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Original Article

The role of CXCL chemokine family in the development and progression of gastric cancer

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Abstract: The chemokine (C-X-C motif) ligand (CXCL) family plays an important role in inflammation. In order to understand the role of CXC chemokine family in carcinogenesis, this study explored a group of early gastric cancer (GC) patients, and assessed the level of CXC chemokine ligand (CXCL) in blood samples of patients representing systemic circulation and tumor microenvironment, detected the expression of CXC chemokine receptor (CXCR) in tumor tissues, and measured tumor infiltrating immune cell subsets. 69 patients with GC were included in a single center prospective study and were followed up for 6 years. The level of CXCL1-14 was determined by ELISA and the concentration gradient of chemokine was calculated. Western blot was used to detect the expression of CXCR1, CXCR2, CXCR3, and CXCR4 in tumor tissue. CXCL1-14 expression was inhibited by siRNA in HGC27 cells and then the migration ability of HGC27 cells was detected by cell scratch test. The results of this study showed that the chemokine concentrations of CXCL1, CXCL2, CXCL5, CXCL8, CXCL11, and CXCL13 in peripheral blood and tumor drainage blood of patients without recurrence after treatment were significantly lower than those before treatment. The concentrations of CXCL1, CXCL2, CXCL4, CXCL5, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, CXCL13, and CXCL14 in peripheral blood and tumor drainage blood were significantly higher than those in patients without recurrence. Patients with low expression of CXCR1 and CXCR3 had lower AFP (alpha fetoprotein), smaller tumor volume, and lower TNM tumor stage. Patients with lower expression of CXCR2 and CXCR4 had higher AFP (alpha fetoprotein) level, larger tumor volume, and higher TNM tumor stage. After down-regulation of CXCLs expression, the migration ability of most cell lines was significantly inhibited. This study suggests that CXCL chemokine family plays an important role in the pathogenesis of GC and can be used as a marker for the development of GC.

Keywords: CXCL, CXCR, gastric cancer, chemokine, prognosis

Introduction

Gastric cancer (GC) is the fourth most common malignant tumor in the world and ranks the third in the mortality rate of male and female cancer. The 5-year survival rate of GC is usually lower than 30% [1]. Although the global incidence of GC has declined dramatically in the past decades, most patients with GC are still in the middle and late stages of the diagnosis. In recent years, cancer treatment strategies tend to the research of tumor molecular targeted therapy [2, 3].

The growth, proliferation, and metastasis of tumor cells is a continuous process, which needs a continuous energy supply. The formation of blood vessels provides a material basis for this process. Understanding the occurrence of cancer requires a holistic approach, including the analysis of epidemiological and clinical data, molecular detection of blood biomarkers, and thorough analysis of tumor cells and immune cell infiltration [4, 5]. The data obtained in the past decades confirmed that cancer, as a disease, has heterogeneity; which means that there is no single perfect biomarker for GC, but a group of multi parameters may be a more objective diagnostic and prognostic tool [6]. Solid tumors like GC contain not only tumor cells, but also various types of stromal cells, such as fibroblasts and endothelial cells [7]. In addition, the tumor is infiltrated by inflammatory cells, including neutrophils, macrophages, and lymphocytes. Tumor cells, stromal cells, and tumor related leukocytes contribute

to the local production of chemokines in tumor and affect the level of systemic circulating chemokines [8]. Chemokines are a kind of cytokines that control cell migration. The abnormal expression and function of chemokines and their receptors will lead to immune cells unable to perform the right function in the right position [9, 10].

In our study, we focused on a group of CXC chemokines, which are members of the cytokine family. Previous studies have shown that CXC chemokines family is involved in tumor growth regulation and metastasis [11, 12]. Our team's previous studies confirmed the diagnostic and prognostic value of CXC chemokines as biomarkers [13]. The in-depth exploration of CXC chemokine family can provide valuable scientific basis for clinical and transformation research of GC patients undergoing surgical treatment and provide opportunities for tumor microenvironment research. In order to better understand the role of CXC chemokines in tumorigenesis, this study detected the CXC chemokine ligand (CXCL) levels in blood samples of patients who represent the systemic and tumor microenvironment to determine whether chemokines are involved in the carcinogenesis process. To ensure the overall treatment, our additional goal is to determine the expression intensity of CXC chemokine receptor (CXCR) in tumor tissue and to evaluate the components of tumor immune cell (TIC) infiltration.

