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Ad-PUMA sensitizes ovarian cancer cells to chemotherapeutic agents

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Abstract. – OBJECTIVE: Ovarian cancer accounted for the first cause of death in female reproductive system tumor even with the operation and chemotherapy. We sought to evaluate the therapeutic potential of p53 up-regulated modulator of apoptosis (PUMA) in ovarian cancer.

MATERIALS AND METHODS: An adenovirus expressing PUMA (Ad-PUMA), alone or in combination with chemotherapeutic agents, was used to treat two different ovarian cancer cell lines. The mechanism of PUMA-mediated growth suppression and apoptosis was investigated by analysis of caspase-9 activation and the change of mitochondrial membrane potential ($\Delta\psi m$).

RESULTS: The exogenous PUMA was expressed 6 h after Ad-PUMA infection, which increased the chemosensitivity of the cancer cells and decreased the IC50 of chemotherapeutic agents compared with uninfected cells. The apoptotic percentage of OVCAR-3 and SKOV3 increased greatly compared with Taxol or Cisplatin alone. There was shear zone in caspase-9 and $\Delta\psi m$ decrease after Ad-PUMA infection which suggested apoptosis started in mitochondrial mediated pathway.

CONCLUSIONS: PUMA plays a role in suppressing tumor growth and sensitizing ovarian cancer cells to anticancer drugs and may be a promising tool for cancer biotherapy.

Key Words:
Chemosensitivity, Gene therapy, Ovarian cancer, PUMA.

Introduction

Ovarian cancer accounts for the first cause of death in female reproductive system tumor even with the operation and chemotherapy. Drug resistance contributes to poor clinical outcomes and it remains a significant obstacle to successful chemotherapy[1]. With the development of the research on tumor molecular mechanism, emerging therapies such as gene therapy will help to improve the effect of tumor treatment.

*p*53 up-regulated modulator of apoptosis (PUMA) was discovered in 2001 by two independent groups[2,3]. As downstream proapoptotic effectors of *p*53, PUMA can result in rapid and complete apoptosis by exogenous expression in a variety of cancer cell lines such as lung, esophagus and head and neck carcinoma[4-7]. PUMA cannot only induce a wide variety of tumor cell apoptosis and inhibit tumor cell proliferation, but also can increase the sensitivity of tumor cells to radiation and chemotherapy. It's reported that PUMA is a direct target of NF-κB and mediates doxorubicin-induced apoptosis *in vitro*[8]. And PUMA expression is also a novel prognostic indicator in hepatitis B virus-related hepatocellular carcinoma and may be a potential target for diagnosis and gene therapy[9].

This study was aimed to build an adenovirus expressing PUMA (Ad-PUMA) and determine whether exogenous expression of PUMA could inhibit the growth of ovarian cancer cell and enhance sensitivity of ovarian cancer cells to chemotherapeutic agents.

Materials and Methods

Cell Culture

The Ovarian cancer cell lines SKOV3 and OVCAR-3 were provided by Institute of Basic Medicine, Peking Union Medical College and Chinese Academy of Medicine Science (Beijing, China). Both cell lines were cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) medium supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) in humidified air at 37°C with 5% CO_2.

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RT-PCR

SKOV3 and OVCAR-3 were infected with Ad-PUMA at 10 multiplicity of infection (MOI) for 24 h. Total RNA was isolated from diverse treated cells with TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed and used as the template for PCR amplification. Sequences of the RT-PCR primers for PUMA were: forward, 5'-GTC-CTCAGCCCTCGCTCT-3'; reverse, 5'-CTGCT-GCTCCTCTTGTCTCC-3'. The product is 209 bp in length. Sequences of the RT-PCR primers for *p*53 were: forward, 5'-TAC TCC CCT GCC CTC AAC AAGA-3'; reverse, 5'-CCA CGG ATC TGA AGG GTG AAA TAT-3'. The product is 626 bp in length. Tubulin is amplified as internal control.

