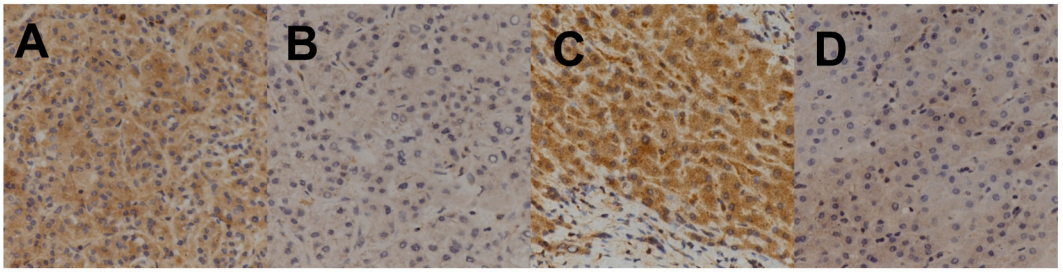

Fig. 2. CXCR7 expression was observed in the cell cytoplasm in HCC and paired non-tumor tissue (brown staining); A) high levels of CXCR7 expression in HCC; B) low levels of CXCR7 expression in HCC; C) high levels of CXCR7 expression in non-tumor tissue; D) low levels of CXCR7 expression in non-tumor tissue (IHC, 400 ×).

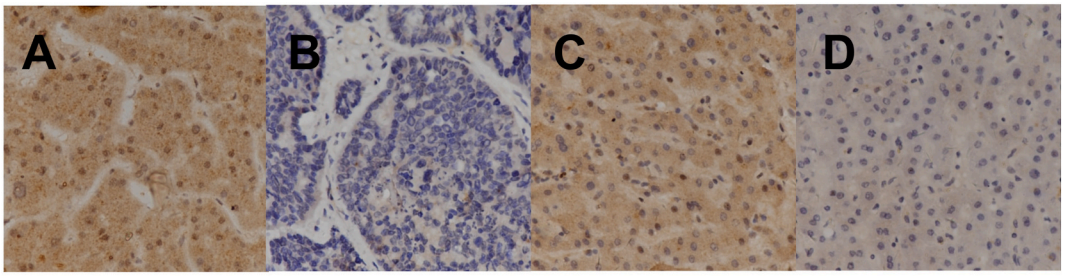

Fig. 3. CXCL12 expression was observed in the cell cytoplasm and nucleus in HCC and paired non-tumor tissue (brown staining); A) high levels of CXCL12 expression in HCC; B) low levels of CXCL12 expression in HCC; C) high levels of CXCL12 expression in non-tumor tissue; D) low levels of CXCL12 expression in non-tumor tissue (IHC, 400 ×).

3.5. Association of CXCL12, CXCR4, and CXCR7 expression with prognosis in patients with HCC

During the follow-up and up to July 31, 2019, the OS time of 86 patients with HCC ranged from 2 to 100 months. The median PFS time was 19.00 ± 2.83 months (95 % confidence interval [CI] 13.45–24.55 months). The survival rates were 82.4 % for one year, 56.5 % for three years, and 45.1 % for five years. No significant difference was observed in PFS and OS between the $CXCR7_{high}$ group and $CXCR7_{low}$ group, as well as between the $CXCR4_{high}$ group and $CXCR4_{low}$ group.

The prognosis of the patients in the $CXCL12_{low}$ group was significantly worse than that of those in the $CXCL12_{high}$ group (Fig. 4A). In the $CXCL12_{low}$ group, the one-year, three-year, and five-year OS rates were 61.1 %, 38.1 %, and 19.0 %, respectively, which were lower than those in the $CXCL12_{high}$ group with OS rates of 88.1 % ($p = 0.005$), 61.2 % ($p = 0.036$), and 51.8 % ($p = 0.006$), respectively. While there was no statistical difference in PFS between the $CXCL12_{high}$ group and the $CXCL12_{low}$ group, the median PFS time of the $CXCL12_{low}$ group was shorter (median PFS 19.0 *vs.* 12.0 m, $p = 0.056$) (Fig. 4D). The subgroup analysis revealed that the survival difference caused by CXCL12 was only manifested in patients with higher CXCR4 expression in HCC contrast with surrounding tissues (three-year OS rate 60.0 % *vs.* 24.2 %, $p = 0.023$; five-year OS rate 50.4 % *vs.* 12.1 %, $p = 0.009$) (Fig. 4B). The difference in patients with lower CXCR4 expression in HCC was less significant (three-year OS rate 66.7 % *vs.* 57.1 %, $p = 0.530$; five-year OS rate 58.3 % *vs.* 28.6 %, $p = 0.174$). Similarly, the influence of CXCL12 on the long-term prognosis of HCC patients was mainly attributed to the patients with high expression of CXCR7 in HCC (five-year OS rate 56.3 % *vs.* 20.8 %, $p = 0.011$) (Fig. 4C); and no significant difference was observed in patients with low CXCR7 expression in HCC (five-year OS rate 33.3 % *vs.* 16.7 %, $p = 0.459$). Simultaneously, it was also observed that PFS of the patients in the $CXCL12_{high}$ group was significantly longer than that in the $CXCL12_{low}$ group, which was only represented in patients with high expression of CXCR7 in HCC (the median PFS 24.0 m *vs.* 5.0 m, $p = 0.015$) (Fig. 4E).

