

p73 Gene Overexpression Induces Apoptosis and Increases Chemosensitivity in Human Lung Adenocarcinoma Cells A549

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Citation

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Abstract

Objective: As some NSCLC harbor wild-type p53, it would be important to override the resistance mechanism due to wild-type p53 in NSCLC gene therapy. The p53 family member p73 has significant homology to p53 tumor suppressor. The aim of this work was to observe the degree of apoptosis and chemosensitivity of p53-resistant human lung adenocarcinoma A549 cells following wild-type p73 gene transfer alone or combined with chemotherapeutic agents.

Methods: The pcDNA3-HA-p53 or pcDNA3-HA-p73 plasmids were transferred into in vitro cultured human lung adenocarcinoma cell line A549 with Dospere. The cells resistant G418 were selected. The expressions of exogenous p53 or p73 gene were examined by Western blot. MTT assay were used to analyze the response of transfected cells to Cis-dichlorodiamine platinum (CDDP) or adriamycin (ADM). The drug-induced apoptosis of transfected cells was measured by flow cytometry assay, TUNEL technique and DNA fragmentation. The biological behavior change of cells was observed by colony formation assay.

Results: The transfected lung adenocarcinoma cells A549 could overexpress P53 or P73 protein stably. We found that p73, but not p53, is capable of sensitizing A549 cells to apoptosis induced by CDDP or ADM. Lower concentration of chemotherapeutic agents without inhibition essentially can suppress growth of p73 transfected cells markedly. p73 gene transfer combined with CDDP or ADM was shown to be even more effective in suppressing growth in A549 cell.

Conclusions: Exogenous p73 gene was capable of enhancing the sensitivity of wild-type p53 human lung adenocarcinoma cells A549 to chemotherapeutic agents. It suggests the possibility of using p73 gene to improve p53-resistant tumor treatment. The combined treatment with p73 gene and chemotherapy could be an attractive strategy for inhibiting progression of NSCLC through effective induction of apoptosis.

INTRODUCTION

Lung cancer has become the most frequent and most lethal cancer worldwide^[1]. Non-small cell lung cancer (NSCLC) histological types comprise about 80% of all cases. 40% of NSCLC patients are diagnosed in advanced stages and are candidates for systemic chemotherapy^[2]. However, most NSCLC are usually chemoresistant, it is important to develop methods of improvement of the clinical response to chemotherapy^[3]. Gene therapy for malignant diseases is one of several promising approaches.

The tumor suppressor gene p53, one of the most intensively

studied in human cancer biology. Many studies have indicated that p53 alteration is responsible for the differences in clinical characteristics of NSCLC. Recent studies suggest that wild-type p53 has been combined with various anti-cancer drugs to reverse the resistance and increase the sensitivity to chemotherapeutic agents^[4]. Combination of the p53 gene therapy and chemotherapy get better effect than either agent alone in clinical trial for patients with NSCLC^[5]. However, human cancer cells containing the wild-type p53 gene are generally resistant to p53 gene therapy both in vitro and in vivo models^[6,7,8]. Wild-type p53 protein could not induce apoptosis in all cases due to the resistance of the

tumors to exogenous p53 [9]. To overcome these restrictions, genes that promote apoptosis by p53-independent mechanisms are particularly useful. The recently identified p53 family member, p73, represents such a molecule [10]. Several studies have clearly shown that p73 can induce apoptosis in tumor cells that lack functional p53 [11,12]. However, it is not known whether it can enhance the effect of anti-cancer agents in cancer cells with wild-type p53.

In this study, the exogenous wild-type p53 or p73 gene were transferred into human NSCLC cells A549 with wild-type p53 gene, growth inhibition and apoptosis were observed following transfer of gene alone or in combination with chemotherapeutic agents. We evaluated whether p73 could enhance the chemosensitivity of p53-resistant NSCLC cell line to two common anti-cancer agents.

