

RESEARCH ARTICLE

Radiosensitization Effect of Overexpression of Adenovirus-mediated SIRT6 on A549 Non-small Cell Lung Cancer Cells

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Abstract

Objective: To explore the radiosensitization effect of overexpression of silent information regulator 6 (SIRT6) on A549 non-small cell lung cancer (NSCLC) cells. **Methods:** Adenovirus vector Ad-SIRT6 causing overexpression of SIRT6 was established. Western blotting and MTT assay were adopted to detect the level of SIRT6 protein and the inhibitory rate of A549 cell proliferation after different concentrations of adenovirus transduction (0, 25, 50, 100, 200, and 400 pfu/cell) for 24 h. Control group, Ad-null group and Ad-SIRT6 group were designed in this experiment and virus concentration of the latter two groups was 200 pfu/cell. Colony formation assays were employed to test survival fraction (SF) of the 3 groups after 0, 2, 4, 6, 8, 10 X-ray irradiation. Flow cytometry was used to detect the status of cell cycle of 3 groups after 48 h of 4Gy X-ray irradiation and Western blotting was used to determine the expression of apoptosis-related genes of 3 groups after 48 h of 4Gy X-ray irradiation. **Results:** In the range of 25~400 pfu/cell, the inhibitory rate of A549 cell proliferation increased as adenovirus concentration raised. The inhibitory rates under the concentrations of 0, 25, 50, 100, 200, and 400 pfu/cell were 0%, 4.23±0.34%, 12.7±2.57%, 22.6±3.38%, 32.2±3.22% and 47.8±5.58% and there were significant differences among groups ($P<0.05$). SF in Ad-SIRT6 group was lower than Ad-null and control groups after 4~10Gy X-ray irradiation ($P<0.05$) and the sensitization enhancement ratio (SER) was 1.35 when compared with control group. Moreover, after 48 h of 4Gy X-ray irradiation, there appeared a significant increase in G1-phase cell proportion, up-regulated expression of the level of apoptosis-promoting genes (Bax and Cleaved caspase-3), but a obvious decline in S-phase and G2-phase cell proportion and a significant decrease of the level of apoptosis-inhibiting gene (Bal-2) in the Ad-SIRT6 group ($P<0.05$). **Conclusion:** The over-expression of adenovirus-mediated SIRT6, which has radiosensitization effect on A549 cells of NSCLC, can inhibit the proliferation of A549 cells and cause G0/G1 phase retardation as well as induce apoptosis of cells.

Keywords: Silent information regulator 6 - overexpression - non-small cell lung cancer - radiosensitization effect

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Introduction

Non-small cell lung cancer (NSCLC), a common type of lung cancer with poor prognosis, is one of the main causes of cancer death all over the world (Jemal et al., 2010). Patients with locally advanced NSCLC who can't receive surgery account for approximately 40% (Vokes, 2007), thus, for whom, although radiotherapy is a commonly-used treatment, adverse reactions caused by high-dose radiotherapy and radioresistance of tumor tissue limit the curative effects, so the valid radiosensitivity-enhancing measures need to be explored. The change of sugar metabolic pathway is the common behavior of tumor cells which lays the foundation of macromolecular synthesis for the growth and proliferation of tumor cells (Vander et al., 2009). Multiple studies have revealed that down-regulated expression of lactic dehydrogenase (LDH) and pyruvate dehydrogenase kinase-1 (PDHK-1) can inhibit the growth and proliferation of the tumor (Bonnet et al., 2007; Fantin, et al., 2006; Le et al., 2010), indicating that the change of tumor cell metabolism is of great

significance to tumor cell growth and proliferation, which now has become a hot issue of prevention and treatment of the tumor. SIRT6 is NAD⁺-dependent histone acetylation enzyme regulating multiple physiological processes of cells such as the growth and apoptosis of cells (Zhong et al., 2010). A study in *Cell* in 2012 elaborated cells with the defect of SIRT6 had oncogenicity, and SIRT6 could regulate the tumor metabolism and be considered as tumor suppressor gene (Sebastián et al., 2012). Therefore, in this study, the radiosensitization effect of overexpression of SIRT6 on A549 cells of NSCLC are explored and the results are reported as follows.

