Regulatory effect of miR-421 on humeral fracture and heterotopic ossification in elderly patients

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Abstract. The present study aimed to investigate the role of miR-421 and bone morphogenetic protein-2 (BMP-2) in the bone tissues and blood of elderly patients with humeral fractures and heterotopic ossification. A total of 38 patients with humeral fractures, including 16 patients who received surgery within 1-7 days of fracture and 22 patients who received surgery within 8-14 days of fracture, were enrolled. An additional 18 patients who had heterotopic ossification and 26 patients who had humeral fracture and not heterotopic ossification were also included. Bone tissues and blood were collected. Reverse transcription-quantitative polymerase chain reaction was performed to determine the miR-421 and BMP-2 mRNA expression levels in the samples. Western blotting and ELISA were performed to detect BMP-2 protein levels in bone tissues and blood, respectively. Dual-luciferase reporter assays were performed to verify whether BMP-2 is the direct target gene of miR-421. Compared with the patients who received surgery 1-7 days after fracture, the patients who accepted the surgery 8-14 days after fracture had significantly increased levels of BMP-2 mRNA and protein in their bone tissues and blood ($P<0.05$). Contrastingly, the expression level of miR-421 decreased in the samples from patients who accepted the surgery 8-14 days after fracture compared with the level in those who received surgery 1-7 days after fracture ($P<0.05$). Compared with the patients without heterotopic ossification, the patients with heterotopic ossification had increased BMP-2 mRNA and protein expression levels in their bone tissues and blood, whereas the expression of miR-421 was significantly decreased ($P<0.05$). The dual-luciferase reporter assay demonstrated that BMP-2 was the direct target gene of miR-421. The upregulation of BMP-2 may be associated with the downregulation of miR-421. miR-421 may regulate the recovery of humeral fracture and heterotopic ossification through BMP-2. The results of the present study may provide a theoretical basis for the diagnosis and treatment of humeral fracture and heterotopic ossification.

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Introduction

Humeral fractures are common and account for 5% of total body fractures (1). Of the total cases of humeral fractures, ~80% are non-displaced or slightly displaced stable fractures that do not require surgical treatment (2). Displaced unstable proximal humeral fractures are usually treated surgically (3). Heterotopic ossification refers to the occurrence of ossification outside the bone tissue, and surgery-complicated or secondary heterotopic ossification is clinically known as traumatic ossifying myositis (4).

Currently, more than a dozen bone morphogenetic proteins (BMPs) have been identified (5,6). BMP-2 was originally recognized as an inducer of bone formation belonging to the transforming growth factor-β family (7), and it serves an important role in bone formation (8,9) and fracture healing (10). BMP-2 also has an important role in the ossification process, as it stimulates the differentiation of pluripotent stem cells into osteoblasts and enhances the function of osteoblasts (6,11,12). There are various ways to regulate the expression of BMP-2, in which microRNA (miRNA) have been extensively studied. For example, miR-98 (13), miR-203 and miR-320 (14) have been reported to regulate the expression of BMP-2.

MiRNAs are a class of small RNA molecules 18-22 nt in length. They regulate mRNA translation through specific binding to the 3'-untranslated region (UTR) of the target mRNA and are important post-transcriptional regulators (15). Previous studies have confirmed that miRNA serve important roles in various diseases, including cancer, cardiovascular and endocrine disease (16,17). MiR-421 is cell-growth associated and was discovered in recent years (18). It serves an important role in proliferation, invasion and metastasis of multiple tumors (18,19).

In the present study, the expression levels of BMP-2 in bone tissues and blood of patients with humeral fractures at different

periods and patients with/without heterotopic ossification following humeral fractures were detected. Bioinformatics analysis was performed to identify miRNA sequences that may regulate BMP-2. To the best of our knowledge, this is the first report to discuss the regulatory effect of miR-421 on BMP-2.

Patients and methods

Clinical data of patients. In order to investigate the role of BMP-2 and miR-421 in bone fracture, 38 patients who received humeral fracture surgery at the Weifang People's Hospital (Weifang, China) between June 2013 and January 2017 were enrolled in the present study. Among them, 16 patients (5 male and 11 female patients) received surgery within 1-7 days of fracture. Their age range was 60-72 years. There were 22 patients who received the surgery within 8-14 days of fracture, including 9 males and 13 females. Their ages ranged from 61-73 years. Reconstructive and fixation surgery was performed according to the fracture site, and complications, including swelling, dislocation and congestion, were treated with respective drugs (Table I). To investigate the role of BMP-2 and miR-421 in the heterotopic ossification process, 18 patients who were diagnosed with heterotopic ossification in the post-surgical follow-up (14-16 months) and required surgery were also enrolled. Among them, there were 5 males and 13 females. Their age ranged from 60-71 years. Presurgical treatment for these patients was the same as in previous treatments of patients with humeral fractures. Another 26 patients with humeral fractures without heterotopic ossification who had received surgery to remove intramedullary nails due to good healing conditions during the same time period were also included. Among them, there were 10 males and 16 females. Their age range was 60-73 years old. These patients were treated as aforementioned (Table II). Patients with rheumatoid arthritis and long-term hormone treatment were excluded from the present study. Bone tissues and blood were collected from all patients. All of the above patients were clearly diagnosed with humeral fractures or heterotopic ossification by pathology analysis. Prior written informed consent was obtained from all patients and the present study was approved by the ethics review board of Weifang People's Hospital.