Materials and methods

Experimental object

From January 2012 to December 2013, 69 patients with GC underwent radical resection and a single center prospective study with clinical, radiological, and pathological stages of adenocarcinoma (n=48) or squamous cell carcinoma (n=21). There was no new adjuvant and/or adjuvant therapy in all patients. The patients were followed up for 6 years. During the follow-up period, the general condition of the patient and information about the outcome and recurrence of the disease were obtained. All patients signed the informed consent for the experimental content.

Program

During the operation, the peripheral blood samples were taken from the elbow vein and put into a 5 ml vacuum tube. The tubes were centrifuged at 1500 g for 10 minutes at room temperature (RT). After centrifugation, the plasma was immediately stored at -80°C for use. Before the ELISA test, the samples were thawed at RT and treated in accordance with the instructions provided by the ELISA kit manufacturer. The level of CXCL1-14 (Abnova, USA) was determined by ELISA Kit with sensitivity of 1 pg/ml and detection range of 1-15000 pg/ml. This was repeated 3 times for each sample. The concentration of CXCLs in plasma was calculated according to the standard curve. The mean value of CXCLs level was stated with 95% confidence interval.

The difference of CXCLs concentration between systemic vascular bed and tumor vascular bed circulation can be described by CXCLs concentration gradient. The gradient of CXCLs was calculated as: [(TCV-PCV)/PCV] × 100, in which TCV was the representative value of tumor circulation and PCV was the representative value of peripheral circulation.

Quantitative real-time PCR

Total RNA was extracted and isolated from tissue samples using Trizol reagent (Invitrogen, USA) following the user manual. SYBR PremixExTaq™ (Takara, Japan) was used in Quantitative real-time PCR (qRT-PCR) on BIO-RAD MY IQ (USA). All primer sequences are shown in **Table 1**. The relative expression of CXCLs was shown as fold difference relative to GAPDH.

Western blot assays

In order to study the relationship between CXC chemokine receptor (CXCR), immune cells, and GC, western blot was used to detect the expression of CXCRs. The total protein was extracted by RIPA method and the samples were electrophoretic by 10% SDS-PAGE. The amount of sample on each pore was 20 μL. After the membrane was transferred by semi dry electric transfer instrument, 5% skimmed milk powder was sealed for 1 hour, then the diluted Rabbit anti CXCR1 (1:1000), CXCR2 (1:1000), CXCR3 (1:5000), and CXCR4 (1:1000) antibody was added. After incubation at night, 4°C, 0.1% TBST washed the membrane three times, 5 min/time, added 1:10000 diluted Goat anti rabbit IgG, incubated in the greenhouse for 30 min, washed the membrane, added the chemiluminescent solution, devel-

