

Nivolumab effectively inhibit platinum-resistant ovarian cancer cells via induction of cell apoptosis and inhibition of ADAM17 expression

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Abstract. – OBJECTIVE: Nivolumab is an anti-PD-1 (anti-programmed death-1) monoclonal antibody. It has achieved an overall response rate of 17% in Phase 1 clinical trial for patient with platinum-resistant ovarian cancer (PROC). However, its underlying mechanism has not been fully explored yet. The aim of the study is to investigate the efficiency of nivolumab to inhibit PROC cells and its possible mechanism.

MATERIALS AND METHODS: Firstly, methylthiazolyl tetrazolium bromide (MTT) assay was performed to determine the IC₅₀ values of cisplatin in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. The results showed that IC₅₀ (half maximal inhibitory concentration) values of cisplatin were significantly decreased in a time-dependent manner in A2780, A2780/DDP, SKOV3, and SKOV3/DDP cells. Secondly, MMT assay was used once again to measure anti-tumor effects of nivolumab in A2780/DDP cells. The results showed that anti-tumor effects of nivolumab increased in a dose- and time-dependent manner. Thirdly, A2780/DDP cells were treated with nivolumab in combination with cisplatin for 48 h.

RESULTS: The results demonstrated that nivolumab increased the anti-tumor effects of cisplatin in A2780/DDP cells. Notably, the combined treatment effectively reversed cisplatin resistance in PROC cells. Also, nivolumab induced cell apoptosis and cell-cycle arrest in G₀/G₁ phase in PROC cells. FACS and Western blot were performed to measure cell apoptosis and Bcl-2 and Bax expression respectively. The results showed that combined treatment significantly increased cell apoptosis rate, down-regulated Bcl-2, and unregulated Bax expression in PROC cells. Additionally, the expression levels of ADAM17 were significantly decreased in a dose-dependent manner in PROC cells, which were treated with nivolumab.

CONCLUSIONS: Therefore, all the results demonstrated that the combined treatment with nivolumab and cisplatin effectively inhibited PROC cells via induction of cell apoptosis and inhibition of ADAM17 expression.

Key Words:

Nivolumab, Cisplatin, Platinum-resistant ovarian cancer (PROC), Drug resistance, Cell apoptosis.

Introduction

Ovarian cancer is one of the most lethal gynecological cancers among females¹. Ovarian cancer primarily has three types, which are epithelial tumor, germ cell tumor, and stromal tumor². Approximately 85-90% of ovarian cancers belong to epithelial tumor^{3,4}. Due to its nature, only 25% of the patients were diagnosed at the early stage. Most of patients were diagnosed at the advanced stage with pelvic metastases or lymph node metastasis. The five-year survival rate was between 20% and 25%⁵⁻⁸. Nowadays, surgery combined with chemotherapy remains primary method to treat ovarian cancer. Among chemotherapy agents, cisplatin is the first-generation platinum-based agent, which has demonstrated significant anti-tumor effects in a variety of cancers^{9,10}. It has been one of the most commonly used drugs in patients with advanced ovarian cancers. However, the extensive use of cisplatin¹¹ has caused a variety of adverse side effects, including nausea, vomiting, renal toxicity, neurotoxicity, etc. More importantly, long-term use of cisplatin would inevitably result in drug resistance, which remains a major cause of platinum-resistant ovarian cancer (PROC)¹²⁻¹⁴. Therefore, to explore an effective way to decrease the drug resistance of cisplatin in PROC has significant value in clinical practice.

Nivolumab is an anti-PD-1 monoclonal antibody, which has been reported to have anti-tumor effects and widely used in clinical treatment of various tumors. It has achieved

an overall response rate of 17% in Phase 1 clinical trial for patient with PROC^{15,16}. Qi et al¹⁷ reported that nivolumab effectively induced cell apoptosis of pancreatic cancer cells. Gao et al¹⁸ found that nivolumab could induce cell apoptosis in colorectal cancer cells via by increasing hydrogen peroxide and inducing glutathione depletion. Also, nivolumab suppressed mTOR signaling and the growth of lung cancer tumors^{19,20}. In the present study, we used two PROC cell lines A2780/DDP and SKOV3/DDP to explore the anti-tumor effect and its possible underlying mechanism of combined treatment of nivolumab and cisplatin.

