Overexpression of CXCR4 is significantly associated with cisplatin-based chemotherapy resistance and can be a prognostic factor in epithelial ovarian cancer

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The chemokine receptor 4 (CXCR4) plays an important role in the growth, angiogenesis and metastasis of various cancers, including epithelial ovarian cancer (EOC). Therefore, it is important to understand the molecular mechanism of chemotherapeutic drug resistance, particularly cisplatin-based therapy, in EOC.

The chemokine receptor 4 (CXCR4) is a seven-transmembrane G protein-coupled receptor. It is also known as a receptor for chemokine (C-X-C motif) ligand 12 (CXCL12, also called stromal-derived growth factor-1, SDF-1). A growing body of evidence has demonstrated that CXCR4 is expressed on multiple cell types including lymphocytes, hematopoietic stem cells, endothelial and epithelial cells, and cancer cells (4). It has been shown to play important roles in regulating the expression of genes involved in tumor progression, angiogenesis, metastasis, and survival in diseases such as gastric cancer, breast cancer and colorectal cancer (5-7). High expression of CXCR4 in several human tumors and cancer cell lines indicates that CXCR4 is critical for tumorigenesis and progression (8, 9). Interfering with the expression of CXCR4 or the blockade of the CXCR4/SDF-1 axis by small interfering RNA (siRNA) or some other specific inhibitor, such as plerixafor, TN14003, or AMD3100, significantly reduces invasion, migration and adhesion of cancer cells in vitro. Our data suggest that CXCR4 is one of the key molecules in cisplatin-based chemotherapy for EOC patients and that CXCR4 inhibition is a potential strategy to address the chemoresistance of EOC. [BMB Reports 2014; 47(1): 33-38]

INTRODUCTION

Epithelial ovarian cancer (EOC), accounting for more than 85% of human ovarian cancer, is the fifth leading cause of death in female cancer patients and has the highest mortality rate of all gynecological cancers worldwide (1). The overall 5-year survival rate of ovarian cancer patients diagnosed at an advanced stage is less than 30% (2). The poor survival is mainly attributed to the high resistance of EOC to current chemotherapeutic regimens (3). Therefore, it is important to understand the molecular mechanism of chemotherapeutic drug resistance, particularly cisplatin-based therapy, in EOC.

In the present study, we investigated the expression of CXCR4 and its correlation with sensitivity to chemotherapy agents and clinical outcomes of cisplatin-based therapy among EOC patients. Furthermore, to confirm the results we obtained from the clinic data, we inhibited the expression of CXCR4 by siRNA in ovarian cancer cells and analyzed the effect of CXCR4 inhibition on chemosensitivity, proliferation and apoptosis to determine if CXCR4 is one of the key factors in cisplatin-based chemotherapy of EOC.
RESULTS

Correlation of CXCR4 expression and response to cisplatin-based chemotherapy and prognosis of EOC patients

As shown in Fig. 1A, CXCR4 was ubiquitously expressed in EOC tissues. The results show that the expression of CXCR4 in EOC was correlated with histological grade and the International Federation of Gynecology and Obstetrics (FIGO) stage (P < 0.05). Moreover, CXCR4 expression was significantly associated with response to cisplatin-based chemotherapy.

The Kaplan-Meier method, the log-rank test, and Cox regression analysis were used to describe the relationship between the progression-free survival (PFS) and overall survival (OS) of EOC patients and CXCR4 expression (Fig. 1B). The data showed that the mean PFS for the high-CXCR4 expression group was only 14.3 months, compared with 34.7 months for the low-CXCR4 expression group (Supplementary Table S2). The median OS time for the low-CXCR4 group was 40.8 months, compared with 23.4 months for the high-CXCR4 group (Supplementary Table S3). In the log-rank test analysis, patients with a higher CXCR4 expression had a significantly shorter PFS time and OS time (P < 0.001). Remarkably, according to the multiple Cox regression analysis, the expression of CXCR4 was an independent predictive factor for poor PFS and OS in EOC patients (PFS, relative risk: 3.393, P < 0.001; OS, relative risk: 3.290, P < 0.001) (Supplementary Table S2 and S3).