Table 1. Primer sequences of products expression

Gene name	Primer name	Primer sequence
CXCL1	forward primer	5' CGCTACAGCGACGTGAAGAA 3'
	reverse primer	5' GTTCCAGGCGTTGTACCAC 3'
CXCL2	forward primer	5' GCTTGTC TCAACCCCGCATC 3'
	reverse primer	5' TGGATTTGCCATTTTTCAGCATCTT 3'
CXCL3	forward primer	5' GCAGGGAATTCACCTCAAGA 3'
	reverse primer	5' TTTTCGATGATTTTCTGAACCA 3'
CXCL4	forward primer	5' TGAAGAATGGAAGGAAAATTTGC 3'
	reverse primer	5' CAAATGCACACACGTAGGCAGCT 3'
CXCL5	forward primer	5' AGCTGCGTTGCGTTTGTTTAC 3'
	reverse primer	5' TGGCGAACACTTGCAGATTAC 3'
CXCL6	forward primer	5' AGAGCTGCGTTGCACTTGTT 3'
	reverse primer	5' GCAGTTTACCAATCGTTTTGGGG 3'
CXCL7	forward primer	5' CTGGCTTCCTCCACCAAAGG 3'
	reverse primer	5' GACTTGGTTGCAATGGGTTCC 3'
CXCL8	forward primer	5' CTTTGTCCATTCCCACTTCTGA 3'
	reverse primer	5' TCCCTAACGGTTGCCTTTGTAT 3'
CXCL9	forward primer	5' CCAGTAGTGAGAAAGGGTCGC 3'
	reverse primer	5' AGGGCTTGGGGCAAATTGTT 3'
CXCL10	forward primer	5' GTGGCATTCAAGGAGTACCTC 3'
	reverse primer	5' TGATGGCCTTCGATTCTGGATT 3'
CXCL11	forward primer	5' GACGCTGTCTTTGCATAGGC 3'
	reverse primer	5' GGATTTAGGCATCGTTGTCCTTT 3'
CXCL12	forward primer	5' ATTCTCAACACTCCAAACTGTGC 3'
	reverse primer	5' ACTTTAGCTTCGGGTCAATGC 3'
CXCL13	forward primer	5' GCTTGAGGTGTAGATGTGTCC 3'
	reverse primer	5' CCCACGGGGCAAGATTTGAA 3'
CXCL14	forward primer	5' CGCTACAGCGACGTGAAGAA 3'
	reverse primer	5' GTTCCAGGCGTTGTACCAC 3'
GAPDH	forward primer	5' ATGTCGTGGAGTCTACTGGC 3'
	reverse primer	5' TGACCTTGCCCACAGCCTTG 3'

oped, and analyzed the strip light density with ImageJ software.

Construction of CXCLs low expression cell line

CXCL1 (Gen bank gene ID: 2919), CXCL2 (Gen bank gene ID: 2920), CXCL3 (Gen bank gene ID: 2921), CXCL4 (Gen bank gene ID: 5196), CXCL5 (Gen bank gene ID: 6374), CXCL6 (Gen bank gene ID: 6372), CXCL7 (Gen bank gene ID: 5473), CXCL8 (Gen bank gene ID: 3576), CXCL9 (Gen bank gene ID: 4283), CXCL10 (GenBank gene ID: 3627), CXCL11 (Gen bank gene ID: 6373), CXCL12 (Gen bank gene ID: 6387), CXCL13 (Gen bank gene ID: 10563), CXCL14 (Gen bank gene ID: 9547) gene sequences were designed by Invitrogen online software. The designed siRNA was synthesized by Ribobio. The siRNA was transfected into HGC27 cells by Lipofectamine 2000 (Invitrogen). After 6 hours of cell culture, the transfection effect was verified by western blot.

Cell scratch test

The cells were verified to obtain CXCLs low expression cell lines. After the cells were cultured for 12 hours, a 2 mm wide scratch was scratched along the middle line of the culture plate with a 1 ml pipette head. The culture plate was washed with PBS buffer solution and no cells were found in the scratches through observation under the microscope. After adding the culture medium, the culture continued for 24 hours. Suck the culture supernatant, add 1 ml of 4% paraformaldehyde into each hole, and take photos under the optical microscope 30 minutes later.

Statistical analysis

Data are presented as mean ± SEM. A Pearson chi-squared test was applied to determine clinicopathological correlations. The Kaplan-Meier method was used to calculate the survival curves. Significance was determined by the log-rank test. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the Student's t-test using GraphPad software version 5.0 (GraphPad Software, CA). Differences were considered significant when $P<0.05$.