Sample collection. Fracture site bone tissues and heterotopic ossification bone tissues were obtained from surgery. The non-heterotopic ossification bone tissues were obtained from the fracture tissues of the fixed plate when it was removed. The bone tissues were immediately stored in liquid nitrogen (-196˚C) and ground into powder for further use. Peripheral blood (30 ml) was collected from the patients following fasting on the day of surgery. Serum was isolated from peripheral blood by centrifugation (200 x g, 4˚C, 10 min).

Reagents. miRcute miRNA isolation kit, miRcute miRNA first-strand cDNA synthesis kit, miRNA miRcute fluorescent quantitative detection kit (FP401), SuperReal PreMix (SYBR Green) and miRNA first-strand cDNA synthesis kit were all purchased from Tiangen Biotech Co., Ltd., (Beijing, China). Rabbit anti-human BMP-2 primary antibody (ab14933), β-actin primary antibody (ab8227), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab6721) and enhanced chemiluminescence (ECL) liquid (ab65623) were all purchased from Abcam (Cambridge, MA, USA). TRIzol agent (10606ES60) was purchased from Yisheng Biological Co., Ltd., (Shanghai, China). A bicinchoninic acid assay (BCA) kit (RTP7102) was purchased from Real-Times Beijing Biotechnology Co., Ltd., (Beijing, China). An miRNeasy Serum/Plasma kit (JL217184) was purchased from Guangzhou Jianlun Biological Technology Co., Ltd., (Guangzhou, China). A Human BMP-2 ELISA kit (ab119581) was purchased from Abcam. Image Lab software (v. 3.0) was free from Bio-Rad Laboratories, Inc., (Hercules, CA, US). All plasmids/agomiR were synthesized by Sangon Biotech Co., Ltd., (Shanghai, China). The pMIR-REPORT™ miRNA Expression Reporter Vector System (AM5795) was purchased from Ambion (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissues or blood samples using TRIzol reagent. The integrity of RNA bands was examined by 1% agarose gel electrophoresis, and the RNA purity was measured by a 260/280 ratio using a spectrophotometer. Following this, RNA was reverse transcribed into cDNA using a miRNA first-strand cDNA synthesis kit. The qPCR reaction solution consisted of 10 μl SuperReal PreMix Plus (SYBR-Green), 0.5 μl upstream primers, 0.5 μl downstream primers, 2 μl cDNA and 7 μl ddH_2O. The following primer sequences were used: BMP-2 forward, 5'-CCTATATGCTCGACCTGTAC-3' and reverse, 5'-CCCACTCATTTCTGAAAGTTC-3'; and GAPDH forward, 5'-GCACAGTCAAGGCTGAGAAT-3' and reverse, 5'-TGAAGACGCCAGTAGACTCC-3'. The qPCR reaction conditions for BMP-2 were as follows: Pre-denaturation at 95˚C for 30 sec, followed by 39 cycles of 5 sec at 95˚C and 20 sec at 60˚C. miRNAs were extracted using a miRcute miRNA isolation kit and reverse transcribed with miRcute miRNA first-strand cDNA synthesis kit. For the measurement of miR-421, qPCR was performed using a miRNA miRcute fluorescent quantitative detection kit and the primers listed in Table III. The reaction conditions were as follows: Pre-denaturation at 95˚C for 5 min, followed by 40 cycles of 15 sec at 95˚C, 15 sec at 60˚C and 10 sec at 72˚C. GAPDH and U6 were used as internal controls. The results were calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. Total proteins were extracted from the bone tissues and heterotopic ossification tissues using an E.Z.N.A®Total DNA/RNA/Protein kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and the protein concentrations were determined using a BCA protein assay kit. Following this, proteins (50 μg) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk at room temperature for 1 h. Subsequently, rabbit anti-human BMP-2 primary antibody (1:1,000) and anti-β-actin primary antibody (1:5,000) were added and incubated at 4˚C overnight. Following washing with TBST, the secondary antibody (1:3,000) was added and incubated at room temperature for

Table I. Characteristics of patients with humeral fractures.

	No. of days after which surgery was performed following fracture	
Clinical characteristics	1-7	8-14
Case number	16	22
Age (years)	65.5±6.7	66.7±6.2
Sex		
Male	5	9
Female	11	13
Fracture cause		
Fall	6	7
Car accident	12	8
Bruise	3	2
Treatment	Mannitol dehydration, traction, promoting blood circulation with safflower injection	Mannitol dehydration, traction, promoting blood circulation with safflower injection

Table II. Characteristics of patients with and without HO.