Materials and Methods

Agents and Cell Lines

Nivolumab and cisplatin were obtained from the Shifeng Biocorporation (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The PROC cell lines A2780/DDP and SKOV3/DDP were obtained from West China Medical Center, Sichuan University (Chengdu, Sichuan, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Beijing, China) at 37°C and 5% CO₂.

MTT Assay

The inhibitory effects of nivolumab alone, cisplatin alone, nivolumab combined with cisplatin for PROC cell lines A2780/DDP and SKOV3/DDP cells were measured by MTT assay. Briefly, cells were cultured in cisplatin-free medium for 3 days in advance. The cells (1.0×10^4 cells/well) were plated into 96-well plates. Cells were allowed to attach to the bottom overnight, and then treated with different concentrations of nivolumab or cisplatin for 24 h, 48 h and 72 h, respectively. Control cells received an equal amount of dimethyl sulfoxide (DMSO) only. Before test, 20 μ L of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37°C in the dark. After removing the supernatant, formazan crystals formed were dissolved in 100 μ L DMSO and mixed thoroughly before reading on a microplate reader. The absorbance was measured at 490 nm. All *in vitro* experiments were carried out three times and calculated the averages.

Apoptosis

FACS was performed to detect the apoptosis rate. A2780/DDP cells (2×10^4 cells/well) were plated into 6-well plates. They were cultured for 6 to 8 h and treated with cisplatin alone, nivolumab alone, or cisplatin in combined with nivolumab for 48 h. The cells were analyzed after being treated with RNase (Sigma-Aldrich, St. Louis, MO, USA) and stained with Annexin v and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) before test.

Western Blotting

Western blots were performed as described previously²¹⁻²³. Briefly, total protein extract for each tissue sample or cell line was dissolved in lyses buffer and equal amounts of protein (60 μ g) were analyzed by immunoblotting. Rabbit polyclonal antibodies against Bcl-2 and Bax, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) was obtained from Abcam Corporation (Cambridge, MA, USA).

Statistical Analysis

SAS 6.12 statistics software (SAS, Cary, NC, USA) was adopted to carry out the data processing and analyses, the measurement data were measured by $\bar{x} \pm s$ and the variable data by ratio or percentage. Differences of measurement data in-group were compared with paired *t*-test and the variable data use χ^2 -test. Statistical significance was designated at $p < 0.05$.

Results

The IC₅₀ Value of Cisplatin is Determined in Ovarian Cancer Cells

To detect the anti-tumor effects of cisplatin in ovarian cancer cells, the human ovarian cancer cell lines A2780 and SKOV3 and its PROC sublines A2780/DDP and SKOV3/DDP were used. The ovarian cancer cells were treated with increasing concentration of cisplatin and the inhibitory rate was determined by MTT assay. Cisplatin had anti-tumor effects in human ovarian cancer cell line A2780 and A2780/DDP in a dose and time-dependent manner. As shown in Figure 1, the IC₅₀ values of cisplatin were 88.89 μ M, 13.20 μ M and 9.55 μ M for 24 h, 48 h and 72 h in sensitive cell line A2780, respectively. However, the IC₅₀ values were 350.5 μ M, 50.96 μ M and

