

lncRNA WT1-AS is upregulated in osteoporosis and regulates the apoptosis of osteoblasts by interacting with p53

CHAOQUN WANG^{1*}, QUAN XIE^{2*}, WEN SUN², YING ZHOU² and YU LIU³

¹Department of Nuclear Medicine, Hainan General Hospital, Haikou, Hainan 570311; ²Department of Nuclear Medicine; ³Cancer Institute Of Hainan Medical University, The First Affiliated Hospital of Hainan Medical University, Haikou, Hainan 570100, P.R. China

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Abstract. In cervical cancer, cellular tumor antigen p53 (p53) interacts with long non-coding WT1 antisense RNA (WT1-AS) and this protein serves an important role in osteoporosis. The present study aimed to investigate the role of WT1-AS in osteoporosis. WT1-AS was upregulated in the plasma of patients with osteoporosis and was positively correlated with p53 expression. Altered expression of WT1-AS and p53 separated patients with osteoporosis from healthy controls. Expression levels of WT1-AS and p53 decreased with prolonged treatment. In osteoblasts, WT1-AS overexpression resulted in increased p53 expression, while WT1-AS small interfering RNA (siRNA) silencing resulted in decreased p53 expression. In addition, WT1-AS overexpression resulted in increased apoptosis rate, while WT1-AS siRNA silencing resulted in decreased apoptosis rate in osteoblasts. p53 overexpression attenuated the effects of WT1-AS siRNA silencing on cell apoptosis. Therefore, WT1-AS was upregulated during osteoporosis and regulated the apoptosis of osteoblasts by interacting with p53.

Introduction

As a common type of skeletal disorder, osteoporosis is characterized by abnormal bone architecture and low bone mineral density, resulting in increased risk of fracture (1). Osteoporosis was once considered an inevitable disorder in the elderly population. Recently, multiple prevention and treatment approaches have been developed for osteoporosis (2). However, with the growth of the aging population, the incidence of osteoporosis is increasing worldwide, alongside increased treatment

costs (3). Therefore, novel therapeutic approaches with higher efficiencies are required to improve the treatment outcomes of osteoporosis. Osteoblasts and osteoclasts serve critical roles in bone formation and resorption, respectively (4,5). However, molecular mechanisms that are associated with this disease remain poorly understood (4,5), leading to difficulties in the development of novel therapeutic regimens.

p53 signaling is a well-studied pathway that plays pivotal roles in diverse cellular processes, such as cell cycle progression, genomic stability and cell apoptosis (6-8). p53 can regulate the apoptosis of osteoblastic cells (9), which plays a key role in the pathogenesis of osteoporosis (10). Inhibition of p53 suppresses the apoptosis of osteoblasts, thereby contributing to recovery from osteoporosis (11). It has been reported that the development and progression of osteoporosis also requires the involvement of long (>200 nt) non-coding RNAs (lncRNAs), which participates in diverse biological processes by regulating gene expression (12). It has been previously reported that WT1-AS can upregulate p53 in cervical cancer to inhibit cancer progression (13). Therefore, WT1-antisense RNA (WT1-AS) may also interact with p53 to participate in the development of osteoporosis. The present study was performed to explore the possible interaction between WT1-AS and p53 in osteoporosis.

Materials and methods

Research subjects. The present study included 60 patients with osteoporosis (23 males and 37 females; age range 33-66 years; mean age, 49.2±6.1 years) and 60 healthy volunteers (23 males and 37 females, 32-66 years; mean age, 49.6±6.3 years). All patients and healthy volunteers were admitted to the First Affiliated Hospital of Hainan Medical College between March 2015 and March 2018. The inclusion criteria of patients were as follows: i) Newly diagnosed cases; and ii) no initiated therapies. The exclusion criteria of patient were as follows: i) Recurrent cases; and ii) complications with other bone disorders or other types of diseases. All patients were informed of the experimental principles, and gave their signed informed consent. The Ethics Committee of the First Affiliated Hospital of Hainan Medical College approved this study prior to the admission of subjects. No significant differences in age, gender, body mass index, or smoking and drinking history were indicated between the two groups (data not shown). The T-score

