

Notoginsenoside R1 promotes differentiation of human alveolar osteoblasts in inflammatory microenvironment through inhibiting NF- κ B pathway and activating Wnt/ β -catenin pathway

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Abstract. Alveolar bone is vital for dental implantation and periodontal treatment. Notoginsenoside R1 (NTR1) may promote the differentiation of human alveolar osteoblasts (HAOBs), but the underlying molecular mechanisms remain unclear. The present study investigated the pro-differentiation function of NTR1 on HAOBs in order to find new methods of dental treatment. HAOBs were surgically obtained from dental patients and the cells were isolated, cultured and identified under an inverted phase contrast microscope. The cells were treated with different concentrations of NTR1 alone or further stimulated by TNF- α . An alkaline phosphate (ALP) activity assay and alizarin red staining were performed to detect ALP activity and mineralization of the cells, respectively. Cell viability was assayed using an MTT assay. The expressions of osteogenic-related factors and the factors associated with the NF- κ B and Wnt/ β -catenin pathways were examined by reverse transcription-quantitative PCR or western blot analysis. Successfully passaged HAOBs presented blue granules and red calcium deposits after staining. The viability of HAOBs was unchanged following treatment with NTR1 at $\leq 20 \mu\text{mol/l}$ and/or TNF- α , but slightly reduced by $40 \mu\text{mol/l}$ NTR1. TNF- α -induced decreases of calcium nodules and ALP activity were decreased by NTR1 in HAOBs. TNF- α also regulated the expressions of runt-related transcription factor 2, osteopontin (OPN), osteocalcin (OCN), p50, phosphorylated p65, AXIN2, Dickkopf-related protein 1 and β -catenin, while the regulatory effect was reversed by NTR1. NTR1 promoted the differentiation of HAOBs in the TNF- α -induced inflammatory microenvironment through inhibiting the NF- κ B pathway and activating the Wnt/ β -catenin pathway.

Introduction

Alveolar bones in the mouth-jaw system are unique and dynamic. They can not only distribute occlusal forces and regulate tooth eruption, but also connect the external environment to periodontium and the interior of the body through the periodontium (1). Accordingly, external mechanical stimuli, such as tooth extraction, will trigger cellular and morphological changes of alveolar bones, accompanied by inflammation (2,3). These properties are vital in the remodeling process of alveolar bones and the quality and quantity of alveolar bones are also essential for the success and maintenance of dental implant treatment for partially or fully edentulous patients (4,5) and for maintaining the health of stomatognathic system (6). Although dental implantation and cures possess a high success rate at present (5), problems and risks still exist, for example, 1-2% of patients have primary implant failure in the first few months due to inadequate bone integration (7). Therefore, understanding the molecular mechanism of alveolar bone remodeling may provide a new way for effective treatment of periodontitis.

Notoginsenoside R1 (NTR1) is the main functional ingredient derived from *Panax notoginseng*, a traditional Chinese herbal medicine (8). Previous studies have shown that NTR1 has cardio-protective and neuro-protective effects (9-11). In addition, NTR1 has the function of promoting osteoblastogenesis through regulating runt-related transcription factor 2 (RUNX2) and activating the Smad-independent signaling pathway p38/MAPK (12). It can also inhibit osteolysis, osteoclastogenesis and bone absorption by suppressing the receptor activator of NF- κ B ligand (RANKL)-regulated MAPK signaling pathway and NF- κ B signaling pathway (13). NTR1 has an anti-inflammatory effect on inflammatory bowel disease by elevating the expressions of xenobiotic-metabolizing enzymes and downregulating the activity of the NF- κ B signaling pathway (14). Furthermore, NTR1 can mediate inflammation induced by oxidized low-density lipoprotein and endotoxin and involving the NF- κ B signaling pathway (8,15). However, studies on the association between NTR1 and alveolar osteoblasts are limited.

The Wnt/ β -catenin signaling pathway serves an essential role in embryo growth and tissue homeostasis (16). It has been confirmed that the Wnt/ β -catenin signaling pathway not only regulates whole-body energy metabolism

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in osteoblasts involving glucose homeostasis, energy expenditure and fat accumulation (17), but also modulates the viability and health of the skeleton (18) and predominantly regulates osteoblast differentiation (19). It has been reported that parthenolide promotes the differentiation of osteoblasts by activating the Wnt/ β -catenin pathway and inhibiting the NF- κ B/p50 and ERK signaling pathways (20). NTR1 promotes the growth of cortical neurons of neonatal rats via the Wnt/ β -catenin signaling pathway (21), but whether NTR1 has an effect on the differentiation of human alveolar osteoblasts (HAObSs) involving this signaling pathway remains to be elucidated.

The present study hypothesized that NTR1 could function as a regulator in the differentiation process of HAObSs. To the best of the authors' knowledge, the present study the first to investigate the regulatory effects of NTR1 on the differentiation of HAObSs through regulation of the NF- κ B and Wnt/ β -catenin signaling pathways in the inflammatory microenvironment induced by TNF- α , which may bring new understanding of dental implantation and periodontal treatment in clinical dentistry, to the benefit of dental patients.