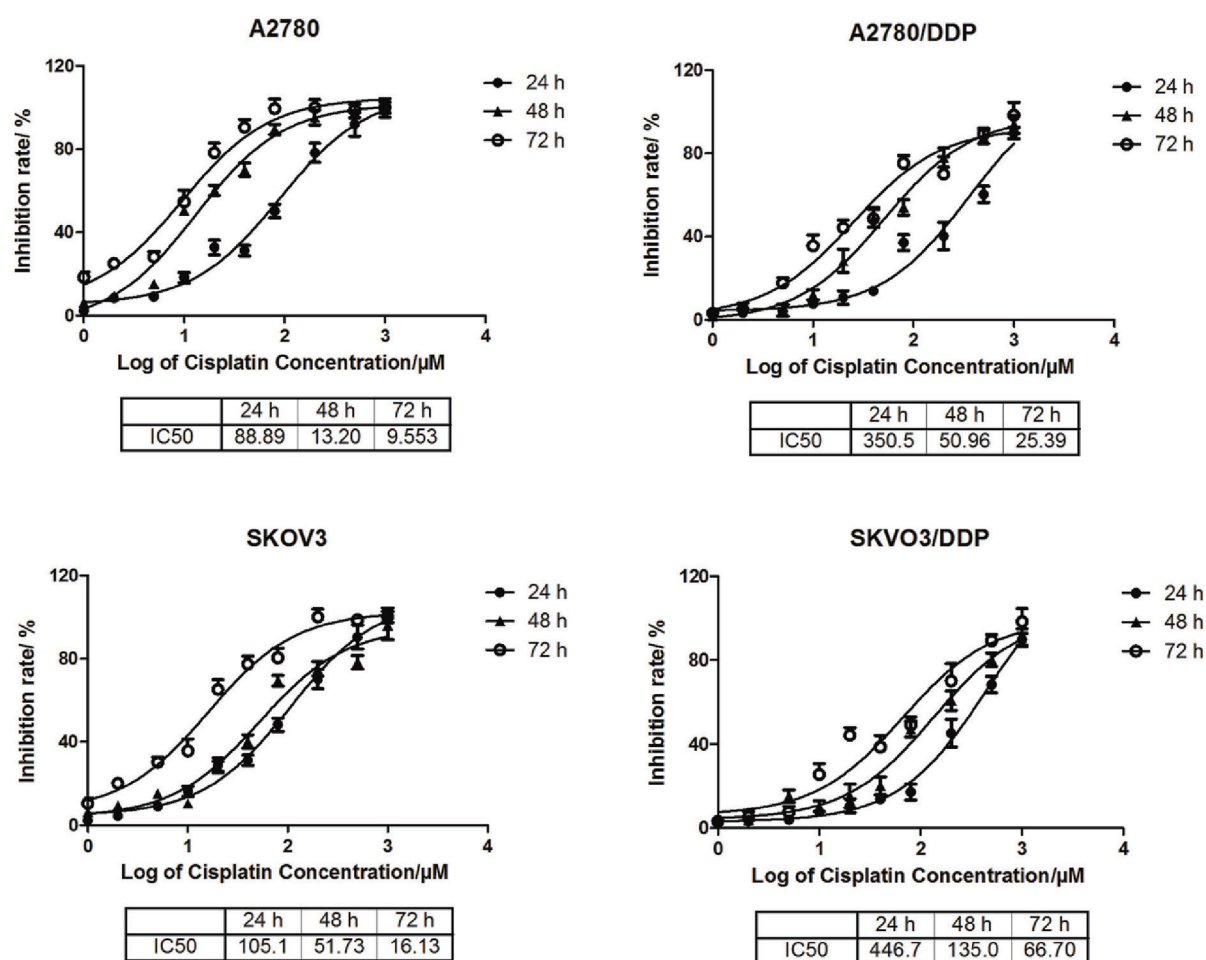


Figure 1. IC₅₀ values of cisplatin on ovarian cancer cells are determined by MTT assay. The ovarian cancer cells A2780, A2780/DDP, SKOV3, and SKOV3/DDP cells (3×10^4 cells/per well) were plated into 96 wells and the cells were exposed for increasing concentrations of cisplatin for 24 h, 48 h and 72 h, respectively. **A**, The proliferation of ovarian cancer cells is determined by MTT assay and IC₅₀s were calculated. **B**, The histogram of IC₅₀ values was shown at 24 h, 48 h and 72 h. All the data were detected three times on duplicates. $^{**}p < 0.01$, compared with cisplatin sensitive cells A2780 or SKOV3.