Results

The relationship between CXCLs concentration and prognosis of GC patients

The final data collection in December 2018 showed that out of 69 GC patients who received surgical treatment, a total of 31 patients recurred. For further analysis, we divided the patients into two groups according to the follow-up data, taking the recurrence of cancer as a reference. We compared CXCL concentrations in paired blood samples representing sys-

Table 2. Concentration of CXCLs in peripheral and tumor draining blood samples (pg/mL) ± SD

CXCLs	Before treatment (n=69)		After treatment (n=69)			
			No recurrence (n=38)		Relapse (n=31)	
	Peripheral	Tumor draining blood	Peripheral	Tumor draining blood	Peripheral	Tumor draining blood
CXCL1	156.23±47.36	137.46±32.57	36.78±9.87*	26.22±11.45*	239.13±103.27#	198.47±34.21#
CXCL2	246.89±35.12	309.82±49.44	50.17±12.89*	66.17±25.13*	451.213±89.24#	486.53±157.16#
CXCL3	486.79±108.55	389.21±67.15	419.21±75.11	513.16±65.24	637.99±79.46	789.31±109.44
CXCL4	2672.28±389.77	2107.26±416.34	2145.35±479.39	1954.17±581.1	3657.71±897.33#	4016.64±1023.38#
CXCL5	568.79±108.11	541.26±200.37	357.65±79.15*	311.37±127.68*	986.32±177.64#	1089.21±208.42#
CXCL6	301.35±63.17	289.47±78.21	219.68±111.29	256.17±101.35	416.37±109.57	468.18±148.39
CXCL7	1255.27±227.34	1536.15±242.45	1419.88±239.65	1549.58±309.17	1002.41±218.29#	889.53±108.57#
CXCL8	58.24±9.78	76.29±7.88	21.89±11.13*	29.15±7.43*	164.45±17.66#	186.64±29.69#
CXCL9	754.14±109.85	656.67±98.33	538.22±125.75	678.26±218.31	1176.11±210.45#	1258.17±117.46#
CXCL10	86.46±19.89	112.29±23.73	56.54±11.24	64.76±11.68	189.43±35.51#	214.61±45.28#
CXCL11	35.26±5.79	46.28±11.37	86.18±27.78*	96.27±33.18*	67.56±8.91	72.79±11.61
CXCL12	26.21±6.76	31.27±10.07	56.57±27.63	76.62±18.97	15.11±4.38#	19.41±8.19#
CXCL13	489.11±87.43	556.17±109.46	201.77±49.78*	196.39±57.66*	866.15±78.93#	796.84±174.51#
CXCL14	87.27±20.31	96.64±19.39	138.23±27.76	186.78±50.33	36.16±7.95#	29.69±9.61#

Compared with before treatment, *P < 0.05, Compared with no recurrence, #P < 0.05.

temic circulation and tumor bed circulation, as shown in **Table 2**. Compared with before treatment, the concentrations of CXCL1, CXCL2, CXCL5, CXCL8, CXCL11, and CXCL13 in peripheral blood and tumor drainage blood of patients without recurrence after treatment were significantly lower. The concentrations of CXCL1, CXCL2, CXCL4, CXCL5, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, CXCL13, and CXCL14 in peripheral blood and tumor drainage blood were significantly higher than those in patients without recurrence.

The relationship between the expression of CXCRs and the prognosis of GC

Using the method of retrospective cohort study, qRT-PCR was used to detect the expression level of CXCRs in 69 pairs of GC tissues and matched adjacent tissues. Compared with the adjacent tissues, the expression level of CXCRs was significantly different in GC tissues. According to the middle value of CXCRs expression level, the studied population was divided into two parts: CXCRs high expression and CXCRs low expression. As shown in **Table 3**, there is no significant correlation between CXCRs expression and age and gender in GC patients. Patients with low expression of CXCR1 and CXCR3 had lower AFP (alpha fetoprotein), smaller tumor volume, and lower TNM tumor stage. Patients with lower expression of CXCR2 and CXCR4 had higher AFP (alpha fetoprotein) level, larger tumor volume, and higher TNM tumor stage.

