Original Article
Decursin inhibits tumor growth, migration, and invasion in gastric cancer by down-regulating CXCR7 expression

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Abstract: CXC chemokine receptor 7 (CXCR7) is highly expressed in various type of cancers and promotes cancer progression and metastasis. However, the biological role and regulation of CXCR7 in gastric cancer remains unclear, and little is known about compounds that modulate CXCR7. Here, we investigated the role of CXCR7 in gastric tumorigenesis, and the effects of decursin, which is derived from Angelica gigas Nakai, on CXCR7. Our results showed that CXCR7 significantly promoted growth of gastric cancer cells and increased migration and invasion, which was mediated by the STAT3/c-Myc pathway. We also confirmed that decursin had an antitumor effect through down-regulating the expression of CXCR7 in gastric cancer. Furthermore, apoptotic cell death was induced through the reduction of anti-apoptotic factors such as Bcl-2 in vitro and in vivo. Our findings show that CXCR7 in gastric cancer promotes cancer progression through the STAT3/c-Myc pathway and that decursin is a natural compound that may target CXCR7 in gastric cancer treatment.

Keywords: CXCR7, decursin, growth, migration, invasiveness, gastric cancer

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of death worldwide [1]. Although great efforts have been made to discover therapeutic targets to reduce gastric cancer mortality, the overall prognosis of gastric cancer remains poor, and the 5-year survival rate is less than 40% [2]. Therefore, there is an urgent need to understand the molecular mechanisms of gastric cancer and to find novel therapeutic targets. Chemokine signaling systems, chemokine receptors, and chemokines mediate various biological processes such as metabolism, immunity, infection, and cancer [3-5]. Chemokine receptors are differentially expressed in many solid tumors and are involved in cancer progression and metastasis [6-9]. CXC chemokine receptor 7 (CXCR7) is a member of the G-protein-coupled receptor family and binds the chemokines CXCL11/I-TAC and CXCL12/SDF-1 [10, 11]. Growing evidence indicates that CXCR7 is differentially expressed and increases tumor aggressiveness in various types of cancer, including non-small cell lung-, bladder-, and breast cancer, and is closely related to tumor progression [12-14]. However, the role of CXCR7 in gastric cancer remains largely unknown. Therefore, it is necessary to investigate the role of CXCR7 in gastric cancer progression and its potential as a therapeutic target.

Decursin is derived from the roots of the herb Angelica gigas Nakai, which grows in various countries across Asia, including Korea, China, and Japan. Different parts of A. gigas have been screened for their therapeutic potential. Studies have shown that the roots of A. gigas contain therapeutically active components, including decursin. Decursin has been shown to exhibit anti-oxidant, anti-bacterial, anti-inflammatory, and anti-amnestic activities [15, 16]. Several studies also have shown that decursin exhibits antitumor activity against lung-, colon- and breast cancer, as well as myeloma and leukemia. Additionally, decursin has been reported to be involved in pathological conditions by con-
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Trolling various signaling pathways such as those involving the TGF-β receptor, estrogen receptor, and reactive oxygen species [15, 17]. However, little is known about the effects of decursin on the chemokine signaling system, especially CXCR7 in gastric cancer. In this study, we evaluated the biological role of CXCR7 in gastric cancer and assessed whether decursin exerts an antitumor effect by regulating CXCR7.

Materials and methods

Preparation of decursin

Decursin was prepared from decursinol which was isolated from the roots of A. gigas as described previously [18]. Briefly, for decursin synthesis, decursinol was added to a solution of dicyclohexylcarbodiimide, 3, 3-dimethylacrylic acid, and 4-dimethylaminopyridine with 350 ml of dry methylene chloride. The mixture was stirred at room temperature for 24 h and then filtered. The filtrate was evaporated under low pressure and purified using flash chromatography to yield decursin as a white powder. The structure of decursin was confirmed by comparing the spectra from nuclear magnetic resonance and mass spectrometry. The decursin was dissolved in dimethyl sulfoxide (DMSO) and in all the experiments the concentration of DMSO was limited to 0.1%.