25.39 μ M in PROC cell lines A2780/DDP, being treated for 24 h, 48 h and 72 h, respectively. The IC₅₀ values of SKOV3 and SKOV3/DDP were consistent with that of A2780 and A2780/DDP. IC₅₀ values in SKOV3 and SKOV3/DDP were 105.10 μ M, 51.73 μ M, 16.13 μ M and 446.7 μ M, 135.0 μ M, 66.70 μ M, respectively, for 24 h, 48 h and 72 h.

Nivolumab Synergistically Increases the Antitumor Effects of Cisplatin in A2780/DDP Cells

The inhibitory effects of nivolumab in ovarian cancer cells were also detected by MTT assay. The PROC cell line A2780/DDP was treated with 10 μ M, 20 μ M, 40 μ M, 80 μ M, and 160- μ M ni-

volumab for 24 h, 48 h and 72 h, respectively. As shown in Figure 2A, the inhibitory effects increased in a dose-dependent manner.

To detect whether nivolumab could play the synergistic anti-tumor role with cisplatin in ovarian cancer cells, we chose 20 μ M as the appropriate concentration for nivolumab and 5 μ M, 10 μ M, 25 μ M, 50 μ M, and 100 μ M as the appropriate concentration for cisplatin. Compared with the group treated with nivolumab only, the inhibitory effects were enhanced as the increasing concentrations of cisplatin in combination with 20 μ M of nivolumab for 48 h (Figure 2B), which demonstrated that nivolumab and cisplatin showed synergistical anti-tumor effects in PROC cell lines.

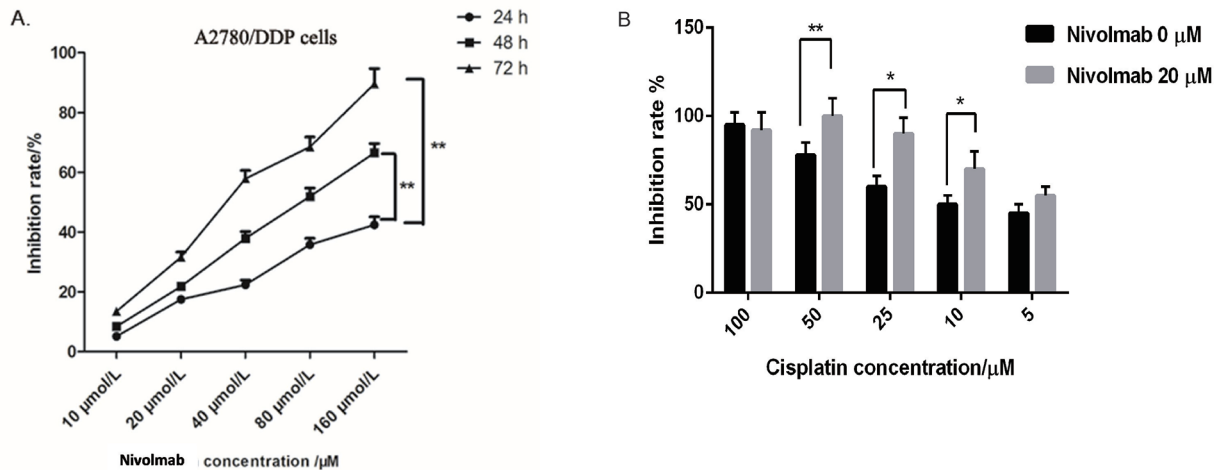


Figure 2. The proliferation of A2780/DDP cells is determined by MTT assay. **A**, The PROC cell line A2780/DDP (3×10^3 cells/per well) were plated into 96-well plates and treated with different concentrations of nivolumab for 24 h, 48 h, and 72 h. Data are shown as the mean values $\pm$ SD. $**p < 0.01$, compared with the values of 24 h. **B**, A2780/DDP cells were treated with 20 μ M of nivolumab in combination with different concentrations of cisplatin for 48 h. The inhibition rate of nivolumab in combination with cisplatin was determined by MTT assay. Here, the cells treated without nivolumab were used as control. $*p < 0.05$, $**p < 0.01$, compared with control.

