Original Article

Inhibitory Effects of 5-Aza-2'-Deoxycytidine and Trichostatin A in Combination with *p53*-Expressing Adenovirus on Human Laryngocarcinoma Cells

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ABSTRACT

Objective: To investigate the effects of 5-Aza-2'-deoxycytidine (5-Aza-Cdr) and trichostatin A (TSA) combined with *p53*-expressing adenovirus (Ad-*p53*) on Hep-2 cell line *in vivo* and *in vitro*, in order to explore its possibility in biological treatment of laryngocarcinoma.

Methods: Effects of 5-Aza-Cdr and TSA in combination with Ad-*p53* on Hep-2 cell line *in vivo* were determined by Cell Counting Kit-8 (CCK-8) assay. The effect of drug combination was calculated by Jin's formula. Effects on the cell line *in vitro* were investigated by establishing the nude mice model.

Results: 5-Aza-Cdr and TSA showed inhibitory effects on the proliferation of Hep-2 cells in dose- and time-dependent manner. Ad-*p53* can inhibit the growth of Hep-2 cells *in vivo* and *in vitro*. However, the combination of epigenetic reagents (5-Aza-Cdr/TSA) and Ad-*p53* was less effective than individual use of Ad-*p53*. 5-Aza-Cdr and Ad-*p53* inhibited the growth of transplanted tumors and reduced the volume of tumors, and the tumor volume of Ad-*p53* group was significantly smaller than that of the control group ($P<0.05$).

Conclusion: Both epigenetic reagents (5-Aza-Cdr/TSA) and Ad-*p53* can suppress cell proliferation on Hep-2 *in vivo* and *in vitro* and there may be some antagonistic mechanism between Ad-*p53* and epigenetic reagents (5-Aza-Cdr/TSA).

Key words: 5-Aza-2'-deoxycytidine; trichostatin A; *p53*-expressing adenovirus; Hep-2 cell line

INTRODUCTION

Genetic and epigenetic changes are the main mechanisms which can cause malignant tumor. Epigenetic modifications mainly involve DNA methylation and histone acetylation[1]. DNA methylation and histone acetylation have some synergies on gene transcription inhibition[2, 3]. 5-Aza-2'-deoxycytidine (5-Aza-Cdr) is a DNA methyltransferase (DNMT) inhibitor, which could specifically inhibit DNA methyltransferase, reverse gene methylation, restore the function of tumor suppressor genes and inhibit the growth of tumor cells. Trichostatin A (TSA) is a histone deacetylase (HDAC) inhibitor, which could inhibit the reverse activity of histone deacetylase, induce tumor cell cycle block, differentiate apoptosis, therefore, achieve an anti-tumor effect. *p53* gene, which has the highest relativity with human tumor, has been discovered (*p53* is an important tumor suppressor). *p53* gene mutation is associated with more than 50% human carcinomas[4-6]. Previous studies have discovered that wild-type *p53* is closely related to regulation of cell cycle and transformation and induction of apoptosis. This study adopted the method of combining 5-Aza-Cdr and TSA with *p53*-expressing adenovirus (Ad-*p53*) to act on Hep-2 cell line *in vivo* and *in vitro*, in order to explore its possibility in biological treatment of laryngocarcinoma.

Laryngocarcinoma is one of the most common malignant tumors in the head and neck regions, and the mechanism involves genetics and epigenetics. Many

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reports indicated DNA methylation patterns in the CpG island and aberrant histone acetylation modification lead to inactivation of some tumor suppressor genes in human laryngeal cancer. At the aspect of *p53* gene, large numbers of research is made, but there is a few works about its combination with epigenetic reagents. Therefore, we make a study on the combination of Ad-*p53* with DNMT inhibitor 5-Aza-Cdr and HDAC inhibitor TSA on the growth of human laryngeal cancer Hep-2 Cells in order to investigate the value of biological therapy of laryngeal cancer.

