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PM2.5 exposure induces alveolar epithelial cell apoptosis and causes emphysema through p53/Siva-1

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Abstract. – OBJECTIVE: This study aims to investigate whether PM2.5 exposure is involved in the induction of alveolar epithelial cell apoptosis and the progression of emphysema in mice, and to further explore its specific molecular mechanism.

MATERIALS AND METHODS: A certain number of PM2.5 exposed mice and normal control mice were selected, and a lung resection operation was performed to collect the pulmonary tissue samples, which were then analyzed by hematoxylin and eosin (H&E) staining assay. Subsequently, the total protein in the pulmonary tissues of mice in PM2.5 exposure group and control group was extracted, and the p53 protein level was detected by Western blot. Meanwhile, in A549 cells, after treatment of different doses of PM2.5, the protein levels of p53, caspase3, and clv-caspase3 were examined by Western blot while the mRNA levels of p53, Siva-1, and clv-caspase3 were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively. In addition, flow cytometry was carried out to measure the incidence of cell apoptosis, while chromatin immunoprecipitation (ChIP) assay was performed to verify whether p53 binds to the Siva-1 promoter region and thus regulates its transcription process.

RESULTS: H&E staining revealed that PM2.5 exposure caused pathological damage in the pulmonary tissues and the expansion of the spatial structure of alveoli, which led to emphysema in mice. Moreover, p53 protein expression in pulmonary tissue of mice in PM2.5 exposure group was remarkably higher than that in the control group. Subsequently, A549 cells were treated with 0, 25, 50, 100 μg/ml PM2.5 for 48 h, and it was found that, with the increase of PM2.5 exposure dose, the p53 protein level, Siva-1 mRNA level and cell apoptosis rate were all found increased in a dose-dependent manner, which could be partially reversed by transfection of si-p53 in A549 cells. In addition, CHIP experiments confirmed that p53 can bind to the Siva-1 promoter region and directly regulate Siva-1 transcription. In A549 cells, PM2.5 exposure increased the expression of the clv-caspase3 protein, which was reversed by the knockdown of p53; however, simultaneous overexpression of Siva-1 could further increase the clv-caspase3 protein level. Additionally, flow cytometry also revealed that PM2.5 exposure induced apoptosis of alveolar epithelial cells, while the knockdown of p53 reduced that, which could be promoted by the overexpression of Siva-1.

CONCLUSIONS: PM2.5 exposure can promote the transcription of Siva-1 to induce apoptosis of alveolar epithelial cells and accelerate the progression of emphysema in mice by enhancing p53 protein expression.

Key Words:
PM2.5, P53, Siva-1, Emphysema, Alveolar epithelial cells, Apoptosis.

Introduction

Emphysema refers to the pathological state of airway elasticity at the distal end of the terminal bronchioles; it occurs with excessive expansion, inflation, increased lung volume, and airway wall destruction[1,2]. Smoking, infection, and air pollution cause bronchiolitis, stenosis or obstruction[3]. When inhaling, the bronchioles dilate and the air enters the alveoli; when exhaling, the lumen shrinks, the air stays, and the alveolar pressure increases continuously, causing the alveoli to over-expand or even rupture[4,5].

PM2.5 causes epigenetic changes in lung cancer and chronic airway inflammatory diseases, including dysregulation of microRNA, DNA methylation, increased cytokines, and increased levels of inflammatory cells, and activation of related signaling pathways[6,7]. Recent studies have found autophagy and apoptosis of alveolar epithelial cells in chron-

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ic obstructive pulmonary disease associated with PM2.5 exposure[8]. Many molecular mechanisms, including PM2.5-induced cytokine release and oxidative stress, can be involved in triggering and aggravating asthma and chronic obstructive pulmonary disease (COPD)[6]. This research was designed to investigate the specific potential molecular mechanisms of PM2.5-induced apoptosis of alveolar epithelial cells, as wells as the occurrence of COPD.