	HO	
Clinical characteristics	Yes	No
Case number	18	26
Age (years)	64.5±7.2	65.1±7.8
Sex		
Male	5	10
Female	13	16
Cause of surgery	HO	Intramedullary nail removal
Treatment	Untreated prior to surgery	Untreated prior to surgery
Time of sampling	2-12 weeks after surgery	2-12 weeks after surgery

HO, heterotopic ossification.

Table III. The primers used in this study.

Name	Sequence (5'-3')
U6 forward	GCAAATTCGTGAAGCGTTCCAT
U6 reverse	GGGATCAACAGACATTAATT
miR-421 forward	TGGAAAACTTCCCGAAGAAC
miR-421 reverse	GGGATCAACAGACATTAATT

miR, microRNA.

1 h. The membrane was developed with ECL solution. Image Lab v. 3.0 software was used to analyze the protein bands. The ratio of the gray value of the target protein band to that of the β-actin band was determined as the relative content of the target protein.

ELISA. ELISA was conducted with a Human BMP-2 ELISA kit, according to the manufacturer's protocol. Briefly, 10 μl serum and 40 μl diluent were added. Except for the blank wells, 100 μl horseradish peroxidase-labeled antibody was added to the wells. The plate was sealed with film and incubated at 37˚C for 1 h. Following five washes with the provided washing reagent, 50 μl of substrate A and B were added to each well and incubated at 37˚C for 15 min. Then, 50 μl termination solution was added to each well, and the optical density value at 450 nm was measured within 15 min.

Prediction of the upstream regulatory miRNA of BMP-2. The target gene prediction software of DIANAmT (Ensemble v84; diana.imis.athena-innovation.gr/DianaTools/index.php?r=mrmicrot/index) (21), miRanda (last modified, August 2010; http://34.236.212.39/microrna/home.do) (22), miRDB (last modified, May 2016; mirdb.org/) (23), miRWalk (v2.0; zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) (24), RNAhybrid (last modified, September 2017; bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (25), PICTAR4 and PICTAR5 (v4.5; pictar.mdc-berlin.de/) (26), PITA (v6; genie.weizmann.ac.il/pubs/mir07/mir07_data.html) (27), RNA22 (v2.0; cm.jefferson.edu/rna22/) (28) and TargetScan

(v6.2; targetscan.org) (29) were used to predict the upstream regulatory miRNA of BMP-2.

Dual-luciferase reporter assay. The mutant binding sequence was designed as described previously by Guo *et al* (30). The wild-type and mutant seed regions of the 3'-UTR of the BMP-2 gene containing the miR-421 binding site were chemically synthesized *in vitro*. Following the addition of the cleavage sites of *Spe*I and *Hind*III at both ends, they were cloned into the pMIR-REPORT luciferase reporter plasmid. The mutated 3'-UTR seed region was used as a control. The seed region sequence for the double luciferase reporter plasmid was constructed as follows: 5'-TAATCAAAAGAAGTATCGGGT TTGTACATAATTTTCCAAAAATTGTAGTTGTTTTCA GTTGTGTGTATTTAAGATGAAAAGTCTACATGGAAG GTTACTCTGGCAAAGTGCTTAGCACGTTTGCTTTTT TGCAGTGCTACTGTTGAGTTCACAAGTTCAAGTCCA GAAAAAAAAAGTGGATAATCCACTCTGCTGACTTTC AAGATTATTATATTATTCAATTCTCAGGAATGTTGCAGA GTGATTGTCC-3'. The bold letters represent the target region, and the mutant sequence was GACAACT in the experiments. The plasmid containing the wild-type or mutant 3'-UTR sequences were transfected into 293T cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) using liposomes (Lipofectamine® 3000; Thermo Fisher Scientific Inc.) and then the agomiR-421-containing plasmid was transfected (100 nM) into the cells. After 24 h of incubation at 37˚C, each group of cells was lysed according to the manufacturer's instruction of the Dual-Luciferase Reporter Assay system (Promega Corporation, Fitchburg, WI, USA). The luciferase reporter activity was recorded by a GloMax 20/20 luminometer (Promega Corporation), using *Renilla* luciferase activity as the internal standard.

