Chemokine CXCL-2 Regulates Resistance to Platinum in Epithelial Ovarian Cancer by Mediating ATR/CHK1 Signaling Pathway

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Abstract

**Backgrounds:** Platinum-resistance remains a challenge to recurrence and metastasis of epithelial ovarian cancer (EOC) and the mechanisms are unidentified. Tumor microenvironment (TME) and chemokine might play a key role in tumor chemoresistance. This study is designed to reveal the important role of CXCL-2 in causing EOC platinum-resistance.

**Materials and Methods:** Differently expressed chemokines were selected based on the GSE114206 dataset of GEO database. Chemokines levels of platinum-sensitive and platinum-resistant EOC. Cell assays were performed and the cell stem characteristics were investigated for exploring the roles of CXCL-2 in EOC chemoresistance. We further explored the downstream signaling pathway regulated by CXCL-2 by detecting the expression of related molecules and rescue assay with the signaling inhibitor.

**Results:** CXCL-2, CXCL-11 and CXCL-13 were found up-regulated in platinum-resistant EOC based on GSE114206 databases, and CXCL-2 was identified as key chemokine by validation. The cell assays showed overexpressing CXCL-2 and co-culturing with recombinant human CXCL-2 promoted cancer cell chemoresistance. Conversely, knocking down CXCL-2 and co-culturing with neutralizing antibody to CXCL-2 reduced cisplatin-resistance in cisplatin-resistant EOC cells. CXCL-2 levels regulated the stemness of cancer cells and activated ataxia telangiectasia and Rad3-related (ATR)/cell cycle checkpoint kinase1 (CHK1) signaling pathway. The cancer cell chemoresistance can be saved by CXCL-2 receptor inhibitor (SB225002) and CHK1 inhibitor (SAR-020106) in vitro.

**Conclusion:** These results identified a CXCL-2 mediated platinum-resistance mechanism in EOC, and provided a novel target for chemoresistance prevention and treatment strategies.

1. **Background**

Epithelial ovarian cancer (EOC) is the first cause of gynecological malignancy-related death. According to statistics of American Cancer Society in 2020, there is estimated numbers of 21750 ovarian cancer cases and 13940 deaths in the United States in 2020. The currently accepted treatment for EOC is the satisfied tumor reductive surgery and platinum-based chemotherapy. Although the tremendous progress has made in comprehensive therapy, the survival rate of patients with advanced tumor are poor even in high-resource countries such as the United States and Canada, remained at only 47%. EOC is most commonly treated with a combination of platinum and paclitaxel. Though initial response rates are 60-80%, majority of EOC patients acquire platinum-resistant during treatment with subsequent relapses and metastasis. Platinum resistance remains an urgent challenge for patients with EOC, and the mechanism remains unidentified. There is lack of effective approach to prevent and against resistance for patients with EOC. Therefore, revealing the molecular mechanism contributing to platinum resistance and exploring therapeutic target to overcome the platinum resistance of EOC are clinical significances.
Chemokines contains a group of about 50 small (8–14kDa) secreted proteins, which regulate cell biological processes, including in malignancies progression \(^4,5\). These secreted proteins work by interacting with the corresponding receptors, a family of about 20 seven-transmembrane G-protein-coupled receptors \(^6\). Previous studies have suggested chemokines and their receptors are involved in malignancies progression mainly in the three mechanisms: attracting cancer cells for metastasis; mobilization of hematopoietic cell populations from the bone marrow to colonize at the tumor site and regulate tumor processes; acting as growth factors, which supporting tumor growth through an autocrine pathway \(^7,8\). Increasing evidences have supported that using immune checkpoint inhibitors that targeting chemokines and their receptors can be a novel approach in cancer therapy \(^9-11\). The anti-CCR4 monoclonal antibody and the CXCR4 receptor inhibitor are already in the clinical practice for hematological malignancies \(^12-16\). CXCL-10 was suggested as an immune checkpoint molecule in cancer, displaying a positive autocrine effect and directly suppressing tumor growth \(^17,18\). Increased CXCL-17 expression was demonstrated that associated with lung cancer and hepatic cancer. In addition, the recent studies have also proved chemokines and their receptors participate in the cancer chemoresistance. Ren Ying et al. demonstrated CXCR3 regulated AMPK pathway activity in hepatocellular carcinoma, which resulting in metabolic alteration during the chemoresistance to agent-sorafenib \(^19\). Zhang Guolin et al. identified CXCL-13 mediated 5-Fu resistance in colorectal cancer \(^20\). These studies suggested that chemokines and their receptors might be potential novel therapeutic target in cancer chemoresistance.