Western Blotting

SKOV3 and OVCAR-3 were collected after infected by Ad-PUMA at 5 MOI for different time. The cell lines were prepared for western blot. Whole-cell protein extracted was prepared and quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein (80 μg/lane) was denatured, resolved on 12% SDS-PAGE gels and semi-dry transferred at 12 V for 3 h onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were probed with polyclonal antibodies against PUMA (Calbiochem, Billerica, MA, USA) overnight at 4°C, followed by incubation with secondary goat anti-rabbit IgG conjugated to horseradish peroxidase and detection using SuperSignal ECL (Applygen Technologic Inc., Beijing, China). To control protein loading, membranes were stripped and reprobed with anti-β-actin antibody at 1:5000 (Santa Cruz Biotech, Santa Cruz, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide (MTT) Assay for Assessing Cell Viability of Ad-PUMA

SKOV3 and OVCAR-3 were seeded in a 96-well plate and incubated overnight. Ad-PUMA at different MOI was added to the medium before the cells were incubated with 0.5 mg/ml MTT (Sigma Aldrich, St. Louis, MO, USA) 48 h or 72 h later. The formazan precipitate was dissolved in 150 μl DMSO (Sigma Aldrich, St. Louis, MO, USA) and the absorbance at 492 nm was measured by a benchmark microplate reader (Bio-Rad, Hercules, CA, USA). At least three independent experiments were done and the average rates of inhibition were calculated according to the OD values. The IC50 of Ad-PUMA was calculated according to the average inhibition rates.

MTT Assay for Assessing Cell Viability of Ad-PUMA in Combindation with Taxol and Cisplatin

To determine whether exogenous expression of PUMA could enhance sensitivity of SKOV3 and OVCAR-3 to chemotherapeutic agents, we tested the effect of the combined treatments. SKOV3 was infected with Ad-PUMA at 1 MOI before the medium containing 10% FBS and chemotherapeutic agents of various concentrations were added to the wells 12 h later. While 6 MOI Ad-PUMA was added before Taxol and Cisplatin (Sigma Aldrich, St. Louis, MO, USA) added for OVCAR-3. The cells were incubated for 48 h before MTT was added. The IC50 of chemotherapeutic agents was calculated according the OD values.

Apoptosis Assays

Sub-G_1 > Measurement

SKOV3 and OVCAR-3 were seeded at 5×10^5 cells per dish (35 mm^2). After overnight incubation, the cells were infected with Ad-PUMA at 1 MOI (for SKOV3) or 6 MOI (for OVCAR-3) before Taxol and Cisplatin added for 24 h. After treatment, attached and floating cells were harvested. After washed twice with phosphate buffered saline (PBS), the cells were fixed with 70% ethanol at 4°C overnight. Then incubated in RNase A/PBS (100 μg/ml) at 37°C for 30 min. Intracellular DNA was labeled with 50 μg/ml propidium iodide (PI, Sigma Aldrich, St. Louis, MO, USA) and analyzed with a FACS Calibur fluorescence-activated cell sorter using Cell Quest software (BD Biosciences, San Jose, CA, USA). The percentage of sub-G_1 nuclei in each population was determined from at least 1×10^4 cells.

Rhodamine 123 (Rh123) Staining to Assay Mitochondrial Membrane Potential ($\Delta\psi m$) of Ovarian Cancer Cell

A total of 1×10^6 SKOV3 and OVCAR-3 cells were seeded into each well and incubated overnight. After the culture medium was replaced by 1640 free of FBS supplemented with Ad-PUMA, complete 1640 were added 90 min later. The cells were incubated for 12 or 24 h at

37°C. Rh123 (Sigma Aldrich, St. Louis, MO, USA) was then added to give a final concentration of 10 g/l. The cells were further incubated for 30 min, washed twice with ice-cold PBS, harvested with trypsin, and suspended in PBS. Using the FACS Calibur system, the fluorescence intensity of Rh123 was measured at excitation and emission wavelength of 485 nm and 530 nm, respectively.

Diamidino-phenyl-indole (DAPI) Staining

SKOV3 and OVCAR-3 were infected with Ad-PUMA at 1 MOI (for SKOV3) or 6 MOI (for OVCAR-3) before Taxol and Cisplatin added for 48 h. Cells (attached cells plus those floating in the medium) were harvested at indicated time points and fixed in a solution containing a final concentration of 3.7% formaldehyde, 0.5% Nonidet P-40, and 10 g/ml DAPI (Sigma Aldrich, St. Louis, MO, USA) in PBS. Apoptosis was assessed through microscopic visualization of condensed chromatin and micronucleation. At least two independent experiments were carried out for each condition, and a minimum of 300 cells were counted in each measurement.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software. The values in the figures and tables are presented as the mean ± standard deviation (SD). *p* values were calculated by Student's *t* test or two-way ANOVA.