Cell culture and stable cell line establishment

Two human gastric cancer cell lines-SNU484 and SNU216- were purchased from the Korea Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI-1640 medium (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1 × penicillin/streptomycin in a humidified incubator at 37°C with 5% carbon dioxide. Overexpression of CXCR7 in gastric cancer cells was produced by lentivirus-mediated transduction of full-length human CXCR7 sub-cloned into a pLVX-EF1α-IRES-Puro lentiviral vector (Clontech, Mountain View, CA, USA). To generate the CXCR7 overexpression lentivirus, the lentiviral vector was co-transfected with psPAX2 viral packaging and a PMD2.G envelope plasmid using Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. CXCR7 expression levels were analyzed using Western blot and flow cytometry.

Small interfering RNA (siRNA) targeting CXCR7

We obtained the siCXCR7 (5'-CCU GCU CUA CAC GCU CUC TT-3') and non-target siRNA control from Bioneer (Daejeon, Korea). Cells were transfected using Lipofectamin RNAiMax (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, cells were lysed or used in the experiments.

Western blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were washed twice in cold phosphate-buffered saline (PBS) and whole cell protein extracts were obtained using a ProEX™ CETi Lysis buffer (TransLab, Korea) containing protease and phosphatase inhibitors. The cell lysates were incubated on ice for 15 min and then centrifuged at 13,000 rpm for 20 min at 4°C to remove any cell debris. Protein samples were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane and analyzed using primary antibodies. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using qPCR RT Master Mix (TOYOBO, Osaka, Japan). RT-PCR was performed using EmeraldAmp Master Mix (TaKaRa Bio, Shiga, Japan).

Flow cytometry

Cells were dissociated using an enzyme-free cell dissociation solution (Millipore, Burlington, MA, USA) for 5 min and washed using 0.1% bovine serum albumin (BSA) in PBS. Next, 10 µl of conjugated CXCR7-PE (R&D Systems, Minneapolis, MN, USA) or mouse IgG2A-PE antibodies (negative control) were added into the 1 × 10^5 cell suspension and incubated for 1 h at room temperature or for 2-3 h at 4°C. After the antibodies had bound, any remaining antibodies were removed by washing three times with the same buffer. Cell sorting was performed using a MoFlo cell sorter (Beckman Coulter, Brea, CA, USA) and the resultant histograms were created using the Kaluza analysis program (ver. 1.2; Beckman Coulter).

Cell proliferation and anoikis assay

The cells were seeded at 3 × 10^3 cells/well in a 96-well plate and treated with different doses of decursin. Cell proliferation was determined...
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using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Santa Clara, CA, USA) and absorbance was measured at 450 nm on an ELISA reader (Molecular Devices, San Jose, CA, USA). In the anoikis assay, cells were plated onto an ultra-low attachment plate (Corning, NY, USA) and incubated for ~5-7 days.

Clonogenic assay

For this assay, we seeded $3 \times 10^3$ cells onto a 6-well plate. The cells were treated with each concentration of decursin and washed three times with PBS after 24 h. Once the appropriate colony size had formed, the plates were rinsed with PBS three times and fixed with 10% formalin overnight at 4°C. Colonies were then stained with 0.1% crystal violet at room temperature for 1 h and observed under a microscope.

Wound healing assay

Wound healing and migration assays were conducted by seeding $5 \times 10^4$ cells into a Culture-Insert 2 well (Ibidi, Madison, WI, USA) and waiting until cells had attached. After 24 h, the media was carefully removed and the decursin was replaced with diluted media and decursin. After 24 h, the diluted media was removed, and the culture-insert was removed using forceps. The migration of cells to the edge of the scratch was analyzed at 0, 8, and 24 h, and microscopic images of the cells were captured.

