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Elevated expression of lncRNA SNHG15 in spinal tuberculosis: preliminary results

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Abstract. – OBJECTIVE: The incidence and disability rate of spinal tuberculosis is high. The role of the expression of lncRNA SNHG15 in spinal tuberculosis and related mechanisms remains unclear.

PATIENTS AND METHODS: Spinal tuberculosis and normal control tissues were collected, and lncRNA SNHG15 level was analyzed by real-time PCR. Mouse RAW264.7 cells were cultured and divided into control group, tuberculin (PPD) group, si-SNHG15, and PPD+ si-SNHG15 group followed by analysis of lncRNA SNHG15 level, cell proliferation by MTT assay, formation of osteoclasts by TRAP staining, levels of interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) by ELISA, as well as expression of RANK and RANKL by Western blot.

RESULTS: The lncRNA SNHG15 expression in spinal tuberculosis tissues was significantly increased compared with that in the control group ($p < 0.05$). The expression of lncRNA SNHG15 was increased in RAW264.7 cells in the PPD group with increased cell proliferation, TRAP-positive cells, IL-6 and TNF-α secretion, as well as elevated RANK and RANKL expression which were statistically different compared with the control group ($p < 0.05$). Transfection of lncRNA SNHG15 siRNA in the PPD model significantly inhibited the expression of lncRNA SNHG15, decreased cell proliferation, TRAP staining positive cells, IL-6 and TNF-α secretion, as well as reduced RANK and RANKL expression. Compared with the PPD group, the differences were statistically significant ($p < 0.05$).

CONCLUSIONS: The expression of lncRNA SNHG15 was significantly increased in spinal tuberculosis tissues. The downregulation of lncRNA SNHG15 expression could inhibit the secretion of inflammatory cytokines by regulating the RANK/RANKL pathway, thereby regulating osteoclasts.

Key Words:
Spinal tuberculosis, LncRNA SNHG15, Osteoclast, Inflammatory factor, RANK, RANKL.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* infection is a chronic, consumptive infectious disease that occurs in young and middle-aged people. The most important infectious organ is lung tissue[1]. Due to factors, such as reduced lifestyle and immunity and immunodeficiency, the incidence of tuberculosis is increasing. The infectious and refractory problems of tuberculosis have made it one of the diseases that threaten human health[2,3]. Increased population movements and the drug resistance of tuberculosis leads to a further increase in the spread of tuberculosis and treatment difficulties[4,5]. The incidence of tuberculosis is concealed, the latent period is long, the progress is slow, and it is easy to be ignored. Therefore, *M. tuberculosis* can enter the skin, meninges, peritoneum, intestines, and bones with blood circulation and lymphatic circulation to form extrapulmonary tuberculosis[6]. Bone tuberculosis is a devastating lesion caused by *Mycobacterium tuberculosis* invading bone or joint[7]. Bone tuberculosis occurs in the spine, hip, knee, foot, elbow, hand, and other parts, affecting the weight and activity of the bone or joint[8]. In tuberculosis, the high incidence of extrapulmonary tuberculosis is the spine. The incidence of spinal tuberculosis accounts for more than half of the bone and joint tuberculosis, and the disability rate is high, which seriously affects the quality of life of patients[9]. Spinal tuberculosis usually causes destruction of the vertebral body and intervertebral disc, which leads to instability of the spine and spinal cord compression, resulting in paraplegia and kyphosis, causing great pain to patients[10].

In the past few years, with the progress of sequencing research, it was confirmed that only 1-2% of the human genome encodes pro-

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teins, and most of them are transcribed into non-coding RNAs (ncRNAs)[11]. Different types of ncRNAs are expressed in different biological processes and cellular pathways, and play different roles, including small ncRNAs (microRNAs, piRNAs and siRNAs) and long ncRNAs containing at least 200 nucleotides in length, and non-coding RNAs that can be spliced, called lncRNAs, which are classified into introns, intergenic, and antisense lncRNAs depending on the location of protein-encoding genes[12,13]. LncRNA plays an important role in various biological activities, such as cell proliferation and differentiation, and is involved in the progression of various diseases[14]. LncRNA SNHG15 is one of the newly discovered lncRNAs with abnormal expression in a variety of diseases[15,16]. However, the role of lncRNA SNHG15 in spinal tuberculosis and related regulatory mechanisms have not yet been elucidated.

