LncRNA AWPPH participates in the development of non-traumatic osteonecrosis of femoral head by upregulating Runx2

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Abstract. AWPPH is a newly discovered long noncoding (lnc)RNA that plays an oncogenic role in development of several types of malignancies, whiles its involvement in non-traumatic osteonecrosis of femoral head (ONFH) is unknown. Therefore, the present study aimed to investigate the functionality of AWPPH in non-traumatic ONFH. Blood and mesenchymal stem cells (MSCs) were obtained from both non-traumatic ONFH patients and healthy controls, and expression of AWPPH in those tissues was detected by RT-qPCR. Receiver operating characteristic curve analysis was performed to investigate the diagnostic value of lncRNA AWPPH expression for non-traumatic ONFH. Bone morphogenic protein (BMP-2) was used to treat MSCs to induce osteogenic differentiation and the effects on lncRNA AWPPH expression was detected by RT-qPCR. LncRNA AWPPH overexpression and short hairpin (sh)RNA silencing cell lines were established and the effects on runt-related transcription factor 2 (Runx2) expression were detected by western blotting. It was demonstrated that AWPPH was significantly downregulated in non-traumatic ONFH patients compared with in healthy controls in both MSCs and serum. Expression of AWPPH in MSCs and serum is a sensitive diagnostic marker for non-traumatic ONFH. Expression of AWPPH exhibited no significant correlation with patients' age, gender and living habits, but was significantly correlated with course of disease. BMP-2 treatment significantly increased the expression level of AWPPH in human MSCs from bone marrow (hMSC-BM). AWPPH overexpression promoted, while AWPPH short hairpin RNA silencing inhibited the expression of Runx2 expression in hMSC-BM cells. Therefore, it was concluded

that lncRNA AWPPH may participate in the development of ONFH by upregulating Runx2.

Introduction

As a bone-destructive disease that is caused by a disorder of the coagulation and fibrinolysis system and insufficient blood supply, osteonecrosis of the femoral head (ONFH) is a rare but disabling condition that usually leads to progressive femoral head collapse and secondary arthritis necessitating total hip arthroplasty (1). ONFH can be categorized into traumatic and non-traumatic types. As a subtype of ONFH, non-traumatic ONFH often occurs after the treatment of inflammatory diseases by corticosteroid therapy (2). It has been reported that the onset and development of non-traumatic ONFH is closely correlated with various factors including human immunodeficiency virus infection, autoimmune diseases, alcohol abuse, use of glucocorticoids and coagulopathies (3). However, up to now, the pathogenesis of this disease remains unclear.

In addition, messenger RNA (mRNA) that encode protein products of the human genome also transcribe large sets of non-coding RNAs that have no protein-coding ability (4). It has been reported that the development of non-traumatic ONFH is usually accompanied by changes in the expression of different types of non-coding RNA, such as microRNAs (miRNAs/miR) (5). However, the involvement of long non-coding (lnc)RNAs, which is a subgroup of non-coding RNAs composed of >200 nucleotides and with pivotal roles in both normal physiological and pathological processes (6) is largely unknown. AWPPH is a newly discovered lncRNA that plays an oncogenic role in the development of hepatocellular carcinoma (7) and bladder cancer (8), whiles its involvement in non-traumatic osteonecrosis of femoral head (ONFH) is unknown. Significantly downregulated expression of AWPPH in ONFH patients compared with in healthy controls was observed in the authors' preliminary microarray analysis (data not shown), indicating the possible involvement of AWPPH in ONFH. Therefore, a systemic investigation on the functionality of AWPPH in non-traumatic ONFH was carried out. It was demonstrated that lncRNA AWPPH can inhibit the development of non-traumatic ONFH by upregulating Runx2 expression. The present study provided new insights for the diagnosis and treatment of non-traumatic osteonecrosis.

