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An experimental study of cyclinD1 expression in human epithelial ovarian cancer cells

Jun Dai[1], Peishu Liu[1], Yangyun Liu[2], Wei Cai[3]

[1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan*
[2]*Department of Otolaryngology, The 163th Hospital of PLA, Changsha*
[3]*Department of Orthopaedics, The 163th Hospital of PLA, Changsha (China)*

Summary

Purpose of investigation: As an important oncogene, cyclinD1 has close relationship with carcinomars. This study aims to investigate the correlation between cyclinD1 expression and ovarian cancer cell growth capability and apoptosis. *Materials and Methods:* Epithelial ovarian cancer (EOC) cell line 3AO was cultured and incubated with different concentrations (0, 1, 5, and 10 ug/ml) of cisplatin. RT-PCR and FCM analysis were used to detect cyclinD1 mRNA and protein expression, 3AO cell cycles, cell growth capability, and apoptosis were assessed by FCM. *Results:* EOC cell 3AO presented anti-apoptosis capabilities and cyclinD1 overexpression. After treatment with cisplatin, low cyclinD1 expression, decelerated cell growth capability, and high cell apoptosis levels were detected. *Conclusion:* CyclinD1 expression has closely correlated with EOC cell growth and apoptosis. CyclinD1 may be a good biomarker for monitoring the progress of EOC.

Key words: Epithelial ovarian cancer; CyclinD1; RT- PCR; Flow cytometry.

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological cancers, with has a five-year survival rate of 35-40%. Screening and early detection could probably reduce mortality rate [1]

Disturbance in cell cycle regulation can lead to tumor development. The progression of cell cycle is known to be critical for tumor survival. Previous studies indicated that cyclinD1 was an important factor that induces cell cycle G1/S phase transition, and demonstrated that upregulated cyclinD1 expression promotes malignant tumor growth [2, 3]. A close correlation between cyclinD1 and many malignant tumor growths had been found, such as in gastric cancer and liver cancer [4-10]. However, few are known on the cyclinD1 in ovarian carcinoma.

It is known that cisplatin can restrain tumor cell proliferation and induce cell apoptosis. In the present study, the authors focused on the correlation of cyclinD1 expression with cell proliferation and apoptosis on EOC cells treated by cisplatin, aiming to provide a theoretical foundation for cyclinD1 to be used as a predictor for tumor growth in EOC.

Materials and Methods

Ovarian cancer cell line 3AO was utilized. 3AO cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C with 5% CO_2 in a humidified incubator. Protease cell culture bottle filter tube. Cryopreserved cells were utilized.

Five years of relevant literature reports were used as reference to determine the peak blood concentration of chemotherapeutic drug cisplatin [11], and concentrations of cisplatin (1, 5, and 10 µg/ml) were determined. A week before the experiment, cisplatin was diluted with physiological saline to a concentration of 1,000 µg/ml and then stored in a refrigerator at −20 °C

The samples were divided into five experimental groups: A, B, C, D, and E. Group A was assigned as the blank control group, group B was assigned as the negative control group, and chemotherapy groups were C, D, and E. Each group had six samples, and each sample had a cell density of 5×10^7. The cell culture was incubated with different cisplatin concentrations for 56 hours. Then, cells were collected after incubation.

Mouse monoclonal antibody against human cyclinD1 was added into tumor cells from a single cell suspension at room temperature. The 3AO cells were incubated, washed twice with PBS, and added with secondary resistance of 100L fluorescent tags. After incubation for 45 minutes at room temperature, cells were rinsed twice with PBS and stained with ethyl bromide. FACS FCM instrument and ModiFit software were used for data processing. The mean

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Table 1. — *3AO cell apoptosis, cell cycle, and cyclin D1 protein expression 56 hours after treatment with cisplatin.*