Currently, gene expression profiles have been increasingly used in studies to identify candidate significant genes in various diseases, especially in malignancies \(^21\). Public genomics data repositories provided powerful systems biology approaches to detect the association between genes and cancers. In the present study, we devoted to explore the roles of chemokines in platinum-resistant EOC. We used bioinformatics methods to screened differentially expressed chemokines by comparing gene expression profiles based on platinum sensitivity status and PARP levels. Then, differentially expressed chemokines were validated the expression levels in EOC samples and cell lines to identify candidate chemokines eventually. Functional assays were further performed to investigate the molecule roles in EOC. In addition, we further explored the downstream signaling pathway which might influence the process of DNA repairment.

## 2. Materials And Methods

### 2.1 Differentially expressed genes (DEGs) screening

The gene expression profile was obtained from GSE114206 dataset of GEO database, which contained 6 EOC samples with low PARP and platinum-resistant and 6 EOC samples with high PARP and platinum-sensitive. We use the “limma” R package to screen the DEGs between the platinum-resistant and sensitive samples. The adjusted \(P\)-value < 0.05 and |log2 fold change (FC)| > 1, were chosen as the cut-off threshold. The candidate genes were visualized by “circos” package (https://www.omicstudio.cn/tool).
2.2 Patients and clinical sample collection

All the ovarian cancer tissues and serum samples were obtained from patients who were diagnosed and underwent surgery in the First Affiliated Hospital with Nanjing Medical University between January 2010 to January 2019. Surgically resected specimens were immediately flash-frozen in RNA latter (AM7020, Invitrogen, USA) at −80°C for nucleic acid and protein extraction was performed. In the present study, 15 platinum-sensitive EOC samples and 9 platinum-resistant EOC samples were collected to investigate the molecular expression. Meanwhile, blood samples of 6 platinum-resistant and 9 platinum-sensitive EOC patients were collected to detect levels of chemokine in serum. All patients signed informed consent before using clinical specimens, and the use of specimens for this study has been proved by the ethics committee of the First Affiliated Hospital with Nanjing Medical University.

2.2. Cell culture and chemicals

The platinum-sensitive EOC cell line HO8910, A2780 and the resistant cell lines A2780-DDP, HO8910-DDP, which construed by exposure to increasing concentration of cisplatin, were used for research. Cells were cultured in RPMI1640 (C11875500BT, Gibco, USA) containing 10% FBS (FB25015, CLRAK Bioscience, USA) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C supplied with 5% CO2.

2.3 Plasmid and siRNA interference

The plasmid encoding transcript of CXCL-2 was designed and synthesized by Tsingke (Nanjing, China). The siRNA targeted CXCL-2 and the negative control (NC) were purchased from GemmaPharma (Shanghai, China). Plasmid and siRNA transfected cells by Lipofectamine 3000 Transfection Reagent (L3000150, Invitrogen, USA) as manufacturer’s instructions. Cells were treated with the indicated agents for analyses 24 or 48 hours after transfection. The CXCL-2 siRNA sequence was described in previous study 22.

2.4 Cell viability assay

Preprocessed EOC cells were plated at a density of 6000-8000 cells per well in 96-well plates. A series of cisplatin (P4394, Sigma-Aldrich, USA) concentrations (0, 5, 10, 15 and 20μM) were added. After 48 hours treating, cell viability was qualified by Cell Counting Kit-8 (CCK8) assay (A31102, Vazyme, China). Absorbance was measured at 450 nm on a microplate reader (TECAN, Infinite M200 PRO, Switzerland), and IC$_{50}$ were analyzed in Graphpad 8.0. The recombinant human CXCL-2 (ab268433, Abcam, USA), CXCL-2 neutralizing antibody (ab89324, Abcam, USA) and IgG antibodies (ab188776, Abcam, USA) as control were used to regulate CXCL-2 level in supernatant of cell culture. The inhibitor of CXCR2, SB225002 (MedChemExpress, USA) and the inhibitor of CHK1, SAR-020106 (Selleck Chemicals, USA) were also used in cell viability assay.