Statistical analysis. All experiments were performed in triplicate independently. All reported data were processed by SPSS 18.0 (SPSS, Inc., Chicago, IL, USA), and the data was presented as the mean ± standard deviation. Normality tests were performed for the data. Student's t-tests were used for the comparison between two groups. Multiple groups of measurement data were analyzed by one-way analysis of variance. If the variance was homogeneous, the least significant differences and Student-Newman-Keuls post hoc methods were used; if the variance was heterogeneous, Tamhane's T2 or Dunnett's T3 post hoc methods were used. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression difference of each indicator at different time periods following fracture

Changes of BMP-2 mRNA expression in bone tissue and blood samples in patients with humeral fractures. To determine the changes of BMP-2 mRNA expression in each sample, RT-qPCR was performed. Compared with the patients who received surgery 1-7 days after fracture, the patients who accepted surgery 8-14 days after fracture demonstrated significantly increased BMP-2 mRNA expression levels in their bone tissues (P<0.05) and blood (P<0.01) (Fig. 1A). The different levels of BMP-2 mRNA expression suggests that BMP-2 is upregulated over time within the two weeks after humeral fracture, which may serve a particular regulatory role in humeral fractures.

Changes of BMP-2 protein expression in bone tissue and blood samples in patients with humeral fractures. To determine the expression of BMP-2 protein in the bone tissue of humeral fractures, western blotting was performed. The patients who accepted surgery 8-14 days after fracture had significantly upregulated BMP-2 protein levels in their bone tissues compared to those observed in patients who received surgery 1-7 days after fracture (P<0.01) (Fig. 1B).

To determine the expression of BMP-2 protein in the serum of patients with humeral fractures, ELISA was performed. Compared with the patients who received surgery 1-7 days after fracture, the patients who accepted surgery 8-14 days after fracture had significantly elevated BMP-2 protein levels in their serum (P<0.01) (Fig. 1C), which was consistent with the upregulation trend of mRNA expression. The upregulated BMP-2 protein expression level in the blood may indicate that BMP-2 is released from the injured bone tissues.

Changes of miR-421 expression in bone tissue and blood samples in patients with humeral fractures. To determine the changes of miR-421 expression in each sample, RT-qPCR was performed. The patients who accepted surgery 8-14 days after fracture had significantly decreased miR-421 expression levels in their bone tissue samples (P<0.01) and blood samples (P<0.05) compared to those in the patients who received surgery 1-7 days after fracture (Fig. 2). This result suggests that miR-421 may serve a regulatory role in the recovery of the humeral fracture.

Expression difference of each indicator between heterotopic and non-heterotopic ossification

Changes of BMP-2 mRNA expression in bone tissue and blood samples in patients with heterotopic ossification. To determine the BMP-2 mRNA level in each sample, RT-qPCR was performed. Compared with the patients without heterotopic ossification, the patients with heterotopic ossification demonstrated significantly upregulated BMP-2 mRNA expression levels in their bone tissues and blood (P<0.01) (Fig. 3A). This suggests that BMP-2 is upregulated in heterotopic tissues following humeral fracture, and may serve a particular regulatory role in the disease.

Changes of BMP-2 protein expression in bone tissue and blood samples in patients with heterotopic ossification. To determine the expression of BMP-2 protein expression in heterotopic ossification bone tissue following humeral fracture, western blotting was performed. Patients with heterotopic ossification demonstrated significantly upregulated BMP-2 protein expression levels in their bone tissues, compared with those observed in patients without heterotopic ossification (P<0.05) (Fig. 3B), which was consistent with the upregulation trend of mRNA.

To determine the expression level of BMP-2 protein in the serum of patients with heterotopic ossification, ELISA was performed. As demonstrated in Fig. 3C, compared with