Western blot results showed that before treatment, the expression of CXCR1 and CXCR3 was significantly up-regulated, while that of CXCR2 and CXCR4 was significantly down regulated in GC tissue samples compared with the adjacent tissues (**Figure 1**). After treatment, the patient was divided into two parts. In the relapse free group, no tumor sample was taken, so the expression of CXCRs in tumor tissue could not be detected. However, in the tumor tissues of the recurrent group, the expression of CXCR1 and CXCR3 was significantly up-regulated, while that of CXCR2 and CXCR4 was significantly down regulated. This trend is more obvious than before treatment. This suggests that cancer cells may develop immune tolerance.

The relationship between CXCRs expression and tumor invasion

The expression intensity of CXCR1 was related to the level of CXCL6 and CXCL13, the number of macrophage infiltrated tumor and tumor infiltrated immune cells. The expression intensity of CXCR2 was related to the level of CXCL1, CXCL2, CXCL5, and CXCL8 in the whole body and the infiltration of tumor plasma cells. The expression intensity of CXCR3 was correlated with the tumor infiltration level of CXCL4, CXCL9, CXCL10, CXCL11, B cells, T helper cells,

Table 3. Correlation of the expression of CXCRs with clinicopathologic features

Clinicopathologic features	n (69)	CXCR1 expression		*P*	CXCR2 expression		*P*	CXCR3 expression		*P*	CXCR4 expression		*P*
		Low	High		Low	High		Low	High		Low	High	
Age (year)													
<50	28	15	13	0.669	11	18	0.835	12	16	0.581	13	16	0.617
≥50	41	19	22		23	18		21	20		18	23	
Sex													
Male	37	20	17	0.812	16	21	0.396	15	22	0.327	22	15	0.723
Female	32	14	18		21	11		19	13		16	16	
Serum AFP level(ng/mL)													
<20	21	15	6	0.015*	3	18	0.026*	17	10	0.037*	13	7	0.029*
≥20	48	9	41		30	18		11	37		26	22	
Tumor size (cm)													
<5	31	23	8	0.023*	12	19	0.052	15	16	0.039*	13	18	0.041*
≥5	38	11	27		21	17		9	28		29	9	
TNM tumor stage													
I+II	45	31	14	0.008*	9	36	0.026*	24	21	0.043*	13	32	0.035*
III+IV	24	3	21		19	5		6	18		20	4	

The asterisk * indicate P<0.05.

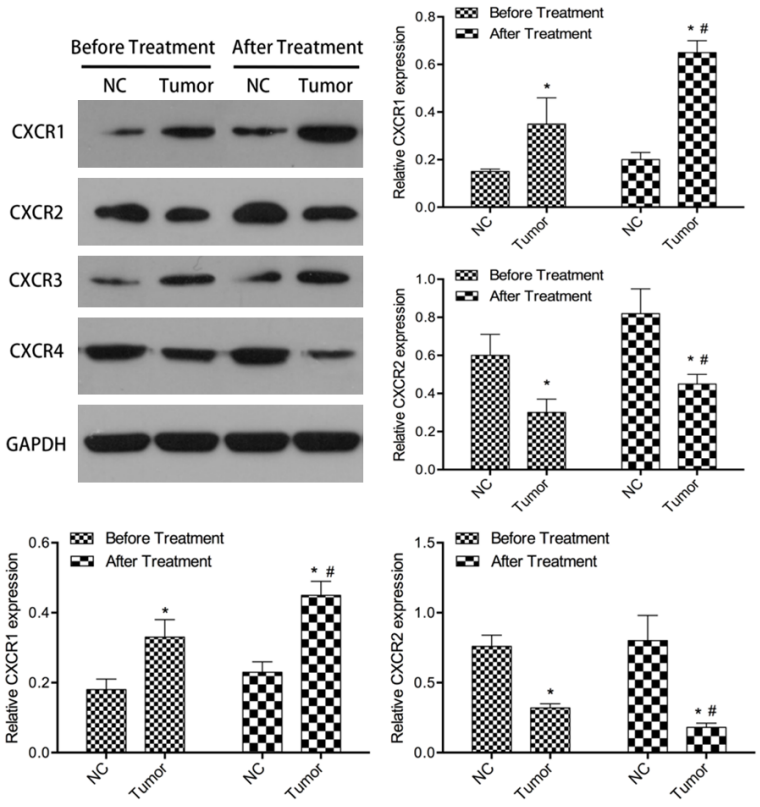

Figure 1. Relative CXCRs expression levels in GC tissues. CXCRs were detected in 69 pairs of GC tissues with western blot. NC, normal control tissues, Tumor, GC tissues. Compared with NC *P<0.05. Compared with before treatment #P<0.05.

