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Original Article

Expression of chemokine receptor CXCR7 and its effects on gastric cancer tissues and cell lines

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Abstract: Gastric cancer (GC), the most common and malignant tumor of the digestive system, exhibits high invasive capacity that escapes immune attack. Chemokine receptor CXCR7 plays an important role in the development of cancer. Therefore, the aim of the present study is to investigate the expression of CXCR7 in GC tissues and to evaluate the role of CXCR7 in tumor growth, apoptosis, and invasion of GC cells. The expression status of CXCR7 was detected in 65 primary GC tissues by immunohistochemistry. The correlation of CXCR7 expression with the clinicopathologic parameters and prognostic factors of GC was analyzed. Further, the effects of CXCR7 knock-down by CXCR7-shRNA lentiviral vector on the proliferation, apoptosis, and invasion of GC cells were explored *in vitro*. MTT assay, FCM analysis, and transwell chamber test were employed. The expression of CXCR7 was significantly higher in GC tissues than in normal tissues. CXCR7 expression was correlated with the degree of differentiation, depth of invasion, and lymph node metastasis. Transfection of MKN-45 cells with CXCR7-shRNA lentiviral vector resulted in a significantly decreased expression of CXCR7 at an mRNA and protein level. Tumor invasiveness was suppressed *in vitro* by silencing of CXCR7 in MKN-45 cells. However, no obvious suppression in cell proliferative activity and apoptosis were observed. The expression of CXCR7 could be used as a biomarker to predict prognosis and metastasis of GC. LV-CXCR7-shRNA constructs effectively inhibited the expression of CXCR7 RNA, thus reducing the migration and invasion capacity of human GC cells *in vitro*.

Keywords: Gastric cancer, chemokine receptor CXCR7, invasion, lentivirus-mediated CXCR7 gene silencing

Introduction

Gastric cancer (GC) is currently the third cause of cancer-related deaths worldwide, and is particularly prevalent in Asia [1]. Local and distant metastases after curative gastrectomy are critical events affecting the prognosis of GC patients [2]. Since the molecular mechanisms underlying metastasis and recurrence of GC have not been defined, an effective approach towards prevention and cure is not yet available. Therefore, it is necessary to elucidate the molecular mechanisms related to progression of the malignant potential of GC. CXC chemokine ligand CXCL12, also called stromal cell-derived factor (SDF)-1, is a member of the CXC sub-family and mediates hematopoiesis [3], vascular formation [4], and neurogenesis [5] through interaction with receptor CXCR4. It has recently been discovered that CXCL12 also binds to the receptor CXCR7 with high affinity. CXCR7 plays a crucial role in several cancers, including colon cancer [6], liver cancer [7], and bladder cancer [8]. In addition, studies have shown that CXCL12-CXCR7 axis promotes tumor aggressiveness and metastasis [9].

The importance of CXCR7 in cancer is further clarified by the fact that malignant tumors (in contrast to benign tumors) overexpress CXCR7, which is correlated with metastasis to distant organs and reduced overall survival [10]. Expression of CXCR7 and CXCR4 can assist in mTOR signaling transduction pathway in cancer cells, and mediate the implication of CXCR7 in tumorigenesis [11]. Upregulation of CXCR7 not only regulates the development of atherosclerosis, autoimmunity, and HIV infection [12], but also participates in tumor proliferation, invasion, and metastasis [9]. Thus, strategies

involving interventions of CXCR7 and related signaling pathways may offer new therapeutic opportunities in cancer. In a previous study, CXCR7 was found to be highly expressed in patients with GC, which was additionally associated with lifestyle risk factors, such as alcohol consumption [13]. Although the role of CXCR4 in the promotion of GC invasive growth is well documented, the significance of CXCL12/CXCR7 axis in regulating GC biological behavior has not been completely described. We, therefore, hypothesized the involvement of CXCR7 in the malignant properties of GC. Herein, we investigated the expression of CXCR7 in primary GC by immunohistochemistry. Furthermore, we explored the effects of CXCR7 knock-down by CXCR7-shRNA lentiviral vector on the biological behavior, including proliferation, apoptosis, and invasion of human GC cells.

Materials and methods

Ethics

The Ethics Committee of Gansu Provincial Hospital, Lanzhou, China approved the protocol of the present study. Informed written consent was obtained from all patients for the acquisition and use of patient tissue samples and anonymized clinical data.

