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The mechanism and inhibitory effect of recombinant human P53 adenovirus injection combined with paclitaxel on human cervical cancer cell HeLa

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Abstract. – OBJECTIVE: To investigate the effect of recombinant human p53 adenovirus injection combined with paclitaxel on human cervical cancer HeLa cell proliferation, apoptosis and expression of vascular endothelial growth factor (VEGF).

MATERIALS AND METHODS: Detection of effects of paclitaxel, rAd-p53, and drug combination on the proliferation of HeLa cells by MTT method. Detection of effects of paclitaxel, rAd-p53, and drug combination on the proliferation of HeLa cells by diamidino-phenyl-indole (DAPI) staining. Detection of effects of paclitaxel, rAd-p53, and drug combination on the expression of VEGF of HeLa cells by Western blotting.

RESULTS: Paclitaxel, rAd-p53 alone or in combination could inhibit the proliferation of HeLa cells in 24-72 h. The inhibition is time dependent and dose dependent. Inhibition of HeLa cells in the combination group was significantly higher than single use of paclitaxel group and rAd-P53 group ($p < 0.05$). The combined effect of the coefficient of drug interaction (CDI) value was CDI < 1, showing that the two have a synergistic effect. Cell inhibition rate combined group of RAd-P53 (5 × l07 vp/mL) and paclitaxel (3 μg/mL) 48 h after application is higher than that of monotherapy group [(54.0 ± 0.92) % vs. (31.8 ± 0.58) %, (27.2 ± 0.55) %, $p < 0.05$]. The apoptosis rate of HeLa cells in combination group was significantly higher than that in paclitaxel group, rAd-p53 alone group [(83 ± 0.07) % vs. (11 ± 0.01) %, (36 ± 0.04) %, (62 ± 0.05) %, $p < 0.05$]. Expression of VEGF in HeLa cells of combination group was significantly lower than that of the two single drug groups. Expression of VEGF in HeLa cells was decreased by (81 + 0.08) %, (45 + 0.07) % and (60 + 0.06) % ($p < 0.05$), respectively.

CONCLUSIONS: The effect of rAd-p53 and paclitaxel inhibiting HeLa cell proliferation and induction of apoptosis is better than the single drug. Its mechanism may be related to the down-regulation of VEGF.

Key Words:
rAd-p53, Paclitaxel, Cervical cancer, HeLa cell, VEGF.

Introduction

Cervical cancer is the most common gynecologic malignant tumor. Early stage cervical carcinoma can be cured by operation or radiotherapy, and terminal cervical cancer lost operation opportunity. Radiation treatment alone showed poor effect with high recurrence rate. Endocrine function damage occurred to young patients after radiotherapy, causing vaginal epithelial fibrosis and vaginal contracture that had serious impact on patients' quality of life. Since application of the new adjuvant chemotherapy (NACT) in cervical cancer in clinic, large amounts of data[1-6] show that preoperative local chemotherapy can effectively reduce the size of tumor in advanced cervical cancer, to down-staging, to create the conditions for operation treatment, and to improve the effect of operation. But drug resistance during chemotherapy affects the treatment result. Study[7,8] proved that wild type *p53* gene mutations play an important role in the occurrence and development of cervical cancer, and resistance to chemotherapy is related to *p53* gene mutation in tumor cell. This study intends to use the recombinant human adenovirus-p53 (rAd-p53) infection of cervical cancer cell HeLa to observe whether reconstruction of *p53* gene in tumor cells can improve the sensitivity of it to chemotherapy. We will discuss the mechanism and the effect of rAd-p53 combined with paclitaxel for cervical cancer by detecting the changes of vascular endothelial growth factor (VEGF) expression on HeLa cells after paclitaxel treatment.

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Table I. Effect of paclitaxel and rAd-p53 alone or in combination on the proliferation of HeLa cells.

Time (%) Group Inhibition	24 h	48 h	72 h
Paclitaxel (μg/mL)			
1.5	13.9 ± 0.44	16.3 ± 0.21	11.2 ± 0.13
3	27.6 ± 0.68	31.8 ± 0.58	29.2 ± 0.14
6	36.3 ± 0.13	40.2 ± 0.23	39.3 ± 0.36
rAd-p53 (vp/mL)			
5×10^6	10.4 ± 0.27	15.0 ± 0.77	19.5 ± 0.45
5×10^7	26.0 ± 0.31	27.2 ± 0.55	30.4 ± 0.57
5×10^8	34.7 ± 0.59	42.6 ± 0.25	47.2 ± 0.16
rAd-p53 (vp/mL) + Paclitaxel (μg/mL)			
$5 \times 10^6 + 1.5$	34.6 ± 0.20*	43.2 ± 0.72*	46.8 ± 0.51*
$5 \times 10^7 + 3$	47.8 ± 0.10$^+$	54.0 ± 0.92$^+$	62.4 ± 0.16$^+$
$5 \times 10^8 + 6$	68.8 ± 0.43§	78.8 ± 0.25§	84.9 ± 0.28§

