EJGO European Journal of Gynaecological Oncology

Silencing S100A4 gene promoted platinum sensitivity in ovarian cancer

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Summary

Objective: To explore the relationship of S100A4 and platinum resistance in ovarian cancer. *Materials and Methods:* S100A4 expression level in tissue slices from 35 cisplatin-sensitive ovarian cancer patients and 28 cispatin-resistant patients were analyzed by immunohistochemistry. Platinum-resistant cells CP70 transfected with S100A4 siRNA were set as experimental group, negative control siRNA, and transfection reagent was also transfected cells and set as negative control group and blank group, respectively. After transfection, S100A4 transcription level of cells was determined by qRT-PCR, the expression levels of S100A4 and PKC/p53 pathway associated proteins were determined by Western-blot; cells 'cisplatin sensitivity and cells' invasion ability were analyzed by MTT method and Transwell assay and the cell cycle and apoptosis was determined by flow cytometry. *Results:* S100A4 expression level of cisplatin-sensitive patients was lower than cisplatin-resistant patients; after transfection, IC_{50} of blank group, negative control group and experimental group was 75.96, 66.73 and 40.31 μmol/L, respectively; the cells' percentage in G1 phase of experimental group was higher than other groups while in S phase it was lower than other groups ($p < 0.05$); the expression level of PKC-δ/ε and p-p53 increased significantly ($p < 0.05$). *Conclusions:* Silencing S100A4 gene in CP70 cells could u-regulate the expression of PKC-δ/ε and p-p53, thus promoting apoptosis by PKC-δ/ε in coordination with p-p53 and increasing chemosensitivity of cells.

Key words: Ovarian cancer; S100A4 siRNA; Platinum resistance; Cisplatin sensitivity.

Introduction

Ovarian cancer is the sixth most common cancer in women and the second most lethal gynecologic cancer among the female population in developed areas [1]. Cisplatin (cis-Dichlorodiamineplatinum, DDP) is the drug of first choice for ovarian cancer. The combination of cisplatin/carboplatin and taxane are regarded as the "gold standard" of chemotherapy after operation of the ovarian cancer patients. At present, the combinations of paclitaxel and carboplatin, cisplatin and cyclophosphamide, and paclitaxel and cisplatin are the common based chemotherapy regimens for treating epithelial ovarian cancer in China. However, drug resistance has become a major challenge associated with successful DDP treatment of cervical carcinoma and DDP-resistance is the important cause of cancer metastasis and recurrence, while the molecular basis for platinum-resistance remains unclear [2].

S100A4 belongs to the S100 family of proteins that contain two Ca2+-binding sites, including a canonical EF-hand motif [3]. S100A4 involved in the regulation of a wide range of biologic effects, including cell motility, survival, differentiation, and contractility [4]. In human patients with cancer, increased expression of S100A4 is positively associated with an increased incidence of metastasis, invasiveness, aggressiveness, and a worse prognosis [5-7]. Liang *et al.* found that neutrophil-promoting tumor progression could be blocked by S100A4 downregulation in vitro and in vivo and S100A4 depletion increased the effectiveness of anti-VEGF therapy in glioma [8]. Mencia *et al.* [9] found that S100A4 over-expression decreases the sensitivity of HT29 colon cancer human cells to methotrexate, but its knockdown causes chemosensitization toward methotrexate.

In order to verify the role that S100A4 plays in the platinum-resistance of ovarian cancer, the present authors observed the drug-resistance in platinum-resistant ovarian cancer cell line CP70 and cells biological characteristics after silencing S100A4 gene, aiming to explore how S100A4 mediates the development of platinum-resistance in ovarian cancer.

Material and Methods

Tissues were collected from epithelial ovarian cancer patients undergoing surgical treatment as first treatment from January 2008 to June 2014 in the present hospital. None of patients received preoperative radiotherapy and chemotherapy, and all patients were given combined chemotherapy based on platinum drugs after operation. According to the suggestion given by Gynecology Oncology Group, all patients were divided into platinum-sensitivity group (n=35) and platinum-resistance group (n=28). Judgment criterions of recurrence of ovarian cancer were as follows: (1) pelvic mass could be touched by physical examination or gynecologic examination; (2) increasing CA125 content was detected in fol-

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Revised manuscript accepted for publication February 24, 2016

low-up examination; (3) pelvic/abdominal mass was identified by B ultrasonic, distant metastases/tumor was identified by CT/PET-CT; (4) clinical symptoms: ascites, ileus. and so on.

