

# Experimental studies on the protective effects of the overexpression of lentivirus-mediated sirtuin 6 on radiation-induced lung injury

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## Conflict of interest

None declared

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## Abstract

**Background.** Sirtuin 6 (*SIRT6*) can increase the radiosensitivity of non-small cell lung cancer and exert protective effects on radiation-induced lung injury.

**Objectives.** To investigate protective effects of *SIRT6* overexpression on radiation-induced lung injury in rats.

**Material and methods.** Male Wistar rats ( $n = 72$ ) were randomly divided into 3 groups. Models were made by radiating both lungs with a 6MV X linear accelerator. Each group was injected through the tail vein with normal saline (the control group and radiation group) and lentivirus carrying overexpressed *SIRT6* (the Lent-*SIRT6* group) on the same day as the modeling. Routine blood indexes (white blood cells (WBC), red blood cells (RBC), neutrophils and lymphocytes) were recorded; the rats were sacrificed and their lung tissues taken; pathological changes in the lungs were evaluated using hematoxylin and eosin (H&E) staining; and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 6 (IL-6) and interleukin 1 $\beta$  (IL-1 $\beta$ ) were detected with enzyme-linked immunosorbent assay (ELISA) 8 weeks after radiotherapy.

**Results.** The lung structure including alveolar walls and interstitium in the control group was normal, but the alveolar walls in the radiation group were obviously thickened and a large amount of hyperplastic fibrous tissue was found in the alveolar interstitium. The thickness and interstitial fibrosis of the alveolar walls were more alleviated in the Lent-*SIRT6* group than in the radiation group. Compared with those in the 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 the liver all were increased, while WBCs and lymphocytes were decreased in the radiation 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 the liver were all decreased, and WBCs and lymphocytes were increased after injection with lentivirus carrying overexpressed *SIRT6*.

**Conclusions.** Sirtuin 6 inhibits inflammation and alleviates radioactive pneumonia and lung injury. Therefore, *SIRT6* can exert certain protective effects on lung injury.

**Key words:** inflammation, lung injury, sirtuin 6, radioactive pneumonia

## Cite as

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## Introduction

At present, lung cancer has become one of the common malignant diseases with the highest incidence and mortality worldwide. The global annual death toll of cancer is up to 1.2 million, and radiotherapy is the main treatment for late-stage or postoperatively recurring lung cancer. Radiation-induced lung injury not only affects lung functions but sharply reduces the patients' quality of life, which is a common complication of lung cancer after radiotherapy. It is of great significance to protect the lungs of patients undergoing radiotherapy from damage. Sirtuin is a NAD<sup>+</sup>-dependent histone deacetylase that can alter the activity of the target protein by lysine deacetylation.<sup>1</sup>

The 7 widely expressed sirtuin subtypes (*SIRT1*–*SIRT7*) encoded in mammalian genomes play an important role in various physiological processes, including cell growth and apoptosis.<sup>2</sup> Several studies have reported that *SIRT1* exerts protective effects on lung injury of different types<sup>3,4</sup> and plays a crucial role in this process.<sup>5</sup> Our previous study found that *SIRT6* could increase the radiosensitivity of non-small cell lung cancer, and that it exerts protective effects on radiation-induced lung injury.<sup>6</sup>

Pulmonary inflammation plays a crucial role in the occurrence and development of radiation-induced lung injury, and various inflammation factors are involved in this process, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6). The aim of this study was to transfect lentiviral vectors of overexpressed *SIRT6* in models of rats with radiation-induced lung injury and observe the protective effects of *SIRT6* on radiation-induced lung injury. In the present study, lung injury was induced by routine radiation of the thoracic cavity, and changes in lung pathologies, plasma and pulmonary inflammation were compared randomly between 2 groups: 1 with overexpressed *SIRT6* lentivirus and 1 with no treatment.

## Material and methods

### Main reagents

The overexpressed lentivirus vector construction of *SIRT6* was completed by the Shanghai Genechem Company Ltd. (Shanghai, China); the enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and interleukin 1 $\beta$  (IL-1 $\beta$ ) were purchased from the R&D Systems Inc. (Minneapolis, USA); and ELISA kits for TNF- $\alpha$  were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Animals and grouping

Male 150–200-gram specific pathogen-free (SPF)-grade Wistar rats ( $n = 72$ ) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing,

China). They were given national-standard rodent food ad libitum. We randomly chose 48 of the rats to be radiated with a 6MV X linear accelerator for the lung models. The radiated rats were randomly divided into 2 groups of 24: the radiation group and the Lent-*SIRT6* group. The rats in the Lent-*SIRT6* group were injected with  $5 \times 10^7$  TU overexpressed *SIRT6* lentivirus through the tail vein, while those in the radiation group received the same volume (100  $\mu$ L) of normal saline. The other 24 animals were only injected with the same volume of normal saline, without X-ray radiation.

