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A study of CCND1 with epithelial ovarian cancer cell proliferation and apoptosis

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Abstract. – OBJECTIVE: Ovarian cancer is a gynecological malignancy with high mortality rates all over the world. Markers for diagnosis, prognosis and therapy are urgently required to improve the mortality rates. As a key proto-oncogene, CCND1 is known to be amplified in many different carcinomas, including breast cancer, esophageal cancer, bladder cancer, endometrial cancer and ovarian cancer, etc. CCND1 plays an important role in cancer development and progression. However, its function and mechanism have not been completely elucidated in ovarian cancer.

MATERIALS AND METHODS: In the present study, we use cisplatin *in vitro* to inhibit the cell proliferation and promote cell apoptosis in epithelial ovarian cancer cell line SKOV-3. CCND1 expression, cell proliferation and cell cycle analysis were carried out by real-time PCR, CCK-8 and flow cytometry respectively.

RESULTS: Our results demonstrated that cisplatin could inhibit the expression of CCND1 in human epithelial ovarian cancer cell line, which is related to the decreased cell proliferation and increased cell apoptosis.

CONCLUSIONS: This study demonstrated that CCND1 is a potential therapeutic target for epithelial ovarian cancer treatment.

Key Words:
CCND1, Ovarian cancer, Cell proliferation, Apoptosis.

Introduction

Ovarian cancer (OC) is the leading cause of death for patients with gynecological cancers, with a mortality rate ranging from 40% to 50%. In most patients with OC, metastasis have occurred within the peritoneum by the time of diagnosis[1-4]. Despite the combined of surgery, chemotherapy and radiotherapy for treatment, the 5-years survival of OC patients is still less than 30%. The etiology and development mechanisms of OC are still unclear. Therefore, new bio-markers for early diagnosis and treatment for OC patients are urgently needed to be explored.

CCND1 plays an important role in cell cycle regulation. It is localized on intron 11 at 11q13, encoding protein cyclinD1, which played an important role in regulating the cell cycle. Therefore, CCND1 plays a biological role in the occurrence and development of tumors at the transcriptional level. CCND1 was firstly characterized by studies in parathyroid adenoma and a translocation t (11: 14) in B-cell lymphomas[5]. Afterward, the importance of CCND1 as an oncogene has been extensively reported. Overexpression of CCND1 in the mammary gland is sufficient for the induction of mammary cancer[6]. CCND1 was a regulatory subunit of the cyclin-dependent kinases CDK4/6 that are positive regulators of cell proliferation[7-9]. Besides, CCND1 was also associated with many metastatic cancers[10-13]. Many works suggested that CCND1 was not restricted to the nucleus but also associated with the cytoplasmic membrane, where it can be activated cytoplasmic targets involved in cell invasion[14-17].

Little is known about CCND1 in the progression of ovarian carcinoma. In this study, CCND1 was over-expressed in epithelial ovarian cancer cell line. We investigated the CCND1 expression level, cell proliferation and cell cycle in epithelial ovarian cancer cells treated with cisplatin. We hypothesize that CCND1 could be an important therapeutic target for ovarian cancer.

Materials and Methods

Cell Culture and Treatment

Ovarian cancer cell line SKOV-3 was obtained from Shandong University School of Oncology (Jinan, China) and maintained in RPMI 1640 medium (Invitrogen, Jinan, China) supple-

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mented with 10% fetal bovine serum (Hyclone, Jinan, China), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37 °C and 5% CO_2 in a humidified incubator. The medium was changed daily. Upon confluence, the cells were collected enzymatically. Cells were directly cryopreserved in a refrigerator at -20°C for real-time PCR (qRT-PCR) analysis. Cellular precipitation was fixed with 70% alcohol at 4°C refrigerator for flow cytometry detection.

The SKOV-3 cells were divided into five experimental groups: blank control group (no cells), negative control group (cells without cisplatin treatment) and cells treated with a concentration of cisplatin (1, 5, 10 μg/ml) respectively. Each group had six samples with a cell density of 5×10^7. The treatment concentration of cisplatin (0, 1, 5, 10 μg/ml) was determined by the peak blood dose of the cisplatin according to recently relevant literature reports[18-20].

CCK-8 Assay

Ovarian cancer cells SKOV-3 were seeded in 96-well plates at a concentration of 3500 cells per well. After cell adhesion, the medium was replaced with the fresh media with different concentration of cisplatin (0 μg/ml, 1 μg/ml, 5 μg/ml and 10 μg/ml) were added to the medium for 24, 48 and 72 hours respectively. Each treatment was repeated for four wells. Measurement of cell viability was performed using CCK-8 analysis (Beyotime Biotechnology, Shanghai, China). Specifically, 10 μL CCK-8 and 100 μL RPMI1640 were added to each well and incubated for 2 hours at 37°C. The absorbance was measured at a wavelength of 490 nm using a microplate absorbance reader (model 680; Bio-Rad laboratories Inc, Hercules, CA, USA) to calculate cell growth rate. Each experiment was repeated three times. The cell viability was estimated using cell growth curve.