Results

The mRNA Expression of PUMA and p53

There was difference in expression of PUMA and *p*53 between SKOV3 and OVCAR-3. PUMA was expressed much higher in SKOV3 than OVCAR-3 (Figure 1). As for *p*53, there was much higher expression in OVCAR-3 than SKOV3. The result may explain the different effect of PUMA in two cell lines.

Western blot showed PUMA Induce Apoptosis in Mitochondrial Pathway

Figure 2 showed PUMA was expressed in high level than control at 6 h. This suggested that the Ad-PUMA can effectively induce the expression of PUMA in ovarian cancer cells. There was shear zone in caspase-9 after 6 h after Ad-PUMA' infection which indicated apoptosis started in mitochondrial mediated pathway.

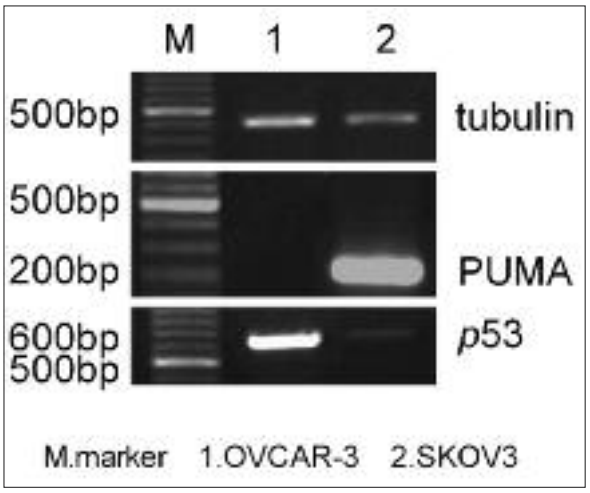

Figure 1. The mRNA expression of PUMA in SKOV3 and OVCAR-3. The results showed that PUMA was expressed lower in SKOV3 than in OVCAR-3 and p53 was expressed higher in OVCAR-3 than in SKOV3.

Ad-PUMA Profoundly Suppressed Growth of both SKOV3 and OVCAR-3

Ad-PUMA inhabited the growth of both SKOV3 and OVCAR-3 with time and concentration. Ad-PUMA showed more powerful effect on SKOV3. The IC50 of Ad-PUMA for SKOV3 and OVCAR-3 at 48 h was 6.6 and 24.5 MOI respectively (Table I).

Ad-PUMA Sensitized Ovarian Cancer Cell to Chemotherapeutic Agents

To evaluate whether Ad-PUMA can enhance the cytotoxicity of chemotherapeutic agents in treating cancer, we carried out MTT assay for assessing cell viability. The results indicated the cells were inhibited more considerably in both cell lines when treated with Ad-PUMA and Taxol or Cisplatin combined together than those

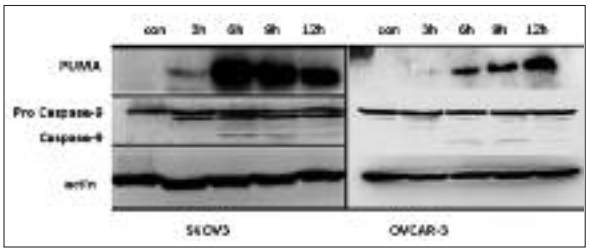

Figure 2. The expression of caspase-9 as a result of Ad-PUMA infection. PUMA was expressed in high level than control at 6 h and there was shear zone in caspase-9 after 6 h after Ad-PUMA' infection.

Table I. Inhibitory rate of Ad-PUMA on SKOV3 and OVCAR-3 (%).

