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Original Article

Regulation of exogenous P53 combined with Dickkopf-1 on human osteosarcoma MG-63 cells

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Abstract: Objective: To regulate the growth of human osteosarcoma cell strain MG-63 *in vitro* through the combined drugs of exogenous P53 gene and Dickkopf-1 and to explore its possible mechanisms in order to provide the reference for clinical treatment of osteosarcoma. Methods: Human osteosarcoma MG-63 cell strain was subcultured *in vitro*, and exogenous P53 and Dickkopf-1 were used separately or jointly to act on MG-63 cells. The proliferation and apoptosis of MG-63 cells were respectively detected by trypan blue exclusion and flow cytometry; the expression levels of tumor suppressor gene P53 and oncogene c-fos and c-myc were detected by real-time fluorescent quantitative and western blot in mRNA and protein levels. Results: The proliferation test results showed that the combination of exogenous P53 and Dickkopf-1 could significantly inhibit the growth of MG-63 cells, compared with the single drug using ($P<0.05$), and cell cycle assay found that the combined drugs caused the cell cycle arrest at G0/G1 phase ($P<0.05$). Flow cytometry assay indicated the apoptosis of MG-63 cells caused by the combined drugs was significantly higher than that of using exogenous P53 or Dickkopf-1 alone, and the results of western blot manifested the combined drugs could down-regulate the expression levels of c-fos and c-myc whichwere related to the proliferation and differentiation of MG-63 cells, and increase the expression of tumor suppressor gene P53. Conclusion: The exogenous P53 gene transfer combined with Dickkopf-1 may induce cell apoptosis and inhibit cell proliferation by inhibiting the expression levels of c-fos and c-myc and promoting the expression of tumor suppressor gene P53. Thus, it can play an inhibitory role in osteosarcoma *in vitro*. The combined drugs are expected to become a new treatment method for osteosarcoma.

Keywords: Human osteosarcoma cell strain MG-63, P53, Dickkopf-1, apoptosis, P53

Introduction

Osteosarcoma is a malignant bone tumor which has a high incidence in children and adolescents. According to statistics, the annual incidence rate is about 0.00003% and osteosarcoma accounts for 4% of primary malignant tumors among children and adolescents in China [1, 2]. Before 1970, osteosarcoma was generally treated by amputation, but the incidence of lung metastasis was relatively high and the survival rate was extremely low [3]. Until 1993, G Rosen used a large dose of methotrexate to treat osteosarcoma, which started with the medication treatment for osteosarcoma [4]. However, the chemical drugs in conventional treatment all have the characteristics of strong cytotoxicity and severe side effects in liver and kidney, which seriously affects the survival and quality of life of patients with osteosarcoma. Therefore, the research on the regulation of drugs on proliferation of osteosarcoma cells can help to find an effective combination therapy with low side effects, which is of great significance for the treatment of human bone tumor diseases.

P53 is an important tumor suppressor gene in cells which was found in 1979, with a very short protein half-life period [5-7]. When the human body encounters external stimuli, P53 protein has the functions of maintaining genome stability, organizing cells into proliferation cycle, promoting the apoptosis of cells and inhibiting tumor growth [5, 8]. Gendicine, exogenous P53, a cancer gene therapy drug which is the world's first approved gene therapy in the clinical treatment, is recombined by tumor suppressor gene P53 in normal human bodies and adenovirus type 5. The adenoviral-mediated P53 gene is

transferred into tumor cells to express P53 protein to produce anti-tumor effects [8].

Currently, Dickkopf-1 (DKK1) is the most frequently studied DKK family member. Studies have shown that DKK1 plays an important role in inducing tumor cells apoptosis and inhibiting tumor cell growth and proliferation [9, 10]. Chamorro et al found that DKK1 could regulate the growth of endometrioid adenocarcinoma cells through Wnt signaling pathway [11]. Patil et al also found DKK1 had high expression in hepatocellular carcinoma through DNA microarray [12]. However, there are no related reports about the combined drugs of P53 and DKK1 now.

In this study, we investigate the regulatory effects of combined use of exogenous P53 and Dickkopf-1 on human osteosarcoma cell strain MG-63, so as to provide other feasible therapeutic methods for the treatment of osteosarcoma.

