CXCL12/CXCR4 axis induces proliferation and invasion in human endometrial cancer

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Abstract: Objective: Since that we have previously found CXCL12/CXCR4, an important biological axis is highly transcribed in several cancer cells. We aim to investigate whether CXCL12/CXCR4 axis regulates critical processes in neoplastic transformation that affects endometrial cancer cell biology. Methods: The expression levels of CXCR4 were analyzed in human normal endometrial tissue, simple hyperplasia, atypical hyperplasia and endometrial cancer cells by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR). Serum CXCL12 was measured by Enzyme-Linked Immunosorbent Assay (ELISA) in Ishikawa endometrial cancer cell line. To study the biological function of CXCL12/CXCR4 in endometrial cancer, short interfering RNA silencing of CXCR4 was established to analyze the roles of CXCL12/CXCR4 in proliferation, migration, invasion and apoptosis of Ishikawa cells in vitro. Results: The expression level of CXCR4 in endometrial cancer tissue was higher as compared to atypical hyperplasia, simple hyperplasia and normal cycling endometrium cells. Ishikawa cells secreted CXCL12 spontaneously and continuously for 96 hrs in culture. The proliferation, migration and invasion of Ishikawa cells was significantly induced, and the apoptosis was significantly reduced by CXCL12 in combination with CXCR4. Moreover, CXCR4 silencing could significantly antagonize all these functions. Conclusions: CXCL12/CXCR4 axis plays an important role in the proliferation, invasion and metastasis of endometrial cancer, indicating that CXCR4 could be the target for the treatment of endometrial cancer.

Keywords: CXCL12, CXCR4, endometrial cancer, proliferation, invasion, apoptosis

Introduction

Endometrial cancer is the most common gynecological malignancy in many developed countries, affecting about 2-3% of women [1]. Endometrial cancer possesses a complicated chemokine network that could influence the extent and phenotype of leukocyte infiltrates angiogenesis, tumor cell growth, survival and migration. The prognosis of endometrial cancer is closely related to some factors in the tumor microenvironment, such as cytokines and chemokines [2]. Recent advances in the molecular genetics of endometrial cancer have revealed that molecular changes play an important role in the development of endometrial cancer [3].

Chemokines are small signaling cytokines act as chemoattractants through interaction with G-protein [4, 5]. CXCL12, also known as SDF-1, functions as a growth factor for B cell progenitors [6], a chemotactic factor for T cells and monocytes, a regulator of hematopoiesis, and a chemoattractant for tissue-committed stem cells [7, 8]. CXCR4 is extensively expressed in several cell types, including hematopoietic stem cells, peripheral blood leukocytes, endothelial and epithelial cells. In addition, CXCR4 is the most common chemokine receptor found in cancer cells, which is associated with aggressive disease course, metastasis and poor prognosis [9].

CXCR4 is over-expressed in 23 cancer cell lines and many human tumor types, including lung, breast, stomach, liver, bladder, and ovarian cancer [10]. However, its role in endometrial cancer is largely unknown. Understanding the function of CXCR4 in endometrial cancer could provide clues to endometrial carcinogenesis, and also offer therapeutic alternatives. To detect the function and regulation of CXCL12/CXCR4 in endometrial cancer, we investigated the expressions of CXCL12 and CXCR4, as well
CXCL12 plays a critical role in human endometrial cancer

as their impacts on proliferation, invasion, migration and apoptosis of Ishikawa cells.

**Materials and methods**

**Tissue selection**

A total of 146 samples of paraffin-embedded tissues, including 78 endometrial adenocarcinoma tissues, 14 atypical hyperplasia tissues, 32 simple hyperplasia tissues and 22 normal cycling endometrium tissues were collected from the Affiliated Hospital of Qingdao University. The cancer group consisted of 27 cases of Grade 1 tumors, 39 of Grade 2, and 12 of Grade 3. Among which, 53 cases were categorized as Stage Ia, 14 cases as Stage Ib, and 11 cases as Stages II-III. The average age of the cancer patients was 55 years (range: 26-78 years). Histological diagnosis and tumor grading were based on the International Federation of Gynecology and Obstetrics (FIGO) classification system [11]. None of the selected patients was undergoing hormone replacement therapy, radiotherapy and chemotherapy. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Informed consent was obtained from each patient.