After DAB staining, S100A4 protein expression and localization in epithelial ovarian carcinoma tissues and normal epithelial tissues were observed. Yellow brown granules were positive signal, negative results were judged by the method of Norihiko Kikuchi [10]. Judgment criterions in cytoplasm (vascular smooth muscle cells with determinate S100A4 expression was the positive control): negative, positive cells percentage ≤ 10% weakly positive, positive cells percentage > 10%, and ≤ 50%; strongly positive, positive cells percentage > 50%. Judgment criterions in nucleus: negative, positive cells percentage ≤ 5%; weakly positive, positive cells percentage > 5 and ≤ 20%; strongly positive, positive cells percentage > 20%.

For transfection, two ml of CP70 cells was seeded in a six-well plate at the concentration of 4~5×10^4cells/ml for 16 hours. Then the medium was changed to serum-free medium. S100A4 expression was knocked down by transfection with siRNA at the final concentration of 100nM. A siRNA duplex that shared no homologous sequences with the target gene was used as a negative control. Transfection was performed using a DNA transfection reagent according to the manufacturer's instructions. Cells in wells were added the transfection reagent, siRNA complexes, and the negative control siRNA were grouped as the blank cells (CP70 cells), S100A4 siRNA cells, and negative control cells.

Forty-eight hours after transfection, cells were collected for extracting total RNA by Trizol method. Then cDNA was synthesized according to the reverse transcription kit. Primers were as follows (GAPDH was reference gene): F-5'-CACTGGCGTCTTCACC-ACCATGGAG-3', R-5'-CTGCCAAATATGATGACATCAAGG-3'. S100A4 gene PCR primers were as follows: F-5'-GCAUCGCCAUGAUGUGUAATT-3', R-5'-UUACACAU-CAUGGCGAUGCTT-3' .

Forty-eight hours after transfection, cells in six-well plates were collected, washed, split, and centrifuged. Then, the upper clear liquid was obtained for Western-blot analysis, β-actin protein was the control. Primary antibodies were added (rabbit anti-human S100A4 monoclonal antibody (McAb); mouse anti-human PKC-α/PKC-δ/PKC-ε McAb and mouse anti-human p53, p-p53 McAb were also purchased, the secondary antibodies were HRP labeled goat anti-rabbit or anti-mouse IgG; rabbit anti-human β-actin McAb was also purchased.

The cytotoxic effects of DDP on CP70 cells were determined using the MTT assay. Cells were seeded in 96-well plates and transfected according to 48 hours after transfection, then cells were exposed to different concentrations of DDP (0, 2.5, 5, 10, 20, 40, 80, and 160 μmol/L). Cell viability was measured at 48 hours by MTT assay at 490 nm (OD readings) after DDP treatment. Cell viability = treated cells OD490/untreated cells OD490 ×100% and the inhibition rate = 1−cell viability, through calculating the inhibition rate, the IC50 was obtained. All the experiments were performed in triplicate.

Cells were transfected according to the above methods, and then using the same method to process cells by DDP at the concentration measured 24 hours later; cells were then harvested and made into single cell suspension and adjusted to 5×105 cells/ml. After three times of washing by PBS, the cells were resuspended in 200 μl binding buffer. Then, ten-μl Annexin V-FITC and ten μl propidium iodide (PI) solution were added and mixed gently. After 15 minutes of incubation at room temperature in dark, 300 μl binding Buffer was added. One hour later, cell apoptosis rate and cell cycle distribution were determined by flow cytometry.

For cell invasion assay, CP70 cells were transfected according to 48 hours later; matrigel invasion assay was then done using membranes coated with matrigel matrix. CP70 cells at 5×10^5 cells/ml were suspended and then seeded in upper chambers of 24-well transwell plates with FBS-free medium. Culture medium containing 10% FBS was deposited in the lower chambers. After 24 hours, cells that migrated were stained by 0.5% crystal violet solution for 15 minutes and counted.

The result was analyzed statistically by SPSS16.0 software. The mean of two groups were compared using *t*-test. The grouped data were compared using χ^2 test, and when $p < 0.05$, the differences were statistically significant.