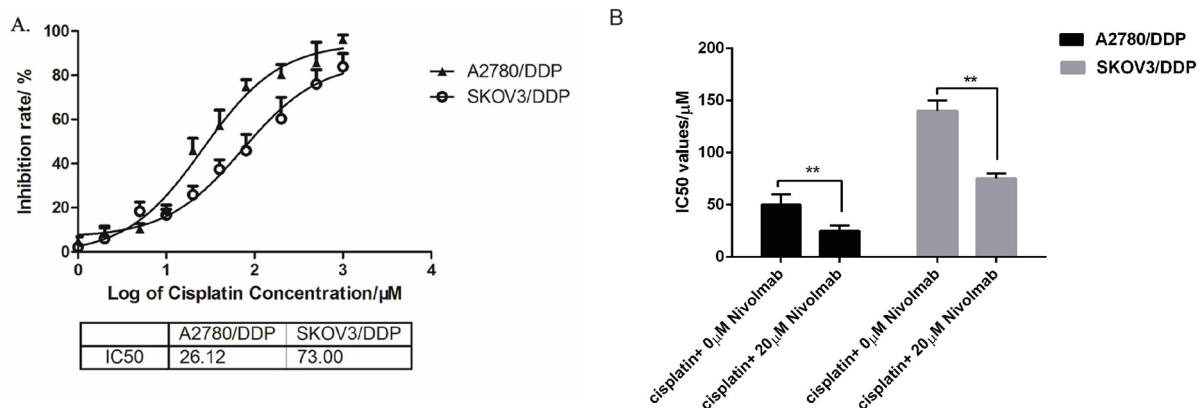


Figure 3. The IC₅₀ values of cisplatin are decreased in combination with 20- μ M nivolumab in A2780/DDP and SKOV3/DDP cells. **A**, PROC cell lines A2780/DDP and SKOV3/DDP (3×10^3 cells/per well) were plated into 96-well plates and treated with different concentrations of nivolumab for 48 h. MTT assay was performed to measure proliferation of PROC cells. IC₅₀ values were calculated and the data were shown as the mean values $\pm$ SD. **B**, The histogram of IC₅₀ values in A2780/DDP and SKOV3/DDP cells. The PROC cells were treated with 20 μ M of nivolumab in combination with different concentrations of cisplatin for 48 h. MTT assay was used to measure the inhibition rate of nivolumab in combination of cisplatin. The cells treated with cisplatin only were used as control. $**p < 0.01$, compared with the control group.

The IC₅₀ Values of Cisplatin is Decreased in Combination with 20 μ M Nivolumab in PROC Cell Lines

We demonstrated that nivolumab synergistically increased the anti-tumor effects of cisplatin in PROC cell lines A2780/DDP and SKOV3/DDP. The cells were treated with different concentrations of cisplatin in combination with 20- μ M nivolumab for

48 h. All of the samples were measured twice. The untreated cells were used as control. As shown in Figure 3, the IC₅₀ values were calculated as 26.12 μ M and 73.00 μ M for 48 h. The ratio of IC₅₀ values was decreased by approximately 1.95-fold and 1.84-fold in cisplatin combined with 20 μ M nivolumab, which demonstrated that nivolumab synergistically decreased the resistance to cisplatin in PROC cell lines.