Overexpression of CXCR4 in human ovarian cancer cisplatin-resistant cells

In order to investigate the potential role of CXCR4 in EOC, we first examined its expression in both paired isogenic cisplatin-sensitive cell line A2780 and cisplatin-resistant cell line A2780/cis using both qRT-PCR and Western blot (Fig. 2A and B). The CXCR4 mRNA levels in A2780/cis cell lines were approx-
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Fig. 3. The effect of CXCR4 siRNA on cell proliferation and sensitivity of A2780 and A2780/cis cells. (A) The effect of siRNA depletion of CXCR4 on proliferation of A2780 and A2780/cis cells by MTT assay. (B) The effect of siRNA depletion of CXCR4 on sensitivity of A2780 and A2780/cis cells to cisplatin. All experiments were done in triplicate.

DISCUSSION
Cisplatin is the most widely used crosslinking drug for killing ovarian cancer cells, and cisplatin-based chemotherapy improves patient survival. Unfortunately, like other anticancer drugs, chemoresistance remains a significant obstacle to its clinical success. Therefore, the identification of new markers with a predictive value in response to cisplatin treatment could be helpful for the development of individualized treatment strategies to further improve the efficacy and minimize the side ef-

approximately increased fourfold compared with the A2780 cell line. The A2780/cis cells also demonstrated higher expression protein levels of CXCR4 than the A2780 cells. We also investigated the subcellular location of CXCR4 in ovarian cancer cells using immunofluorescence. Fluorescence microscope revealed CXCR4 was mainly expressed in the cytoplasmic, with only a small proportion in the nuclei in A2780/cis cells, while the predominant pattern of CXCR4 expression in A2780 cells was cytoplasmic (Fig. 2C).

To examine the correlation between CXCR4 expression and cisplatin resistance, cells were incubated with cisplatin at a concentration of between 10 to 40 μM for 48 h. As shown in Fig. 2D, after treatment with different concentrations of cisplatin, CXCR4 content increased in a dose-dependent manner in both cell lines (P < 0.05). The changes of CXCR4 content in A2780/cis cells are more significant than in the A2780 cells. These data indicated a correlation between the expression of CXCR4 and varying concentrations of cisplatin in A2780 and A2780/cis.

CXCR4 siRNA effectively reduces CXCR4 protein levels in ovarian cancer cells
To detect the effect of siRNA targeting CXCR4 expression in A2780 and A2780/cis cells, qRT-PCR and Western blot were performed to evaluate the expression of CXCR4 mRNA and protein. As shown in Supplementary Fig. S1, transient transfection of CXCR4-specific siRNA resulted in the suppression of CXCR4 protein and mRNA expression (P < 0.05). These data indicated that siRNA CXCR4 could effectively inhibit the expression of CXCR4 in EOC cells.

MTT assays were performed to determine whether the knockdown of cellular CXCR4 level inhibited cell proliferation. The cell growth curves showed that CXCR4 siRNA could suppress the growth of EOC cells more than that of the other two cell lines. The inhibition occurred at 36h and became more significant after that point (Fig. 3A).

A significantly decreased cell survival was observed in cells transfected with CXCR4 siRNA compared with cells transfected with scrambled siRNA (Fig. 3B). The A2780 cells transfected with CXCR4 siRNA had IC50 values 5.19 ± 1.47 μM cisplatin compared with 13.65 ± 1.77 μM for the control A2780 cells. The A2780/cis cells transfected with CXCR4 siRNA had 2.17- to 5.37-fold enhancement in cisplatin sensitivity (P < 0.05), which indicates a protective effect of CXCR4 on cisplatin-induced apoptosis.