Univariate and multivariate analyses were performed using a Cox proportional hazard model to compare the impact of CXCL12, CXCR4, and CXCR7 expression with other clinicopathological factors on the prognosis of patients with HCC. The univariate Cox regression analysis revealed that TNM stage, venous or bile duct cancer thrombus, satellite nodule, and tumor pseudo-capsule were significantly related to the five-year PFS. Additionally, CXCL12 expression, TNM stage, venous or cholangiocarcinoma thrombus, satellite nodule, extrahepatic tissue invasion, AFP level, and tumor pseudo-capsule were significantly related to the five-year OS (Tables 4 and 5). The multivariate Cox regression analysis indicated that TNM stage and tumor embolus were independent predictors for five-year PFS, and that tumor embolus, extrahepatic tissue invasion, and CXCL12 expression were independent predictors for five-year OS (Tables 4 and 5), which suggested that CXCL12 expression might be useful for predicting the prognosis of HCC patients. The abdominal administration of fluorouracil implants in operations had no effect on PFS and OS.

Table 2
Relationship between CXCR7 expression and clinicopathologic parameters of HCC patients.

Characteristics	Number of cases	CXCR7 expression		p value[b]
		High (n = 66)	Low (n = 20)	
Age (years)	86	54.8 ± 8.01	57.8 ± 10.32	0.178
Gender				0.609
Male	70	55	15	
Female	16	11	5	
Tumor size				0.877
<5 cm	40	31	9	
≥5 cm	46	35	11	
Tumor number				0.866
Single	72	56	16	
≥2	14	10	4	
HBV load				0.699
$<10^3$	33	28	5	
$\geq 10^3$	32	26	6	
HBSAg				0.092
Negative		14	8	
Positive		52	12	
AFP level				0.838
<200 ng/mL	49	38	11	
≥200 ng/mL	37	28	9	
TNM stage[a]				0.768
I	54	42	12	
II, III	32	24	8	
Embolus				0.487
No	66	49	17	
Yes	20	17	3	
Satellite lesions				0.225
No	72	53	19	
Yes	14	13	1	
Extrahepatic invasion				0.676[c]
No	83	64	19	
Yes	3	2	1	
Pseudo-capsule(69)				0.916
Yes	48	36	12	
No	21	16	5	
Histologic grade				0.344
Well	27	19	8	
Mid + Poorly	59	47	12	
Portal hypertension				0.667
Yes	18	15	3	
No	68	51	17	

[a] Tumor stage was obtained according to AJCC TNM criteria(8th).
[b] all statistical tests were two sided, significance level: $p < 0.05$.
[c] Mann-Whitney Test.

4. Discussion

More than 90 % of HCC in China is associated with HCV/HBV infections and cirrhosis. It is widely believed that the up-regulation of CXCL12 and CXCR4 expression in the liver due to HCV and HBV infection and cirrhosis is involved in the development of hepatocarcinogenesis [20–23]. Furthermore, it was discovered that in HCC tissues, the CXCR4 expression was up-regulated and higher than that in liver cirrhosis tissues [19]. Some studies even suggested that CXCR4 was exclusively expressed in HCC tissues, but not in normal liver tissues [17,24]. However, in another study, the expression of CXCR4 was found to be only higher in 47.7 % of the HCC tissues, while 22.1 % of the HCC tissues had lower expression of CXCR4 [25]. In contrast, some studies suggest that not CXCR4 but CXCR7 is closely related to tumorigenesis, which the expression of CXCR4 is not up-regulated but only the transcription and protein expression of CXCR7 is up-regulated in HCC, the mRNA level of which is five–ten times higher than that in normal liver tissue [26,27]. Compared with normal liver tissues, the expression of CXCR7 is significantly up-regulated in HCC and pericancerous fibrosis liver tissues [26]. Elsewhere, the expression of CXCR7 was found to be higher in 46.5 % of the HCC tissues than it was in pericancerous tissues, and only 9.3 % of the HCC had a lower expression of CXCR7 [25].

