Original Article
Impact of chemokine receptor 7 (CXCR7/ACKR3) gene silencing on inhibiting invasion of bladder cancer

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Abstract: The aims of this study were to investigate the impact of chemokine receptor 7 (CXCR7/ACKR3) silencing on inhibiting the biological behaviors of bladder cancer (BC) bladder carcinoma in situ. The siRNA sequence of CXCR7/ACKR3 was synthesized and transfected into the BIU-87 cells so as to screen the interference group (I). The impact of CXCR7/ACKR3 on the proliferation, and invasion of the BC cells were then determined. The CXCR7/ACKR3 mRNA and protein interfered by CXCR7-siRNA-1 were downregulated by 86.0% and 58.4%, respectively, and the gene silencing effect was the best. The proliferation speed of CXCR7/ACKR3 in Group I was significantly slower than the control group (C), with the mean inhibitory rate of 40.92% (P < 0.05). Compared with the control group, the number of transmembrane cells in Group CXCR7-siRNA-1 at 48 h was significantly reduced (P < 0.05). The 12 h and 24 h migration distance of the BC cells in Group I were 0.51±0.13 μm and 1.18±0.19 μm, respectively, which were significantly less than those in Group C (1.02±0.21 μm and 1.86±0.16 μm, respectively) (P < 0.05). Silencing the CXCR7/ACKR3 gene can significantly inhibit the proliferation, invasion, and migration of the BC BIU-87 cells, suggesting that CXCR7/ACKR3 is a potential molecular target for the treatment of human BC.

Keywords: Bladder cancer, CXCR7/ACKR3, cell scratch test, RNAi, transwell

Introduction
Bladder cancer is a malignant cancer derived from the urinary bladder transitional epithelium, the incidence of which ranks the first in Chinese male genitourinary malignancies. It directly threatens patients’ lives [1]. BC has a higher recurrence rate and malignant behavior features like easy recurrence, higher recurrent malignancy, invasion, or metastasis; with the environmental pollution increased due to industrial developments, the incidence of BC is also increased year by year, and the ages of onset also continuously broadens [2]. It will have great significance and is also an urgent problem for clinical medical personnel to analyze and solve the pathogenic mechanism of BC, especially the molecular mechanism, which can improve the early diagnostic rate of BC, improve the patients’ quality of life, and prolong their survival periods through active treatment [3].

Among gene therapies which have been significantly focused on and given high hopes to, RNA interference (RNAi) is a kind of gene silencing technologies [4], referring to the phenomenon of double-stranded RNA-induced highly-efficient specific degradation of its complementary homologous mRNA during the evolutionary process. RNAi is ubiquitous in a variety of organisms, can inhibit the violation of homologous genes or viruses, and can effectively reduce or shut down the expression of specific genes [5]. The RNAi technology can be used to find the functions of certain genes, thus providing theoretical basis for gene therapies of malignant tumors.

Chemokines are directionally movable small protein molecules secreted by immune cells or tissue cells under the stimulation of growth factors, interferon, or other substances [6]. Chemokines are slightly or not expressed in normal human tissues, but highly expressed in such malignant tumors as human ovarian cancer,
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breast cancer, prostate cancer, or bladder cancer, among which chemokine receptor 12 (CXCR12) and its receptor CXCR4 are the key factors of the proliferation, invasion, or metastasis of BC. Previous studies showed that the interaction between CXC chemokine ligand 12 (CXCL12) and CXC chemokine receptor 4 (CXCR4) was the key biological behavior of tumor invasion, metastasis and proliferation. Recent researches found that tumor proliferation and metastasis were only partially blocked by inhibiting CXCR4 expression, and there must be some other factors control the proliferation and tissue-specific metastasis of malignant tumors, then another chemokine receptor of CXCL12, CXC chemokine receptor 7 (CXCR7), was found, which would enhance the proliferation, invasion and metastasis of tumor cells [7-9]. In 2005, the high-affinity receptor of CXCL12, CXCR7/ACKR3, was discovered; CXCR7/ACKR3 belongs to the GPCR family (G-protein coupled receptor) and is a specific receptor of SDF1α. Studies have shown that the activation of the SDF1α pathway is the potential mechanism of anti-tumor biological agents [10]. This study used the RNAi silencing technology to transfecit into the human BC cell line BIU-87, aiming to investigate the impact of RNAi-silencing-CXCR7 on inhibiting the biological behaviors, such as invasion, of BC and to lay a foundation for the gene therapies of BC.