MATERIALS AND METHODS

REAGENTS

Dosper liposome transfection kit and TUNEL kit were purchased from the Roche Company. Western blot kit, polyclonal anti-HA antibody was bought from Santa Cruz. MTT was purchased from Huamei Company. G418 was Clontech Company's product. Plasmid maxi prep kit was purchased from Omega. Annexin V-FITC and PI were obtained from Becton Dickinson. CDDP and ADM were bought from the Qilu Company and Hisun Company respectively. The drugs were dissolved according to the manufacturer's instructions and diluted in culture medium before addition to the cell cultures.

PLASMID

The plasmids pcDNA3-HA-p53 and pcDNA3-HA-p73 were a kind gift from Prof. Gerry Melino and Dr. Vincenzo De Laurenzi in University of Rome 'Tor Vergata' (Rome, Italy). The plasmids were used to generate cell lines that inducible express p53 or p73. Both P53 and P73 protein were tagged at their N-termini with an influenza hemagglutinin (HA) peptide. Empty vector pcDNA3 was provided by Professor Dirk Gruendemann in University of Cologne (Cologne, Germany). Plasmids were amplified in coli. DH5, and the extraction, purification and identification of the plasmid followed the instruction of the kit and the routine.

CELL CULTURE, TRANSFECTION AND SCREENING

The human NSCLC cell lines A549 was donated by Doctor

Shi Tong-dong in the Immunological Academe of the Third Military Medical University (Chongqing, China). The A549 cells have wild-type p53 but do not express endogenous p73 protein. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, and 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were culture in a 5% CO₂ incubator at 37°C, and the medium was changed every 3 days. Cells were split as needed with 0.25% trypsin-0.02% EDTA. All of the transfections were performed in six-well plates with Dosper according to the manufacturer's instructions (DNA/Dosper ratio 1:3). The day before transfecting, Approx 1x10⁵ A549 cells were seeded on every well in 2 ml medium, at 70-80% confluence, we used Dosper reagent to perform transfection. 2 µg plasmid DNA and 6 µl Dosper reagent were incubated with cells for 6 hours. The cells were divided into 4 groups, including the non-transfection group (A549), the group transfected with pcDNA3 (A549/pcDNA3), the group transfected with pcDNA3-HA-p53 (A549/p53) and the group transfected with pcDNA3-HA-p73 (A549/p73). After the growth of the transfected cells for 48 hours, we split the cells in every well at the cell density of 1/3, and screened out the resistant clones with 1 000 µg/ml G418, non-transfected cells as the control. The screening was sustained by the G418 medium 400 µg/ml and the subculture of the cells was continued and amplified. Individual clones were screened for inducible expression of P53 or P73 protein by western blot analysis.

WESTERN BLOT ANALYSIS FOR P53 OR P73 EXPRESSION

Western blot analysis was performed using the ECL western blot detection system and protocol. Briefly, cells were scraped from the plates, wash with PBS, and were lysed with RIPA solution [1% NP-40, 0.5% sodium deoxycholate, 10% sodium dodecylsulfate (SDS), 3 µl/ml aprotinin and 5 µg/ml leupeptin in PBS, pH7.4]. The total protein was obtained by centrifuging at 10 000 rpm (10 600 g) at 4 °C; 10 µg/slot of proteins were boiled in a sample buffer for 1 min at 100 °C, electrophoresed in 10% SDS/polyacrylamide gel and then transferred to PVDF membranes. Nonspecific antibody bindings were blocked by preincubation of the membranes with 5% non-fat milk overnight. Then the membranes were incubated for 1 h with the polyclonal rabbit anti-human HA antibody (1:200), followed by further incubation 1 h with goat anti-rabbit IgG (1:2500). After each antibody incubation the membranes were washed three times by Tris-buffer saline. To visualize the protein bands, enhanced chemiluminescence western blot detection reagents were

used and the membranes were exposed to X-ray film.