Correspondence to: Dr Ying Zhou, Department of Nuclear Medicine, The First Affiliated Hospital of Hainan Medical University, 31 Longhua Road, Haikou, Hainan 570100, P.R. China
E-mail: tkfqttau642923@126.com

*Contributed equally

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was calculated using the following formula: T-score = (bone mineral density-reference bone mineral density)/reference standard deviation (14). The T-score of the patients ranged from -2.5 to -4.7 (mean score, -3.3 ± 0.4), while the T-score for healthy volunteers ranged from -0.9 to 3.3 (mean score, 1.3 ± 0.5). T-score was significantly lower in patients compared with controls (data not shown). The disease duration of patients ranged from 2.2 to 13.8 years, with a mean of 8.1 ± 2.4 years.

Patient treatment and plasma sample preparation. All 60 patients with osteoporosis were treated with bisphosphonates, including alendronate, risedronate, zoledronic acid and ibandronate. Estrogen was only used in females. Bisphosphonates attenuate bone loss, and estrogen was used in female patient to control postmenopausal symptoms (15). Drug doses were determined according to patients' health conditions, disease severity and mid-term treatment outcomes. Blood (5 ml) was extracted from each healthy volunteer and patient under fasting conditions prior to therapy initiation (1-3 days). The same amount of fasting blood was also extracted from each patient with osteoporosis at 3 months after therapy initiation. Blood samples were centrifuged at $1,200 \times g$ (room temperature) for 15 min to prepare plasma samples.

Transient transfection of osteoblasts. *In vitro* experiments were performed using primary osteoblasts purchased from Sigma-Aldrich; Merck KGaA. Primary osteoblasts were cultivated in Osteoblast Growth Medium (PromoCell GmbH) at 37°C. All subsequent experiments were performed using cells from passage 4 or 5. WT1-AS and p53 expression vectors (pcDNA3.1) and empty pcDNA3.1 vector, as well as small interfering RNA (siRNA) negative control and WT1-AS siRNA, were synthesized by Guangzhou RiboBio Co., Ltd. Osteoblasts (1×10^6) were transfected with 10 nM WT1-AS or p53 expression vector, 10 nM empty pcDNA3.1 vector (negative control, NC group), 40 nM WT1-AS siRNA, or 40 nM siRNA NC (NC group). Non-transfected osteoblasts acted as the control (C) group. Osteoblast RNA and protein were harvested at 24 h post-transfection for use in subsequent experiments.

ELISA. Human p53 ELISA kit (cat. no. ab46067; Abcam) was used to measure levels of p53 in plasma samples. All steps were performed following manufacturer's instructions. All plasma levels of p53 were expressed as pg/ml.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). A total of 1×10^6 osteoblasts and 2 ml of plasma was mixed with 1 ml of RNAzol (Sigma-Aldrich; Merck KGaA) to extract total RNAs. DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) was used to digest genomic DNA in all RNA samples at 37°C for 1 h. Following that, a qScript cDNA Synthesis kit (Quantabio) was used to perform RT (25°C for 10 min, 55°C for 20 min and 85°C for 10 min). qPCR reaction mixtures were prepared using a KAPA SYBR FAST qPCR Master Mix kit (Roche Diagnostics). GAPDH was used as an endogenous control. Primer sequences were: WT1-AS, 5'-GCC TCTCTGTCCTCTCTTTG-3' (forward), 5'-GCTGT GAGTC CTGGTGCTTA-3' (reverse); GAPDH, 5'-TTGGCATCGTTG AGGGTC-3' (forward), 5'-AGTGGGAACACGGAAAGC-3' (reverse); P53, 5'-AGAGTCTATAGGCCACCCC-3' (forward),

Table I. Association between patients' clinical data and WT1-AS and p53 expression.