Materials and methods

Design and synthesis of CXCR7/ACKR3 interference plasmids

The whole sequence of the CXCR7/ACKR3 mRNA was searched in the GENEBANK database, and based on this, the on-line design software (www.dhannaeon.com) combined with the corresponding design principles of siRNA was used to design the CXCR7-specific siRNA sequence, the secondary structure of which was evaluated using RNAstructure4.4. Finally, 3 pairs of interference sequences and a pair of negative sequences were obtained and ensured no other homologous sequence through the BLAST searching (NIH, http://www.ncbi.nlm.nih.gov/BLAST). After guaranteed the specificity of the amplification, the primers (Table 1) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai).

Reverse transcription polymerase chain reaction (RT-PCR)

Expression of CXCR7/ACKR3 mRNA after 72 h transfection. The total RNA was extracted from the transfected BIU-87 cells using the Trizol base, and based on this, the on-line design software (www.dhannaeon.com) combined with the corresponding design principles of siRNA was used to design the CXCR7-specific siRNA sequence, the secondary structure of which was evaluated using RNAstructure4.4. Finally, 3 pairs of interference sequences and a pair of negative sequences were obtained and ensured no other homologous sequence through the BLAST searching (NIH, http://www.ncbi.nlm.nih.gov/BLAST). After guaranteed the specificity of the amplification, the primers (Table 1) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai).

Cell culture and transfection

In this study, the expressions of CXCR7/ACKR3 in several BC cell lines (purchased from the Cell Bank of Type Culture Collection Committee, Chinese Academy of Science) were quantitatively analyzed, among which the human BC cell line BIU-87 with high expression of CXCR7/ACKR3 was screened and it was chosen to continue follow-up experiments. After resuscitated from cryopreservation, the BIU-87 cells were inoculated in 10% FBS-containing DMEM medium (containing 100 μg/mL streptomycin and 100 U/mL penicillin) at 37°C and 5% CO2. The culture medium was changed every other 2 to 3 days. After the cells covered about 90% of the flask wall, they were digested and passaged using 0.25% trypsin, and inoculated into new culture flasks. The cells in logarithmic growth phase were then seeded in 6-well plates with the density as 2 × 104 cells/well. When the cells covered 90% of the plate, the CXCR7-siRNA synthesized using the above method was transfected using the Lipofectamine 2000 liposome method according to the instructions (Gibco, USA). CXCR7-siRNA fragment diluted by OPTI-MEM low serum culture medium and diluted Lipofectamine 2000 liposome method according to the instructions (Gibco, USA). CXCR7-siRNA fragment diluted by OPTI-MEM low serum culture medium and diluted Lipofectamine 2000 were incubated at room temperature for 20 min, then was incubated in 5% CO2 at 37°C for 4-5 h after transfection, which was further cultured in DMEM 4ml containing 5% FBS for 24 h, then the proportion and distribution of cells were observed by microscope.

Reverse transcription polymerase chain reaction (RT-PCR)

Expression of CXCR7/ACKR3 mRNA after 72 h transfection. The total RNA was extracted from the transfected BIU-87 cells using the Trizol

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR7-siRNA-1-F</td>
<td>GGATCCATGTCGGGACCCGTGCC</td>
</tr>
<tr>
<td>CXCR7-siRNA-1-R</td>
<td>GAATTCTTACTGCTGACGCAG</td>
</tr>
<tr>
<td>CXCR7-siRNA-2-F</td>
<td>CTCGCTCCTCAATATGAG</td>
</tr>
<tr>
<td>CXCR7-siRNA-2-R</td>
<td>GGAAGTAGAAGAAGACGATA</td>
</tr>
<tr>
<td>CXCR7-siRNA-3-F</td>
<td>CCUGUGUAACCGCUCUUAATT</td>
</tr>
<tr>
<td>CXCR7-siRNA-3-R</td>
<td>UAAAGAGGCUUCAUACGGTT</td>
</tr>
<tr>
<td>NC-F</td>
<td>UCUCCGGAACGUGUACGUGTT</td>
</tr>
<tr>
<td>NC-R</td>
<td>ACGUGACCGUUCGAGAATT</td>
</tr>
<tr>
<td>CXCR7/ACKR3-F</td>
<td>CTGCTCACAATAGAG</td>
</tr>
<tr>
<td>CXCR7/ACKR3-R</td>
<td>GGAAGTAGAAGAAGACGAT</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>ACGGACAGATTGACAGATT</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GCCACTTGTCCTCTGAAGAA</td>
</tr>
</tbody>
</table>