Patients and samples

The present study included 65 patients with primary GC, all of whom underwent gastrectomy between 2011 and 2013 at the Department of General Surgery, Gansu Provincial Hospital. Gastric cancer was pathologically diagnosed after operation in the cancer group. This group included 51 men and 14 women ranging from 35 to 74 years of age (mean, 55 years). No patient was treated with local ablative therapy, chemotherapy, or immunotherapy prior to surgery. Control tissues were obtained from 20 benign gastric ulcer patients, who received gastrectomy. Human gastric tissue samples were formalin-fixed and paraffin-embedded. According to AJCC and UICC criteria, there were 7 (10.8%), 15 (23.1%), 40 (61.5%), and 3 (4.6%) cases at stage I, II, III, and IV, respectively. The median follow-up period was 11 months (range, 2-24). Pathological characteristics were collected and classified. All specimens were pathological and were reassessed independently by two gastroenterology pathologists blinded to the clinical data.

Immunohistochemistry

To assess the CXCR7 protein expression in gastric cancer and normal tissue, monoclonal rabbit anti-human CXCR7 (P25106; Abgent, San Diego, CA, USA) was used. Paraffin-embedded sections (5-μm thick) were fixed in freshly prepared 10% paraformaldehyde for 5 min. After blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide in TBS for 15 min, the sections were immersed in horse serum (diluted 1:10 in TBS) for 30 min to reduce any nonspecific binding, and were subsequently incubated with the primary antibody (1:100) overnight at 4°C after washing with TBS. The sections were then incubated with biotinylated goat anti-rabbit IgG for 30 min, and avidin-biotin-peroxidase complex for 30 min. After each step in the staining procedure, the sections were given three 5-min washes in TBS. Immunoreactivity (IR) was visualized using 1 mg/ml diaminobenzidine (DAB) as chromogen and 0.01% hydrogen peroxide as substrate. The peroxidase reaction was stopped after 5 min with distilled water, and the sections were counter-stained with Toluidine blue, dehydrated, and then mounted with Entellan. Negative controls were obtained by substituting the primary antibody with phosphate-buffered saline.

Slides were evaluated under a light microscope (× 400 magnifications). For digital image analysis, Adobe Photoshop software version 7.0 was used. Results were scored by two independent pathologists. The proportion score reflected the fraction of positive staining cells (score 0, <25%; score 1, 25-50%; score 2, 50-75%; and score 3, >75%), and the intensity score represented the staining intensity (score 0, no staining; score 1, weak positive; score 2, moderate positive; and score 3, strong positive). The two scores were averaged. Based on the analysis, CXCR7 was regarded as negatively expressed in GC tissues if the score was <2, and positively expressed with the score ≥2.

Synthesis of siRNA

Four pairs of siRNA targeting CXCR7 were synthesized: sequence A (siCXCR7-A): 5'-gaAGGCCTTCATCTTCAAGTA-3' (sense) and 3'-TACTTGAAGATGAAGGCC Ttc-5' (antisense); sequence B (siCXCR7-B): 5'-acGCTCTCCTTCATTTACATT-3' (sense) and 3'-AATGTAAATGAAGGAGAGCgt-5' (antisense); sequence C (siCXCR7-C): 5'-ctGGGTGAATATCCAGGCCAA-3' (sense) and 3'-TTG-

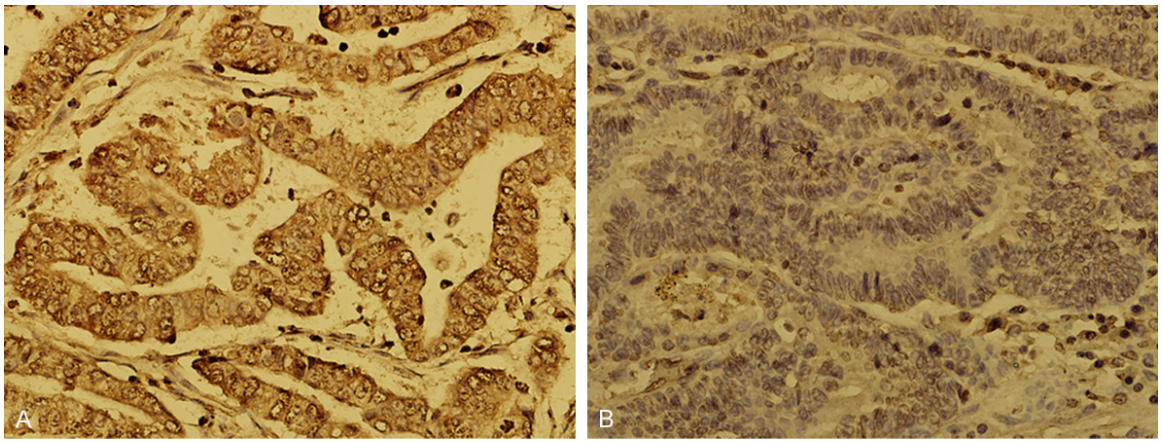

Figure 1. Immunohistochemistry for CXCR7 in gastric cancer tissues and normal gastric tissues (magnification × 400). A. CXCR7 is strongly detected in the membranes and cytoplasm of cancer cells; B. CXCR7 is not detected in normal gastric cells.