Materials and methods

Materials

Experimental cells: Human osteosarcoma cell strain MG-63 was purchased from Shanghai Institutes for Biological Sciences Cell Resource Center (ATCC number: CRL-1427).

Main reagents: We purchased $1*10^{12}$ VP/mL of Exogenous P53 (Gendicine) from Shenzhen Saibainuo Company and 4 μg/mL of DKK1 from AmyJet Scientific Inc (PRO-1566). Dulbeco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from American Gibco Company. Trypan blue was from Beyotime. Penicillin and streptomycin antibiotics and dimethyl sulfoxide were purchased from American Solarbio Company. Exogenous P53 and DKK1 were supplied by Shanghai Biolight Technology Company. Primary antibodies including β-actin, c-fos, c-myc, and P53 and secondary antibody including goat anti-rabbit antibody which contains horseradish peroxidase (HRP) were bought from American Abcam Company.

Methods

Cell thawing and culture: The human osteosarcoma cell strain MG-63 was put in a 37°C constant temperature water bath and was thawed. After that, it was added into DMEM medium (complete medium) containing 10% FBS, double antibiotics (containing 100 U/mL penicillin and 100 U/mL streptomycin) and they were fully mixed. After the strain centrifuged for 5 minutes at the speed of 1000 rpm and supernatant removed, it was added in complete medium, fully mixed, put into a 25 cm^2 sterile culture bottle with the concentration of $1*10^6$/mL, and incubated in a 37°C incubator containing 5% CO_2. The cells at logarithmic phase were used for experiments.

Growth curve determination: Cells were seeded in 25 mL culture flasks with the density of $5*10^4$/mL and placed in a 37°C incubator containing 5% CO_2 for 24 h. The control group was replaced with fresh complete medium, and the experimental group was replaced with the culture medium containing P53 alone, Dickkopf-1 alone and the combined drugs. While the cells were cultured for 1-7 days, three bottles were taken from each group every day, trypan blue exclusion was used to count viable cells and cell growth curve was drawn.

Cell cycle determination: When cells grew adhering to the wall until 80% fusion, trypsin was used to digest them, and the cells were collected after centrifugation for 5 minutes at the speed of 1000 rpm. Pre-cold PBS was used to wash the cells twice, which were then incubated in 100 μg/mL RNase for 20-30 min at 37°C, and then immediately put in an ice bath. Meanwhile, 0.5% v/v propidium iodide (PI) was added in the tube to stain in the dark surroundings for 30 minutes. Then flow cytometry was used to take a cell cycle dynamic detection and analyze the phase distribution of cell cycle (Flowjo Software).

Flow cytometry: Annexin V and 7 aminoactinomycin D (7-ADD) reagent kits (BD-Pharmingen 559763) were used to take a flow cytometer detection. We washed the cultured cells twice with cold PBS. A total of 100 μL to 1.5 mL of 1× Binding Buffer resuspended cells (10^6/mL) were put in an EP tube, and 5 μL of Annexin V and 5 μL of 7-AAD were added into it. After a light vortex, the cells were incubated in the dark surroundings for 15 minutes at room temperature. Then 400 μL of binding buffer was added, and a flow cytometer detection was performed (BD Accuri C6).

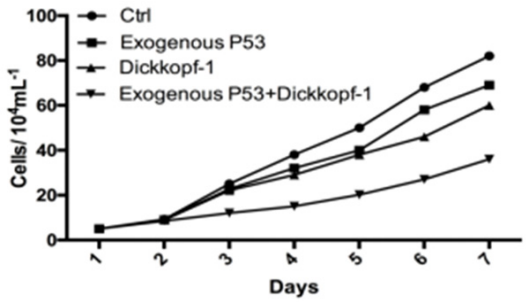

Figure 1. MG-63 cells growth curve after treated with exogenous P53 and Dickkopf-1.

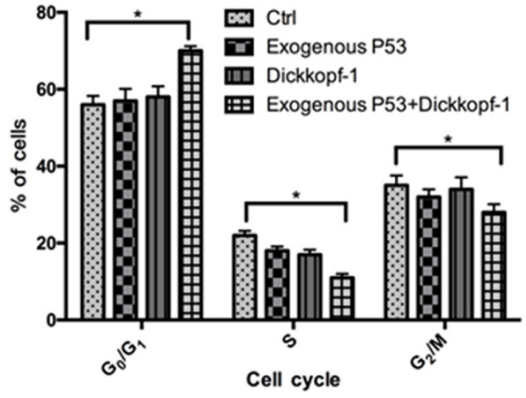

Figure 2. Effects of exogenous P53 and Dickkopf-1 on the cell cycle of MG-63 cells, *P<0.05.