and T cytotoxic cells. The expression intensity of CXCR4 was related to the level of CXCL14 in the whole body and the infiltration of tumor plasma cells.

CXCL1 gradient is related to the absolute number of B cells; CXCL2 gradient is related to the absolute number of B cells; CXCL3 gradient is related to the absolute number of macrophages and the total number of tumor infiltrating immune cells; CXCL4 gradient is related to the absolute number of macrophages and the total number of tumor infiltrating immune cells; CXCL5 gradient is related to the absolute number of T-helper cells, the percentage of T-helper cells, and the infiltration of t-toxic cells. CXCL6 gradients were correlated with the absolute number of B cells, CXCL7 gradients with the absolute number of T helper cells, CXCL8 gradients with the absolute number of T cytotoxic cells and the total number of tumor infiltrating cells. There was no significant correlation between CXCL9 gradient and immune cell subsets. There was no significant correlation between CXCL10 gradient and immune cell subsets. CXCL11 gradients were associated with the absolute number of T-cell toxic cells and the total number of tumor infiltrating cells. There was no significant

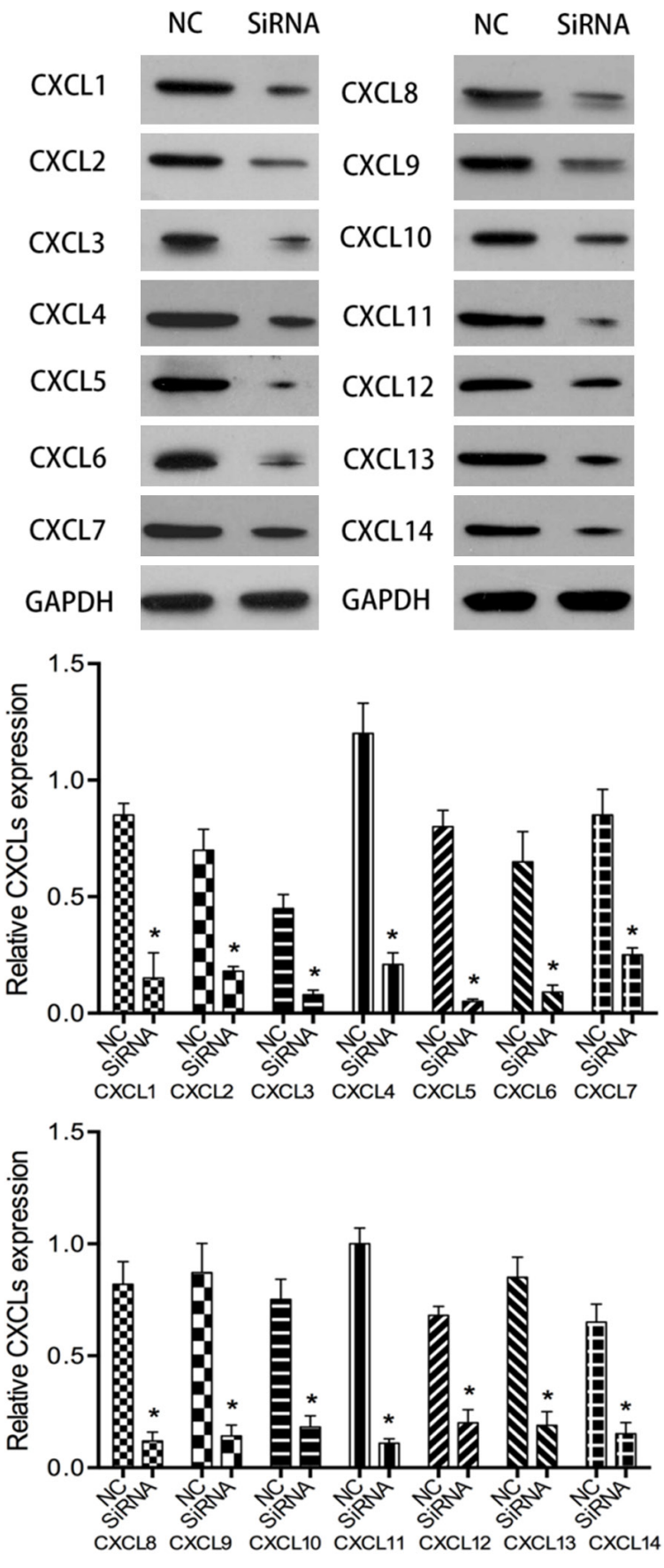

Figure 2. Western blot was used to verify the transfection effect of CXCLs siRNA. NC, normal control HGC27 cells. siRNA, HGC27 cells transfected with CXCLs siRNA. Compared with NC *P<0.05.

correlation between CXCL12 gradient and T helper cell subsets. CXCL14 gradients were correlated with the absolute number of macrophages and the total number of tumor infiltrating immune cells.

Detection of cell migration in vitro by scratch test

After 6h of transfection, western blot was used to verify the transfection effect. The experimental results showed that the CXCLs low expression cell line was successfully obtained (**Figure 2**). After 24 hours of cell culture, cell scratch test was used to detect the migration ability of HGC27 inhibited by CXCLs siRNA. The experimental results showed that after the down-regulation of CXCLs expression, the migration ability of most cell lines was significantly inhibited, but the migration ability of some cell lines did not change in detail (**Figure 3**).

Discussion

It is very important for the clinical study of cancer to explore the therapeutic target of cancer. GC is a kind of malignant tumor with high mortality and poor prognosis. Although the clinical prevention and treatment of GC has achieved important results, the early diagnosis of GC is still a big challenge [14-16]. The occurrence of GC is a very complex process. Irregular work and rest, eating habits, and unhealthy food are important external factors to induce GC.