Transwell assay

Migration and invasion abilities were examined in 8-µm-pore Transwell chambers (Corning, New York, NY, USA). Briefly, the lower surface of the insert was coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) and the upper side was coated with 50 µg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Fresh medium containing 10% FBS was placed in the lower chamber as a chemottractant. We loaded 100 µl cell suspension into each of the upper wells and the chamber was incubated at 37°C for 24 h. After incubation, inserts were fixed with 10% formalin and stained with 0.1% crystal violet. The number of cells in six random fields was counted for each assay.

Apoptosis detection

Cell apoptosis was detected with an apoptosis/necrosis detection kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions. The cells were treated with varying concentrations of decursin. After 24 h, the cells were washed twice with cold PBS before being incubated with Apopxin-FITC for 1 h at room temperature. The cells were then washed with 0.1% BSA in cold PBS and the level of apoptosis was examined using fluorescence-activated cell sorting.

Immunofluorescence

Cells were transferred onto gelatin-coated coverslips in a 6-well plate. Samples were washed with cold PBS and fixed in 10% formalin for 20 min at room temperature or overnight at 4°C. After removing the fixation solution and washing with PBS, the cell membrane was permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) for 20 min. Samples were blocked with 4% BSA in PBS for 1 h at room temperature and incubated with rabbit anti-CXCR7 (Abcam) and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After washing to remove the primary antibodies, the samples were incubated with the appropriate fluorescence-conjugated secondary antibodies for 1 h in the dark. The samples were then mounted using fluorescence mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).

Immunohistochemistry

Sections of 4-mm-thick paraffin-embedded mouse tumor tissue were prepared for immunohistochemical staining. The slides were deparaffinized in xylene and then rehydrated in a graded alcohol series. Sections were washed in water before antigen retrieval was performed by microwaving the sections in Antigen Retrieval Buffer with pH 9.0 (Dako, Carpinteria, CA, USA). Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide buffer for 10 min. Slides were blocked for 20 min with protein block buffer (Dako), Primary antibodies were diluted in blocking solution and incubated for 1 h at room temperature. Subsequently, the slides were incubated with the secondary antibody for 30 min and were then counterstained with hematoxylin.

Mouse model

Balb/c-nude mice (4-week-old females) were purchased from DooYeol Biotech (Seoul, Korea).
and were maintained in a pathogen-free environment. In the experiment, SNU484-CXCR7 cells cultured on the plate were pre-treated with DMSO or 40 µM decursin for 24 hours before being injected into the mice. The cells were washed three times with cold PBS to minimize the effect of the remaining compound. We injected 5 × 10^6 cells subcutaneously into both mice flanks with a 1:1 ratio of Matrigel. On day 47, the mice were sacrificed, and the dissected tumor masses were fixed in 10% formalin (Figure S1). The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Chungnam National University School of Medicine (Daejeon, Korea).

Statistical analyses

Data from the experiments are expressed as means ± the standard error of the mean (SEM). Differences between groups were analyzed using the Student's t-test. A P-value < 0.05 was considered to indicate statistical significance. Each mean was calculated from at least three independent experiments.

Results

**CXCR7 promotes tumor growth, migration, and invasion in gastric cancer**

We established stable gastric cancer cell lines that overexpressed CXCR7 using lentivirus-mediated transduction of an expression vector for full-length human CXCR7. Relative expression of CXCR7 in two different gastric cancer cell lines-SNU484 and SNU216- was confirmed using flow cytometry, Western blotting, RT-PCR, and immunofluorescence staining (Figures 1A and S2). Overexpression of CXCR7 significantly increased the proliferation of gastric cancer cells (Figure 1B). CXCR7 overexpression also en-
Enhanced anchorage-independent growth, as determined by the number and size of colonies in the anoikis assay (Figure 1C). To assess the role of CXCR7 on gastric cancer cell motility, Transwell migration- and invasion assays were performed. Migration and invasion levels were significantly higher in CXCR7-overexpressing cells than in control cells, based on numbers of migrated (Figure 1D) and invaded cells (Figure 1E).

