CXCR6 predicts poor prognosis in gastric cancer and promotes tumor metastasis through epithelial-mesenchymal transition

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Abstract. Chemokines and their receptors have been confirmed to be involved in several types of cancer. However, little is known concerning the role of CXCL16 and its receptor CXCR6 in gastric cancer (GC) progression and metastasis. In the present study, expression of CXCL16 and CXCR6 in GC tumor and peritumoral tissues was detected by immunohistochemistry (IHC) in a cohort of 352 GC patients who underwent gastrectomy, and the correlation between CXCL16/CXCR6 expression and clinicopathological characteristics was further analyzed. To evaluate the function of CXCR6, we overexpressed and knocked down CXCR6 in GC cell lines. Results showed that expression of CXCR6, but not CXCL16, was significantly upregulated in GC tumor tissues, and was significantly correlated with lymph node and distant metastases, and advanced clinical stage in the GC patients. Survival analysis showed that large tumor size (>5 cm), elevated preoperative serum carcinoembryonic antigen (CEA) level, advanced TNM stage and high CXCR6 expression indicated worse overall survival (OS) and disease-free survival (DFS) in GC, and CXCR6 was an independent predictor for both OS and DFS in GC. *In vitro* experiments showed that CXCR6 overexpression induced cell migration and invasion ability, and promoted epithelial-mesenchymal transition of GC cells by upregulation of mesenchymal markers and inhibition of epithelial markers. In contrast, knockdown of CXCR6 in GC cells resulted in inhibition of cell proliferation, migration and invasion ability, and reversal of epithelial-mesenchymal transition (EMT) phenomenon. Our results demonstrated that CXCR6 is an independent prognostic factor for poor survival in GC patients, and may promote GC metastasis through EMT.

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Introduction

Globally, the incidence of gastric cancer (GC) ranks fourth in men and fifth in women, and affects more than one million individuals per year. The death rate for GC is next to lung cancer, and the number of deaths caused by GC ranks third in men and fourth in women among total cancer-related deaths (1,2). Accurate figures vary in regards to different populations and world regions (3,4). Despite the declining morbidity and improved standardized treatment, GC carries a poor prognosis with the mortality-to-incidence ratio ranging from 0.35 to 0.8 (5). This situation probably results from the fact that patients with GC are often diagnosed at the advanced stage, along with a high incidence of metastasis and recurrence.

Carcinomas arising from epithelial tissues progress to higher pathological grades of malignancy, as reflected in local invasion and distant metastasis. Along with this process, the associated cancer cells develop alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM). Loss of epithelial-cadherin (E-cadherin) by carcinoma cells, a key cell-to-cell adhesion molecule, is well characterized in this alteration. Inactivation of E-cadherin induces expression of transcriptional repressors such as Snail and ZEB family numbers, subsequently inducing epithelial-mesenchymal transition (EMT) which is a hallmark of tumor progression (6,7).

Rudolf Virchow described leukocyte infiltrates within tumors in the 19th century (8), and now it is clear that the infiltrates can exert both tumor-suppressive and tumor-promoting effects. Chemokines and chemokine receptors, which were initially researched for their role in the regulation of leukocyte trafficking to inflammatory sites, were found to be involved in enhancing the immunity of tumor-associated antigens, regulating new blood vessel

formation, promoting cancer cell proliferation and directing cancer cell metastasis (9-11). CXCR6 was reported to be positively correlated with Gr-1$^+$ neutrophil infiltration and microvessel growth, to lead to a protumor inflammatory microenvironment, and to predict poor prognosis in hepatocellular carcinoma (HCC) (12). GC is inflammation-related and *Helicobacter pylori* infection increases the risk of GC 3- to 6-fold (13). However, a recent study showed that blockade of CXCR6 reduced the migration and invasion of GC cells (14). Thus, CXCR6 is possibly involved in gastric tumorigenesis and metastasis. We explored CXCR6 expression in a cohort of 352 GC cases, and analyzed the association of CXCR6 expression with clinicopathological parameters and survival in GC.

Materials and methods

Patients and specimens. The present study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (Shanghai, China). Written informed consent was obtained from all of the patients enrolled in the present study. A cohort of 352 surgically resected GC patients recruited between 2010 and 2011 at Fudan University Shanghai Cancer Center were enrolled in the study. Patients did not have signs of distant metastasis nor had they received anticancer therapy before surgery. Tumor stage was determined according to the 2010 American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification system. Conventional clinicopathologic variables, including age, gender, carcinoembryonic antigen (CEA), tumor size, degree of differentiation, vascular invasion, tumor stage, therapy and status, were recorded and are documented in Table I.