Table 1. Expression of CXCR7 protein in GC tissues

Variables	Groups	Cases (N)	Expression levels (n)		Positive rate (%)	χ^2	*P*-value
			Negative	Positive			
CXCR7	GC	65	10	55	84.62%	38.55	<0.01
	NC	20	18	2	10%		

GC, gastric cancer; NC, normal control tissue.

GCCTGG ATATTCACCCag-5' (antisense); sequence D (siCXCR7-D): 5'-ctGCACACACAAC GAACAGTT-3' (sense) and 3'-AACTGTTCGTTGTGTGTGCag-5' (antisense). In addition, the negative control siCXCR7 (CON049) was synthesized by Shanghai Genechem Co. Ltd. (Shanghai, China) for monitoring the influence of exogenous genes.

Construction and identification of LV-shCXCR7

Vector *GV118* was digested with restriction enzymes (*HpaI*/*XhoI*) and siRNA was inserted into the vector. The sequencing of LV-shCXCR7 was carried out by the Shanghai Genechem Co. Ltd. The packing of lentiviral vector was done according to the instructions provided with the Lenti-X Bicistronic Expresion System. Then, the LV-shCXCR7 was tittered using a kit based on the manufacturer's instructions.

Culture of MKN-45 cells and transfection

If not indicated otherwise, all materials were purchased from Gibco (Carlsbad, CA, USA). MKN-45 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and seeded on 6-well plates at a density of 5×10^4 cells/well. At 30% confluence, MKN-45 cells were transfected with recombinant LV-shCXCR7-A, LV-shCXCR7-B, LV-shCXCR7-C, LV-shCXCR7-D, and negative control virus (NC) at different doses (8, 6, and 5 μl). At 24 h after transfection, the medium was removed and serum containing growth medium was added. On day 3 post-transfection, transfection efficiency was measured by the frequency of green fluorescent protein (GFP)-positive cells. Positive cells (>80%) were collected and 1 ml of Trizol reagent was added, and quantitative real time PCR was performed to detect the mRNA levels.

Quantitative real-time PCR

Total RNA was extracted from MKN-45 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Using a first strand cDNA synthesis kit (Promega, Beijing, China), cDNA was generated with Oligo dT primer. Primers for CXCR7 (136 bp) were synthesized by Sangon Inc. (Shanghai, China) - F: AGCATCAAGGAGTGGCT GAT; R: TGTGCTTCTCCTGGTCACTG. GAPDH was used as an endogenous control. The reaction mixture contained 1.0 μl cDNA, 0.5 μl forward primer, 0.5 μl reverse primer, 10 μl SYBR Master Mix, and 8.0 μl RNase-Free H_2O (Takara, Dalian, China). Reaction mixtures were incubated at 95°C for 5 min, and then amplified for 45 cycles

Table 2. Relationship between CXCR7 expression and clinical characteristics in patients with gastric cancer

Items	Patients (n)	CXCR7 Expression		χ^2	*P* values
		Low	High		
Sex					
Male	51	7 (13.73)	44 (86.27)		
Female	14	3 (21.43)	11 (78.57)		
Age					
≥60	27	4 (14.81)	23 (85.19)	0.01	0.92
<60	38	6 (15.79)	32 (84.21)		
Tumor diameter (cm)					
≥5	34	4 (11.76)	30 (88.24)	0.72	0.39
<5	31	6 (19.35)	25 (80.65)		
Tumor differentiation					
WD	6	4 (66.67)	2 (33.33)	13.78	<0.01
MD	41	5 (12.20)	36 (87.80)		
PD	18	1 (5.56)	17 (94.44)		
Localization					
Gastric fundus and body	35	6 (17.14)	29 (82.86)	0.18	0.47
Gastric angles and antrum	30	4 (13.33)	26 (86.67)		
Histopathological type					
Diffuse	25	4 (16)	21 (84)	0.41	0.38
Intestinal	40	9 (22.5)	31 (77.5)		
T classification					
T1-2	9	5 (55.56)	4 (44.44)	12.95	<0.01
T3-4	56	5 (8.93)	51 (91.07)		
N classification					
N0	21	6 (28.57)	15 (71.43)	4.14	0.04
N1-N3	44	4 (9.09)	40 (90.91)		
Distant metastasis					
Presence	5	0 (0)	5 (100)	0.99	0.42
Absence	60	10 (16.67)	50 (83.33)		
TNM stage					
I-II	22	6 (27.27)	16 (72.73)	3.61	0.07
III-IV	43	4 (9.30)	39 (90.70)		

of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C annealing temperature. Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). All experiments were performed in triplicate. Cells transfected with LV-shCXCR7-B showed the lowest expression of CXCR7, and were therefore used in the following experiment.