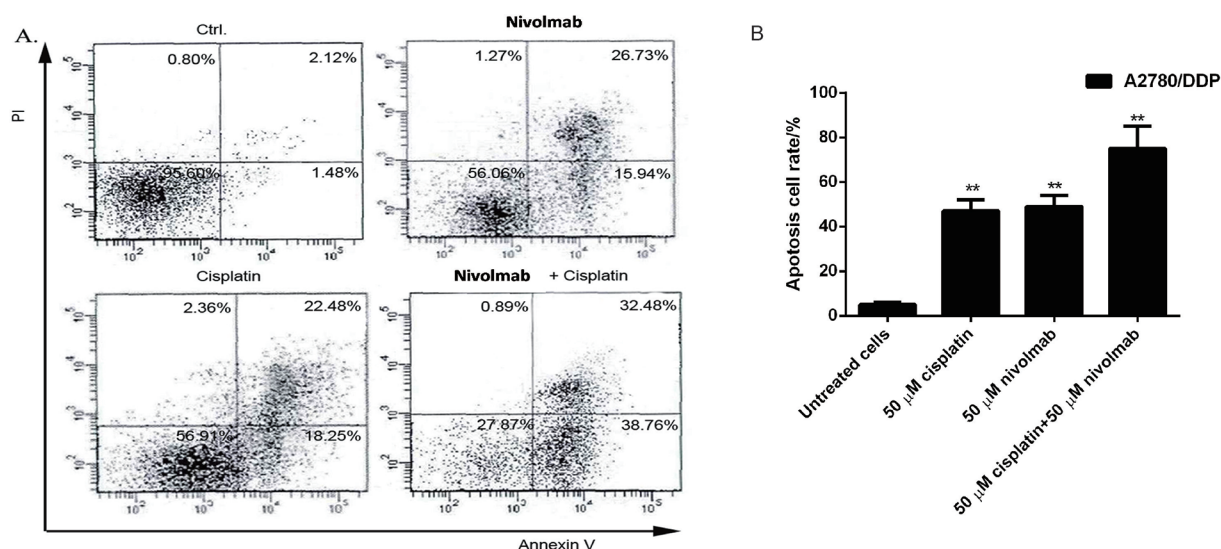


Figure 4. Cell apoptosis rates are detected by flow cytometry of A2780/DDP cells. A2780/DDP cells (2×10^4 cells/well) were plated into 6-well plates. They were cultured for 6 to 8 h and treated with cisplatin only, nivolumab only, and cisplatin in combination with nivolumab for 48 h. Here, untreated cells were used as negative controls. Cell apoptosis rate were detected by FACS analysis with Annexin V and PI dual staining method. **B**, The histogram of cell apoptosis rates was shown. ** $p < 0.01$, compared with untreated cells.

Nivolumab and Cisplatin Synergistically Induce Cell Apoptosis in A2780/DDP Cells

We observed that the cell apoptosis rate significantly increased in the combined treatment group. To identify whether cell apoptosis in A2780/DDP cells could be induced by nivolumab only, cisplatin only, or nivolumab in combination with cisplatin, FACS analysis was performed to detect the cell apoptosis rate in A2780/DDP cells. As shown in Figure 4A, the cells were treated with 50 μ M cisplatin only, 50 μ M nivolumab only, or 50 μ M cisplatin combined with 50 μ M nivolumab for 48 h. The results showed that the apoptosis rate was 32.48% in the combined treatment group, which was significantly higher than that of the nivolumab only group and cisplatin only group. As shown in Figure 4B, the cell apoptosis rates were 42.67%, 40.73% and 71.24% in nivolumab only group, cisplatin only group, and combined treatment group, respectively. All the results demonstrated that nivolumab had a synergistic role with cisplatin in inducing cell apoptosis in PROC cell lines.

Nivolumab Induces Cell Apoptosis of PROC Cells and Causes Cell-cycle Arrest in G0/G1 Phase

To further clarify the possible underlying mechanism of cell apoptosis in PROC cells, the A2780/DDP cells were treated with nivolumab at the concentrations of 10 μ M, 20 μ M, 40 μ M, 80 μ M, and 160 μ M for 48 h. As shown in Figure

5A, the results showed that nivolumab induced cell apoptosis of A2780/DDP cells and the apoptosis rate increased in a dose-dependent manner. The distribution of cell cycle of A2780/DDP cells was also analyzed by propidium iodide-staining method. As shown in Figure 5B, G0/G1 phase arrest was induced and the number of cells in G0/G1 phase increased in a dose-dependent manner as well.