Eight pairs of fresh frozen human GC tumor and matched peritumoral tissues were obtained for western blot analysis. Ethical approval was obtained from Fudan University Cancer Center Research Ethics Committee and written informed consent was obtained from each patient.

Follow-up and postoperative treatment. After surgery, patients with stage II or III were treated with chemotherapy. The adjuvant treatment lasted for 1 year which included oxaliplatin with fluoropyrimidine or oxaliplatin with capecitabine for 6-8 courses, and oral fluoropyrimidine or capecitabine for the rest of the year. Certain patients interrupted the treatment due to toxic side-effects. Follow-up was conducted following our standard protocol (every 3 months for at least 2 years, every 6 months for the next 3 years, and after 5 years every 12 months for the duration of life) (15). Patient monitoring included physical examination, tumor marker assessment, ultrasound, chest radiography, computed tomographic scan and endoscopic examination. A diagnosis of recurrence was based on typical imaging appearance in computed tomography and/or endoscopic examination. The treatment modality after relapse varied among the individuals. OS was defined as the interval between surgery and death, or between surgery and the last observation for surviving patients. Data were censored at the last follow-up for patients without relapse, or death. Follow-up was completed on July 30, 2014. The median follow-up was 40 months (range, 1-59 months).

Tissue microarray and immunohistochemistry. All GC cases were histologically reviewed by hematoxylin and eosin (H&E) staining and representative areas were pre-marked in the paraffin blocks, away from necrotic and hemorrhagic materials. A duplicate of 1.5-mm diameter cylinders was included in each case to ensure reproducibility of the slides. Thus, 8 different tissue microarray blocks were constructed. Sections of 4-μm thickness were placed on 3-aminopropyl-triethoxysilane-coated slides. CXCL16 (1:100) and CXCR6 (1:100) polyclonal antibodies were both purchased from Abcam (Cambridge, UK).

Immunohistochemistry of tissue microarrays was carried out using a two-step protocol. Briefly, paraffin sections were deparaffinized, hydrated and washed in Tris-buffered saline containing Tween-20 (TBST). After microwave antigen retrieval, endogenous peroxidase activity was blocked as required by incubating the slides in 0.3% H_2O_2 and non-specific binding sites were blocked with Protein Block (Novocastra Laboratories, Newcastle upon Tyne, UK). Then, the tissues were incubated with primary antibodies for 12 h at 4˚C, and then washed off. The components of the EnVision Plus detection system were applied (EnVision$^+$/HRP/Mo; Dako, Carpinteria, CA, USA), and the sections were developed in 3,3-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative controls identically treated, but with the primary antibodies omitted were included in all assays.

All slides were independently evaluated by two experienced pathologists. Immunoreactivity scores of CXCR6 and CXCL16 staining were determined by a semi-quantitative method multiplying the proportion and intensity of positively stained tumor cells. The percentage of positive cells was scored as 0 (no positive cells), 1 (<25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The staining intensity score multiplied by the percentage of positive staining was used to define the expression levels of CXCR6. Median value of all scores was used as a cut-off point for classification of protein expression. The GC patients were divided into two groups: a low expression (scores, 0-6) and a high expression group (scores, 6-12), for the CXCR6 protein.

Cell lines and transfection. Gastric carcinoma cell lines HGC-27 and SGC-7901 were obtained from Shanghai Cell Bank, Chinese Academy of Sciences and maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Beit-Haemek, Israel), 100 U/ml penicillin G and 100 μg/ml streptomycin. Lentiviral expression plasmid pCDH-cmV-EF1-copGFP (purchased from System Biosciences, Mountain View, CA, USA) was used to generate CXCR6 (NM_006564) expression plasmid by Genesent Technologies (Shanghai, China). To silence the expression of CXCR6, a short hairpin RNA (shRNA) sequence targeting the CXCR6 gene was purchased from Genesent Technologies. The lentivirus was harvested 48 h after co-transfection of the targeted plasmids, with psPAX2 and pMD2.G or the corresponding empty vector into HEK-293T cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Target cells were

Table I. Clinicopathological features of the GC patients.