2.5 RNA extraction and quantitative Real-time PCR (qRT-PCR)
The total RNA of cultured cells and EOC tissues was extracted with Trizol (15596026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA HiScript Q RT SuperMix for qRT-PCR (R323, Vazyme, China) to prepare cDNA. qRT-PCR was performed with an SYBR Green PCR Kit (Q311, Vazyme, China). The sequences of gene primers used for qRT-PCR were synthesized by Tsingke (Nanjing, China) and showed in Supplementary Table 1. The amplification was performed as the manufacturer’s instructions. The RNA expression level was normalized to GAPDH to calculate the relative gene expression.

2.5 Western blot assay

The total protein of cultured cells and tissues were lysed in RIPA buffer (P0013B, Beyotime, China) with protease inhibitor (P1005, Beyotime, China). Western blot assays were performed by protocol as we previously reported. The antibodies used for western blot assay contained anti-human CXCL-2 (ab89324, Abcam, USA), anti-human NANOG (ab21624, Abcam, USA), anti-human SOX2 (ab218520, Abcam, USA), anti-human OCT4 (ab200834, Abcam, USA), anti-human ATR (ab2905, Abcam, USA), and anti-human CHK1 (ab133277, Abcam, USA). In addition, anti-human β-actin and anti-human GAPDH were used as control.

2.6 Enzyme-linked immunosorbent assay

Platinum-resistant and platinum-sensitive patient’s serum and cell culture supernatants were respectively extracted for cytokine analysis. Human CXCL-2 enzyme-linked immunosorbent assay (ELISA) kits, purchased from Hengyuan Biological Co. Ltd (H-10236, Shanghai, China) were used for analysis as the manufacturers’ instructions. Each sample was duplicated.

2.7 Cell apoptosis assay

For the apoptosis assay, EOC cells were plated in 6-well plates and pretreated. After 48 hours cisplatin treating, 2× 10^4 cells and the cultural supernatant were collected. For cell staining, 5 μl of FITC Annexin V and 5 μl of propidium iodide (BD Biopharmingen, NJ, USA) were added to the collected cells and suspending in 300 μl of binding buffer for 15 min in dark. Cell apoptosis were eventually detected by a flow cytometer (FACScan; BD Biosciences, USA) using Cell Quest software (BD Biosciences, USA).

2.8 Statistical analysis

Each sample was analyzed based on results that were repeated at least three times and analyzed in Graphpad 8.0. The statistical significance of differences between two groups was determined by a standard Student’s t-test. In all cases, differences at p < 0.05 were regarded as statistically significant (*), ones at p < 0.01(**), p < 0.001(***), or p > 0.0001(****) was considered higher statistical significances.

3. Results

3.1 CXCL-2 is up-regulated in platinum-resistant EOC tissues and cell lines
By analyzing the gene expression profiles of GSE114206 dataset, 1269 up-regulated genes and 1140 down-regulated genes were identified as DEGs. Among these DEGs, we investigated the differentially expressed chemokines and found CXCL-2, CXCL-11 and CXCL-13 as candidate genes (Fig. 1A). Next, we detected the mRNA expression level of CXCL-2, CXCL-11 and CXCL-13 respectively in platinum-sensitive and resistant EOC tissues, and found CXCL-2 was up-regulated in platinum-resistant EOC tissues (Fig. 1B). In addition, CXCL-2 was found up-regulated both in A2780-DDP and HO8910-DDP cell lines (Fig. 1C-I). The results of ELISA assays showed CXCL-2 levels in serum of platinum-resistant EOC patients was higher compared to that in platinum-sensitive EOC patients (Fig. 1J).

3.2 CXCL-2 promotes tumor resistance to cisplatin in EOC

EOC cells A2780 and HO8910 were transfected with plasmid overexpressed CXCL-2 (oeCXCL-2) and the negative control (oeNC). The mRNA and protein levels of CXCL-2 was then confirmed by qRT-PCR and western blot (Fig. 2A-2C). The cell viability assay showed that CXCL-2 overexpression significantly promoted cancer cell chemoresistance in cisplatin-sensitive EOC cells (Fig. 2D,2E). Additionally, we used specific siRNA to knockdown CXCL-2 respectively in A2780-DDP and HO8910-DDP cells (Fig. 2F-2H). The cell viability assay illustrated a decreased IC$_{50}$ in cisplatin-resistant EOC cells with CXCL-2 siRNA transfected (Fig. 2I,2J).