Abrogation of CXCR7 reverses CXCR7-induced biological effects

To confirm whether enhanced cell growth, migration, and invasion in CXCR7-overexpressing gastric cancer cells were due to CXCR7, CXCR7 was knocked down in SNU484- and SNU216-CXCR7 cells using siCXCR7 and control siRNA, respectively (Figure 2A). Knockdown of CXCR7 significantly inhibited proliferation, anchorage-independent growth, migration, and invasion (Figure 2B-E), consistent with the images shown in Figure 1. Taken together, these data indicate that CXCR7 plays an important role in tumor growth and metastasis in gastric cancer.

Decursin reduces expression of CXCR7 and inhibits cell growth and survival in gastric cancer

To determine the effect of decursin on CXCR7 and its antitumor activity, we first investigated changes in CXCR7 expression in gastric cancer cells treated with decursin. Treatment with decursin markedly decreased expression of CXCR7 in SNU484-CXCR7 and SNU216-CXCR7 gastric cancer cells in a dose-dependent manner (Figures 3A and S3). Next, cell proliferation, clonogenic, and anoikis assays were performed to assess the biological effects of decursin. Decursin treatment inhibited cell proliferation.
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Figure 3. Decursin reduces expression of CXCR7 and inhibits cell growth and survival in gastric cancer. (A) Expression of CXCR7 in SNU484-CXCR7 and SNU216-CXCR7 cells treated with 20 or 40 µm decursin for 24 h determined using flow cytometry. (B) Proliferation of SNU484-CXCR7 and SNU216-CXCR7 cells treated with 20 or 40 µm decursin assessed using a CCK-8 assay. (C) Clonogenic and (D) anoikis assays of SNU484-CXCR7 and SNU216-CXCR7 cells treated with 20 or 40 µm decursin. Scale bar = 200 µm. (E) SNU484-CXCR7 cells pretreated with DMSO or 40 µM decursin were subcutaneously injected into both flanks of BALB/c nude mice. (Left panel) Excised xenograft tumors of the control (n = 5) and decursin-treated mice (n = 5). (Right panel) Representative photomicrographs of the excised tumors of the control and treated mice (x 40). *P < 0.05; **P < 0.01; ***P < 0.001.
in a dose-dependent manner in both SNU484-CXCR7 and SNU216-CXCR7 cells (Figure 3B). Consistent with the proliferation assay, the clonogenic and anoikis assays showed that decursin treatment decreased proliferative cell numbers (Figure 3C, 3D). To further validate the effect of decursin on tumor growth, a tumor formation assay was performed in nude mice using SNU484-CXCR7 cells. As expected, the decursin pre-treatment group had smaller tumors than the control group (Figure 3E, left panel). Consistent with the in vitro cell proliferation assay, the number of living cells in the tumor mass was much higher in the control group than in the decursin pre-treatment group (Figure 3E; right panel). These results suggest that decursin reduced cell survival and growth through the down-regulation of CXCR7 in vitro and in vivo.

**Decursin inhibits migration and invasion and induces apoptosis in gastric cancer**

Treatment with decursin markedly inhibited wound healing ability in a dose-dependent manner in SNU484-CXCR7 and SNU216-CXCR7 cells (Figure 4A). In addition, cell migration and invasiveness were greatly reduced in gastric cancer cells treated with decursin (Figure 4B, 4C). To determine whether decursin down-regulates CXCR7 and induces apoptosis in gastric cancer cells, apoptosis rates were measured using Apopxin-FITC. The results revealed that apoptosis rates increased 3-fold in cells treated with decursin compared to control cells (Figure 4D). Expression of Bcl-2, an anti-apoptotic molecule, was also significantly reduced in gastric cancer cells treated with decursin in vitro and in vivo (Figure 4E, 4F). These findings suggest that decursin inhibits not only the motility and invasiveness of gastric cancer cells but also induces apoptosis by down-regulating CXCR7 expression.

