

## **Experimental studies on the protective effects of the over-expression of lentivirus-mediated SIRT6 on radiation-induced lung injury.**

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### **Abstract**

**Objective:** To investigate the protective effects of the over-expression of SIRT6 on radiation-induced lung injury in rats.

**Methods:** 72 male Wistar rats (150-120 g) were randomly divided into 3 groups (n=24). Models were made through radiating both lungs with a 6 MV-X linear accelerator. Each group was injected through the tail vein with normal saline (the control group and radiation group) and lentivirus carrying over-expressed SIRT6 (Lent-SIRT6 group) on the exact day of modeling. Changes in respiratory rates, body weight and levels of TNF- $\alpha$  and IL-6 in serum were measured respectively at 1, 2, 4 and 8 weeks after radiation. Blood routine indexes (RBCs, neutrophils and lymphocytes) were recorded, rats were sacrificed with their lung tissues taken, pathological changes of lungs were evaluated by HE staining and TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were detected with ELISA at 8 weeks after radiotherapy.

**Results:** The lung structure including alveolar walls and interstitium in control group were normal, but alveolar walls in radiation group were obviously thickened and a large amount of hyperplastic fibrous tissues were found in alveolar interstitium, while the thickness and interstitial fibrosis of alveolar walls were more alleviated in Lent-SIRT6 group than in radiation group. Compared with those in control group, the respiratory rates, levels of TNF- $\alpha$  and IL-6 in serum, neutrophils and levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in liver all were increased, while WBCs and lymphocytes were decreased in radiation group. The differences were statistically significant (P<0.05). The respiratory rates, levels of TNF- $\alpha$  and IL-6 in serum, neutrophils and levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in liver were all decreased, and WBC and lymphocytes were increased after injection with over-expressed SIRT6. The differences were statistically significant (P<0.05).

**Conclusion:** SIRT6 inhibits inflammation and alleviates radioactive pneumonia and lung injury. Therefore, SIRT6 can exert certain protective effects on lung injury.

**Keywords:** SIRT6, Radioactive pneumonia, Lung injury, Inflammation.

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### **Introduction**

At present, lung cancer has become a malignant disease with highest incidence and mortality worldwide. The global annual death toll of cancers is up to 1.2 million, while radiotherapy is the main treatment for late-stage or postoperatively recurred lung cancer. Radiation-induced lung injury, a common complication of lung cancer after radiotherapy, not only affects lung functions but sharply reduce patients' quality of life, so it is of great significance to protect lungs of patients with radiotherapy from damages. Sirtuin is a NAD<sup>+</sup>-dependent histone deacetylase that can alter the activity of the target protein by lysine deacetylation [1-4]. The 7 widely expressed sirtuin subtypes (SIRT1-SIRT7) are encoded in mammalian genome play an important role in various physiological processes, such as cell growth and apoptosis [5]. It has been reported that SIRT1 exerts protective effects on lung injury of

different types [6,7], and the pathways SIRT1 participates in play a crucial role in this process [8]. Previous studies of this project found that SIRT6 can increase the radiosensitivity of non-small cell lung cancer [9], and exerts protective effects on radiation-induced lung injury. Therefore, the aim of this study is to transfect lentiviral vectors of over-expressed SIRT6 in the models of rats with radiation-induced lung injury and observe the protective effects of SIRT6 on radiation-induced lung injury. The research was sponsored by Natural Science Foundation of Shanghai (15ZR1434300).

### **Materials and Methods**

#### ***Main reagents***

The over-expressed lentivirus vector construction of SIRT6 was completed by the Shanghai Genechem Co., Ltd; ELISA

kits for IL-6 and IL-1 $\beta$  were purchased from the R&D Systems, USA; and ELISA kits for TNF- $\alpha$  were purchased from the Nanjing Jiancheng Bioengineering Institute.

### **Animals and grouping**

Male 150–200 g Wistar rats were selected and radiated with a 6 MV-X linear accelerator for lung models. The 48 radiated rats were randomly divided into 2 groups (n=24): the radiation group and the Lent-SIRT6 group. Rats in Lent-SIRT6 group were injected with  $5 \times 10^7$  TU over-expressed ISIRT6 lentivirus through tail vein, while those in radiation group only received normal saline of the same volume (100  $\mu$ l). The other 24 Wistar rats were only injected with normal saline of the same volume but with no X-ray radiation. Rats in this experiment were all in SPF grade and purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. They were given national standard feed for rodents and free to eat.