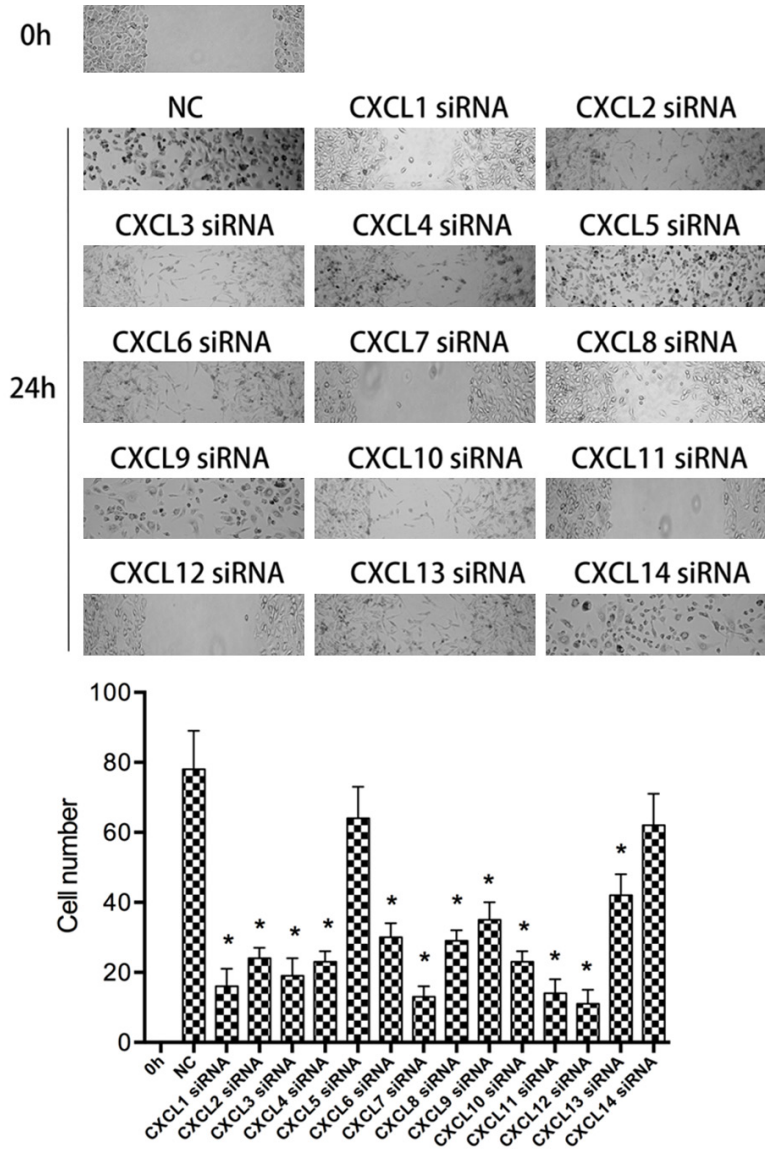

Figure 3. Scratch test was used to detect the migration ability of CXCLs siRNA to HGC27 cells. NC, normal control HGC27 cells. CXCL1-14 siRNA, HGC27 cells transfected with CXCLs siRNA. Compared with NC *P<0.05.

The disorder of human internal environment and the imbalance of cell self-monitoring mechanism are important internal factors to induce GC [17, 18]. Therefore, it is very important to develop a new biomarker for early diagnosis of GC. Chemokine family plays an important role in the occurrence and development of a variety of tumors. The basic function of chemokines is the directional chemotaxis of cells expressing corresponding chemokine receptors [19-21].

Chemokines and their receptor families are important mediators for leukocyte migration to inflammatory sites. Long term inflammation may provide an ideal microenvironment for tumor cell development and growth [22]. Chemokine family can stimulate or inhibit the development of tumor by attracting tumor promoting and anti-tumor cells. Chemokines affect tumor transformation, survival, growth, invasion, and metastasis by regulating angiogenesis and tumor leukocyte interaction [23, 24]. The function of chemokines is very complex. Some chemokines may be beneficial to the growth and development of tumor, while others may enhance the anti-tumor immunity [25, 26].

In this study, we identified the level of differentially expressed proteins in a complex proteomic context. The concentration of CXCL in tumor tissue is a dynamic parameter. On the one hand, the circulating CXCL produced by other tissues may combine with the CXCR expressed by tumor or tumor infiltrating immune cells, so as to