Western blot: MG-63 cells were spread in a 6-well plate with the density of $1*10^6$/mL and cultured for 16 hours. Each was given exogenous P53 protein alone, DKK1 alone or the combined drugs, with the complete medium without drugs as a control, and the cells were collected after cultured for 24 hours. A total of 80 μL of radio immunoprecipitation assay (RIPA) protein lysis buffer was added in each well to extract total protein, and 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate proteins, which were transferred to a polyvinylidene fluoride (PDVF) membrane, then they were incubated with primary antibodies like c-fos, c-myc, P53 (the diluted concentration was 1:1000) and internal reference β-actin (the diluted concentration was 1:600) at 4°C overnight, and washed with Tirs-buffered Satine with Tween 20 (TBST) for three times and 15 minutes each time. The proteins and secondary antibody (the diluted concentration was 1:3000) were incubated for 90 minutes at room temperature. We used enhanced chemiluminescence to expose and develop the image after membrane rinsing with TBST, took photos and recorded (Image Lab).

Statistical processing: Graphpad Prism 6 software was applied to analyze the data. The measurement data were expressed as $\bar{x}\pm S$. Comparison between two groups was compared by t test (ANOVA). P<0.05 was considered as statistically significant.

Results

Inhibition effects of exogenous P53 combined with DKK1 on MG-63 cells growth

The growth curve obtained by trypan blue exclusion showed the rapid proliferation of human osteosarcoma cell strain MG-63. After seven days, the number of inoculated cells in the control group increased from $5.0*10^4$/mL to $82.0*10^4$/mL, which increased by nearly 16 times, and the doubling time was 48 h. However, the growth rate of MG-63 cells was inhibited when they were treated with exogenous P53 or Dickkopf-1, the number of cells increased from $5.0*10^4$/mL to $69*10^4$/mL and $60*10^4$/mL respectively, and the number of cells was still more than 10 times than the original number. But the growth rate of MG-63 cells was significantly inhibited by the combined use of exogenous P53 and DKK1. The cell number was $36*10^4$/mL, and the inhibition ratio was 43.9% at the seventh day (**Figure 1**).

Effects of exogenous P53 and DKK1 on the cell cycle of MG-63 cells

Cell cycle determination results showed that after the combined treatment of exogenous P53 and DKK1, cell cycle distribution of the human osteosarcoma cell strain MG-63 appeared obvious changes, G0/G1 phase of the control group accounted for 49.6%, S phase accounted for 19.5%, and G2/M phase accounted for 30.9%. After the combined use, G0/G1 phase accounted for 64.2% which indicated a significant block compared with the control group (P<0.05), S phase accounted for 10.1%, and G2/M phase accounted for 25.7% which showed a decrease compared with the control group. However, compared with the control group, there was no obvious difference after the use of exogenous P53 or DKK1 alone (**Figure 2**).

