Original Paper

A MicroRNA-124 Polymorphism is Associated with Fracture Healing via Modulating BMP6 Expression

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Key Words

MicroRNA-124 • rs531564 • Polymorphism • Fracture healing • BMP6 • Apoptosis

Abstract

Background: miR-124-3p has been reported to be involved in the pathogenesis of many diseases by modulating a variety of signaling pathways. In this study, we aimed to understand the impact of miR-124-3p expression level on the fracture healing in the patients of metaphyseal *fracture* of distal tibia, who received minimal invasive percutaneous plate osteosynthesis. **Methods:** We firstly collected 195 patients of metaphyseal *fracture* of distal tibia, and the genotype of rs531564 was determined: GG (n=124) and GC+CC (n=71). We collected information of the participants including age, gender, total in-hospital time, smoking and alcohol consumption. Subsequently, we searched the miRNA database online to identify the possible binding sequence of miR-124-3p located within the 3'-UTR of the target gene. We did correlation analysis and luciferase to understand the regulatory relationship between miR-124-3p and BMP6. Meanwhile, we also conducted real time PCR and western blotting analysis to study the mRNA and protein expression level of BMP6 in different genotype groups. We then treated the cells with scramble control, miR-124-3p mimics, BMP6 siRNA and miR-124-3p inhibitors to investigate the influence of miR-124-3p on the expression of BMP6, viability and apoptosis of cells. **Results:** Total in-hospital time was significantly longer in GC+CC group than GG group. MiR-124-3p was up-regulated in GG group than GC and CC groups. BMP6 was virtual target of miR-124-3p. There existed negative regulatory relationship between miR-124-3p and BMP6. The mRNA and protein expression level of BMP6 decreased in GG group. MiR-124-3p decreased the expression of BMP6. MiR-124-3p negatively interfered with the viability of cells and BMP6 positively interfered with the viability of cells. MiR-124-3p reduced apoptosis and BMP6 promoted apoptosis. **Conclusion:** These data proved the expression of miR-124-3p was associated with the healing of metaphyseal *fracture* of distal tibia, and could be recognized as a biomarker to predict the healing of metaphyseal *fracture* of distal tibia.

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Introduction

Fractures are among the most common traumatic injuries in humans and osteoporosis related fractures are the fastest growing health care problem of aging [1]. The vast majority of fractures sustained worldwide are either untreated (e.g. patients in many third-world countries) or are treated in a manner that involves a periosteal response and a process of endochondral bone formation [2]. Despite the intrinsic ability of the body to heal fractures, patient risk factors can significantly impair skeletal repair [3]. The rate of delayed fracture healing or non-union is highest amongst subpopulations with specific risk factors such as smoking, advanced age, steroid use, use of certain pharmaceuticals (i.e. anti-cancer drugs) and metabolic diseases such as diabetes mellitus (DM) [3]. An increased mechanistic understanding for impaired osseous healing associated with specific high-risk populations will provide fundamental information necessary to design a regenerative approach for fracture patients with specific risk factors for non-union.

A variety of genetic and environmental factors have been reported to play an important role in the control of the apoptosis of osteoblasts, which is believed to be involved in the determination of fracture healing [4, 5]. Upregulation of BclXL in osteoblasts suppressed the apoptosis of the host cells, and promoted the regeneration or the strength of the bone [4]. Some other apoptosis repressors were also found to participate in the control of osteogenic differentiation of osteoblast cells, and zoledronate administration could enhance the survival of osteoblasts and positively contributed to the regeneration of the bone tissue [5]. On the other hand, induction of the apoptosis of osteoblasts could consistently compromised the osteogenesis, and IL-1A treatment could promote the apoptosis of osteoblasts through JNK and p38 MAPK signaling pathway [6]. Meanwhile, the apoptosis of other cell types in bone is also considered to be part of osteogenesis [7]. Apoptosis of chondrocytes is associated with secretion of osteogenic growth factors such as transforming growth factor β (TGF- β) and angiogenic factors such as fibroblast growth factor 2 (FGF-2) and may be important in regulating the early steps in bone formation as cartilage matrix is resorbed [7, 8].