### **Establishment of models of radiation-induced lung injury**

Rats were intraperitoneally injected with pentobarbital sodium and then placed on the radiating table in the supine position after full anesthesia. The radiation field adjusted according to the location of both lungs was up to 5 cm  $\times$  4 cm, from armpit midpoint of the forelimbs down to the xiphoid process. A 6MV-X linear accelerator was used to perform a single chest radiation, with the parameters of the total dose were 20 Gy at 300 cGy/min. Rats in control group were placed with the radiating table but with no radiation. All procedures above were performed in the Department of Radiotherapy of the Affiliated Shanghai Pulmonary Hospital of Tongji University.

### **Specimen collection and processing**

Six rats were selected every time point at 1,2,4 and 8 weeks after radiation. Respiratory rates and weight records followed intraperitoneal injection of pentobarbital sodium according to their weight. Their hearts were opened in the supine position and then their right auricles were cut for 5ml blood. At the same time, 6 rats at 8 weeks after radiation were selected and fixed with blood pincers for ligation of lungs. After removal, the left lungs were fixed with 4% formaldehyde solution and stained with HE for observation of pathological changes of lungs, while the right lungs were preserved with liquid nitrogen to detect the levels of pulmonary inflammation factors.

### **Detection indexes**

Changes in respiratory rates, body weight and levels of TNF- $\alpha$  and IL-6 in serum were measured respectively at 1, 2, 4 and 8 weeks after radiation. Blood routine indexes (WBCs, RBCs, neutrophils and lymphocytes) were recorded at 8 weeks after radiation, rats were sacrificed with their lung tissues taken, pathological changes of lungs were evaluated by HE staining and levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were detected with ELISA at 8 weeks after radiation.

### **Statistical treatment**

All statistical analyses were performed with SPSS software (version 20.0). Data in this study were all expressed with “X +SD”. T test was used for comparison between every two groups and univariate analysis for comparison among various groups with  $\alpha=0.05$ .  $P<0.05$  showed statistical significance.

## **Results**

### **Pathological changes in lung tissues of rats among groups**

The lung structure including alveolar walls and interstitium in control group were normal, but alveolar walls in radiation group were obviously thickened and a large amount of hyperplastic fibrous tissues were found in alveolar interstitium, while the thickness and interstitial fibrosis of alveolar walls were more alleviated in Lent-SIRT6 group than in radiation group, but were still more serious than those in control group.

### **Comparison of respiratory rates after routine radiation in rats among groups**

Compared with the control group without radiation and Lent-SIRT6 transfected rats, the respiratory rates of rats in radiation group increased at 1 week after radiation, reached the highest value at 2 weeks and remained high from 4 to 8 weeks after radiation. There was statistically significant in each time point ( $P<0.05$ ). The respiratory rates of rats in Lent-SIRT6 group were lower than those in radiation group, and the differences were statistically significant ( $P<0.05$ ). The respiratory rates in radiation group were higher than those in control group except the one at 8 weeks after radiation ( $P<0.05$ ) (Table 1).

**Table 1.** Respiratory rates of rats among groups at different time points.

Group	1 w	2 w	4 w	8 w
Control group	115 $\pm$ 12	117 $\pm$ 13	121 $\pm$ 11	118 $\pm$ 12
Radiation group	143 $\pm$ 15 <sup>a</sup>	152 $\pm$ 17 <sup>a</sup>	147 $\pm$ 18 <sup>a</sup>	136 $\pm$ 16 <sup>a</sup>
Lent-SIRT6 group	127 $\pm$ 0.18 <sup>ab</sup>	131 $\pm$ 16 <sup>ab</sup>	132 $\pm$ 15 <sup>ab</sup>	124 $\pm$ 14 <sup>a</sup>

Compared with the control group: <sup>a</sup> $P<0.05$ ; compared with the radiation group: <sup>b</sup> $P<0.05$ .

### **Changes in body weight of rats among groups after routine radiation**

Changes in body weight among 3 groups at 1, 2, 4 and 8 weeks after radiation were in the normal range, and differences among groups were no statistically significant ( $P>0.05$ ) (Table 2).