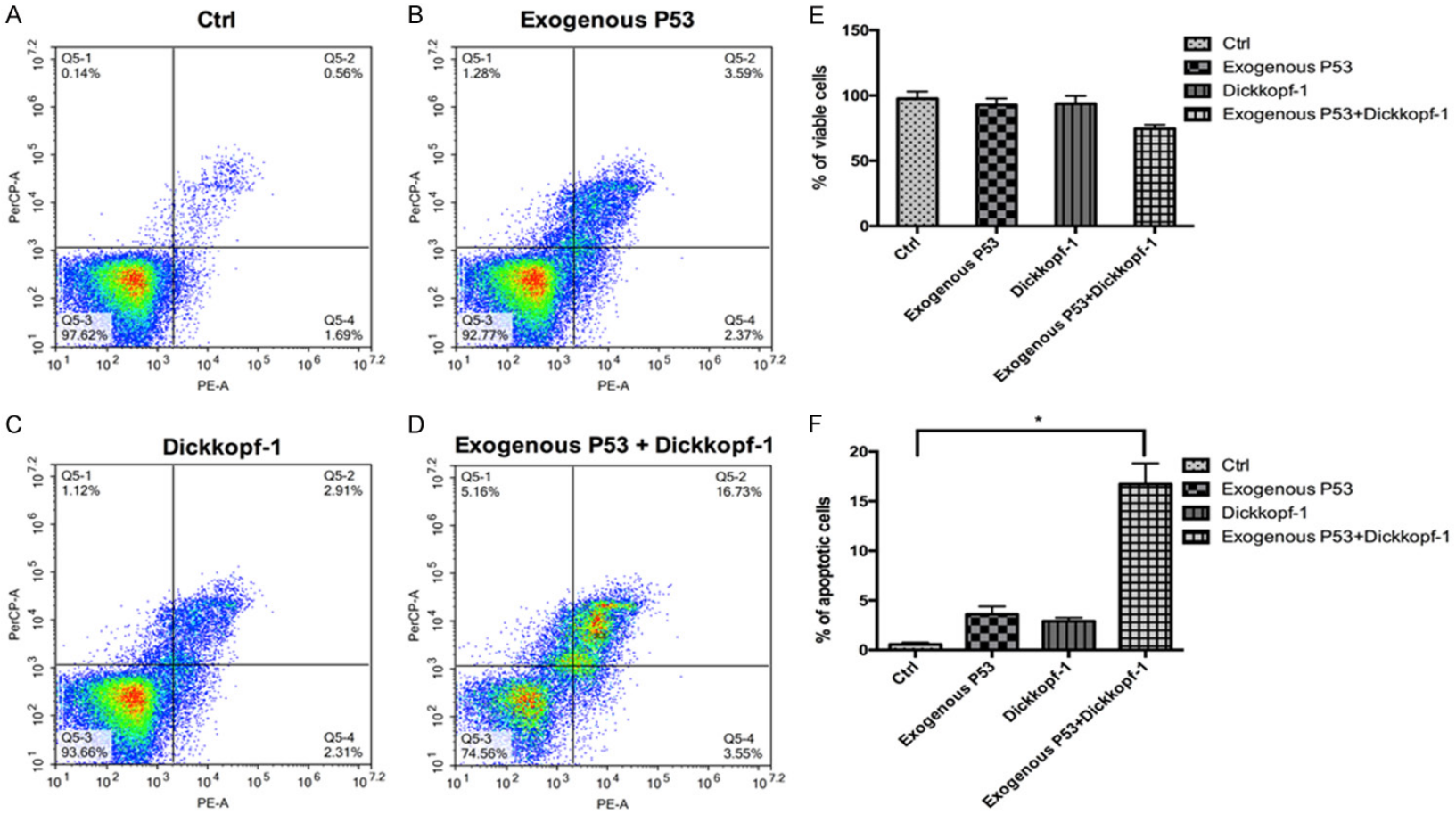

Figure 3. Effects of exogenous P53 and Dickkopf-1 on the apoptosis of MG-63 cells. A-D: Apoptosis flow patterns in the four groups (PE-A: Annexin V; PerCP-A: 7AAD); E: Annexin V(-)7AAD(-) living cell ratio in each flow pattern; F: Annexin V(+)7AAD(+) apoptotic cell ratio in each flow pattern.

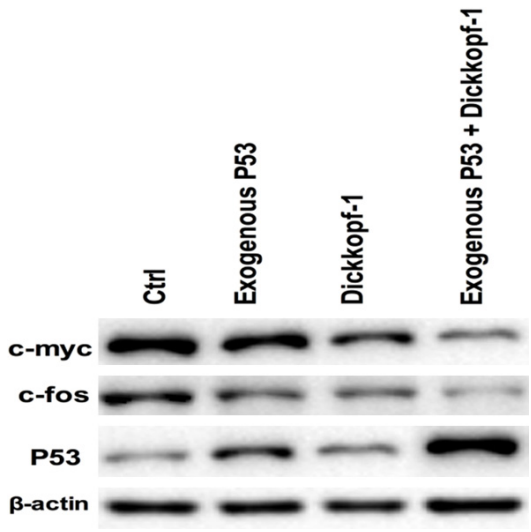

Figure 4. Effects of exogenous P53 and Dickkopf-1 on the gene expression of c-myc, c-fos, P53 in MG-63 cells.