MTT ASSAY

MTT assay is also used for the measurement of cytotoxicity of drug alone or in combination treatments. Exponentially growing cells of A549, A549/pcDNA3, A549/p53 and A549/p73 were harvested and resuspended to a final concentration of $(1\sim 2)\times 10^5$ cells/ml in fresh medium. Cell suspensions (200 μ l) were dispensed into wells of 96-well culture plates. After 24 h culture at 37 °C for cell adherence to the plate, the solutions of CDDP or ADM were simultaneously added to the medium, the finally concentrations were 0, 0.01, 0.05, 0.25, 1.25, 6.25 and 31.25 μ mol/l, respectively, and subsequently incubated 24 hours. Then 20 μ l MTT (5 mg/ml) was added to each well then incubated for 4 hours under the same condition. After incubation, culture medium in each well was discarded and replaced with 200 μ l DMSO. After 10 minutes' shaking, the absorbance of each well was determined by a spectrophotometer with a 490nm wavelength. The wells treated by the complete medium without chemotherapeutic drug served as a control. The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Chemotherapeutic drugs' IC₅₀ was determined as a chemotherapeutic drugs' concentration showing 50% cell growth inhibition as compared with control cell growth. The experiments were repeated in triplicate.

APOPTOSIS ASSAY

Cell apoptosis was monitored by three methods. First, cell apoptosis was measured by flow cytometry. exponentially growing cells of A549, A549/pcDNA3, A549/p53 and A549/p73 were divided into treatment group and non-treatment group, respectively. Each bottle was then incubated for 48 h with cDDP (final concentration 6.25 μ mol/L) or ADM (final concentration 0.25 μ mol/L). Then both floating dead cells in the medium and live cells on the wall of bottle were collected and washed twice with cold PBS. The cells were resuspended in 0.1 ml of PBS to a density of 1×10^6 /ml. Cells were stained with 5 μ l Annexin V-FITC and 10 μ l PI (50 mg/L), and incubated at room temperature for 30 min in the dark. Then 400 μ l staining buffer was added to bring the total volume to 500 μ l prior to FCM analysis. At least 10 000 cells of each sample were analyzed each time. Second, cell apoptosis can also be reflected by TUNEL analysis. In brief, after the same treatment schedule described above. Slides with cells were

then washed, and fixed for 30 min in 4% paraformaldehyde, incubated in 3% H₂O₂ in methanol for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 for 2 min. After incubation with reaction buffer containing terminal deoxyribonucleotidyl transferase according to the manufacturer's protocol (Roche Molecular Biochemicals), the slides were stained with ABC reagent. After this, the slides were incubated with DAB/H₂O₂ solutions and retained with hematoxylin after complete washing. Data shown are representative of three independent experiments. Third, the harvested cells were lysed in a solution containing 100 mmol/L NaCl, 10 mmol/L Tris, 25 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate. After the centrifugation, the supernatant was incubated with 300 μ g/ml proteinase K for 3 hours at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 volume of 3 mol/L sodium acetate, and the DNA was precipitated with 2.5 volumes of ethanol. After treatment with 100 μ g/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination.

CLONE FORMATION ASSAY

Cells were plated in log phase into six-well plates with 5 ml medium and grown at 37 °C and with 5% CO₂ silently. After 48 h culture for cell adherence to the plate, rinsed with fresh medium, and then CDDP (final concentration 6.25 μ mol/L) or ADM (final concentration 0.25 μ mol/L) were added to each well. After cells were incubated for 48 h with chemotherapeutic drug, Cells were then washed twice with prewarmed PBS, trypsinized, diluted and 500 cells per treatment were seeded into six-well plates in triplicate with chemotherapeutic drug-free medium. The medium was changed every 5 days. Colonies were allowed to grow for 10 \pm 14 days. The medium was discarded and each well was washed twice by PBS carefully. The cells were fixed about 10 minutes by 1:3 acetic-methanol, and then stained with Giemsa for 10 \pm 30 minutes. Finally, positive colony formation (more than 50 cells/colony) was counted. The survival fraction for each cell line was expressed as the ratio of plating efficiency of treated cells to that of untreated control cells.