Materials and methods

Alveolar osteoblasts specimen collection. The present study enrolled 163 patients with normal gums who needed tooth extraction due to impacted teeth or orthodontic treatment between March 2016 and March 2019. X-rays were used to further screen the patients without periapical tissue lesions and alveolar bone resorption. In addition, patients suffering from systemic diseases, infectious diseases, diseases that affect bone metabolism, or being treated with drugs that affect bone metabolism were excluded. Finally, a total of 40 patients (19 males and 21 females aged 25-40 years old; median age, 32.5 years) were included. Next, 0.5 cm² alveolar bone tissues were surgically collected from the alveolar socket wall of the individuals. The current study was approved by the Ethics Committee of the Jingmen Number 1 People's Hospital (Jingmen, China; approval no. JMPH20160123). All patients signed informed consent and agreed to their tissues being used for clinical research.

Cell isolation and cell culture. Alveolar bone samples were cleaned and maintained in phosphate buffer saline (PBS; Thermo Fisher Scientific, Inc.) containing 100 units/ml penicillin (TargetMol) and 100 μ g/ml streptomycin (TargetMol). The alveolar bone granules were then digested in 0.25% of trypsin digestion solutions (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 20 min and further digested in a mixture of 0.1% collagenase type I (Sigma-Aldrich; Merck KGaA) and 0.1% collagenase type II (Sigma-Aldrich; Merck KGaA) at a 1:1 ratio. The digested alveolar bone granules were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin (TargetMol) and 100 μ g/ml streptomycin (TargetMol) at 37°C for 12 h. Osteogenic medium (OM) was used for osteoblast differentiation, which consisted of high-glucose DMEM supplemented with 10% FBS,

100 units/ml penicillin, 100 μ g/ml streptomycin, 10 mM β -glycerophosphate (APeXBio Technology LLC), 50 mM ascorbic acid (BioVision, Inc.) and 100 mM dexamethasone (TargetMol), subsequently, the granules were incubated under saturated humidity and 5% CO₂ at 37°C for 14 days and the medium was replaced by new medium every three days. Third generation cells were used for further experiments. Images of cell passages were captured using an inverted phase contrast microscope IX53 (Olympus Corporation). The third generation cells were stained using an alkaline phosphate (ALP) staining assay and Alizarin Red S staining assay for identification.

ALP staining assay. ALP staining assay was performed using an Alkaline Phosphatase Stain kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's instructions. HAObSs of third generation were used to make cell slides. Following fixation with 4% formaldehyde for 4 h at room temperature, the cultured cells were treated with a staining solution containing 3 ml buffer solution, 10 μ l 300XBCIP reagent and 20 μ l 150XNBT reagent. The cells were incubated at room temperature for 5-30 min in the dark. Finally, the staining solution was removed and the cells were examined using an Invitrogen EVOS M700 Imaging system (Invitrogen; Thermo Fisher Scientific, Inc.).

Alizarin Red S staining assay. Alizarin Red S (Sigma-Aldrich; Merck KGaA) was used to identify and evaluate the mineralization of HAObSs. For cell identification, HAObSs of third generation were produced as a cell smear and stained at 37°C for 5 min with 1% Alizarin Red S and examined under an Invitrogen EVOS M7000 Imaging system after being rinsed with distilled water.

For mineralization evaluation, HAObSs were divided into five groups: Control group (the cells treated with DMEM for 14 days at 37°C); OM group (the cells treated with OM for 14 days at 37°C); TNF- α group (the cells stimulated by 10 ng/ml TNF- α for 14 days at 37°C); TNF- α + NTR1 2.5 group (the cells were pre-treated with 2.5 μ mol/l NTR1 and stimulated by 10 ng/ml TNF- α for 14 days at 37°C) and TNF- α + NTR1 20 group (the cells were pre-treated with 20 μ mol/l NTR1 and stimulated by 10 ng/ml TNF- α for 14 days at 37°C). The treated cells were fixed with 4% formaldehyde for 4 h at room temperature and then stained with 1% Alizarin Red S at room temperature for 20 min. Microscopic examinations were performed under an inverted phase contrast microscope IX53 (Olympus Corporation) after the cells had been washed with distilled water.

ALP activity assay. Alkaline Phosphatase Activity Detection kit (Shanghai Yeasen Biotechnology Co., Ltd.) was used to measure the ALP activity of HAObSs treated with TNF- α and NTR1 according to the manufacturer's instructions. The cells were pre-treated by OM for osteoblast differentiation and then stimulated with 10 ng/ml TNF- α for 7 and 14 days at 37°C. The cells were lysed in 0.2% Triton X-100 and then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant of the medium was collected for the detection of ALP activity under a microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 405 nm.

MTT assay. MTT assay was performed to detect cell viability during cell culture process. HAOBs were treated with different concentrations of NTR1 (2.5, 5, 10, 20 and 40 $\mu\text{mol/l}$) for 1, 3 and 7 days at 37°C. Cytotoxicity was assessed to confirm the influence of NTR1 on cell viability. The combined effects of NTR1 and TNF- α on the cells were also tested. A total of 10 μl MTT solution (5 mg/ml, Sigma-Aldrich; Merck KGaA) was added to each well, followed by incubation with 5% CO₂ at 37°C for 4 h. Formazan crystals were dissolved in 100 μl DMSO at 37°C. Optical density (OD) value was measured at 495 nm with a microplate reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Gene expression changes caused by NTR1 and TNF- α were analyzed using RT-qPCR. HAOBs (1×10^6) were pre-treated with or without 2.5 and 20 $\mu\text{mol/l}$ of NTR1 and then placed into an inflammatory microenvironment induced by TNF- α . Total RNAs were extracted from the cells using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols, and then the concentration of the total RNAs was determined using UV spectrophotometer DR3900 (Hach). A TaqMan microRNA reverse transcription kit (Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions to synthesize first-strand cDNAs. RT-qPCR was then performed with BlazeTaq™ SYBR Green RT-qPCR Mix 2.0 (BioCat GmbH) according to the manufacturer's protocols using PCR instrument CFX96 Touch (Bio-Rad Laboratories, Inc.), and the reaction system with 10 μl total volume was prepared. The conditions for RT-qPCR were 1 cycle of 95°C for 30 sec, followed by 40 cycles of at 95°C for 10 sec and at 60°C for 30 sec. The comparative cycle threshold method ($2^{-\Delta\Delta C_q}$) was used to calculate the relative expression of each mRNA (22). The experiment was repeated three times. The primer sequences of the genes used in the experiment are listed in Table I.