**Decursin down-regulates c-Myc expression by inhibiting CXCR7-mediated STAT3 signaling**

Next, we investigated the mechanism by which CXCR7 contributed to gastric cancer progression. Using gastric cancer cell lines, several signaling molecules related to cancer biology were screened by western blotting. As depicted in Figure S4A, we observed that overexpression of CXCR7 increased phosphorylation of STAT3. Public RNAseq data also showed positive correlation between CXCR7 and STAT3 (Figure S4B). We therefore assumed that CXCR7 would influence cancer progression through the STAT3 pathway. CXCR7 overexpression increased phospho-STAT3 and subsequently increased downstream c-Myc in the gastric cancer cells (Figure 5A). In addition, phospho-STAT3 and c-Myc were restored by knockdown of CXCR7 in CXCR7-overexpressing cells (Figure 5B), consistent with results displayed in Figure 5A. Furthermore, as expected, phospho-STAT3 and c-Myc expression were reduced by decursin treatment in CXCR7-overexpressing gastric cancer cells in a dose-dependent manner (Figure 5C). Moreover, the expression of cleaved PARP and cleaved caspase-3 were up-regulated after CXCR7-overexpressing gastric cancer cells were treated with decursin (Figure 5C).

**Discussion**

In this study, we demonstrated that CXCR7 increased cell proliferation, growth, migration, and invasion in gastric cancer via the up-regulation of STAT3/c-Myc signaling. In addition, we showed that a natural product derivative—decursin-inhibited gastric cancer progression by down-regulating CXCR7 expression. Our results suggest that CXCR7 may be a potential therapeutic target for gastric cancer treatment because it plays an important role in cancer progression. We also demonstrated that decursin could be a new CXCR7-inhibiting antitumor agent to treat gastric cancer.

Cancer cells grow much faster than normal cells and exhibit abnormal migration and invasion, finally metastasizing to other organs, which is a very important characteristic directly related to the prognosis of patients with cancer. In particular, gastric cancer remains common despite a steady decrease in incidence, and is the second major cause of cancer-related deaths [19]. The most powerful risk factor for gastric cancer is *Helicobacter pylori*. It is estimated that nearly 20% of the world’s population have been infected by *H. pylori*, which is related to more than 60% of gastric cancer cases [20, 21]. Although *H. pylori* is a very important factor in gastric cancer, there are also many other risk factors involved in its promotion [22]. For example, a variety of chemokine receptors are overexpressed in gastric cancers and facilitate the progression of cancer in relation to cancer growth, metastasis, angiogenesis, and inflammation [4, 16, 23].
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A

Control  20uM  40uM  Control  20uM  40uM

0h  8h  24h

SNU484-CXCR7  SNU216-CXCR7

B

Control  20uM  40uM  Control  20uM  40uM

SNU484-CXCR7  SNU216-CXCR7

C

Control  20uM  40uM  Control  20uM  40uM

SNU484-CXCR7  SNU216-CXCR7

D

Count

Control  20uM  40uM

SNU484-CXCR7

7.56%  14.19%  20.13%

Apoxin-FITC

22.34%  58.28%

E

Bcl-2

DAPI

Merge

Control  40uM  Control  40uM

SNU484-CXCR7  SNU216-CXCR7

F

Control  Decursin

Bcl-2

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Among them, the role of CXCR7 and its therapeutic potential remains relatively unknown in gastric cancer.

CXCR7 promotes cell growth of breast cancer, induces TGFβ1-mediated epithelial-mesenchymal transition, and presents the characteristics of tumor infiltration in lung cancer [14, 24]. In addition, the interaction with its ligand-CXCL12 contributes to the invasive phenotype of pancreatic cancer, making CXCR7 expression a potential therapeutic target [25, 26]. Results from microarray analyses, immunohistochemistry, and reverse-transcription PCR have revealed that CXCR7 expression was elevated in cases of gastric cancer. CXCR7 is up-regulated in tumor tissues compared to normal tissues and CXCR7 overexpression is reported to be associated with a poor prognosis [27]. In our study, CXCR7 increased the proliferation and growth of gastric cancer cells and promoted cell motility, migration, and invasiveness.