MicroRNAs are non-protein-coding RNAs which are involved in gene regulation at the post-transcriptional level [9]. First discovered as critical regulators of developmental timing in *Caenorhabditis elegans*, microRNAs have since been found to function in almost all cellular processes in animals and plants [10-12]. MicroRNAs are approximately 22 nucleotides in length, generated from large primary transcripts that form imperfect stem loop structures. MicroRNAs regulate gene expression by binding to target mRNAs and promoting transcript degradation or inhibiting translation [13].

A variant (rs531564) located in the miR-124 has been found to interfere with the mature processing of the miRNA, resulting in a reduced expression of mature miRNA. On the other hand, BMP6 is believed to be functionally involved in the control of fracture healing by regulating the apoptosis of osteoblasts, and we also identified BMP6 as a virtual target of miR-124 using in-silicon analysis. In this study, we confirmed the regulatory relationship between miR-124 and BMP6 in OS-732 cells, and studied the association between miR-124 rs531564 polymorphism and fracture healing [14].

Materials and Methods

Sample collection

A total of 195 patients with metaphyseal fracture of distal tibia who have received surgical intervention in the Department of Traumatic Orthopedic Surgery, the General Hospital of Jinan Military Command (Jinan, China) were enrolled in this study during Sept 2013 and Nov 2014, and all signed written consent prior to donation of 5 ml peripheral blood. Among them, bone tissue samples were collected from 62 patients, and the samples were stored in -80°C for future use. The study protocols were approved by the Ethics Committee of the General Hospital of Jinan Military Command.

Genotyping by taqman genotyping kit

Qiagen Genomic DNA extraction kits (Valencia, CA, USA) were used to extract DNA from leucocytes according to protocol. Taq-Man genotyping technology (Applied Biosystems, Foster city, CA) was used to perform the SNP genotyping (rs531564) based on the instruction from the supplier. Taqman Genotyper software (Applied Biosystems, Foster city, CA) was used to analyze the genotype calling. All experiments were performed at least three times.

RNA isolation and real-time PCR

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate the whole RNA from OS-732 cells according to the manufacturer's description. Genomic DNA contamination was excluded using the RNase-free DNase (Promega, Madison, WI, USA), and NANODROP 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify the concentration of the RNA as described before. Agarose gel electrophoresis was used to examine the integrity of the RNA with values of A260/A280 from 1.8 to 2.0 were accepted. The DNase-treated total RNAs was subjected to reverse transcribed with oligo primer (Takara, Japan) containing the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in accordance with manufacturer's recommendation. CFX96Touch™ Real-Time PCR Detection System (Bio-Rad, USA) with FastStart Essential DNA GreenMaster (Roche, Indianapolis, IN, USA) was used to detect the levels of BMP6 mRNA and miR-124-3p using the primer sets: BMP6, 5'-CCGTGTAGTATGGGCCTCAGA-3' and 5'-TCACAACCCACAGATTGCTAGTG-3'. miR-124-3p, 5'-CTAGCCTGCAGGAGCTGCGGTAGGAAGGATGC-3' and 5'-ATCCGGCCGGCCAGGCTATTTCTGCCGCG-3'. The cycle parameters were set up at 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s. The house keeping gene (GAPDH for mRNA and U6 for miRNA) was carried out in parallel with all samples to normalize the cDNA loading. The relative mRNA expression for the BMP6 mRNA and miR-124 was determined using the 2^{-ΔΔCT} method. All reactions were carried out three times.

Cell culture and transfection

DMEM medium (Invitrogen, Carlsbad, CA, USA) with 100 µg/mL streptomycin, 100 U/mL penicillin and 10% FBS (fetal bovine serum) (Gibco Invitrogen, Grand Island, NY) was used to incubate the OS-732 cells in an atmosphere of 5% CO₂ in air at 37°C. When the cells were grown to 70-80% confluence, lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to perform the transfection following the protocol by the supplier, and we used the fresh medium (DMEM/F12) supplemented 10% FBS instead of the medium 4 hours after transfection. All experiments were performed in triplicate.

