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Research Paper

A study of the expression of p53 in posttransfection cells with rAdp53 gene and inhibitory activity *in vitro*

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Abstract

Objective: To investigate the inhibitory effect and IC_{50} (50% inhibiting concentration) of the recombinant adenoviral p53 gene (rAdp53) in colorectal cancer cells *in vitro* and to guide clinical practice. **Methods**: We evaluated the efficiency (IC_{50}) of the rAdp53 and six kinds of anti-cancer drugs (5-fluorouracil, tegafur, mitomycin c, cisplatin, oxaliplatin, paclitaxel) in human colorectal cancer cell line-174 through the cell culture and MTT chemosensitivity assay to make sure the anti-cancer capability of rAdp53. Expression of p53 protein in transfection cells of colorectal cancer line-174 with rAdp53 was evaluated by immunohistochemical staining. **Results**: The rAdp53 is a dose- and time-dependent anti-cancer drug, its IC_{50} is 5.73×10^{11} VP/ml, but its effect was not obvious when compared with other anti-cancer drugs. In control group, the immunohistochemistry stain was negative. However, rAd-p53 of five different concentrations were all positive in infected colorectal cancer cells with rAd-p53 and the earliest positive result would present 24 hours after infection. **Conclusion**: The rAdp53 has good anti-cancer efficacy is colorectal cancer cell line-174 *in vitro*. But its anti-cancer efficacy was less than those of the classical chemical medicine mitomycin c, 5-fluorouracil and cisplatin etc., when it was used alone.

Keywords: rAdp53; chemosensitivity; gene transfection; immunohistochemistry stain

INTRODUCTION

Presently, most researches have been revolved around p53 tumor suppressor gene in gene therapy field[1,2]. Alterations of p53, including mutation and / or dysfunction are common genetic events in cancers, especially with a high occurrence of 75% in colon cancers[3]. In 2004, China took the lead to apply rAdp53 injection, the first gene therapeutic medicine in the world for head and neck cancers. The purpose of our research was to compare the inhibitory efects of rAdp53 injection with those of other six kinds of chemical therapeutic medicines for the colorectal cancer cell line-174 *in vitro* and understand the expression of p53 protein in transfection cells.

MATERIALS AND METHODS

Cell line preparation

Colorectal cancer cell line-174 was obtained from Professor Wang Yili (Xi'an Jiaotong University). It was grown in complete minimal essential medium with 10% heat-inactivation fetal bovine serum, and its most favorable inoculation density was $1.5\text{-}5.0\times10^4$ cells/mL, logarithm growth phase took place 3-5 days after inoculation[4].

Inhibitory effect of rAdp53 injection and other six kinds of chemical therapeutic medicines in colorectal cancer cell line-174 *in vitro* evaluation (MTT chemosensitivity assay)

The colorectal cancer cells were plated at a density of 1.0×10^4 cells/well in 96-well plates in triplicate, and then maintained in the incubator at 37°C. Forty-eight hours later, 100 μL rAdp53 injection with different concentrations (1×10^{10}VP, 1×10^9VP, and 1×10^8VP) was added to each well of experimental group. Other drugs were added to each experi-

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mental group respectively. In the control group, 100 μL 0.9% sodium chloride solution was added to each well. The assay was performed for 4d.

The method of dissolving coloration and determination of light absorption value refer to The Cell Culture[5].

Transfection of colorectal cancer cells by the rAdp53 *in vitro*

The glass slide was immersed in polylysine, and preserved after high pressure disinfection.Colorectal cancer cells were plated at a density of 3.0×10^4 cells/well in 12-well plates in triplicate; 72 h later, cells were infected with rAdp53 (rAdp53 was added into each well at the quantity of 1×10^{11}VP/well, 1×10^{10}VP/well, 1×10^9VP/well, 1×10^8VP/well, 1×10^7 VP/well).Glass slides were taken out at 1 h,2 h,6 h, 12 h, 24 h, 48 h, and 96 h after transfection and fixed with 4°C ethanol for 10 min. In the control group, only 100 μL 0.9% sodium chloride solution was added.