STATISTICAL ANALYSIS

The data were analyzed with SPSS 10.0. The results were expressed as the mean \pm standard deviation $\bar{x}(_) \pm s$. Statistical significance was determined by t test. A P value of 0.05 or

less was considered statistically significant.

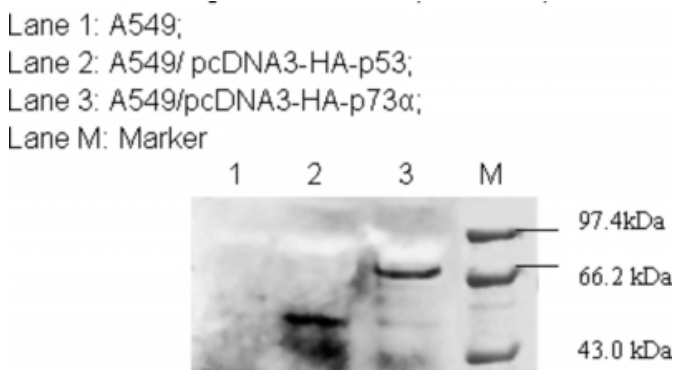
RESULTS

EXOGENOUS PROTEIN STABLE EXPRESSION ACHIEVED IN A549 CELLS

By tagging p73 and p53 with an HA epitope, the relative amount of the exogenous P73 and P53 proteins can be qualitative and quantified by Western blot analysis with rabbit anti-HA polyclonal antibody. The expression of HA was detected in cells transfected with pcDNA3-HA-p53 and pcDNA3-HA-p73 by western blot (as shown in Fig 1), demonstrating that the transfection of pcDNA3-HA-p53 and pcDNA3-HA-p73 were successful. The normal level of p73 is undetectable in A549 cells. However, a high level of expression of P73 protein was achieved in A549/p73 by western blot assay. Plasmid pcDNA3 was not tagged by HA epitope, but it could express neo gene. Therefore, we used G418 to screen A549 and A549/pcDNA3 cells. Screened by G418 medium 1 00 mg/L for 4 days, most of the A549 cells died, while A549/pcDNA3 cells grew normally, which proved that the transfection of pcDNA3 was successful.

Figure 1

Figure 1: Detection of exogenous P73-HA protein in parent and transferring cell line



MTT ASSAY

Similar viability of A549 and A549/pcDNA3 acted by the same drug concentration were observed, there was only slight growth inhibition in the cells transfected with pcDNA3-HA-p53. However, the viability of A549/p73 was significant lower than those of A549, A549/pcDNA3 and A549/p53. This suggested that p73 was more cytotoxic than pcDNA3 and p53 in A549 cells, consistent with a p73 specific effect. 6.25 μmol/L CDDP or 0.25 μmol/L ADM could only damage A549 or A549/pcDNA3 cells under 50%, a subtoxic concentration. By contrast, A549/p73 cells

responded to the subtoxic concentration chemotherapeutic drugs (6.25 μmol/L CDDP or 0.25 μmol/L ADM) sensitively, over 50% cells were killed (as shown in Fig 2). Furthermore, the concentrations of agents that caused 50% inhibition of growth (IC₅₀) was determined, p73 could cause a decrease in IC₅₀ of cDDP and ADM (as shown in Tab.1). The cell growth inhibitory effects of the combined treatment were synergistic and more significant compared with p73 or chemotherapeutic drug alone.