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Materials and methods

Subjects. A total of 36 patients with ONFH were enrolled at the Luoyang Orthopedic Hospital of Henan Province from January 2015 to January 2017. Those patients included 20 males and 16 females, and were aged between 30 to 67 years, with a mean age of 48.3±7.9 years. The duration of disease ranged from 1 year to 12.5 years, with a mean duration of 5.8±2.1 years. At the same time, 30 healthy people with similar age and gender distributions were also included to serve as the control group. The control group included 16 males and 14 females, and were aged between 27 to 70 years, with a mean age of 49.1±6.6 years. All participants signed informed consent. This study has been approved by the ethics committee of Luoyang Orthopedic Hospital of Henan Province.

Specimen collection. EDTA-PBS (2 mM) was used to dilute bone marrow aspirates and Ficoll-Hypaque density gradient centrifugation was performed to isolate mononuclear cells. Cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1% antibiotic-antimycotic solution (Invitrogen; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in an incubator (37℃, 5% CO_2). Cells were harvested when mesenchymal stem cells (MSCs) reached 80-90% confluence. Then cells were incubated with 0.25% trypsin for passage. Whole blood (20 ml) was extracted from each participant on the day of admission. Serum samples were prepared by keeping whole blood at room temperature for 90 min, followed by centrifugation at 1,250 x g at room temperature for 20 min.

Cell line and cell culture. Human MSCs from bone marrow (hMSC-BM) was provided by ScienCell Research Laboratories. Cells were cultured in high-glucose DMEM containing 1% antibiotic (streptomycin)-antimycotic solution (Invitrogen; Thermo Fisher Scientific, Inc.) and 10% FBS in an incubator (37℃, 5% CO_2). MSCs were harvested when 80-90% confluence was reached. Cells were then incubated with 0.25% trypsin for passage. Serum was not added in case of drug treatment. After transfection, cells were culture for 48 h before subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MSCs and serum derived from patients were mixed with TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) to extract total RNA. In cases of bone morphogenetic protein-2 (BMP-2) treatment, MSCs were treated with BMP-2 (0, 25, 100 and 200 ng/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 12 h before use. NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, Inc.) was used to measure RNA concentration. RNA samples with a A260/A280 ratio between 1.8 and 2.0 were subjected to reverse transcription to synthesize cDNA using PrimeScript RT Reagent Kit (Takara Bio, Inc., Tokoyo, Japan) through following thermal conditions: 25℃ for 5 min, 55℃ for 20 min and 75℃ for 5 min. PCR mixtures were prepared using SYBR® Green Realtime PCR Master Mix (Toyobo Life Science, Tokoyo, Japan) PCR reactions were performed using primers listed below: 5'-CTGGATGGTCGCTGCTTTTTA-3' (forward) and 5'-AGGGGGATGAGTCGTGATTT-3' (reverse) for human lncRNA AWPPH; 5'-CGGCCCTCCCTGAAC TCT-3' (forward) and 5'-TGCCTGCCTGGGGTCTGTA-3' (reverse) for human Runx2; 5'-GACCTCTATGCCAACACA GT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for human β-actin. PCR reaction conditions were: 95℃ for 35 sec, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 42 sec. Data were processed using $2^{-\Delta\Delta Cq}$ method (9) and expression of AWPPH and Runx2 was normalized to β-actin.

Establishment of AWPPH overexpression and short hairpin (sh)RNA silencing cell lines. AWPPH cDNA was inserted into pIRSE2-EGFP vector (Clontech Laboratories, Inc., Mountainview, Palo Alto, CA, USA) to construct AWPPH expression vector. AWPPH shRNA expression vector and scrambled shControl were provided by Shanghai GenePharma Co., Ltd., (Shanghai, China). The target site for AWPPH shRNA was GGTCTGGTCGGTTTCCCATTT. hMSC-BM cells were cultured overnight to reach 80-90% confluence and transfection was performed using Lipofectamine 2000 reagent (11668-019; Invitrogen; Thermo Fisher Scientific Inc.) to transfect 10 nM vectors into 5x10⁵ cells. Empty pIRSE2-EGFP vector and scrambled shControl (5'-CCTAAGGTTAAGTCG CCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3') were as negative controls. Cells without transfection were used as control.

