Chemokine (C-X-C motif) receptor 4 RNA interference inhibits bone metastasis in breast cancer

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Abstract. Chemokine (C-X-C motif) receptor 4 (CXCR4) has been found to closely correlate with the incidence, development, treatment and prognosis of breast cancer. The aim of the present study was to investigate the effects of CXCR4 on bone metastasis in breast cancer and to explore the mechanisms of this process. CXCR4 small interfering RNA was transfected into the breast cancer cell line, MDA-MB-231BA-rfp, and the cell proliferation and invasion abilities of the cells were measured using cell counting kit-8 cell proliferation and Transwell assays. A mouse model of breast cancer with bone metastasis was prepared and the bone metastasis was confirmed using micro-positron emission tomography. The associated proteins were detected by western blot analysis and the results showed that CXCR4 RNAi inhibited the cell proliferation and invasion ability of the MDA-MB-231BA-rfp cells. In addition, CXCR4 RNAi inhibited the duration and extent of bone metastasis in the MDA-MB-231BA-rfp cells in the mouse model, while the inhibition of CXCR4 RNAi blocked the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (AKT)/matrix metalloproteinase (MMP)-9 pathway.

In conclusion, the present study demonstrated that CXCR4 RNAi inhibits bone metastasis and the cell proliferation and invasion abilities of breast cancer cells. Furthermore, the CXCR4/PI3K/AKT/MMP-9 pathway may be important in the bone metastasis of breast cancer.

Materials and methods

Experimental materials. The human breast cancer cell line, MDA-MB-231BA-rfp, was stored frozen in liquid nitrogen. Dulbecco’s modified Eagle’s medium with 10% fetal calf serum was purchased from Gibco-BRL (Carlsbad, CA, USA). The rabbit polyclonal antibodies against human CXCR4, phosphatidylinositide 3-kinase (PI3K), protein kinase B (AKT), and matrix metalloproteinase (MMP)-9 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The western blot analysis kits were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). This study was approved by the Institutional Review Board of Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China).

CXCR4 small interfering RNA (siRNA) construction and transfection. The two CXCR4 siRNA oligonucleotide sequences purchased from Dharmacon, Inc., (Lafayette, CA, USA) were identified and matched with the following CXCR4 cDNA sequences obtained from GeneBank through a BLAST search: Sense, 5’-UAAACUUCUGCCGACCAT3’ for siRNA1; and sense, 5’-GGAAGCUGUUGGCUGAAAdTdT-3’ for siRNA2. In addition, a negative control siRNA sequence was formulated and synthesized, as follows: 5’-UUCUCGAACGUGUACGUUUGGUCGAAAdTdT-3’ for siRNA2. The MDA-MB-231BA-rfp cells (1x10⁵ cells/ml) were then transfected with 100 nM CXCR4 siRNA mediated by oligofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA).
Western blot analysis. The MDA-MB-231BA-rfp cells in the exponential growth phase were centrifuged at 30,000 x g at 4°C for 5 min to separate the supernatant from the cellular debris (centrifugation radius, 4 cm) following the application of radioimmunoprecipitation assay protein lysis buffer (Wuhan Boster Biological Technology, Ltd.). Next, the level of protein expression was determined using the bicinchoninic acid assay method. Subsequently, 50 µg of protein was harvested and 2X loading buffer was added to the protein samples, which were then then heated to 100°C for 5 min. Next, following SDS-PAGE separation, the samples were loaded onto a nitrocellulose filter and then combined with the specific antibodies and corresponding diantibodies. Finally, the samples were stained using enhanced chemiluminescence kit (Wuhan Boster Biological Technology, Ltd.) prior to the X-ray films being exposed, developed and fixed. Gray scale images were also captured and analyzed using BandScan software (Glyko, Novato, CA, USA).

Cell invasion assay in vitro. Transwell chamber models (Chemicon, Temecula, CA, USA) were performed to prepare a cell suspension containing 1x10⁶ cells/ml, of which 50 µl was added to the upper chamber. At 24 h post-incubation, the cells located on the inner layer of the chamber were removed and the remaining cells were fixed using 10% formalin. Giemsa stain was used to count the number of invasive cells that had migrated through the membrane.