Figure 2

Figure 2: Alteration of cisplatin and adriamycin chemosensitivity caused by the transfer of p73 or p53 gene in A549 cell line

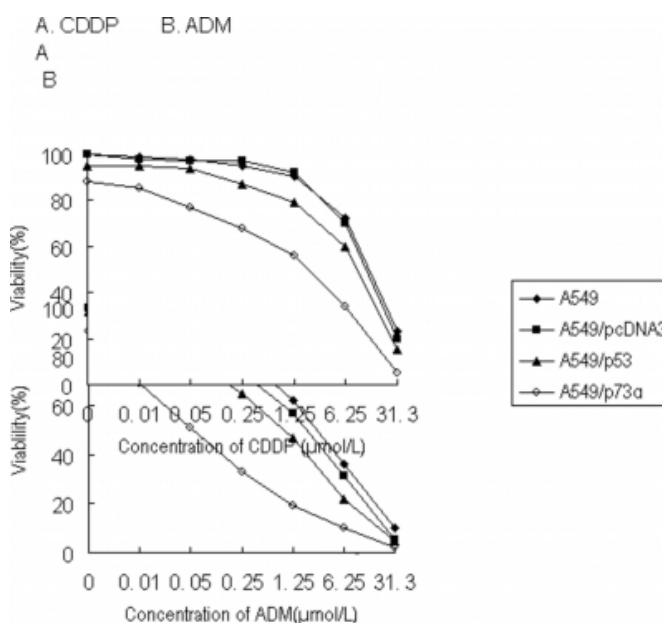


Figure 3

Table 1: IC₅₀ Values of Cisplatin and Adriamycin in A549 Cells before and after p73 Gene Transfection

	A549	A549/pcDNA3-HA-p73	D of IC ₅₀
cDDP(μmol/L)	22.650	3.750	0.166
ADM(μmol/L)	4.200	0.060	0.014

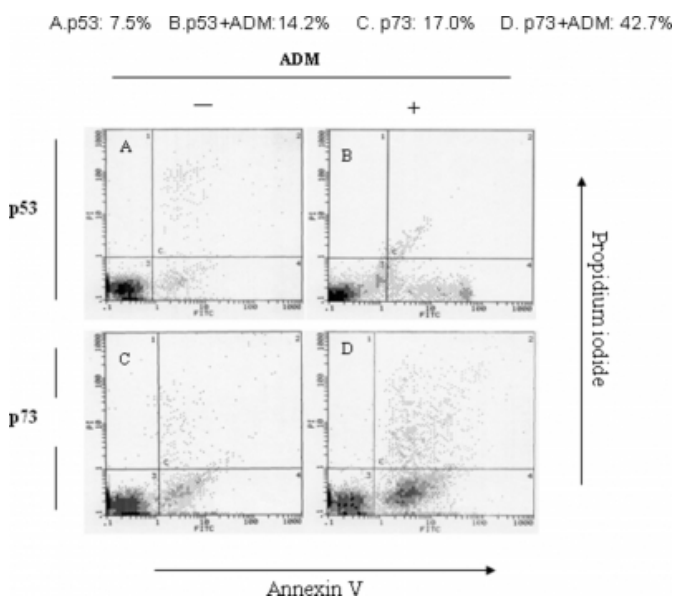
IC₅₀ values were obtained graphically by determining the dose that caused a 50% reduction in the control values. D of IC₅₀, combination index, D<1 indicates as synergistic effect, D=1 as additive effect, D>1 means antagonistic effect

APOPTOSIS ASSAY

Flow cytometry showed that much more apoptotic cells were observed in the group treated by the combination compared with those treated by p73 or chemotherapeutic drug alone (as shown in Fig 3).

Figure 4

Figure 3: Apoptosis was measured by flowcytometry analysis of Annexin V-FITC double-labeled cells



A significant difference ($P < 0.01$) was observed between p73 gene transfected or chemotherapy alone and a combination of the two approaches (as shown in Tab.2).