	Blank control	**Negative control**	**cisplatin (1 µg/ml)**	**cisplatin (5 µg/ml)**	**cisplatin (10 µg/ml)**	***p*-value**
Apoptosis rate (%)	0.001	0.69	7.83	19.41	36.97	<0.05
Mean(average fluorescence intensity coefficient)	1.05	109.43	33.28	17.31	3.15	<0.05
G0/G1 (%)	0.001	59.20	56.06	48.67	61.65	<0.05
S(%)	0.003	38.94	43.73	21.34	18.49	<0.05
PI (%)	0.003	40.63	48.19	30.15	28.61	<0.05
Cell proliferation activity (%)	1.04 ± 0.77	79.55±2.31	51.03 ± 3.11	9.17 ± 1.54	6.52 ± 5.43	<0.05

value was used as an assessment index, to evaluate cyclinD1 protein expression in cell line 3AO.

Cell cycle and apoptosis of 3AO cells incubated with or without cisplatin were evaluated to determine whether cisplatin regulates the growth and apoptosis of epithelial ovarian cells. Cells were trypsinized, centrifuged at 300 ×g (1,000 rpm) for five minutes, resuspended (1×10^6 cells/ml), and then fixed with 70% ice-cold ethanol for 30 minutes. Subsequently, cells were centrifuged, washed, and resuspended in 500 µl of PBS containing 10 µl of DNase-free RNase (final concentration was 1%). After 30 minutes of incubation, pyridine iodide (PI, 0.05 mg/ml) was added to the cell suspension, incubated for five minutes in the dark, and filtered by a nylon mesh to remove cell clusters. The fluorescence of PI was measured using FACS calibur flow cytometer. Cell subpopulations were studied by gating analysis based on the differences in DNA content. At least 2×10^4 cells were analyzed per sample. Cell proliferation characteristics were indexed by ratio in the S-phase.

Total RNA of human ovarian cancer cell line 3AO was extracted using the TRIzol method. RNA was reverse-transcribed using a commercially available kit. The PCR mixture (25 µl in total) consisted of 0.5 µl of cDNA, 0.5 U of Taq DNA polymerase, 2.5 µl of 10× PCR buffer, 2.5 ul of dNTP mixture, and 50 PM of each sense and antisense primers. CyclinD1 was analyzed using the following primers: cyclinD1 forward, 5′-GAACAGAAGTGCGAGGAGGAG-3′ and reverse primer 5′-AGGCGGTAGTAGGACAGGAAG-3′; β-actin: forward 5′-GGCACCCAGCACAATGAA-3′, and reverse 5′-TAGAAGCATTTGCGGTGG-3′.

The reaction mixture was subjected to 30 cycles of PCR. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 60 seconds. Then, 10 µl of each PCR product was analyzed by electrophoresis on 1.5% agarose gels containing 0.5% ethidium bromide. The computation was performed according to the relative coefficient histogram or statistical processing.

All experiments were repeated independently for three times and data were expressed as mean ± SD. SPSS version 17.0 was used for statistical analysis. *T*-test was used for comparisons of continuous data between two groups. Qualitative data (cyclinD1 expression) were investigated with chi-square test. $P < 0.05$ was considered statistically significance.

Results

After incubating 3AO cells with 1 µg/ml cisplatin for 56 hours, cell apoptosis rate was found to be significantly higher, compared to cells in the control group ($p < 0.01$), and the G0/G1 phase cell quantity ratio further decreased (Figure 1). The cell proliferation index (PI) {[G0 / G1 (S + G2 / M)] + S + G2 / M } ×100% significantly increased (Figure 1), and the mean value of the protein expression of cyclinD1 was significantly lower than those in the control group (Table 1). As shown in Table 1, cisplatin affected cyclinD1 protein expression, as well as cell apoptosis' cell cycle and cell growth activity. As the concentration of cisplatin increased, 3AO cell cycle changed, cell growth capability decreased, cyclinD1 expression protein decreased, and cell apoptosis had an upward trend.