Cell counting kit (CCK)-8 cell proliferation assay. The cells were digested with 0.25% pancreatic enzyme (Wuhan Boster Biological Technology, Ltd.), which resulted in a cell suspension containing 1.2x10⁶ cells/ml. Next, the cell suspension was seeded into 96-well plates (200 µl per well) and separated into the following three groups: Control group, lipopolysaccharide (1.0 µg/ml) intervention group and Toll-like receptor-4 intervention group. After 24 h, CCK-8 (10 µl/well) was added and the cells were incubated for another 2 h. The absorbance at 450 nm was measured using a microplate reader and the proliferation ability of the mesenchymal stem cells of different mice were analyzed.

Tumorigenesis in nude mice. In total, 24 C57BL/6 nude mice were kept in a biologically clean animal laboratory at a temperature of 23±1°C, with a relative humidity of 55-60%. The mice were housed six per cage in polycarbonate cages (8x13.5x8.1 cm in size). The dry sawdust bedding was sterilized and replaced every five days, and sterile distilled drinking water was provided. Animals were randomized into intervention (n=12) and control (n=12) groups following one week of acclimation to the same conditions. MDA-MB-231BA-rfp cells in the exponential growth phase were dissociated to prepare a single cell suspension, of which the cell density was adjusted to 1x10⁶ cells/ml, with a cell viability of >95%. Briefly, 2 ml of the cell suspension was injected into the caudal veins of the mice. The suspension included MDA-MB-231BA-rfp cells transfected with CXCR4 siRNA for the intervention group and MDA-MB-231BA-rfp cells without CXCR4 siRNA transfection for the control group.

Micro-positron emission tomography (PET) detection of bone metastasis in nude mice. The mice were deprived of food and water for 8 h and anesthetized via inhalation of 2% isoflurane prior to microPET. At 40 min after the injection of the radioactive tracer, fludeoxyglucose, into the tail vein, the mice were placed in a prone position and imaging was performed for 10 min. Bone metastasis was analyzed using ASIPro (Siemens Medical Solutions USA, Inc., Knoxville, TN, USA) to determine the region of interest (ROI), and the maximum standard uptake value (SUV) was used for the statistical analysis. The ROI was evaluated by a researcher blinded to the experimental schedule and groupings.

Statistical analysis. All data are presented as the mean ± standard deviation and were analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) by two-tailed t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Interference effects of CXCR4 siRNA. The results of the western blot analysis revealed that the expression of CXCR4 in the MDA-MB-231BA-rfp cell line was significantly down-regulated in the S1 and S2 groups compared with the Con-B, Con-A and Sn groups at 24 h after the transfection with 100 nM CXCR4 siRNA. The interference efficiency was calculated using the following formula: Interference efficiency = (down-regulation range of CXCR4 in control group - downregulation range of CXCR4 in intervention group) / downregulation range in control group. The results showed interference efficiencies of 83 and 92% in the S1 and S2 groups, respectively. Therefore, since the S2 group exhibited a relatively higher interference efficacy than the S1 group, the S2 group was selected to be
CXCR4 siRNA inhibition of cellular proliferation and invasion. Based on the finding that the S2 group was found to be the most effective in reducing CXCR4 expression, S2 was selected as the CXCR4-specific sequence to proceed with in the study. The CCK-8 proliferation assay revealed that the proliferation rate of the S2-transfected cells decreased with increasing concentrations of the transfection reagent (0, 3.125, 6.25, 12.5, 25, 50 and 100 nM; Fig. 2A). At 48 h after the transfection of the breast cancer cells with the varying S2 concentrations, the results of the Transwell migration assay also indicated that the number of cancer cells that migrated through the filter membrane substantially decreased with increasing siRNA concentration (Fig. 2B).

CXCR4 RNAi inhibition of bone metastasis in nude mice. The nude mice in the intervention group were injected with MDA-MB-231BA-rfp cells that had been transfected with 100 nM S2 for 48 h (n=8), and the control group were injected with an equal amount of non-transfected MDA-MB-231BA-rfp cells (n=8). MicroPET analysis revealed that the number of metastatic foci in the intervention group was significantly reduced compared with the control group.

Figure 2. CXCR4 siRNA inhibits cellular proliferation and invasion. (A) The cell counting kit-8 (CCK-8) cell proliferation assay revealed that the proliferation rate in the siRNA2-transfected cells decreased with increasing concentrations of transfection reagent. (B) The Transwell migration assay indicated that the number of cancer cells that migrated through the filter membrane substantially decreased with increasing siRNA concentration. CXCR4, chemokine (C-X-C motif) receptor 4; siRNA, small interfering RNA.