Figure 5

Table 2: Apoptosis Index by Flowcytometry Analysis of A549 Treated with Cisplatin or Adriamycin before and after Gene Transfection(\pm s)

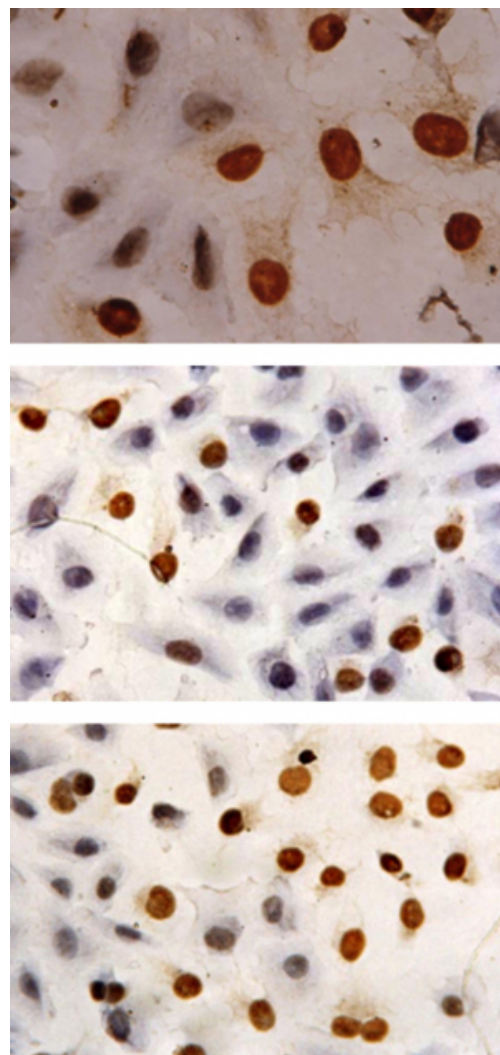
Cell	Apoptosis index		
	Control	cDDP(6.25 μ mol/L)	ADM(0.25 μ mol/L)
A549	4.5 \pm 1.1	10.6 \pm 2.7	13.0 \pm 3.5
A549/pcDNA3	5.3 \pm 1.4	11.4 \pm 3.6	13.7 \pm 2.9
A549/p53	7.0 \pm 2.5	14.3 \pm 4.1	17.2 \pm 2.6
A549/p73 α	15.5 \pm 2.0	36.8 \pm 2.7*	41.1 \pm 1.9*

* $p < 0.01$

Compared to A549 cells, the apoptosis rate increased 2.4 and 2.9 fold after treated by 6.25 μ mol/L CDDP or 0.25 μ mol/L ADM respectively. However, the apoptosis rate of the A549/p73 increased 8.2 and 9.1 fold after treated by 6.25 μ mol/L CDDP or 0.25 μ mol/L ADM respectively. Thus, there was significant apoptosis following p73 gene transfer, subsequent chemotherapy made the process even more effective. To examine this further, we performed TUNEL assay. The results are consistent with the data obtained by the Flow cytometry assay (as shown in Fig 4).

Figure 6

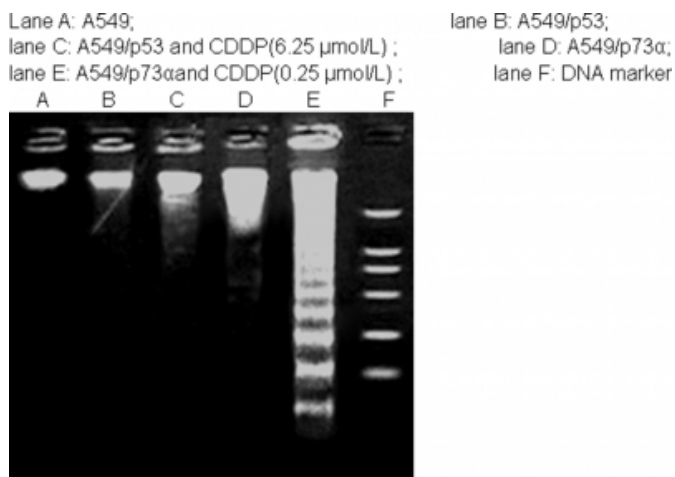
Figure 4: The CDDP(6.25 μ mol/L) induced apoptosis of A549 cells before and after p73 gene transfection was measured by TUNEL. The Apoptosis index was significantly higher after p73 gene transfection. A: Apoptotic cells are brown and dispersed(x400). B. before p73 gene transfection(x200). C. after p73 gene transfection(x200).