Table 1. — *The comparison of basic clinical data between platinum-sensitivity and platinum-resistance groups.*

Clinical index	Platinum-sensitivity group (n)	Platinum-resistance group (n)	χ^2 value	*p* value
Age (years)				
< 60	27	20	0.268	0.605
≥ 60	8	8		
Tumor grade				
High differentiation	7	5	0.086	0.958
Moderate differentiation	19	15		
Poor differentiation	9	8		
Pathological pattern				
Serous cystadenocarcinoma	20	15	0.564	0.754
Mucinous cystadenocarcinoma	10	7		
Endometrial cystadenocarcinoma	5	6		
FIGO Stage				
II	9	6	0.158	0.924
III	19	16		
IV	7	6		
S100A4 protein expression level				
Low expression	20	5	10.03	0.002
High expression	15	23		
S100A4 protein subcellular localization				
Cytoplasm	26	7	15.149	0
Nucleus	9	21		

Results

Table 1 shows that there was no difference between two groups regarding age, tumor grades, pathological patterns, and FIGO stages ($p > 0.05$), but S100A4 protein expression level and subcellular localization of two groups showed obvious difference ($p < 0.01$).

Forty-eight hours after transfection, S100A4 mRNA and protein expression level of experimental group were higher than blank group and negative control group ($p < 0.05$), and there was no difference between blank group and negative control group ($p > 0.05$) (Figure 1).

To investigate whether S100A4 downregulation alters chemosensitivity in CP70 cells, the authors tested DDP in cells transfected with S100A4 siRNA. The cell viability is shown in Figure 2, through calculating cell inhibition rate, the authors obtained IC_{50} in blank group, negative control group, and experimental group to be 75.96, 66.73, and 40.31 μmol/L, respectively. These results indicated that in-

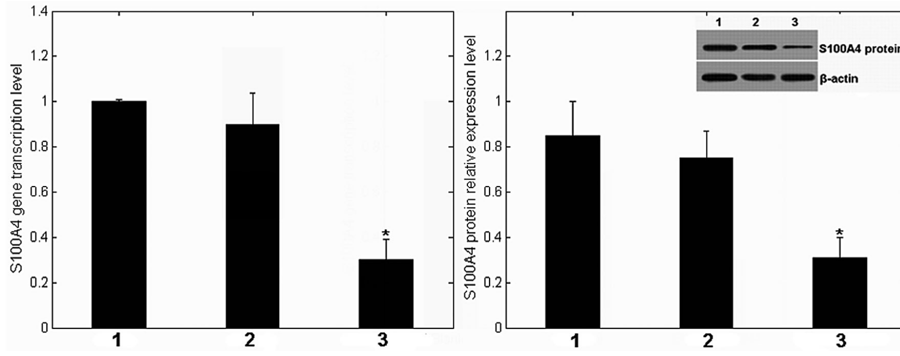

Figure 1. — RT-PCR and Western-blot analysis of S100A4 expression in CP70 cells after transfection.
1: blank group cells; 2: negative control group cell; 3: experimental group cells. The same applies to the following figures.

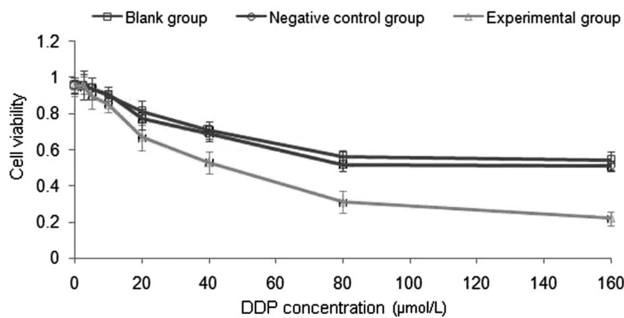

Figure 2. — Cell viability after transfection at different concentrations of DDP.

hibiting S100A4 expression could enhance the DDP-sensitivity of CP70 cells.

Results of flow cytometry showed that apoptosis rate of experimental group was higher than other groups ($p < 0.05$) (Figure 3c), which implies that downregulation of S100A4 could reverse drug-resistance of CP70 cells through promoting apoptosis. Cells percentage in G1 phase of experimental group was higher than other groups, while in S phase of experimental group it was lower than other groups ($p < 0.05$) (Figures 3a, 3b). Cell invasion assay also demonstrated that cell invasion ability of experimental group was lower than other groups ($p < 0.05$) (Figure 3d).