*$p < 0.05$ vs. paclitaxel alone or rAd-P53 group; $^+p < 0.05$ vs. paclitaxel alone or rAd-P53 group; $^§p < 0.05$ vs. paclitaxel alone or rAd-P53 alone group.8-oxoguanine (pg/ml)

Materials and Methods

Main Materials

Cervical cancer cell line HeLa is from blood disease research Chinese Tianjin Medical Sciences Institute, cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) medium containing 10% fetal bovine serum (FBS) in incubator of 5% CO_2, 37°C with saturated humidity, and passaged using 0.25% trypsin for digestion. The cells were in logarithmic growth phase. Recombinant human p53 adenovirus injection (1×10^{12} vp/tube) was purchased from Shenzhen SiBiono Gene Technologies Co. Ltd. (No. 20090201), diluted with phosphate buffered saline (PBS).

Paclitaxel (size 5 mL: 30 mg) was purchased from Yangtze River Pharmaceutical Company (No. 09102901). Mouse anti-human VEGF monoclonal antibody was purchased from Bioworld Technology Inc. (St. Louis Park, MN, USA). General S-P9000 kit was purchased from Beijing Zhongshan Biotechnology Company. Protein electrophoresis and power transfer device were purchased from Bio-Rad Company (Hercules, CA, USA). The image processing apparatus was purchased from American Gene Company (Genetech's South S. Francisco, CA, USA). Fluorescence microscope was purchased from Olympus Corporation, Tokyo, Japan.

Detection of Effects of Paclitaxel, rAd-P53, and Drug Combination on the Proliferation of HeLa cells by MTT Method

Cell concentration of HeLa in logarithmic growth phase was adjusted to 5×10^4/mL, cultured in a 96-well plate with 200 μL per well. After culture for 24 h, the supernatant was discarded. Add 200-μL medium containing paclitaxel and rAd-p53, respectively. For combination group, add paclitaxel before adding the rAd-p53, and cultured for 24, 48, 72 h. The control group was added only with medium and without drugs.

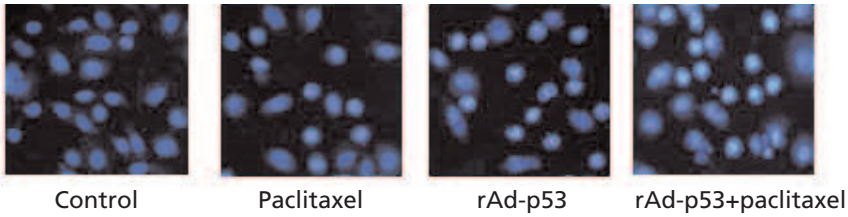

Figure 1. Effect of paclitaxel and rAd-p53 alone or in combination on the apoptosis of HeLa cells.

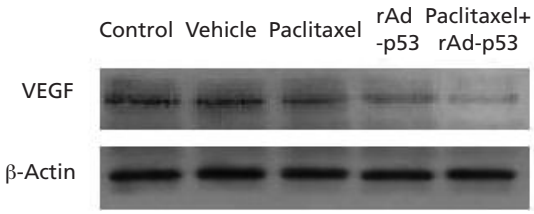

Figure 2. Effect of paclitaxel and rAd-p53 alone or in combination on the expression of VEGF in HeLa cells. $^{*}p < 0.05$ vs. control or vehicle groups; $^{\#}p < 0.05$ vs. paclitaxel or rAd-p53 groups.

Each group had four repeats. Add to each well 20 μL of fresh medium with 0.5 mg/mL MTT 4 h before termination and continue incubation for 4 h. Add 200 μL Dimethyl Sulfoxide (DMSO) to each well. We took values for each well using 492 nm optical density (OD). The experiment was repeated for three times. Cell inhibition rate for each group was calculated according to the following formula: Cell inhibition rate (%) = (1 − OD of drug group/OD of control group) × 100%. Coefficient of drug interaction (CDI) was used to evaluate properties of two drug interactions. CDI = $AB/(A \times B)$. *AB* is the ratio of OD value of the two drugs combination group and control group. *A* or *B* is the ratio of OD value of the single drug group and control group. CDI < 1 means that the two medicine had synergistic effect. If CDI = 1, effect of two drugs effect was additive. When CDI > 1, the two drug effect had antagonistic properties.