Variables	Results
Age (years): median (range)	62 (21-84)
Gender: male/female	275/77
CEA (preoperative) (ng/ml): median (range)	2.07 (0-539.26)
Tumor size (cm): median (range)	5 (0.9-15)
Vascular invasion: absence/presence	161/191
Tumor differentiation: well or moderate/poor	171/181
AJCC/UICC TNM stage: I/II/III/IV	20/65/204/63
Adjuvant therapy: none/chemotherapy	26/326
Alive with recurrence (without recurrence)/death due to tumor (non-tumor)	26 (222)/66 (38)

GC, gastric cancer; CEA, carcinoembryonic antigen; AJCC, American Joint Committee on Cancer; UICC, International Union Against Cancer; TNM, tumor-node-metastasis.

infected with the filtered lentivirus plus 6 μg/ml Polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Western blot analysis. Expression levels of CXCR6 in tumor and peritumoral tissues were evaluated via western blot analysis. Total protein was extracted in lysis buffer for 30 min on ice. Equal amounts of protein were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The primary antibody against CXCR6 (1:1,000; Abcam) was used. A monoclonal antibody against GAPDH (1:1,000) was used as an internal control. Each experiment was repeated at least 3 times.

Transwell assays. The migration ability of the tested cells was evaluated in 24-well Corning chambers (8-μm pore size) (Corning, NY, USA). A total of $5x10^4$ cells in serum-free medium was added to the upper chamber. Invasion assay was conducted using the Transwell inserts coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Cells ($2x10^5$) were seeded into the upper chambers in serum-free medium. After 48 h of incubation at 37˚C in 5% CO_2, the cells that had invaded were fixed and stained in dye solution (Beyotime, NKG, China). The cells that had migrated/invaded were counted in 5 random fields at a magnification of x100, and imaged using an IX71 inverted microscope (Olympus Corp., Tokyo, Japan). Each experiment was carried out in triplicate.

Cell proliferation assay. Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Tested cells were seeded into 96-well plates (Corning) at a density of 1,000 cells/well. Cells were allowed to grow for 1, 2, 3, 4, 5, 6 and 7 days, and then 10 μl of CCK-8 solution was added to each well and incubated at 37˚C for 4 h. Cell viability was detected by measurement of absorbance at 490 nm using a microplate reader (ELx800NB; BioTek Instruments, Winooski, VT, USA).

Statistical analysis. Statistical analyses were conducted using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistically significant differences were analyzed by the Chi-square test for categorical variables and the Student's t-test for continuous variables. Cumulative survival rates were calculated by the Kaplan-Meier method, and differences between the survival curves were analyzed by the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional-hazards model. Statistically significant differences were determined at a P-value of <0.05.

Results

CXCR6 is correlated with lymph node and distant metastasis, and advanced clinical stage in patients with GC. To investigate the role of CXCR6 in GC, we first examined CXCR6 protein in 8 pairs of frozen GC tumor and peritumoral tissues by western blotting, and found that CXCR6 expression was elevated in the tumor tissues (Fig. 1A). Further study was conducted in 352 GC specimens with immunohistochemical staining for CXCR6. The results showed that in GC tissues, CXCR6 staining was strong in 58, moderate in 152, weak in 100 and negative in 42 cases, located diffusely in the cytoplasm and cell membrane, while in paired peritumoral tissues, CXCR6 expression was moderate or weak (Fig. 1B). Moreover, in patients with distant metastasis (DM) (n=63), CXCR6 staining densities were significantly higher than that in patients without DM (n=289; Mann-Whitney test; $P<0.0001$; Fig. 1C). For further analysis, patients were classified into a CXCR6-low (negative and weak; n=142) or -high (moderate and strong; n=210) group. Clinicopathologic analysis revealed that expression of CXCR6 was positively correlated with larger tumor size (≥5 cm; P=0.004), poor differentiation status (P=0.001), LN metastasis (P=0.001), DM (P=0.011) and advanced TNM stages (stage III and IV; P=0.006) (Table II). Taken together, CXCR6 was significantly correlated with LN metastasis, DM and advanced TNM stage in patients with GC.