3.3 CXCL-2 promoted tumor resistance to cisplatin in EOC by autocrine mechanism

We firstly used cell culture supernatants of A2780-DDP and HO8910-DDP cells to culture A2780 and HO8910, respectively (Fig. 3A,3B). The CXCL-2 levels in cell culture supernatants were also detected in cisplatin-resistant and sensitive EOC cells and cells transfected CXCL-2 specific siRNA or oeCXCL-2 plasmid. We found CXCL-2 level is higher in cisplatin-resistant EOC cells, and were consistently regulated by CXCL-2 expression, which suggested CXCL-2 expression might regulate the CXCL-2 level in TME by autocrine (Fig. 3C,3D). Moreover, the addition of recombinant CXCL-2 could promote cell resistance to cisplatin in A2780 and HO8910 (Fig. 3E,3F), while the IC$_{50}$ of cisplatin decreased after adding CXCL-2 neutralizing antibody in A2780-DDP and HO8910-DDP (Fig. 3G,3H). These findings showed that the CXCL-2 in the conditional culturing promote cell resistance to cisplatin. In addition, inhibition of CXCR2, the CXCL-2 receptor, decreased the IC$_{50}$ in cisplatin-resistant EOC cells (Fig. 3I, 3J).

3.4 CXCL-2 regulated the cell apoptosis induced by cisplatin and the cell stemness in EOC

The results of cell apoptosis assay showed cell apoptosis induced by cisplatin were decreased after transfecting oeCXCL-2 plasmid or adding recombinant CXCL-2 in A2780 and HO8910 (Fig. 4A-F). On the contrary, transfecting CXCL-2 specific siRNA or addition of CXCL-2 neutralizing antibody promoted cell apoptosis in cisplatin-resistant EOC cells (Fig. 4G-N). Meanwhile, we investigated the cell stem characteristics in EOC. The expression level of cell stem characteristics, including Nanog, SOX2 and OCT4, were also found be regulated by CXCL-2 (Fig. 5).
3.5 CXCL-2 promoted tumor resistance to cisplatin in EOC by mediating ATR/CHK1 signaling pathway

Given that ataxia telangiectasia and Rad3-related (ATR)/cell cycle checkpoint kinase1 (CHK1) signaling pathway has recently been proved to play a significant role in DNA repair, as a PARP-independent approach. We detected the expression of ATR and CHK1 respectively in platinum-resistant and sensitive EOC samples and cell line. The results showed that ATR and downstream molecule CHK1 expressions were up-regulated in platinum-resistant EOC tissues and cisplatin-resistant EOC cells (Fig. 6A-F). The addition of CHK1 inhibitor reduced tumor resistance in vitro (Fig. 6G and 6H). Then, we further investigated the regulation of CXCL-2 and ATR/CHK1 signaling pathway. By detecting ATR and CHK1 expression, we found CXCL-2 expression could activating ATR/CHK1 signaling pathway, while knocking down CXCL-2 expression could suppress it (Fig. 6I-R). The cell function rescue assays suggested inhibiting ATR/CHK1 signaling pathway by using SAR-020106 reduced cell chemoresistance in cisplatin-sensitive cells with oeCXCL-2 transfection (Fig. 6S and 6T).

4. Discussions

TME significantly influences therapeutic response and clinical outcomes. According to previous studies, chemokines in TME are associated with cancer cell proliferation, migration, invasion and stemness, which could partially explain the resistance of cancer cells to chemotherapeutic drugs. In the present study, we explored the chemokines contributed to platinum resistance in EOC. As a result, we identified candidate chemokines by bioinformatics analysis. We then demonstrated that the CXCL-2 expression is significantly up-regulated in platinum-resistant EOC tissues and cisplatin-resistant EOC cell lines. Cell functional assays showed CXCL-2 could promote tumor resistance to cisplatin in vitro and reduce cell apoptosis induced by cisplatin. Mechanistically, we found CXCL-2 promotes tumor resistance to cisplatin in EOC by mediating ATR/CHK1 signaling pathway.