Immunohistochemical stain

40 μL H_2O_2 (3%) was added to each glass slide, incubated for 10min at room temperature,and washed 3min×3times. The primary monoclonal antibody(50 μL) was added to each glass slide, incubated for 60min at 37°C,and washed 3min×3times. The secondary antibody was added to each glass slide, incubated for 15min at 37°C, and washed 3min×3times; 100 μL DAB solution was added to each glass slide, and we observed their stain through microscope. It was washed with water and stained by hematoxylin.

Statistical analysis

Data were expressed as the mean±SD. The t test was used. *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Inhibitory activities of different concentrations of rAdp53 *in vitro*

As shown in ***Tab 1***, MTT chemosensitivity assay indicted that different concentrations of rAdp53 resulted in different tumor suppression. The intervals when rAdp53 worked also affected tumor suppression in a dose- and time-dependent manner. Tumor suppression demonstrated direct proportion relation with medicine concentration and interval. According to y=ax+b [5], IC_{50} was figured out as 5.73×10^{11}VP/mL. In the course of culture, cells would age and die finally. In the previous experiment, we observed that logarithm growth phase of colorectal cancer cell line-174 took place within 3-5 d after inoculation. ***Fig 1*** showed that 96 h after transfection, i.e. 6 d after inoculation, the cells were aging and began to die. This event didn't influence the overall growth basically.

Tab 1 **Colorectal cancer cell survival rates after being transfected by varied concentrations of rAdp53 within different intervals** ($\bar{x}\pm s$,%)

Time (after transfected)	Concentrations of rAdp53		
	1×10^9 V.P/mL	1×10^{10} V.P/mL	1×10^{11} V.P/mL
12 h	94.27 ± 1.23	95.19 ± 1.47	94.54 ± 1.21
24 h	98.98 ± 1.65	93.89 ± 1.68	87.81 ± 2.11
48 h	95.74 ± 1.46	92.06 ± 2.01	68.99 ± 1.02
72 h	90.44 ± 1.37	77.51 ± 1.74	39.69 ± 1.76
96 h	8.40 ± 1.10	8.40 ± 0.99	8.73 ± 1.12

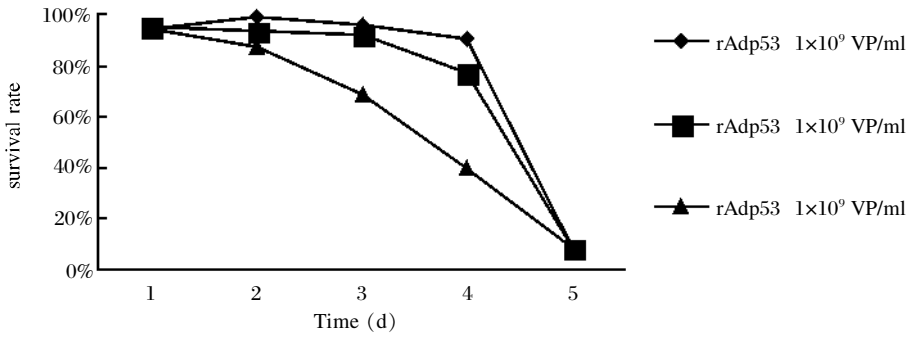

Fig 1 Growth curve after transfection with rAdp53

Inhibitory effect of rAdp53 Vs. chemical medicines in cell strain-174

Imax% stands for survival rate of residual tumor cells while the chemical therapeutic medicines peaked the suppression. The higher Imax% was, the more tumor cells survived, and the more insignificantly tumor suppression showed. There existed significant differences of inhibition among different medicines, as shown in ***Tab 2***.

