

Original Article

Silencing of CXCR2 and CXCR7 protects against esophageal cancer

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Abstract: This study was aimed to investigate the functional roles of cytokine receptor (CXCR) CXCR2 and CXCR7 in esophageal cancer (EC). Specific small interfering RNAs (siRNA) against CXCR2 and CXCR7 were transfected into EC cell lines TE-1, EC9706, and EC109 cells. Expression of CXCR2 and CXCR7 was validated, along with cell viability, chemotaxis, apoptosis rate, and ERK1/2 pathways associated protein after transfection. Moreover, EC9706 cells treated with or without CXCR2/7 siRNA were injected into athymic nude mice. Tumor volumes were measured. Besides, immunohistochemical (IHC) staining was performed to investigate the expression of CXCR2/7 in adjacent normal tissues and tumor tissues from esophageal squamous cell carcinoma (ESCC) patients. Also, the associations between CXCR2/7 expression and clinicopathological features and progression were explored. The mRNA levels of CXCR2 and CXCR7 were significantly reduced after transfection. Silencing of CXCR2 and CXCR7 statistically decreased cell viability and chemotaxis, and increased apoptotic rate. Cells invasion was significantly reduced by silencing of CXCR2, however, no significance was found in silencing of CXCR7. The protein levels of pERK1/2 were significantly decreased by silencing of CXCR2 and CXCR7. Besides, silencing of CXCR2 and CXCR7 significantly reduced tumor growth *in vivo*, and associated with clinicopathological features and progression. Silencing of CXCR2 and CXCR7 protects against EC by inhibiting cell growth and chemotaxis, and inducing apoptosis through ERK1/2 pathways. Silencing of CXCR2 and CXCR7 has potentially therapeutic target for EC.

Keywords: Esophageal cancer, CXCR2, CXCR7, siRNA, clinicopathological features, progression

Introduction

Esophageal cancer (EC) is the eighth most common malignancy worldwide and the sixth leading cause of cancer mortality [1]. Whereas, EC has been ranked as the fourth most common cause of cancer death in China [2], and it accounts for approximately one-tenth of all malignancies mortality [2]. More than 90% EC patients fail to be diagnosed as a pre-malignant condition [3, 4]. A randomized control trial indicated that endoscopic screening and surveillance for patients with esophageal neoplasia were unpractical due to the expensive cost and relatively low benefit for patients [5]. Most patients develop into advanced stage before this disease could be suspected or diagnosed. In spite of considerable progresses have been made in diagnosis and management, the overall prognosis of EC is still poor [6-9]. The 5-year

survival rate of EC is about 15-25% [2]. Therefore, a better and a deeper understanding of the molecular mechanisms underlying EC is helpful to search novel and tailored therapeutic strategies.

It has been well demonstrated that both chemokines and their receptors play significant roles in tumor development [10-13]. Among the chemokines, chemokine ligand (CXCL) 12/stromal-derived factor (SDF)-1 and interleukin (IL) 8/CXCL-8 have been reported to be involved in tumor progression and metastasis of various entities [14, 15]. Recently, the functional roles of CXCL12 and its receptor chemotaxis cytokine receptor (CXCR) 4 or CXCR7, and CXCL-8 and its receptor CXCR2 in EC have been well investigated [16, 17]. Although several studies have investigated the function and clinical significance of CXCR2 and CXCR7 in EC, however,

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rare studies have been done concerning the impact of silencing of CXCR2 and CXCR7 on EC cells.

In the present study, we performed the small interfering RNAs (siRNA) to silence the expression of CXCR2 and CXCR7 into the three EC cell lines (TE-1, EC9706, and EC109 cells). The cell growth, chemotaxis, invasion, and apoptosis of these cells were explored, as well as the potential associated mechanism. Besides, animal experiment was used to analyze the effect of silencing of CXCR2 and CXCR7 on tumor growth *in vivo*. In addition to cell and animal researches, the associations between CXCR2/7 expression in human EC and clinicopathological features and progression were explored.

Materials and methods

Cell lines and cultures

The human EC cell lines TE-1, EC9706, and EC109 were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Dualbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 100 µg/µL streptomycin (Gibco, Gaithersburg, MD, USA) and 100 µg/µL penicillin (Gibco, Gaithersburg, MD, USA) at 37°C in a humidified incubator with 5% CO₂.