As a tumor suppressor gene, P53 mutations have been found in more than 50% of all malignant tumors[9]. The protein encoded by this gene is a transcriptional factor that controls the initiation of the cell cycle[9,10]. In addition, p53 gene plays a monitoring role in cell division under normal conditions[11]; however, the function of p53 protein in the development of alveolar epithelial cell apoptosis and emphysema disease remains unclear.

As an apoptosis-inducing factor, Siva1 is a typical apoptotic protein activated by p53 tumor suppressor protein, which has pro-apoptotic activity in various cell systems[12,13]. This study revealed the functional role of p53/Siva-1 in the progression of emphysema in mice caused by PM2.5 exposure-induced apoptosis of alveolar epithelial cells. A previously unknown molecular regulatory mechanism has established that PM2.5 exposure increases the protein expression of p53 in alveolar epithelial cells, which can promote Siva-1 transcription and induce cell apoptosis, leading to the occurrence of emphysema. The p53/Siva-1 pathway may serve as a potential clinical therapeutic target for COPD, which provides clues and evidence for clinical diagnosis and treatment.

Materials and Methods

Construction of PM2.5 Exposure Mouse Model

BALB/c mare mice, 6-8 weeks old, were purchased and housed in experimental animal centers. All animal experiments were approved by the Animal Ethics Committee. Animals were treated humanely, and pain relieved according to protocols approved by the Animal Care and Use Committee. To determine the effect of PM2.5 on alveolar epithelial cell apoptosis and emphysema, 12 BALB/c male mice (6-8 weeks old) were randomly assigned to two groups, which were treated with PM2.5 exposure and normal air. Briefly, mice were exposed to the PM2.5 systemic exposure system for 60 minutes each time, twice daily, 4 hours apart, 5 days per week for a total of 4 weeks. The humidity, temperature, and CO_2 concentration in the exposed system were continuously measured during the exposure.

Cell Culture and Transfection

The lung cancer cell line A549 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) low glucose complete medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. A549 cells with good growth condition were selected and uniformly plated in a six-well plate according to the density standard of about 10^4/well. After the cells are covered with the bottom surface of the six-well plate, transfection was performed according to the manufacturer's instructions. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 1.5 mL of serum-free medium, 500 μL of si-p53, and pcDNA-Siva-1 were added to the cultured cells, and the culture was continued at 37°C in an incubator. After 6 h, the medium was replaced with complete medium and further operations were carried out according to the purpose of the experiment.

RNA Extraction and quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

For both cell and tissue samples, the total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol. The extracted RNA was stored at -80°C after being measured at a concentration of a micronuclear quantifier. The complementary deoxyribose nucleic acid (cDNA) was obtained by reverse transcription, and the SYBR Green method (TaKaRa, Komatsu, Japan) was used for PCR detection. The PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 30 s. siva-1: F: CAAGCGACTCCTGTTCCTCG; R: GTCTGGTCCAATCAGCATCTG. Caspase 3: F: CATGGAAGCGAATCAATGGACT; R: CTGTACCAGACCGAGATGTCA. p53: F: CAGCACATGACGGAGGTTGT; R: TCATCCAAATACTCCACACGC.

Western Blot Assay

Tissue and cell samples were collected for protein extraction with radioimmunoprecipitation assay (RIPA) cell lysate, and total protein concentration was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA).

50 μg of sample protein was taken and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), and blocked with 5% skim milk powder for 1 h at room temperature. The primary antibodies were added for incubation with the membrane overnight at 4°C shaker. In the next day, the membrane was rinsed 3 times with Tris-Buffered Saline and Tween (TBST) and incubated with second antibody for 1 h at room temperature. After that, the protein samples on the membrane were finally developed and analyzed with enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Hematoxylin and Eosin (H&E) Staining

Mice pulmonary tissues were fixed with 4% paraformaldehyde and embedded in paraffin. To assess pathological damage in the lung tissue of mice and to observe the alveolar spatial structure, serial 5-μm lung sections were placed on glass slides, and H&E staining (Boster, Wuhan, China) was performed. Alveolar spatial area was quantified to estimate pathological damage in pulmonary tissue samples.