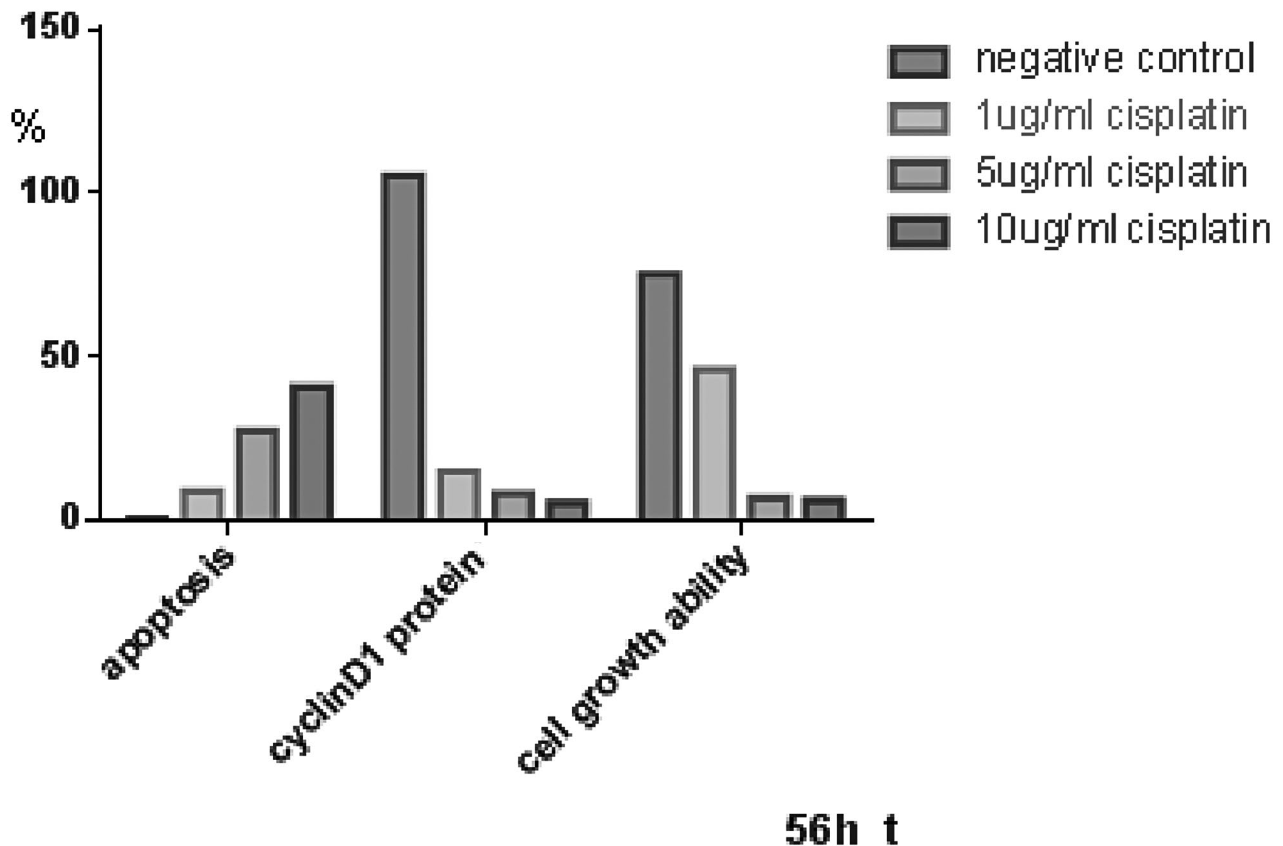


Figure 1. — 3AO cell growth ability, apoptosis, and cyclinD1 protein expression after treatment with cDDP for 56 hours.

After incubating 3AO cells with 5 µg/mL of cisplatin for 56 hours, the apoptosis rate increased from 1.44% to 3.41%, the G0/G1 phase cell quantity ratio decreased from 48.67% to 13.2%, and the mean value of cyclinD1 protein expression decreased from 37.1 to 1.19 (Figure 1). The same results were found in groups with high concentrations of cisplatin. The cyclinD1 protein expression level decreased significantly as cisplatin concentration increased.