In the present study, the CXCR4 and CXCR7 expression was generally higher in the HCC tissues than in the non-tumor tissues. Moreover, in 65.12 % of cases, the expression of both CXCR4 and CXCR7 in HCC tissues was not less than that in non-tumor tissues. Only 22.09 % of the cases showed a lower expression of CXCR4 in the HCC tissues compared to the non-tumor tissues, and similarly, only 23.26 % of the cases exhibited a lower expression of CXCR7 in the HCC tissues compared to the non-tumor tissues. This result suggests that both CXCR4 and CXCR7 may be involved in hepatocarcinogenesis, demonstrating that there are also no differences in the protein expression of CXCR4 and CXCR7 in HCC. This is consistent with the fact that both of them had no differences in mRNA levels

Table 3
Relationship between CXCL12 expression and clinicopathologic parameters of HCC patients.

Characteristics	Number of cases	CXCL12 expression		*p* value[b]
		High (n = 68)	Low (n = 18)	
Age (years)	86	55.3 ± 8.70	56.3 ± 8.57	0.656
Gender				1.000
Male	70	55	15	
Female	16	13	3	
Tumor size				0.207
<5 cm	40	34	6	
≥5 cm	46	34	12	
Tumor number				1.000
Single	72	57	15	
≥2	14	11	3	
HBV load				0.061
$<10^3$	33	29	4	
$\geq 10^3$	32	22	10	
HBSAg				1.000
Negative	22	17	5	
Positive	64	51	13	
AFP level				0.227
<200 ng/mL	49	41	8	
≥200 ng/mL	37	27	10	
TNM stage[a]				0.207
I	54	45	9	
II, III	32	23	9	
Embolus				0.844
No	66	53	13	
Yes	20	15	5	
Satellite lesions				0.260
No	72	59	13	
Yes	14	9	5	
Extrahepatic invasion				0.049[c]
No	83	67	16	
Yes	3	1	2	
Pseudo-capsule(69)				0.302
Yes	48	41	7	
No	21	15	6	
Histologic grade				0.710
Well	27	22	5	
Mid + Poorly	59	46	13	
Portal hypertension				0.862
Yes	18	15	3	
No	68	53	15	

[a] Tumor stage was obtained according to AJCC TNM criteria(8th).
[b] all statistical tests were two sided, significance level: $p < 0.05$.
[c] Mann-Whitney Test.

[18]. Different from the upregulation of CXCR4 and CXCR7 expression, the expression of CXCL12 protein or mRNA is decreased in HCC. It has been reported that the expression of the CXCL12 protein is positive in the HCC tissues in 64.2 % of the cases [18,25]. Even, it was reported that the expression of CXCL12 was not detected in HCC, only in metastatic lymph nodes [24]. However, our results indicated that only 20.93 % of the cases had a lower expression of CXCL12 in the HCC tissues than in the surrounding tissues.

The above indicates that the CXCL12-CXCR4/CXCR7 signaling pathway in HCC has certain abnormalities that may be related to the hepatocarcinogenesis. An active CXCL12-CXCR4/CXCR7 pathway is associated with tumor differentiation, invasion, metastasis, and poor prognosis [28]. Several studies reported that CXCR4 expression was significantly related to the histological grade and hepatic cirrhosis, and the overexpression of CXCR4 was considered to be an independent risk factor for the tumor-free survival and OS of patients with HCC [17]. But it was also reported that only nuclear expression of CXCR4 is an independent prognostic factor for the OS of HCC [29]. The high expression of CXCR7 in HCC was associated with microvascular invasion and lung metastasis [30]. Not only is the CXCR7 protein overexpressed in HCC compared to normal liver tissue, but the expression of the CXCR7 protein is also higher in HCC with lung metastasis than in those without metastasis [31]. However, various studies reported that there is no significant correlation between the expression of CXCR4 and CXCR7 and the clinicopathological features of HCC [18], and that only CXCR4 affects the prognosis of HCC, with CXCR7 having no significant effect [25]. The expression of CXCL12 in HCC was decreased, which was related to the tumor grade of the HCC. Moreover, the OS of those with negative nuclear expression of CXCL12 is lower [18]. It is well known that CXCL12 exerts its biological effect by coupling with CXCR4 or CXCR7. The liver is an organ with a high concentration of CXCL12 that is produced by biliary endothelial cells, HSC, and liver sinusoidal endothelial cells, as well as by malignant cells. Moreover, the chronic inflammation caused by HCV and HBV infections induces up-regulation of CXCL12 expression in the surrounding environment of HCC associated with viral hepatitis [32]. Therefore, when analyzing the relationship between