Nivolumab and Cisplatin Synergistically Down-regulated the Expression of Bcl-2 and Upregulated the Expression of Bax

Western blot was performed to measure the expression of Bcl-2 family proteins. As shown in Figure 5C, treatment with nivolumab resulted in down-regulation of Bcl-2 and up-regulation of Bax. More importantly, A2780/DDP cells were treated with 20 μ M nivolumab and 10 μ M cisplatin for 48 h, the ratio of bax/Bcl-2 was significantly higher than that treated with cisplatin and nivolumab only. All the data revealed that nivolumab and cisplatin synergistically down-regulated expression of Bcl-2 and up-regulated expression of Bax.

The Expression Level of ADAM17 Decrease in Dose-dependent Manner with Nivolumab

ADAM17 (A Disintegrin and Metalloprotease Domain 17) is involved in the proliferation

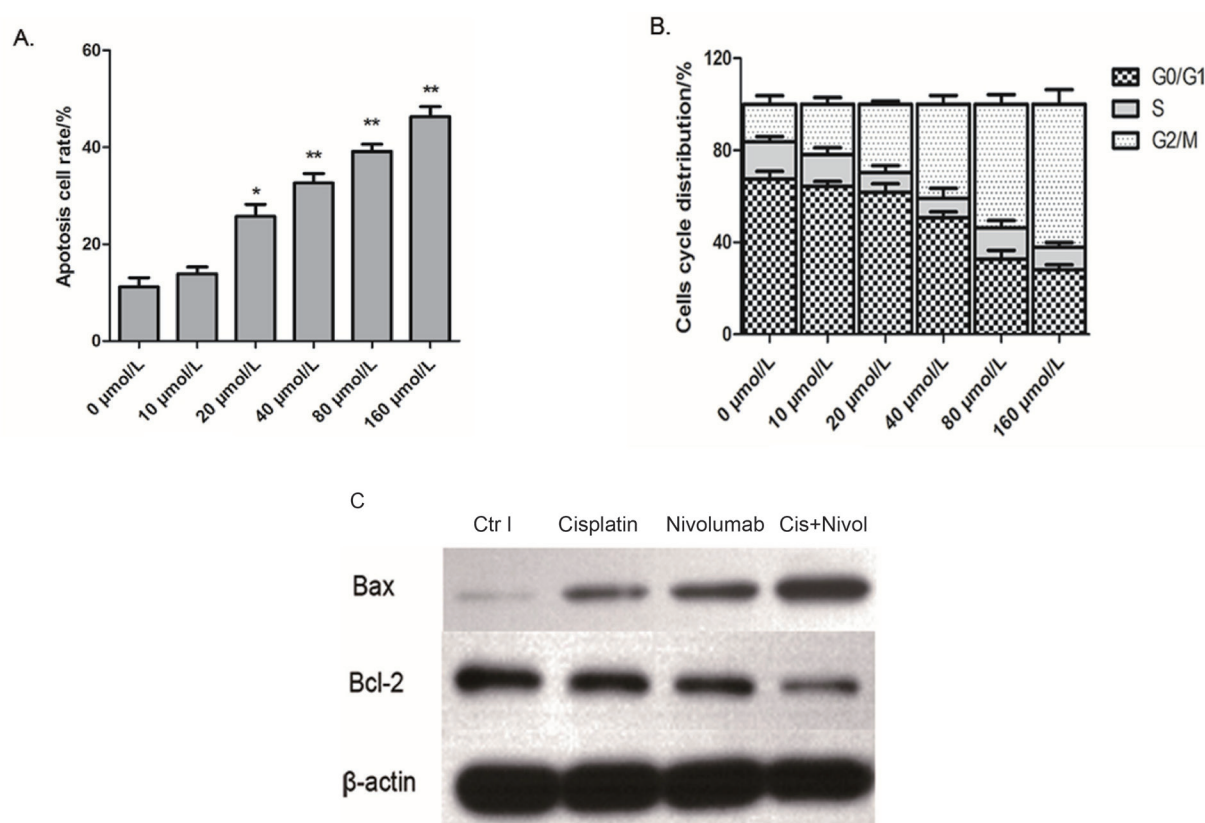


Figure 5. The cell apoptosis rate and cell cycle of A2780/DDP cell is determined. **A**, A2780/DDP cells were treated with different concentrations of nivolumab for 48 h, and the untreated cells were used as the control. Cell apoptosis rate was determined by FACS analysis with Annexin V-FITC and PI dual staining. * $p < 0.05$, ** $p < 0.01$, compared with untreated cells. **B**, Cell cycle of A2780/DDP cells was determined by propidium iodide-staining method. A2780/DDP cells were treated with different concentrations of nivolumab for 48 h. Proportion of the cells in each phase was determined. The cell distribution was shown in the histogram. **C**, Expression of apoptosis related proteins in A2780/DDP cells was detected by Western blot. A2780/DDP cells were treated with 20 μM of nivolumab and 10 μM of cisplatin for 48 h. Expressions of Bax and Bcl-2 in A2780/DDP cells were measured by Western blot. The experiment was repeated for three times.

and progress of several human malignancies. Therefore, we measured the expression levels of ADAM17 in A2780/DDP cells treated with different concentrations of nivolumab. Briefly, A2780/DDP cells were treated with different concentrations of nivolumab for 48 h. The concentrations of nivolumab were 10 μM, 20 μM, 40 μM, and 80 μM, respectively. The expression level of ADAM17 was decreased as the concentrations of nivolumab were increasing, which suggested that nivolumab could suppress proliferation and progression of PROC cells.

Discussion

Drug resistance to chemotherapy agents remains one of the major problems in ovarian