In order to discuss the molecular mechanism of chemosensitivity of CP70 cells influenced by S100A4, combining related literature, the authors analyzed the change of activity of PKC/p53 pathway after S100A4 gene silencing by

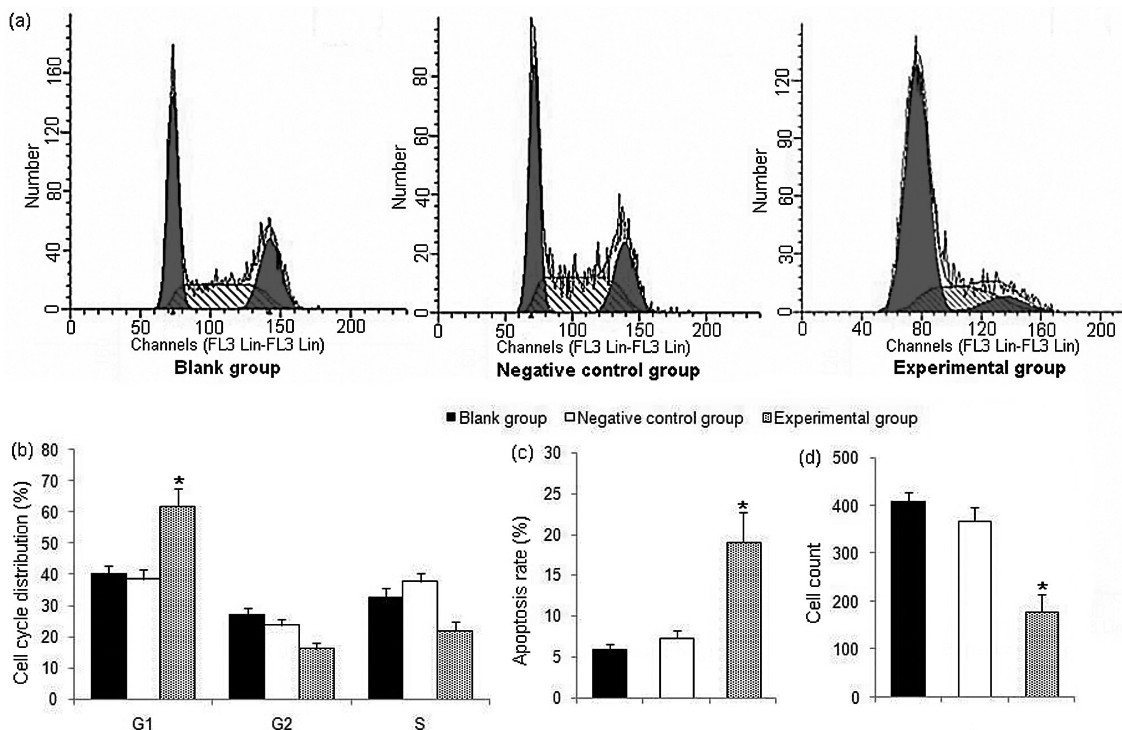

Figure 3. — Determination of CP70 cell cycle distribution, apoptosis rate, and cell invasion ability.

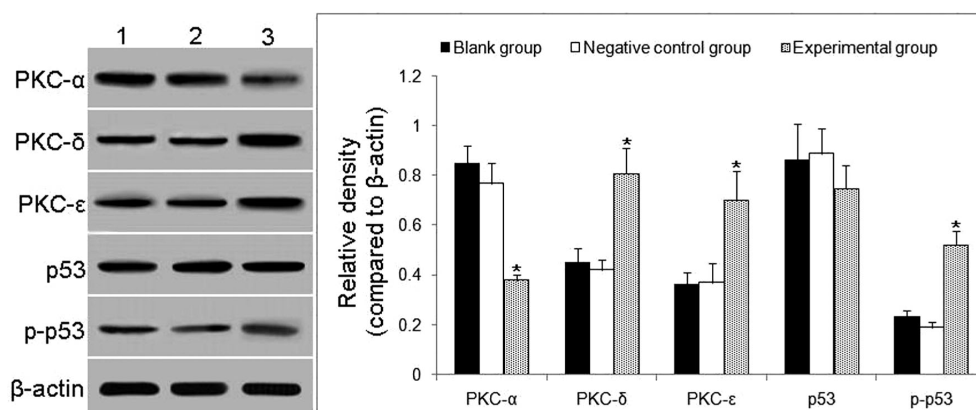

Figure 4. — Expression level of PKC pathway associated proteins after silencing S100A4 gene.