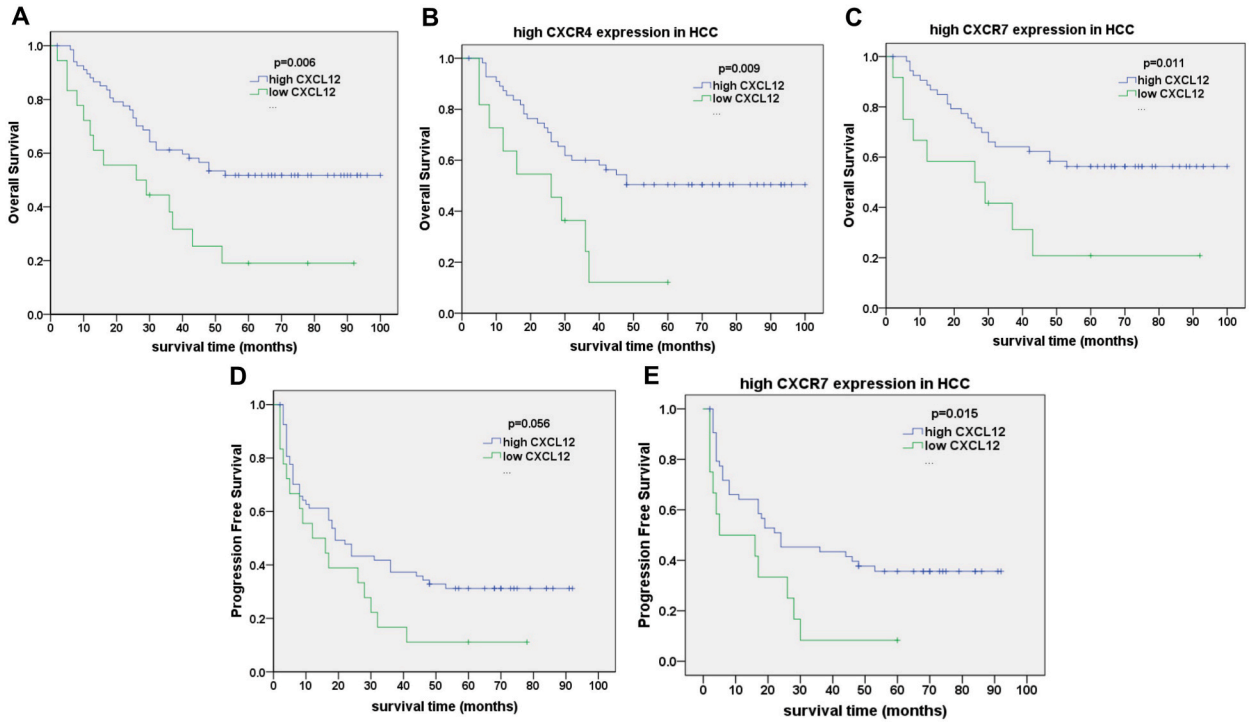

Fig. 4. Only CXCL12 was associated with the postoperative survival of patients with HCC in the CXCL12-CXCR4/CXCR7 axis; A) patient prognosis in the $CXCL12_{low}$ group was significantly worse than in the $CXCL12_{high}$ group, as represented in lower one-year, three-year, and five-year OS rates; B) and C) the survival difference associated with CXCL12 was only manifested in patients with higher CXCR4 or CXCR7 expression in HCC compared with surrounding tissues; D) and E) the patients in the $CXCL12_{low}$ group had a shorter PFS, which was represented in cases with high expression of CXCR7 in HCC (the median PFS = 24.0 vs. 5.0 m).