Similar significant differences in cell apoptosis and cyclinD1 protein expression level were found in groups D and E. Furthermore, the authors found a more significant sublipliod apoptotic peak appeared before G1 phase (Figure 1).

Significant differences in cyclinD1 protein expression were found in 3AO cells with or without cisplatin treatment (Table 1). The average fluorescence intensity coefficient, (or "mean" value) represents cyclinD1 protein expression level. The higher the protein expression level, the higher the mean value. The mean values for negative control, low-concentration chemotherapy, lower concentration chemotherapy and high-concentration chemotherapy groups were 109.43, 33.28, 17.31, and 3.15, respectively (Figure 1). These values indicated that cyclinD1 protein expression levels were significantly down-regulated with increasing cisplatin concentration ($p < 0.01$). PI is 3AO cell proliferation index. $PI = [G0 / G1 (S + G2 / M) + S + G2 / M] \times 100\%$.

Figure 2 shows the agarose gel electrophoresis results for the mRNA expression of cyclinD1 in 3AO cells. The band of cyclinD1 mRNA was detected at 438 KB except in blank control. However, as cisplatin concentration increased, the density and width of the bands of cyclinD1 mRNA decreased. Significant changes in cyclinD1 mRNA expressions were found before and after cisplatin treatment.

Discussion

Many oncogenes and pathways are involved in the occurrence and development of cancers. A number of reports have indicated that disturbance in cell cycle regulation led to tumorigenesis, and that cyclinD1 promoted the progression of the cell cycle. CyclinD1, an important member of