Western blot assay. HAOBs were cultured in OM or DMEM, pretreated with 2.5 and 20 $\mu\text{mol/l}$ NTR, alone or further stimulated with TNF- α for 14 days at 37°C. Then western blotting was performed to measure the expression levels of proteins related to NF- κB pathways (at 30- and 60-min culture) and Wnt/ β -catenin pathways (at 3- and 7-day culture). HAOBs were stimulated with TNF- α , with or without NTR1 (2.5 and 20 $\mu\text{mol/l}$) for 14 days at 37°C. Total proteins were extracted from HAOBs using Pierce RIPA buffer (Thermo Fisher Scientific, Inc.) for 30 min on ice. Then centrifugation was performed for 10 min at 14,000 $\times g$ at 4°C. A bicinchoninic acid protein quantitative kit (Sigma-Aldrich; Merck KGaA) was used to determine protein concentration according to the manufacturer's instructions. Protein (10 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Thermo Fisher Scientific, Inc.), placed in a water bath with boiling water for 3 min and transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). The transferred membranes were blocked with 5% non-fat dried milk for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. The primary antibodies were p50 (1:1,000; cat no. 13586; Cell Signaling Technology, Inc.), phosphorylated (p-)p65 (1:1,000; cat no. 3039; Cell Signaling Technology, Inc.), p65 (1:1,000; cat no. 8242; Cell

Signaling Technology, Inc.), GAPDH (1:1,000; cat no. 4292; Cell Signaling Technology, Inc.), β -catenin (1:1,000; cat no. 9562; Cell Signaling Technology, Inc.), Dickkopf-related protein 1 (DKK1) (1:1,000; 29 kDa; cat no. ab61275; Abcam) and AXIN2 (1:1,000, cat no. ab109307, Abcam); GAPDH served as an internal reference. The membranes were washed three times and then incubated for 1 h with corresponding fluorescent secondary antibodies [Goat Anti-Mouse IgG H&L (HRP); 1:5,000; cat no. ab6789; Abcam]. Finally, the grey values of the strips were calculated by ImageJ software (version 5.0; National Institutes of Health).

Statistical analysis. All data were expressed as mean \pm standard deviation unless otherwise shown. Student's t-test was used to analyze statistical differences between two groups and one-way ANOVA was used to analyze statistical differences between >2 groups, followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HAOBs passage and identification. Morphological changes of HAOBs during cell passage were observed under an inverted phase contrast microscope. After 5 days of culture, primary osteoblasts started to extend from the edge of alveolar bone granule (Fig. 1A1). The first and the second passages of osteoblasts were usually in irregular shapes similar to triangle, spindle and polygon (Fig. 1A2-A3). When osteoblasts passaged to the third generation, most of them appeared to be triangular and fusiform (Fig. 1A4). At this stage, osteoblasts were large in size, rich in cytoplasm and had more protuberances compared with the second passages of osteoblasts with which to connect with each other. Large oval single nuclei with one to three nucleoli were located in the center of HAOBs cells. As the osteoblast number increased, the protuberances radially expanded to the surrounding areas. Then the osteoblasts fused and overlapped, which further disclosed the white mineralized nodules to the naked eye. Next, conventionally cultured osteoblasts were stained with ALP. The positive expression of HAOBs was identified by a cluster of blue granules in the cytoplasm (Fig. 1B). The osteoblasts were authenticated through mineralization assay. After staining with Alizarin Red S, red calcium deposits of various sizes and shapes could be observed (Fig. 1C). These results indicated that HAOBs were successfully isolated and cultured.

Effects of NTR1 and TNF- α on HAOBs. HAOBs were pre-treated with different concentrations of NTR1 in medium and then detected by MTT assay. It was identified that the viability of the cells remained unchanged with 0-20 $\mu\text{mol/l}$ of NTR1, but was slightly inhibited by 40 $\mu\text{mol/l}$ NTR1 after 3-day's culture ($P < 0.05$ vs. Control; Fig. 2A). Next, the cells were treated with NTR1 (2.5 and 20 $\mu\text{mol/l}$) alone, or further stimulated by TNF- α . As shown in Fig. 2B, TNF- α could significantly inhibit the viability of HAOBs ($P < 0.05$, $P < 0.01$ vs. Control or TNF- α), thus demonstrating that TNF- α could create an inflammatory microenvironment. NTR1 (2.5 and 20 $\mu\text{mol/l}$) and TNF- α had no effect on the proliferation of HAOBs. These results suggested that 40 $\mu\text{mol/l}$ NTR1 affected cell viability.

Table I. Primers used in reverse transcription-quantitative PCR analysis.