Taken together, these results demonstrate that siRNA-mediated CXCR4 inhibition could result in an obvious decrease in cell viability and enhance the cisplatin sensitivity of EOC cells.

CXCR4 regulates ovarian cancer cell cycle and apoptosis
In order to further define the role of CXCR4 in determining the proliferation rate of ovarian cancer cells, we next analyzed cell cycle of A2780 and A2780/cis following siRNA depletion of CXCR4 at 48 hours after transfection by flow cytometry. A2780 and A2780/cis cells treated with CXCR4 siRNA for the same period of time showed a significant increase in the proportion of cells in G1 phase and a significant decrease in the proportion of cells in S phase in both A2780 and A2780/cis cells compared with that of the cells transfected with scramble siRNA (P < 0.05) (Fig. 4A and B) (Supplementary Table S4 and S5).

We also investigated the impact of CXCR4 siRNA and cisplatin combination therapy on A2780 and A2780/cis cells by flow cytometry. Cells were treated with Scrambled siRNA or CXCR4 siRNA, and cisplatin (IC50 concentration)-treated, respectively. As shown in Fig. 4C, the inhibitory effect on the expression of CXCR4 promotes cells apoptosis, while CXCR4 siRNA increased significantly cell apoptosis induction by cisplatin. These results demonstrate that the combination of CXCR4 siRNA and cisplatin induces cell apoptosis in A2780 and A2780/cis cells in a supra-additive manner. These data indicate that down-regulated the expression of CXCR4 results in G1/S phase arrest and an increase in apoptosis.
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In several retrospective studies, CXCR4 expression was investigated for its relationship with prognosis in several human tumor types. In 71 primary cutaneous melanoma specimens, CXCR4 expression was correlated in multivariate analysis to an unfavorable prognosis when compared to CXCR4 negative subjects (20). In this study, we used IHC staining to investigate the relationship between CXCR4, resistance, and prognosis toward cisplatin-based chemotherapy in EOC. Our research indicated that the overexpression of CXCR4 was associated with chemotherapy responses, the histological grade and an advanced FIGO stage (III+IV). These findings suggest that CXCR4 expression might be involved in tumor progression and chemoresistance and might be an independent prognostic factor with regard to both PFS and OS. Consistent with our results, Sekiya et al. (9) showed that the overexpression of CXCR4 was an independent prognostic factor for worse overall survival and progression-free survival of patients with clear cell carcinoma of the ovary.

Chemotherapy can also lead to the specific enrichment of CXCR4-expressing chemoresistant tumor cells, as shown in an orthotopic metastatic melanoma model (21). We also found that the expression levels of CXCR4 were correlated with the response to cisplatin-based chemotherapy in vitro. This was an intriguing finding because of CXCR4's well-known role in tumor metastasis. CXCR4 expression levels significantly increased in A2780/cis cells when compared with the parental A2780 cells. Our findings here demonstrate that the cisplatin drug-resistance decreased in cells transfected with CXCR4 siRNA, which indicates that reduction of CXCR4 expression dramatically enhances the chemosensitivity to cisplatin in EOC cells. In addition, our current work showed that CXCR4 silencing had a powerful effect on increasing the proportion of cells in G1 phase and causing G1/S phase arrest as well as increasing the rate of cell apoptosis in EOC cells, which is consistent with previous studies (11).

In summary, our study demonstrated that the overexpression of CXCR4 in EOC is not only correlated with lower degree of differentiation and advanced FIGO stage but also is closely related to chemotherapy resistance. The expression of CXCR4 can be used to predict the sensitivity to cisplatin-based chemotherapy in EOC patients. Reduction of CXCR4 expression can suppress cell proliferation, increase apoptosis and dramatically enhance the chemosensitivity to cisplatin in EOC cells. Although the exact molecular mechanism needs to be further studied, the results imply that CXCR4 is an important regulator of cisplatin-based chemotherapy resistance in EOC and that strategies that target CXCR4 may provide novel therapeutic treatments for cisplatin resistance in EOC.