MATERIALS AND METHODS

Materials

5-Aza-Cdr and TSA were obtained from Sigma Chemicals, Co. 5-Aza-Cdr was dissolved in phosphate buffered saline (PBS, Thermo Fisher Scientific) as 20 mg/ml stock solution and stored at −20˚C. TSA was dissolved in 1 ml dimethyl sulfoxide (DMSO) as 5 mg/ml stock solution and stored at −20˚C.

Ad-*p53* (Gendicine®) was obtained from Shenzhen Sibiono Bentech, China. It was stored at −20˚C at a concentration of 1×10^{12} viral particles (VP)/ampoule. Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories, USA. RPMI 1640 culture medium and fetal bovine serum (FBS) were obtained from Thermo Scientific HyClone, USA. BALB/c mice (male, 11−13 g and 4 weeks old) raised under SPF conditions were obtained from Animal Laboratory, Capital Medical University, Beijing, China. The animal experiments were approved by the Animal Care and Use Committee of Beijing Institute of Otorhinolaryngology.

Cell lines and Culture Conditions

Human laryngeal carcinoma cell line Hep-2 was obtained from Cytobiology Laboratory of Beijing Institute of Otorhinolaryngology and cultured in RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37˚C.

Drugs Treatment

Exponentially growing cells were randomly assigned into 7 groups (5-well in each group): blank control group (added in RPMI-1640 medium containing 100 g/L serum), negative control group (drug-free medium), 5-Aza-Cdr group (treated with 5-Aza-Cdr at different final concentrations of 0.5, 1.0 and 2.0 mg/L for 72 h, and fresh 5-Aza-Cdr was replaced every 24 h), TSA group (treated with TSA at different final concentrations of 50, 100 and 200 μg/L for 72 h, and fresh TSA was replaced every 24 h), 5-Aza-Cdr+TSA group (treated with 5-Aza-Cdr at different final concentrations of 0.5, 1.0 and 2.0 mg/L for first 24 h, then with TSA at different final concentrations of 50, 100 and 200 μg/L for later 48 h, fresh TSA was replaced every 24 h), Ad-*p53* group (treated with Ad-*p53* at final concentration of 10^{10} VP for 72 h), 5-Aza-Cdr+TSA+Ad-*p53* group (treated with 5-Aza-Cdr at different final concentrations of 0.5, 1.0 and 2.0 mg/L for first 24 h, then with TSA at different final concentrations of 50, 100 and 200 μg/L for the second 24 h and with Ad-*p53* at final concentration of 10^{10} VP for the last 24 h, drugs and fresh medium were replaced every 24 h).

In Vitro Cytotoxicity Assays

Hep-2 cells in logarithmic growth phase were inoculated in 96-well plate, with the amount of 100 μl per well and the cell suspension density of 5×10^4/ml. After 24 h of incubation, the cells were randomly divided into the control group and the test groups medially with five duplicates per group, and drugs and fresh medium were replaced every 24 h. All samples were taken every 24 h for 3 d. Accompanying with every sampling, 10 μl of CCK-8 was added to each well. After 2 h of incubation, the absorption value A of each well was detected at the wavelength of 450 nm in μQuant spectrophotometer (Bio-Tek Instruments, USA). Cell inhibition rate (I%) was calculated using the following equation:

$$I\% = (A_{control} - A_{treated}) / (A_{control} - A_{blank}) \times 100\%$$

The results from the assays were analyzed for the combination effect between 5-Aza-Cdr and TSA according to Jin's[7] method. In this method, $Q<0.85$ indicates antagonism, $0.85\leq Q<1.15$ indicates additivity, and $Q\geq1.15$ indicates synergism. The formula is $Q=Ea+b/(Ea+Eb-Ea\times Eb)$, where Ea+b, Ea and Eb are the average effects of the combination treatment, 5-Aza-Cdr only, TSA only, respectively. All treatments were performed in quadruplication and the experiments were repeated three times.