CXCR6 is an independent prognostic factor for OS and DFS in GC. Survival analysis was conducted to determine prognostic factors for overall survival (OS) and disease-free survival

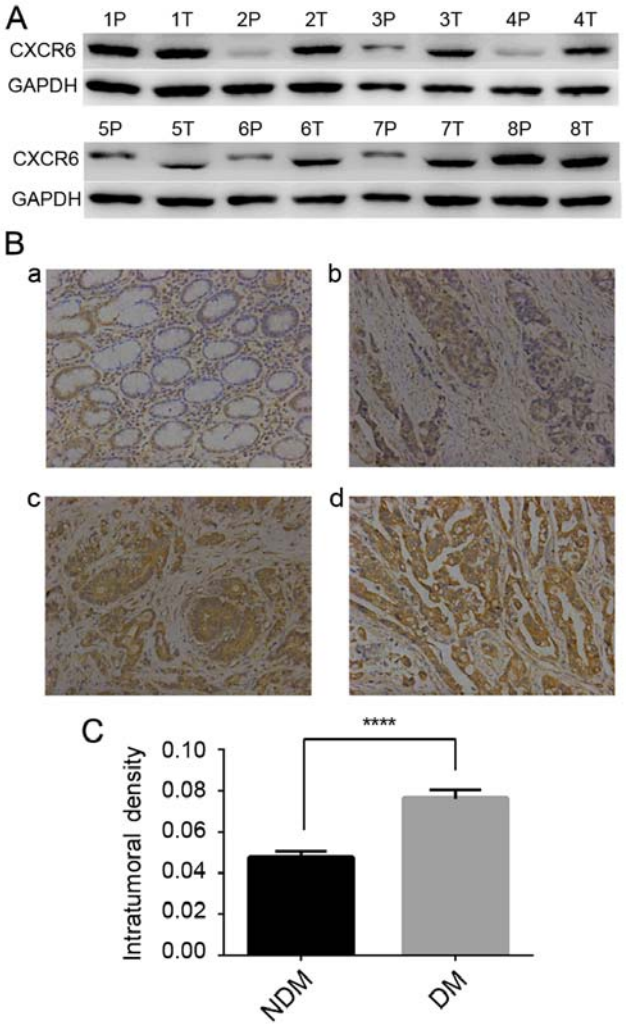

Figure 1. CXCR6 expression in gastric cancer (GC) tumor tissues. (A) Examination of CXCR6 protein in GC tumor (T) and paired peritumoral tissues (P), by western blotting (n=8). (B) Immunostaining of CXCR6 using 3,3'-diaminobenzidine (DAB; brown) is shown for peritumor normal gastric tissues and GC tissues: a, negative and weak staining of CXCR6 in peritumoral tissues; b, weak staining of CXCR6 in cancer tissues; c, moderate staining of CXCR6 in tumor tissues; d, strong staining of CXCR6 in tumor tissues. Magnification, x200. (C) CXCR6 densities were compared (Mann-Whitney test) in the intratumoral tissues of patients without distant metastasis (NDM; n=289) or with distant metastasis (DM; n=63); ****P<0.0001.

(DFS) in GC patients. Univariate analysis showed that larger tumor size (P=0.010), elevated preoperative serum CEA level (P=0.012), advanced TNM stage (P<0.001), and high CXCR6 expression (P=0.030) indicated both worse OS and DFS in GC. Nevertheless vascular invasion predicted shorter OS time, but had no impact on DFS in GC (Table III). As shown in Fig. 2, patients in the CXCR6-high group had significantly shorter OS and DFS time than those in the CXCR6-low group. Results remained significant in cases with stage III and IV (median DFS time; 29 vs. 40 months; P<0.001). On the basis of these results, multivariate Cox regression analysis was conducted, TNM stage and CXCR6 expression were verified to be independent prognostic factors for both OS and DFS in GC, and preoperative serum CEA level was an independent prognostic factor for DFS in GC (Table IV). Hence, conclusions were drawn that CXCR6 expression was an independent prognostic factor for both OS and DFS in GC.

Table II. Correlation between tumor CXCR6 expression and clinicopathological features of the gastric cancer cases.

Characteristics	CXCR6 expression: High (n=210) n (%)	CXCR6 expression: Low (n=142) n (%)	P-value
Age, years			0.155
<60	82 (56.2)	64 (43.8)	
≥60	128 (62.1)	78 (27.9)	
Gender			0.115
Male	159 (57.8)	116 (42.2)	
Female	51 (58.6)	26 (41.4)	
Tumor size (cm)			**0.004**
<5	99 (52.9)	88 (47.1)	
≥5	111 (67.3)	54 (32.7)	
Differentiation status			**0.001**
Well/moderately	123 (69.5)	54 (30.5)	
Poor	87 (49.7)	88 (50.3)	
Vascular invasion			0.069
Absent	103 (64.3)	57 (35.7)	
Present	107 (56)	84 (44)	
Infiltration depth			0.193
T1, T2	20 (69)	9 (31)	
T3, T4	190 (58.8)	133 (41.2)	
Lymph node metastasis			**0.001**
Absent	61 (77.2)	18 (21.8)	
Present	149 (54.6)	124 (45.4)	
Distant metastasis			**0.011**
Absent	164 (56.7)	125 (43.3)	
Present	46 (73)	17 (27)	
TNM stage			**0.006**
I, II	61 (71.8)	24 (28.2)	
III, IV	149 (53.8)	118 (46.2)	

Bold indicates significance at P<0.05. TNM, tumor-node-metastasis.