A, WT1-AS			
Index	Expression		P-value
	High (n=30)	Low (n=30)	
Age			>0.05
≥50 years	14	15	
<50 years	16	15	
Sex			>0.05
Male	13	10	
Female	17	20	
Smoking			>0.05
Yes	11	10	
No	19	20	
Disease duration			<0.001
≥8 years	22	6	
<8 years	8	24	
T score			
≥-3.5	19	6	
B, p53			
Index	Expression		P-value
	High (n=30)	Low (n=30)	
Age			>0.05
≥50 years	14	15	
<50 years	16	15	
Sex			>0.05
Male	13	10	
Female	17	20	
Smoking			>0.05
Yes	11	10	
No	19	20	
Disease duration			<0.001
≥8 years	22	6	
<8 years	8	24	
T score			
≥-3.5	19	6	0.001

WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

5'-GCTCGACGCTAGGATCTGAC-3' (reverse). PCR cycling conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 58°C for 50 sec. The expression levels of WT1-AS and p53 mRNA were normalized using the $2^{-\Delta\Delta C_q}$ method (16).

Western blot analysis. Osteoblasts were lysed with 1 ml RIPA buffer (Guangzhou RiboBio Co., Ltd.) to extract total

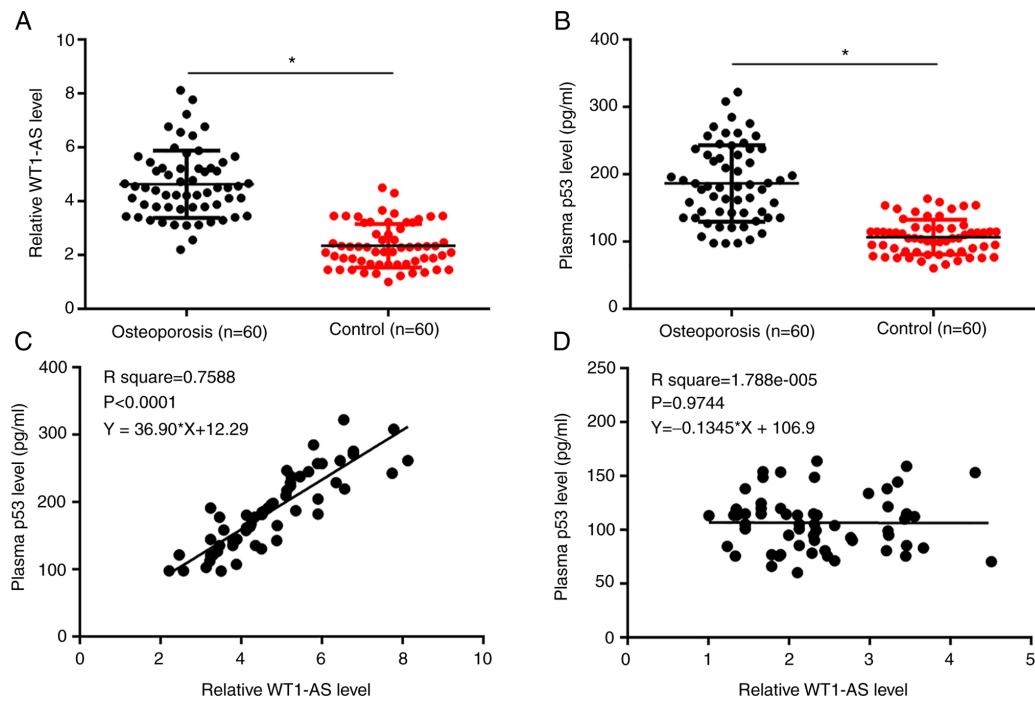


Figure 1. WT1-AS and p53 expression levels are positively correlated in patients with osteoporosis. Plasma levels of (A) WT1-AS and (B) p53 in plasma were measured using ELISA. Data between osteoporosis and control groups were compared by performing an unpaired t-test. Correlations between WT1-AS and p53 in (C) patients with osteoporosis and (D) healthy controls were analyzed using Pearson's correlation coefficient. * $P < 0.05$. WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

protein. Protein samples were quantified using a bicinchoninic acid kit (Sangon Biotech Co., Ltd.), followed by denaturation in boiling water for 5 min. Electrophoresis was performed using 12% SDS-PAGE to separate proteins (30 μg per well) according to their molecular weights. Proteins were transferred to a PVDF membrane and blocking was carried out in 5% non-fat milk for 2 h at room temperature. Primary antibodies of rabbit GAPDH (1:1,200; cat. no. ab181602; Abcam) and p53 (1:1,200; cat. no. ab131442; Abcam) were used to incubate the membranes for 15 h at 4°C. Horseradish peroxidase goat anti-rabbit (immunoglobulin G; 1:1,100; cat. no. ab6721; Abcam) secondary antibody was then used to blot the membranes further at room temperature for 2 h. The ECL Chemiluminescence Detection kit (Sangon Biotech Co., Ltd.) was used to develop protein signals. Gray values were normalized using ImageJ version 1.46 (National Institutes of Health).