Gene	Primer sequence
Runt-related transcription factor 2	Forward: 5'-TGGTTACTGTCATGGCGGGTA-3' Reverse: 5'-TCTCAGATCGTTGAACCTTGCTA-3'
Osteopontin	Forward: 5'-GGGAATTCATGAGATTGGCAGTG-3' Reverse: 5'-AAGGATCCGTTGACCTCAGAAGA-3'
Osteocalcin	Forward: 5'-GGGGTACCCAGAGCAGTAATTTCCGGCC-3' Reverse: 5'-GAAGATCTCGAACCGAACCGCCTTATA-3'
GAPDH	Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'
AXIN2	Forward: 5'-CAACACCAGGCGGAACGAA-3' Reverse: 5'-GCCCAATAAGGAGTGTAAGGACT-3'
Dickkopf-related protein 1	Forward: 5'-CCTTGAACCTCGGTTCTCAATTCC-3' Reverse: 5'-CAATGGTCTGGTACTTATTCCCG-3'
β -actin	Forward: 5'-GTGACGTTGACATCCGTAAAGA-3' Reverse: 5'-GCCGGACTCATCGTACTCC-3'

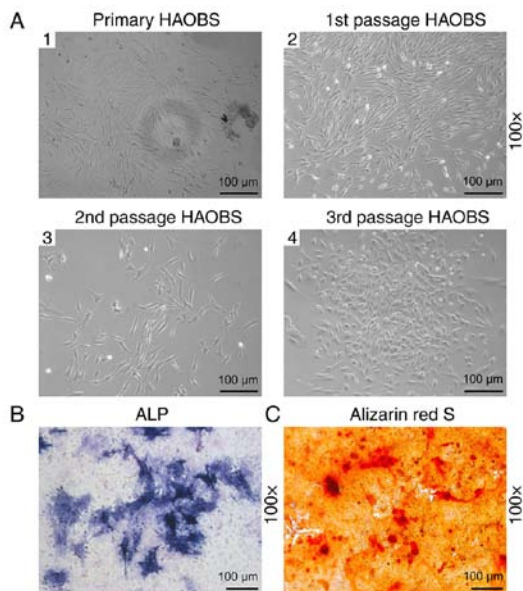


Figure 1. HAOBs were isolated and verified. (A1) Primary HAOBs were cultured for 5 days. (A2) HAOBs of generation 1 were cultured for 3 days. (A3) HAOBs of generation 2 were cultured for 3 days. (A4) HAOBs of generation 3 were cultured for 3 days. (B) ALP staining was used to observe the cytoplasm of positively expressed HAOBs. (C) Alizarin Red S was used to stain the HAOBs. n=3, magnification, $\times 100$, scale bars=100 μm . HAOBs, human alveolar osteoblasts; ALP, alkaline phosphatase.

NTR1 promotes the differentiation of HABOs in the inflammatory microenvironment induced by TNF- α . Using Alizarin Red S staining, red mineralized nodules could be detected in OM, whereas the calcium deposits were largely reduced by TNF- α . Nevertheless, NTR1 at an concentration of $\leq 20 \mu\text{mol/l}$ could abrogate the function of TNF- α in a dose-dependent manner; TNF- α plus 20 $\mu\text{mol/l}$ NTR1 resulted in more calcium deposits compared with TNF- α plus 2.5 $\mu\text{mol/l}$ NTR1 (Fig. 3A). In addition, the ALP activity in HAOBs co-treated with TNF- α and NTR1 (2.5 and 20 $\mu\text{mol/l}$) was significantly higher compared with that in HAOBs stimulated with TNF- α

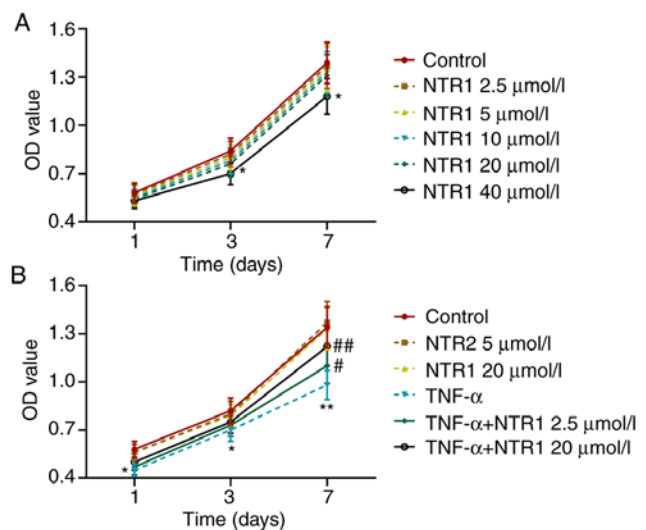


Figure 2. The effects of TNF- α and NTR1 on HAOBs. (A) MTT assay was used to detect the viability of HAOBs treated with NTR1 (2.5, 5, 10, 20 and 40 $\mu\text{mol/l}$) on days 1, 3 and 7. (B) MTT assay was used to detect the viability of HAOBs treated with 2.5 or 20 $\mu\text{mol/l}$ NTR1 and TNF- α alone or in combination. * $P < 0.05$, ** $P < 0.01$ vs. control, # $P < 0.05$, ## $P < 0.01$ vs. TNF- α . n=3. NTR1, notoginsenoside R1; HAOBs, human alveolar osteoblasts.