Table 1. CXCR7-shRNA primer sequence list
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After determined the RNA concentration using one ultra-trace UV spectrophotometer and adjusted the concentration to 1 μg/μL, 2 μg of total RNA was reversely transcribed to cDNA, which was then used as the template for performing real-time quantitative PCR toward CXCR7/ACKR3 and the internal reference GAPDH. Each sample was repeated three times. The PCR reaction system was 20 μL, and the amplification conditions were as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 40 s, annealing at 58°C for 30 s, extension at 72°C for 40 s, a total of 35 cycles, followed by extension at 72°C for 5 min. The amplification results were then performed agarose gel electrophoresis and analyzed by the gel scanning system. The results of electrophoresis were analyzed by gel electrophoresis (TaKaRa, Inc., Japan).

Western blot

After harvested, the transfected BIU-87 cells were washed three times with phosphate buffered saline (PBS), added the RIPA protein lysate to extract the total proteins, and determined the protein concentration using the BCA protein concentration assay kit. After denatured at 100°C for 10 min and isolated by 10% polyacrylamide gel electrophoresis, the products were transferred onto polyvinylidene fluoride (PVDF) membranes, closed in 5% non-fat milk powder-TBST for 2 h, and incubated with the primary antibody (1:1000, rabbit anti-human monoclonal antibody, Abcam, UK) and the monoclonal GAPDH antibody at 37°C overnight; after washed three times using TBST, the products were incubated with the horseradish peroxidase-labeled secondary antibody (1:2000, goat anti-rabbit polyclonal antibody) for 2 h. After washed with TBST, the ECL chemiluminescent reagent A and B (1:1) were mixed with the membrane, developed and photographed in one darkroom. The ultraviolet irradiation (UVi) gel imaging system was then used for photography, and he integrated optical densities (IOD) of CXCR7/ACKR3 protein and GAPDH protein were then calculated using the Image-Pro Plus8.0 software. The ratio of these two proteins was set as the relative expression of the CXCR7/ACKR3 protein so as to screen the RNAi interference sequence with the highest silencing rate for the subsequent experiments. (Invitrogen Co., USA).

Methyl thiazolytetrazolium (MTT)

The above-mentioned BIU-87 cells were inoculated into 96-well cell culture plates with the density as 1 × 10^3 cells/well (200 μl per well), together with the zero well and the control well (3 reduplicate wells for each group). The wells were incubated at 37°C and 5% CO₂ for 24 h, followed by adding 10 μl of MTT solution (5 mg/ml) per well for another 4 h culture. After carefully removed the supernatant, 150 μl of dimethylsulphoxide (DMSO) was added into each well and shaken at low speed for 10 min so as to fully dissolve the crystals. The absorbance value of each well was then determined at 490 nm using one 550 microplate reader. The cell growth curve was then drawn with the
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Table 2. Expression comparison of CXCR7 mRNA after transfection

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative expression of mRNA (2ΔΔCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CXCR7-siRNA-1 0.1015±0.0024&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>CXCR7-siRNA-2 0.3614±0.0032&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>CXCR7-siRNA-3 0.4479±0.0150&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>NC 0.7223±0.0120</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup> compared with the control group, the expressions of CXCR7 mRNA are reduced (P < 0.05); <sup>*</sup> compared with Group 2 and 3, the CXCR7 mRNA is downregulated the most (P < 0.05).

Figure 2. Expressions of CXCR7 protein by Western blotting. A: Expressions of CXCR7-siRNA-1 GADPH and CXCR7 protein; B: Expressions of CXCR7-siRNA-2 GADPH and CXCR7 protein; C: Expressions of CXCR7-siRNA-3 GADPH and CXCR7 protein; D: Expressions of NC GADPH and CXCR7 protein.

time as abscissa and the absorbance value as the ordinate for calculating the cell proliferation inhibition rate as well as performing the statistical analysis (Sigma, USA).

Inhibition rate (%) = (1 - OD of Group I/OD of Group C) × 100%

Transwell

The human BC cells were firstly diluted into the single cell suspension (5 × 10<sup>5</sup> cells/ml) using serum-free medium and then divided into two parts. 100 mL of the cell suspension (5 × 10<sup>5</sup> cells) was added into the upper Transwell chamber together with 200 mL of serum-free DMEM medium, and 500 μl of chemokine was added into the lower Transwell chamber, followed by 24 h incubation at 37°C and 5% CO<sub>2</sub>. After gently wiped the non-invasive cells on the polycarbonate membrane with a moist swab, the upper chamber was carefully removed and fixed in pre-cold methanol for 30 min, followed 5 min Giemsa staining and gradient dehydration using 80%, 95%, and 100% ethanol. After carefully removed from the bottom of the upper chamber, the polycarbonate membrane was placed on a glass slide for closure using neutral resin. The cells attached onto the lower surface of the polycarbonate membrane were then counted at a high magnification (× 200) in 5 randomly selected fields, and each group was repeated three times of the average. The number of migrated cells was the performed quantitative and statistical analysis. The inhibitory rate of silencing CXCR7/ACKR3 on the BC cell invasion was calculated as follows.