Cell apoptosis analysis. A total of 6×10^4 osteoblasts were mixed with 1 ml serum-free aforementioned cell culture medium to prepare single-cell suspensions. Osteoblasts were cultivated at 37°C and 5% CO_2 in a 6-well plate with 2 ml of cell suspension in each well. Cells were incubated at 37°C for 48 h before being harvested and digested with 0.25% trypsin. Cells were subsequently stained using Annexin V-FITC (Thermo Fisher Scientific, Inc.) and propidium iodide (Thermo Fisher Scientific, Inc.) at 4°C for 20 min in dark. Apoptotic cells were analyzed using a flow cytometer. Data were processed using FCSalyzer Version 0.9.18 (SourceForge; DHI Group, Inc.).

Statistical analysis. The mean \pm SD values of three biological replicates of each experiment were calculated and used for all comparisons. Statistical power was calculated using Origin

software version 10 (OriginLab Corp.) and a statistical power of ~ 0.9 was obtained. Differences between patients and controls were measured using an unpaired t-test. Differences between two time points in the patient group were analyzed using a paired t-test. Differences among different cell transfection groups were investigated using a one-way ANOVA followed by Tukey's test. Correlations were analyzed using Pearson's correlation coefficient. A receiver operating characteristic (ROC) curve was plotted for diagnostic analysis. Patients with disease are grouped in the true positive class and healthy controls are grouped in the true negative class. Patients were divided into high and low WT1-AS/p53 level groups ($n=30$), using the mean expression levels of WT1-AS/p53 in osteoporosis as a cutoff score. Any association between patients' clinical data and WT1-AS/p53 expression was analyzed using a χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

WT1-AS and p53 levels are positively correlated in patients with osteoporosis. WT1-AS and p53 plasma levels were measured. Plasma levels of WT1-AS (Fig. 1A) and p53 (Fig. 1B) were significantly higher in patients with osteoporosis compared with the control group ($P < 0.05$). Correlations between WT1-AS and p53 were analyzed using Pearson's correlation coefficient. WT1-AS and p53 levels were significantly and positively correlated in patients with osteoporosis (Fig. 1C, $P < 0.0001$), but not in healthy controls (Fig. 1D, $P = 0.9744$). The T-score of the patients ranged from -2.5 to -4.7 . χ^2 test revealed that expression levels of WT1-AS and p53 were not associated with patients' age, sex and smoking habits, but were closely associated with T-score and disease duration (Table I).

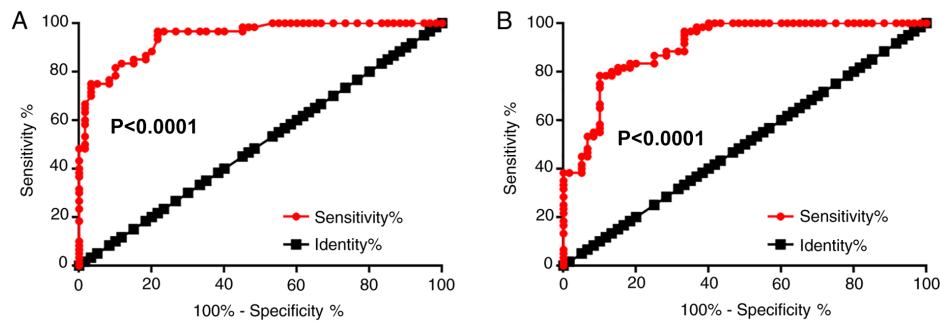


Figure 2. Altered expression levels of WT1-AS and p53 separate osteoporosis patients from healthy controls. The potential application of plasma (A) WT1-AS and (B) p53 for the diagnosis of osteoporosis was explored by plotting a receiver operating characteristic curve. WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