To confirm the finding that p73 gene could enhance the apoptotic response induced by chemotherapeutic drugs, DNAs were isolated from A549, A549/p73 or A549/p53 cells treated with or without chemotherapeutic drugs and electrophoresed for DNA ladder formation. Using the same treatment schedule described above, the characteristic apoptotic DNA ladder was observed only after combined treatment with p73 and chemotherapy, but not with either agent alone (as shown in Fig 5).

Figure 7

Figure 5: DNA fragmentation in parent and transferring cell line 2% agarose gel electrophoresis of DNA

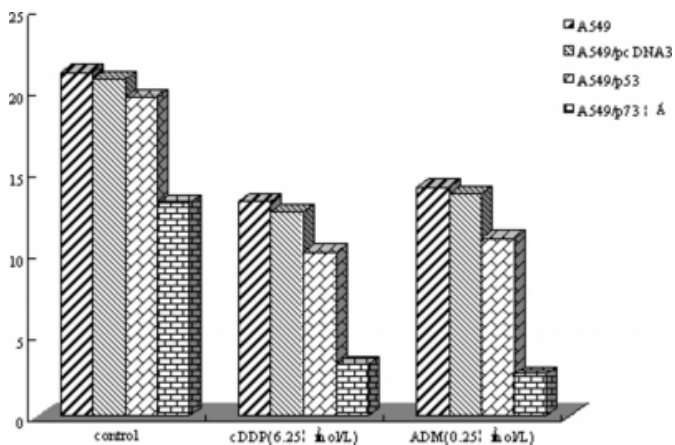


CLONE FORMATION ASSAY

We examined the effect of p73 expression on cell biological behavior change by colony formation assay. After incubated for 48 h with 6.25 μmol/L CDDP or 0.25 μmol/L ADM, transfection of p73 result in significant or dramatic decrease in the number of colonies compared with A549, A549/pcDNA3 or A549/p53 (as shown in Fig.6).

Figure 8

Figure 6: The colony formation ratio of H1299 treated with CDDP or ADM before and after gene transfection



Compared to their respective non-treatment cells, the clones of A549, A549/pcDNA3, A549/p53 and A549/p73 cells exposed to 6.25 μmol/L CDDP or 0.25 μmol/L ADM decreased 37.4%, 39.6%, 46.7%, 76.3% and 33.5%, 33.8%, 44.4%, 80.5%, respectively. p73 gene transfection significantly inhibited the cells' clonogenicity after the treatment with chemotherapeutic drugs. A statistically significant difference (P<0.01) was observed between p73

transfer or drug exposure alone and a combination of the two treatments. However, A549/p53 partially retained their capacity.

DISCUSSION

Despite the development of aggressive protocols utilizing chemotherapy, radiation therapy, and surgery, the overall survival of lung cancer has changed little, with less than 15% of patients surviving longer than five years [13]. Even though most NSCLC are usually chemoresistant, about 40% of NSCLC patients are candidates for systemic chemotherapy [14]. However, the efficacy of cancer chemotherapy is restricted by the ability of tumors to resist or develop resistance to treatment. Although the cellular resistance mechanisms involved in cancer chemoresistance are not yet clearly defined, study indicate that the blockade of the cellular apoptotic pathway may be a contributing factor [15,16,17,18]. It was reported that the resistibility of the tumor cells to apoptosis determined their chemosensitivity [19]. Therefore, transduction of proapoptotic genes that sensitize tumor cells to chemotherapeutic agents represents a promising strategy for cancer gene therapy. The tumor suppressor gene p53 plays a critical role in apoptosis induced by DNA-damage agents, including many chemotherapeutic agents [20]. Thus, Combination of the p53 gene therapy and chemotherapy get better effect than either agent alone in clinical trial for patients with NSCLC [21].