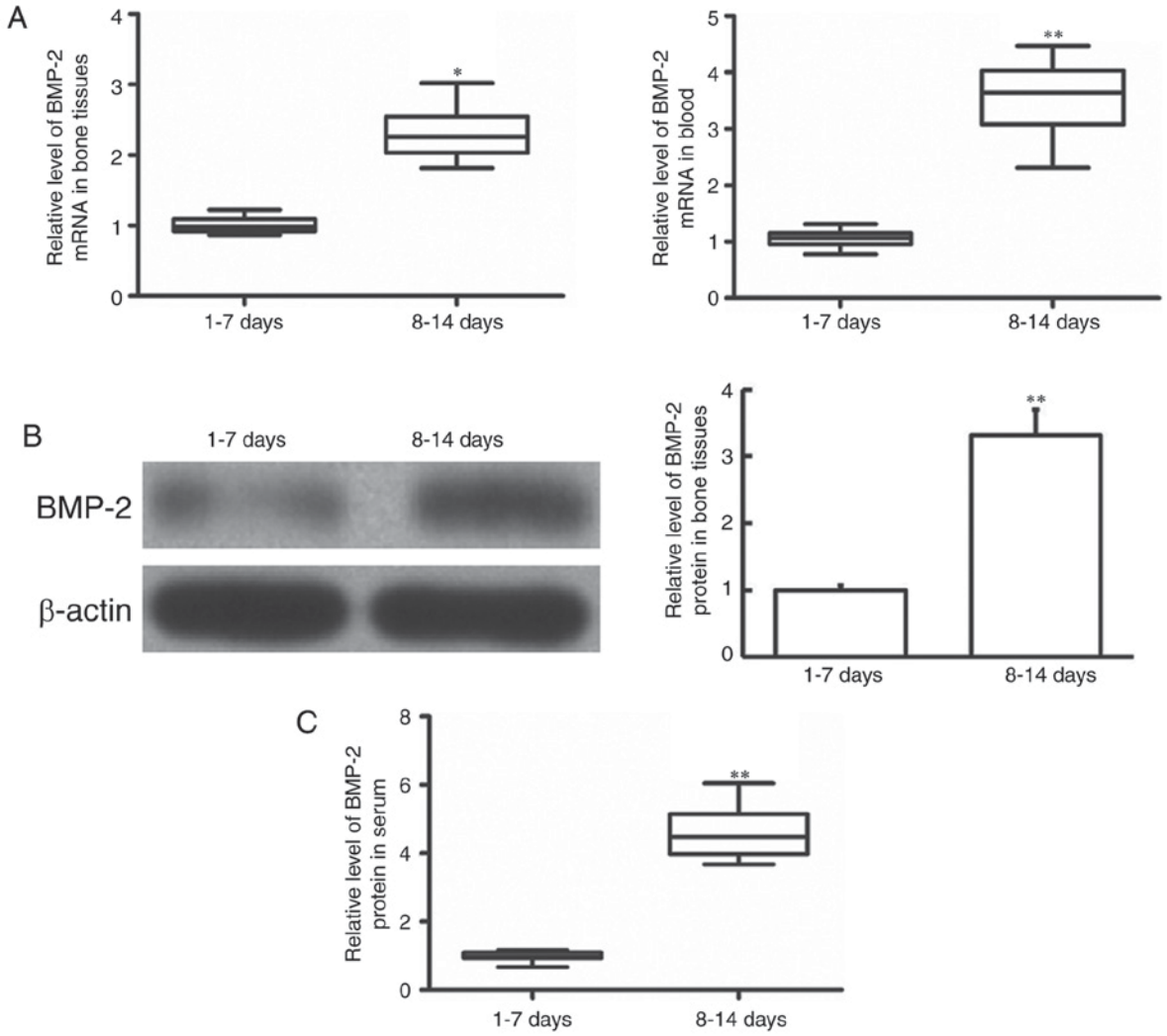

Figure 1. Expression levels of BMP-2 mRNA and protein in bone tissues and blood of patients with humeral fractures. Bone tissues and blood were collected from patients who received surgery 1-7 days after fracture and from patients who received surgery 8-14 days after fracture. (A) The expression changes of BMP-2 mRNA in the fracture bone tissues and blood. The expression changes of BMP-2 protein in the (B) fracture bone tissues and (C) serum. Student's t-tests were used for the comparison between two groups. *P<0.05 and **P<0.01 vs. patients who received surgery 1-7 days after fracture. BMP-2, bone morphogenetic protein-2.

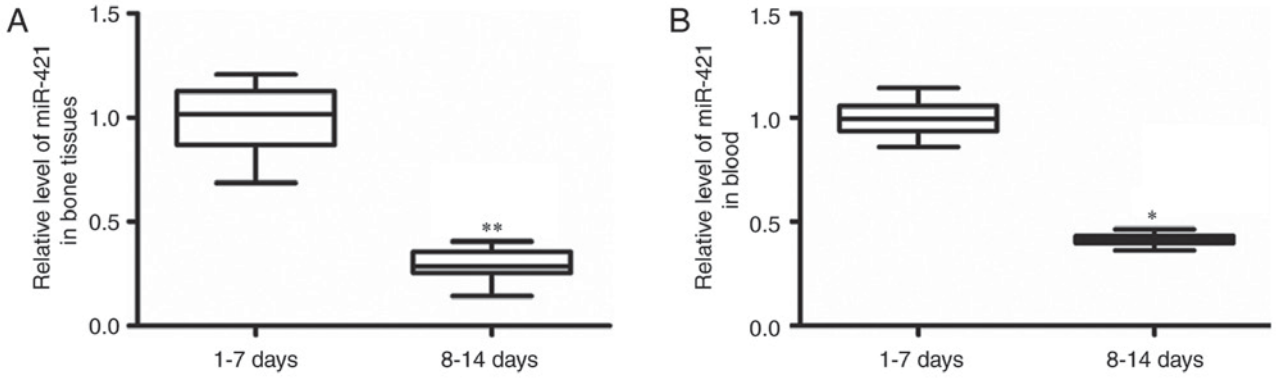

Figure 2. Expression levels of miR-421 in bone tissues and blood of patients with humeral fractures. Bone tissues and blood were collected from patients who received surgery 1-7 days after fracture and patients who accepted surgery 8-14 days after fracture. The expression changes of miR-421 in the (A) fracture tissues and (B) blood are shown. Student's t-tests were used for the comparison between two groups. *P<0.05 and **P<0.01 vs. patients who received surgery 1-7 days after fracture.

patients that did not have heterotopic ossification, the BMP-2 protein levels were significantly higher in the serum of the patients with heterotopic ossification (P<0.05). These results suggest that BMP-2 may serve a regulatory role in the recovery of heterotopic ossification at the transcription and protein function levels.