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

The rats were intraperitoneally injected with pentobarbital sodium and then placed on the radiating table in a supine position after full anesthesia. The radiation field, adjusted according to the location of both lungs, was up to  $5 \times 4$  cm, from armpit midpoint of the forelimbs down to the xiphoid process. A 6MV X linear accelerator was used to perform a single round of chest radiation with a total dose of 20 Gy at 300 cGy/min. The rats in the control group were placed on the radiating table without being subjected to radiation. All these procedures were performed in the Department of Radiotherapy of the Affiliated Shanghai Pulmonary Hospital of Tongji University (Shanghai, China).

### Specimen collection and processing

Six rats were selected at every time point 1, 2, 4, and 8 weeks after radiation. Respiratory rates and weights were recorded, followed by intraperitoneal injection of pentobarbital sodium according to their weight. Their hearts were opened in a supine position and their right auricles were cut for 5 mL of blood. Eight weeks after radiation, 6 rats were selected and fixed with blood pincers for ligation of the lungs. After removal, the left lungs were fixed with 4% formaldehyde solution and stained with hematoxylin and eosin (H&E) for observation of pathological changes in the 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 at 1, 2, 4, and 8 weeks after radiation, respectively. Blood routine indexes (white blood cells (WBC), red blood cells (RBC), neutrophils, and lymphocytes) were recorded at 8 weeks after radiation. The rats were sacrificed with their lung tissues taken and pathological changes of lungs were evaluated using H&E staining; the levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were detected using ELISA at 8 weeks after radiation.

## Statistical analysis

All statistical analyses were performed with the IBM SPSS Statistics for Windows software v. 20.0 (IBM Corp., Armonk, USA). Data in this study was expressed as  $X \pm$  standard deviation (SD). The t-test was used for comparisons between every 2 groups, and univariate analysis for comparisons among various groups;  $\alpha = 0.05$ . The threshold of statistical significance was  $p < 0.05$ .

## Results

### Pathological changes in lung tissues of different groups of rats

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

### Comparison of respiratory rates in different groups of rats after routine radiation

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

Table 1. Respiratory rates of the 3 groups of rats at different time points

Group	1 week	2 weeks	4 weeks	8 weeks
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- <i>SIRT6</i> 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 the body weight of different groups of rats after routine radiation

As shown in Table 2, changes in body weight among the 3 groups at 1, 2, 4, and 8 weeks after radiation were in the normal range, and the differences among groups were not statistically significant ( $p > 0.05$ ).

Table 2. The body weight [g] of the 3 groups of rats after routine radiation (3 min)

Group	1 week	2 weeks	4 weeks	8 weeks
Control group	62 $\pm$ 17	89 $\pm$ 15	141 $\pm$ 13	169 $\pm$ 23
Radiation group	71 $\pm$ 16	87 $\pm$ 21	136 $\pm$ 20	172 $\pm$ 19
Lent- <i>SIRT6</i> group	65 $\pm$ 11	94 $\pm$ 18	139 $\pm$ 17	165 $\pm$ 21

### Comparison of serum TNF- $\alpha$ levels in different groups of rats after routine radiation

As shown in Table 3, compared with the control group without routine radiation and the Lent-*SIRT6* transfected rats, the serum TNF- $\alpha$  levels in the radiation group increased at 1, 2, 4, and 8 weeks after radiation. The differences were statistically significant ( $p < 0.05$ ). The serum TNF- $\alpha$  levels in the Lent-*SIRT6* group were lower than those in the radiation group, and the differences were statistically significant ( $p < 0.05$ ). The serum TNF- $\alpha$  levels in the Lent-*SIRT6* group were higher than those in the radiation group, and these differences were also statistically significant ( $p < 0.05$ ).

Table 3. Comparison of serum TNF- $\alpha$  levels in the 3 groups after routine radiation [ng/L]

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

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

### Comparison of serum IL-6 levels in different groups of rats after routine radiation

As shown in Table 4, compared with the control group without routine radiation and the Lent-*SIRT6* transfected rats, the serum IL-6 levels in the radiation group increased at 1, 2, 4, and 8 weeks after radiation. The differences were

**Table 4.** Comparison of serum IL-6 levels in the 3 groups of rats after routine radiation [ng/L]

Group	1 week	2 weeks	4 weeks	8 weeks
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- <i>SIRT6</i> 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>

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

statistically significant ( $p < 0.05$ ). The serum IL-6 levels in the Lent-*SIRT6* group were lower than those in the radiation group, and the differences were statistically significant ( $p < 0.05$ ). The serum IL-6 levels in the Lent-*SIRT6* group were higher than those in the control group, and these differences were also statistically significant ( $p < 0.05$ ).