Detection of Effects of Paclitaxel, rAd-p53, and Drug Combination on the Proliferation of HeLa cells by DAPI Staining

HeLa cells were cultured in 24-well plate and were added with Paclitaxel (3 g/mL), rAd-p53 (5 × $l0^7$ vp/mL), and the combination of two drugs, respectively, when the confluence is up to 50%. The control group was not added any drugs. After culture for 48 h, Hela was DAPI stained for detecting the apoptosis. The protocol was that the HeLa cells were washed three times with PBS, and then fixed with 4% paraformaldehyde (dissolved in 0.1 mmol/L PB, pH 7.4) for 15 min at room temperature. Wash again with PBS for three times. Add 10 μg/mL DAPI. Incubated for 30 min at 37°C. The cells were observed under a fluorescence microscope. Determination method for apoptotic cells is irregular margin of the nucleus, nuclear chromosome concentration, heavy coloring, and accompanied by chromatin condensation and increased nuclear fragmentation. The calculation of apoptosis rate: randomly select 10 fields in each well under the vision of 400 times, and calculate the percentage of apoptotic cell number to total cell number.

Detection of Effects of Paclitaxel, rAd-p53, and Drug Combination on the Expression of VEGF of HeLa Cells by Western Blotting

HeLa cell in the logarithmic growth phase was cultured, digested, centrifugated, and adjusted to the concentration of 1 × 10^6/mL. Cells were added with paclitaxel (final concentration 6 g/mL), rAd-p53 (final concentration of 5 × 10^8vp/mL), both rAd-p53 and paclitaxel in combination group 24 h after culturing in 4-mL flask. The control group was without drugs. Cultured for another 48 h and then collect lysed cells. The lysates were measured for protein concentrations. Equal amounts of protein samples were added for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose (NC) membrane using semi-dry transfer method. NC membrane was blocked by 3% bovine serum albumin (BSA). Incubate with primary antibody (anti-VEGF antibody, 1:1000) overnight at 4°C. Wash nitrocellulose with three 5-min washes. Incubate with Goat antirabbit (IgG-AP, 1:2000) of about 10 mL 3 h at 37°C. Wash nitrocellulose with three 5-min washes. Show the colored liquid in the freshly prepared AP using the NBT/BCIP kit. Water washing terminates reaction. The staining result was analyzed by image analysis software. β-Actin was used as internal parameters in order to guarantee the accuracy of experimental results.

Statistical Analysis

The data were analyzed by SPSS 18 statistical software (SPSS Inc., Chicago, IL, USA). Data of each group was shown as, one-way ANOVA. $p < 0.05$ or $p < 0.01$ means it has statistically significant difference.

Results

Drug Combination of rAd-p53 and Paclitaxel Significantly Inhibited the Proliferation of HeLa cells

MTT test results (Table I) shows that drug use of different concentration of paclitaxel, rAd-p53, rAd-p53 in combination with paclitaxel could all inhibit

the growth of HeLa cells after 24, 48, 72 h. The drug effect is time-dependent and dose-dependent pattern. HeLa cell proliferation inhibition rate under different concentrations of rAd-p53 combined with paclitaxel in the 24, 48, 72 h was significantly higher than that of single use of paclitaxel or rAd-p53. CDI < 1 calculating of the combined effect of different concentration of rAd-p53 and paclitaxel, so the two have a synergistic effect.

Drug Combination of rAd-p53 and Paclitaxel Induces Apoptosis of HeLa cells

The experimental results (Figure 1) show that only a few apoptotic cells are shown in the control group. However, after treatment of paclitaxel (3 g/mL), rAd-p53 ($5 \times l0^7$vp/mL) alone or combination to HeLa cells, cell chromatin agglutination, fluorescence-staining enhancement, condensed lumpy or granular fluorescence of the chromatin and the formation of apoptotic bodies are all shown in cells. The statistical analysis showed: HeLa apoptosis rate was (11 + 0.01) % in control group. Apoptosis rate was (36 + 0.04) % in paclitaxel group. Cell apoptosis rate was (62 + 0.05) % in rAd-p53 group rAd-p53. Apoptosis rate was (83 + 0.07) % in group of rAd-p53 combined with paclitaxel. Thus, cell apoptosis rate increased in group of combination of rAd-p53 and paclitaxel compared with the single drug group ($p < 0.05$). This study also detected other dosage and time, finding that a lower dose of paclitaxel and shorter delivery time have little effect on promoting apoptosis of HeLa cells. While HeLa cell death is enormous when it was administered with higher dosage and longer time, in which condition it cannot do DAPI detection (Data not shown).