Plasmid Construction and Transfection

After constructing the pcDNA3.0-Siva-1 vector-based on amplification with specific primers, the cDNA of Siva-1 was cloned into the mammalian expression vector pcDNA3.0 (Invitrogen, Carlsbad, CA, USA). The pcDNA3.0-Siva-1 vector overexpression plasmid was transfected into A549 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 4-6 hours of transfection, A549 cells were collected for further experimental research.

Chromatin Immunoprecipitation (ChIP)

The cells (1 x 10^7) were incubated for 24 hours and fixed in 1% formaldehyde for 10 minutes. After cell lysis, the chromatin was fragmented to an average size of 500 bp. Dynal beads (Invitrogen, Carlsbad, CA, USA), conjugated antibodies against p53 or H3K27me3 or isotype IgG at 4°C were added to the formulation and the preparations were incubated overnight. The cross-linking of the enriched and input DNA was then reversed, and the DNA was washed with RNase A (0.2 mg/mL) and proteinase K (2 mg/mL) prior to phenol/chloroform purification. The sequence of the immunoprecipitated and input DNA was determined by PCR primers for the upstream region APC promoter.

Flow Cytometry Analysis to Detect Apoptosis Rate

The cells were collected first, and the suspended cells were directly collected into a centrifuge tube, centrifuged, and the culture solution was discarded. The number of cells per sample was (1-5) × 10^6. After washing with phosphate-buffered saline (PBS), the cells were resuspended with 100 μL of the labeling solution and incubated for 10 to 15 minutes at room temperature in the dark. After centrifuged at 500-1000 r/min for 5 min, the precipitated cells were washed once with incubation buffer, and fluorescent (SA-FLOUS) solution was added to incubate for 20 min at 4°C. Flow cytometry excitation wavelength was 488 nm. Fluorescein isothiocyanate (FITC) fluorescence was detected with a passband filter with a wavelength of 515 nm, and another filter with a wavelength greater than 560 nm was used to detect Propidium Iodide (PI). As a result, it was judged that apoptotic cells were resistant to all dyes for cell activity identification such as PI, and necrotic cells were not. The DNA of cells with damaged cell membranes can be stained with PI to produce red fluorescence, while the cells with intact cell membranes do not produce red fluorescence.

Statistical Analysis

Each procedure was repeated at least three times. The data were expressed as mean ± SD (standard deviation). The comparisons between multiple groups were performed by One-way analysis of variance (ANOVA) and comparisons between groups were performed using multi-range least significant differences (LSD). p-value <0.05 was considered statistically significant. Statistical evaluation was performed using Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA).

Results

PM2.5 Exposure Caused a Significant Increase in p53 Levels and Led to Enlarged Alveolar Space and Emphysema in Mice

H&E staining revealed that PM2.5 exposure caused pathological damage in the lungs of mice, and the spatial structure of alveoli expanded, which led to emphysema in mice (Figures 1A and 1B). Western blot analysis showed that the level of p53 protein in the pulmonary tissues of PM2.5 exposed mice was remarkably higher than that of