Transfection

After 24 h post-culturing in DMEM at an appropriate concentration (about 60-80%), the TE-1, EC9706, and EC109 cells were plated in 6-well plates, respectively. Then the specific siRNA against CXCR2, CXCR7, and nonspecific (NS)-siRNA (GenaPharma Shanghai, China) were respectively transfected into TE-1, EC9706, and EC109 cells at a final concentration of 60 nM using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 4 h of incubation, normal media was added. In addition, cells in control group received no special treatment. Then the cells were harvested after 48 h for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was respectively extracted from TE-1, EC9706, and EC109 cells by TRIzol reagent

(Invitrogen Corp., Carlsbad, CA) using a commercially available kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Complementary DNA (cDNA) was synthesized using the Reverse Transcription System (Promega, Madison, WI). The products of PCR were identified by an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a reference control. PCR amplification was carried out using LightCycler - FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan). PCR conditions included 1 predenaturation cycle of 1 min at 95°C, 50 cycles of 95°C for 30 s, 58-62°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The mRNA levels were expressed as threshold cycle (CT). The amount of target was measured using 2^{-ΔΔCT} method. Reactions were carried out in triplicate.

The primers sequences used were as follows: CXCR2, 5' primer-AGGCAC AGTGAAGACATCGG-3' and reverse, 5' primer-CAGCAGGCTCAGCAGGAATA-3' [18]; CXCR7, 5' primer-TGGGTG-GTCAGTCTCGT-3' and reverse, 5' primer-CCGG CAGTAGGTCTCAT-3' [19]; and GAPDH, 5' primer-CGGAGTCAACGGATTG GTC-3' and reverse, 5' primer-AGCCTTCTCCAT GGTCGTGA-3'.

Cell proliferation, malignant and metastasis assays

For proliferation rate, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, TE-1, EC9706, and EC109 cells were respectively placed in 96-well plates at a final concentration of 5×10⁴ per mL in 100 µL medium. Then the plates were maintained at 37°C in 5% CO₂ incubator. After 24 h incubation, MTT (20 µL; 5 g/L) was added to each well and the plates were incubated at 37°C for another 4 h. The absorbance at 570 nm was determined with a microplate reader (Model Benchmark). Experiments were performed 3-5 times.

For cell invasion assays, Transwell chamber (Costar, Cambridge, MA) were used. Briefly, cells were harvested and then were suspended in serum-free dulbecco's modified eagle medium (DMEM) media at a density of 1×10⁵ cells/mL on 24-Transwell membranes (8 µm pores). The upper chamber of the transwell was coated

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with 70 μ L Matrigel (1 mg/mL), and the lower chamber was filled with 10% FBS. Cell suspensions (100 μ L) were added to the upper side, and then were disassembled after incubation at 37°C in 5% CO₂ for 24 h. The membranes were fixed in 1% glutaraldehyde and then stained with 0.5% crystal violet reagent. The remaining cells were counted in eight random microscopic fields per membrane.

For chemotaxis assays, a modified Boyden chamber was performed. In brief, polycarbonate filter (8 μ m pores) coated with collagen type IV was placed on a 48-well chamber. TE-1, EC9706, and EC109 cells (1×10^5 cells/well) were loaded into the upper chamber. The filter was disassembled after incubation at 37°C in 5% CO₂ for 6 h. Then the cells were fixed and stained. The number of cells that moved through the chamber was counted by measuring absorbance at 595 nm.

Flow cytometry (FCM) detection

For the apoptosis rate, Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (Biosea, China) was used in accordance with the manufacturer's instruction. Briefly, cells (5×10^6) were harvested, mixed with 10 μ L Annexin, and incubated at room temperature in the dark for 15 min. Then 5 μ L 10 mg/L propidium iodide (PI) was added. Finally, the cells were read by FCM (Becton Dickinson, San Jose, CA, USA) with excitation wavelength at 488 nm and emission wavelength at 635 nm. CELLQuest 3.0 software (Becton Dickinson, San Jose, CA) was performed to analyze the numerical values.

Western blotting for signaling pathway analysis

For Western blotting analysis, TE-1 cells were harvested for protein extraction. Protein density was determined using a BCA assay kit (TaKaRa BIO INC, Japan). Proteins samples (20 μ L) were resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bedford, MA, USA). The membranes were blocked with 5% defatted milk powder in phosphate buffer saline (PBS) for 2 h at room temperature and incubated overnight at 4°C with the following antibodies: anti-pERK1 (1:1,000), anti-pERK2 (1:1,000), anti-MMP-9 (1:1,000) and anti- β -

actin (1:1,000). The membranes were then incubated with an appropriate secondary antibody for 2 h at room temperature. All the antibodies were purchased from Santa Cruz Biotechnology. Finally, enhanced chemiluminescence and densitometric analysis were performed according to the manufacturer's instructions.

Mouse xenograft study

Male athymic nude mice (NCI-nu, 8-12 weeks of age) were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences. The mice were housed in a pathogen-free barrier facility. The use of animal was approved by the local Ethics Committee and was in line with the Guide for Care and Use of Laboratory Animals published by the China National Institutes of Health. Each mouse was subcutaneously injected in the right flank through a 22-gauge needle with EC9706 (1×10^6) cells treated with or without CXCR2 siRNA and CXCR7 siRNA. Mice injected with cell lines expressing green fluorescent protein (GFP) siRNA were regarded as controls. The mice (six in each group) were weighed, and tumor mass volumes were measured at 7, 14, 28, and 35 days throughout the study. The tumor mass volumes were recorded as follows: length \times width²/2 with a vernier caliper. Five weeks after cell inoculation, the mice were killed by CO₂ asphyxiation. The tumor xenografts were excised, weighed and recorded.