Cell Cycle and Apoptosis

To assess the influences of cisplatin on epithelial ovarian cancer cells' cell cycle and apoptosis, SKOV-3 cells were harvested with 0.25% trypsin and washed with phosphate-buffered saline (PBS). After centrifugation, the cells were stained with annexin V-FITC/propidium iodide Apoptosis Detection Kit (BD, Franklin Lakes, NJ, USA). Analysis of the cell cycle and apoptosis was performed on a flow cytometer (BD, Franklin Lakes, NJ, USA). The experiment was performed in triplicate.

CyclinD1 Protein Expression

Mouse monoclonal antibody against human cyclin D1 was added into SKOV-3 cells at room temperature. SKOV-3 cells were incubated, washed twice with PBS, and then incubated with a secondary antibody labeled by fluorescent tags. After incubation for 45 min, the cells were washed twice with PBS and then stained with ethyl bromide. FACS FCM instrument (BD, Franklin Lakes, NJ, USA) and ModiFit software were used to evaluate the cyclin D1 protein expression in ovarian cancer cells.

Quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted from SKOV-3 cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was reversely transcripted using a reverse conversion kit. PCR was performed on a qPCR device (MiniOpticon, Bio-Rad, Shanghai, China) using SYBR Green Super Mix. The results were calculated with $2^{-\Delta\Delta CT}$ methods and presented as a percentage of the internal control β-actin. The primers using in this study was as follows: CCND1, forward, GAGACCATCCCCCTGACGGC, reverse, TCTTCCTCCTCCTCGGCGGC. β-actin: forward, TGACGGGGTCACCCACACTGTGCCCATCTA, reverse, CTAGAAGCATTTGCGGTGGACGATGGAGGG.

Statistical Analysis

All experiments were repeated independently three times, and the data were expressed as mean ± SD. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. *t*-test was used for comparisons between two groups. Qualitative data (CCND1 expression) were investigated with χ^2-test. $p < 0.05$ was considered as the level of significance.

Results

Cell Proliferation Assay

In this study, the inhibited effects of cisplatin with different doses (1, 5, 10 μg/ml) and times (24h, 48h, 72h) on epithelial ovarian cancer cell growth capability were measured using CCK-8 assay. As shown in Figure 1, the SKOV-3 cell growth ability reduced after treatment with cisplatin, and the stronger inhibited effect was observed with the increased dosage and longer duration of cisplatin.

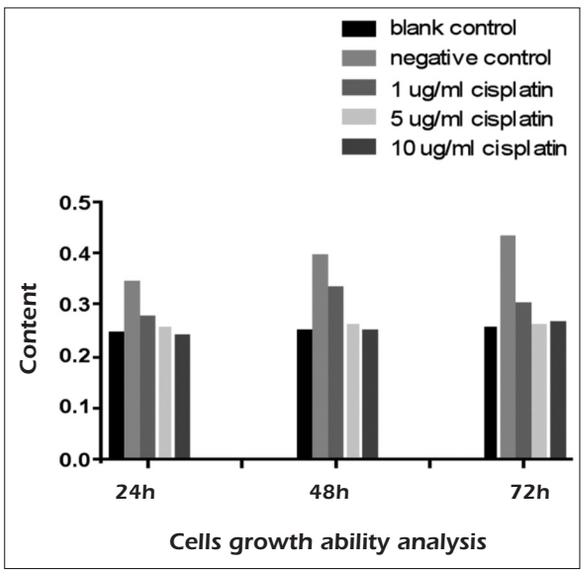

Figure 1. SKOV-3 cells growth ability with cispilatin by CCK-8.

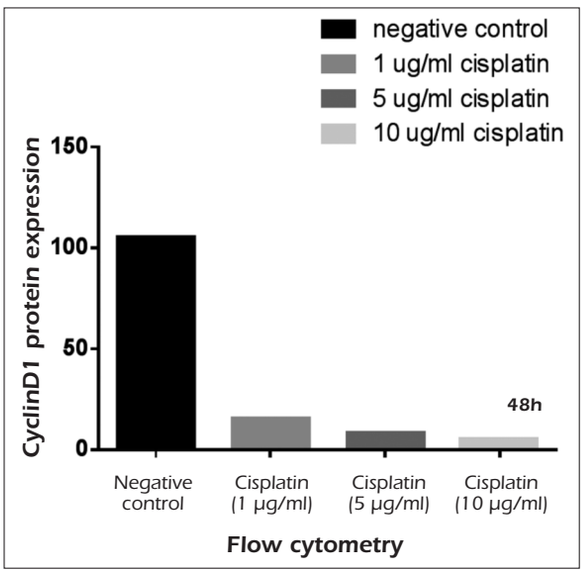

Figure 2. CyclinD1 protein expression in SKOV-3 cells after 48 hours treatment with cisplatin by flow cytometry.