Regulatory effects of exogenous P53, DKK1 or the combined drugs on apoptosis of MG-63 cells

The results of flow cytometer detection showed that the percentage of apoptotic cells was 16.73% in the human osteosarcoma cell strain MG-63 treated with exogenous P53 and DKK1, which was significantly higher than that of the control group, 0.56% ($P<0.05$). Compared with the control group, apoptotic cells increased slightly after the treatment of exogenous P53 or DKK1 alone (**Figure 3**).

Effects of exogenous P53 and DKK1 on the expression of oncogenes and tumor suppressor gene associated with proliferation and differentiation

Western blot detection results showed that the combined use of exogenous P53 and DKK1 could significantly inhibit the protein expression of the cancer suppressor genes c-myc and c-fos, promote the protein expression of cancer suppressor gene P53, and these effects on protein also existed after the treatment of P53 or DKK1 alone, but, which was less strong than those after the treatment of the combined drugs (**Figure 4**).

Discussion

Osteosarcoma is a kind of malignant tumor originated from mesenchymal cells, which is common in adolescents and children with the characteristic of early metastasis, bad prognosis and high morality. Research found that cell proliferation and apoptosis were two important factors that could not be ignored in the occurrence and development of tumor, and continuous proliferation and division were important biological characteristics which differed tumor cells from normal cells [13-15]. Therefore, we explored the inhibitory effects of the exogenous P53 and DKK1 on the proliferation of human osteosarcoma cell strain MG-63 cells which can be considered as an effective means to find anti-tumor drugs.

In this study, the cell growth curve showed that the growth of MG-63 cells increased by 16 times after 7 days. After the treatment of exogenous P53 combined with DKK1, the growth rate of MG-63 cells was significantly inhibited, and the inhibition rate was 43.9% in the seventh days (**Figure 1**), indicating the drug combination therapy can significantly inhibit the proliferation of osteosarcoma cell strain MG-63, and the difference was statistically significant. Through PI staining and flow cytometer detection, we found that combined use of exogenous P53 and DKK1 significantly blocked MG-63 cells in G0/G1 phase (**Figure 2**, $P<0.05$), and it was consistent with what HanKi Park et al reported that green tea polyphenols could cause contact inhibition of osteosarcoma cells and the increase of cell distribution in G0/G1 phase [16]. We also found that the percentage of apoptosis of MG-63 cells treated by exogenous P53 combined with DKK1 (16.73%) was significantly increased (**Figure 3**, $P<0.05$), compared with the control group (0.56%), which further proved that the combined use of exogenous P53 and DKK1 could inhibit the growth and proliferation of osteosarcoma cells.

With the process of molecular biology and molecular technology, the research on the disease develops gradually from cells to molecular mechanisms. Different cancer related genes exist in normal cells of human body, and they are divided into proto-oncogenes and tumor suppressor genes, which maintain dynamic balance to regulate cell growth and differentiation, and henogenesis. The proto-oncogene c-myc was found firstly in the v-myc gene separated from chicken virus, and it is a kind of nuclear transcription factor, proto-oncogene, which can promote cell proliferation and inhibit cell differ-

entiation, and play an important role in the regulation of cell proliferation, differentiation and apoptosis, meanwhile, there are c-myc overexpression phenomena in a variety of human cancer tissues [17]. C-fos, a kind of endonuclear proto-oncogene, is called transient gene for its quick expression to external stimulus in the cell. Related research showed c-fos played a key role in the occurrence and development of multiple tumors [18, 19]. But expression of c-myc and c-fos in osteosarcoma is rarely reported [20, 21], so this study explored the effects of the combined use of exogenous P53 and DKK1 on the protein expression of proto-oncogene. The results shows that the combined use of P53 and DKK1 could obviously inhibit the expression of cancer gene c-myc and c-fos protein, and increase the expression of tumor suppressor gene P53 protein, which suggests that exogenous P53 combined with DKK1 could regulate cell proliferation by affecting the expression of cancer related genes to strengthen its anti-tumor effects.

In summary, exogenous P53 combined with DKK1 has obvious inhibitory effects on the proliferation and differentiation of human osteosarcoma MG-63 cells, which might be closely related with the inhibition of c-myc and c-fos gene and the increasing expression of tumor suppressor gene P53. The specific regulation mechanisms required in-depth study.

Disclosure of conflict of interest

None.

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