Human tissue and immunohistochemistry (IHC)

The use of human tumor specimen was approved by the local Ethics Committee and informed consents were obtained from all patients. Between April 2008 and December 2014, a total of 156 patients with esophageal squamous cell carcinoma (ESCC) who underwent surgery at our hospital were enrolled into the retrospective study. Samples including carcinoma tissue and paired adjacent normal tissues were selected from patients after obtaining the patients' complete information on clinicopathologic characteristics. The ages of the patients ranged from 48 to 86 years (median, 67 years). Of the patients, 91 were men and 65 were women. Patients were diagnosed with ESCC according to the clinical pathological findings. None of the patients received preopera-

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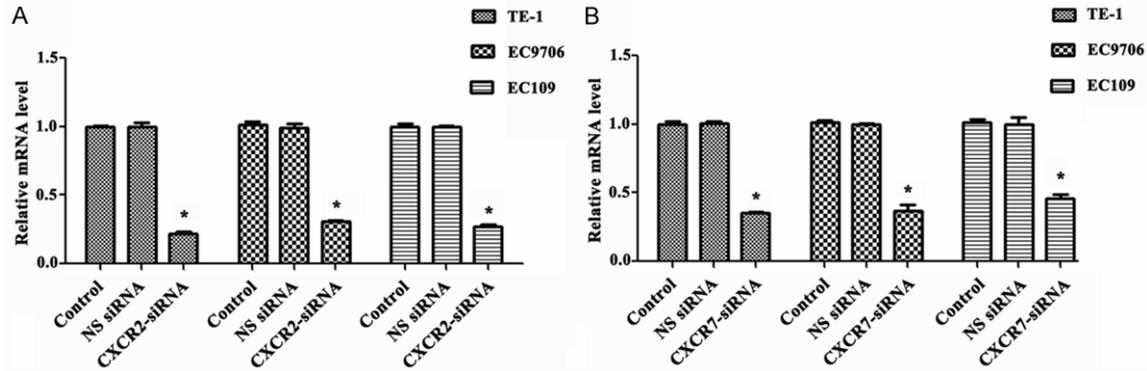


Figure 1. SiRNA reduces the expression of CXCR2 and CXCR7 in TE-1, EC9706 and EC109 cells. A. Relative expression of mRNA levels after transfection with CXCR2 siRNA; B. Relative expression of mRNA levels after transfection with CXCR7 siRNA. SiRNA, small interfering RNAs; CXCR, chemotaxis cytokine receptor. * $P < 0.05$ compared with control group and NS siRNA group.

tive special treatment (e.g. radiotherapy, chemotherapy, neoadjuvant or adjuvant treatment). According to Tumor Node Metastasis (TNM)/Union for International Cancer Control (UICC) classification 6th edition, the number of I:II_A:II_B:III was 32:20:48:56. The follow up for survival were recorded from all patients. By April 2015 (the time of data analysis), 66 patients had died and 90 patients were alive. The median survival time was 64 months.

The samples both from the tissue and surrounding normal tissue were embedded in paraffin. Tissue sections (4 μ m) were prepared for IHC analyses of CXCR2 and CXCR7. In brief, the sections were de-paraffinized and, rehydrated, and were then subjected to antigen retrieval. Endogenous peroxidase was eliminated by the use of 3% H₂O₂ in PBS for 10 min. The slides were then incubated with normal horse or goat serum in PBS and anti-CXCR2 (1:100) or anti-CXCR7 (1:100) diluted in PBS overnight at 4°C. After being washed with PBS, the samples were incubated with a biotinylated second antibody (Horse anti-mouse IgG or Goat-anti rabbit IgG) (GBI) for 30 min at room temperature, developed with 3, 3'-diaminobenzide tetrahydrochloride (DAB, Vector Laboratories Inc., Burlingame, CA, USA), counterstained with Mayer's haematoxylin solution (Sigma) for 30 s, and scored under a microscope.

The staining intensity (0, 1⁺, 2⁺, 3⁺) and the positivity was quantified according to a previous study [20]. CXCR2/7 was recorded as negative when the spots without staining and spots with a staining intensity of 1⁺ accounted for $\leq 20\%$ of

tumor cells, and while was considered as positive when spots with a staining intensity of 1⁺ accounted for $> 20\%$ tumor cells, or spots with staining intensity ≥ 2 .