Western-blot. RIPA buffer (Cell Signaling Technology, Inc.) was mixed with *in vitro* cultured hMSC-BM cells to extract total protein. Protein concentration was measured using bicinchoninic acid method. Protein samples were denatured and subjected to 10% SDS-PAGE gel electrophoresis (20 µg per lane), followed by transmembrane to PVDF membrane. After blocking in PBS containing 5% non-fat milk for 2 h at room temperature, incubation with primary antibodies including rabbit anti-Runx2 antibody (1:2,000; cat. no. ab23981; Abcam) and anti-GAPDH (1:2,000; cat. no. ab8245; Abcam) was performed overnight at 4℃. After washing, membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc.) at room temperature for 1 h. After washing, Amersham™ ECL™ Western Blotting Reagent (Sigma-Aldrich, Merck KGaA) method was used to develop signal. Image J v1.46 software (National Institutes of Health, Bethesda, MD, USA) was used to normalize relative expression of Runx2 to GAPDH.

Statistical analysis. SPSS19.0 (IBM Corps., Armonk, NY, USA) was used for all statistical analyses in this study. Count data (basic clinical data) were processed using a Chi-square test. Comparisons of measurement data between two groups and among multiple groups were performed by unpaired Student's t-test and one-way analysis of variance followed by least significant difference test, respectively. Receiver operating characteristic (ROC) curve analysis was performed using the default parameters and the software automatically output images. P<0.05 was considered to indicate a statistically significant difference.
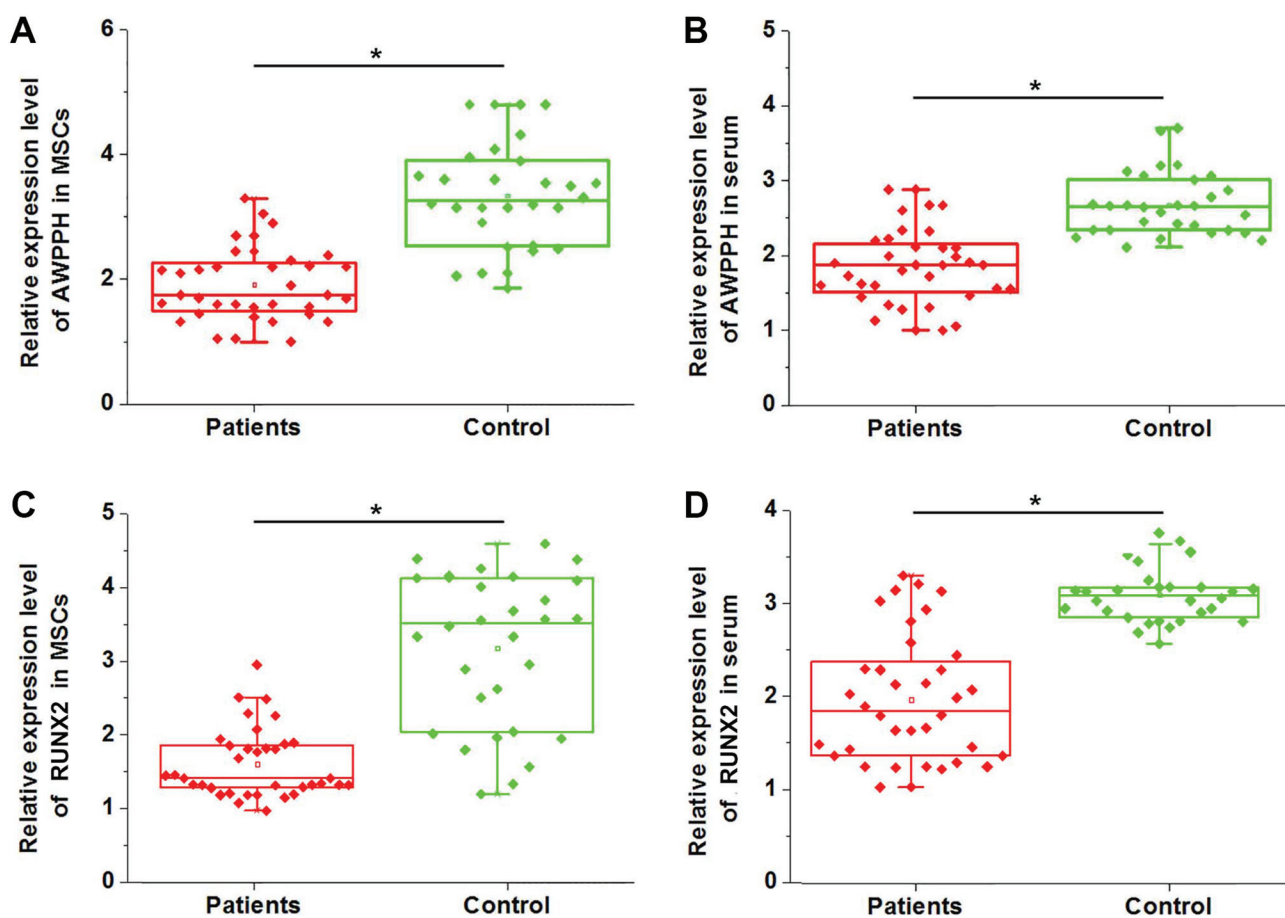
Figure 1. Expression of lncRNA AWPPH and Runx2 mRNA in MSCs and serum derived from non-traumatic ONFH patients and healthy people. This figure presents the expression of lncRNA AWPPH in (A) MSCs and (B) serum and the expression of Runx2 mRNA in (C) MSCs and (D) serum collected from patients with non-traumatic ONFH and healthy people. *P<0.05. MSCs, mesenchymal stem cells; Runx2, runt-related transcription factor 2; lnc, long noncoding; ONFH, osteonecrosis of femoral head.