CXCL-2, which was known as growth-related oncoregene-2/β or macrophage inflammatory protein-2α, is 90% identical in amino acid sequence. CXCL-2 works in cells via the corresponding CXCR2, which is known as interleukin 8 receptor β, have been extensively studied with regard to their role in malignancies. CXCL-2/CXCR2 axis was reported to promotes chemotaxis of neutrophils and endothelial cells to contribute to angiogenesis, chemoresistance, growth, and transformation of tumors. Natsume Makoto et al. suggested omental adipocytes trigger gastric cancer cells proliferation, migration and capacity to induce angiogenesis by CXCL2 secretion. Monocytes-derived CXCL-2 and CXCL-8 as main factors in regulating the recruitment of neutrophils into tumor milieu of hepatocellular carcinoma, which could inhibit then cancer cell apoptosis. Previous study has also suggested that concentrations of CXCL-2 were higher in serum of ovarian adenocarcinoma patients with chemoresistance. Overexpression of CXCR2 has been demonstrated promote ovarian cancer progression. However, there is lack of research about the role of CXCL-2 and the regulation mechanism in platinum-resistant EOC. Herein, we systematically explored the molecule biological function in cisplatin-resistant EOC. As the results illustrated, high expression level of CXCL-2 in TME of EOC was related to the cisplatin resistance of
tumor. We demonstrated that CXCL-2 overexpression in EOC cell could protect from cell apoptosis induced by cisplatin and maintain cancer cell stemness, which might result in cell chemoresistance phenotype.

As we known, cisplatin exerts anti-tumor effects via multiple mechanisms, yet its most prominent mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response. Interfering with cell cycle checkpoint signaling is a significant approach to modulate DNA repair activity in platinum resistant tumor. In the present study, we found CXCL-2 might regulate ATR/CHK1 signaling pathway to promote platinum-resistance in EOC. ATR and its downstream kinase CHK1, can be activated by DNA damage and DNA replication stress. Activating ATR/CHK1 signaling pathway results in arresting cell cycle, which allowing time for DNA replication and appropriate DNA repairment. In addition, activating ATR/CHK1 signaling pathway stabilizes replication forks and prevents collapse into DNA double strand breaks (DSBs). Those functions of ATR/CHK1 provided potential therapeutic targets. ATR/CHK1 inhibitors have been developed and are currently used either as single agents or paired with radiotherapy or a variety of genotoxic chemotherapies in preclinical and clinical studies, including in EOC. Catherine J Huntoon et al suggested that inhibiting ATR/CHK1 broadly sensitizes ovarian cancer cells to chemotherapy independent of BRCA status. Our finding suggested a novel regulation mechanism of CXCL-2 in tumor microenvironment and ATR/CHK1 signaling pathway. However, it is possible that CXCL-2 may also promote EOC chemoresistance in ATR/CHK1-independent pathways.

**Conclusions**

In summary, our findings revealed the role of CXCL-2 in EOC cisplatin chemoresistance and demonstrated a new regulatory mechanism of CXCL-2 mediating ATR/CHK1 signaling pathway in platinum-resistant EOC. Our results suggest that inhibition the expression of CXCL-2 and the specific receptor and regulatory pathways represents a potential strategy to overcome platinum resistance in EOC.

**Abbreviations**

EOC: Epithelial ovarian cancer

TME: Tumor microenvironment

DEGs: Differentially expressed genes

CCK8: Cell Counting Kit-8

NC: Negative control

qRT-PCR: Quantitative real-time PCR
ELISA: Enzyme-linked immunosorbent assay

ATR: Ataxia telangiectasia and Rad3-related

CHK1: Cell cycle checkpoint kinase1

DSBs: DNA double strand breaks

**Declarations**

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**Authors’ contributions**

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**Data Availability**

They could be achieved upon reasonable request to the authors.

**Ethics approval and consent to participate**

All patients signed informed consent before using clinical specimens, and the use of specimens for this study has been proved by the ethics committee of the First Affiliated Hospital with Nanjing Medical University.

**Authors’ contributions**

Authors Wenjun Cheng and Sipei Nie designed the project. Authors Sipei Nie, Yicong Wan and Hui wang contributed on experiments, data analysis and prepared the main manuscript. All authors reviewed the manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Written informed consent for publication was obtained from all authors.

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