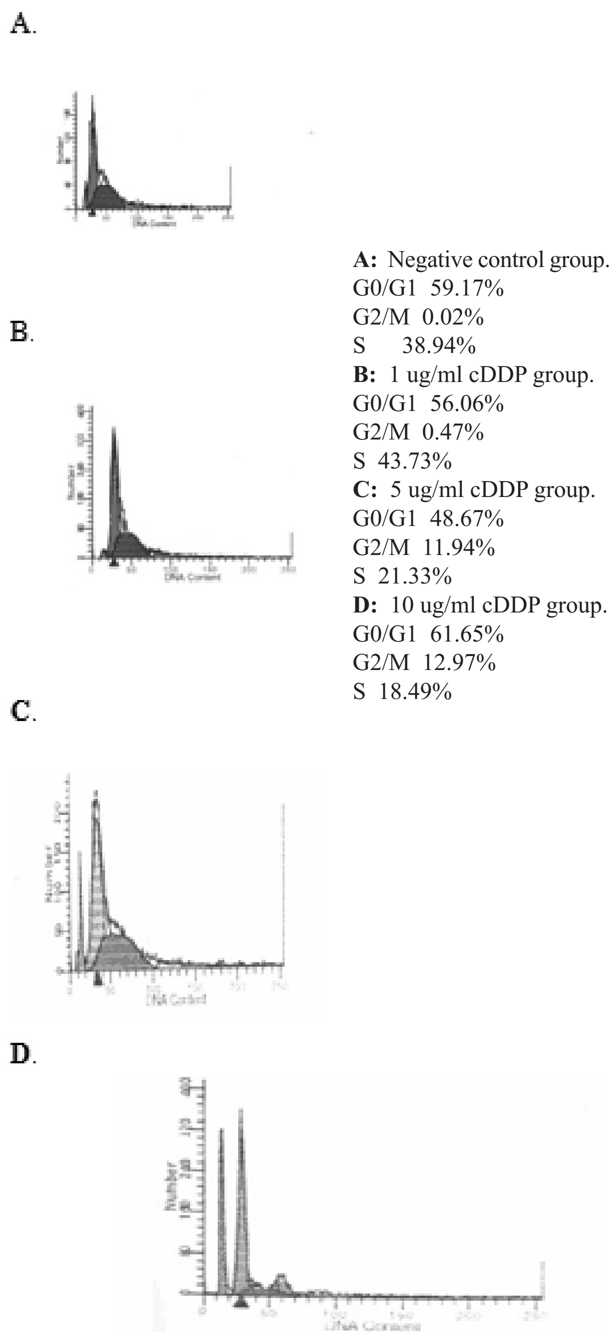
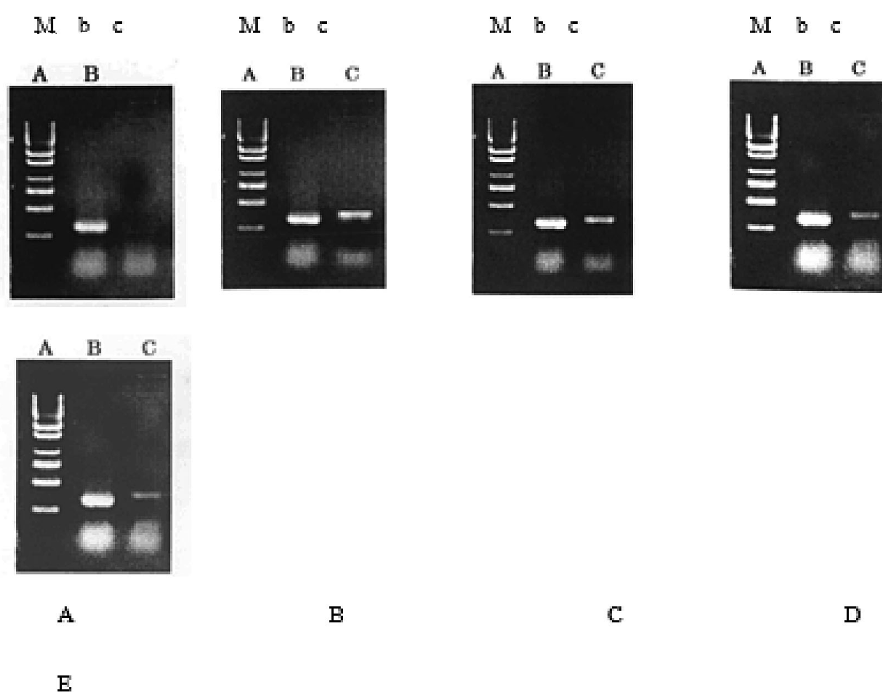


Figure 2. — 3AO cells apoptosis and cell cycle analysis by flow cytometry after treatment with cisplatin for 56 hours.

cell cycle regulators, is located in chromosome 11q13. It has a length of 15 KB with five extrons and four introns. CyclinD1 protein consists of 295 amino acids with a relative molecular mass of 34,000. CyclinD1, in the G1 phase S phase transformation, plays an important role in the positive control. Under physiological conditions, cyclinD1 is initially synthesized in the G0 phase. Its synthesis increases to a maximum level in the G1 phase and reduces in the S phase. CyclinD1 expression remains at a low level in other phases of the cell cycle [12-14]. Previous researches revealed that cyclinD1 expression was closely correlated with EOC cell growth capability and apoptosis, which promotes the progression of the cell cycle and is related to cell proliferation [15-19].