Results

Expression of lncRNA AWPPH and Runx2 mRNA in MSCs and serum derived from non-traumatic ONFH patients and healthy people. Expression of lncRNA AWPPH and mRNA in MSCs and serum collected from non-traumatic ONFH patients and healthy people was detected by RT-qPCR. As presented in Fig. 1A, expression of lncRNA AWPPH in MSCs was significantly decreased in patients with non-traumatic ONFH compared with in healthy people (P<0.05). Similarly, expression of lncRNA AWPPH in serum was also significantly downregulated in patients with non-traumatic ONFH compared with in healthy people (P<0.05; Fig. 1B). In addition, expression of Runx2 in MSCs (Fig. 1C) and serum (Fig. 1D) was also downregulated in ONFH patients compared with in the controls. Those data suggest that downregulation of lncRNA AWPPH and Runx2 is likely to be involved in the pathogenesis of non-traumatic ONFH.

Diagnostic value of lncRNA AWPPH expression in MSCs and serum for patients with non-traumatic ONFH. ROC curve analysis was performed to analyze the diagnostic value of lncRNA AWPPH expression in MSCs and serum for patients with ONFH. As presented in Fig. 2A, the area under the curve (AUC) of the use of lncRNA AWPPH expression in MSCs

for the diagnosis of non-traumatic ONFH was 0.8259 with 95% confidence interval of 0.7417 to 0.9100 (P<0.0001; data not shown). In addition, AUC of the use of lncRNA AWPPH expression in serum for the diagnosis of non-traumatic ONFH was 0.8177 with 95% confidence interval of 0.7310 to 0.9044 (P<0.0001).