Patients and Methods

Patients

A sample of 3 patients with spinal tuberculosis diagnosed in our hospital from January 20 to December 2018 was collected, including 2 males and 1 female, aged 24-38 years, with an average age of 26.5±5.2 years. Inclusion criteria[17]: pathological examination confirmed typical tuberculosis pathological changes in spinal tuberculosis or surgery; bacterial test to determine Mycobacterium tuberculosis; based on typical clinical symptoms, signs and imaging examination; anti-tuberculosis treatment is effective. It can be diagnosed by having one of the three items mentioned above. Exclusion criteria[17]: complicated with other infectious diseases, malignant tumors, severe diabetes, severe liver and kidney disease, pulmonary fibrosis, bone metabolic diseases, systemic immune diseases, and malignant tumor complications. Three non-bone tuberculosis specimens were collected as a control group, including 2 males and 1 female, aged 23-41 years, with an average age of 28.5±6.5 years. There was 1 case of bone tumor, 1 case of rheumatoid arthritis, or traumatic arthritis. There were no statistical differences in the general clinical conditions between the two groups, such as gender and age. The study obtained informed consent, and both the patients and the families signed informed consent. The study was approved by the Medical Ethics Committee of the Hospital.

Main Reagents and Instruments

Mouse RAW264.7 cells were kept in our laboratory and stored frozen in liquid nitrogen. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and cyan chain double-antibody were purchased from HyClone (San Angelo, TX, USA). Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA digest, tuberculin pure protein derivative PPD, and TRAP staining reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Life Sciences (Port Washington, NY, USA), ethylenediaminetetraacetic acid (EDTA) was purchased from HyClone (San Angelo, TX, USA), Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), rabbit anti-mouse RANK monoclonal antibody, rabbit anti-small Murine RANKL mAb, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell signaling technology (Danvers, MA, USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). IL-6 and TNF-α ELISA kits were purchased from R&D (Minneapolis, MN, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Waltham, MA, USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China).

Mouse RAW264.7 Cell Culture and Grouping

The mouse RAW264.7 cell line was stored in liquid nitrogen, thawed in a 37°C water bath until the cells were completely thawed, centrifuged at 1000 rpm for 3 min, and resuspended in 1 ml of fresh DMEM medium, transferred to a 50 ml cell culture flask, followed by addition of 4 ml fresh DMEM medium and incubation for 24-48 hours in a 37°C, 5% CO_2 incubator. QBC939 cells were seeded in 6-well plates at a density of 1×10^5 cells/cm^2. The culture medium consisted of 10% FBS, 90% high glucose DMEM medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin). The experiment used 3-8 genera-

tion logarithmic growth phase cells. RAW264.7 cells were randomly divided into four groups, control group, PPD group, in which RAW264.7 cell model of tuberculosis pure protein derivative PPD was used to infect mice; si-SNHG15 group (transfection of si-SNHG15 to normal cells), and PPD+si-SNHG15 group which was transfected with SNHG15 siRNA on a PPD basis.

Liposome Transfection of SNHG15 siRNA in RAW264.7 Cells

SNHG15 siRNA was transfected into RAW264.7 cells. The SNHG15 siRNA sequence was 5'-GATGATACTGAAGTACAG-3', and the siRNA negative control primer sequence: 5'-ACGATTCCGTATCTACT-3'. The cell density was fused to 70-80%. The SNHG15 siRNA liposome was added to 200 μl of serum-free DMEM medium, and the negative control liposome was added to another 200 μl of serum-free DMEM medium, mixed well, and incubated at room temperature for 15 min. The mixed Lipofectamine 2000 was mixed with FRα siRNA and negative control dilutions, respectively, and incubated for 30 min at room temperature. The serum of the cells was removed, and the PBS was gently rinsed. 1.6 ml of serum-free DMEM medium was added, and each system was added to each system, and cultured in a 5% CO_2 incubator at 37°C for 6 hours, the culture medium containing 10% serum DMEM was replaced, and cell culture was further continued for 48 hours.

Real-Time PCR Analysis of the Expression of SNHG15, RANK, and RANKL

The total RNA was extracted using TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed by PrimerPremier 6.0 according to each gene sequence and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real-time PCR was performed for detection of the gene of interest with the reaction conditions as follows: 92°C 30 s, 58°C 45 s, 72°C 35 s, a total of 35 cycles. GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using the 2-ΔCt method.