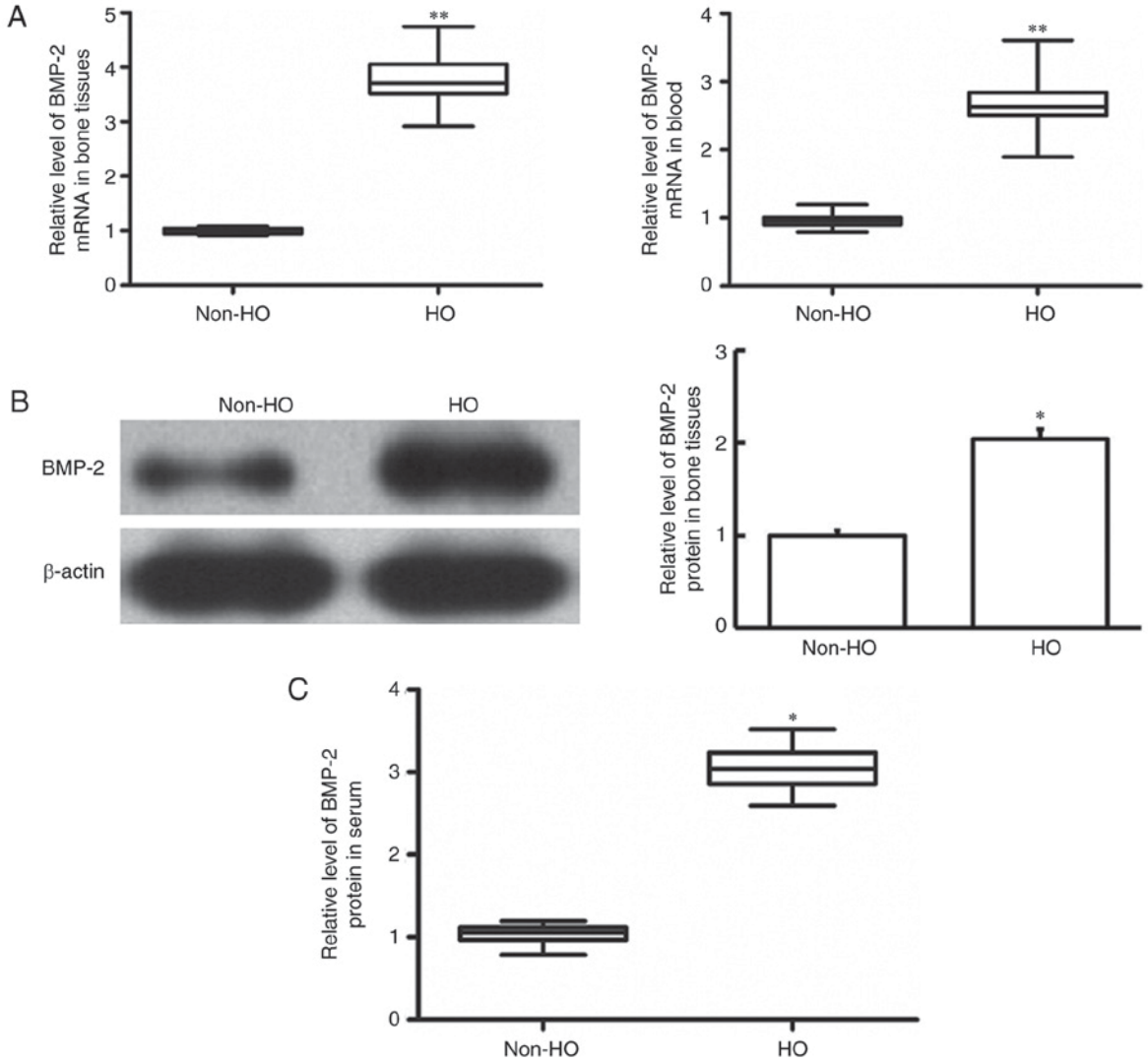

Figure 3. Expression levels of BMP-2 mRNA and protein in bone tissues and blood of patients with and without HO. Bone tissues and blood were collected from patients with HO and from those without HO (non-HO). (A) The expression changes of BMP-2 mRNA in the fracture bone tissues and blood. The expression changes of BMP-2 protein in the (B) fracture bone tissues and (C) serum. Student's t-tests were used for the comparison between two groups. *P<0.05 and **P<0.01 vs. non-HO. BMP-2, bone morphogenetic protein-2; HO, heterotopic ossification.