Association between lncRNA AWPPH expression in MSCs and serum with the clinicopathological data of patients with non-traumatic ONFH. Chi-square analysis was performed to analyze the association between lncRNA AWPPH expression in MSCs and serum and the clinicopathological data of patients with non-traumatic ONFH. As presented in Tables I and II, lncRNA AWPPH expression in MSCs and serum exhibited no significant association with age, gender and living habits of patients with non-traumatic ONFH (P>0.05). However, a significant association between AWPPH expression and course of disease was observed (P<0.05).

BMP-2 induces the expression of lncRNA AWPPH in hMSC-BM cells. BMP-2 is an inducer of osteoblastic differentiation. In the present study, different concentrations of BMP-2 (0, 25, 100 and 200 ng/ml) were used to treat hMSC-BM cells for 12 h and expression of lncRNA was detected by RT-qPCR. As presented in Fig. 3, BMP-2 significantly upregulated the
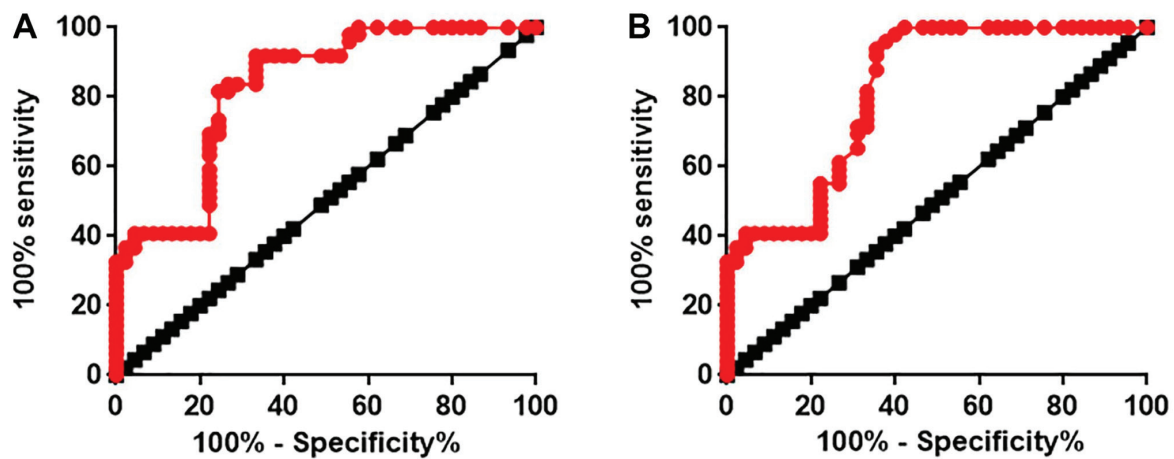
Figure 2. Diagnostic value of lncRNA AWPPH expression in MSCs and serum for patients with non-traumatic ONFH. This figure demonstrated the receiver operating characteristics curve of the uses of AWPPH expression in (A) MSCs and (B) serum for the diagnosis of non-traumatic ONFH. MSCs, mesenchymal stem cells; lnc, long noncoding; ONFH, osteonecrosis of femoral head.

expression of lncRNA AWPPH in a dose-dependent manner (P<0.05).

Effects of AWPPH overexpression and shRNA silencing on expression of Runx2 in hMSC-BM cells. Effects of AWPPH overexpression and shRNA silencing hMSC-BM cell lines were constructed and confirmed by measuring the expression level of AWPPH by RT-qPCR. Runx2 is a marker of osteoblastic differentiation. Therefore, the effects of altered AWPPH overexpression on Runx2 expression were investigated. The results demonstrated that AWPPH overexpression significantly promoted (P<0.05; Fig. 4A) and shRNA silencing significantly inhibited (P<0.05; Fig. 4B) the expression of Runx2 in hMSC-BM cells.