reduce the concentration of CXCL in tumor tissue blood drainage. On the other hand, CXCL can be produced by tumor or tumor infiltrating immune cells and then released to tumor tissue blood drainage in addition, the concentration of CXCL in tumor tissue was increased [27]. This phenomenon can be explained by the classical hypothesis of immune editing, that is, by recognizing tumor specific antigen, the immune system can not only clear tumor cells and protect the host, but also edit tumor genome to shape tumor development and generate tumor variants with reduced immunogenicity. In this process, immune cells and CXCL are in a state of dynamic balance [28]. In our study, we found that most chemokines (CXCL1, CXCL2, CXCL4, CXCL5, CXCL7, CXCL9, CXCL10, CXCL12, CXCL13, and

CXCL14) concentrations changed significantly in patients with GC without postoperative recurrence. In these patients with GC recurrence, most of the chemokines in the blood concentration did not change significantly. After the next stage of tumor development, escape stage, patients will experience immunosuppression, so that tumor spread. We also found that the levels and gradients of chemokines were related to the expression of CXCR and the number of tumor infiltrating immune cell subsets, thus promoting the development of GC immune core.

Our study shows that CXCL family plays an important role in the pathogenesis of GC. It can be used as a marker for the development of GC. This method has a wide range of applications and can be included in the study of GC transformation.

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Disclosure of conflict of interest

None.

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