only on both day 7 and 14 (both $P < 0.001$ vs. Control, OM or TNF- α ; Fig. 3B). RT-qPCR was used to measure the expression of gene markers of osteoblasts. After 3-day culture, the relative mRNA expression levels of RUNX2, OPN and OCN were significantly inhibited by TNF- α compared with those in the OM group at day 3 and 7 (all $P < 0.001$ vs. Control, OM or TNF- α ; Fig. 3C and D). It was identified that 2.5 $\mu\text{mol/l}$ NTR1 had no significant effect on TNF- α -induced results, while 20 $\mu\text{mol/l}$ NTR1 reversed the effect of TNF- α on HAOBs ($P < 0.001$; Fig. 3C). On the 7th day of osteoblast culture, mRNA expressions were also inhibited by TNF- α . However, both 2.5 and 20 $\mu\text{mol/l}$ NTR1 could reverse the inhibitory effect of TNF- α to a large extent ($P < 0.001$ vs. Control, OM or

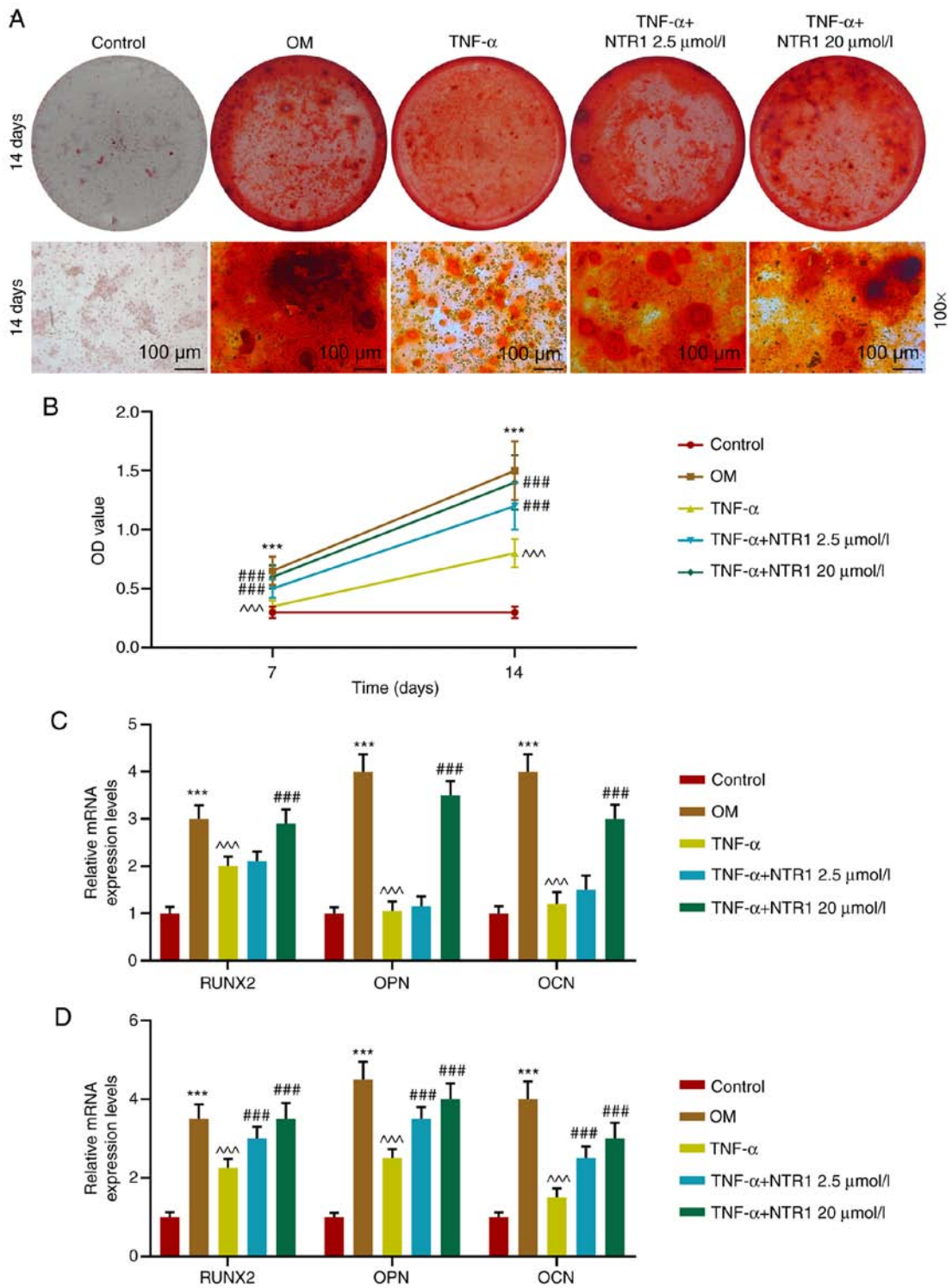


Figure 3. NTR1 promoted the differentiation of HAOBs in the inflammatory microenvironment created by TNF- α . (A) Alizarin Red S staining was performed for calcium deposition (magnification, $\times 100$) and (B) ALP activity was measured by ALP staining on day 7 and 14. Reverse transcription-quantitative PCR was performed to detect the relative mRNA expression levels of HAOBs on (C) day 3 and (D) day 7 of cell culture. $^{***}P < 0.001$ vs. control, $^{***}P < 0.001$ vs. OM, $^{###}P < 0.001$ vs. TNF- α , n=3. NTR1, notoginsenoside R1; HAOBs, human alveolar osteoblasts; ALP, alkaline phosphatase; OM, osteogenic medium; RUNX2, runt-related transcription factor 2.