Tab 2 **Imax% of rAdp53 Vs. chemical medicines in cell strain-174** ($\bar{x} \pm s$,%)

Concentrations	rAdp53	5-FU	tegafur	cisplatin	oxaliplatin	MMC	paclitaxel
10 times	38.29 ± 1.03*	28.19 ± 1.67*	49.82 ± 1.78*	21.93 ± 0.98*	22.93 ± 1.97*	14.20 ± 1.77**	33.64 ± 1.02*
1 time	86.67 ± 1.16$^{\triangle}$	33.97 ± 1.88$^{\triangle\triangle}$	69.11 ± 1.36$^{\triangle}$	66.74 ± 1.56$^{\triangle}$	51.60 ± 1.47$^{\triangle}$	47.76 ± 0.96$^{\triangle}$	46.46 ± 1.35$^{\triangle}$
0.1 time	86.81 ± 2.10$^{\#}$	62.99 ± 1.90$^{\#}$	89.29 ± 1.22$^{\#}$	76.47 ± 1.65$^{\#}$	82.82 ± 0.98$^{\#}$	84.26 ± 1.24$^{\#}$	50.25 ± 1.94$^{\#\#}$

The difference of **and*, $^{\triangle\triangle}$and$^{\triangle}$, $^{\#\#}$and$^{\#}$ was of great significance, respectively. ($P < 0.05$)

Tab 2 showed that 10 times, 1 time and 0.1 time of plasm peak concentration (PPC), mitomycin c (MMC), 5-fluorouracil(5-FU)and paclitaxel shored the strongest inhibition in tumor. Thus, the oxaliplatin, mitomycin c, and cisplatin were considered dose-dependent, whose tumor inhibition demonstrated direct proportion relation with medicine concentration. While 5-fluorouracil and oxaliplatin were sensitive to tumor, they performed the strongest inhibition at a low concentration.

Through IC_{50}, we compared the sensitivity intensity of colorectal cancer cell line-174 responding to different medicines. The lower IC_{50} was the stronger sensitivity the tumor cell had to the medicine. Otherwise, the tumor had less strong sensitivity, as shown in ***Tab 3***.

Tab 3 **IC_{50} of rAdp53 Vs. chemical medicines in cell strain-174**

Concentration	rAdp53	5-FU	tegafur	cisplatin	oxaliplatin	MMC	paclitaxel
PPC (μg/mL)	10^{11}	250	250	50	100	10	4
IC_{50} (times of PPC)	5.73$^{\#\#}$	0.28*	9.77$^{\#\#}$	2.36$^{\triangle\triangle}$	1.14$^{\triangle\triangle}$	0.86**	0.53**

The unit of rAdp53 was VP/ml. The difference of *and** can be ignored ($P > 0.05$); but the difference of *and$^{\triangle\triangle}$, *and$^{\#\#}$ was of great significance, respectively($P < 0.05$).

Tab 3 showed that in the same situation, recombinant adenoviral p53 had less inhibition in colorectal cancer cell line-174, compared with those of 5-fluorouracil, paclitaxel and mitomycin c which showed that the strongest inhibition with reference to IC_{50} 0.28 time of PPC 5-fluorouracil could reach half of tumor inhibition. Compared with 5-fluorouracil, cisplatin and oxaliplatin had less strong inhibition for tumor, but had stronger inhibition than that of recombinant adenoviral p53 and tegafur.

Immunohistochemistry stain after transfection with rAdp53

The immunohistochemistry stain after transfection was shown in the ***Fig 2***, ***3***, ***4***, ***5*** and ***6***. Among positive cells, p53 protein was detected by immunostaining against light brownish yellow and dark brown. However, All rAdp53 with five different densities, had positive results in infected colorectal cancer cells with rAdp53 and the earliest positive result was present at the 24h after infection. The only difference was in the number of positively stained cells. The immunohistochemistry stain in control group was negative (***Fig 7***).

DISCUSSION

Colorectal cancer is a malignant tumor, which ranks the second among malignant tumors in terms of disease incidence in the world. In China, with the increase of colorectal cancer incidence [6], to find a proper treatment has become a focus in medical fidd.