**Cell culture**

Human endometrial cancer cell line Ishikawa was obtained from the central laboratory of the Affiliated Hospital of Qingdao University (Shandong Province, China). Ishikawa cells was cultured in DMEM (Gibco BRL) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂.

**Immunohistochemical analysis**

Immunohistochemical assays were performed as previously published. Briefly, deparaffinized sections were sequentially blocked with methanol containing 3% H₂O₂ and 7% horse normal serum, and incubated with rat anti-CXCR4 antibody (MAB171, 1:80 dilution; R&D, USA) and rat IgG, respectively, overnight at 4°C. The sections were then improved with avidin-biotin histostain kit. Slides were stained with 3, 3'-diaminobenzidine (DAB), and counterstained with hematoxylin. Immunohistochemical results were evaluated by a pathologist. The mean percentage of positive tumor cells was determined in at least 10 random fields at 400 X magnification for each section, and graded as 0 score (<10%), 1 score (10-49%), 2 score (50-74%), and 3 score (≥75%). The intensity of the CXCR4 immunoreaction was graded as no (0 score), weak (1 score), moderate (2 score), and intense (3 score). The mean percentage of positive tumor cells and the staining intensity were then combined. Cases that graded “2 score = weak, 3-4 score = moderate, and 5-6 score = strong” were considered positive for CXCR4, while others were considered negative. Reviewers of IHC staining were blinded to the pathology results.

**RT-PCR analysis**

Trizol (Invitrogen) was used to extract total RNA from the Ishikawa cells. The RNA was reverse-transcribed into cDNA using a reverse transcription kit (TaKaRa, Japan). Primers for CXCR4 were 5’-GAA CCT CCT ATG CAA GGC AGT CC-3’ (sense) and 5’-CCA TGA TGC TGA AAC TGG AAC-3’ (antisense). The PCR product was 302 bp long. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The GAPDH primers were 5’-GGC ATG GAC TGG TGG TCA TGA G-3’ (sense) and 5’TCA TGG GTG TGA ACC ATG AGA A-3’ (antisense), and the PCR product was 146 bp long. PCR conditions were as follows: 95°C for 5 min, 36 cycles of 98°C for 10 sec, 59°C for 30 sec, 72°C for 1 min, and 72°C for 10 min. PCR products were detected by 2% agarose electrophoresis. The intensities of CXCR4 and GAPDH were evaluated. The relative levels of CXCR4 were expressed as the ratio of CXCR4/GAPDH.

**Enzyme-linked immunosorbent assay**

Ishikawa cells were seeded in a 96-well plate at densities of 2×10⁴, 5×10⁴, 6×10⁴, and 8×10⁴ cells/ml. Cell supernatants were collected after 12, 24, 36, 48, 60, 72, 84 and 96 h of culture. The supernatants were centrifuged at ×2000 g and stored at -80°C. Human CXCL12 ELISA kit (R&D Systems, UK) was used to measure chemokine production in each supernatant according to the manufacturer’s instructions.

**Western blot analysis**

Cells were harvested and dispersed with 200 µl of RIPA and PMSF (RIPA; PMSF = 1:1) buffer. 50 µg of each soluble protein sample was separated by 12% or 15% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane, blocked in
CXCL12 plays a critical role in human endometrial cancer

5% skimmed milk, and probed with each primary antibody overnight at 4°C. Immunoreactive proteins were visualized using an Enhanced Chemiluminescence (ECL) System (GE Healthcare UK Ltd., UK). The optical densities of CXCR4 (molecular weight: \(43 \times 10^3\) kD) and GAPDH (molecular weight: \(36 \times 10^3\) kD) were analyzed, and quantified using Quantity One 4.6 software. Primary antibody against CXCR4 was purchased from R&D Systems (MAB171, 1:80 dilution).