Discussion

The onset of non-traumatic ONFH requires the involvement of multiple non-coding RNAs. In a study on corticosteroid-induced non-traumatic ONFH, Li *et al* (10) identified 11 differentially expressed miRNAs, indicating the involvement of miRNAs in the development of this disease. In another study, Wei *et al* (11) found that miR-17-5p was downregulated and lncRNA HOTAIR was upregulated in non-traumatic ONFH, and lncRNA HOTAIR overexpression inhibited the expression miR-17-5p to participate in the development of non-traumatic ONDH by regulating osteogenic differentiation and proliferation. AWPPH is a novel lncRNA with significantly upregulated expression in the development of hepatocellular carcinoma (7) and bladder cancer (8). In the present study, expression levels of lncRNA AWPPH in MSCs and serum were identified to be increased in non-traumatic ONFH patients compared with healthy people. The results of the present study suggest that, in addition to cancer, lncRNA AWPPH may also participate in the pathogenesis of other human diseases, such as non-traumatic ONFH.

As a rare bone-destructive disease, non-traumatic ONFH is sometimes misdiagnosed by physicians using traditional diagnostic methods, such as preoperative radiograph images, magnetic resonance images (12). Therefore, highly sensitive biomarkers are needed to improve the diagnosis of this disease. It has been reported that serum levels of α-melanocyte stimulating factor (MSH) were significantly decreased in non-traumatic ONFH patients compared with in healthy people and were further decreased with the progression of disease, and the reduced expression level of α-MSH is a sensitive diagnostic marker for non-traumatic ONFH (13). In the present study, ROC curve analysis demonstrated that expression of lncRNA AWPPH in both MSCs and serum can be used to effectively distinguish non-traumatic ONFH patients from healthy people, indicating that lncRNA AWPPH expression may serve as a promising diagnostic marker for non-traumatic ONFH. In addition, as a less invasive technique, detection of lncRNA AWPPH in serum through blood extraction should be a preferred method. Expression of lncRNAs can be altered under certain conditions, such as aging (14), alcohol abuse (15) and tobacco consumption (16), which may affect the accuracy of the use of lncRNAs in the diagnosis of diseases. In the present study, lncRNA AWPPH expression in MSCs and serum demonstrated no significant correlations with the age, gender and smoking and drinking habits of patients with non-traumatic ONFH, indicating the high accuracy of the use of AWPPH in the diagnosis of non-traumatic ONFH. However, it is worth noting that altered expression of lncRNA AWPPH has been observed in different types of human diseases (7,8). There, multiple markers should be used to improve the specificity of the diagnosis of non-traumatic ONFH.

Altered osteoblastic differentiation is a key pathological change during the development of non-traumatic ONFH (17). BMP-2 induces osteoblastic differentiation (18). In this study, BMP-2 induces the expression of lncRNA AWPPH in hMSC-BM cells in a dose-dependent manner, indicating the involvement of lncRNA AWPPH in the regulation of osteoblastic differentiation. Runx2 is marker of osteoblastic differentiation (19) and increased expression level of Runx2 promotes osteoblastic differentiation (20), while downregulation of Runx2 may promote the progression of non-traumatic ONFH (17). In this study, AWPPH overexpression promoted and shRNA silencing inhibited the expression of Runx2 in hMSC-BM cells. The results of the present study suggest that

Table I. Correlation between long noncoding RNA AWPPH expression in mesenchymal stem cells and the clinicopathological data of patients with non-traumatic osteonecrosis of femoral head.

Variables	Groups	Cases	High-expression	Low-expression	χ^2	P-value
Gender	Male	20	11	9	0.45	0.5
	Female	16	7	9		
Age	>45 (years)	21	12	9	1.03	0.31
	<45 (years)	15	6	9		
Course of disease	>5 years	17	12	5	5.45	0.02
	<5 years	19	6	13		
Smoking	Yes	16	9	7	0.45	0.5
	No	20	9	11		
Drinking	Yes	14	6	8	0.47	0.49
	No	22	12	10		

Table II. Correlation between long noncoding RNA AWPPH expression in serum and the clinicopathological data of patients with non-traumatic osteonecrosis of femoral head.