Statistical analysis

The Kolmogorov-Smirnov (K-S) one-sample test was performed to test the normal distribution for all collected data. Chi-square test or rank-sum test was used to analyze enumeration data. Student t-test was used to evaluate the difference between two groups, while analysis of variance (ANOVA) was conducted to compare the difference for more than three groups. Post-hoc Tukey test was performed to examine significant pairwise comparisons. Univariate survival analysis was determined with Kaplan-Meier method, and log-rank test was performed to assess the difference between the survival curves. All the statistical analysis was determined by statistic package for social science (SPSS, version 17.0, Chicago, IL). A statistical significance was defined when $P < 0.05$.

Results

SiRNA reduced the expression of CXCR2 and CXCR7 in EC cell lines

To examine the impact of silencing of CXCR2 and CXCR7 on EC cells (TE-1, EC9706, and EC109 cells), CXCR2 siRNA and CXCR7 siRNA were used to down-regulate the expression of CXCR2 and CXCR7 in TE-1, EC9706 and EC109 cells. After 48 h transfection, expression mRNA levels of CXCR2 and CXCR7 was validated by

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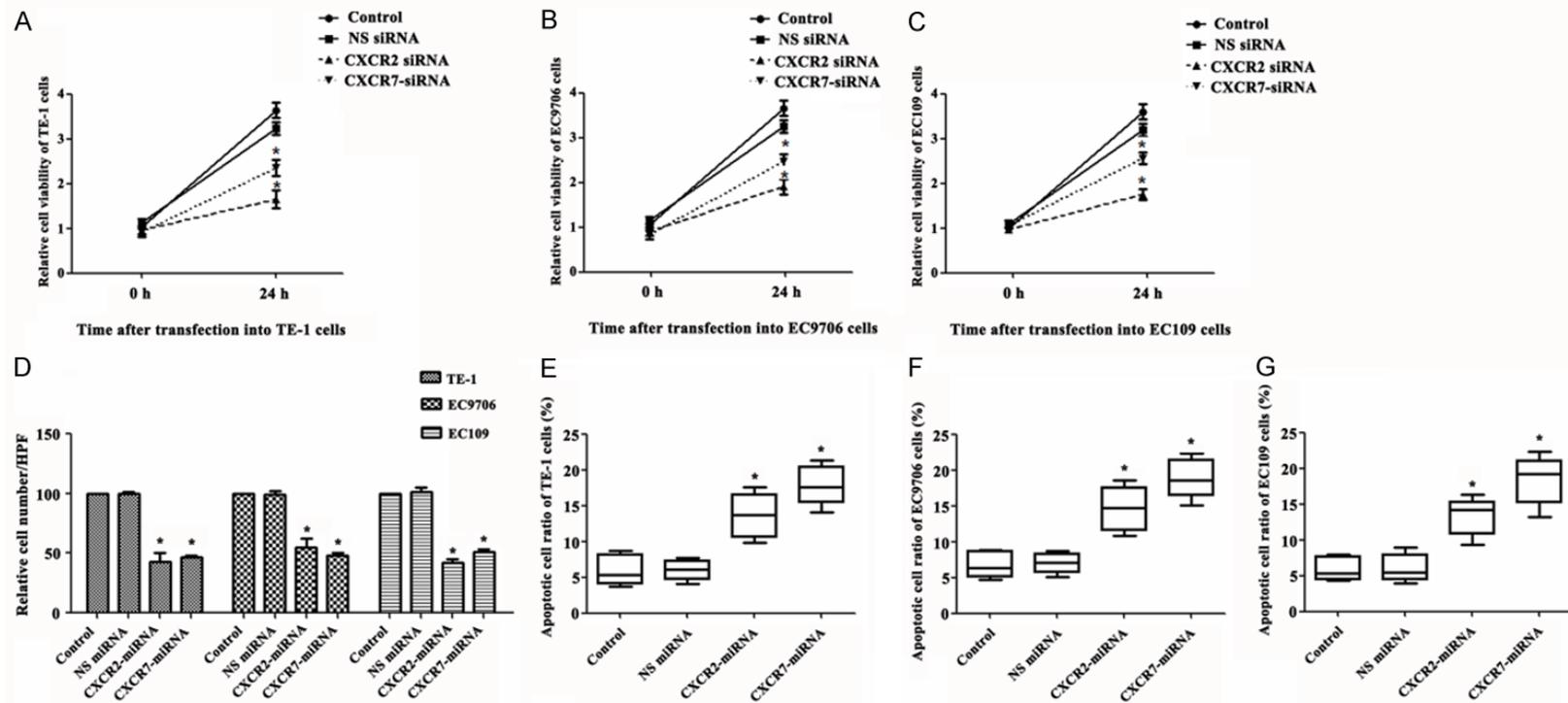


Figure 2. Silencing of CXCR2 and CXCR7 decreases cell viability and chemotaxis, and increases apoptotic rate. A-C. Silencing of CXCR2 and CXCR7 to TE-1, EC9706 and EC109 cells decreases cell viability; D. Silencing of CXCR2 and CXCR7 to TE-1, EC9706 and EC109 cells decreases cell chemotaxis; E-G, Silencing of CXCR2 and CXCR7 to TE-1, EC9706 and EC109 cells increases apoptotic rate. SiRNA, small interfering RNAs; CXCR, chemotaxis cytokine receptor; NS, nonspecific. * $P < 0.05$ compared with control group and NS siRNA group.