Cell proliferation assay

Suspended cells were seeded at \(5 \times 10^4\) cells/ml in 96-well plates in 100 μl of serum-free medium, and the medium was then replaced with DMEM containing 10% heat-inactivated FBS. All cells were cultured at 37°C in a humidified atmosphere with 5% CO\(_2\). The samples were divided into four groups, Group A: CXCR4-siRNA; Group B: Blank-control; Group C: siRNA-control; Group D: vector-control. Each group had six parallel control samples. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer’s instructions. After transfection, 20 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemicals, USA] and 180 μl of DMEM with 10% heat-inactivated FBS were added for 24, 48 and 72 h, respectively. After continuously culturing for 4 h, cells were washed with serum-free medium. Then, 150 μl of DMSO was added for 15 min to shock the cells. The absorbance of each well was measured at 490 nm using a microplate reader.

Migration assay

24-well transwells with fibronectin-coated polycarbonate membrane inserts (6.5 mm in diameter with 8.0 μm pores) were used for the migration assay. The samples were divided into four groups, and each group had three parallel samples. Group A: 100 μl of \(5 \times 10^4\)/ml suspended Ishikawa cells were added to the upper chambers, and the lower wells were filled with 600 μl of medium containing 1% heat-inactivated FBS. Group B: The upper chambers were filled with the same sample as group A, and the lower wells were filled with the same sample as group C. The cells were allowed to invade for 12 h in 5% CO\(_2\) at 37°C. We then removed the cells attached to the upper surface of the filter by scrubbing with a cotton swab. The cells on the lower surface were fixed in 95% alcohol for 20 min at room temperature, and stained with 0.1% crystal violet for 5-8 min. For quantification, the cells that had migrated to the lower surface were counted under a light microscope in five predetermined fields at 200 X magnification. The assay was repeated three times, and the results were expressed as a percentage of the mean of three controls.

Matrigel invasion assays

A 24-well transwell plate was used for the invasion assay with the filter coated with 75 μl of pure Matrigel, and air-dried. Before use, the Matrigel was rehydrated with 100 μl of warm DMEM for 2 h. The samples were divided into four groups, and each group had three parallel samples. Group A: 100 μl of \(5 \times 10^4\)/ml suspended Ishikawa cells were added to the upper chambers, and the lower wells were filled with 600 μl of medium containing 5% heat-inactivated FBS and 10 ng/ml rhCXCL12. Group B: The upper chambers were filled with the same sample as group A, and the lower wells were filled with 600 μl of medium containing 5% heat-inactivated FBS and 100 ng/ml rhCXCL12. Group C: 100 μl of \(5 \times 10^4\)/ml suspended Ishikawa cells cultured with AMD3100 for 4 h were added to the upper chambers, and the lower wells were filled with the same sample as group B. Group D: The upper chambers were filled with the same sample as group A, and the lower wells were filled with 600 μl of medium containing 5% heat-inactivated FBS. The remaining procedure was the same as the migration assay.

Design and preparation of constructs

Three siRNA sequences targeting human CXCR4 were designed by Jima (Shanghai, China). The three siRNAs were named Cxcr4-homo-960, Cxcr4-homo-370 and Cxcr4-homo-1609, and their sequences were sense 5′-CUG UCC UGC UAU UGC AUU ATT-3′, antisense 5′-UAA UGC UGC UAU GCU ATT-3′.
CXCL12 plays a critical role in human endometrial cancer

AAU AGC AGG ACA GTT-3'; sense 5'-GGG ACU AUG ACU CCA UGA ATT-3', antisense 5'-UUC AUG GAG UCA UGA ATT-3', and sense 5'-CGU GGU AGG ACU GUA GAA ATT-3', antisense 5'-UUU CUA CAG UCC UAC CAC GTT-3', respectively. The FAM (sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'), which had no significant homology to any known human mRNA in the databases, was used as a negative control. GAPDH (sense 5'-GUA UGA CAA CAG CCU CAA GTT-3', antisense 5'-CUU GAG GCU GUU GUC AUA CTT-3') was used as a positive control.