Cell proliferation assay

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] assay was used to measure the proliferation of OS-732. 2 days after transfection, the incubated medium was replaced with new one and the cells was incubated for another 4 hours. And then added 50 µL of DMSO into each well. A plate reader was used to measure the A540 of each sample after incubating for 10 min at 37°C. Each test was repeated three times.

Luciferase assay

The 3'-UTR of BMP6 was amplified through PCR with carefully designed primers, then the PCR products were inserted into the pGL3 (Promega, Madison, WI, USA), and the 3'-UTR of BMP6 mutagenesis was inserted using site directed mutagenesis (Stratagene). And QIAGEN plasmid purification kit (QIAGEN, Hilden, Germany) was used to prepare the plasmids for transfection according to the manufacturer's recommendation. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transiently transfect the OS-732 cells with the recombination plasmids in accordance with the manufacturer's protocol. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure the activities of Firefly and Renilla luciferase 24 hours after transfection based on the standard protocol.

Western blot analysis

Pre-chilled PBS was used to wash the OS-732 cells, ice-cold lysis buffer (Biosharp, Hefei, China) including proteinase inhibitor (Roche, Indianapolis, IN, USA), 5 g/L sodium deoxycholate, 0.2 g/L Na₃N, 1% NP-40, 150 mmol/L NaCl, 100 µg/mL, 0.1% SDS (sodium dodecyl sulfate), phenylmethylsulfonyl fluoride, 1

µg/mL aprotinin, and 50 mmol/L Tris-HCl (pH 7.4) was used to lyse cells according to the manufacturer's introduction. The DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify the concentration of proteins, and hypotonic buffer (Invitrogen, California, USA) was used to dilute the proteins to an equal concentration. 4-20% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to size-fractionate the protein, and then transferred to nitrocellulose membranes (Invitrogen, California, USA). TBST containing 5% non-fat dry milk (Merck, Darmstadt, Germany) was used to block the membranes for 60 min. primary antibody against BMP6 (1:1,000; Santa Cruz Biotechnology, Beijing, China) was used to hybridize the membranes at 4°C for 12 hours. TBST was used to wash the membranes three times. HRP (horseradish peroxidase)-conjugated anti-mouse or rabbit secondary antibody (1:10,000, Santa Cruz Biotechnology, Beijing, China) was used to hybridize the membranes for another 2 hours. TBST was used to wash the membranes three times. The enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the target protein by chemiluminescence. Quantity One software (Bio-Rad Laboratories) was used to quantify the optical density of the immunoblots based on the internal control (β-actin). All experiments were carried out three times.

Apoptosis analysis

The flow cytometer (FACScalibur, Becton-Dickinson, France) was used to analyze the apoptosis of the OS-732 cells according to the manufacturer's protocol. Briefly, PI staining was performed on the fixed cells followed by flow cytometry using 488nm to excite the cells and emission was observed at 585 nm to detect the extent of absorbance.

Statistical analysis

All data were shown as the mean ± SE (standard error). One way analysis of variance (ANOVA) was used to estimate the comparisons among different groups, and then Dunnett's post hoc test was also used to estimate the comparisons among different groups. Two-way ANOVA was used to determine the statistical significance for different treatments, and then Bonferroni post hoc test was also used to determine the statistical significance for different treatments. Logistic regression analysis was used to determine the SNP of the genotyping.

Results

Demographic and clinicopathological characteristics of the patients enrolled in this study

We first collected 195 demographic and clinicopathological characteristics of patients enrolled in this study. We divided them into two groups by the genotype: GG (n=124) and GC+CC (n=71). We collected information of age, gender, Total in-hospital time, smoking and alcohol consumption. Then we conducted *t* test of age, gender and total in-hospital time and Chi-square test of smoking and alcohol consumption. Only total in-hospital time had significant difference between the two groups (approximately 2.5 times longer in GC/CC than GG), other variables are comparable. The details were shown in Table 1.