Western blot

Nuclear protein extracts of MKN-45 cells were prepared in SDS lysis buffer containing protease inhibitors. Cell extracts were denatured in laemmli buffer (10% glycerol, 2% SDS, 0.1 m dithiothreitol, 50 mm Tris, 0.01 mg/ml bromophenol blue; pH 6.8) at 90°C for 5 min, and were separated by SDS-PAGE. Separated proteins were transferred onto a PVDF membrane in Trans-blot wet buffer (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk powder. The primary antibody against CXCR7 (P25106; Abgent, USA) was diluted according to the instructions and incubated overnight at 4°C. Horseradish peroxidase-conjugated goat anti-rabbit IgG were then added for 1 h at room temperature, and washed with 1 × TBS three times. Membranes were treated with ECL plus Western blotting detection kit (Transgen, Beijing, China), and bands were detected using STORM 840v20-05 with ImageQuant software (GE, Pittsburgh, PA, USA). The relative protein level in different groups was normalized to β-actin concentration. Three experiments were performed for each clone.

Cell proliferation assay

Cell proliferation was analyzed with the MTT assay. Briefly, cells infected with LV-shCXCR7 were incubated in 96-well plates at a density of 1×10^5 cells per well. Cells were treated with 20 μl MTT dye (5 mg/ml). The color reaction was measured at 490/570 nm with enzyme immunoassay analyzer (Biotek, Winooski, VT, USA). The proliferation activity was calculated for each clone.

Cell apoptosis assay

To detect cell apoptosis, cells were trypsinized, washed with PBS, and re-suspended in 1 ×

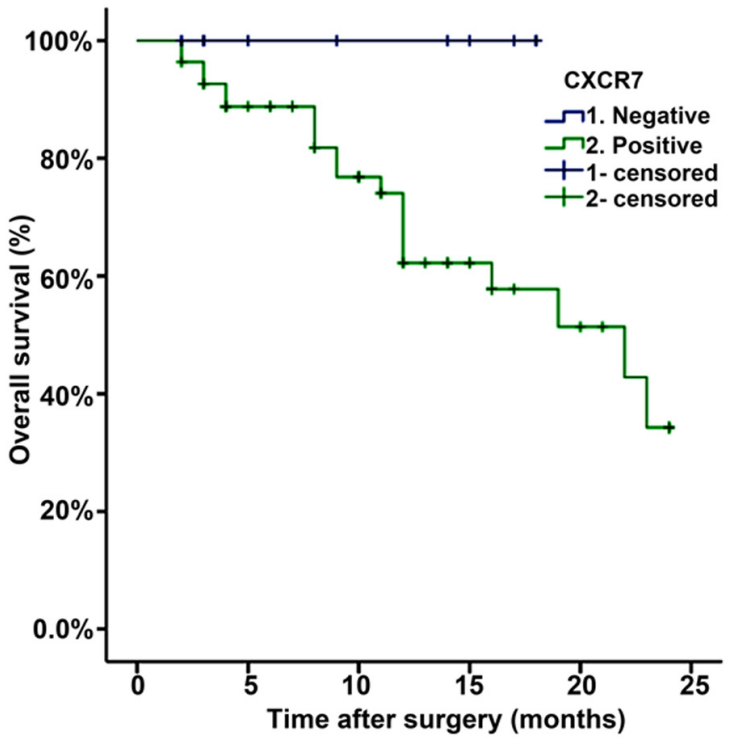

Figure 2. Analysis of overall survival according to the expression of CXCR7 in gastric cancer patients.