In vitro transfection

Transfection of siRNAs was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 5×10^5 Ishikawa cells/well were plated onto 6-well plates and grown for one day by when the cells were 75-85% confluent. The siRNA and Lipofectamine 2000 were each diluted in 250 μl of serum-free DMEM (Hyclone), and incubated for 5 min at room temperature. Next, the diluted siRNA and Lipofectamine 2000 were combined at a 1:1 ratio (4 pmol of siRNA with 4 μl of Lipofectamine 2000). This combination was mixed gently, and incubated for 20 min at room temperature to form siRNA/Lipofectamine 2000 composite. 400 μl of the combination was added to each well in a final volume of 2 ml/well. The cells were incubated for another 48 h before the experiments were conducted.

Cell cycle and apoptosis analysis after CXCR4 silencing

Cell cycle distribution was determined by measuring the cellular DNA content using flow cytometry. Cells were washed with PBS, digested with 0.25% trypsin, adjusted to 1-5×10^5 cells/ml, and fixed with 70% alcohol at 4°C overnight. The cells were washed again and suspended in 3 ml PBS. The cells were filtered through a 400 mesh screen, stained with 1 ml propidium iodide, and then kept in a darkroom for 30 min at 4°C. Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit I (BD Pharmingen, USA) was used to identify apoptotic and viable cells according to the manufacturer’s instructions. The percentage of early apoptotic (FITC-positive and propidium iodide-negative) cells was determined by flow cytometry.

Statistical analysis

Results were analyzed by SPSS 17.0 to compare the differences among different groups. X^2 test was used for multiple comparisons among three or more groups. Student’s t-test was used to compare the means between two groups. Pearson’s correlation analysis was used to...
CXCL12 plays a critical role in human endometrial cancer


Results

CXCR4 mRNA expression in endometrial cancer as compared to normal endometrium

We analyzed CXCR4 mRNA expression in endometrial cancer and normal cycling endometrium by semi-quantitative RT-PCR, and found that CXCR4 mRNA expression level was significantly higher in endometrial cancer cells as compared to normal endometrium cells (P<0.01), with the ratios of the band intensities of CXCR4 to GAPDH being 0.503 and 0.281 (mean values), respectively (Figure 1).

CXCR4 protein expression in endometrial cancer

Immunohistochemical staining showed that CXCR4 protein expression was positive in 69.23% (54 of 78) of endometrial cancer samples, 57.14% (8 of 14) of atypical hyperplasia samples, 46.88% (15 of 32) of simple hyperplasia, and 31.82% (7 of 22) of normal endometrium samples. As compared to those in atypical hyperplasia, simple hyperplasia and normal endometrium cells, CXCR4 protein expression was significantly higher in endometrial cancer cells. (P<0.05). The staining intensity was also stronger in tumor cells as compared to atypical and normal cells (Figure 2A-D). However, no correlation was found between CXCR4 protein expression and clinicopathological parameters, including patient age, tumor grade, and tumor stage (Table 1), suggesting that CXCR4 overexpression is an early event in endometrial carcinogenesis.

Secretion of CXCL12 by Ishikawa cells

Ishikawa cells constitutively secreted CXCL12 at a nearly constant and high rate in 48 h culture, after which the secretion rate decreased weakly (Figure 2E). Serum CXCL12 level was correlated positively with the plating density. CXCL12 secretion level of Ishikawa cells was 3.187±0.399 ng/ml after culturing for 96 h at a density of 2×10^4 cells/ml, which was significantly lower than those seeded at 5×10^4 cells/ml.
CXCL12 plays a critical role in human endometrial cancer

Table 1. Relationship between CXCR4 expression and clinico-pathological features of endometrial cancer

<table>
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<tr>
<th>Characteristic</th>
<th>Total Number</th>
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<th>Number of CXCR4 positive</th>
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<tr>
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*P<0.05 as compared to normal endometrium.

CXCL12 induced Ishikawa cell proliferation in a dose-dependent manner. The number of viable Ishikawa cells increased significantly as compared to the negative control with 10 ng/ml of CXCL12. Higher proliferation of Ishikawa cells was observed after treatment with 100 ng/ml and 300 ng/ml of CXCL12. Meanwhile, addition of AMD3100 completely inhibited the stimulatory effect of exogenously administered CXCL12 (Figure 3A).