Effect of 5-Aza-Cdr and Ad-*p53* on Hep-2 Transplanted Tumor Growth in Nude Mice

Forty male BALB/c mice received subcutaneous injection with 0.1 ml of 2×10^7/ml Hep-2 cells. Three weeks later, 10 unqualified mice were weeded out, and the remained 30 mice were divided into 5 groups randomly, with 6 mice in each group. Group 1 (*p53* group) was treated with Ad-*p53* (0.1 ml to each one, every 3 d). Group 2 (combination group) was treated with Ad-*p53* (0.1 ml each one, every 3 d) and 5-Aza-Cdr (0.1 mg/ml, 0.1 ml, three times a week). Group 3 (epigenetic group) was treated with 5-Aza-Cdr (0.1 mg/ml, 0.1 ml, three times a week). Group 4 (control group) was treated with normal saline (three times a week). Group 5 (blank group) was treated with nothing. Tumor size was monitored weekly by measuring the largest and smallest diameters of tumor and estimated according to the formula[8]: volume = 1/2 × (largest diameter) × (smallest diameter)^2. On the 20th

day, we sacrificed the mice, took out the tumors to weigh and calculated the inhibition rate of tumor.

Statistical Analysis

All statistical analyses were carried out using SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). All data were expressed as $\bar{x}\pm s$ and cell proliferation was compared with one-way analysis of variance (ANOVA) in different groups, and the tumor volume in groups was also analyzed with one-way ANOVA, and the pairwise comparison was performed with least significant difference (LSD) *t* test. $P<0.05$ was considered statistically significant.

RESULTS

Effect of Drugs Inhibition on Cell Proliferation

Hep-2 cells *in vitro* were treated with different concentrations of drugs, and the cell growth inhibition rate of Hep-2 was assessed by CCK-8 assay. The results showed that the inhibitory effect on the proliferation of Hep-2 cells increased with the increase of concentration and time. Different experimental groups (Ad-*p53*, 5-Aza-Cdr, TSA and 5-Aza-Cdr+TSA) all inhibited Hep-2 cell growth significantly, in which the inhibition action strengthened with the raised drug concentration and prolongation of the treatment. In each group, the inhibition rate was significantly different between different concentrations acting for the same period ($P<0.05$), and it was also significantly different from each other ($P<0.05$) at the same concentration for different periods. The relationship between the concentrations and time in cell proliferation is listed in Table 1−5.

Analysis of Combination Effect

We analyzed the combination effect according to the Jin's method after calculating the inhibition rate of cell proliferation. Table 6 presents analysis on combination effect between 5-Aza-Cdr and TSA, We found all Q values were under 0.85, so it seemed to be an antagonistic effect. However, with the increment of the drug concentration and prolongation of the exposed time, Q value became higher and higher even to 0.85 and then the two drugs tended toward an additive effect. Table 7 presents analysis on combination effect

Table 1. Effects of 5-Aza-Cdr on Hep-2 cell proliferation

5-Aza-Cdr (mg/L)	A450 ($\bar{x}\pm s$)			Inhibition rate (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	0.749±0.034	1.200±0.104	1.902±0.058	0	0	0
0.5	0.673±0.049	1.067±0.077	1.663±0.055	10.1	11.1	12.6
1	0.580±0.042	0.943±0.052	1.571±0.071	22.6	23.6	55.8
2	0.549±0.077	0.712±0.105	0.830±0.047	26.7	40.7	56.4

Table 2. Effects of TSA on Hep-2 cell proliferation

TSA (μg/L)	A450 ($\bar{x}\pm s$)			Inhibition rate (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	0.749±0.034	1.200±0.104	1.902±0.058	0	0	0
50	0.449±0.047	0.514±0.012	0.760±0.147	40.1	57.2	60.0
100	0.395±0.426	0.481±0.081	0.738±0.100	47.3	59.9	61.0
200	0.373±0.472	0.340±0.031	0.354±0.007	50.2	71.7	81.4