CXCR6 promotes proliferation, invasion and migration in GC cells. Considering the clinical significance of CXCR6 in GC patients, we examined the biological effects of CXCR6 in GC cell lines. CXCR6-shRNA and CXCR6 expression vector, and the corresponding negative control vectors were transfected into the GC cell lines, SGC-7901 and HGC-27, respectively. Western blotting showed that CXCR6 was upregulated in the HGC-27-CXCR6-OV cells (Fig. 3E and F), and was reduced in the SGC-7901-CXCR6-sh cells (Fig. 4C and D). CCK-8 assay was used to examine cell proliferation. Results showed that CXCR6 overexpression significantly increased GC cell proliferation (Fig. 3B), while CXCR6 reduction inhibited GC cell proliferation (Fig. 4E). Transwell assays were used

Table III. Univariate analysis of prognostic variables for gastric cancer.

Factors	OS		DFS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years) (<60 vs. ≥60)	0.95 (0.65-1.41)	0.806	0.94 (0.64-1.38)	0.742
Gender (male vs. female)	1.04 (0.65-1.67)	0.867	1.03 (0.65-1.66)	0.867
Tumor size (cm) (<5 vs. ≥5)	1.66 (1.23-2.45)	**0.010**	1.62 (1.09-2.39)	**0.015**
Differentiation status (well vs. poor)	0.83 (0.57-1.22)	0.352	0.82 (0.56-1.20)	0.352
Vascular invasion (absent vs. present)	1.57 (1.05-2.35)	**0.030**	1.47 (0.98-2.18)	0.057
CEA (ng/ml) (<5.2 vs. ≥5.2)	1.69 (1.12-2.55)	**0.012**	1.78 (1.18-2.68)	**0.012**
TNM stage (II vs. III/IV)	0.13 (0.05-0.32)	**0**	0.13 (0.05-0.32)	**0**
CXCR6 expression (low vs. high)	2.32 (1.49-3.64)	**0.001**	2.39 (1.53-3.37)	**0.001**

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis. Bold indicates significance at P<0.05.

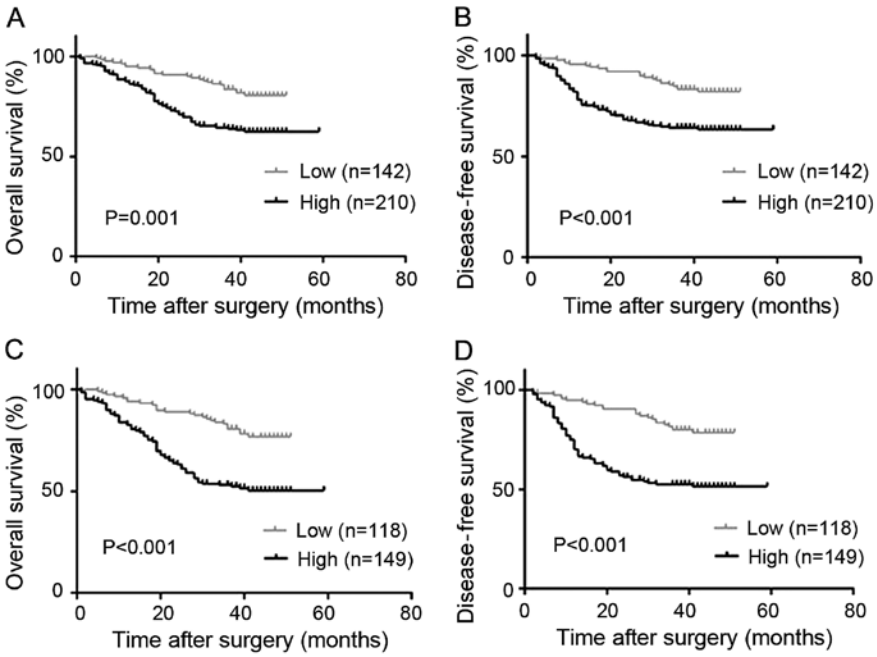

Figure 2. Kaplan-Meier curves for gastric cancer (GC) patients with different intratumoral CXCR6 levels. (A and B) Patients with high expression of CXCR6 exhibited significantly shorter OS and DFS time than those with low CXCR6 expression. (C and D) Stage III/IV patients with high expression of CXCR6 exhibited significantly shorter OS and DFS time than those with low CXCR6 expression.