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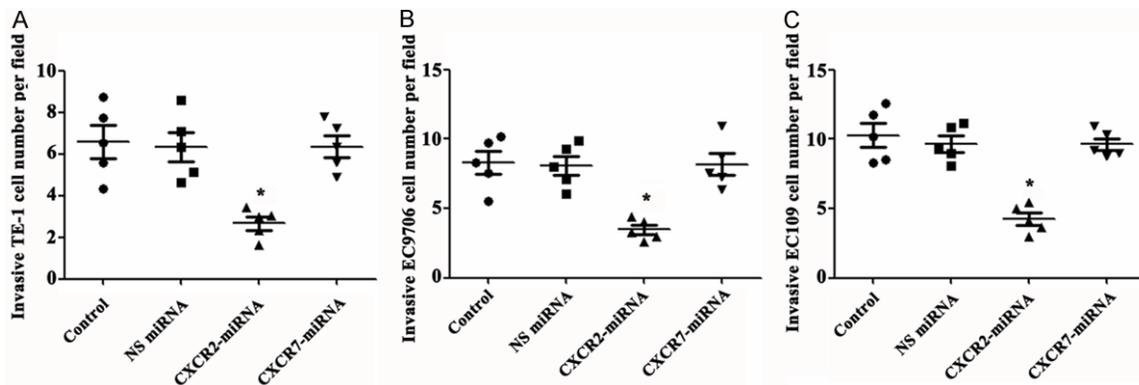


Figure 3. Silencing of CXCR2, but not CXCR7, induces cells invasion. A-C. Silencing of CXCR2, but not CXCR7, induces cells invasion in TE-1, EC9706 and EC109 cells, respectively. SiRNA, small interfering RNAs; CXCR, chemotaxis cytokine receptor; NS, nonspecific. * $P < 0.05$ compared with control group and NS siRNA group.

qRT-PCR. The results showed that both the expression mRNA levels of CXCR2 and CXCR7 in TE-1, EC9706 and EC109 cells were significantly reduced after transfection of CXCR2 siRNA and CXCR7 siRNA, respectively ($P < 0.05$) compared with NS-siRNA and control group, respectively ($P < 0.005$). Besides, these cells transfected with NS siRNA had no evidence of knockdown of CXCR2 and CXCR7 (**Figure 1A** and **1B**).

Silencing of CXCR2 and CXCR7 decreased cell viability and chemotaxis, and increased apoptotic rate

To investigate the impact of silencing of CXCR2 and CXCR7 on EC cells, cell viability, chemotaxis, and apoptotic rate were evaluated in TE-1, EC9706 and EC109 cells. We found that both silencing of CXCR2 and CXCR7 could significantly decrease the cell viability (**Figure 2A-C**) and chemotaxis (**Figure 2D**) and increase the apoptotic rate (**Figure 2E-G**) compared with control group and NS siRNA group ($P < 0.05$).

Silencing of CXCR2, but not CXCR7, induced TE-1 cells invasion

To explore the invasion ability of silencing of CXCR2 and CXCR7 on TE-1, EC9706 and EC109 cells, we used the matrigel invasion assay. We found that silencing of CXCR7 failed to induce these three kinds of cells invasion. There were no significant differences among the control group, NS siRNA group and CXCR7 siRNA group. However, silencing of CXCR2 induced significant invasion of these three kinds of cells compared with the control group and NS siRNA group (**Figure 3A-C**).

Silencing of CXCR2 and CXCR7 to TE-1 cells activated ERK1/2 pathways

To confirm whether silencing of CXCR2 and CXCR7 could induce ERK1/2 pathway activation in TE-1 cells, we measured associated protein expression of ERK1/2 pathway after transfection with siRNA. The results demonstrated that silencing of CXCR2 and CXCR7 both significantly decreased the expression protein levels of pERK1 and pERK2 compared with control group and NS siRNA group, indicating silencing of CXCR2 and CXCR7 was involved in activation of ERK1/2 pathways. In addition, the expression of MMP-9 was closely related with silencing of CXCR2, but was not related with silencing of CXCR7 (**Figure 4A-D**).