MiR-124 was up-regulated in GG group

We then collected 62 blood sample among these patients and extracted RNA from the blood sample. We then performed real time PCR, the results showed that the expression level of miR-124 was more than 2 times higher in GG group (Fig. 1) than GC and CC group.

BMP6 is a direct downstream target of miR-124-3p

Using publicly available databases, we searched candidate target genes

Table 1. Demographic and clinicopathological characteristics of the participants of this study

Variables	GG (n=124)	GC+CC (n=71)	P
Age (years)	35.3 ± 2.3	37.3 ± 2.6	0.08
Male gender (%)	68.5	69.1	0.85
Total in-hospital time (days)	18.6 ± 3.2	45.9 ± 9.6	<0.001
Smoking			
Non-smoking	202 (81.5)	108 (76.1)	0.37
Smoking	46 (18.5)	34 (23.9)	
Alcohol consumption			
No	136 (54.8)	72 (50.7)	0.58
Yes	112 (45.2)	70 (49.3)

Fig. 1. The expression level of miR-124-3p was higher in GG group than GC and CC group (** $P < 0.01$ compared with the control).

Fig. 2. BMP6 as the candidate target gene of miR-124-3p in cells with the 'seed sequence' in the 3'UTR.

Fig. 3. Luciferase activity reporter assay was conducted to verify BMP6 as the direct target gene of miR-124-3p (** $P < 0.01$ compared with the control).

Fig. 4. The correlation between the expression level of miR-124-3p and BMP6 mRNA.

Fig. 5. The expression of BMP6 mRNA (Fig. 5A) and protein (Fig. 5B) decreased in GG group compared with GC and CC group (** $P < 0.01$ compared with the control).

of miR-124-3p, of all the candidates, BMP6 exhibited the most complementary structure with miR-124-3p (Fig. 2). We thus performed luciferase activity reporter assay in the cells to furthermore understand the regulatory relationship between miR-124-3p and BMP6. As shown in Fig. 3, the intensity of fluorescence from the cells co-transfected with miR-124-3p

Fig. 6. When transfected with the cells with scramble control, miR-124 mimics (30nM and 60nM), BMP6 siRNA (60nM) and miR-124-3p inhibitors (30nM and 60nM), the expression level of miR-124-3p treated with miR-124-3p mimics (30nM and 60nM) and BMP6 siRNA increased (Fig. 6A), while cells treated with miR-124-3p inhibitors (30nM and 60nM) decreased (Fig. 6D); the expression level of BMP6 protein (Fig. 6B and E) and mRNA (Fig. 6C and F) treated with miR-124-3p mimics (30nM and 60nM) and BMP6 siRNA decreased, while cells treated with miR-124-3p inhibitors (30nM and 60nM) increased (** $P < 0.01$ compared with the control).

and wild-type BMP6 or mutant2 BMP6 3'UTR decreased significantly (about 50% lower, Fig. 3), while cells co-transfected with miR-124-3p and mutant BMP6 3'UTR were comparable compared with scramble control (Fig. 3). The results confirmed that BMP6 could bind to miR-124-3p, causing a significant decrease in luciferase activity in cells.