MTT Detection of Cell Proliferation

The logarithmic growth phase hepatobiliary carcinoma RAW264.7 cell line was digested, counted, and seeded in a 96-well plate at 3000 cells/well, and then, treated as mentioned above. Five replicate wells were designed for each group. After adding relevant factors according to each group design, the cells were cultured for 48 hours, followed by the addition of 20 μl of 5 g/L MTT solution in each well and culture for 4 hours in the incubator. The supernatant was removed, 150 μl/well of DMSO was added, and shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was measured at a wavelength of 570 nm, and the cell proliferation rate was calculated. The experiment was repeated 3 times (n=3).

Western Blot Analysis of the Expression of RANK and RANKL Proteins

The total protein of RAW264.7 cells in each group of hepatobiliary carcinoma was extracted: lysate was added, the cells were lysed on ice for 15-30 min, and the cells were disrupted by sonication for 5 s × 4 times, centrifuged at 4°C, 10 000 × g for 15 min, and the supernatant was transferred. In the new Eppendorf (EP) tube, the protein was quantified by bicinchoninic acid (BCA) assay and stored at -20°C for Western blot analysis. The isolated protein was electrophoresed on a 10% SDS-PAGE, transferred to a PVDF membrane by a semi-dry transfer method, 100 mA, 1.5 h, blocked with 5% skim milk powder for 2 h and incubated with primary antibodies RANK or RANKL (1:1000, 1:2000) and β-actin (1:2000) at 4°C overnight. After washing with PBST, corresponding horseradish peroxidase

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
SNHG15	TCATCGACACCTCCAGT	GGGTCCAGCTGTCATATAT
RANK	GACTCGCTCATTACAC	CCATTGTTCACTTCTCA
RANKL	AGCTTTGGTCAGCCTTG	CAGCGTCAGATTAGC

(HRP)-conjugated secondary antibodies diluted in different proportions were added, incubated for 30 min, washed with PBST, and developed by chemiluminescence for 1 min. X-ray exposure imaging was performed to observe the results. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

TRAP Stained Osteoclasts

The cells were sectioned and deparaffinized to water wash; fixed with 80% alcohol and washed with distilled water 3 times for 3 min each time. According to the kit instructions, 37°C distilled water was added for 30 s; incubated at 37°C for 50-60 min; washed with deionized water for 3 min; stained with hematoxylin for 40 s; tap water washing for 10 min to return blue followed by sealing with glycerin gelatin. The experiment was repeated three times.

Statistical Analysis

Statistical analysis was performed using the Statistical Product and Service Solution (SPSS) 19.0 software (IBM Corp, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation (SD). The comparison of multiple groups of samples was performed by One-way ANOVA. The LSD test was used for comparison between the two groups. $p < 0.05$ indicates statistical difference.

Results

Expression Analysis of LncRNA SNHG15 in Spinal Tuberculosis

The expression of lncRNA SNHG15 was analyzed by real-time PCR after collecting spinal tuberculosis clinical samples and normal control tissues. The expression of lncRNA SNHG15 was significantly increased in spinal tuberculosis compared with that in the control tissues ($p < 0.05$) (Figure 1).

Expression of LncRNA SNHG15 in RAW264.7 Cells

The expression of lncRNA SNHG15 in osteoclast RAW264.7 cells was analyzed by real-time PCR. The results showed that the expression of lncRNA SNHG15 was significantly increased in the PPD group ($p < 0.05$). However, the transfection of lncRNA SNHG15 siRNA into normal cultured cells and PPD cell models significantly decreased the expression of lncRNA SNHG15 compared with that in the control group and PPD group ($p < 0.05$) (Figure 2).

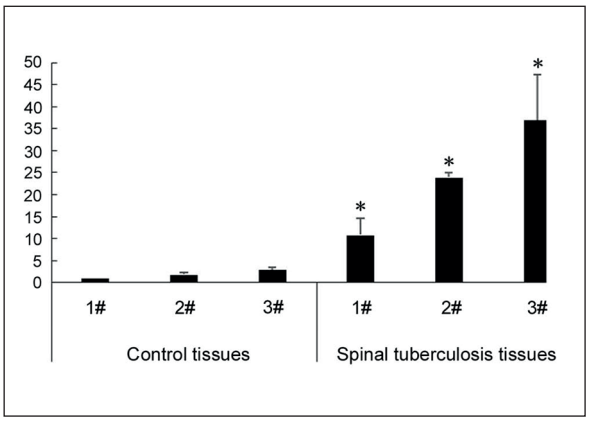

Figure 1. Expression of SNHG15 in spinal tuberculosis samples and control tissues. Compared with the control group, $*p < 0.05$.