Silencing of CXCR2 and CXCR7 reduced in vivo tumor growth

To investigate whether silencing of CXCR2 and CXCR7 was associated with *in vivo* tumor growth in EC cells, we tested tumor sizes of mouse xenograft tumor growth after inoculation. As indicated in **Figure 5**, the subcutaneous tumor sizes in mice (**Figure 5A** and **5B**) were both significantly declined in EC9706 cells treated with CXCR2 siRNA and CXCR7 siRNA compared with the controls (GFP siRNA).

Silencing of CXCR2 and CXCR7 associated with progression

To explore the role of CXCR2 and CXCR7 in EC, we collected 156 EC samples with paired adjacent normal tissues and performed IHC. The results showed that both CXCR2 and CXCR7 positive staining were observed in cytoplasm

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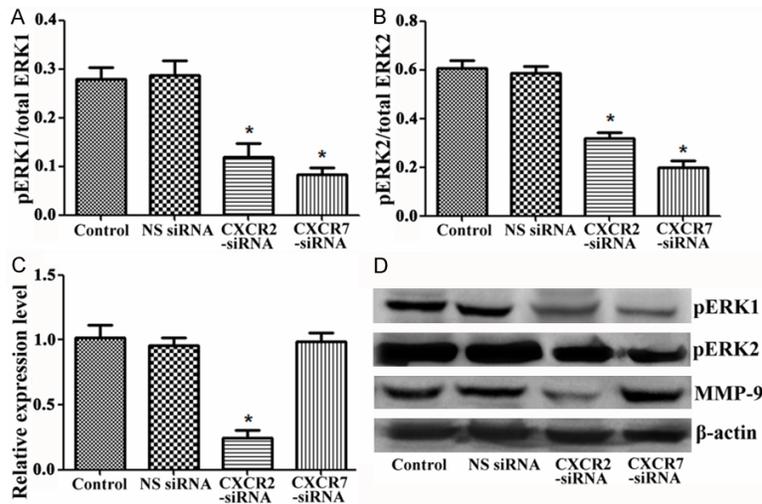


Figure 4. Silencing of CXCR2 and CXCR7 to TE-1 cells activates ERK1/2 pathways. A. pERK1/total ERK1 protein level after transfection with siRNA; B. pERK2/total ERK2 protein level after transfection with siRNA; C. Relative expression level of MMP-9 after transfection with siRNA; D. The photo of Western blotting. SiRNA, small interfering RNAs; CXCR, chemotaxis cytokine receptor; NS, nonspecific; MMP, matrix metalloproteinases. * $P < 0.05$ compared with control group and NS siRNA group.

and membrane of EC cells (**Figure 6A-D**). Both the protein expression levels of CXCR2 ($P = 0.006$) and CXCR7 ($P = 0.02$) were significantly up-regulated in carcinoma tissues compared with adjacent normal tissues (**Figure 6E**). In addition, the results of follow-up data showed that the survival rate of patients with high expression of CXCR2 and CXCR7 was significantly lower than that with low expression of CXCR2 ($P = 0.016$) and CXCR7 ($P = 0.023$), respectively (**Figure 6F** and **6G**).

Silencing of CXCR2 and CXCR7 associated with clinicopathological features of EC

To explore the relationship between CXCR2/CXCR7 protein expression and EC progression, we evaluated the correlation between CXCR2/CXCR7 expression and clinicopathological features of ESCC. For the clinical TNM/UICC stage, the strongly positive rate of CXCR2 protein was 10.6%, 30.6%, 46.9%, and 73.8% in stage I, II_A, II_B, and III, respectively. The strongly positive rate of CXCR7 protein was 8.4%, 25.9%, 44.7%, and 69.6% in stage I, II_A, II_B, and III, respectively. The strongly positive rate of CXCR2 and CXCR7 in stage II_A, II_B, and III were significantly higher than those in stage I ($P = 0.000$ and $P = 0.021$, respectively). These results suggested that the high expression of CXCR2 and CXCR7 were

both closely associated with higher histological grade, advanced clinical stage in patients with ESCC.

Discussion

In the present study, we demonstrated that silencing of CXCR2 and CXCR7 could inhibit cell growth, reduce metastasis, induce apoptosis in EC cell lines, and reduce tumor growth *in vivo*. Besides, we found that high expression of CXCR2 and CXCR7 were closely associated with higher histological grade, advanced clinical stage, and lower survival in patients with ESCC. Our results suggest that silencing of CXCR2 and CXCR7 protects against EC.

The effects may be considered to be associated with activation of ERK1/2 pathways.