Negative regulatory relationship between miR-124-3p and BMP6

The blood sample of three different groups (GG, $n=36$, GC, $n=21$, CC, $n=5$) were used to further explore the impacts on the interaction between miR-124-3p and BMP6 3'UTR. We first analyzed the correlation between the expression level of miR-124-3p and BMP6 mRNA among the blood samples ($n=62$), they showed negative regulatory relationship (Fig. 4, $r=-0.3875$, $p<0.01$). We then performed real time PCR and densitometry analysis to detect the expression level of BMP6 mRNA and protein, we found them 2 times decreased in GG compared with GC and CC group (Fig. 5), confirming the negative regulatory relationship between miR-124-3p and BMP6. We further transfected with the cells with scramble control, miR-124-3p mimics (30nM and 60nM), BMP6 siRNA (60nM) and miR-124-3p inhibitors (30nM and 60nM). 48 hours after transfection, as shown in Fig. 6, compared with scramble control, up-regulation of miR-124-3p significantly decreased the expression of BMP6 mRNA and protein in a stepwise manner (Fig. 6B and C). We also found reduced the expression of BMP6 in the cells in a dose dependent manner; While down-regulation of miR-124-3p significantly increased the expression of BMP6 mRNA and protein level in the cells transfected with 60 nM miR-124-3p inhibitors but not in 30 nM (Fig. 6E and F). The observations indicated that miR-124-3p negatively regulated the expression of BMP6.

Fig. 7. Cells transfected with miR-124-3p inhibitors showed evidently reduced viability (Fig. 7A), while cells transfected with miR-124-3p mimics and BMP6 siRNA showed higher viability; cells transfected with miR-124-3p mimics and BMP6 siRNA, reduced apoptosis while cells transfected with miR-124-3p inhibitors promoted apoptosis (Fig. 7B) (** $P < 0.01$ compared with the control).

MiR-124 and BMP6 interfered with the viability in cells

To further validate whether BMP6 is a functional target of miR-124-3p, we transfected with cells with scramble control, 30 and 60nM miR-124-3p mimics, 60nM BMP6 siRNA and 30 and 60nM miR-124-3p inhibitors. The results showed that cells treated with 60 nM miR-124-3p mimics and 60 nM BMP6 siRNA displayed evidently up-regulated viability (approximately 2.5 times elevation, Fig. 7A), and transfection of 30 nM miR-124-3p mimics or inhibitors had minimal effect on viability. Meanwhile, the cells transfected with 60 nM miR-124-3p inhibitors reduced viability to about 30% of the control (Fig. 7A), suggesting miR-124-3p positively interfered with the viability of cells and BMP6 negatively interfered with the viability of cells.

MiR-124 and BMP6 interfered with the apoptosis in cells

We then investigated the relative apoptosis of cells when transfected with scramble control, 30/60nM miR-124-3p mimics, 60nM BMP6 siRNA and 30/60nM miR-124-3p inhibitors. When transfected with miR-124-3p mimics and BMP6 siRNA, the number of live cells were less and the number of apoptotic cells were 2 times more than the scramble controls (Fig. 7B), and 30 nM miR-124-3p mimics or inhibitors had minimal effect on apoptosis. Meanwhile, the cells transfected with 60 nM miR-124-3p inhibitors showed about 50% less live cells and more apoptotic cells. The results indicated miR-124-3p suppressed apoptosis and BMP6 promoted apoptosis.

Discussion

Fracture healing is a complex biological process involving four stages: inflammatory response, soft callus formation, hard callus formation, and bone remodeling [15]. The regeneration process of fracture healing is precisely organized by hormones, cytokines, chemokines, growth factors, and other regulators in each stage [16]. MiR-124-3p is commonly down-regulated in several types of cancer. Aberrant expression of miR-124-3p is related to methylation status of its locus [17]. In hepatocellular carcinoma, miR-124-3p is involved in an inflammatory feedback loop where it suppresses the expression of IL-6R and inhibits STAT3 activation. Systemic administration of miR-124-3p prevents hepatocellular carcinogenesis by inducing tumor-specific apoptosis [18]. In glioblastoma, miR-124-3p is implicated in T-cell-mediated antitumor immune response [19]. It has also been shown that miR-124-3p is significantly down-regulated in colon cancer patients with ≥ 5 -year survival compared to patients with < 5 year survival. The level of miR-124-3p is associated with poor prognosis of CRC [20]. Zhang *et al.* also reported that miR-124-3p is significantly down-regulated in colon cancer tissue as compared with that of adjacent non-tumor colorectal tissue [21]. miR-124-3p has also been shown to directly suppress iASPP (inhibitor of apoptosis stimulating protein of p53) protein expression and up-regulate NF- κ B level, which suppresses cell proliferation of colon cancer cells [22]. Over-expression of miR-124-3p leads to increased apoptosis of colon