Materials and Methods

Reagent

A549 cell line was purchased from American Type Culture Collection (ATCC). Methyl thiazolyl tetrazolium (MTT), dimethylsulfoxide (DMSO), methylene double acryloyl, lauryl sodium sulfate and Tris were available from Sigma-Aldrich. Glycine, RNase and acrylamide were

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provided by Amresco Inc. Company. Cell lysis buffer, Protein Quantitative Reagent Kit-BCA Method and ECL Chemiluminescence Detection Kit were bought from Beyotime Institute of Biotechnology. RPMI 1640 medium and trypsin were purchased from Gibco and penicillin, streptomycin and fetal calf serum (FCS) from Hyclone Company, PI Cell Cycle Detection Kit from Nanjing KeyGEN Biotech. Co., Ltd, Rabbit anti-GAPDH, Rabbit anti-Bal-2 and Sheep anti-Cleaved Caspase-3 Polyclonal Antibody from Cell Signaling Technology, Inc., Rabbit anti-Bax and Sheep anti-SIRT6 Polyclonal Antibody from Santa Cruz Biotechnology, Inc., adenovirus vector Ad-SIRT6 of overexpressed SIRT6 from SinoGenoMax Co., Ltd.

Transfection efficiency test

Western blotting was used to detect the expression of SIRT6 after 24 h of Ad-SIRT6 transfection. Logarithmic phase A549 cells after digested by 0.25% trypsin were inoculated in 6-well culture plate, 1×10^6 in each well, collected after via the different levels of adenovirus transduction (0, 25, 50, 100, 200, and 400 pfu/cell) for 24 h, added with cell lysis buffer for cell lysis on the ice. The concentration of extractive protein was detected by BCA method and the equivalent protein supernatant taken from each well was performed with 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), added with the proper quantity of first antibody of SIRT6 with dilution rate being 1: 200 for overnight incubation at 4°C, then added with the second antibody of SIRT6 with dilution rate being 1: 3 000 for 2 h of incubation at , and lastly proceeded by ECL developing. Image-Pro Plus 5.1 software was used to analyze optical density of developing bands and the last result was expressed as the ratio of SIRT6 to internal reference GAPDH band optical density.

Detection of cell proliferation

Logarithmic phase A549 cells were prepared to single-cell suspension, which was incubated into 96-well culture plate, 1×10^5 each well, 200 μ L and after cultivation for 24 h, transduced by the different levels of adenovirus (0, 25, 50, 100, 200, and 400 pfu/cell), then after cultured with 5% CO₂ at 37°C for 24 h, added with 10 μ L MTT (5 mg/mL-1) for cultivation and stopped after 4 h. Absorbance A value in groups was detected by ELIASA at 492 nm wavelength. The concentration of 0 pfu/cell was used as control group while the other concentrations were considered as experimental group. Each concentration was designed with 6 parallel repeated wells and the experiment was repeated for 3 times. According to formula for calculating the inhibitory rate of cell proliferation, inhibitory rate (%) = (A value of control group - A value of experimental group)/A value of control group $\times 100\%$.

Conditions of cell irradiation

Electron linear accelerator was employed to irradiate cells. Below the cell culture plate was 5 cm thick water tank and above the cell culture plate was 1.55 cm equivalent tissue gel. The source-target distance was 100 cm and the field of 10 cm \times 10 cm was given 6 MV-X irradiation with dosage rate of 200 cGy/min. Cells after given different

dose of irradiation were put into the incubator with 5% CO₂ and saturation humidity for cultivation at 37°C.

Clone formation experiment

A549 single-cell suspension was prepared to be inoculated into 24-well plate according to the cell density of 200/well and divided into control group, Ad-null group and Ad-SIRT6 group. The latter two groups with virus concentration of 200 pfu/cell were irradiated by 0, 2, 4, 6, 8 and 10Gy X-ray after 24 h of cell transfection, 10 d later, fixed by absolute ethyl alcohol for 15 min and then performed with crystal violet staining for 20 min. The number of cell cloning more than 50 was counted under the inverted microscope for calculating the cloning efficiency (CE): CE (%) = (Clone formation average of treatment group/Inoculated cell number) $\times 100\%$. Survival fraction (SF) = (irradiated group CE/non-irradiated group CE) $\times 100\%$. The experiment was repeated 3 times for calculating the average. According to multi-target single-hit model [SF=1- (1-e^{-D/D0})^N], cell survival curve was drawn for calculating the sensitization enhancement ratio (SER). SER = control group D0/Ad-SIRT6 group D0.