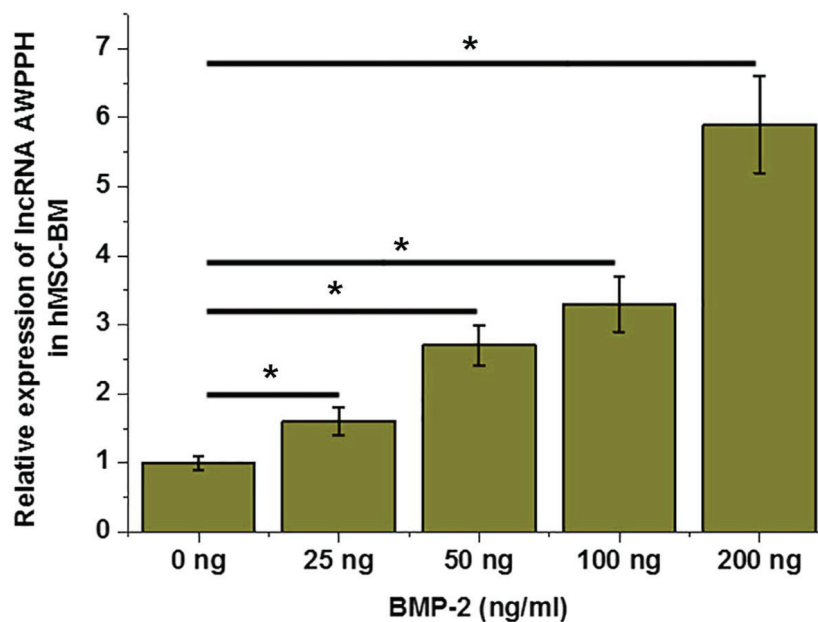| Items	Groups	Cases	High-expression	Low-expression	χ^2	P-value
Gender	Male	20	12	8	1.8	0.18
	Female	16	6	10		
Age	>45 (years)	21	12	9	1.03	0.31
	<45 (years)	15	6	9		
Course of disease	>5 years	17	12	5	5.45	0.02
	<5 years	19	6	13		
Smoking	Yes	16	10	6	1.8	0.18
	No	20	8	12		
Drinking	Yes	14	6	8	0.47	0.49
	No	22	12	10		