Table 3. Effects of Ad-*p53* on Hep-2 cell proliferation

Ad-*p53* (VP)	A450 ($\bar{x}\pm s$)			Inhibition rate (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	0.749±0.034	1.200±0.104	1.902±0.058	0	0	0
10^{10}	0.447±0.064	0.257±0.025	0.329±0.063	40.3	78.6	82.7

Table 4. Effects of 5-Aza-Cdr and TSA on Hep-2 cell proliferation

5-Aza-Cdr+TSA (mg/L+μg/L)	A450 ($\bar{x}\pm s$)			Inhibition rate (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
0+0	0.749±0.034	1.200±0.104	1.902±0.058	0	0	0
0.5+50	0.673±0.049	0.953±0.125	1.489±0.092	10.1	20.6	21.8
1+100	0.580±0.042	0.769±0.026	0.598±0.020	22.6	35.9	68.6

2+200	0.549±0.077	0.684±0.011	0.412±0.058	26.7	43.0	78.3

Table 5. Effects of 5-Aza-Cdr and TSA combined with Ad-*p53* on Hep-2 cell proliferation

5-Aza-Cdr+TSA+Ad-*p53*	A450 ($\bar{x}$±*s*)			Inhibition rate (%)		
(mg/L+μg/L+VP)	24 h	48 h	72 h	24 h	48 h	72 h
0+0+0	0.749±0.034	1.200±0.104	1.902±0.058	0	0	0
0.5+50+10^{10}	0.673±0.049	0.953±0.125	1.172±0.045	10.1	20.6	38.4
1+100+10^{10}	0.580±0.042	0.769±0.026	1.071±0.014	22.6	35.9	43.7
2+200+10^{10}	0.549±0.077	0.684±0.011	0.982±0.036	26.7	43.0	48.4

between epigenetic reagents (5-Aza-Cdr and TSA) and Ad-*p53,* all of the Q values were less than 0.85, and the maximum one is only 0.5. It showed an antagonistic effect as well.

Effect of 5-Aza-Cdr and Ad-*p53* on Hep-2 Transplanted Tumor Growth in Nude Mice

After successfully making the model (Figure 1), we used 5-Aza-Cdr and/or Ad-*p53* to treat implanted tumors, then observed every group's tumor volume. All mice were sacrificed after 20 d, then we took out the tumors (Figure 2). These groups were significantly different from each other (P<0.05), except the control group and blank group. The tumor grew slowest in the *p53* group but fastest in both control group and blank group (Table 8). And the tumor growth speed of combination group was slower than that of epigenetic group. The inhibition rates of tumor growth were calculated as[9]: Inhibition rate=(1−average tumor weight in the experimental group/average tumor weight of control group) ×100%. From this, we found that the inhibition rate of *p53* group was 50.55%, which was superior to that of combination group (39.83%). And the inhibition rate of epigenetic group was the lowest (14.94%).

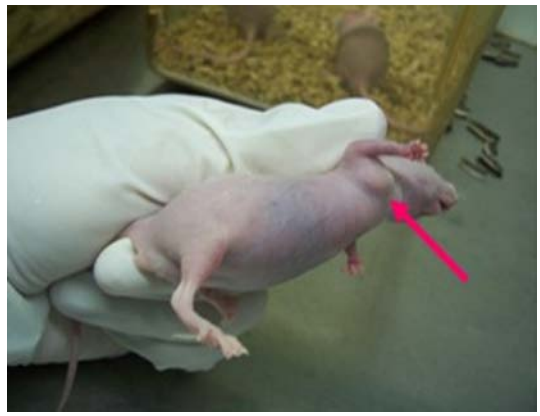

Figure 1. Obvious tumor at right axilla of nude mouse.