MOI	2	5	10	20	50	100
SKOV3	0.21 ± 0.06	0.42 ± 0.06	0.71 ± 0.04	0.76 ± 0.11	0.93 ± 0.06	0.98 ± 0.02
OVCAR-3	0.01 ± 0.03	0.07 ± 0.06	0.16 ± 0.1	0.38 ± 0.11	0.78 ± 0.07	0.97 ± 0.04

Values are presented as means ± SD (n = 3).

treated with anticancer drugs alone. Overexpression of PUMA increased the chemosensitivity of the cancer cells and decreased the IC50 of chemotherapeutic agents compared with uninfected cells (Table II). The combination index was smaller than 1 which suggested Ad-PUMA had synergy effect when combined with anticancer drugs. To achieve same inhibition effect to ovarian cancer cell, small doses of Ad-PUMA can obviously reduce the dose of Taxol and Cisplatin.

Apoptosis Induced by Ad-PUMA or Combination with Chemotherapeutics

Ad-PUMA induced apoptosis of SKOV3 and OVCAR-3 with concentration dependence. Rhodamine 123 can assay the change of mitochondrial membrane potential ($\Delta\psi m$) related to apoptosis. The $\Delta\psi m$ decreased significantly 12 h after Ad-PUMA given to SKOV3 or OVCAR-3 (Figure 3). The result was consistent with the sub-G_1 measurement. Ad-PUMA induced apoptosis of SKOV3 and OVCAR-3 with concentration dependence (Figure 4).

SKOV3 cells were collected after treatment of Cisplatin or Taxol combined with Ad-PUMA for 24 h, and then the sub-G_1 was tested. The combined treatments of the Ad-PUMA and anticancer drugs increased the sub-G_1 percentage of SKOV3 (78.7% with Cisplatin, 74.7% with Taxol) compared with the anticancer drugs alone at 24 h exposure (24.0% with Cisplatin, 15.1% with Taxol). While in OVCAR-3, the sub-G_1 percentage increased from 13.7% to 72.1% for Cisplatin and from 5.46% to 67.7% for Taxol.

We counted the apoptotic cells by DAPI staining after cells were treated with Cisplatin or Taxol alone and combined with Ad-PUMA for 48 h (Figure 5). The morphological change of nucleus detected by DAPI staining can evaluate apoptosis. The apoptotic percentage of OVCAR-3 resulted from chemotherapeutic agent alone was 19.6% (Taxol) or 16.3% (Cisplatin), respectively at 48 h. When combined with Ad-PUMA, their apoptotic percentages increased to 66.3% and 78.3% respectively. There was similar result in SKOV3. The apoptotic percentage of cells resulted from chemotherapeutic agent alone was 15.4% (Taxol) or 13.9% (Cisplatin) respectively at 48 h. When combined with Ad-PUMA, their apoptotic percentages increased to 76.5% and 78.1%, respectively.

Discussion

Apoptosis, also called programmed cell death, is a genetically encoded program that disposes of unwanted cells. Disruptions of this pathway have been implicated as a cause of cancer and its resistance to drug-induced apoptosis[10]. Therefore, genetic restoration of the

Table II. Ad-PUMA sensitized SKOV3 and OVCAR-3 to chemotherapeutic agents.

Cell line	Anticancer drugs	IC_{50}		CI[a]
		Ad-PUMA+	Ad-PUMA-	
SKOV3	DDP (μM)	1.83	8.98	0.36
	TAX (nM)	2.87	20.59	0.29
OVCAR-3	DDP (μM)	2.23	6.8	0.57
	TAX (nM)	3.55	53.88	0.31

[a]CI = combination index.