Table IV. Multivariate analysis of prognostic variables.

Factors	OS		DFS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size (cm) (<5 vs. ≥5)	1.28 (0.86-1.89)	0.214	1.24 (0.84-1.83)	0.283
Vascular invasion (yes vs. no)	1.10 (0.73-1.65)	0.623		
CEA (ng/ml) (<5.2 vs. ≥5.2)	1.45 (0.96-2.20)	0.730	1.54 (1.02-2.32)	**0.040**
TNM stage (I/II vs. III/IV)	0.12 (0.05-0.30)	**0.000**	0.12 (0.05-0.29)	**0.000**
CXCR6 expression (low vs. high)	2.78 (1.77-4.37)	**0.003**	2.85 (1.81-4.47)	**0.010**

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis. Bold indicates significance at P<0.05.

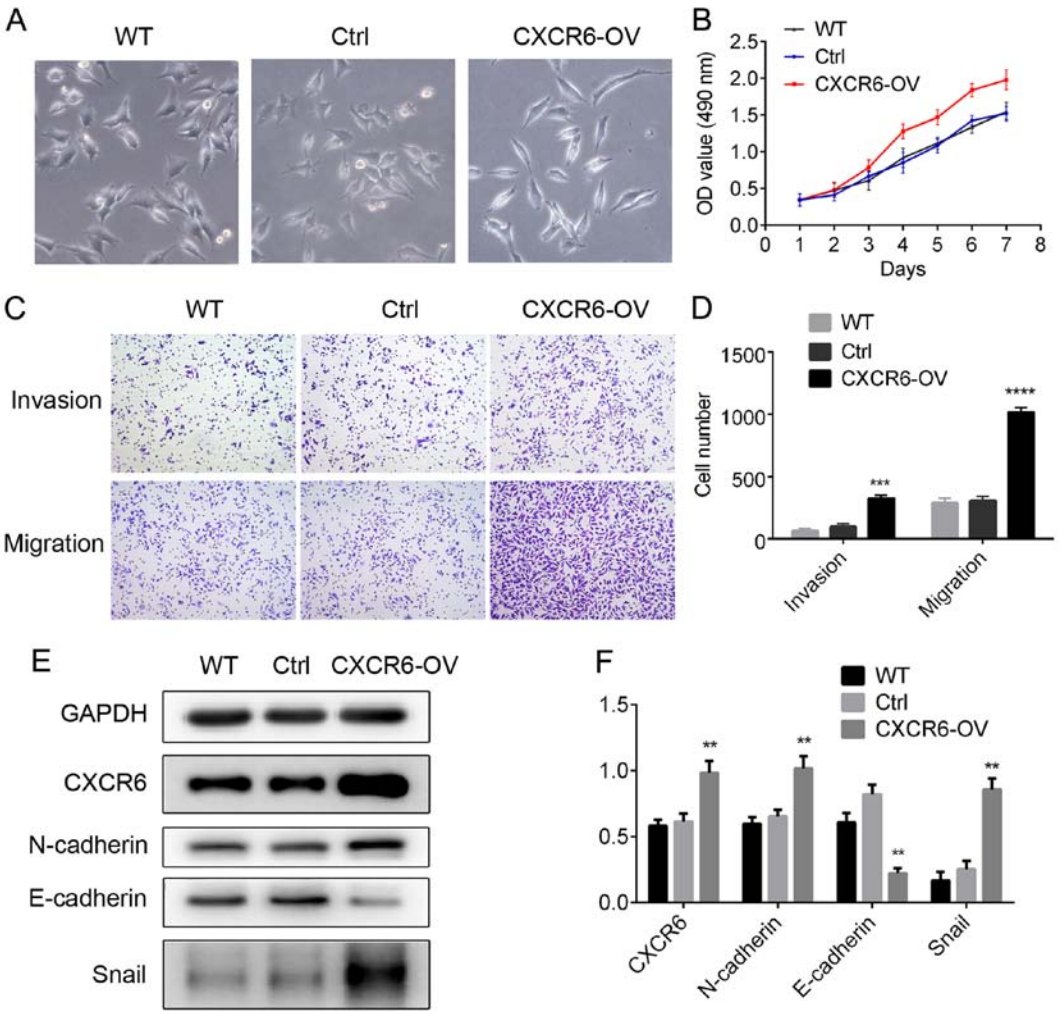

Figure 3. CXCR6 overexpression promotes proliferation, invasion, migration and epithelial-mesenchymal transition (EMT) in gastric cancer (GC) cells. (A) CXCR6 overexpression in HGC27 cells induced the EMT phenomenon with morphological transformation and alterations in cellular configuration in HGC-27 cells. Magnification, x200. (B) CXCR6 overexpression promoted the proliferation of HGC-27 cells. (C and D) CXCR6 overexpression significantly increased invasion and migration in HGC-27 cells. ***P<0.001, ****P<0.0001. Magnification, x40. (E and F) CXCR6 overexpression increased N-cadherin and Snail expression, and decreased E-cadherin expression in the HGC-27 cells. **P<0.01. Three independent experiments were conducted.

to assess the migration and invasion abilities of the GC cells. Results showed that HGC27-CXCR6-OV cells exhibited higher migration and invasion abilities compared with the control cells (P<0.001 and P<0.0001; Fig. 3C and D), while SGC7901-CXCR6-sh cells displayed decreased migration and invasion abilities compared with the control cells (P<0.001; Fig. 4A and B). In conclusion, CXCR6 increased proliferation, and promoted the migration and invasion of GC cells.