TNF- α ; Fig. 3D). These results indicated that NTR1 promoted differentiation of HAOBs in an inflammatory microenvironment induced by TNF- α .

TNF- α -activated NF- κ B signaling pathway is restrained by NTR1. After HAOBs were stimulated with TNF- α for 30 and

60 min, western blotting was performed to determine the relative protein expression levels of components of the NF- κ B signaling pathway (Fig. 4). The protein expressions of p50 and p-p65 were both increased by TNF- α , while that of p65 remained stable. Adding NTR1 (2.5 and 20 μ mol/l) resulted in the downregulation of p50 expression in osteoblasts after

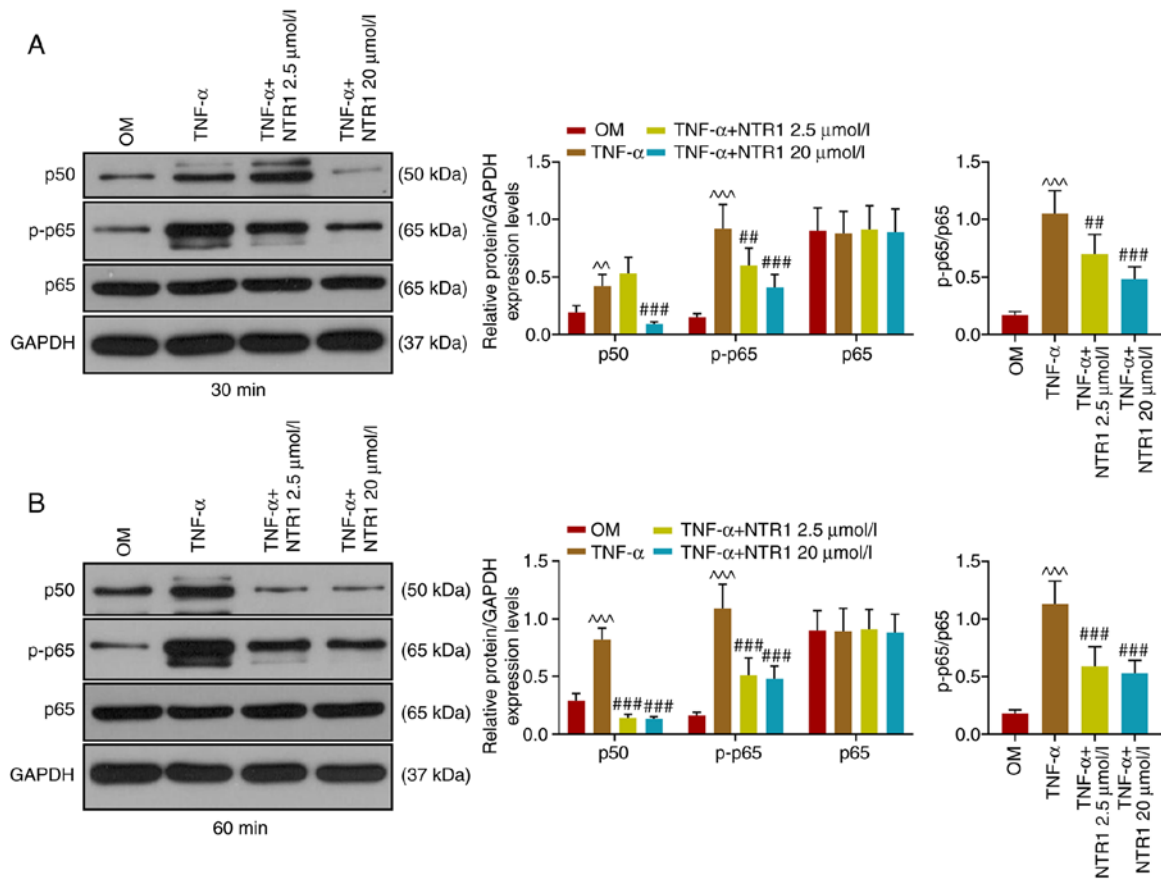


Figure 4. TNF- α -induced NF- κ B pathway was inhibited by NTR1. After HAOBs were treated for (A) 30 min and (B) 60 min, western blotting was conducted to examine the expression levels of p50, p-p65 and p65. $^{^^}$ P<0.01, $^{^^^}$ P<0.001 vs. OM, $^{##}$ P<0.01, $^{###}$ P<0.001 vs. TNF- α . n=3. NTR1, notoginsenoside R1; HAOBs, human alveolar osteoblasts; OM, osteogenic medium-, phosphorylated.

60-min TNF- α treatment compared with the TNF- α group (P<0.001 vs. Control, OM or TNF- α). These results indicated that TNF- α -activated NF- κ B signaling is restrained by NTR1.

NTR1 rescues the impaired Wnt/ β -catenin signaling pathway caused by TNF- α . The expression levels of the Wnt/ β -catenin signaling pathway-related proteins in HAOBs were determined using western blotting, after which the expressions of corresponding mRNAs were detected by RT-qPCR. Measurements were taken on day 3 and 7 of osteoblast treatment. Notably, the gene expression patterns of AXIN2, DKK1 and β -catenin of both NTR1 groups presented a similar trend. Compared with the TNF- α only group, NTR1 significantly upregulated the expression levels of AXIN2 and β -catenin and downregulated DKK1 expression on the 7th day (P<0.001 vs. Control, OM or TNF- α ; Fig. 5A-D). These results indicated that NTR1 rescued the impaired Wnt/ β -catenin signaling pathway caused by TNF- α .