### Comparison of blood routine indexes of different groups of rats at 8 weeks after routine radiation

As shown in Table 5, compared with the control group without routine radiation and the Lent-*SIRT6* transfected rats, the number of lymphocytes in the radiation group decreased, while the number of neutrophils increased. The differences were statistically significant ( $p < 0.05$ ). The number of lymphocytes in the Lent-*SIRT6* group was higher than that in the radiation group, while the number of neutrophils was higher than in the radiation group. These differences were also statistically significant ( $p < 0.05$ ). The differences in the number of neutrophils and lymphocytes between the Lent-*SIRT6* group and the control group were also statistically significant ( $p > 0.05$ ). The differences in RBCs among the 3 groups were statistically significant as well.

**Table 5.** Comparison of blood routine indexes of the 3 groups at 8 weeks after routine radiation [ $\times 10^9/L$ ]

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- <i>SIRT6</i> 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>  $p < 0.05$ .

### Comparison of levels of pulmonary inflammation factors in different groups of rats at 8 weeks after routine radiation

As shown in Table 6, compared with the control group without routine radiation and the Lent-*SIRT6* transfected rats, the IL-6 and IL-1 $\beta$  levels in the radiation group

**Table 6.** Comparison of levels of pulmonary inflammation factors in the 3 groups after routine radiation [pg/mg]

Group	TNF- $\alpha$	IL-6	IL-1 $\beta$
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- <i>SIRT6</i> 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>  $p < 0.05$ .

increased after radiation. The differences were statistically significant ( $p < 0.05$ ). The IL-6 and IL-1 $\beta$  levels in the Lent-*SIRT6* group were lower than those in the radiation group, and higher than those in the control group; these differences were all statistically significant ( $p < 0.05$ ).

## Discussion

The therapeutic effect of radiotherapy for lung cancer is dose-dependent. High-dose radiotherapy can improve the remission rate and local control rate of tumors, but at the same time can bring about serious side effects.<sup>7</sup> The incidence of radiation-induced lung injury is 6–20%. It has been found that gene activation during radiotherapy leads to early lung injury, including tissue and cellular dysfunction, increased vascular permeability and radiation-induced inflammation which may be caused by cytokines secreted in macrophages and leukocytes.<sup>8,9</sup> As a stimulus, inflammatory responses in radiation-induced lung injury can promote the initiation of collagen genes and stimulate fibroblast hyperplasia.<sup>10</sup>

In this study, we found that the respiratory rates in the radiation group increased after X-ray radiation. The most obvious increase occurred at 2 weeks after radiation, which was consistent with the results of related studies.<sup>11</sup> However, the increase in the respiratory rates of rats receiving overexpressed Lent-*SIRT6* were a little lower and tended to become slower. These results suggested that the rats in the radiation group had significant lung injury, whereas rats in the overexpressed Lent-*SIRT6* group could alleviate the dyspnea caused by radiation-induced lung injury.

Pathological changes in the lungs are the most direct evidence for evaluating lung injury.<sup>12</sup> In this study, H&E staining showed that the rats in the radiation group had obvious lung injury compared with the control group; it was mainly manifested by thickened alveolar walls and fibrosis, and a large amount of hyperplastic fibrous tissues in the alveolar interstitium. In the group administered with overexpressed Lent-*SIRT6*, these pathological changes were improved, which provided direct evidence for the protective effects of *SIRT6* against lung injury.

The TNF- $\alpha$  can promote inflammatory responses, while IL-6 can induce lung fibrosis by preventing the apoptosis of lung fibroblasts.<sup>13</sup> Therefore, this study analyzed changes in both the process of radiation-induced lung

injury and the overexpression of *SIRT6*. The results showed that the levels of TNF- $\alpha$  and IL-6 increased at 1 week after radiation, and could last through 8 weeks after radiation, which indicated that inflammatory responses were sustained throughout the entire process of lung injury. However, in the group of animals administered with overexpressed Lent-*SIRT6*, the levels of TNF- $\alpha$  and IL-6 decreased and had protective effects on pulmonary inflammation during the process of lung injury. In our experiments, increased Lent-*SIRT6* levels also decreased the levels of pulmonary inflammation factors. Blood tests also suggested that overexpressed Lent-*SIRT6* could improve radiation-induced neutrophils, decrease the content of lymphocytes, and exert certain effects on inflammation, which was one of the possible ways to improve the pathological changes in lung injury.

## Conclusions

In summary, *SIRT6* could effectively inhibit inflammatory responses and alleviate radiation-induced lung injury, exerting certain protective effects on lung injury. However, the mechanisms and pathways of this process call for further study.

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