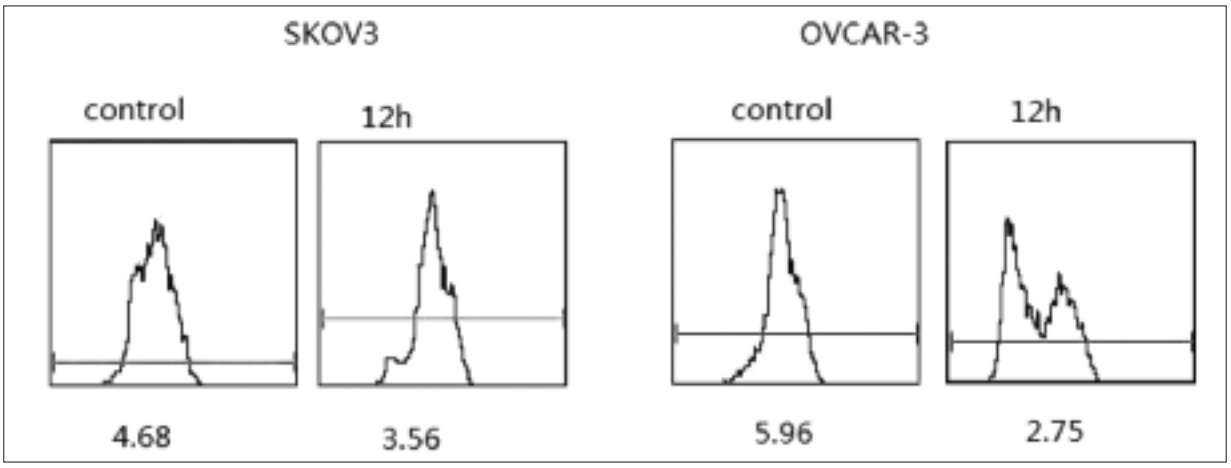

Figure 3. The decrease of Δψm caused by Ad-PUMA. The Δψm was measured by the mean fluorescence intensity after Rh123 staining. The Δψm decreased significantly 12 h after Ad-PUMA given to SKOV3 or OVCAR-3.

apoptotic pathway or introduction of pro-apoptotic molecules is an attractive approach for treating tumors. The *p*53-mediated apoptosis is the principal mechanism by which most chemotherapeutic drugs kill tumor cells. And it's reported that the effect of rAd-*p*53 and paclitaxel inhibiting HeLa cell proliferation and induction of apoptosis is better than the single drug[11]. In the great majority of human tumors, the *p*53 pathway is disarmed. Restoration of the *p*53 pathway by activating *p*53 itself or *p*53 downstream targets has been explored to improve efficacy of anticancer therapies[12]. Therefore, it is necessary for seeking alternative candidates for effective cancer gene therapy, in particular under conditions where *p*53 is ineffective.

As downstream proapoptotic effectors of *p*53, the exogenous expression of PUMA can result in rapid and complete apoptosis and induce release of apoptogenic mitochondrial proteins Cytochrome C, and activate procaspases-3 and 9 in