Effect of LncRNA SNHG15 on Proliferation of RAW264.7 Cells

The effect of lncRNA SNHG15 on the proliferation of RAW264.7 cells was detected by MTT assay. The results showed that the proliferation of RAW264.7 cells was promoted in the PPD group, and the difference was statistically significant ($p < 0.05$). Transfection of lncRNA SNHG15 siRNA into the PPD cell model significantly inhibited RAW264.7 cell proliferation, and the difference was statistically significant compared with the PPD group ($p < 0.05$) (Figure 3).

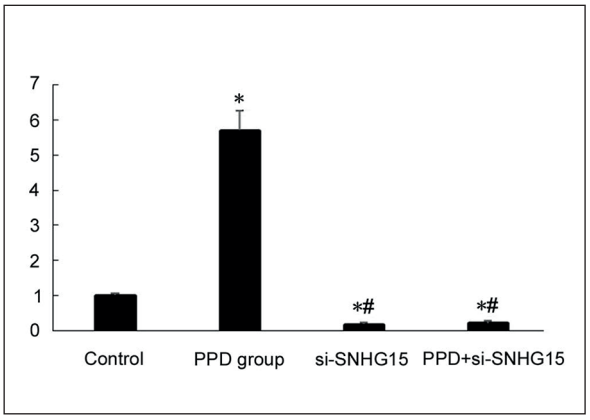

Figure 2. Expression of LncRNA SNHG15 in RAW264.7 cells. Compared with the control group, $*p < 0.05$; compared with the PPD group, $^{\#}p < 0.05$.

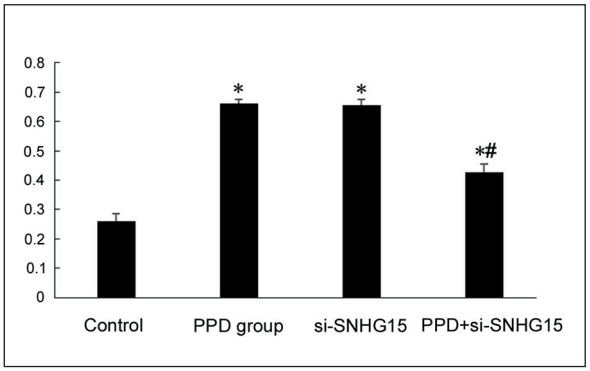

Figure 3. Effect of LncRNA SNHG15 on proliferation of RAW264.7 cells. Compared with the control group, $*p < 0.05$; compared with the PPD group, $^{\#}p < 0.05$.

Effect of LncRNA SNHG15 on the Formation of RAW264.7 Cells

The effect of lncRNA SNHG15 on the formation of RAW264.7 cells was analyzed by TRAP staining. The results showed that the formation of RAW264.7 cells was promoted in the PPD group, and the number of TRAP-positive cells was significantly increased compared with the control group ($p < 0.05$). Transfection of lncRNA SNHG15 siRNA significantly inhibited the formation of RAW264.7 cells and decreased the number of TRAP-positive cells ($p < 0.05$) (Figure 4).

Effect of LncRNA SNHG15 on the Secretion of Inflammatory Factors

The effect of lncRNA SNHG15 on the secretion of inflammatory factors IL-6 and TNF-α in RAW264.7 cells was analyzed by ELISA. The results showed that the secretion of the inflammatory factors IL-6 and TNF-α was significantly increased in the PPD group, and the difference was statistically significant ($p < 0.05$). Transfection of lncRNA SNHG15 siRNA significantly reduced the secretion of inflammatory factors IL-6 and TNF-α in RAW264.7 cells ($p < 0.05$) (Figure 5).