Cyclin D1 Protein Expression

CyclinD1 protein expression in SKOV-3 cells was detected by flow cytometry after treated with different concentrations of cisplatin (0, 1, 5, 10 μg/ml) 48 hours later. As shown in Figure 2, over-expression of cyclinD1 protein was detected in SKOV-3 cells, while expression of cyclinD1 is negative in normal ovarian cells. After treatment with cisplatin, the cyclinD1 protein expression level decreased. Furthermore, the inhibited effects of cisplatin on an expression of cyclinD1 protein, was concentration-dependent. The "mean" value in Table I represents the average fluorescent strength of cyclinD1 protein expression in SKOV-3 cells.

Cell Cycle and Apoptosis Analysis

After treated with cisplatin for 48 hours, cell cycle arrest and apoptosis analysis in SKOV-3 cells were detected by flow cytometry. As shown in Figure 3, treatment with cisplatin induced more cells arrested in the G0/G1 phase compared with control ($p < 0.05$). Similarly, a higher incidence of apoptosis in SKOV-3 cells was detected compared to control cells ($p < 0.05$) and the effects of cisplatin were dosage-dependent. Moreover, a hypodiploid apoptotic peak was detected with increased cisplatin concentration at G1 phase (Figure 3). The results of flow cytometry analysis revealed that cisplatin treatment increased cell apoptosis and enhanced cyclinD1 protein expression in SKOV-3 cells compared to control.

Table I. SKOV3 cell apoptosis, cyclin D1 protein expression, cell cycle,and cell proliferation activity 48h after treatment with

	Blank control	Negative control	Cisplatin (1 μg/ml)	Cisplatin (5 μg/ml)	Cisplatin (10 μg/ml)	*p*-value
Apoptosis rate (%)	0.001	0.63	9.08	27.41	40.69	< 0.05
Mean (average fluorescence intensity coefficient)	1.29	105.23	15.40	8.35	5.38	< 0.05
G0/G1 (%)	0.001	61.17	55.57	72.60	83.44	< 0.05
G2/M (%)	0.001	0.006	0.49	11.94	12.97	< 0.05
S (%)	0.005	38.83	43.94	15.47	11.32	< 0.05
PI (%)	0.005	38.83	44.43	36.41	32.52	< 0.05
Cell proliferation activity (%)	4.04 ± 0.65	74.5 ± 5.13	46.0 ± 1.14	7.0 ± 1.57	6.13 ± 1.12	< 0.05