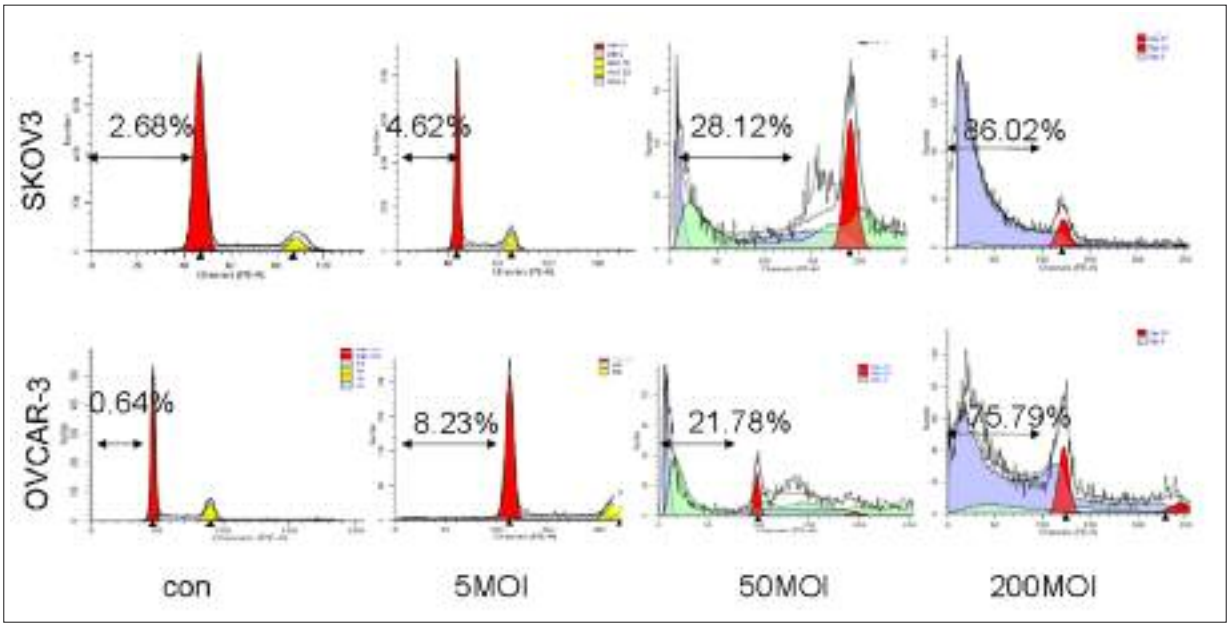

Figure 4. The change of sub-G1 caused by Ad-PUMA. Apoptosis was assayed by FACS to measure the percentage of sub-G1. The combined treatments of the Ad-PUMA and anticancer drugs increased the sub-G1 percentage of SKOV3 or OVCAR-3.