Drug Combination of rAd-p53 and Paclitaxel Reduced Significantly the Expression of VEGF in HeLa cells

When Hela cell was treated with paclitaxel (6 g/mL), rAd-p53 (5×10^8 vp/mL), and the combination of two drugs, respectively, it was shown in Western blotting (Figure 2) that the level of VEGF expression in HeLa cells decreased (45 + 0.07) % in paclitaxel group compared with control group, and the expression level of VEGF was lower (60 + 0.06) % in rAd-p53 in group than that in the control group. The expression level of VEGF decreased (81 + 0.08) % in the combination therapy group than the control group ($p < 0.05$). The experiments were also carried out HeLa cells treatment with paclitaxel (1.5 g/mL), rAd-p53 (5×10^6 vp/mL), and combined administration, and paclitaxel 3 g/mL, rAd-p53 of 5×10^7vp/mL and the co-administration. But the decreased expression of VEGF is lower which is less than paclitaxel 6 g/mL, rAd-p53 5×10^8vp/mL monotherapy group and two drug combination group (Data not shown).

Discussion

Zhang et al's study[9] showed that the mutation in the *p53* gene is related not only with cervical cancer morbidity but also with the cervical cancer cells sensitivity to chemotherapeutic. Some research[5,10,11] has shown that the combined application of recombinant p53 and radiation therapy or phototherapy in cervical carcinoma can improve the treatment effect of radiotherapy and phototherapy. Recombinant human adenovirus p53 combined with chemotherapy also showed stronger tumor suppression effects in the treatment of various tumors than chemotherapy treatment alone[12-15]. Recombinant human adenovirus p53 product rAd-p53 is with adenovirus as the carrier to transfect of *p53* gene into tumor cells, which will induce the exogenous *p53* gene highly expressed in cells. It will not only fulfill the G2/M phase block of the cell cycle, but also promote apoptosis of tumor cells and improve the sensitivity of various tumor cells to chemotherapy and radiotherapy through the activation of *Bax* gene, inhibition of *Bcl-x* gene, and expression of apoptosis genes *Puma, Bak*, and *Fas*[16,17].

Paclitaxel is a kind of antitumor drug extracted from the Pacific Taxus mountain mahogany. Polymerization and depolymerization of microtubules is essential for chromosome segregation in cell mitosis. Paclitaxel binding to microtubules β position specifically hinders the microtubule motor causing the cells fail to mitotic and apoptosis[18]. Paclitaxel has antitumor activity on human ovarian cancer, cervical cancer, and other malignant tumors. But as other chemotherapy drugs, paclitaxel is also faced with the problem of drug resistance in tumor cells. It needs to maintain the stability of p53 in tumor cells to reduce the paclitaxel resistance. The mechanism may be related to paclitaxel's function of causing cells cycle arrest in the G1 phase, mediating cell apoptosis selectively[8]. Wild type *p53* gene promotes the cell cycle arrest and apoptosis induced by the chemotherapeutic, while *p53* gene mutation will lose this function which is one of the important causes of tumor cells to chemotherapy

resistance. The study found that drug combination of rAd-p53 and paclitaxel will induce higher HeLa cell growth inhibition rate and apoptosis rate compared with the single drug group ($p < 0.05$) by MTT method and DAPI staining assay. Calculation of the combined effect of different concentration of rAd-p53 and paclitaxel CDI display that CDI < 1, indicating that rAd-p53 and paclitaxel have synergistic effect. The mechanism should be that paclitaxel and rAd-p53 effect on cell proliferation cycle through different target, causing cell proliferation arrest and apoptosis to a greater extent which means multiple target effect is better than the single target. And the *p53* gene was transfected into HeLa cells by adenovirus vector to maintain the stability of p53 in cells, to reduce the resistance to paclitaxel and to enhance the role of chemotherapy drugs.

Tumor angiogenesis is one of the key steps in the process of tumor development. Lots of research have been confirmed[19] that VEGF is most critical factors stimulating tumor angiogenesis growth. Expression of VEGF in cervical cancer tissues and cancer adjacent tissues was significantly higher than that in normal tissues[20], and also higher than that of chronic cervicitis and cervical intraepithelial neoplasia tissue[21]. Another study confirmed[22] that VEGF-C antigen positive expression rate decreased in cervical cancer after receiving neoadjuvant chemotherapy (NACT). In this study, we conclude that the expression of rAd-p53 or paclitaxel can inhibit VEGF expression in HeLa cells, and the inhibitory effect on the expression of VEGF in HeLa cells is more evident in rAd-p53 in combination with paclitaxel group than that of single drug group. VEGF expression was inhibited in combination group, which means that the tumor vascular growth was significantly inhibited, so as to the proliferation of HeLa cells was inhibited, and apoptosis is more present. It suggests that rAd-p53 may down regulate expression of VEGF in HeLa cells, inhibiting tumor angiogenesis and cervical carcinoma HeLa cell proliferation, promoting apoptosis of HeLa cells, and enhance the effect of taxol at the same time.

Conclusions

The results of the study provide a reliable experimental basis for the clinical application of combined gene therapy and chemotherapy for cervical cancer. A reasonable combination plays a synergistic role in improving the efficacy of drugs through multiple channels and links. At the same time, it can decrease the drug concentration, and reduce adverse reaction delaying the emergence of drug resistance.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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