CXCL12-CXCR4/CXCR7 and clinicopathological features and the prognosis of HCC, we chose the high or low expression of CXCL12-CXCR4/CXCR7 in HCC tissues relative to the surrounding tissues and analyzed the influence of CXCL12 on the prognosis of HCC under different expressions of its receptors. The results indicated that there was no correlation between CXCR4 or CXCR7 expression and the clinicopathological characteristics of HCC, including the size and number of tumors, stage, differentiation, envelopes, satellite foci, vascular tumor embolus, extrahepatic invasion, viral load, AFP level, and portal hypertension. Overall, CXCL12 is associated with extrahepatic invasion of HCC but not with other clinicopathological features.

The survival analysis revealed that there was no correlation between CXCR4 or CXCR7 and the PFS and OS of HCC. However, the OS of the $CXCL12_{low}$ group was significantly lower than that of the $CXCL12_{high}$ group. Additionally, the median PFS of the $CXCL12_{low}$ group tended to be lower than that of the $CXCL12_{high}$ group (12.0 vs. 19.0 m, $p = 0.056$). The subgroup analysis demonstrated that the OS of the $CXCL12_{low}$ group was significantly lower than that of the $CXCL12_{high}$ group when the CXCR4 or CXCR7 expression in the HCC was higher than in the corresponding surrounding tissues. There was no significant difference when the CXCR4 or CXCR7 expression in the HCC was lower than in the corresponding surrounding tissues, which suggested that the effect of the CXCL12 on the prognosis of HCC is affected by its receptor status. In the univariate analysis of the prognostic risk factors, the tumor stage, a vascular tumor embus, a tumor without pseudo-capsule, and a tumor with multiple satellite foci were found to be the risk factors influencing HCC PFS; for CXCL12 and tumor stage, a vascular tumor thrombus, a tumor without pseudo-capsule, a tumor with multiple satellite foci, an extrahepatic invasion, and the AFP level were the risk factors influencing the HCC OS. The multivariate analysis indicated that relative to CXCL12, tumor stage and vascular tumor thrombus were independent risk factors for PFS of HCC, and vascular tumor thrombus and extrahepatic invasion were independent risk factors for the OS of HCC. These results suggest that CXCL12 may play the most important role when evaluating the prognostic value of the CXCL12-CXCR4/CXCR7 axis in HCC and the effect of CXCL12 may be more pronounced for HCC with overexpression of CXCR4 or CXCR7.

While our results are not completely consistent with those obtained in previous studies, there was some agreement in that whether the increased expression of the receptor CXCR4/CXCR7 or a decreased expression of ligand CXCL12 can be all negative factors in the prognosis of HCC. When the expression of either the CXCR4 or the CXCR7 receptor is up-regulated in HCC tissues, or the ligand CXCL12 is highly expressed in the surrounding tissues (CXCL12 is relatively lower in HCC), CXCL12 in the surrounding environment can induce the directional migration of HCC cells [24,27]. The higher the concentration of CXCL12 in the surrounding environment, the more obvious the migration effect of hepatoma cells [24]. In short, CXCL12 may recruit CXCR4-positive cancer cells to migrate into the vasculature and infiltrate the peritumoral capsule. This tendency of migration results in an accumulation of CXCR4-positive cancer cells near the tumor boundary [33]. Furthermore, CXCL12/CXCR7 is also involved in tumor metastasis and invasion process,

Table 4
Univariate and multivariate analysis of clinicopathological factors for PFS in HCC.

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p^a value	HR (95%CI)	p^a value
Tumor number ≥2/Single	1.769(0.964–3.244)	0.065		
Tumor size ≥5cm/<5 cm	1.227(0.744–2.026)	0.423		
Stage AJCCII+III/I	2.549(1.523–4.264)	0.000	2.265(1.045–4.910)	0.038
Embolus Present/Absent	2.471(1.412–4.324)	0.002	2.373(0.982–5.735)	0.055
Satellite lesions Yes/No	2.497(1.358–4.591)	0.003		
Extrahepatic invasion Yes/No	3.014(0.923–9.846)	0.068		
Grade Mid + poorly/well	1.422(0.821–2.465)	0.209		
Pseudo-capsule No (21), yes (48)	2.174(1.207–3.915)	0.010		
AFP(ng/ml) ≥200/<200	1.382(0.836–2.284)	0.207		
HBV load ≥E3/<E3(32/33)	0.872(0.483–1.573)	0.649		
Portal hypertension Yes/no	1.031(0.559–1.901)	0.922		
Fluorouracil implant No/yes	0.860(0.520–1.421)	0.556		
CXCR4 Low/High	1.473(0.783–2.772)	0.229		
CXCR7 Low/high	0.777(0.444–1.362)	0.379		
CXCL12 Low/high	1.712(0.966–3.036)	0.066		