cancer therapy²⁴. Especially, platinum resistance is a major obstacle in the treatment of ovarian cancer and results in PROC^{4,25}. In the present study, we have investigated a new method to reverse the platinum resistance via the combined treatment of nivolumab and cisplatin. Two PROC cell lines A2780/DDP and SKOV3/DDP were used. Our results showed that nivolumab had a synergistical role with cisplatin in inhibiting proliferation and inducing cell apoptosis of PROC cells. Firstly, the combined treatment of nivolumab and cisplatin has a synergistical anti-tumor effect. It is effective to inhibit growth and proliferation of PROC cells at a relatively lower dose. The results demonstrated that cell death significantly increased in the combined treatment group mainly because cell apoptosis were induced

by nivolumab combined with cisplatin. The apoptosis rates were 71.24% in the combined treatment group, which was significantly higher than 42.67% and 40.73% in nivolumab only group, and cisplatin only group, respectively. This was consistent with the results from Western blot. The down-regulation of Bcl-2 and up-regulation of Bax demonstrated that the combined treatment of nivolumab and cisplatin could induce cell apoptosis in PROC cells. Also, FACS was performed to detect cell phases and the results demonstrated that nivolumab induces cell-cycle arrest in G0/G1 phases. All of the results have demonstrated that the combined treatment is an effective way to inhibit the proliferation of PROC cells. Most importantly, the combined treatment of nivolumab and cisplatin could effectively reverse platinum resistance. The IC₅₀ values were significantly decreased from 50.97 μ M to 26.12 μ M and from 135.20 μ M to 73.00 μ M in A2780/DDP and SKOV3/DDP cells respectively.

Also, the inhibitory effect might be at least partly attributed to the inhibition of ADAM17. Previous studies have shown that ADAM17 is involved in proliferation and progression of cancer cells. In the present study, we found that the expression level of ADAM17 was decreased in A2780/DDP cells treated with different concentrations of nivolumab. The data revealed that nivolumab could have the ability to suppress proliferation and progression of PROC cells.

Conclusions

The combined treatment of nivolumab and cisplatin was a useful method to treat PROC. Both agents exerted synergistic anti-tumor effects and effectively reverse platinum resistance in PROC cells. Therefore, it would be a beneficial and useful way to treat PROC in clinical practice.

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Conflict of interest

The authors declare no conflicts of interest.

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