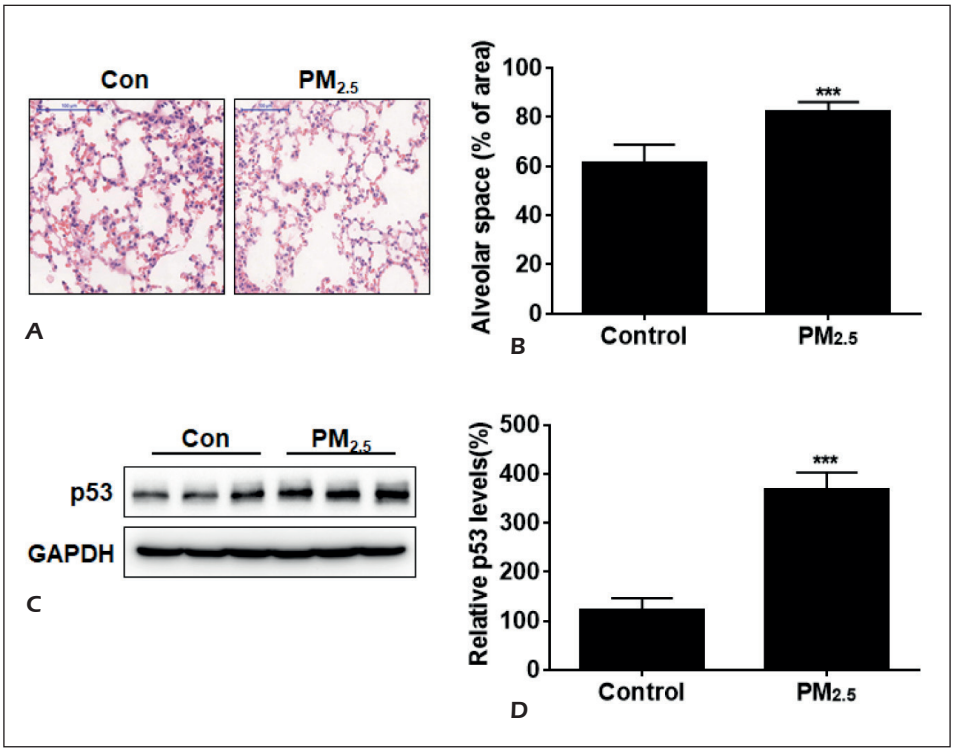

Figure 1. PM2.5 causes emphysema in mice and increases p53 expression. **A**, H&E staining showed that PM2.5 exposure caused emphysema in mice (magnification: 40×). **B**, The alveolar space of mice exposed to PM2.5 was enlarged. **C**, Western blot results showed that the level of p53 protein in pulmonary tissues of PM2.5 exposed mice was significantly increased. **D**, Quantification of p53 protein expression.

the control group (Figures 1C and 1D). In summary, PM2.5 exposure could increase p53 protein levels in mice pulmonary tissues, enlarge alveolar space, and ultimately led to emphysema.

PM2.5 Exposure Resulted in Increased Levels of p53 and Siva-1 in Alveolar Epithelial Cells, and Increased Apoptosis Rate

To investigate the role of p53 and Siva-1 in the development of emphysema, A549 cell line was selected and cultured for related experimental studies. First, A549 cells were treated with different doses of PM2.5. As a result, the p53 protein level and Siva-1 mRNA level were found increased in a dose-dependent manner (Figures 2A, 2B, 2C); meanwhile, the result of flow cytometry showed that the incidence of apoptosis of A549 cells also increased with the increase of PM2.5 exposure dose (Figure 2D). The above results revealed that PM2.5 exposure remarkably increased the expression of p53 and Siva-1, as well as the apoptotic rate of alveolar epithelial cells.

p53 Induces Alveolar Epithelial Cell Apoptosis by Promoting Siva-1 Transcription

To further explore the ways in which p53 is involved in the regulation of emphysema, we first constructed the interfering sequence of p53 and transfected it into A549 cells. It was found that the transfection of si-p53 remarkably reduced the elevated levels of p53 and Siva-1 induced by PM2.5 exposure (Figures 3A and 3B). In addition, CHIP assay confirmed that p53 can bind to the Siva-1 promoter region and directly regulate the transcription of Siva-1 (Figure 3C). Later, flow cytometry was performed to examine cell apoptosis and it was found that the knockdown of p53 remarkably reduced the incidence of A549 cell apoptosis induced by PM2.5 exposure (Figure 3D).