Effect of LncRNA SNHG15 on RANK/RANKL Expression

The effects of lncRNA SNHG15 on RANK/RANKL expression in RAW264.7 cells were analyzed by real-time PCR and Western blot and

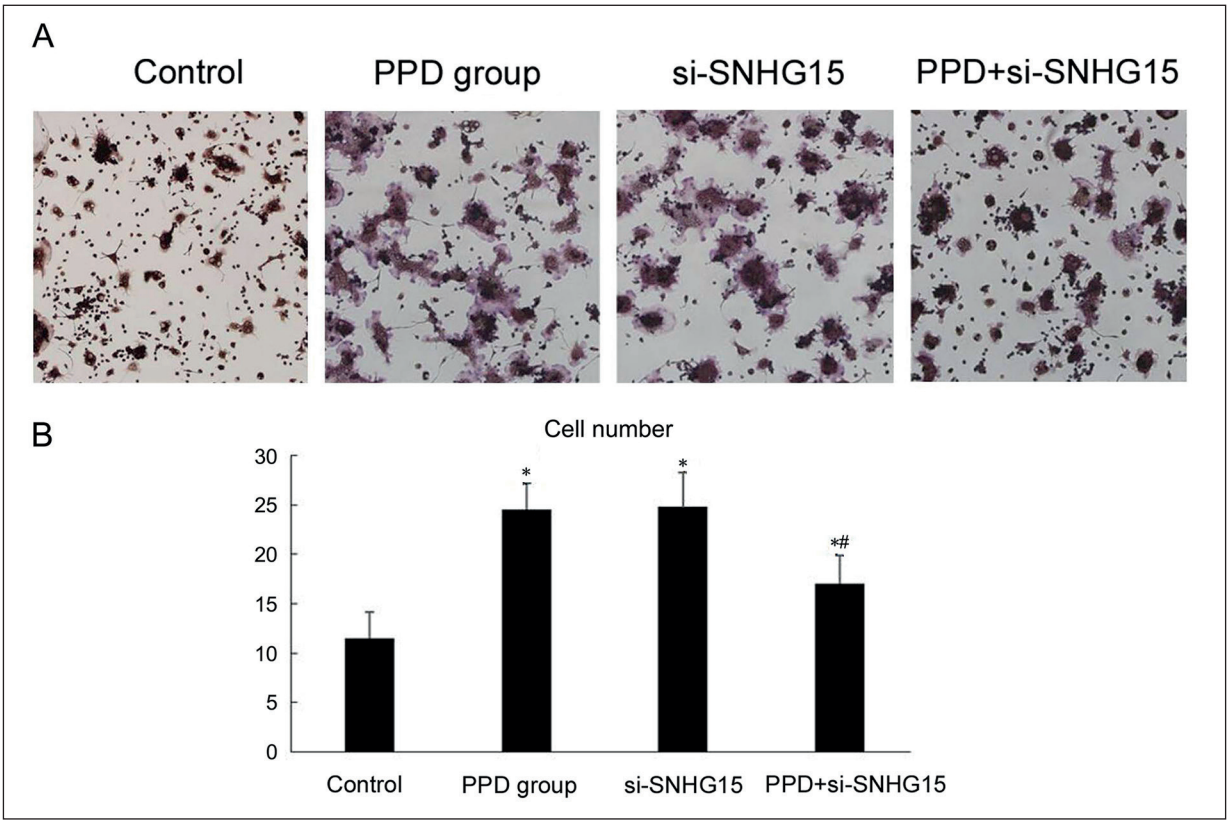

Figure 4. Effect of lncRNA SNHG15 on the formation of RAW264.7 cells. (**A**) TRAP staining analysis of the effect of lncRNA SNHG15 on the formation of RAW264.7 cells (200x); (**B**) regulation of the effect of lncRNA SNHG15 on the formation of RAW264.7 cells, compared with the control group, $*p < 0.05$; compared with the PPD group, $^{\#}p < 0.05$.