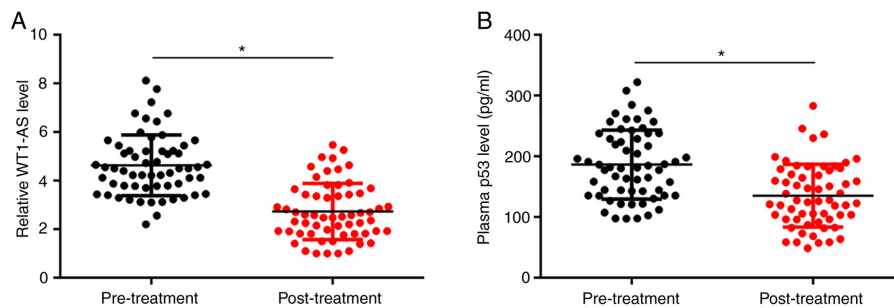


Figure 3. Bisphosphonate therapy downregulates WT1-AS and p53 plasma levels in osteoporosis patients. Plasma levels of (A) WT1-AS and (B) p53 were measured pre-treatment and at 3 months post-treatment. Plasma levels of WT1-AS and p53 were compared between the two time points by performing a paired t-test. * $P < 0.05$. WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

Altered expression of WT1-AS and p53 separates patients with osteoporosis from healthy controls. The potential application of plasma WT1-AS and p53 for the diagnosis of osteoporosis was explored by performing a ROC curve analysis. The area under the curve (AUC) of plasma WT1-AS was 0.94 (95% confidence interval, 0.91-0.98; standard error, 0.019; $P < 0.0001$; Fig. 2A), while the AUC of plasma p53 was 0.91 (95% confidence interval, 0.85-0.96; standard error, 0.026; $P < 0.0001$; Fig. 2B).

Bisphosphonate therapy downregulates WT1-AS and p53 plasma levels in patients with osteoporosis. Plasma levels of WT1-AS and p53 were measured during pre-treatment and at 3 months post-treatment. Levels of WT1-AS (Fig. 3A) and p53 (Fig. 3B) significantly decreased at 3 months post-treatment compared with pre-treatment levels ($P < 0.05$).

WT1-AS positively regulates p53 expression in osteoblasts. Osteoblasts were transfected with WT1-AS and p53 expression vectors and WT1-AS siRNA. WT1-AS and p53 expression was significantly altered compared with NC and C groups at 24 h post-transfection ($P < 0.05$; Fig. 4A). Moreover, WT1-AS overexpression resulted in upregulated p53 expression, while WT1-AS siRNA silencing resulted in downregulated p53 expression at mRNA and protein levels compared with the control and NC groups ($P < 0.05$; Fig. 4B).

WT1-AS promotes osteoblast apoptosis through p53. WT1-AS and p53 overexpression resulted in significantly increased apoptosis rates, while WT1-AS siRNA silencing resulted in

significantly decreased rates of osteoblast apoptosis compared with NC and C groups. In addition, p53 overexpression was indicated to attenuate the effect of WT1-AS siRNA silencing on cell apoptosis in comparison to cells with WT1-AS siRNA transfection alone ($P < 0.05$; Fig. 5).

Discussion

To date, the functions of WT1-AS have only been investigated in a few types of cancer (13,17,18). The participation of WT1-AS in cancer biology is mainly mediated by its roles in regulating cell behaviors such as proliferation, apoptosis and invasion (3,17,16). It is known that cell death in osteoblasts contributes to the pathogenesis of osteoporosis (19). The present study investigated the roles of WT1-AS in osteoporosis. WT1-AS was upregulated in osteoporosis and exhibited diagnostic values. In addition, WT1-AS was revealed to serve a role in osteoblast apoptosis and indicated an association with p53, which may indicate an interaction between the genes. Therefore, overexpression of WT1-AS may promote the progression of osteoporosis by promoting osteoblast apoptosis.