To further explore the role of CXCL12 in Ishikawa cell proliferation, three recombinant CXCR4 siRNAs (Cxr4-homo-960, Cxr4-homo-370 and Cxr4-homo-1609) and one negative control (FAM-neg) were successfully constructed and tested for their ability to knockdown CXCR4 expression in Ishikawa cells. Transfection with Cxr4-homo-960 resulted in a reproducible decrease in the expression level of CXCR4 mRNA and protein, whereas transfection with FAM-neg did not alter the mRNA and protein expression levels of CXCR4. Results showed that transfection with Cxr4-homo-960 significantly reduced the proliferation of Ishikawa cells in 48 h and 72 h cultures (Figure 3B), suggesting that CXCL12 induced cell proliferation in combination with its receptor CXCR4.

AMD3100 inhibits the migration of Ishikawa cells induced by CXCL12

To evaluate the role of CXCL12 in Ishikawa cell migration, AMD3100 was used to block the association between CXCL12 and CXCR4. As shown in Figure 4, 100 ng/ml of CXCL12 significantly increased Ishikawa cell migration, and this effect was significantly blocked by AMD3100. Stimulation of Ishikawa cell migration was readily apparent upon microscopic examination of the lower surface of the filter. Therefore, we speculate that CXCL12 could induce migration of Ishikawa cells by combining with its receptor CXCR4.

CXCR4 silencing inhibits the invasion of Ishikawa cells induced by CXCL12

As shown in Figure 5A, CXCL12 caused a specific dose-dependent increase in Ishikawa cell invasion. This stimulatory effect on cell invasion was first seen with 10 ng/ml of CXCL12 (P<0.05), and was approximately 1.9-fold higher with 300 ng/ml of CXCL12. The stimulation of Ishikawa cell invasion by CXCL12 was completely inhibited by AMD3100. To confirm the role of CXCL12/CXCR4, we then inhibited the expression of CXCR4 using three recombinant CXCR4 siRNAs (Cxr4-homo-960, Cxr4-homo-370 and Cxr4-homo-1609). The invaded cells...
CXCL12 plays a critical role in human endometrial cancer

CXCL12 was shown to be over-expressed in all types of human cancers, including endometrial cancer. In this study, we compared the expression levels of CXCR4 in endometrial cancer with atypical hyperplasia endometrium, simple hyperplasia endometrium and normal endometrium. We demonstrated that the mRNA and protein levels of CXCR4 were significantly up-regulated in endometrial cancer as compared to atypical, simple hyperplasia and normal endometrium. Furthermore, continuous secretion of CXCR4 ligand, CXCL12 was found in Ishikawa cells. These findings indicate that CXCR4 plays an important role in endometrial carcinogenesis.

RNAi uses short RNA duplexes of defined sequences to silence a target gene [12], and is an important technique for knocking down gene expression to study gene function. For our study, we designed and successfully constructed three CXCR4 gene expression vectors with double-stranded RNAi and verified that three of them (Cxcr4-homo-960, Cxcr4-homo-370, Cxcr4-homo-1609) were effective, with Cxcr4-
CXCL12 plays a critical role in human endometrial cancer

CXCL12 plays a critical role in human endometrial cancer

CXCL12 plays a critical role in human endometrial cancer

homo-960 being the most effective in knocking down CXCR4 gene expression in Ishikawa cells. In contrast, the negative sequence (FAM-neg) did not knockdown CXCR4 gene expression.