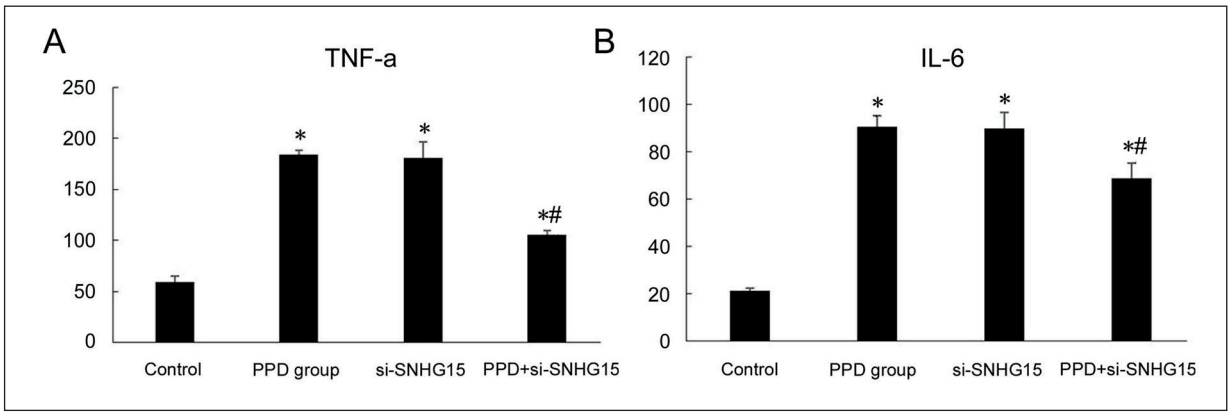

Figure 5. Effect of lncRNA SNHG15 on the secretion of the inflammatory factors in RAW264.7 cells. (**A**) ELISA assay for TNF-α secretion; (**B**) ELISA for IL-6 secretion, compared with the control group, $*p < 0.05$; compared with the PPD group, $^{\#}p < 0.05$.

showed that the expression of RANK/RANKL gene and protein was significantly increased in RAW264.7 cells in the PPD group, and the difference was statistically significant ($p < 0.05$). Transfection of lncRNA SNHG15 siRNA significantly inhibited the RANK/RANKL gene and protein expression in RAW264.7 cells, and the difference was statistically significant ($p < 0.05$) (Figure 6).

Discussion

Spinal tuberculosis can cause spinal deformities and even paraplegia. Spinal tuberculosis is more harmful to the human body than other types of bone and extrapulmonary tuberculosis[17]. LncRNAs, as long-chain non-coding RNAs, are involved in the regulation of several biological processes, including cell development, proliferation, differentiation, apoptosis, and cell death, although they are not involved in protein coding[18]. However, the role of lncRNA SNHG15 in spinal tuberculosis and its related regulatory effects have not been reported. This study confirmed that the expression of lncRNA SNHG15 in spinal tuberculosis tissues was significantly increased, suggesting that lncRNA SNHG15 may be involved in the occurrence and development of spinal tuberculosis.

Further, the aim of this study is to analyze the regulatory mechanism of lncRNA SNHG15 in spinal tuberculosis as osteoclasts play an important role in the infection of *Mycobacterium tuberculosis*, and the related mechanisms are very complicated[19]. On the one hand, it can partially play a role in resisting *Mycobacterium tuberculosis*. On the other hand, osteoclasts can be associated with *Mycobacterium tuberculosis*, causing them to survive in the body, thereby promoting latent and infection[20,21]. *Mycobacterium tuberculosis* can reduce the osteoclast apoptosis, inhibit necrosis, and reduce the sensitivity of osteoclasts to stimulating responses, thereby promoting the escape of *M. tuberculosis* and against the bactericidal effects of immune cells[22]. *Mycobacterium tuberculosis* is a typical intracellular parasite that can survive in osteoclasts, thereby evading immune system clearance[23]. The RANKL and RANK proteins are the most important pair of ligands and receptors in the osteoclast signaling system, and their increased expression promotes osteoclast formation and proliferation[24,25]. Therefore, this study was to analyze the regulation of lncRNA SNHG15 on PPD-acting osteoclast RAW264.7 cell line and showed that the expression of SNHG15 was increased in RAW264.7 cells in the PPD group, cell proliferation was increased, TRAP staining positive cells were increased, IL-6 and TNF-α secretion were increased, RANK and RANKL genes and protein expression were increased. However, PPD downregulation by transfection of SNHG15 siRNA can inhibit osteoclast proliferation, decrease TRAP staining positive cells, inhibit its formation, reduce inflammatory factor secretion, and reduce RANK and RANKL genes and proteins. These results suggest that by regulating PPD-infected osteoclasts, RANK/RANKL signaling can be inhibited, thereby inhibiting inflammatory factors secretion and reducing osteoclast formation and proliferation.