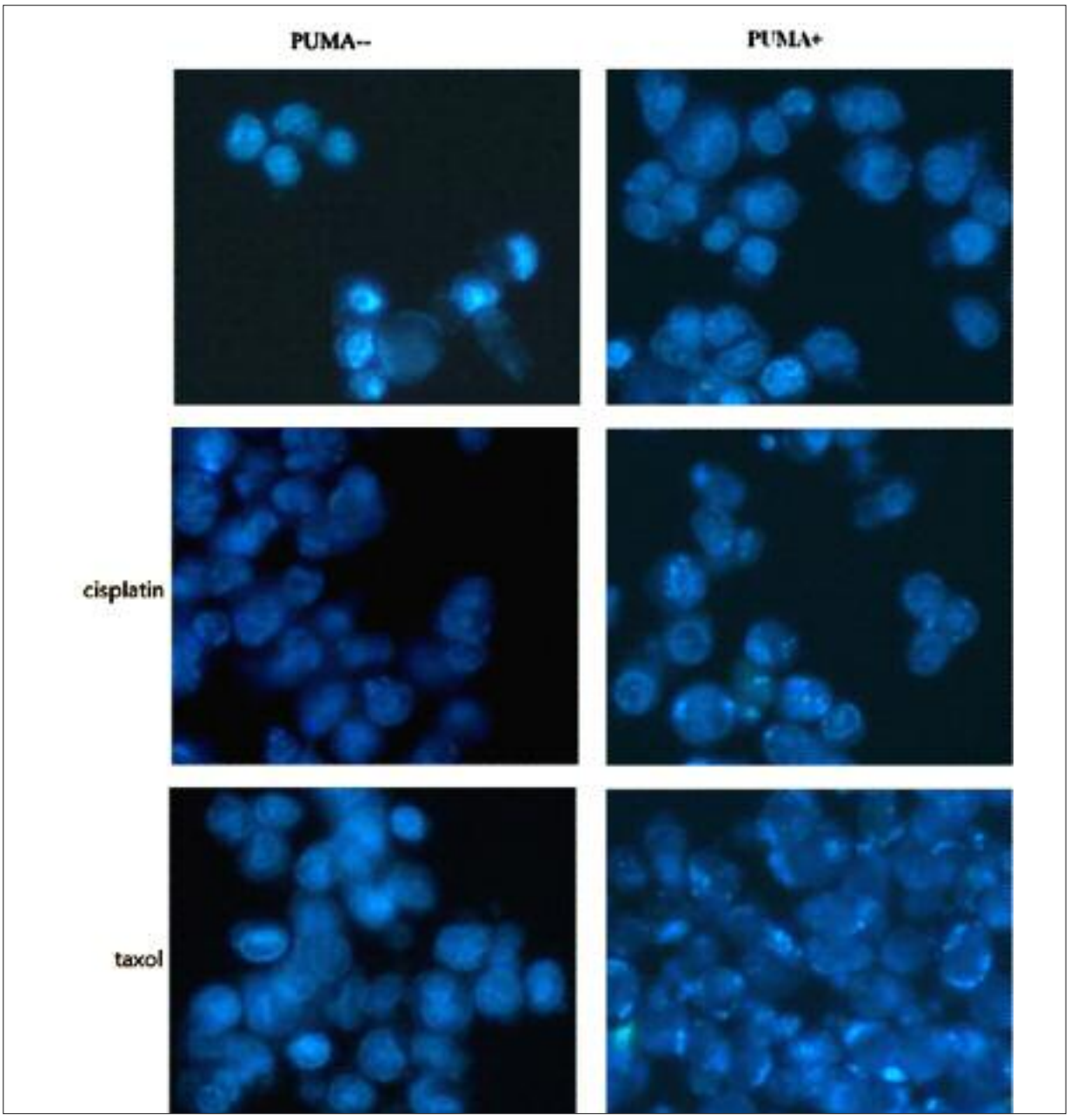

Figure 5. The apoptosis detected by DAPI staining. The morphological change of nucleus was detected by DAPI staining to evaluate apoptosis. When combined with Ad-PUMA, the apoptotic percentages increased compared to chemotherapeutic agent alone.

a variety of cancer cell lines, suggesting a prominent role in the intrinsic apoptotic pathway[3]. In our study, exogenous overexpression of PUMA significantly inhabited the growth of SKOV3 and OVCAR-3 cells. Similarly, it has been reported that PUMA could induce apoptosis more efficiently than *p*53 in malignant glioma cells regardless of *p*53 status through the human telomerase reverse transcriptase (hTERT) promoter system[13]. The consistent conclusion was drawn in human melanoma cell lines.

Drug resistance of tumor cell represents a significant obstacle to successful chemotherapy as the result of abnormal pathway including mutation or deletion of *p*53 in apoptosis[14]. Accordingly, PUMA might be an effective alternative for tumors in which exogenous delivery of *p*53 is ineffective. In our study, mRNA expression of

PUMA was down-regulated in OVCAR-3, while the inhibition effect of Ad-PUMA was different between SKOV3 and OVCAR-3. The *a*denoviral delivery of PUMA sensitized SKOV3 and OVCAR-3 to chemotherapeutic agents especially in OVCAR-3. We chose the concentration of Ad-PUMA according its inhibition rate at about 10% and IC50 was greatly decreased for Taxol and Cisplatin combined with Ad-PUMA in SKOV3 and OVCAR-3. Ad-PUMA had synergy effect when combined with anticancer drugs and small doses of Ad-PUMA reduced the dose of Taxol and Cisplatin.

Ad-PUMA facilitated apoptosis induced by anticancer drug treatment. From sub-G_1 measurement, the $\Delta\psi m$ and DAPI staining, we could see combination increased the percentage apoptotic cells greatly compared with Cisplatin or Taxol alone. This indicated that the potent inhibitory effect on the cells viability by the combination of Ad-PUMA and anticancer drugs may result from abundant apoptosis induction. There are two major approaches for apoptosis, receptor way and mitochondrial way. PUMA is located on the mitochondrial membrane and belongs to Bcl-2 family, which promotes or inhibits apoptosis by homologous or heterologous dimmers[15]. With other Bcl-2 family member, PUMA promotes Cytochrome C released from mitochondrial and induces procaspase-9 self-catalytic[16]. Activated caspase-9 is the main executor of cell apoptosis and leads to apoptosis later. In our study, there was not only PUMA in high level, but also a shear zone in caspase-9 and decrease of $\Delta\psi m$ after infection, which suggested apoptosis starts in mitochondrial pathway.

Delivery of PUMA into OVCAR-3 through adenoviral gene transfer resulted in apoptosis and enhanced sensitivity to chemotherapeutic agents, suggesting that adequate level of PUMA is crucial for DNA damage and enhancing growth suppression and apoptosis when combined with DNA-damaging agents (etoposide) and chemotherapeutic agents (5-FU and MTX). Our observation is consistent with the study of Yu et al[5], which suggested that PUMA was rather a broad-spectrum chemosensitizer and radiosensitizer of cancer cells. The recent studies showed that PUMA can regulate diverse apoptotic pathways by antagonizing all known antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1, which are frequently overexpressed in cancer cells, suggesting that PUMA may be useful for targeting a variety of apoptotic defects in cancer cells[17,18].

Conclusions

These results demonstrated that low dose of Ad-PUMA significantly improved the sensitivity of ovarian cancer cells to chemotherapeutic agents, in addition to its role in inhibiting tumor growth. Thus adenoviral delivery of PUMA may be a promising tool for cancer biotherapy with or without combination with chemotherapeutic agents.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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