Inhibition rate = (Group C - Group I)/Group C × 100%

Cell scratch test

Firstly, one marker pen was used to draw evenly distributed parallel lines (about 0.5~1 cm in width and crossing wells, and each well should contain at least five lines) on the back of 6-well culture plates together with three parallel controls. Each well was seeded approximately 5 × 10<sup>5</sup> cells depending on the cell types, and the seeding principle was set as that the overnight cell density should reach 100%. After 24 h incubation at 37°C and 5% CO<sub>2</sub>, the cells were performed siRNA transfection and continuously cultured using the serum culture medium 24 h later until the adherence rate reached 100%. After that, one pipette tip was used to draw strictly vertical lines along one ruler, and after washed away the scratched cells 3 times with PBS, the cells were added the serum-free medium and cultured at 37°C and 5% CO<sub>2</sub>. Photos were taken 12 h and 24 h later to calculate the average distance among cells so as to compare the cell migration rate.

Statistical analysis

SPSS19.0 (IBM, Chicago, USA) was used in this study to analyze the experimental data. The data were expressed as mean ± standard deviation (X ±s). The OD values were compared using the single factor ANOVA between the two groups, and were compared by t test between two groups, with P < 0.05 considered as statistical significance.

Results

Expression of CXCR7/ACKR3 mRNA

It can be seen from Figure 1 that the expressions of CXCR7/ACKR3 mRNA interfered by CXCR7-siRNA-1, CXCR7-siRNA-2, and CXCR7-siRNA-3 were significantly downregulated by 86.0%, 59.0% and 38.0% than that in Group C,
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The results of the Transwell invasion assay showed that after being silenced for 48 h, the cells that penetrated the membrane in Group C and CXCR7-siRNA-1 were 146.2±17.63 and 70.1±13.97, respectively, and the number in Group CXCR7-siRNA-1 was significantly decreased than Group C (P < 0.05) (t-test, comparison of count of transmembrane cells between the two groups), indicating that silencing the CXCR7/ACKR3 gene by CXCR7-siRNA-1 can significantly inhibit the invasion of the BIU-87 cells, and the inhibitory rate was 52.10% (Table 5, Figure 4).

Impact of silencing CXCR7/ACKR3 on BC cell migration

The results showed that after silencing CXCR7/ACKR3 for 12 h, the growth rate of BC cells in Group C was faster than Group CXCR7-siRNA-1; 24 h later, the scratches in Group C were basically covered by cells, but those in Group CXCR7-siRNA-1 were still separated by a certain distance. The relative migration distance was measured by Photoshop cs3 and shown in Table 6. The statistics showed that the migration distances in Group CXCR7-siRNA-1 were 0.51±0.13 μm and 1.18±0.19 μm after 12 h and 24 h, respectively, which were much less than those in Group C (1.02±0.21 μm and 1.86±0.16 μm, respectively, P < 0.05), indicating that silencing the CXCR7/ACKR3 gene can significantly reduce the BC cell migration distance, so silencing the CXCR7/ACKR3 gene can effectively inhibit the migration of BC cells (Table 6, Figure 5).

Discussion

Bladder cancer (BC) is a biological process caused by the co-effects of multiple steps, multiple factors, and multiple genes [11]. The occurrence and development of tumors is relat-
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CXCR7/ACKR3 was previously known as “orphan protein-coupled receptor” (RDC1), similar to CXCR4 [13], is composed of 362 amino acids, and is expressed highly conservatively. CXCR7/ACKR3 locates on human 2q37 Chromosome, with its extracellular N-terminal region binding with the ligands and its intracellular domain coupling with the G protein. Its C-terminal contains serine/threonine, the phosphorylation of which may participate in signal transduction. It is widely accepted that CXCR4 is the only receptor of CXCL12, but recent studies have shown that CXCR7/ACKR3 is also a CXCL12-specific receptor and highly compatible with CXCL12. The affinity of CXCR7/ACKR3 and CXCL12 is higher than that of CXCR4, and CXCR7/ACKR3 could bind to CXCL11, but the affinity of CXCR7/ACKR3 and CXCL12 is 10~20 times of CXCL11. Studies have shown that CXCR7/ACKR3 is highly expressed in some cancer cells and is closely related to tumor angiogenesis, as well as the proliferation, invasion, and migration of tumor cells [14].