PM2.5 Induces A549 Cell Apoptosis Through the p53/Siva-1 Pathway

Transfection of the Siva-1 overexpression plasmid into A549 cells resulted in a significant increase in Siva-1 mRNA level (Figure 4A). In A549 cells, PM2.5 exposure was found to be able to enhance clv-caspase3 protein expression, which could be conversely reduced by the knockdown of p53; meanwhile, simultaneous overexpression of Siva-1 further elevated the expression of clv-caspase3 protein (Figures 4B and 4C). Next, the results of flow cytometry revealed that PM2.5 exposure induced an increase in the incidence of A549 cell apoptosis, which could be partially reversed by the knockdown of p53. However, the overexpression of Siva-1 promoted the apoptosis of A549 cells (Figure 4D).

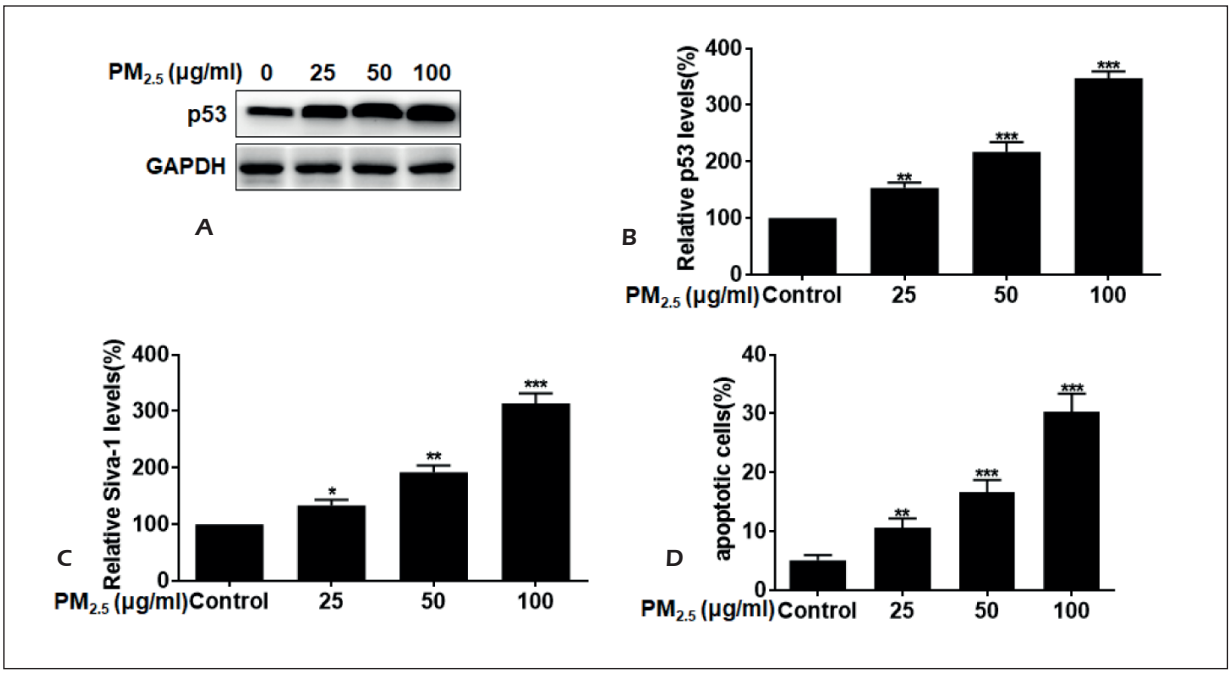

Figure 2. PM2.5 induces an increase in p53 protein expression and the incidence of apoptosis in alveolar epithelial cells. A549 cells were treated with 0, 25, 50, 100 μg/ml PM2.5 for 48 h, **A**, Western blot results showed that the level of p53 protein in A549 cells increased with the increase of PM2.5 concentration. **B**, Quantification of p53 protein. **C**, The level of Siva-1 mRNA in A549 cells increased with the increase of PM2.5 concentration and had a dose-effect relationship. **D**, With the increase of PM2.5 concentration, the apoptosis rate of A549 cells increased, and it had a dose-effect relationship.