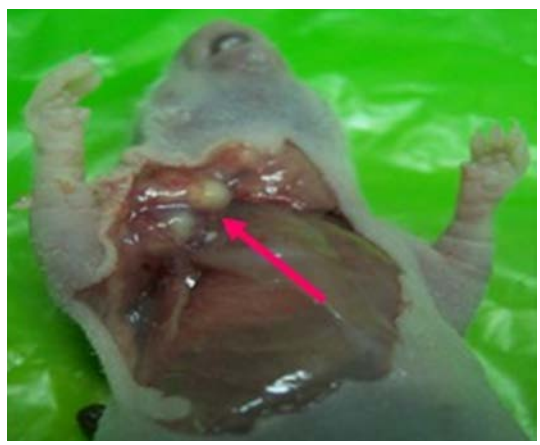

Figure 2. Anatomical tumor tissue.

Table 6. Relationship between combination effect and different dose and time

5-Aza-Cdr+TSA	Q		
(mg/L+μg/L)	24 h	48 h	72 h
0.5+50	0.22	0.33	0.34
1+100	0.38	0.52	0.83
2+200	0.42	0.52	0.85

Table 7. Analysis on combination effect between epigenetic reagents and Ad-*p53*

Group	Concentration	72 h inhibition rate (%)	Q
Control	0	0	0
Ad-*p53* (VP)	10^{10}	82.0	
5-Aza-Cdr+TSA	0.5+50	21.8	
(mg/L+ug/L)	1+100	68.6	
	2+200	78.3	
Ad-*p53*+5-Aza-Cdr+TSA	10^{10}+0.5+50	38.4	0.24

(VP+mg/L+ug/L)	10^{10}+1+100	43.7	0.46
	10^{10}+2+200	48.4	0.50

Table 8. Comparison among tumor volume of different groups

Group	*P*					Tumor volume (mm^3)
	Ad-*p53*	Ad-*p53*+5-Aza-Cdr	5-Aza-Cdr	Control	Blank	
Ad-*p53*	/	0.001	0	0	0	120.0887±24.3976
Ad-*p53*+5-Aza-Cdr	0.001	/	0	0	0	146.1111±22.4860
5-Aza-Cdr	0	0	/	0.001	0.001	206.5502±40.1077
Control	0	0	0.001	/	0.997	242.8406±57.0162
Blank	0	0	0.001	0.997	/	247.8339±54.0919

DISCUSSION

Epigenetic modification is an important form of gene-expression regulation, including DNA methylation and histone deacetylation. This abnormal modification inhibits gene transcription, which leads to antioncogene inactivation. Luckily, gene can be silenced by DNA methylation and histone deacety-lation, that is to say – could be re-expressed after treatment with demethylating reagent and acetylating reagent[10, 11].

5-Aza-Cdr is the most important DNMT inhibitor. It is a pyrimidine analogue and can bind to DNMT in a covalent complex to specifically inhibit the activity of DNMT and the growth of tumor cells[12, 13]. TSA derives from metabolites of streptomyces. It was first used as an antifungal agent. Since Japanese scholar Yoshida first discovered it could inhibit non-competitively the activity of HDAC in breast carcinoma cells of mouse, Nowadays, as a HDAC inhibitor, it has been mostly studied. It had the characteristic of a HDAC inhibitor and could inhibit reversibly the activity of HDAC, induce cell cycle arrest, differentiation and apoptosis and inhibit the growth of tumor cells[14, 15]. It also showed HDAC inhibitors may also contribute to increased DNA demethylation while increasing the histone acetylation levels, so there is close relationship between DNA methylation and histone acetylation[16, 17]. Thus, the combination of 5-Aza-Cdr and TSA could fully exert the synergistic effect, reduce the methylation levels in the promoter region, achieve the re-expression of the silent tumor-suppressor genes and realize the purpose of tumor therapy finally[18]. In recent years, as the representative drugs of DNMT inhibitor and HDAC inhibitor, 5-Aza-Cdr and TSA have been a hot spot for tumor treatment research. Related study showed that silenced O6-methylguanine DNA methyltransferase (*MGMT*) gene was re-expressed after treatment with 5-Aza-Cdr in laryngeal cancer Hep-2 cell line. Some researchers also used 5-Aza-Cdr acting on laryngeal cancer in nude mice and the result showed the median tumor volume of nude mice decreased obviously, compared with the control group.