MATERIALS AND METHODS

Patients and tissues
This study recruited 124 patients consecutively diagnosed as EOC at the Department of Obstetrics and Gynecology of Xijing Hospital between January 2004 and December 2007 (Supplementary Table S1). Institutional review board approval
was obtained from the Fourth Military Medical University, and informed consent was acquired. No patient had undergone either chemotherapy or radiotherapy before surgery. Cisplatin sensitivity was defined by the response to cisplatin-based chemotherapy or a progression-free interval > 6 months off treatment. Patients were considered to have progressed during treatment if they had a stable disease in response to initial cisplatin-based therapy, and those who relapsed within 6 months were considered to have cisplatin-refractory disease. All the specimens had been histologically diagnosed by Department of Pathology, Xijing Hospital. Overall survival is defined as the time elapsed from surgery to death of patients. Death of participants was ascertained by reporting from the family and verified by review of public records. Follow-up information of all participants was updated every 3 months by telephone and questionnaire letters. This study was approved by the Ethics Committee of the Fourth Military Medical University.

**Immunohistochemistry**

Tissue specimens were taken intraoperatively, fixed in 10% formalin and embedded in paraffin. Consecutive sections were used for immunohistochemical staining with the CXCR4 antibody (1:100, Sigma-Aldrich). The intensity of CXCR4 staining was scored as 0 = no staining, 1 = weak, 2 = moderate, and 3 = marked. Percentage scores were assigned as 1 (1-25%); 2 (26-50%); 3 (51-75%); and 4 (76-100%). The scores of each tumor sample were multiplied to give a final score between 0-12, which was scored as 0 = no staining, 1 = weak, 2 = moderate, and 3 = marked. Percentage scores were assigned as 1 (1-25%); 2 (26-50%); 3 (51-75%); and 4 (76-100%). The scores of each tumor sample were multiplied to give a final score between 0-12, and the tumors were finally classified as either negative (−), score 0; lower expression (+), score 1-4; or high expression (+++), score ≥ 9. In this study, we grouped all of the samples into a high expression group (++ or +++) and a low expression group (− or −−) according to the amount of protein expression. Two of the pathologists independently graded the staining intensity in all cases without prior knowledge of the clinical data.

**Cell culture**

Paired isogenic cisplatin-sensitive human ovarian cancer cell line A2780 and cisplatin-resistant cells A2780/cis were kindly provided by Dr. Jia Luo (Institute for Nutritional Science, Shanghai, China) and cultured in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine. A2780/cis cells were grown with cisplatin (1 μM) every 3 passages to maintain resistance. Cisplatin was purchased from Sigma-Aldrich.

**siRNA transfection**

A2780 and A2780/cis cells were seeded onto 6-well plates at 1 × 10^4 cells/well and transfected with siCXCR4 or scrambled siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. 48 h later, cells were harvested. si-CXCR4 (5'-UCUUGGGCCUUCUAUCAG GCUU-3') was synthesized by Gene Pharma (Shanghai, China). Scrambled siRNA oligos (Scr) was used as a negative control.

**qRT-PCR**

RNA was extracted from cells using the RNeasy Kit (Takara, Dalian, China) following the manufacturer’s protocol. Reverse transcription was performed using PrimeScript RT-PCR Kit (Takara). CXCR4 and GAPDH expression were analyzed by SYBR GREEN-based qRT-PCR (LightCycler480 system, Roche, WI, USA). All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co, Ltd. The primer sequences were as follows: CXCR4, 5’-ACTACACCGAG GAAATGGGCT-3’ (sense) and 5’-CCCACAATGCCAG TAAG AAG A-3’ (antisense).