Detection of cell cycle

A549 cells were inoculated into 6-well plate with the density of 1×10^6 /well and the grouping was the same as the above-mentioned clone formation experiment. After transduced by the corresponding viral vector and irradiated by 4Gy X-ray for 48 h, according to the instruction of PI Cell Cycle Detection Kit, cell cycle was detected by flow cytometry (FCM) after cells were washed by PBS for one time, added with PI for 15 min of staining.

Detection of the expression of apoptosis-related gene

Western blotting was adopted to detection the protein levels of apoptosis-promoting genes (Bax and Cleaved caspase-3) and apoptosis-inhibiting gene (Bal-2). Cells from control group, Ad-null group and Ad-SIRT6 after irradiated by 4Gy X-ray for 48 h were performed with protein extraction and SDS-PAGE. The operational approach was the same as the above-mentioned Transfection efficiency test. Dilution rate of Bax and Caspase-3 were 1 : 200 and dilution rate of bcl-2 was 1: 300. And the final result was expressed as the ratio of SIRT6 to internal reference GAPDH band optical density.

Statistical data analysis

Windows SPSS16.0 software was used for data analysis. Quantitative data was expressed by the mean \pm standard deviation ($\bar{x} \pm s$). One-way Anova was used for comparison of multiple groups and SNK method was used for pair-wise comparison. A value of $P < 0.05$ was considered to be significant.

Results

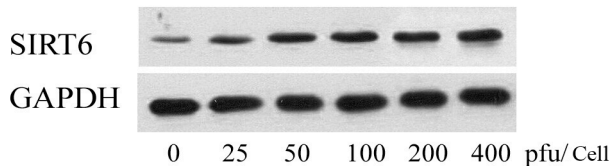
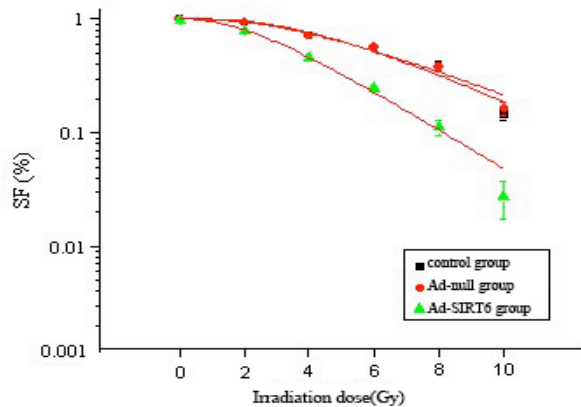
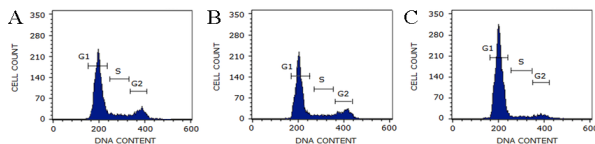
Effect of Ad-SIRT6 transfection on the expression of target gene

In the range of 25~400 pfu/cell, SIRT6 level of A549 cells increased as adenovirus concentration raised. The

Table 1. SF of 3 Groups in Different Irradiation Doses (%)

Groups	Irradiation dose (Gy)					
	0	2	4	6	8	10
Control group	100.00±1.32	93.16±1.74	72.33±2.25	55.37±1.79	39.82±3.11	14.28±1.46
Ad-null group	99.01±2.16	94.82±2.45	70.48±1.97	56.42±2.23	37.69±2.57	16.33±2.15
Ad-SIRT6 group	97.82±2.27	78.65±1.86	45.70±2.53 ^{ab}	24.59±1.82 ^{ab}	11.24±1.69 ^{ab}	2.75±1.02 ^{ab}

Compared with control group, ^a*P*<0.05; Compared with Ad-null group, ^b*P*<0.05

**Figure 1. Effect of Ad-SIRT6 Transfection on Expression of the Target Gene****Figure 2. Multi-Target Single-Hit Model Fitting Survival Curve****Figure 3. FCM Detection Figure of Cell Cycle of 3 Groups. A: control group; B: Ad-null group; C: Ad-SIRT6 group**

relative expression levels under the concentrations of 0, 25, 50, 100, 200, and 400 pfu/cell were (0.12±0.02), (0.15±0.03), (0.21±0.04), (0.38±0.04), (0.54±0.02) and (0.67±0.04) and there were statistical differences among groups (*P*<0.05) (Figure 1).