Discussion

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death, accounting for 6% of global deaths[14]. The World Health Organization announced that more than 3 million people died of chronic obstructive pulmonary disease in 2012. However, the relevant molecular mechanisms of COPD are still unclear[15]. Smoking and air pollution are major risk factors for the development of COPD[16]. Fine particulate matter (PM2.5) with an aerodynamic equivalent diameter of 2.5 μm or less is an important component of atmospheric particulates and air pollution[17]. PM2.5 is apter to adsorb heavy metal particles, acidic oxides, organic pollutants, bacteria, fungi, and viruses[5]. PM2.5 deposits in the airways and lung tissue and triggers abnormal immune-inflammatory responses[18]. Long-term exposure to lower levels of PM2.5 is associated with accelerated decline in lung function[19,20]. The present study found that PM2.5 exposure can cause pathological damage in mice pulmonary tissues, leading to the expansion of spatial structure of alveoli and the occurrence of emphysema.

PM2.5 is an important component of air pollution. It consists of a large number of heavy metals, organic matter, inorganic substances, and other trace substances, including carcinogenic, and mutagenic substances, such as bap and dioxins[21]. Long-term PM2.5 exposure causes many pathological respiratory diseases such as bronchitis, asthma, COPD[22], and can induce a range of pathological reactions, such as oxidative damage, immune and inflammatory responses, apoptosis, DNA damage, and gene mutations[6,23]. The results of this study revealed that PM2.5 exposure can induce alveolar epithelial cell damage and emphysema in a dose-dependent manner, and cause cell apoptosis by upregulating the expression of caspase-3, an apoptosis-related gene.

P53, a human tumor suppressor gene, also has the function of helping cells to repair defects[24]. The mutants of p53 gene increase carcinogenesis and play a central role in tumor formation. When p53 gene mutation occurs, it loses its regulation on cell growth, apoptosis and DNA repair, and transform from tumor suppressor gene to oncogene[25]. The mutation of p53 gene has been found in more than 50% of human tumor tissues, which is the most common genetic alteration in tumors, indi-