Figure 3. Micro-positron emission tomography (PET) images of the two groups, with a color code indicating the glucose metabolism levels; red indicates high levels and blue indicates low levels (radioactive tracer, 18F-FDG). The bright white region is positively proportional to the uptake volume of 18F-FDG by the tumors in the region. The direct viewing of images demonstrated that the bright white regions in the lower extremities, ribs and spine were considerably larger in the control groups than in the interference group. 18F-FDG, 18F-fluorodeoxyglucose; RNAi, RNA interference.

Figure 4. Western blot analysis revealed that CXCR4 RNAi inhibited PI3K/AKT signaling and the expression of MMP-9. PI3K, phosphatidylinositide 3-kinase; AKT, protein kinase B; MMP-9, matrix metalloproteinase-9; RNAi, RNA interference.
in the lower extremities four weeks after tumor cell injection. By contrast, it was not until the sixth week after tumor cell injection that distinct bone invasion, indicating the emergence of bone metastasis, was identified in the interference group. Direct observation of the microPET images six weeks after the injections revealed that the bright white region in the lower extremities, ribs and spine was much larger in the control group than in the interference group (Fig. 3). Semi-quantitative analysis was performed based on the SUV ratios, which revealed that the SUVmax in the interference group was 9.38±0.54 versus 2.13±0.21 in the control group (P<0.01), indicating that the onset and degree of MDA-MB-231SA-rfp cell bone metastasis may be significantly inhibited by CXCR4 RNAi.

**CXCR4 RNAi downregulates MMP-9 via blockade of the PI3K/AKT signaling pathway.** To investigate the potential mechanism of CXCR4 RNAi controlling breast cancer metastasis to the bone, western blot analysis was used to analyze the expression of PI3K/AKT/MMP-9 following the silencing of CXCR4 by RNAi. As a result, the expression of PI3K/AKT/MMP-9 was reduced by CXCR4 RNAi, with the levels of PI3K, AKT and MMP-9 far lower than those in the control group (0.32±0.06 vs. 0.89±0.12 for PI3K; 0.16±0.03 vs. 0.86±0.10 for AKT; and 0.12±0.02 vs. 1.12±0.16 for MMP-9; P<0.01; Fig. 4).

**Discussion**

CXCR4 is a highly conserved G protein-coupled receptor of the chemokine receptor family that mediates chemotactic activity and is a receptor specific to CXC ligand 12 (CXCL12) (7). Previous studies have demonstrated that CXCR4 is the most common chemokine receptor expressed in tumor cells, and that it plays a predominant role in the migration and invasion of tumors (8-11). In addition, a recent study has revealed that CXCR4 is vital for the migration, invasion, treatment and prognosis of breast cancer (12,13). Furthermore, Gil et al (14) reported that a virus-coated CXCR4 antagonist is effective in the treatment of primary or metastatic breast cancer, functioning by disrupting the internal environment for tumor cell growth and inhibiting the vascularization and expression of CXCL12 and vascular endothelial growth factor (VEGF). Additionally, Ling et al (15) reported that the CXCR4 antagonist, AMD3465, inhibits the growth and migration of breast cancer by partially blocking signal transducer and activator of transcription 3 signaling, which has an impact on tumor and immune cells in the internal tumor environment.

In the present study, the effect of CXCR4 on the bone metastasis of breast cancer by targeting the downregulation of CXCR4 using RNAi techniques was observed (Fig. 1). Firstly, the CKC-8 cell proliferation and Transwell chamber assays were used to detect the oncological characteristics of the breast cancer cells prior to and following CXCR4 suppression. The observations revealed that CXCR4 siRNA significantly inhibits the proliferation and invasion of breast cancer cells (Fig. 2). Therefore, CXCR4 is key in the growth and proliferation of breast cancer cells, indicating that the control of its activity may significantly reduce the proliferation of breast cancer cells in vitro. In addition, as CXCR4 is involved in the motility and chemotaxis of breast cancer cells, the suppression of CXCR4 expression may significantly reduce the migration of breast cancer cells to distant organs (16). Furthermore, Wendel et al (17) reported that CXCR4/CXCL12 is significant in the migration of breast cancer cells by affecting the adhesiveness, morphology and migration of the cells and the regulation of the expression of the protein family in the extracellular matrix.

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