The important role of CXCR7 in cancer development and metastasis implies that its control is one way to suppress the progression of malignancy.
nant tumors. Targeting CXCR7 inhibited glioma cell growth and mobility, and miR-100 also suppressed esophageal cancer growth by down-regulating CXCR7 [28, 29]. In addition, the combination of irradiation and CCX771-a specific CXCR7 inhibitor- has been shown to have beneficial effects on brain tumors [30], and the CXCR7 inhibitor POL6926 inhibited tumor progression in multiple myelomas [31]. LYG-202 synthesized from a natural flavonoid reduces the activity of endothelial cells by inhibiting the CXCL12/CXCR7 axis, thereby reducing tumor gravity and angiogenesis [32]. In our study, decursin derived from a natural substance reduced the expression of CXCR7 and thus decreased tumor growth, migration, and invasion in gastric cancer, as well as induced gastric cancer cell apoptosis. These results suggest that decursin could be a new natural product-derived anticancer drug that exhibits antitumor activity by targeting CXCR7.

Several studies have reported that CXCR7 is involved in gastric cancer progression and is related to a poor prognosis. However, the mechanisms by which CXCR7 contributes to gastric cancer progression are not well-defined. In our study, overexpression of CXCR7 induced phosphorylation of STAT3, thereby increasing growth, migration, and invasiveness of gastric cancer. STAT3 regulates the transcription of genes involved in cell proliferation, apoptosis, metastasis, and immune responses, and is constantly active in human cancers [33-36]. Furthermore, activation of STAT3 has been associated with a poor prognosis. Although STAT3 is widely reported to be involved in the progression of cancer [36, 37]. The development of drugs that inhibit STAT3 have not been developed. Here, we demonstrate that decursin reduces the activity of CXCR7-mediated STAT3 by decreasing the expression of CXCR7. Decursin decreased CXCR7 expression in a dose-dependent manner and thereby inhibited STAT3 and c-Myc, which is located downstream of STAT3. Activation of STAT3 promotes the transcription of many downstream oncogenes, such as CyclinD1, Bcl-XL, and c-Myc, which are involved in proliferation, invasion, and anti-apoptotic effects [37]. Our results showed that CXCR7 induced-activation of STAT3 increased the expression of c-Myc in gastric cancer cells. Decursin treatment reduced the CXCR7/STAT3/c-Myc signaling axis, reversing the cell growth, migration, and invasion processes induced by the activation of this axis in gastric cancer.

In summary, CXCR7 expression increases phospho-STAT3 expression, thereby increasing the expression of c-Myc, which promotes cell invasion and migration, and tumor growth. In addition, CXCR7 inhibits the processing of PARP and caspase-3 and induces an anti-apoptotic effect. Decursin inhibits tumor growth and induces apoptosis through the inhibition of the STAT3 pathway by decreasing the expression of CXCR7. Thus, decursin exhibits anticancer activity in gastric cancer (Figure 5D).

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Disclosure of conflict of interest

None.

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Figure S1. Schematic representation of the animal experiment. SNU484-CXCR7 cells were pretreated with either dimethyl sulfoxide (DMSO) or 40 µM decursin in complete media \textit{ex vivo} for 24 hours and then injected into mice subcutaneously. After 46 days, the mice were sacrificed.

Figure S2. Expression of CXCR7 was determined using (A) reverse-transcriptase-polymerase chain reaction (top two rows), Western blotting (bottom two rows), and (B) immunofluorescence staining using two gastric cancer cell lines-SNU484 and SNU215. GADPH, glyceraldehyde 3-phosphate dehydrogenase.
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Figure S3. Expression of CXCR7 in SNU484-CXCR7 and SNU216-CXCR7 cells treated with 20 or 40 µm decursin for 24 h was determined using immunofluorescence staining.

Figure S4. The relationship of CXCR7 expression and STAT3 expression in gastric cancer. (A) Western blotting of several cancer related pathways and (B) the correlation of CXCR7 (ACKR3) and STAT3 expression in GEPIA stomach cancer dataset.