Inhibiting effect of cell proliferation

In the range of 25~400 pfu/cell, the inhibitory rate of A549 cell proliferation increased as adenovirus concentration raised. The inhibitory rates under the concentrations of 0, 25, 50, 100, 200, and 400 pfu/cell were 0%, (4.23±0.34)%, (12.74±2.57)%, (22.60±3.38)%, (32.18±3.22)%, (38.66±4.09)% and (47.81±5.58)% and there were significantly differences among groups (*P*<0.05).

Radiosensitivity

There was no difference between Ad-null group and control group after via the doses of 0, 2, 4, 6, 8 and 10Gy X-ray irradiation (*P*>0.05). SF in Ad-SIRT group was

Table 2. Cell Distribution of Different Cell Cycle of 2 Groups after 48 h of 4Gy X-ray Irradiation (%)

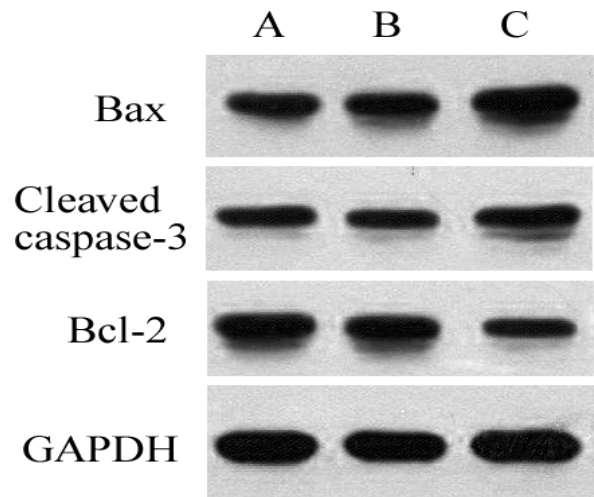
Groups	G1	S	G2
Control group	56.02±2.13	27.16±1.35	16.33±2.02
Ad-null group	54.24±1.86	29.84±2.10	17.48±1.97
Ad-SIRT6 group	80.37±4.46 ^{ab}	14.58±1.14 ^{ab}	6.62±0.94 ^{ab}

Compared with control group, ^a*P*<0.05; compared with Ad-null group, ^b*P*<0.05

Table 3. Effect of Ad-SIRT6 on the Expression Level of Apoptosis-Related Genes

Groups	Bcl-2	Bax	Cleaved caspase-3
Control group	0.52±0.04	0.33±0.05	0.36±0.04
Ad-null group	0.38±0.05	0.47±0.04	0.52±0.06
Ad-SIRT6 group	0.24±0.06 ^{ab}	0.61±0.04 ^{ab}	0.65±0.03 ^{ab}

Compared with control group, ^a*P*<0.05; compared with Ad-null group, ^b*P*<0.05

**Figure 4. Western Blotting Kymogram of Apoptosis-Related Genes of 3 Groups. A: control group; B: Ad-null group; C: Ad-SIRT6 group**

lower than Ad-null and control groups after 4~10Gy X-ray irradiation (*P*<0.05). Multi-target single-hit model fitting survival curve was used for calculating SER (Figure 2). Control group D0 was (3.35±0.48)Gy and Ad-null group D0 was (2.49±0.17)Gy, so the SER was 1.35. (Table 1, Figure 2).

Effect of Ad-SIRT6 transfection on cell cycle

There was no difference in cell distribution of different cell cycle between control group and Ad-null group (*P*>0.05). Compared with control and Ad-null groups, there was relatively higher cell proportion in phase G1 and lower cell proportion in phase S and G2 in Ad-SIRT6 group after 48 h of 4Gy X-ray irradiation (*P*<0.05) (Table 2 and Figure 3).

Effect of Ad-SIRT6 on the cell apoptosis

There was no difference in expression level of apoptosis-related genes between control group and Ad-null group ($P>0.05$). Compared with other 2 groups, there were decreased level of Bal-2 and increased levels of Bax, Cleaved caspase-3 in Ad-SIRT6 group after 48 h of 4Gy X-ray irradiation ($P<0.05$) (Table 3 and Figure 4).