The previous research proved that alterations of p53, including mutation and/or dysfunction in colon cancer were common genetic events in cancers, especially whicha hado high occurrence of 75%. They plays an important role in the progression of cancer[7,8].

Wild- type p53 acts as a "monitor" for the growth of cells, which supervises cell reproduction cycles. Its function comprises maintaining the stable status of gene group, moderating growth of cells, preventing the impaired cells from entering G1 stage, repairing damaged DNA and promoting the fail-to-repair cells to enter the death program [9]. Therefore, the use of wild-type p53 as an adjunct to traditional therapy has become a research focus.

In 1989, the notion whether to create a wild-type p53 as substitute for the p53 defected tumor cell to

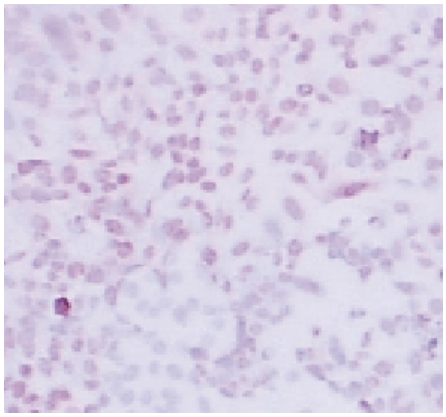

Fig 2 positively stained 1×10^{11}VP(× 40)

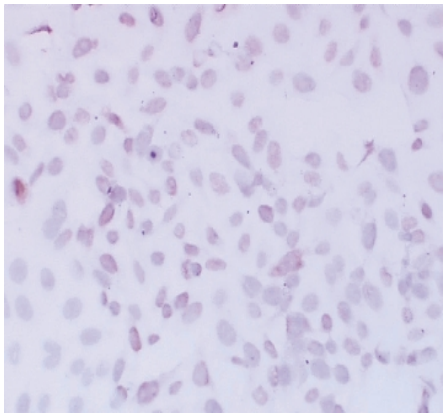

Fig 3 positively stained 1×10^{10}VP(× 40)

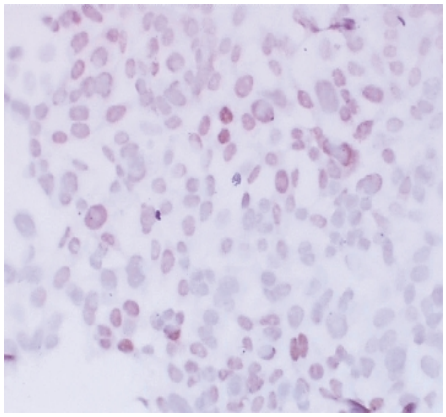

Fig 4 positively stained 1×10^{9}VP(× 40)

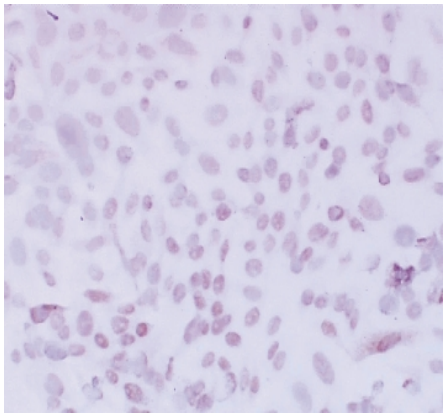

Fig 5 positively stained 1×10^{8}VP(× 40)

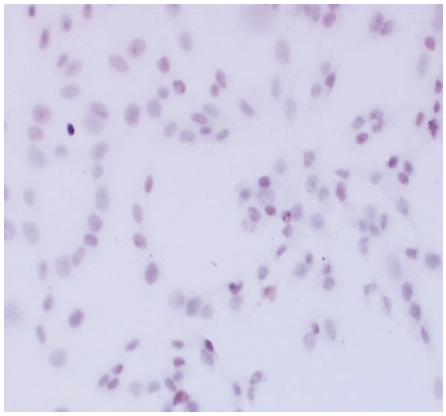

Fig 6 positively stained 1×10^{7}VP(× 40)