CXCR6 promotes EMT in GC cells. We found that HGC27-CXCR6-OV cells displayed a loose cell-cell contact, and spindle-shaped morphology representative of EMT (Fig. 3A). Previous studies have proposed that EMT is associated with cancer cell migration, tumor metastasis and progression. Therefore, we explored levels of EMT markers in the CXCR6 overexpressing/silenced cells. Western blotting showed that in the HGC27-CXCR6-OV cells, expression of mesenchymal markers, N-cadherin and Snail, was significantly upregulated, while expression of epithelial marker, E-cadherin, was suppressed (P<0.01; Fig. 3E and F); while in SGC7901-CXCR6-sh cells, N-cadherin and Snail expression was decreased, while E-cadherin was upregulated (P<0.01; Fig. 4C and D). Considering the changes in representative cell morphology and EMT marker expression, we conclude that CXCR6 promotes EMT in GC cells.

Discussion

We studied the association between CXCR6 expression and gastric cancer (GC) patients after follow-up for at least 3 years. The 3-year OS rate for patients with high CXCR6 expression was 63%, while the 3-year OS rate for patients with low CXCR6 expression was 81.6% (log-rank test: HR, 2.334; 95% CI, 1.469-3.188; P<0.001). Notably, multivariate analysis showed for the first time that CXCR6 is an independent predictor for both OS and DFS in GC. Patients with stage III/IV and high CXCR6 expression exhibited worse OS than patients with low CXCR6 expression (log-rank test: HR, 2.803; 95% CI, 1.771-3.895, P<0.001). However, CXCR6 had no effect on OS of patients with stage I/II GC.