**Western blot**

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail tablets (Roche). Equivalent amounts of cell extracts were separated by 10% SDS-PAGE and transferred onto PVDF. Filters were incubated with anti-CXCR4 antibody (1:1,000) at 4°C overnight. Then, the secondary antibodies were added at a 1:5,000 (Thermo, MA, USA) dilution at room temperature for 1 h and detected by chemiluminescence using ECL Hyperfilm.

**Immunofluorescence staining**

Cells were washed by precooled PBS (4°C) and fixed with 4% paraformaldehyde at 4°C for 15 min. Then, these cells were incubated with CXCR4 antibody (1 : 50) at 4°C overnight. Then, Cy3-conjugated anti-mouse secondary antibodies (Sigma-Aldrich) were incubated with the cells at room temperature for 30 min. To visualize the nuclear material, 4,6-diamidino-2-phenylindole (DAPI) was incubated with the cells at room temperature for 5 min. The expression patterns were visualized using confocal fluorescence microscopy.

**MTT**

Cells were plated at 1,000 cells/well in 96-well plates and transfected with siCXCR4 or scrambled siRNA. 48 h later, MTT was added (20 μl/well), and the solution was incubated for another 4 h. Formazan products were solubilized with DMSO, and the optical densities were measured at 490 nm. All experiments were performed in triplicate.

**Cisplatin sensitivity assays**

After 48 h transfection with siRNA molecules, cisplatin cytotoxicity was measured using the MTT assay. Cells were plated at 1 × 10^4 cells/well in 96-well plates in triplicate for each dose. Cisplatin was added with the desired drug treatment concentrations ranging from 1 to 128 μM and incubated for 48 h. Formazan products were solubilized with DMSO, and the optical densities were measured at 490 nm. IC_{50} was used as the measure of relative cytotoxicity.

**Flow cytometry**

A2780 and A2780/cis cells were plated onto 6-well plates trans-
fected with siCXCR4 and scrambled siRNA and then harvested at 48 h post-transfection. Apoptotic cells and the percentage of cells in each stage of the cell cycle were analyzed by flow cytometry.

Statistical analysis

The data were represented as means ± SD. The correlation between CXCR4 expression levels and the clinical characteristics of the patients was analyzed using the χ^2 test. Survival was analyzed using the Kaplan–Meier method. A Log-rank test was used for univariate analysis of prognostic factors. P < 0.05 was considered statistically significant.

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REFERENCES

1. Siegel, R., Naishadham, D. and Jemal, A. (2012) Cancer statistics, 2012. CA 62, 10-29.
2. Sudo, T. (2012) Molecular-targeted therapies for ovarian cancer: prospects for the future. Int. J. Clin. Oncol. 17, 424-429.
3. Sueblinvong, T., Ghebre, R., Iizuka, Y., Pambuccian, S. E., Isaksson Vogel, R., Skubitz, A. P. and Bazzaro, M. (2012) Establishment, characterization and downstream application of primary ovarian cancer cells derived from solid tumors. PLoS One 7, e50519.
4. Teicher, B. A. and Fricker, S. P. (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. J. Biol. Chem. 287, 12132-12141.
5. Ying, J., Xu, Q., Zhang, G., Liu, B. and Zhu, L. (2012) RNAi targeting CXCR4 inhibits tumor growth through inducing cell cycle arrest and apoptosis. Mol. Ther 20, 398-407.
6. Alitalo, K., Kim, I. and Koh, G. Y. (2010) CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. Cancer Res. 70(635,677),(690,773)(680,677),(736,773)(737,677),(792,773)(793,677),(848,773)(849,677),(904,773)(906,677),(961,773)(384,677),(439,773)(488,677),(543,773). J. Exp. Clin. Canc. Res. 31, 34.
7. Zhou, Z., Chen, Z. W., Yang, X. H., Shen, L., Ai, X. H., Lu, S. and Luo, Q. Q. (2012) Establishment of a biomarker model for predicting bone metastasis in resected stage III non-small cell lung cancer. J. Exp. Clin. Canc. Res. 31, 34.