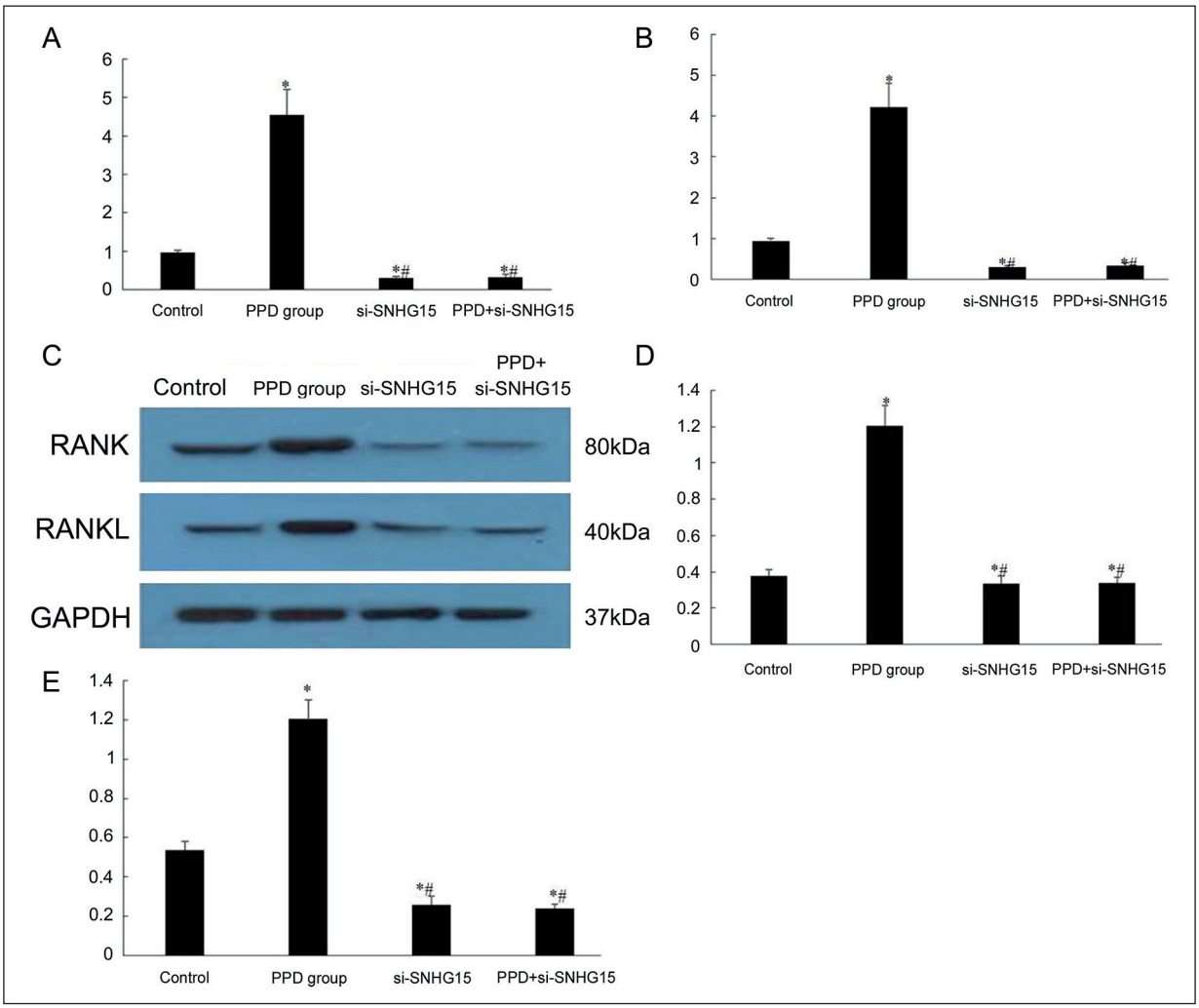

Figure 6. Effect of lncRNA SNHG15 on RANK/RANKL expression in RAW264.7 cells. (**A**) Real-time PCR analysis of lncRNA SNHG15 on RAW264.7 cell RANK gene expression; (**B**) Real-time PCR analysis of lncRNA SNHG15 on RAW264.7 cell RANKL gene expression; (**C**) Western blot analysis of RAW264.7 cell RANK/RANKL protein expression; (**D**) lncRNA SNHG15 analysis of RANK protein expression in RAW264.7 cells; (**E**) lncRNA SNHG15 analysis of RANKL protein expression in RAW264.7 cells; compared with the control group, $*p < 0.05$; compared with the PPD group, $^{\#}p < 0.05$.

However, the target genes and related regulatory effects of lncRNA SNHG15 in spinal tuberculosis remain unclear and need further study and analysis.

Conclusions

The expression of lncRNA SNHG15 in spinal tuberculosis tissues is significantly increased. The downregulation of the expression of SNHG15 in osteoclasts can inhibit the secretion of inflammatory cytokines by regulating the RANK/RANKL pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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