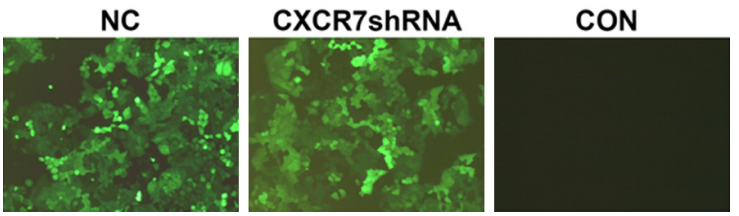

Figure 3. Lenti-CXCR7-siRNA infected MKN-45 cells shows green fluorescence (× 100).

binding buffer according to the instructions provided in the apoptosis kit (eBioscience, San Diego, CA, USA). 10 μl Annexin V-APC was added to the fixed cells for 15 min in darkness at room temperature. Subsequently, apoptosis in treated cells was analyzed by flow cytometry (Guava easyCyte HT, Germany) with Cellquest software (BD Biosciences-Pharmingen, San Diego, CA, USA). Three experiments were performed for each clone.

Transwell invasion assay

Harvested cells (1×10^5) in 100 μl serum-free DMEM were added into the upper chamber. The lower chamber was filled with 600 μl conditioned medium derived from MKN-45 cells as a source of chemoattractant. After 6 h of incubation at 37°C with 5% CO_2, the medium was removed from the upper chamber. The non-invaded cells on the upper side of the chamber were scraped off with a cotton swab. Migrated cells were dyed with 400 μl Giemsa for 20 min. Then, the color reaction was measured at 570 nm with enzyme immunoassay analyzer (Biotek). MTS method measured migration of cells according to the instructions given in the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, China). The transfer rate (OD 570/MTTOD 490) was calculated. Each assay was repeated three times.

Statistical analysis

SPSS 19.0 was used for the statistical analysis. χ^2 test and t test were employed to analyze the expression rate in all the groups. Patient clinical characteristics and disease factors were summarized using descriptive statistics. Cumulative survival time was calculated by Kaplan-Meier method and analyzed by log-rank test. The Cox proportional hazards regression model was used to perform univariate and multivariate analysis. Statistical significance was set at $P<0.05$.

Results

Expression of CXCR7 protein was upregulated in human GC tissues

The expression of CXCR7 protein was evaluated using immunohistochemical (IHC) staining. The positive expression of CXCR7 protein was detected in the cytoplasm and cell membranes of GC tissues. CXCR7 in cancer cells stained more intensely as compared to the normal gastric cells (**Figure 1**). The positive rates of CXCR7 expression were detected in 84.62% (55/65) of

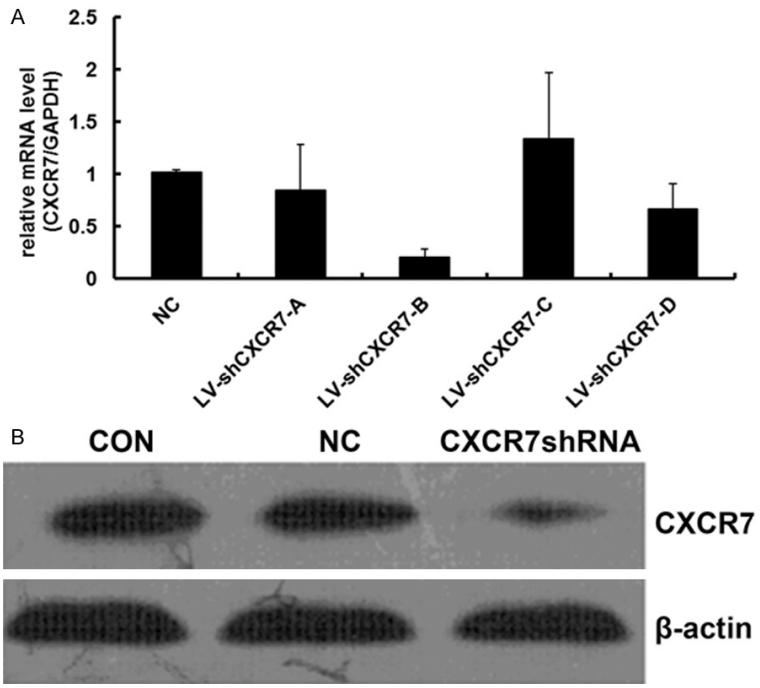

Figure 4. The expression of CXCR7 mRNA and proteins in different LV-shCXCR7 groups and NC group.

GC tissues, while the normal tissues displayed positive staining in 10% (2/20) of the cases; and a significant difference was observed between the two (χ^2=38.55, *P*<0.01; **Table 1**). These observations suggested that CXCR7 expression is increased in GC tissues as compared with normal tissues.