CXCL12 plays a critical role in human endometrial cancer

CXCR4 is a regulator of spindle microtubule function during mitosis, and an inhibitor of apoptosis. CXCL12/CXCR4 axis has been shown to play an important role in targeting cancer cells to metastasis sites. Recently, CXCR4 was found positive in at least 23 malignancies, and its expression level was significantly increased in many malignancies. Dominguez et al. found that CXCR4 expressed during the whole menstrual cycle, and its expression levels changed in different phases of the cycle [13]. Mizokami et al. found that CXCR4 was always expressed in endometrial cancer cell lines and tissues [14], and the expression of CXCL12/CXCR4 was higher in well-differentiated carcinoma than in poorly differentiated carcinoma. Tsukamoto et al. found that the expression of CXCR4 increased along with the tumor infiltration extent in endometrial cancer samples, which was consistent with our previous study.

In the present study, we found that the expression of CXCR4 mRNA in Ishikawa cells was consistent with previous studies. Although CXCR4 is highly expressed during the cell cycle and over-expression of CXCR4 may overcome the checkpoint to enforce progression of cells through mitosis, we observed that knockdown of CXCR4 expression caused S-phase cell cycle arrest.

Figure 5. Invasion capacity of Ishikawa cells significantly increased after CXCL12 treatment (A) and decreased after CXCR4 silencing (B and C). Treatment with CXCL12 significantly induced Ishikawa cell invasion in a dose-dependent manner. This stimulatory effect could be completely inhibited by AMD3100 (A). siRNA-mediated knockdown could reduce the invasion capacity of Ishikawa cells. Results were highly reproducible in five independent experiments, and (B) is a representative example. The penetrating cells in CXCR4-siRNA group reduced significantly as compared to the three control groups (C). *P<0.05, **P<0.01 versus control group. ###P<0.01. Error bars depict the standard error of the mean.
CXCL12 plays a critical role in human endometrial cancer

![Figure 6. Induction of S-phase cell cycle arrest and apoptosis in Ishikawa cells by CXCR4 RNAi. After transfection for 72 h, Ishikawa cells were subjected to fluorescence-activated cell sorting analysis to determine the cell cycle distribution based on DNA content (A) and apoptosis (B). CXCR4-siRNA group showed higher S-phase accumulation (P<0.01) and apoptosis rate (P<0.01) as compared to the control group. Results were highly reproducible in three independent experiments, and (A and B) are representative examples.]
CXCL12 plays a critical role in human endometrial cancer

Thus, CXCR4 could regulate the phase checkpoint in endometrial cancer.

In several recent studies, CXCL12/CXCR4 was blocked using CXCR4 antagonists such as T140, AMD3100 and vMIP-II to change the expression of cytoskeleton, suppress actin polymerization and dissociation, promote pseudopodia formation, and inhibit cell migration in order to suppress the metastasis of malignant cells, but this method is not very specific [15]. Since that RNAi can silence the target gene with a high degree of sequence specificity and efficiency, RNAi serves as an effective and appropriate tool for investigating specific genes. In this study, we transfected siRNAs into endometrial cancer Ishikawa cell line using Lipofectamine 2000. Our results show that overexpression of CXCR4 is involved in endometrial cancer cell proliferation, migration, invasion and metastasis, which is consistent with previous studies [16]. Silencing expression of CXCR4 mRNA by RNAi could suppress the migration and invasion of endometrial cancer cells.

Carcinogenesis is a multi-gene, multi-stage process, which is influenced by environmental factors. CXCL12 activity is mediated by two receptors, CXCR4 and CXCR7, which could also act as rate-limiting elements [17]. CXCR7 is expressed in many tumor cell lines include endometrial cancer. Furthermore, there is evidence to show a crosstalk between CXCR7 and CXCR4 in CXCL12-mediated events, such as cell motility and chemotaxis [18]. Given the central role of CXCR4 in cancer metastasis, it could serve as an important diagnostic target in the detection and treatment of cancer. Future studies should focus on understanding the mechanisms underlying increased CXCR4 expression, and potentially target such pathways in cancer treatment. It is also important to determine the respective contributions of CXCR4 and CXCR7 to the pathological activities of CXCL12 in endometrial cancer. Thus, understanding the mechanisms that regulate CXCR4 expression and function could be useful in treatment and prevention of cancer metastasis. CXCR4 could become a new target for endometrial cancer treatment.

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Disclosure of conflict of interest

None.

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CXCL12 plays a critical role in human endometrial cancer