**Table 2.** Changes in body weight of rats among groups after routine radiation (times/min).

Group	1 w	2 w	4 w	8 w
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Control group	62 ± 17	89 ± 15	141 ± 13	169 ± 23
Radiation group	71 ± 16	87 ± 21	136 ± 20	172 ± 19
Lent-SIRT6 group	65 ± 11	94 ± 18	139 ± 17	165 ± 21

**Comparison of TNF-α levels in serum after routine radiation in rats among groups**

Compared with the control group without routine radiation and Lent-SIRT6 transfected rats, the TNF-α levels in serum in radiation group increased at 1, 2, 4 and 8 weeks after radiation. The differences were statistically significant (P<0.05). The TNF-α levels in serum in Lent-SIRT6 group were lower than those in radiation group, and the differences were statistically significant (P<0.05). The TNF-α levels in serum in Lent-SIRT6 group were higher than those in radiation group (P<0.05) (Table 3).

**Table 3.** Comparison of TNF-α levels in serum of rats among groups after routine radiation (ng/L).

Group	1 w	2 w	4 w	8 w
Control group	41.25 ± 5.47	39.50 ± 6.39	40.77 ± 5.83	43.14 ± 5.02
Radiation group	53.64 ± 6.29 <sup>a</sup>	66.28 ± 6.11 <sup>a</sup>	69.03 ± 7.34 <sup>a</sup>	79.75 ± 8.46 <sup>a</sup>
Lent-SIRT6 group	57.31 ± 5.48 <sup>ab</sup>	55.41 ± 7.36 <sup>ab</sup>	51.43 ± 6.15 <sup>ab</sup>	49.26 ± 6.93 <sup>ab</sup>

Compared with the control group: <sup>a</sup>P<0.05; compared with the radiation group: <sup>b</sup>P<0.05.

**Table 4.** Comparison of IL-6 levels in serum of rats among groups after routine radiation (ng/L).

Group	1 w	2 w	4 w	8 w
Control group	0.91 ± 0.35	1.03 ± 0.44	0.98 ± 0.36	1.15 ± 0.37
Radiation group	1.45 ± 0.62 <sup>a</sup>	1.66 ± 0.77 <sup>a</sup>	1.87 ± 0.71 <sup>a</sup>	1.72 ± 0.84 <sup>a</sup>
Lent-SIRT6 group	1.32 ± 0.49 <sup>ab</sup>	1.24 ± 0.52 <sup>ab</sup>	1.36 ± 0.75 <sup>ab</sup>	1.29 ± 0.55 <sup>ab</sup>

**Comparison of IL-6 levels in serum after routine radiation in rats among groups**

Compared with the control group without routine radiation and Lent-SIRT6 transfected rats, the IL-6 levels in serum in radiation group increased at 1,2,4 and 8 weeks after radiation. The differences were statistically significant (P<0.05). The IL-6 levels in serum in Lent-SIRT6 group were lower than those in radiation group, and the differences were statistically significant (P<0.05). The IL-6 levels in serum in Lent-SIRT6 group were higher than those in control group (P<0.05) (Table 4).

**Comparison of blood routine indexes of rats among groups at 8 weeks after routine radiation**

Compared the control group without routine radiation and Lent-SIRT6 transfected rats, the number of lymphocytes

decreased, while that of neutrophils increased in radiation group. The differences were statistically significant (P<0.05). The number of lymphocytes in SIRT6 group was higher than that in radiation group, while the number of neutrophils was higher than that in radiation group. The differences were statistically significant (P<0.05). The differences in the number of neutrophils and lymphocytes between Lent-SIRT6 group and control group were still statistically significant (P>0.05). The differences in RBCs among the three groups are statistically significant (Table 5).

**Table 5.** Comparison of blood routine indexes of rats among groups at 8 weeks after routine radiation (× 10<sup>9</sup>/L).

Group	RBC	Neutrophil	Lymphocyte
Control group	6.72 ± 0.85	0.61 ± 0.36	4.39 ± 0.37
Radiation group	6.34 ± 0.91	1.28 ± 0.54 <sup>a</sup>	1.65 ± 0.84 <sup>a</sup>
Lent-SIRT6 group	6.80 ± 0.79	0.93 ± 0.47 <sup>ab</sup>	1.29 ± 0.61 <sup>ab</sup>

Compared with the control group: <sup>a</sup>P<0.05; compared with the radiation group: <sup>b</sup>P<0.05.