Western-blot method. Figure 4 shows that after silencing S100A4 gene, the expression level of PKC-α decreased significantly ($p < 0.05$), while the protein expression level of PKC-δ, PKC-ε, and p-p53 increased significantly ($p < 0.05$), p53 having no obvious change. These illustrated that different subtype PKC had different influence on drug resistance of ovarian cancer CP70 cell strains.

Discussion

In order to explore how platinum-resistance occurred in patients with ovarian cancer, the authors analyzed the basic clinical and pathological characteristics of 35 cispatin-sensitive ovarian cancer patients and 28 cispatin-resistant patients and found the S100A4 protein expression level in cispatin-resistant patients was higher than that in cispatin-sensitive patients. Therefore, they downregulated S100A4 expression through silencing the gene in drug-resistant ovarian cancer CP70 cell strain and observed the change of biological characteristics in vitro. The results showed that inhibiting the expression of S100A4 could reverse the cisplatin resistance and arrest the cell cycle in G1 phase.

Platinum-resistance in treating ovarian cancer is one of the main reason that causes high mortality of ovarian cancer. The mechanism of causing ovarian cancer drug resistance is very complex, the primary mechanisms include decreased blood flow in tumor, the decreased drug intake with increased outflow, enhanced DNA repair capacity, the blocked apoptotic pathways, the upregulated expression of drug-resistant molecules and so on [11-13]. After downregulating S100A4 expression by siRNA technique, through calculating cell inhibition rate, the authors obtained IC_{50} in blank group, negative control group, and experimental group to be 75.96, 66.73, and 40.31 μmol/L, respectively. These results indicated that silencing S100A4 gene increased the sensitivity of CP70 cell to cisplatin.

Currently, it is difficult to understand the exact mechanisms underlying S100A4-mediated chemotherapy resistance because multiple pathways are involved in it. Studies in endothelial cells and tumors demonstrate that S100A4 expression can be promoted by TGF-β1, which is well known to induce epithelial-mesenchymal transition [14, 15]. Liang *et al.* concluded that overexpression of S100A4 may be associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells and knockdown of S100A4 enhances the sensitivity to cisplatin of laryngeal carcinoma cells via inhibition of Slug expression [16]. Protein kinase C (PKC) signal transduction pathway has been reported to play a role in modulating cell death mediated by cisplatin [17] because some PKC members are substrates for caspase 3 activated during drug-induced apoptosis and PKCα has been described as an antiapoptotic protein [18].

P53 is a transcription factor that, depending on its phosphorylation status, regulates the cell cycle and apoptosis [19]. Members of the PKC family of serine/threonine kinases are key components of signal transduction pathways that regulate proliferation, cell survival, and malignant transformation [20]. PKC has also been implicated in the acquisition of resistance to anticancer treatments in malignant tumors [21]. Wu *et al.* demonstrated a novel mechanism in which GHRH antagonist-induced cell growth inhibition and apoptosis through PKC-δ-mediated activation of p53/p21 in human endometrial cancer cells [22]. Albihn *et al.* revealed that activation of PKC-δ in response to CPT treatment required Myc and was important in cytotoxic agents' camptothecin-mediated apoptosis signaling [23]. The study of Iioka *et al.* also suggested that PKC delta, in cooperation with p53, possibly regulates cisplatin-induced caspase-3-mediated cell death in gastric cancer [24].

In the study, after S100A4 gene silencing, the authors found the expression level of PKC-δ, PKC-ε and p-p53 protein in CP70 cell increased significantly with the increasing of apoptosis rate and cell cycle arresting in G1 stage ($p < 0.05$). These results implied that the upregulated expression of PKC-δ and PKC-ε may promote the activation of p53 and the phosphorylated p53 could accelerate cell apoptosis in cooperation with PKC-δ and PKC-ε [25].

In conclusion, silencing S100A4 gene in platinum-resis-

tant ovarian cancer CP70 cells could upregulate the expression of PKC-δ, PKC-ε. and p-p53, thus promoting apoptosis, which may be one of the causes that S100A4 siRNA increased the chemosensitivity of CP70 cells.

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