Chemokines are low-molecular-weight cytokines and can be subdivided into CXC and CC chemokines. They could selectively modulate leukocyte extravasation to inflammation areas via chemoattraction [21]. In addition to their roles in inflammatory responses, a good deal of evidence has suggested that chemokines also play significant roles in tumor. Both CXC chemokines and their receptors CXCR regulate tumor development by regulating angiogenesis, activating a tumor-specific immune response and stimulating cell proliferation [22]. Recently, the functional roles of CXCR7 and CXCR-2 in tumorigenesis and tumor development have been widely studied. CXCR7, the second receptor of CXCL12 (SDF-1), is highly conserved in mammals. It is a membrane associated receptor protein located on chromosome 1 in mice and on chromosome 2 with CXCR4, CXCR2, and CXCR1. CXCR-2 is a G-protein-coupled receptor of CXCL-8 (formally known as IL-8) that functions as an autocrine growth factor in many various malignant melanoma cells [23]. It has been well demonstrated that CXCR7 and CXCR-2 express on many human tumor cell lines, such as breast cancer, prostate cancer, lung cancer, EC and so on [17, 24-26]. Wang *et al.*

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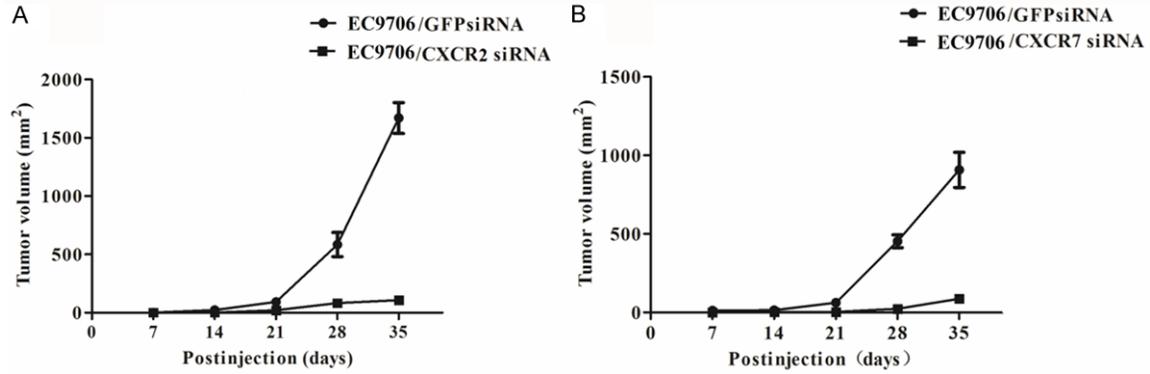


Figure 5. Silencing of CXCR2 and CXCR7 reduces *in vivo* tumor growth. SiRNA, small interfering RNAs; CXCR, chemotaxis cytokine receptor; GFP, green fluorescent protein.