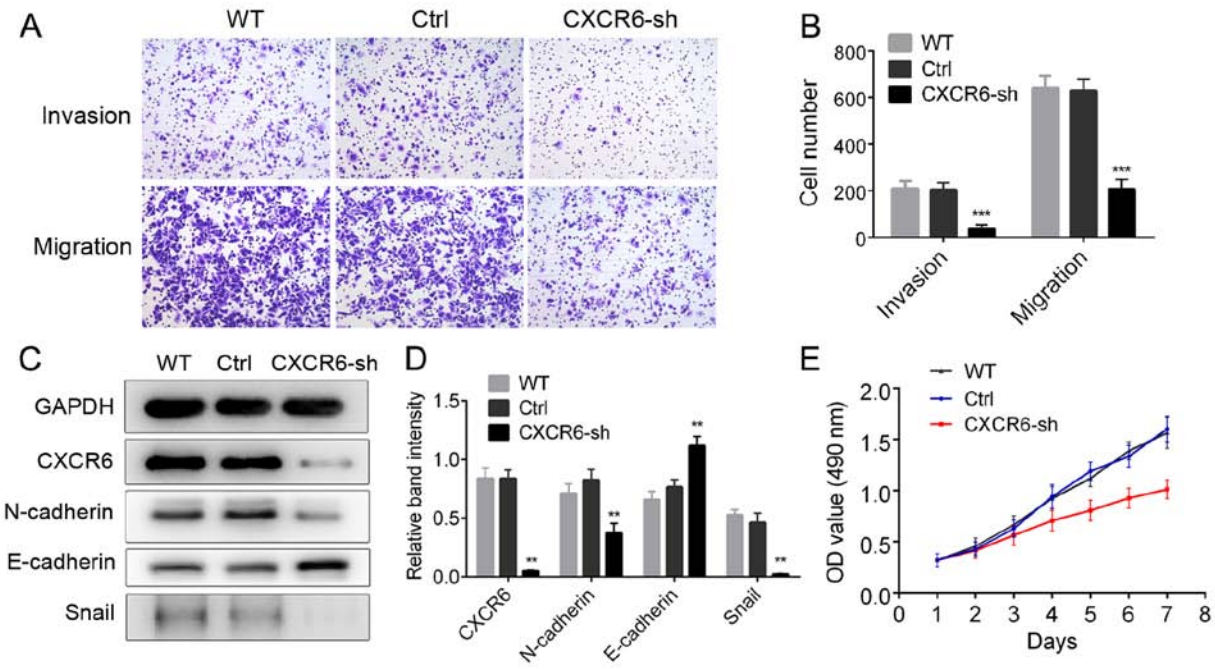

Figure 4. Knockdown of CXCR6 inhibits proliferation, invasion, migration and epithelial-mesenchymal transition (EMT) in gastric cancer (GC) cells. (A and B) CXCR6 knockdown significantly inhibited invasion and migration of the SGC-7901 cells. ***P<0.001. Magnification, x40. (C and D) CXCR6 knockdown increased E-cadherin expression, decreased N-cadherin and Snail expression in the SGC7901 cells. ***P<0.01. (E) CXCR6 knockdown inhibited cell proliferation in the SGC7901 cells. At least three independent experiments were conducted.

In the present study, CXCR6 was also found to be correlated with lymph node and distant metastases of GC. This correlation does not exist in GC alone. In breast cancer (BC), CXCR6 expression was found to be higher in BC nest tissues and metastatic lymph node, and may be responsible for invasion and metastasis (16). Another study showed that CXCR6 promoted HCC invasion and a protumor inflammatory microenvironment, which promoted metastasis and poor patient outcome in HCC (12). In lung cancer, CXCR6 was reported to support metastasis via modulation of metalloproteinase (17). However, in prostate cancer, ovarian cancer and schwannomas, the correlation of CXCR6 with metastasis was not reported (18-20). Mechanisms underlying the metastasis-promoting effects in different cancer types were not uniform. The CXCR6/ERK1/2/RhoA/cofilin/F-actin pathway was identified in BC, while in HCC, Gr-1^{+} neutrophil infiltration and neoangiogenesis were involved.

EMT occurs in carcinoma development. During EMT, epithelial cells lose their characteristic cell-cell adhesion structures, change their polarity, modulate the organization of the cytoskeletal systems, and become isolated, motile and resistant to anoikis (21-24). These alterations facilitate the malignant behaviors of cancer cells. It has been shown that EMT can be induced by the signaling of several growth factor receptors and chemokine receptors (25-27). In the present study, protein markers for EMT were also detected. E-cadherin was decreased in the CXCR6-overexpressing cells. Decreased expression of E-cadherin is well established as a promotor of invasion and metastasis, while induction of its expression is known to antagonize these phenotypes. Furthermore, E-cadherin-inactivating mutations have been detected in diffuse GC, including both germline and somatic mutations (28-30). Meanwhile, N-cadherin was upregulated in the GC cells with CXCR6 overexpression in the present study. The result is also supported by the fact that N-cadherin is upregulated in many invasive carcinoma cell lines, including BC, pancreatic and prostate cancer. N-cadherin is associated with enhanced migration and invasion, leading to increased metastasis and poor prognosis in these carcinomas (31). Snail, a suppressor of E-cadherin and inducer of EMT, was upregulated in the GC cells with CXCR6 overexpression. Briefly, upregulation of CXCR6 in GC cells induced expression of Snail and N-cadherin, and simultaneously suppressed E-cadherin formation. Upregulation of CXCR6 promoted EMT in GC cells.

In conclusion, CXCR6 promoted tumor progression in GC via modulation of EMT. CXCR6 was found to be an independent prognostic factor for GC and may be a potential target for novel therapy.

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