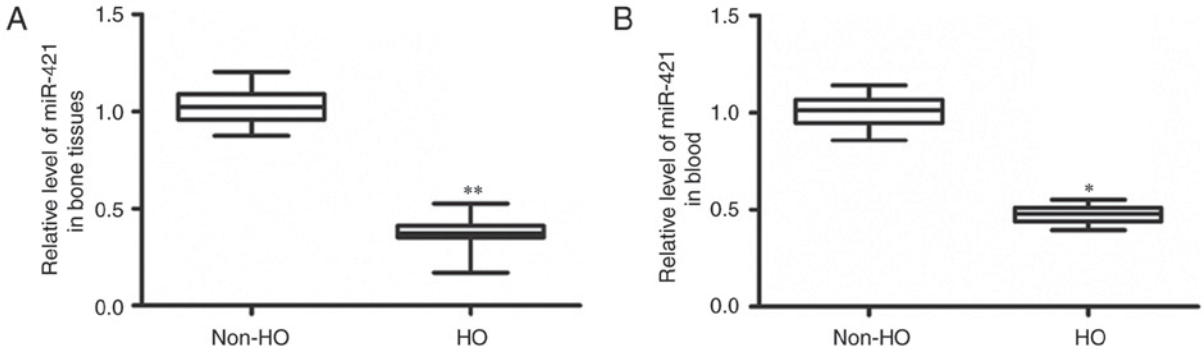

Figure 4. Expression levels of miR-421 in bone tissues and blood of patients with and without HO. Bone tissues and blood were collected from patients with HO and from those without HO (non-HO). The expression changes of miR-421 in the (A) fracture tissues and (B) blood are shown. Student's t-tests were used for the comparison between two groups. *P<0.05 and **P<0.01 vs. non-HO. HO, heterotopic ossification.

Changes of miR-421 expression in bone tissue and blood samples of patients with heterotopic ossification. To determine the expression of miR-421, RT-qPCR was utilized. As indicated in Fig. 4, the patients with heterotopic ossification demonstrated significantly downregulated miR-421 expression levels in their bone tissue samples (P<0.01) and blood (P<0.05) compared with those in the patients without heterotopic ossification. This result indicates that miR-421

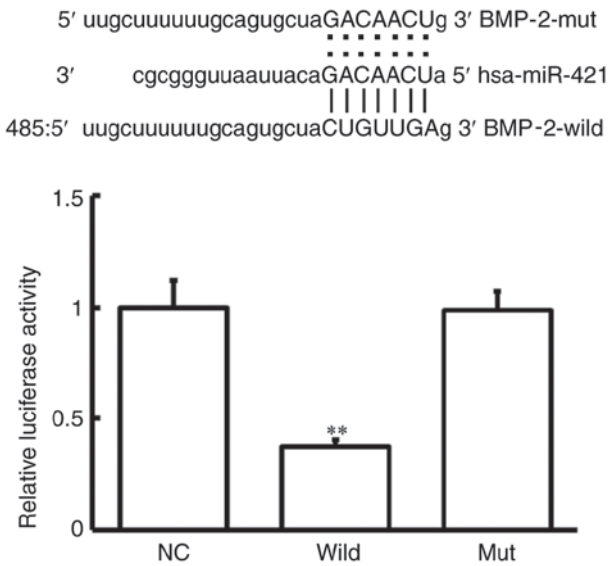

Figure 5. miR-421 targets BMP-2. The wild and mut type binding sequences between BMP-2 and miR-421 are demonstrated on the upper panel. The relative luciferase activity is indicated on the lower panel. One-way analysis of variance was used to compare the measured data of the three groups, and the Student-Newman-Keuls method was used for the pairwise comparison between two groups. **P<0.01 vs. NC group. BMP-2, bone morphogenetic protein-2; wild, wild-type; mut, mutant; NC, negative control.

may serve a regulatory role in the heterotopic ossification process.

Prediction of miRNA sequences that target BMP-2. To identify miRNA sequences that may target BMP-2, 10 databases were screened. There were 99 sequences predicted from DIANAmT, 303 sequences from miRanda, 11 sequences from miRDB, 117 sequences from miRWalk, 0 sequence from RNAhybrid, 0 sequences from PICTAR4, 241 sequences from PICTAR5, 0 sequences from PITA, 62 sequences from RNA22 and 128 sequences from TargetScan, with a total of 961 sequences predicted as the target genes of miR-421. Apart from the three databases that demonstrated no results, the remaining seven databases predicted BMP-2 as the target gene of miR-421. This high predicting proportion suggests that miR-421 may be the upstream gene of BMP-2. In addition, CUGUUGA was a common sequence of the seed region of miR-421 in the positive databases, indicating that this sequence is highly likely to be the seed region.

miR-421 targets BMP-2. To determine whether the BMP-2 gene was the target gene of miR-421, a dual-luciferase reporter assay was conducted. The wild and mutanttypes ofthe binding sequences between BMP-2 and miR-421 are demonstrated in Fig. 5. The mutant type of binding sequence was designed as described previously by Guo *et al* (30). Compared with the negative control, the relative luciferase activity was significantly decreased following co-transfection with agomiR-421 and the wild-type 3'-UTR of BMP-2 ($P<0.05$), while there was no significant difference observed in the mutant group ($P>0.05$). These results indicate that miR-421 may directly bind to the 3'-UTR of BMP-2 and regulate its expression.