In the present study, the authors found that cyclinD1 was overexpressed in ovarian cancer cells. However the expression of cyclinD1 was negative in normal ovarian cells. The present authors cultured ovarian cancer cell line 3AO cells and normal human ovarian surface epithelial cells in vitro, and three chemotherapy groups of 3AO cells were treated with different concentrations of cisplatin. By FCM, the overexpression of cyclinD1 protein in EOC cells was detected, obvious cell cycle changes were observed, the percentages of cells in the G0/G1, G2/M, and S phases significantly changed. After treatment by cisplatin, the authors found that cyclinD1 protein expression was closely correlated with the concentration of cisplatin. As the concentration of cisplatin increased, EOC cell apoptosis increased, the cell proliferation index decreased, cells distribution in S-phase decreased, and cyclinD1 protein expression decreased. This study demonstrated that cyclinD1 expression, cell cycle, and apoptosis in EOC cells were positive correlated. CyclinD1 mRNA was overexpressed in EOC cells by RT-PCR analysis (Figure 3). A clear band of cyclinD1 was detected at 438KB, and this band faded after cisplatin treatment and suggested a positive correlation between cyclinD1 mRNA expression and cisplatin concentration. These results are consistent with previous studies. For example, Zhang *et al.* [8] found that cyclinD1 overexpression was closely associated to the degree of invasion, metastasis, and prognosis in laryngeal squamous cell carcinoma. Hashiguchi *et al.* [19] reported that cyclinD1 is an important regulator correlated with survival in EOC. Masuda *et al.* [4] found that cyclinD1 overexpression prompts the poor prognosis of head and neck squamous cell carcinoma. Gao *et al.* [5] used immunohistochemistry to analyze cyclinD1 in 112 cases gastric cancers. Recently, they revealed that cyclinD1 protein is overexpressed in gastric cancer tissues and that its expression and progress in gastric cancer are positively correlated with the report of Dimova *et al.* [14], in which cyclinD1 overexpression is not associated with tumor grade and stage. Wang *et al.* [16] revealed that cyclinD1 was overexpressed in ovarian serous carcinomas through immunohistochemical staining and that cyclinD1 overexpression was positively correlated to pathological grade and survival rate. Barbieri *et al.* [17] found that ovarian malignant tumor exists in cyclinD1 protein expression (76.5%) and that the malignancy degree and proliferation activity of tumors were positively correlated.

In addition, in the present study, ovarian cancer cells 3AO cell growth activity was examined by cell cycle assay through FCM analysis. This study also revealed that cyclinD1 expression was closely associated with EOC cell growth ability. The authors found that as the concentration



A: Blank control.
B: Negative control.
C: 1 ug/ml chemotherapy group.
D: 5 ug/ml chemotherapy group.
E: 10 ug/ml chemotherapy group.

M: Marker; b: β-actin; c: skov3 at 56 hours after incubation with cis-platinum
Banding from left to right represents PCR marker, beta actin, and cyclinD1 mRNA expression. In this figure, it was found that the banding became gradually dimmed while cDDP concentration increased.

Figure 3. — CyclinD1 mRNA expression in 3AO treated with cisplatin for 56 h by RT-PCR

of cisplatin increased, EOC cell growth ability decreased, cell apoptosis rate increased, and cyclinD1 expression decreased. These findings are consistent with previous studies. CyclinD1 affects ovarian cancer cells in terms of cell morphology, viability, apoptosis. As reported by Cheng *et al.* [15], cyclinD1 affects epithelial-mesenchymal transition in EOC stem cell-like cells.

In summary, this investigation revealed that the cyclinD1 expression has a close relationship with development of EOC cells, which imply that cyclinD1 is a potential biomarker for monitoring EOC progression and treatment, although a further studies, including animal experiment, especially clinical studies, are needed to confirm these findings.

Conclusion

The authors conducted a study on cyclinD1 expression in EOC cells and investigated the relationship between cyclinD1 expression with EOC cell growth capability and apoptosis in vitro. Finally, they concluded that cyclinD1 can serve as a good biological marker for ovarian cancer progression. However, further studies are required to elucidate the function of cyclinD1 and develop cyclinD1 as a concrete target for ovarian cancer therapy.

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Corresponding Author:
JUN DAI, M.D.
Department of Obstetrics and Gynecology
Qilu Hospital, Shandong University
No.107 Wenhua West Road, Lixia District
Jinan, Shandong Province 250012 (China)
e-mail: pl_sci@163.com