Relationship between CXCR7 expression and clinicopathologic characteristics of GC patients

IHC staining for CXCR7 levels was statistically analyzed to determine their relationship with various clinicopathologic features in all the 65 gastric cancer patients. As shown in **Table 2**, there were no significant differences in age, gender, primary tumor location, tumor size, and TNM stage. However, CXCR7 tended to be higher in patients with poor differentiation, deeper invasion, and lymphatic metastasis in comparison to patients with well differentiated tumor, shallow invasion, and no lymphatic metastasis.

Association of CXCR7 expression with overall survival of GC patients

Cancer-specific survival was estimated based on the CXCR7 expression using Kaplan-Meier survival analysis. Patients with high CXCR7 expression showed a reduced two-year survival rate as compared to the patients with low CXCR7 expression (34.2% vs. 100%), as was depicted in the survival curve (*P*=0.0124; **Figure 2**).

LV-shCXCR7 construction and transfection

Purified LV-shCXCR7 was used for the transfection of MKN-45 cells. The transfection outcome was detected using a fluorescence microscope 72 h after the experimental treatment. All infected cells showed green fluorescence (**Figure 3**).

Inhibition of CXCR7 shRNA on CXCR7 mRNA and proteins

In the LV-shCXCR7-A, LV-shCXCR7-B, LV-shCXCR7-C, LV-shCXCR7-D, and NC groups, the level of CXCR7 mRNA was observed to be 0.85±0.44, 0.21±0.08, 1.34±0.64, 0.67±0.24, and 1.02±0.02, respectively. The expression of CXCR7 mRNA was the lowest in the LV-shCXCR7-B group and differed significantly from the NC group (*P*=0.0015; **Figure 4A**). CXCR7 mRNA levels were reduced by 79.4% in LV-shCXCR7-B group, when compared with the control cells. Similar to RT-PCR results, the expression levels of CXCR7 proteins were markedly downregulated in the CXCR7shRNA group in comparison to the CON and NC groups (**Figure 4B**).

Effect of CXCR7 silencing on cell proliferation

To examine the effect of CXCR7 on the growth of GC cells, we investigated the proliferative activities of MKN-45 cells by MTT assay. Silencing of CXCR7 gene did not decrease the proliferative activities of MKN-45 cells in a time dependent manner as compared to the NC groups (**Figures 5A** and **5B**).

Effect of CXCR7 silencing on cell apoptosis

Cell apoptosis was monitored by flow cytometric analysis. As compared with that of the NC group and control group cells, apoptosis rate in

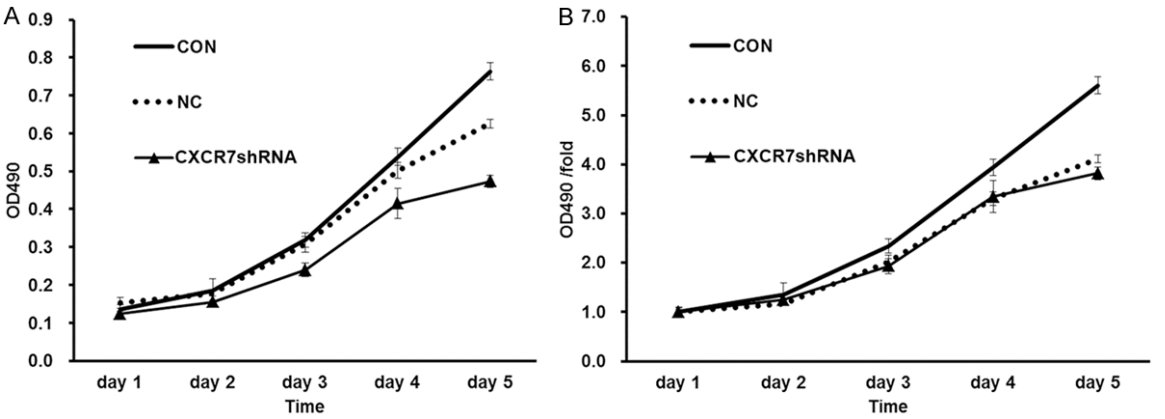

Figure 5. Effect of CXCR7 silencing on cell proliferation. Note: OD490/fold refers to times of OD490 on days 1-5 of all experimental groups compared to the first day, indicating proliferation multiples on different day points.

