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, MTT 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.
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an overall response rate of 17% in Phase 1 clinical trial for patient with PROC\textsuperscript{15,16}. Qi et al\textsuperscript{17} reported that nivolumab effectively induced cell apoptosis of pancreatic cancer cells. Gao et al\textsuperscript{18} 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\textsuperscript{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% CO\textsubscript{2}.

**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 $\times$ 10\textsuperscript{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 mL 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 mL 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$\times$10\textsuperscript{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 previously\textsuperscript{21-23}. Briefly, total protein extract for each tissue sample or cell line was dissolved in lyses buffer and equal amounts of protein (60 μg) 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 $\chi^2$-test. Statistical significance was designated at $p$<0.05.

**Results**

**The IC\textsubscript{50} 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 SKVO3/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 IC\textsubscript{50} values of cisplatin were 88.89 μM, 13.20 μM and 9.55 μM for 24 h, 48 h and 72 h in sensitive cell line A2780, respectively. However, the IC\textsubscript{50} values were 350.5 μM, 50.96 μM and
25.39 μM 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 μM, 51.73 μM, 16.13 μM and 446.7 μM, 135.0 μM, 66.70 μM, 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 μM, 20 μM, 40 μM, 80 μM, and 160-μM nivolumab 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 μM as the appropriate concentration for nivolumab and 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM 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 μM of nivolumab for 48 h (Figure 2B), which demonstrated that nivolumab and cisplatin showed synergistic anti-tumor effects in PROC cell lines.
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The IC50 Values of Cisplatin is Decreased in Combination with 20 μM 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 for 48 h. All of the samples were measured twice. The untreated cells were used as control. As shown in Figure 3, the IC 50 values were calculated as 26.12 μM and 73.00 μM for 48 h. The ratio of IC50 values was decreased by approximately 1.95-fold and 1.84-fold in cisplatin combined with 20 μM nivolumab, which demonstrated that nivolumab synergistically decreased the resistance to cisplatin in PROC cell lines.
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 μM cisplatin only, 50 μM nivolumab only, or 50 μM cisplatin combined with 50 μM 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 and Cisplatin Synergistically Induce Cell Apoptosis in A2780/DDP Cells**

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 μM, 20 μM, 40 μM, 80 μM, and 160 μM for 48h. 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 Induces Cell Apoptosis of PROC Cells and Causes Cell-cycle Arrest in G0/G1 Phase**

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**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 μM nivolumab and 10 μM 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 Metalloprotei-nase Domain 17) is involved in the proliferation
and progress of several human malignancies. Therefore, we measured the expression levels of ADAM17 in A2780/DDP cells 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 therapy. Especially, platinum resistance is a major obstacle in the treatment of ovarian cancer and results in PROC. 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 μM to 26.12 μM and from 135.20 μM to 73.00 μM 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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