Discussion

The present study investigated the function of NTR1 in the inflammatory microenvironment induced by TNF- α . The concentration of NTR1 was controlled at $\leq 20 \mu\text{mol/l}$. NTR1-treated HAOBs were further stimulated with TNF- α . It was identified that NTR1 regulated ALP activity, mineralized nodules and expression levels of osteogenic genes in HAOBs.

In addition, NTR1 abrogated the effects of TNF- α through the NF- κ B and Wnt/ β -catenin signaling pathways and served a pivotal role in osteoblast differentiation in inflammatory microenvironment.

Inflammation is an innate immune response associated with TNF- α , a key factor in the cytokine network (23). Essentially, inflammatory and bone healing processes interact with each other, but prolonged inflammatory process will one way or another hamper bone regeneration (24). For example, ATF3 functions as a regulator to aid TNF- α -mediated inhibition of osteoblast differentiation, while the TNF- α -activated JNK pathway further induces ATF3 expression (25). Another study demonstrated that p53 activity-mediated TNF- α induces apoptosis and inhibits differentiation in cementoblasts (26). In addition, p38, Erk1/2, JNK, PI3K-Akt and NF- κ B pathways are activated to limit the role of TNF- α in inducing apoptosis of cementoblasts (26). As aforementioned, NTR1 is verified to have anti-inflammatory effects. According to a previous study, NTR1 can increase cell viability, suppress the expression of inflammatory cytokines and reverse lipopolysaccharide-induced activation of the NF- κ B signaling pathway in myocardial cells (15). It has also been shown that NTR1 inhibits the expression levels of TNF- α and IL-1 β to protect endothelial cells from oxLDL-induced injury through the suppression of the MAPK and NF- κ B signaling pathways (8). Nevertheless, whether NTR1 could abrogate the negative effects of TNF- α and promote cell differentiation in HAOBs remains to be elucidated.