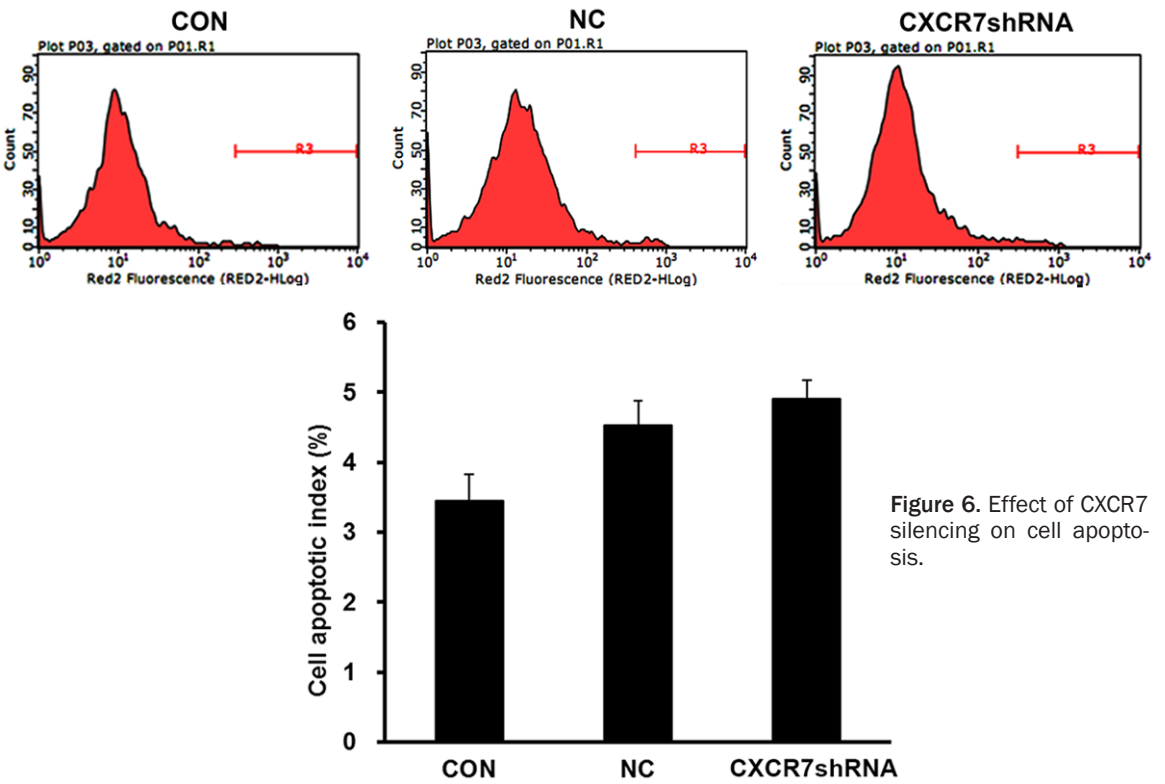

Figure 6. Effect of CXCR7 silencing on cell apoptosis.

the LV-shCXCR7 group cells showed no significant increase (**Figure 6**).

Effect of CXCR7 silencing on cell migration and invasion

To determine the effect of CXCR7 on cell migration and invasion, transwell assay was performed. The invasive potential was determined on the basis of the ability of cells to invade a matrix barrier. Representative micrographs of transwell filters have been presented in **Figure 7A**. The invasive potential of MKN-45 cells was significantly reduced in LV-shCXCR7 group, compared to the NC group ($P<0.05$; **Figure 7B**).

Discussion

As chemoattractant cytokines, chemokines can help in cell activation, differentiation, and trafficking. CXCR7, also known as a chemokine

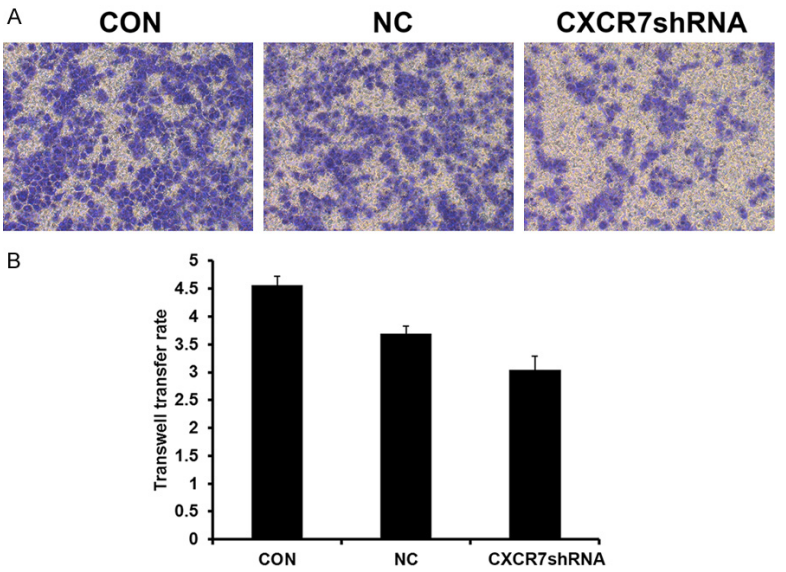

Figure 7. Migration experiment: MKN-45 group (CON); NC group; CXCR7shRNA group (× 100). The number of migrated cells was significantly lower in the lenti-shCXCR7 group than in the MKN-45 group and NC group. Note: KD group refers to normal cells, and cells infected with target genes, in accordance with the CXCR7shRNA group in text.