cancer cells and reduces tumor growth *in vitro* and *in vivo* [21]. In this study, we searched the publicly available databases, such as bioinformatics algorithms TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>) to study candidate target genes of miR-124-3p-3p, of all the candidates, BMP6 exhibited the most complementary structure with miR-124-3p-3p, and further confirmed using luciferase assay, we conducted the luciferase report with wild-type BMP6 3'UTR, mutant1 BMP6 3'UTR and mutant2 BMP6 3'UTR, we found that luciferase activity of the cells co-transfected with wild type BMP6 3'UTR was lower than those transfected with the scramble controls, but comparable with the cell transfected with mutant2 BMP6 3'UTR, while the introduction of mutant1 BMP6 with the potential "seed sequence" in the 3'TUR of BMP6 comparable with the scramble control and blank control.

Bone morphogenetic proteins (BMPs) is composed of a series of proteins that are members of the TGF- β superfamily. Several members of the family are key molecules in events such as odontogenesis and limb bud development [23-25]. BMPs in growth plate are also believed to promote maturation, as well as programmed cell death, or apoptosis [23, 26-28]. Recent research reports have focused on the distribution and role of BMPs in normal growth plate cell dynamics, which is the prototypical model of endochondral ossification [29]. BMP6-expression is significantly lower in proliferating non-malignant (PPCs) or malignant (HMCLs) cells and correlates inversely with the staging of the disease. *In vitro*, BMP6 significantly inhibits proliferation of HMCLs as well as primary MMCs and induces apoptosis in a time-dependent manner [30]. In this study, we investigated the different expression level of BMP6 mRNA and protein in different genotyping groups, and found the BMP6 mRNA and protein levels decreased in GG compared with GC and CC group. We further transfected with the cells with scramble control, miR-124-3p mimics (30nM and 60nM), BMP6 siRNA (60nM) and miR-124-3p inhibitors (30nM and 60nM), and found that the BMP6 mRNA and protein of cells were down-regulated following transfection with 30nM miR-124-3p mimics and, and ever lower when transfection with 60nM miR-124-3p mimics and 60nM siRNA BMP6, on the contrary, the BMP6 mRNA and protein of cells were remarkably up-regulated following transfection with 30nM miR-124-3p inhibitors and, and ever higher when transfection with 60nM miR-124-3p inhibitors. Moreover, we investigated the effect of miR-124-3p on cell viability and apoptosis, and we found that apoptosis of the cells transfected with miR-124-3p mimics, BMP6 siRNA were reduced, while promoted subsequent to transfected with and miR-124-3p inhibitors compared with scramble controls.

MiRNA polymorphisms have been shown to influence the processing and expression of miRNAs, thereby altering target gene expression and influencing the development of diseases [31, 32]. One such polymorphism, miR-124-3p rs531564 (C > G), has been shown to effect the expression of mature miR-124-3p [33]. In this study, we performed western-blot and real-time PCR and revealed the expression of level of miR-124-3p was evidently up-regulated in GG group (Fig. 1) than GC and CC group. Furthermore, we enrolled a total of 195 participants for our research and the genotype of each participant was determined: GG (n=124) and GC+CC (n=71), and found that total in-hospital time was significantly longer in GC or CC group compared with GG group.

Conclusion

These data demonstrated that the expression of miR-124-3p was associated with the healing of metaphyseal *fracture* of distal tibia, and the presence of rs531564 polymorphism downregulated the expression of miR-124-3p increased the in-hospital stay time of the patients of metaphyseal *fracture* of distal tibia. Rs531564 polymorphism might be able to function as a biomarker of fracture healing of metaphyseal *fracture* of distal tibia.

Disclosure Statement

No conflict of interest declared.

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