Discussion

Humeral fractures can be complex, and the fracture may combine with fracture-peripheral nerves or blood vessels and cause soft tissue damage, which may lead to impeded blood circulation and neurotrophic disorders, increasing the difficulty of fracture healing (31). Excessive surgical treatment in the case of complex injuries not only destroys the local blood supply of the fracture, but also fails to achieve effective fracture fixation, eventually resulting in the failure of humeral fracture healing (31).

It is well accepted that BMPs are the strongest osteogenic factor, which promotes mesenchymal cells to differentiate into bone, cartilage, ligamentous, tendon and nerve tissues (32). BMP-2 is able to specifically convert mouse myoblast cell lines into osteoblast cell lines (33,34). It is believed that the level of BMP-2 in osteoblasts may reflect its osteogenesis ability (35,36). In the present study, BMP-2 mRNA and protein expression levels in the bone fracture tissue and blood of the patients who received surgery 1-7 days after fracture were significantly lower than those in the patients who received surgery after 8-14 days. This suggests that the body may respond to fracture damage within 2 weeks and then upregulate BMP-2 expression in blood and fracture tissues to induce osteogenesis and repair fracture damages.

Heterotopic ossification is a very important secondary disease following fracture, in which bone structure forms outside the skeletal system (37). It is characterized by rapid formation of calcified bone in the soft tissue, causing swelling and pain around the joints and disorders in joint movement (38). A study by Qu *et al* (39) suggested that heterotopic ossification required certain incentives, including osteoblasts and an appropriate microenvironment. A study by Mukai *et al* (40) demonstrated that a decrease of BMP-2 alkaline phosphatase activity served a role in the bone differentiation microenvironment and reduced the occurrence of heterotopic ossification (41). This suggests that heterotopic ossification following fracture may be caused by the fracture-induced overregulation of BMP-2. To verify the role of BMP-2 in heterotopic ossification following humeral fracture, the present study measured the expression of BMP-2 in the ossified tissues and blood of patients with heterotopic ossification. A significantly increased expression level of BMP-2 in the ossified tissues and blood of patients with heterotopic ossification following humeral fractures was observed compared with the levels in patients without heterotopic ossification. This suggests that heterotopic ossification may be related to the upregulation of BMP-2.

Many miRNA have become biomarkers of various diseases (41-43), thus the upstream regulatory miRNA of BMP-2 were also investigated in the present study. The upstream regulatory genes of BMP-2 were predicted by bioinformatics, and miR-421 was identified to be one of them. The expression level of miR-421 in gastric cancer has been previously studied (44). In addition, it has been demonstrated that miR-421 was upregulated in neuroblastoma, pancreatic cancer, prostate cancer and other malignant tumors (45-47). miR-421 is closely related to cell growth. A study by Jiang *et al* (18) determined that miR-421 expression in gastric cancer tissue was higher than that in normal tissues, indicating that miR-421 may serve an important role in the early growth

of gastric cancer. A study by Zhou *et al* (19) detected the expression of miR-421 in peripheral blood mononuclear cell lines by RT-PCR and transfected miR-421 inhibitors into animals, and found that tumor growth was inhibited. Significant downregulation of miR-421 expression was observed in the humeral fracture and the heterotopic ossification tissue samples in the present study. As aforementioned, BMP-2 mRNA and protein were upregulated in these samples. This suggests that the upregulation of BMP-2 may be regulated by the downregulation of its upstream regulatory gene, miR-421. In the present study, the BMP-2 dual-luciferase gene reporter plasmid was constructed and transfected into 293T cells together with agomiR-421. It was revealed that overexpression of miR-421 could significantly reduce the luminance of the BMP-2 plasmid, demonstrating that BMP-2 is the direct target gene of miR-421.

In conclusion, BMP-2 levels were increased and miR-421 levels were decreased in bone tissue samples and blood of patients with humeral fractures and heterotopic ossification. BMP-2 was a direct target of miR-421. These results indicate that miR-421 may serve an important role in humeral fracture healing through regulating BMP-2. This study may provide a new therapeutic target for fracture and heterotopic ossification.

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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

CJ, ZL and YJ collaborated to design the study. CJ, ZL and CZ were responsible for performing experiments. CJ and ZL analyzed the data. All authors collaborated to interpret results and develop the manuscript. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Weifang People's Hospital. Written informed consent was obtained from all patients or their families.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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