The diagnosis of osteoporosis mainly relies on the measurement of bone mineral density (BMD) (20). However, the threshold of BMD to diagnose osteoporosis is debated, and there is no way to use BMD to screen people with high risk of osteoporosis (20). In the present study, ROC curve analysis indicated that WT1-AS and p53 expression could be used to distinguish patients with osteoporosis from healthy controls. In addition, increased levels of WT1-AS and p53 were observed after treatment with bisphosphonates or estrogen. Therefore,

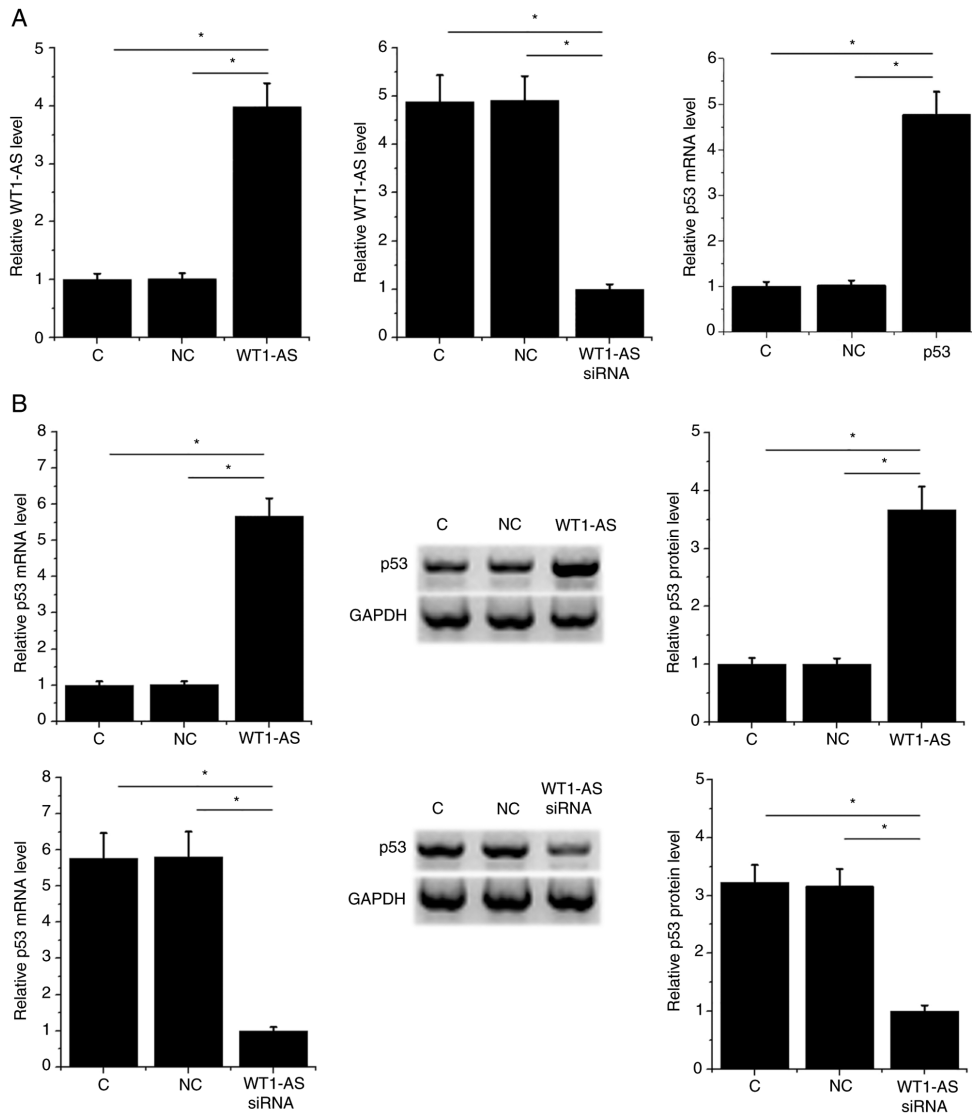


Figure 4. WT1-AS positively regulates p53 expression in osteoblasts. Osteoblasts were transfected with WT1-AS and p53 expression vectors and WT1-AS siRNA. (A) Plasmid and siRNA transfections were confirmed by RT-qPCR analysis. (B) The effects of WT1-AS overexpression and silencing on p53 expression were studied by performing western blotting and RT-qPCR. *P<0.05. siRNA, small interfering RNA; C, control; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