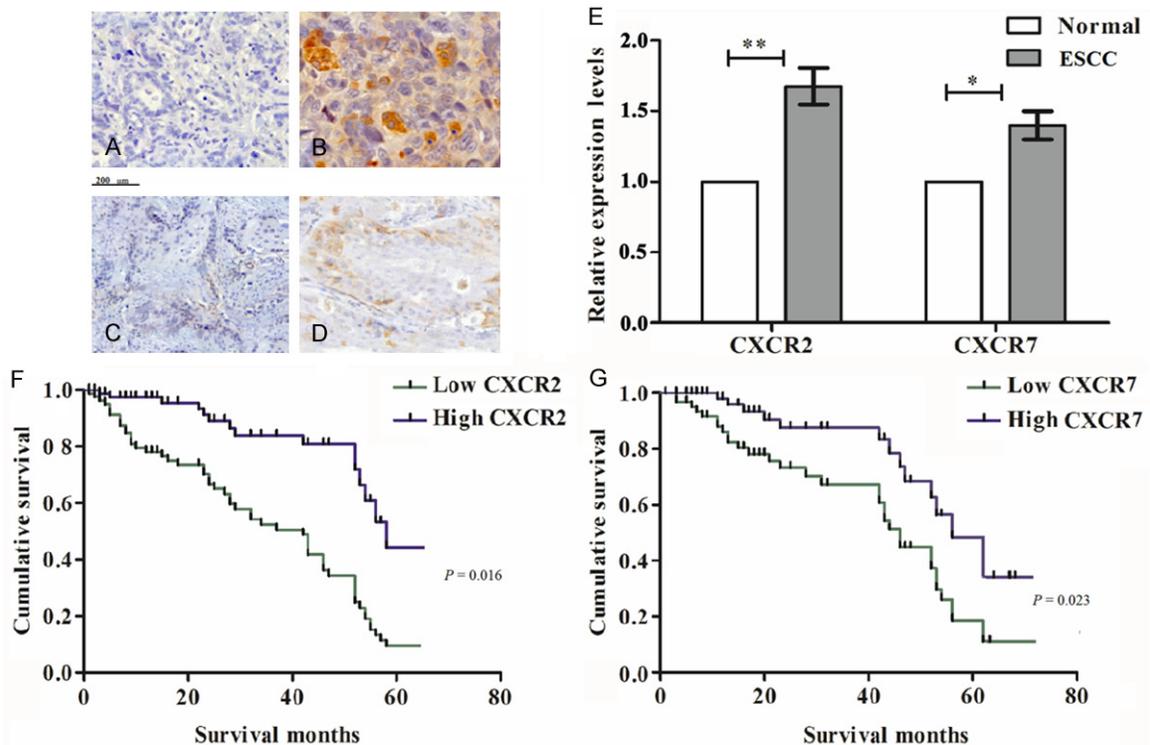


Figure 6. Silencing of CXCR2 and CXCR7 associates with progression. A and B. IHC of CXCR2 in normal tissue and cancer tissue, respectively; C and D. IHC of CXCR7 in normal tissue and cancer tissue, respectively; E. Quantitative analysis of IHC; F and G. Expression of CXCR2 and CXCR7 in EC tissues correlates with a lower cancer patient survival rate, respectively. IHC, immunohistochemistry; CXCR, chemotaxis cytokine receptor; ESCC, esophageal squamous cell carcinoma.

[27] suggested that autocrine growth-related oncogene (GRO) α -CXCR2 and GRO β -CXCR2 signaling pathway may be involved in esophageal carcinogenesis. In addition, Meijer *et al.* [28] proposed that CXCR7 inhibited tumor tumorigenesis mainly in tumor tissue with high expression of CXCL12, in cells with CXCL12 autocrine signaling, or in tumors at specific

stages. Hence, this autocrine signaling pathway contributes significantly to cancer cell proliferation and development.

Previous studies have indicated that CXCR2 is overexpressed in the cytoplasm and plasma membrane of ESCC, and is correlated with ESCC progression and metastasis [16, 29],

while CXCR7 is mainly expressed in ESCC and infrequently in adenocarcinoma [30]. Moreover, some researchers have found that the antagonists of CXCR2 (SCH-527123 and SCH-479833) protects against human colon cancer liver metastasis, indicating that inhibition of CXCR2 might be a potential therapeutic strategy for oncology [31, 32]. Besides, targeting CXCR2 as a treatment of pancreatic cancer has been deeply explored [33, 34]. To confirm the impact of suppression of CXCR2 and CXCR7 on EC, we depleted the expression of CXCR2 and CXCR7 through siRNA technology in TE-1, EC9706, and EC109 cells. Both the MTT and FCM results showed that silencing of CXCR2 and CXCR7 could statistically decrease the cell viability and chemotaxis, and increase the apoptotic rate of EC cell lines. Besides, our animal experimental research showed that silencing of CXCR2 and CXCR7 could significantly decrease the tumor growth at 7, 14, 28, and 35 days. In addition, we performed IHC on human tissues. Both CXCR2 and CXCR7 were expressed on cytoplasm and membrane of EC cells, which suggested that the source of ligands (both CXCR2 and CXCR7) were from the tumor cells. This autocrine signaling may be contributed to the tumor growth. Moreover, the higher expression of CXCR2 and CXCR7, the higher histological grade, advanced clinical stage, and lower survival were found in ESCC patients. These results were in line with previous studies, suggesting that CXCR2 and CXCR7 play significant roles in EC and depletion of CXCR2 and CXCR7 may be potential treatments of EC. However, further studies should be performed to confirm the conclusion.

It has been reported that expression of MMP-9 is involved in the differentiation, vessel permeation and lymph node metastasis of cancers because of its ability on degradation a basement membrane and extracellular matrix [35]. Moreover, some studies suggest the expression of MMP-9 play important roles in its malignant behavior, aggressiveness, and invasiveness on EC cells [36, 37]. MMP-9 expression has been considered as a negative, independent prognostic factor in ESCC and may be a potential biomarker of ESCC diagnosis and treatment [38]. Furthermore, the release of MMP-9 could be significantly reduced by blocking the expression of CXCR2 [39]. In accordance with previous studies, we also found that

expression of MMP-9 was significantly induced by silencing of CXCR2, but could not be induced by CXCR7 in our study. Additionally, many studies have suggested that CXCR2 signaling network is related to the activation of the mitogen-activated protein kinase (MAPK) MEK1/2, ERK1/2, and p38 [40, 41]. Similarly, in our study ERK1/2 pathways were found to be activated by silencing of CXCR2. In addition to the results, we found that ERK1/2 pathways were also activated by silencing of CXCR7.

In conclusion, our results suggest that silencing of CXCR2 and CXCR7 inhibits cell growth, reduces metastasis, and induces apoptosis in EC cell lines, reduces tumor growth *in vivo*. Besides, both CXCR2 and CXCR7 are closely associated with the EC progression. These effects may be involved in activation of ERK1/2 pathways. Our results reflect a potential functional contribution of CXCR2 and CXCR7 in EC, and offer new insights into molecular basis for treatment of EC.

Disclosure of conflict of interest

None.

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