Table 4. Impact of silencing CXCR7 on proliferation of BC cells

<table>
<thead>
<tr>
<th>OD</th>
<th>0 h</th>
<th>12 h</th>
<th>24 h</th>
<th>36 h</th>
<th>48 h</th>
<th>60 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR7-siRNA-1</td>
<td>0.26±0.04</td>
<td>0.44±0.05</td>
<td>0.47±0.08</td>
<td>0.51±0.11</td>
<td>0.6±0.09</td>
<td>0.66±0.15</td>
<td>0.74±0.26</td>
</tr>
<tr>
<td>Control</td>
<td>0.26±0.04</td>
<td>0.53±0.09</td>
<td>0.62±0.17</td>
<td>0.89±0.16</td>
<td>1.09±0.28</td>
<td>1.23±0.24</td>
<td>1.38±0.33</td>
</tr>
</tbody>
</table>

Note: The proliferation rate of CXCR7-siRNA-1 bladder cancer cells in the interference group was significantly slower than the control group. (*P* < 0.05).

Table 5. Comparison of membrane-penetrating cells after silencing CXCR7

<table>
<thead>
<tr>
<th>Group</th>
<th>48-h membrane-penetrating cells</th>
<th>Inhibitory rate</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CXCR7-siRNA-1 70.1±13.97</td>
<td>52.10%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NC 146.2±17.63</td>
<td>52.10%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Migration distance of BC cells after silencing CXCR7

<table>
<thead>
<tr>
<th>Group</th>
<th>12 h</th>
<th>24 h</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.51±0.13</td>
<td>1.18±0.19</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.02±0.21</td>
<td>1.86±0.16</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. The number of transmembrane cells in Group CXCR7-siRNA-1 and control group at 48 h. A: CXCR7-siRNA-1 group; B: Control group.
their proliferation and tumorigenesis, and reducing the CXCR7/ACKR3 expression in nude mice can inhibit the cancerization of cells caused by infection [18]; in breast cancer cell lines, downregulating the CXCR7/ACKR3 expression by 10% using siRNA can significantly reduce the tumorigenicity of breast cancer cells; Burus transfected small molecule CXCR7/ACKR3 antagonist into mice, and found the tumor growth in mice was significantly inhibited [19], after silenced the CXCR7/ACKR3 gene, the growth of colon cancer xenograft was significantly inhibited [20]. Aman found that CXCR7/ACKR3, Wnt/B-catenin, and the FGF signaling pathway can mutually affect each other, thus controlling the selective migration of cells [21]; studies using the microarray technology to regulate CXCR7/ACKR3 also revealed that the overexpression of CXCR7/ACKR3 can cause the changes of such intracellular adherence factors as cadherin CDH11 and CD44, among which the content of CDH11 is positively related to the invasion of tumors; CD44 can regulate the RAS signal, thus achieving the migration and invasion of tumor cells [22]; Yates found that CXCR7/ACKR3 is highly expressed in BC tissue than other normal bladder tissue, and this upregulation is closely related to the tumor grade or metastasis. In addition, more and more studies have shown that CXCR7/ACKR3 is associated with the expressions of ERK, Stat3, and AKL in BC, as well as with the proliferation and migration pathways of many tumors [23]. The results of this study confirmed that the proliferation rate of bladder cancer cells was significantly slowed down after CXCR7/ACKR3 was silenced, which reached 46.4% at 72 h, indicating that the CXCR7/ACKR3 gene can significantly inhibit the proliferation of bladder cancer cells. In addition, the number of transmembrane cells was significantly reduced than the control group after intervened the expression of CXCR7/ACKR3, and the horizontal move distance of bladder cancer cells was significantly decreased, indicating that silencing CXCR7/ACKR3 can significantly inhibit the invasion and migration of the BIU-87 cells.

In order to improve the specificity of target gene silencing and ensure the non-homology with other genes, this study strictly compared the CXCR7/ACKR3 target sequence, but still cannot rule out the off-target effect (non-target gene silencing), so more studies are needed for the further confirmation. However, exploring the impact of RNAi-silencing CXCR7/ACKR3 on the invasion and metastasis of BC can block the invasion and migration of tumor cells and achieve the purpose of treating or even curing tumors, and it will open a new chapter in human BC gene therapies.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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