Discussion

NSCLC patients take up 80%~85% of total cases in lung cancer (Mutlu et al., 2013; Oven et al., 2013; Aydiner et al., 2013; Cai et al., 2013; Kaya et al., 2013; Natukula et al., 2013; Unal et al., 2013; Wang et al., 2013) and patients with locally advanced NSCLC who can't receive surgery were mainly treated with radiotherapy, the effect of which was mainly marked with dose-dependence. Although high-dose radiotherapy can improve the remission rate and local control rate, at the same time, it can bring about severe side effects, such as severe radiation esophagitis and radiation pneumonitis (Feng et al., 2011). Radioresistance in tumor tissues limits its clinical application and hinder the improvement of cure rate of malignant tumors (Ni et al., 2013). Anoxia in solid tumor is the main cause of radiotherapy failure, so overcoming the radioresistance caused by anoxia is the key path for improving the therapeutic effect of the tumor (Wu et al., 2013). In order to adapt the circumstance of anoxia, glycometabolism of cancer cell can transform into glycolysis and production rate of glucose catabolism and lactic acid are improved by upregulating the level and activity of glycolytic enzyme, thus providing the material basis for the growth and malignant development of the tumor (Fantin et al., 2006; Bonnet et al., 2007; Le et al., 2010).

Sirtutin is a kind of NAD⁺-dependent histone deacetylase which is capable of changing target protein activity by lysine acetylation (Zhong et al., 2010; Sebastián et al., 2012). Mammal genome encode 7 broad-expression sirtutin subtypes (SIRT1~SIRT7), SIRT1~SIRT7 which play important roles in multiple physiological processes such as the growth and apoptosis of cells, of which, SIRT6 is a chromosome condensation factor for maintaining genomic stability (Sundaresan et al., 2012). SIRT6, located in telomere of human cells, can control cell aging when combined with the ninth lysine in acetylated histone H3 (Finkel et al., 2009). SIRT6 also plays an important role in energy regulation and can be considered as tumor suppressor gene, and once the defect of SIRT6 occurs, cells have oncogenicity (Sebastián et al., 2012). In this study, the overexpression of SIRT6 by adenovirus vector transection of A549 cells was study and MTT assay showed that in the range of 25~400 pfu/cell, the inhibitory rate of A549 cell proliferation increased as adenovirus concentration raised, revealing inhibiting effect of overexpression of SIRT6 on the growth of A549 cells.

This study employed multi-target single-hit model fitting drawing cell survival curve for calculating SER to evaluate the radiosensitization effect and the result showed SF in Ad-SIRT group reduced after A549 cells were irradiated by 4~10Gy X-ray, suggesting over-expressed

SIRT6 could improve the irradiation effect of X-ray. Moreover, when compared with control group, SER of Ad-SIRT6 was 1.35, which further proved that over-expressed SIRT6 had the radiosensitization effect. Cells in different phase of cell cycle have different sensitivity to radioactive rays. Phase S is the least sensitive, followed by phase G1 and phase G2 and M are the most sensitive (Huang et al., 2008). In this study, compared with control and Ad-null groups, there was relatively higher cell proportion in phase G1 and lower cell proportion in phase S and G2 in Ad-SIRT6 group after 48 h of 4Gy X-ray irradiation ($P<0.05$), showing that over-expressed SIRT can inhibiting the process of cell cycle manifested with retarded phase G1, delayed phase G2 and S, prolonged cell doubling time and slow rate of cell proliferation, consequently enhancing overall radiosensitivity of the tumor.

In addition, the effect of overexpression of SIRT6 on the cell apoptosis was studied in this paper, in which Bax and Cleaved caspase-3 are apoptosis-promoting gene and Bal-2 is common apoptosis-inhibiting gene (Liu et al., 2010; Kim et al., 2012). The levels of these three kinds of proteins are commonly used to evaluate the status of cell apoptosis. The result in this study compared with control group and Ad-null group, there were decreased level of Bal-2 and increased levels of Bax and Cleaved caspase-3 in Ad-SIRT6 group after 48 h of 4Gy X-ray irradiation ($P<0.05$), revealing over-expressed SIRT6 could promote the apoptosis of A549 cells.

In conclusion, in NSCLC cell lines cultured in vitro, over-expressed SIRT6 by adenovirus vector, which has radiosensitization effect on A549 cells, can reduce the ability of cell proliferation, change the cell distribution of cell cycle and induce the cell apoptosis, for those reasons, can be as an adjuvant therapy in the treatment of locally advanced NSCLC, with application prospects.

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