Figure 3. Expression of lncRNA AWPPH in hMSC-BM cells under treatment of different concentrations of BMP-2 (0, 25, 100 and 200 ng/ml). *P<0.05. hMSCs-BM, human mesenchymal stem cells-bone marrow; lnc, long noncoding; BMP-2 bone morphogenic protein-2.
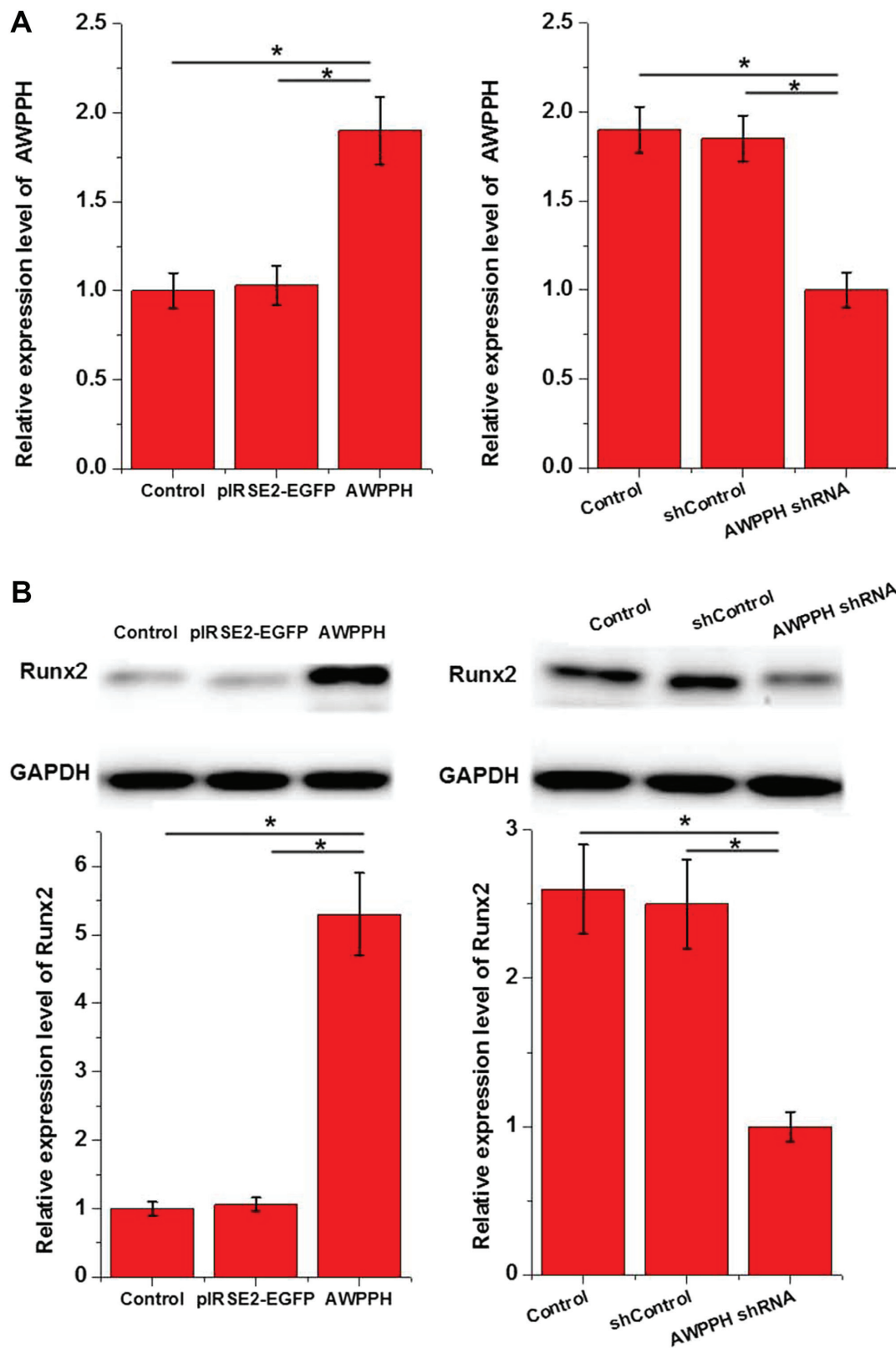
Figure 4. Effects of AWPPH overexpression and shRNA silencing on expression of Runx2 in hMSC-BM cells. *P<0.05; Negative control cells transfected with empty vector (left) or negative control shRNA (right). This figure exhibits the effects of AWPPH overexpression and shRNA silencing on expression of (A) AWPPH and (B) Runx2 in hMSC-BM cells. Sh, short harpin; hMSCs-BM, human mesenchymal stem cells-bone marrow; Runx2, runt-related transcription factor 2.

AWPPH may inhibit the development of non-traumatic ONFH by promoting osteoblastic differentiation through the upregulation of Runx2 expression.

In conclusion, AWPPH was downregulated in non-traumatic ONFH patients compared with in healthy controls. Expression of AWPPH is a sensitive diagnostic marker for non-traumatic

ONFH, especially for patients with longer duration of disease. AWPPH overexpression promoted, while AWPPH shRNA silencing inhibited the expression of Runx2 expression in hMSC-BM cells. Therefore, it was concluded that lncRNA AWPPH may participate in the development of non-traumatic osteonecrosis of femoral head by upregulating Runx2.

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XC designed experiments. XC, JL and DL performed experiments. LZ and QW analyzed data. XC drafted the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

This study has been approved by the ethics committee of Luoyang Orthopedic Hospital of Henan Province. All participants signed informed consent.

Patient consent for publication

All patients signed informed consent for the publication of data in the present study.

Competing interests

The authors declare that they have no competing interests.

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