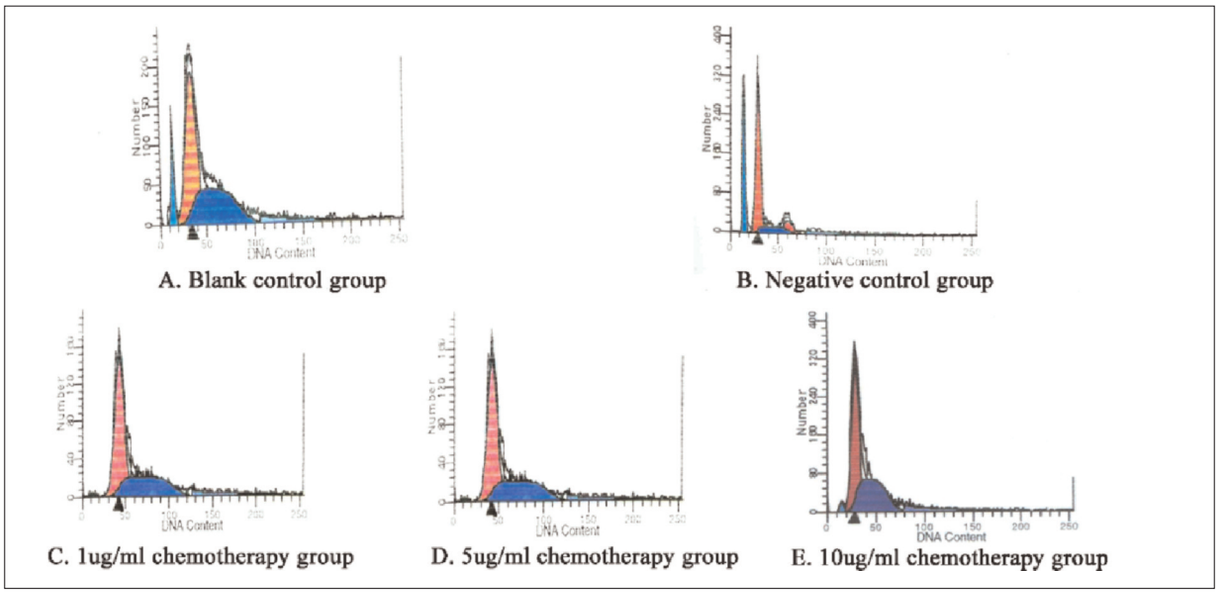

Figure 3. Apoptosis and cell cycle analysis of SKOV-3 cells with treatment of cisplatin. ***(A)*** blank control group ***(B)*** Negative control group; ***(C)*** 1 μg/ml chemotherapy group; ***(D)*** 5 μg/ml chemotherapy group; ***(E)*** 10 μg/ml chemotherapy group.

CCND1 mRNA Expression

To determine the mRNA expression levels of CCND1 in SKOV-3 cells, real-time RT-PCR assay was used. The results showed that CCND1 mRNA expression was significantly upregulated in cell line SKOV-3 compared to control (Figure 4, $p < 0.05$). After treatment with cisplatin, reduced CCND1 mRNA expression was detected. The data revealed a negative correlation between concentration of cisplatin and CCND1 mRNA expression level at 24 hours and 48 hours. Notably, cisplatin could increase the expression of CCND1 at 72 hours. However, the possible mechanisms need further researches.

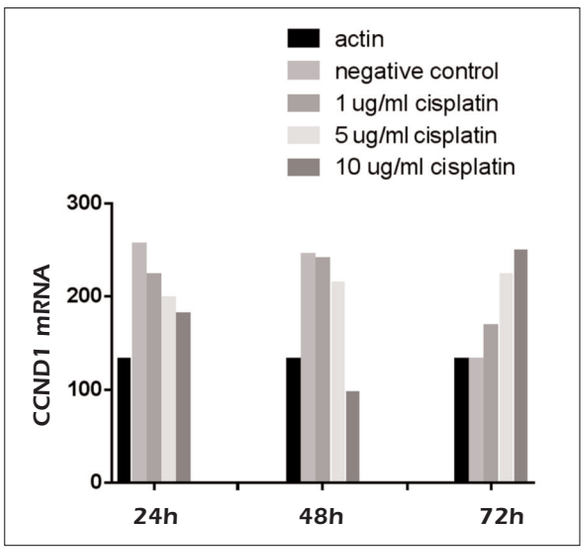

Figure 4. CCND1 mRNA expression of SKOV-3 cells with cisplatin by qRT-PCR

Discussion

CCND1 is one of most amplified genes among the proto-oncogenes in cancers [21]. Since 1991, CCND1 was defined on studies in B-cell lymphomas by Motokura and it was wildly reported as an important oncogene in many cancers, such as head and neck carcinoma [22], breast cancer [23], gastric cancer [24], lung cancer [25], etc. CCND1 also plays an important role in ovarian cancer development. Many gynecological doctors focused on researches about CCND1 with ovarian cancer [26-30]. However, few have been made about relationship between CCND1 and epithelial ovarian cancer cell proliferation and apoptosis.

In this work, we performed CCK-8, flow cytometry, qRT-PCR analysis *in vitro* to investigate the correlation between CCND1 expression and cell proliferation as well as apoptosis in epithelial ovarian cancer cells. Firstly, we found that there existed over-expressions of CCND1 in epithelial ovarian cancer cells compared to normal cells, which was consistent with the previous reports[26]. Secondly, we conducted cisplatin in

our study to inhibit cell proliferation and promote apoptosis in SKOV-3 cells. When treated with cisplatin, SKOV-3 cells had a decreased cell viability, increased apoptosis rate and reduced CCND1 expression levels. We also found that there is a negative correlation with expression levels of CCND1 and dosage of cisplatin. In the study, the cisplatin resistance was detected when SKOV-3 cells were treated with cisplatin for 72 hours. The descender of SKOV-3 cell growth ability became small, and this descender also occurred in CCND1 protein expression level. The results revealed that CCND1 expression decreased along with the decreased cell growth capacity and increased apoptosis in epithelial ovarian cancer cells. We observed that overexpression of CCND1 was consistent with stronger cell growth ability and fewer apoptosis of epithelial ovarian cancer cells. Therefore, CCND1 was suggested to be a good marker for evaluating the development of OC and treatment for OC patients.

However, animal experiment and *in vivo* studies will be performed later in order to conclude a scientific investigation on CCND1 and epithelial ovarian cancer.

Conclusions

CCND1 expression was closely related with cell proliferation ability and apoptosis in epithelial ovarian cancer cells. CCND1 is an important marker for evaluating epithelial ovarian cancer progression and a potential therapeutic target for ovarian cancer.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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