However, We know that about 30% NSCLC harbor wild-type p53 [22]. The tumors with wild-type p53 have defects at some point within the p53 apoptotic pathway [23]. It was reasonable to expect that the NSCLC with wild-type p53 are resistant to p53 replacement gene therapy. In fact, the resistance observed in cells harboring wild-type p53 can be substantially greater than that observed in tumor cells having mutant or null p53 status [24,25]. Therefore, to improve the efficacy of p53 gene therapy, it would be important to overcome the resistance to p53-mediated apoptosis. The p53 family members, p73 share significant sequence homology and a functional similarity with p53 [26]. So, The p73 gene might be one of the candidate genes to overcome this resistance mechanism of NSCLC with wild-type p53. Furthermore, it was shown that p73 is induced by CDDP and ADM, implying that p73 responds to at least a subset of DNA-damaging drugs [27,28]. CDDP and ADM have been the regular chemotherapeutic agents for their strong cell killing ability. But severe systemic toxicity limited their use in the clinical treatment of cancer. Reduction of their dose and

maintenance of their high efficacy will be necessary in the future treatment of tumors. More efforts have been made to explore the combination of chemodrugs with some other agents [29,30]. Therefore, it is anticipated that p73 may be used to sensitize p53-resistance tumor cells to DNA-damaging chemotherapeutic agents.

In the present studies, we transfected p73a and p53 gene into A549 cells successfully and got the cell lines that inducibly express exogenous p73a or p53 gene. Then to evaluated whether p73a can sensitize A549 cells to apoptosis by DNA-damage agents. The result demonstrated that p53 gene transfer to A549 cells did not result in significant levels of cell apoptosis and couldn't improve the chemosensitivity. However, p73 gene transfection significantly inhibited the growth of A549 cells, reduced IC₅₀ of CDDP and ADM by 6 and 70 times, respectively, and significantly inhibited the growth of A549 cells combined with Subtoxic concentration chemotherapeutic agents. P73 gene transfection elevated the apoptotic rate of A549 cells with the treatment of Subtoxic CDDP or ADM by 3.4 and 3.1 times, respectively. DNA Ladder appeared in the DNA electrophoresis of p73 gene transfection A549 cells and p73 gene transfection significantly inhibited the formation of the A549 cell clones. Our results suggest that p73a cooperates with DNA damage to induce apoptosis in A549 that carry an endogenous wild-type p53 gene. p73 gene transfer gene transfer efficiently kills human lung adenocarcinoma cells in vitro by apoptosis. Furthermore, we have demonstrated that the DNA-damage chemotherapeutic agents CDDP and ADM can be used to cooperate with p73-mediated apoptosis. To combination treatment the extent of cellular apoptosis compared to that achieved with p73 overexpression alone or CDDP or ADM treatment alone. Alternatively, it can be considered that p73 sensitizes human lung adenocarcinoma cells to the cytotoxic effects of CDDP and ADM. The findings reported here also indicate that manipulation of the molecular pathways that regulate apoptotic cell death by gene therapy strategies may have efficacy in restoring chemosensitivity to tumor cells that are resistant because of defects in apoptosis. Therefore, lower and less toxic doses of systemic therapy may be needed when p73 gene transfer is used and it might permit a reduction in the dose of chemotherapeutic agents and thereby minimize harmful side-effects.

In short, p73 gene transfer is a potential novel approach for the treatment of NSCLC, particularly for tumors that are resistant to wild-type p53 gene therapy. A combination of

p73 gene therapy and chemotherapy may be an effective strategy for human cancer treatment.

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