receptor, mediates biological effects through seven trans-membrane domain G protein coupled receptors (GPCRs). Additionally, it regulates fundamental biological processes including proliferation, cell survival, and adhesion, which contribute to essential functions of the receptor in multiple aspects of normal development and physiology [14]. CXCR7 also plays a role in cell growth, metastasis, and angiogenesis of certain malignant tumors [15]. The expression of CXCR7 has been found to be correlated with solid tumor size, differentiation, and lymph node status, thereby suggesting its potential function in worse tumor prognosis [16]. Therefore, in the present study, we examined the expression of the chemokine receptor CXCR7 in gastric cancer tissues using IHC and analyzed the relationship between their expression and clinicopathologic features of GC patients. CXCR7, mainly localized in the cytoplasm and membranes, was significantly upregulated in GC tissues as compared to the normal gastric tissues. The positive expression of CXCR7 was observed to be associated with poor differentiation, deeper invasion, and lymphatic metastasis, suggesting its involvement in tumor aggression and differentiation. In addition, we identified high CXCR7 expression as a poor prognostic factor for 2-year overall survival following gastrectomy in GC patients. Other studies have demonstrated that CXCR7 correlates with an alcohol-drinking history in gastric cancer patients; however, it does not correlate with age, gender, smoking history, HP infection, tumor location and pathological type, and an unfavorable survival and prognosis of patients with renal cell carcinoma [13, 17]. Therefore, CXCR7 may serve as a promising biomarker for the detection and diagnosis of cancer.

A greater understanding of the functions of CXCR7 in cancer requires further research. Studies have shown that suppression of the expression or activities of CXCR7 could decrease cell growth, migration, and angiogenesis in pancreatic cancer, colorectal cancer, and glioma [18-20]. Silencing of CXCR7 expression significantly inhibited hepatocellular carcinoma cells invasion, adhesion, and angiogenesis [21]. Furthermore, downregulation of CXCR7 expression caused inhibition of trans-endothelial migration of cancer cells and lymph node metastasis [22], thus implying that CXCR7 could be used as a new targeted therapy for patients with GC. However, functionality of CXCR7 has long been a source of controversy. Burns et al. reported that ligand activation of CXCR7 does not induce typical chemokine responses, such as cell migration and calcium mobilization [14]. In the present study, the function of CXCR7 in GC was manifested using a recombinant lentiviral RNA interference vector of the CXCR7 gene in highly aggressive MKN-45 cells, and a significant repression of the invasion ability of MKN-45 cells was noted *in vitro*. Nevertheless, both proliferation and apoptosis abilities of MKN-45 cells were not affected. These results were consistent with the findings of Zheng et al., which showed that CXCR7 mediates chemotaxis of cancer cells towards CXCL12 [21]. Hence, it is possible to prevent the development of GC metastasis through inhibition of CXCR7. The mechanism of growth, invasion, and metastasis of chemokine CXCL12/CXCR7 axis may involve direct activation of the ERK1/2 signal-

ing pathway in cancer cells [23]. Another report suggested that CXCR7 can induce phosphorylation of MAPK p42/44 and AKT in human rhabdomyosarcoma [24]. We thereby speculated that the different biological effects elicited by CXCR7 may depend on the cell types. However, in this study, we did not describe the molecular mechanisms through which CXCR7 regulates the invasion of GC cells. Therefore, further in-depth studies need to be undertaken to elucidate the role of CXCR7 in invasion and signaling pathways activated by CXCL12/CXCR7 axis.

In conclusion, lymphatic metastasis of GC is a prevalent and serious problem, and an effective therapy is required. Upregulation of CXCR7 expression is associated with poor differentiation, deeper invasion, and lymph node metastasis; whereas silencing of CXCR7 gene represses the migration of gastric cancer cells. This study, therefore, suggested the potential application of lentiviral mediated CXCR7 RNAi as a therapeutic target for the treatment of gastric cancer.

Disclosure of conflict of interest

None.

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