HR: hazard ratio; CI: confidence interval.
[a] all statistical tests were two sided, significance level: $p < 0.05$.

participating in the epithelial mesenchymal transition of tumor cells and enhancing the adhesion between tumor cells and vascular endothelial cells [28]. In addition, in the absence of a downstream signal, the internalization of CXCL12-bound CXCR7 will generate a gradient of chemokine required for the optimal CXCR4 migration response [34]. The coupling of CXCL12 and CXCR4 or CXCR7 can promote the growth and proliferation of tumor cells and tumor angiogenesis, and result in tumor invasion and progression by activating PI3K, the ERK1/2 pathway, the MAPK-ERK1/2 pathway, the JNK/SAPK pathway, the Akt pathway, or the MAPK/VEGF/galectin-3 pathway [7,35–40], and can also promote the directional metastasis of tumor cells by the degradation of the tumor extracellular matrix and the destruction of tissue barriers through up-regulating the expression of the matrix metalloproteinases, MMP-2 and MMP-9, and reducing the secretion of the tissue inhibitors of matrix metalloproteinases [38,40,41]. Therefore, due to its importance in the HCC progression, the CXCL12-CXCR4/CXCR7 axis has become a target for exploring the treatment of HCC.

In conclusion, although more patients need to be evaluated, we have identified a prognostic role for the CXCL12-CXCR4/CXCR7 axis in HCC patients after hepatectomy. This suggests that CXCL12 is a valuable prognostic factor for HCC patients with high expression of CXCR4 or CXCR7 in tumor tissues. Furthermore, this finding may also aid in identifying novel therapeutic targets for HCC. However, since this study is only a retrospective one, and the conclusions may be affected by the sample size， therefore, more prospective research is needed regarding the impact of the CXCL12-CXCR4/CXCR7 axis on HCC.

Ethical approval

The study was approved by the Ethics Review Board of Shengjing Hospital, China Medical University (No.2013PS125K).

Funding

This work was supported by the National Natural Science Foundation of China (No. 81701570) and the Shenyang Science and Technology Project (No. F13-221-9-24).

Data availability

All original data analyzed during this study are available from the corresponding author on reasonable request.

Table 5
Univariate and multivariate analysis of clinicopathological factors for OS in HCC.

Tumor number	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p^a value	HR (95%CI)	p^a value
Tumor number ≥2/Single	1.293(0.623–2.684)	0.490		
Tumor size ≥5cm/<5 cm	1.561(0.863–2.825)	0.141		
Stage AJCCII+III/I	2.141(1.196–3.833)	0.010		
Embolus Present/Absent	2.604(1.411–4.804)	0.002	4.046(1.778–9.207)	0.001
Satellite lesions Yes/no	2.340(1.181–4.636)	0.015		
Extrahepatic invasion Yes/no	3.533(1.082–11.537)	0.037	6.479(1.109–37.847)	0.038
Grade Mid + poorly/well	1.744(0.885–3.437)	0.108		
Pseudo-capsule No (21), yes (48)	2.543(1.330–4.866)	0.005		
AFP(ng/ml) ≥200/<200	1.879(1.050–3.360)	0.034		
HBV load ≥E3/<E3(32/33)	1.294(0.639–2.620)	0.473		
Portal hypertension Yes/no	1.417(0.661–3.038)	0.371		
Fluorouracil implant No/yes	0.915(0.508–1.647	0.766		
CXCR4 Low/high	1.150(0.570–2.319)	0.697		
CXCR7 Low/high	0.631(0.336–1.185)	0.152		
CXCL12 Low/high	2.366(1.258–4.450)	0.008	2.991(1.361–6.573)	0.006

HR: hazard ratio; CI: confidence interval.
[a] all statistical tests were two sided; significance level: $p < 0.05$.

CRediT authorship contribution statement

Yan Lu: Writing – original draft, Investigation, Data curation. **Fei Xing:** Methodology, Funding acquisition, Formal analysis, Conceptualization. **Songlin Peng:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Songlin peng reports financial support was provided by the Shenyang Science and Technology Project Foundation. Fei xing reports financial support was provided by the National Natural Science Foundation of China.

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