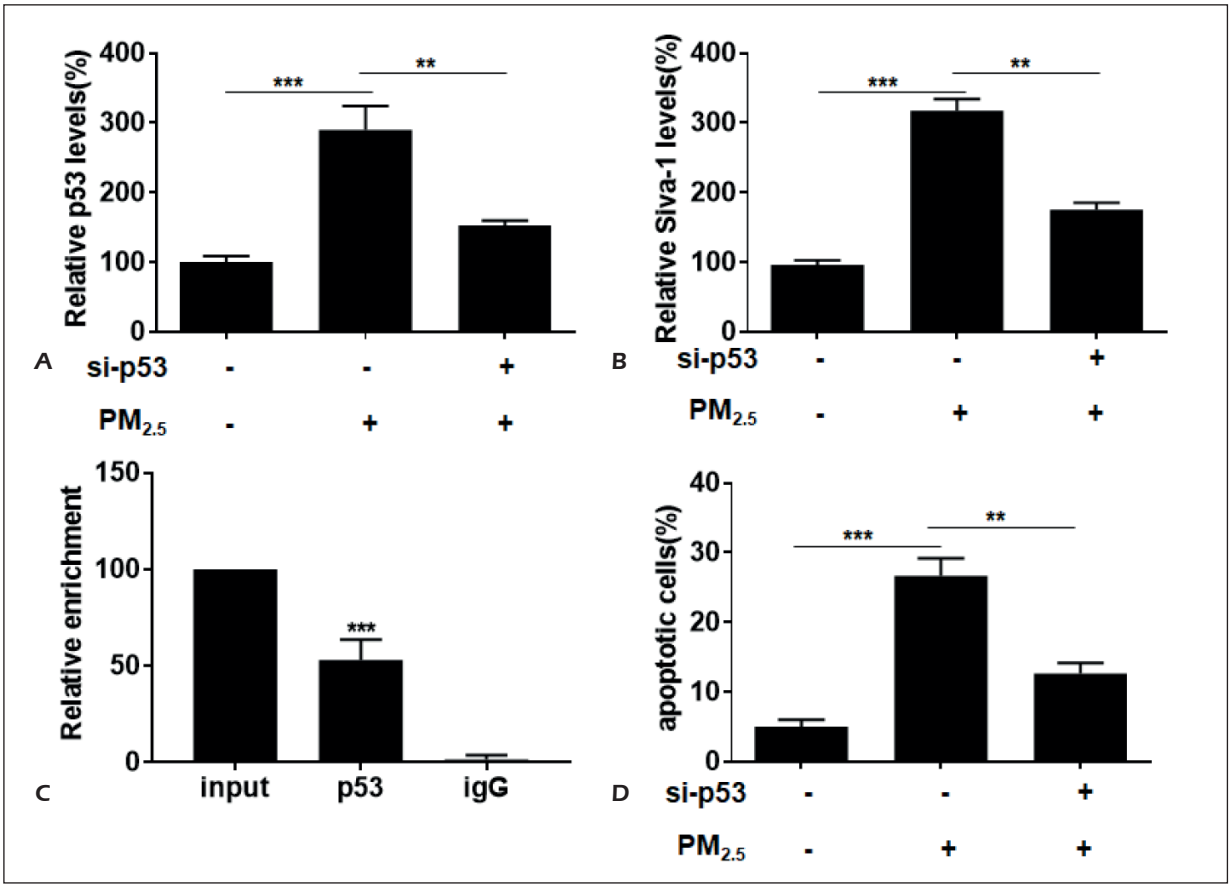

Figure 3. P53 causes apoptosis in alveolar epithelial cells by promoting transcription of Siva-1. **A**, The p53 interference sequence was constructed and the p53 expression level was knocked down in A549 cells. **B**, Treatment of A549 cells with PM2.5 resulted in an increase in the expression level of Siva-1 in cells, while the knockdown of p53 expression at the same time decreased the expression level of Siva-1 in A549 cells. **C**, CHIP experiments showed that p53 binds to the Siva-1 promoter region. **D**, Treatment of A549 cells with PM2.5 resulted in an increase in apoptosis, while the knockdown of p53 expression at the same time decreased the apoptotic rate of A549 cells.

cating that the alteration of this gene is likely to be the main pathogenic factor in human tumors[9,26]. This work revealed that the level of p53 protein in pulmonary tissues of PM2.5 exposed mice was remarkably higher than that of the control group.

The Siva protein contains a domain homologous to the apoptotic domain and induces apoptosis by interacting with the members of the tumor necrosis factor receptor superfamily and anti-apoptotic members of the Bcl-2 protein family[27,28]. Siva also plays a role in the oxidative stress-induced apoptosis and is a transcriptional target of p53[27,29]. Siva-1 protein is mostly localized in the cytoplasm[30], partially localized in mitochondria and nucleus[31]. After infection with Coxsackie B3 virus, Siva protein is strongly activated during cardiac apoptosis, which may lead to heart failure[32]. In this study, the experimental results showed that PM2.5 exposure resulted in an increase in the levels of p53 and Siva-1 in alveolar epithelial cells, as well as the apoptosis rate. In addition, P53 is involved in the induction of apoptosis in alveolar epithelial cells by promoting Siva-1 transcription. In summary, we established a previously unknown molecular mechanism in this experiment, which is, PM2.5 participates in the induction of apoptosis in alveolar epithelial cells through the p53/Siva-1 pathway.

Conclusions

In summary, in alveolar epithelial cells, PM2.5 exposure can increase the protein expression of p53, which further promotes Siva-1 transcription to induce cell apoptosis and emphysema disease.