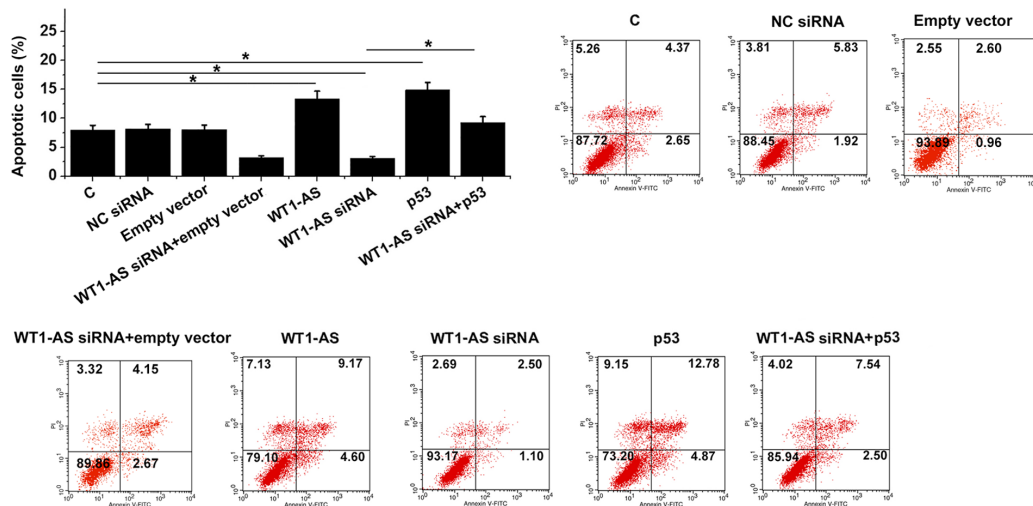


Figure 5. WT1-AS promotes osteoblast apoptosis through p53. The effects of WT1-AS and p53 overexpression as well as WT1-AS siRNA silencing on osteoblast apoptosis were analyzed by performing a cell apoptosis assay. Data among groups were compared by performing one-way ANOVA followed by Tukey's test, *P<0.05. siRNA, small interfering RNA; C, control; NC, negative control; WT1-AS, long non-coding WT1 antisense RNA; p53, cellular tumor antigen p53.

plasma WT1-AS and p53 may be used as a marker to predict osteoporosis. However, clinical trials are required to test sensitivity and specificity.

The results of the current study revealed that WT1-AS positively regulated the expression of p53 in osteoblasts. The lack of significant correlation between WT1-AS and p53 across the healthy controls suggested that the interaction between them was indirect. It is known that lncRNAs can sponge miRNAs to upregulate the expression of their downstream genes (21). Future studies should assess the involvement of miRNAs and its interaction with lncRNAs. WT1-AS can sponge miR-330-5p to regulate p53 in cervical cancer (13). Future studies should try to explore the involvement of miRNAs in this process.

The present study only investigated the expression of WT1-AS in plasma. Future studies should include patient osteoclasts and animal model experiments to further verify the results of the current study. The pathogenesis of osteoporosis is complicated and requires the involvement of multiple factors, such as epigenetic and hormonal factors (22-27). The interactions between WT1-AS and these factors are needed to be further analyzed.

However, the present study has limitations, including the small sample size. Future studies with bigger sampler sizes are required to further confirm the results of the current study. The involvement of p53-related apoptotic factors, including Bax and Bcl-2, was not explored and p53 knockdown experiments were not included. The current study did not include osteoblast functionality and proliferation assays and the analysis of changes in the expression of marker genes. Therefore, future studies are needed to examine these factors.

In conclusion, WT1-AS was demonstrated to be upregulated in osteoporosis and promoted the apoptosis of osteoblasts by upregulating p53.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW, QX and YZ designed experiments. CW and QX performed experiments. WS and YL analysed data. YZ drafted the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The Ethics Committee of the First Affiliated Hospital of Hainan Medical College approved this study prior to the admission of subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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