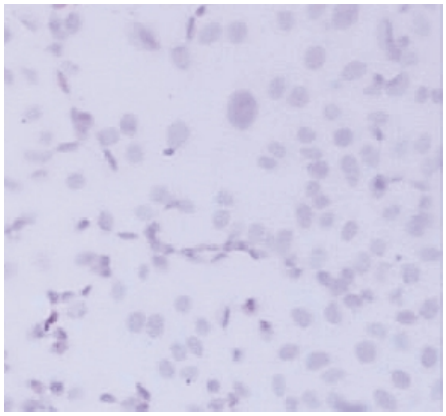

Fig 7 negatively stained(× 40)

treat tumors has been postulated. Thereafter, many researchers, such as Baker[10], Roth[11], showed great enthusiasm in the study of p53, and made a brilliant success in the tumor therapeutic field.

Generally, the carriers used to introduce genes into cells can be classified into virus or non-virus type. rAdp53 injection is made up of homogeneity recombinant p53 gene, carried by V-type reproduction-defected adenovirus. Adenovirus is the most widely used carrier, for it has a wide variety of infections to the host, not only to the cells being produced, but also to the static ones, and thus widening the target cells' selecting options. It is also comparatively safe for adenovirus as carrier, for DNA can not be combined with host's cell chromosome while in fected [12]. Furthermore, the previous researches abroad have proved that rAdp53 had a high transfection rate without being dependent on the status of object cell p53. In other words, the wide type p53 gene introduction could not be influenced whether there was

loss of p53 gene or not. And inhibition in tumors only depended on transfection efficiency of rAdp53 [13-16].

Through MTT assay in vitro, we found that IC_{50} of rAdp53 was 5.73×10^{11}VP/mL, which was dependent on time and dose. ***Fig. 1*** showed that with being longer transfection time, the tumor survival rate decreased sharply especially after 2-3d of transfection. Further, the difference occurs in situations of different transfection concentrations. The lower the concentration of rAdp53 was, the later the decrease survival rate appeared. By immunohistochemical stain assay, we found that 24 h after p53 gene transfection, p53 protein was synthesized and expressed in colorectal cancer cell line-174. The outcome and time of expression were the same as those in nude mouse which was irrelevant with concentration of rAdp53. Besides, through immunohistochemical stain assay, we also found that in positive staining glass slide, there was slight difference of the positive cell count after transfection with different concentrations of rAdp53. The immunohistochemical stain is only an evaluation of quality but not of quantity. Positive cell count should not necessarily indicate transfection efficiency and inhibition intensity. It only proves that anticancer gene p53 can be introduced into cells, and synthesize corresponding protein and serve the function of supervision and inhibition.

It is certain that rAdp53 can inhibit growth of tumor cell *in vitro*. But through MIT chemosensitivity assay, we found that while it was used alone, its inhibition was far from the classical chemical therapy medicines, such as oxaliplatin, 5-fluorouracil, mitomycin c, etc. in terms of Imax% and IC_{50}. Therefore, in clinical practice, the satisfactory effect cannot be achieved if adopting rAdp53 as a means for non-operation colorectal cancer treatment.

rAdp53 can not only inhibit various tumor cell growth independently, but also can cause tumor cells to die by enhancing bax gene, inhibiting bcl-x gene and inducing expression of puma gene, bak gene and fas genes. The real mechanism of p53's inhibition of tumor cells is to increase the sensitivity of tumor cells to chemical therapy, and serve significantly reverted resistance. It is controversial that p53 may cause tumor cells to cease growing instead of apoptosis through inducing mechanisms of p21 gene expression, meanwhile, repair the damaged DNA resulted from chemical and radiation therapy so that it may decrease the sensitivity of tumor cells to chemical and radiation therapy[17]. rAdp53 can be an effective reversion reagent while tumor cells are resistant to chemical and radiation therapy, but how it cooperates with chemical and radiation therapy requires further study.

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