Shaker, et al. showed that the combination of TSA with 5-Aza-Cdr in the treatment of leukemia could reduce side effects and showed a synergistic effect.

In our experiments, we used 0.5 mg/L, 1.0 mg/L and 2.0 mg/L 5-Aza-Cdr acting on Hep-2 cells. And it inhibited the growth of Hep-2 cells, at the concentration of 0.5 mg/L, and the inhibitory effect increased with the augment of concentration and time (P<0.05). After we used 50 ng/ml, 100 ng/ml, 200 ng/ml TSA on Hep-2 cells, we also found the similar inhibitory effect. According to the Q value, the two drugs in combination have an antagonistic effect at lower initial concentration, but the effect becomes weaker and weaker with the increment of the drug concentration and prolongation of the exposed time. When they reach certain concentration and time, the antagonistic effect eventually disappeared and then gradually tended toward an additive effect to each other. It might show that the two drugs' combination was antagonistic to each other at lower concentration and shorter exposed time, only when the concentration and time increased to a certain degree, they could be synergic and additive. So these two drugs could be combined to use.

A large number of experiments elementarily showed that wild-type *p53* gene has a strong potential in the treatment of certain tumors[19, 20]. And related clinical trials also indicated that Ad-*p53* used alone or combinated is believed to be effective in treatment of laryngocarcinoma and other head and neck tumors. Han, et al. have proved the safety of its clinical applications from the phase I clinical trial on Ad-*p53* plus surgical operation for middle or advanced-stage laryngocarcinoma. Clinical tests also have proven that Ad-*p53* was well-tolerated, and did not increase the toxicity when combined with cytotoxic agents[21-23].

In addition, Ad-*p53* has been recently approved by the State Food and Drug Administration of China as the first gene therapy product for head and neck squamous cell carcinoma (HNSCC)[24]. It is adenovirus type 5 carrying *p53* gene , which can regulate cell cycle, induce apoptosis, inhibit tumor angiogenesis, and increase the sensitivity of cancer to chemotherapy or radiotherapy[25, 26]. That also confirmed by Roth, et al.[27]. The possible mechanism of Ad-*p53* includes[28-31]: (1)

blocking tumor cell proliferation cycle and inducing apoptosis to control tumor growth; (2) wild-type *p53* gene transfection can be used to improve the effect of radiotherapy or/and chemotherapy; (3) inhibiting the expression of vascular endothelial growth factor (VEGF), and inducing tumor cell apoptosis; (4) inducing the specific antitumor immunity; and (5) killing tumor cells by regulating immune systems and bystander effect.

5-Aza-Cdr has showed anti-tumor activity to some extent in animal models and cell culture systems. In our experiment, we used 10^{10} VP Ad-*p53* acting alone or in combination with epigenetic drugs on Hep-2 cells *in vivo* and *in vitro*. It was found that Ad-*p53* could obviously inhibit Hep-2 cells either *in vitro* or *in vivo*, compared with the control group. However, we observed that the inhibitory effect became weaker in the combination group of Ad-*p53* and epigenetic drugs (5-Aza-Cdr or TSA). From combination group, all of the Q values were less than 0.85, the maximum one is only 0.5 and this shows antagonistic effect.

Mechanism of tumorigenesis is very complicated, it may be impractical to cure the tumor with one or two agents. From our experiment, there may be some antagonistic mechanism between Ad-*p53* and epigenetic drugs (5-Aza-Cdr or TSA). As for its precisely effect mechanisms, it is very necessary for us to make an further inquiry. In a word, our work is just an attempt and we hope to find out a new target for treating tumors in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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