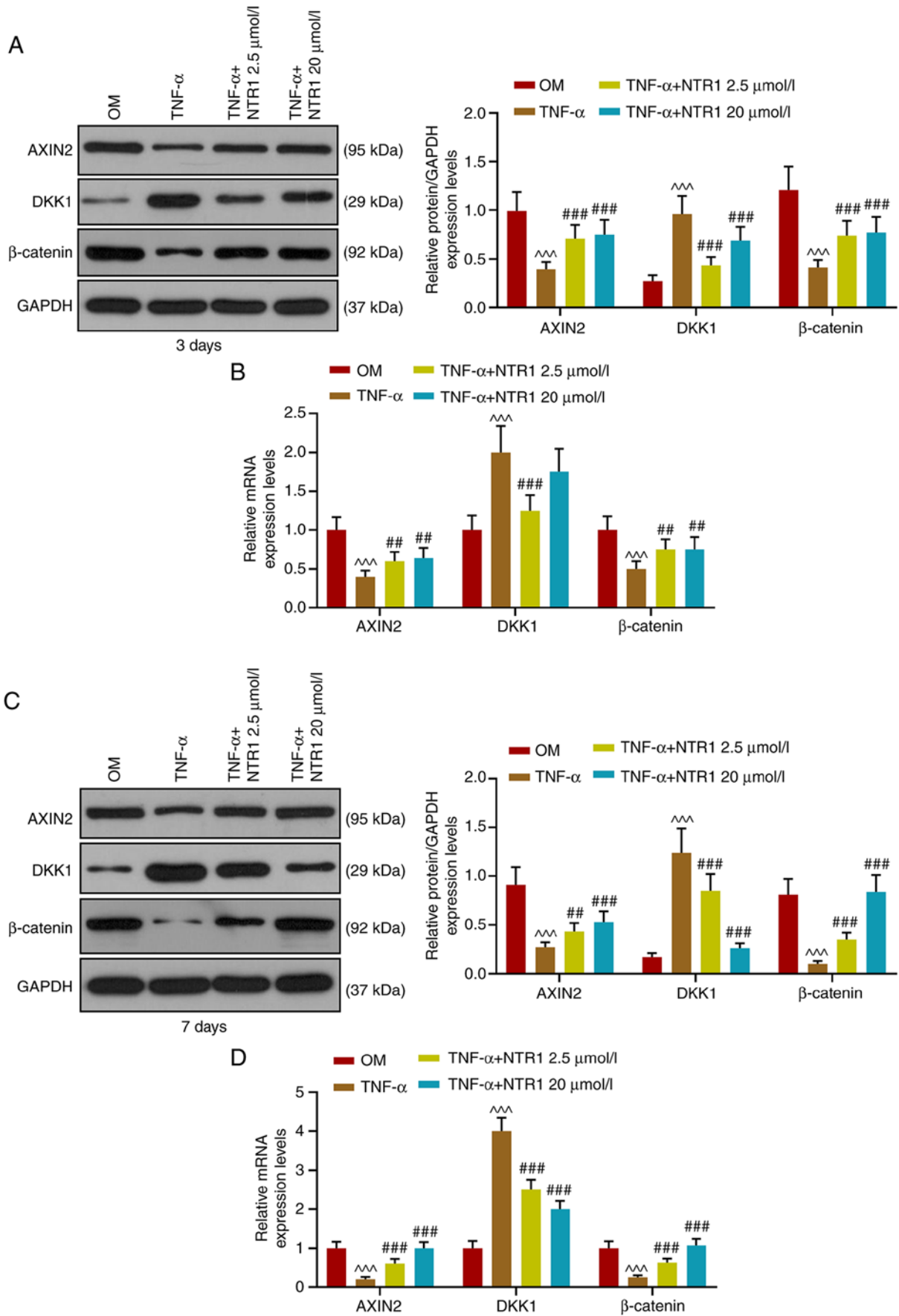


Figure 5. Regulatory effect of TNF-α on the Wnt/β-catenin pathway was abrogated by NTR1. After HAOBs had been treated for 3 days, (A) western blotting and (B) RT-qPCR were used to detect the expression of AXIN2, DKK1 and β-catenin in HAOBs. After HAOBs had been treated for 7 days, (C) western blotting and (D) RT-qPCR assay were used to detect the expressions of AXIN2, DKK1 and β-catenin in HAOBs. ^^^P<0.001 vs. OM, ##P<0.01, ###P<0.001 vs. TNF-α, n=3. NTR1, Notoginsenoside R1; HAOBs, human alveolar osteoblasts; RT-qPCR, reverse transcription-quantitative PCR; OM, osteogenic medium; DKK1, Dickkopf-related protein 1.

As a consequence, the present study first performed cytotoxicity tests to confirm the influence of NTR1 on cell viability. The concentration of NTR1 influenced the effect of NTR1 on cell viability. NTR1 at $\leq 20 \mu\text{mol/l}$ had no effect on HAOBs, whereas $40 \mu\text{mol/l}$ NTR1 slightly decreased the viability of HAOBs. Therefore, the NTR1 dose in subsequent experiments was limited to 2.5 and $20 \mu\text{mol/l}$. Pre-treated osteoblasts were stimulated by TNF- α , which mimicked the inflammatory microenvironment *in vitro*. Osteoblast differentiation and mineralization were detected using an ALP activity assay and Alizarin Red S staining, respectively (26,27). RUNX2 has been demonstrated to induce osteoblast differentiation and, simultaneously, OPN and OCN are defined as mineralization-related genes (28). In the present study, suppression of mineralization caused by TNF- α was largely overcome by NTR1 in HAOBs. Furthermore, under inflammatory conditions, the ALP activity was higher, and the expression levels of osteogenic genes RUNX2, OPN and OCN were higher, in cells treated with NTR1 compared with the TNF- α stimulation group. All these results indicated that NTR1 might regulate osteoblast differentiation by reversing the negative effects of TNF- α .

Previous studies have shown that the NF- κ B signaling pathway serves an important role in the transcription process of inflammatory genes p50, p-p65 and p65 and the increased p65/p50 dimerization can induce gene transcription of pro-inflammatory factors (TNF- α) (29). NTR1 has anti-inflammatory functions by activating the NF- κ B signaling pathway in H9c2 cardiomyocytes (15) and thus the present study investigated whether it has a similar effect in human alveolar bone osteoblasts and the results were encouraging. It was identified that the expression levels of p50, p-p65 and p-p65/p65 in osteoblasts pre-treated with NTR1 were decreased in a time-dependent manner compared with the TNF- α stimulation group. However, no marked influence of NTR1 on HAOBs was observed. The experimental results demonstrated that the TNF- α -activated NF- κ B signaling pathway was abrogated by NTR1.

The Wnt/ β -catenin signaling pathway is of great significance in bone development and regeneration and changes in the pathway involves various genes (16,18,19,30). DKK1 inhibits the Wnt pathway, while β -catenin affects the initiation of target gene transcription and AXIN2 regulates the intensity and duration of Wnt/ β -catenin pathway (31). In the present study, TNF- α hampered the Wnt/ β -catenin signaling pathway in alveolar osteoblasts by increasing DKK1 and decreasing AXIN2 and β -catenin, while NTR1 markedly recovered this pathway by downregulating the expression of DKK1 and upregulating the expressions of AXIN2 and β -catenin in the inflammatory environment created by TNF- α . Therefore, it can be inferred that NTR1 potentially promoted alveolar osteoblast differentiation involving the regulation towards the Wnt/ β -catenin signaling pathway.

Previous studies show that the fundamental part of dental implantation is osseointegration where osteoblast growth is of vital importance (4,5). Inflammation is one of the necessary stages in dental implantation (32). The present study identified that NTR1 was able to reverse inflammatory responses and could promote alveolar osteoblast differentiation *in vitro*, which lays the foundation for experiments *in vivo*.

In conclusion, NTR1 demonstrated the ability to promote the differentiation of HAOBs by antagonizing the activation of NF- κ B signaling pathway and the inhibition of Wnt/ β -catenin signaling pathway, both of which were induced by TNF- α . Thus, NTR1 has the potential to be used to promote bone generation in the treatment of dental implantation or other dental diseases, highlighting the practical significance of NTR1 in clinic. For example, NTR1 could be used as an ingredient in toothpaste for daily care, managing inflammation and activating alveolar bone regeneration.

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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

LH and QL conceptualized and designed the study, collected, analyzed and interpreted data, and drafted and critically revised the article for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The current study was approved by the Ethics Committee of the Jingmen Number 1 People's Hospital (Jingmen, China; approval no. J MPH20160123). All patients signed informed consent and agreed that their tissues would be used for clinical research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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