**Table 6.** Comparison of levels of pulmonary inflammation factors of rats among groups after routine radiation (pg/mg).

Group	TNF-α	IL-6	IL-1β
Control group	19.44 ± 4.52	59.13 ± 6.78	41.92 ± 5.06
Radiation group	46.36 ± 7.16 <sup>a</sup>	82.49 ± 8.63 <sup>a</sup>	60.37 ± 8.42 <sup>a</sup>
Lent-SIRT6 group	27.58 ± 6.24 <sup>ab</sup>	67.80 ± 5.92 <sup>ab</sup>	43.63 ± 0.47 <sup>b</sup>

Compared with the control group: <sup>a</sup>P<0.05; compared with the radiation group: <sup>b</sup>P<0.05.

**Comparison of levels of pulmonary inflammation factors in rats among groups at 8 weeks after routine radiation**

Compared with the control group without routine radiation and Lent-SIRT6 transfected rats, the IL-6 and IL-1β levels in radiation group increased after radiation. The differences were statistically significant (P<0.05). The IL-6 and IL-1β levels in Lent-SIRT6 group were lower than those in radiation group, and the differences were statistically significant (P<0.05). The TNF-α and IL-6 levels in Lent-SIRT6 group were still higher than those in control group (P<0.05) (Table 6).

**Discussion**

The therapeutic effect of radiotherapy for lung cancer is dose-dependent. High-dose radiotherapy can improve remission rate and local control rate of tumors, but at the same time will bring about serious side effects, such as severe radiation-induced lung injury [10]. The incidence of radiation-induced lung injury in the lungs is 6-20%. It was found that gene activation during radiotherapy leads to early lung injury, including tissue and cellular dysfunction, increased vascular permeability and radiation-induced inflammation caused by cytokines secreted

by macrophages and leukocytes [11,12]. As a stimulus, inflammatory responses in radiation-induced lung injury can promote the initiation of collagen genes and stimulate fibroblast hyperplasia [13].

In this research, lungs injury was induced by routine radiation of the thoracic cavity, and changes in lung pathologies, plasma and pulmonary inflammatory were compared randomly between two groups with over-expressed SIRT6 lentivirus and with no treatment respectively. In this research, we found that respiratory rates of radiation group increased after X-ray radiation, and particularly the most obvious increase occurred at 2 weeks after radiation, which is consistent with the result of the related studies [14]. However, the increase in respiratory rates of rats receiving over-expressed SIRT6 were by a decreased margin and tended to become slower. The results above suggests that rats in radiation group have significant lung injury, whereas rats in over-expressed SIRT6 group can alleviate the dyspnea caused by radiation-induced lung injury. Pathological changes of lungs are the most direct evidence for lung injury evaluation. In this study, HE staining showed that rats in radiation group had obvious lung injury compared with the control group, mainly manifested by thickened alveolar walls and fibrosis, and a large amount of hyperplastic fibrous tissues in the alveolar interstitium, while these pathological changes above with over-expressed SIRT6 were improved which provided direct evidence for SIRT6's protective effects on lung injury.

Pulmonary inflammation played a crucial role in the occurrence and development of radiation-induced lung injury, and various inflammation factors were involved in this process. TNF- $\alpha$  can promote the inflammatory responses, while IL-6 can induce lung fibrosis by preventing the apoptosis of lung fibroblasts [15,16]. Therefore, this study analyzes the changes in both the process of radiation-induced lung injury and the overexpression of SIRT6. The results showed that levels of TNF- $\alpha$  and IL-6 obvious increased at 1 week after and could last till 8 weeks after radiation, which indicated that inflammatory responses sustained the entire process of lung injury. However, their levels with over-expressed SIRT6 decreased and had protective effects on pulmonary inflammation during the process of lung injury. In this research, increased SIRT6 levels can also decrease levels of pulmonary inflammation factors. In addition, blood test also suggested that over-expressed SIRT6 could improve the decrease in radiation-induced neutrophils and lymphocytes, and exert certain effects on inflammation, which is one of the possible ways to improve the pathological changes of lung injury.

In summary, SIRT6 can effectively inhibit inflammatory responses and alleviate radiation-induced lung injury. Therefore, SIRT6 can exert certain protective effects on lung injury. However, the pathways during this process call for further studies.

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