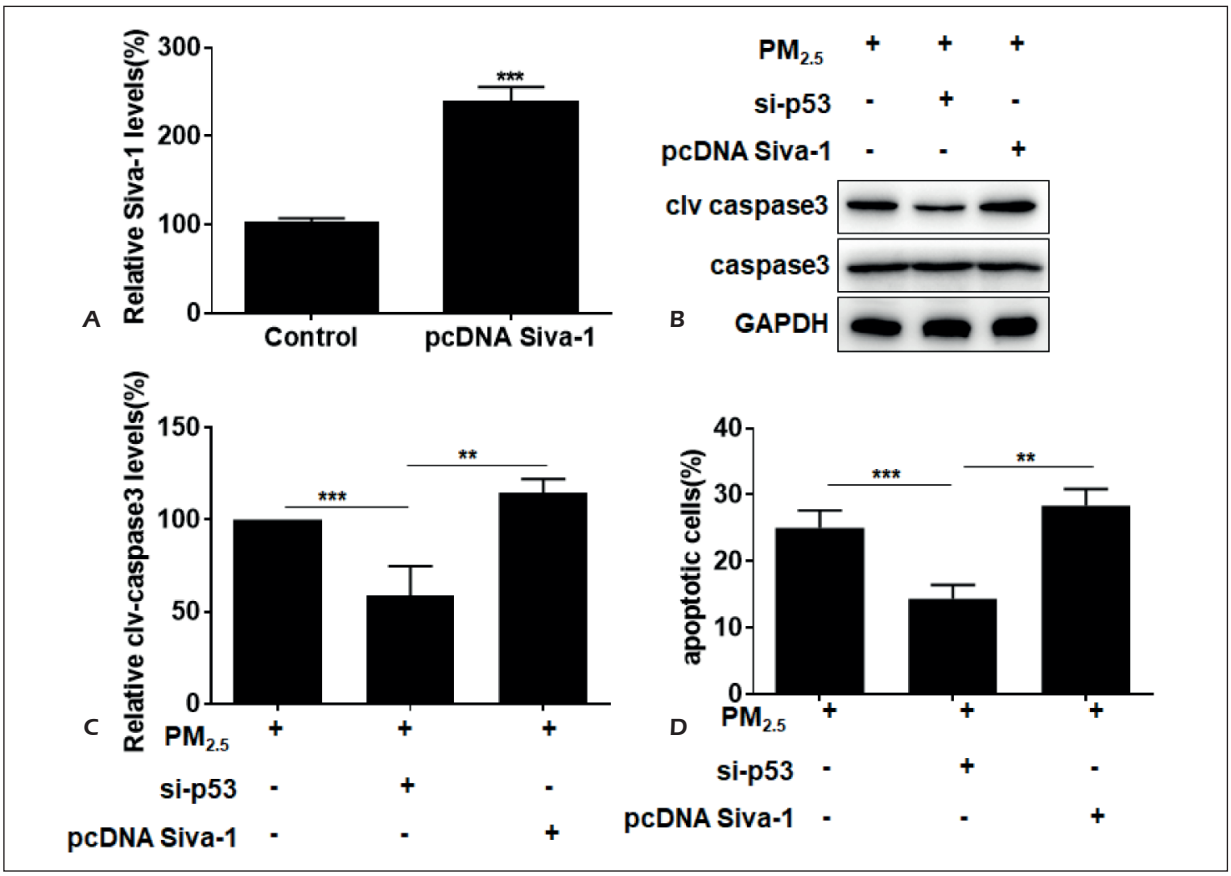

Figure 4. PM2.5 promotes apoptosis of alveolar epithelial cells via p53/Siva-1. **A**, The Siva-1interfering sequence was constructed to reduce the expression level of Siva-1. **B**, In A549 cells, the simultaneous treatment of PM2.5 exposure and knockdown of p53 reduced the clv-caspase3 protein level; meanwhile, further simultaneous overexpression of Siva-1 conversely elevated the expression of clv-caspase3. **C**, Protein quantification of clv-caspase3. **D**, In A549 cells, simultaneous treatment of PM2.5 exposure and knockdown of p53 reduced the cell apoptosis rate; meanwhile, further simultaneous overexpression of Siva-1 conversely elevated the cell apoptosis rate.

Conflict of Interests

The authors declared no conflict of interest.

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