miR-519d-3p promotes TGFβ/Smad mediated postoperative epidural scar formation via suppression of BAMBI

LONGBIAO YANG1, QINGYUAN GAO1, FENG LV1, MIN GUO2 and DONG ZHAO1

Departments of 1Orthopaedics and 2Geriatrics, Central Hospital of Zibo Mining Refco Group Ltd., Zibo, Shandong 255120, P.R. China

Received October 22, 2017; Accepted March 13, 2018

DOI: 10.3892/mmr.2019.10630

Abstract. To investigate the role of microRNA (miR)-519d-3p in postoperative epidural scar formation and its regulation of the bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI), miR-519d-3p and BAMBI expression levels in the lumbar disc of patients who had undergone laminectomy were detected with reverse transcription-quantitative polymerase chain reaction and western blotting. The results demonstrated that miR-519d-3p expression was significantly increased, whereas BAMBI expression was sharply reduced in the lumbar discs of patients suffering from epidural scars. Subsequently, the miR-519d-3p mimic was transfected into primary fibroblasts isolated from epidural scar tissues. Flow cytometric and Cell Counting Kit-8 analyses indicated that overexpression of miR-519d-3p promoted the proliferation of fibroblasts, the production of tumor necrosis factor-α and interleukin (IL)-1α, and the expression of type I collagen (col I), α-smooth muscle actin (α-SMA) and fibronectin (FN). Downregulation of miR-519d-3p by the miR-519d-3p antagonist had the opposite effect. Bioinformatics and luciferase reporter gene analyses demonstrated that BAMBI is a target gene of miR-519d-3p: miR-519d-3p directly binds to the 3'-untranslated region (UTr) of mRNA molecules and suppressed BAMBI protein expression. Finally, the pcDNA-BAMBI vector and BAMBI small interfering RNA were respectively transfected into primary fibroblasts to overexpress and knockdown the BAMBI gene. It was demonstrated that BAMBI overexpression suppressed fibroblast proliferation, TNF-α and IL-1α production, and the expression of col I, α-SMA and FN proteins, whereas BAMBI knockdown had the opposite effect. In conclusion, it was noted that BAMBI is a target of miR-519d-3p and miR-519d-3p promotes transforming growth factor β/mothers against decapentaplegic homolog 9-mediated postoperative epidural scar formation via suppression of BAMBI.

Introduction

Spinal epidural adhesions and scars are implicated as key factors of failed back surgery syndrome, which may cause dura-mater compression or epidural tethering, resulting in persistent backache and leg pain (1). Epidural fibrosis (EF) is thought to be the main cause of spinal epidural adhesion and scar formation in patients undergoing laminectomy (2). Therefore, the prevention of fibrosis and scar formation is key to reducing postoperative recurrence and sequelae and improving patient quality of life. EF regulation is a complex mechanism. Hypernomic proliferation of fibroblasts, accumulation of collagens and the production of pro-inflammatory factors have been considered to be the primary causes of EF formation (3,4).

MicroRNAs (miRs) are relatively conserved tiny non-coding RNA molecules of 18-25 nucleotides in length, which negatively regulate gene expression by targeting specific motifs at the 3'-untranslated region of mRNA molecules (5). miRs serve important roles in diverse biological processes including carcinogenesis (6,7), immune reactions (8,9), intervertebral disc degeneration (10) and EF (5). A previous study demonstrated that miR-519d overexpression is associated with the accumulation of lipids (11). A high fat diet induces fibrosis of the heart in mice. It was demonstrated that miR-519d is associated with transforming growth factor (TGF)β signaling; a master gene in the regulation of fibrosis (12). However, the association between miR-519d and EF remains unclear.

Bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) exhibited an inverse expression pattern with TGF-β1 and collagen I (col I) mRNAs, suggesting an inhibitory role of BAMBI in the expression of these two genes (13). BAMBI overexpression and injection experiments suppress the growth of human keloid and excessive accumulation of col I in vitro and in vivo (13). BAMBI is a component of a rheostat-like mechanism that is controlled by TGF-β and interleukin (IL)-2 signaling strength (14). In lung disease, the overexpression of BAMBI has been observed and hypothesized to control local inflammation (15). However, the function of BAMBI in EF formation and its regulation have not yet been comprehensively studied.
In the present study it was demonstrated that miR-519d-3p was highly expressed in postoperative patients suffering from epidural scarring, compared with patients without scars. Up- and downregulation of miR-519d-3p altered secretion of inflammatory factors and expression of fibrotic markers in fibroblasts from postoperative epidural scar patients. It was also demonstrated that the effect of miR-519d-3p on epidural scar formation was associated with the BAMBI-mediated TGF-β signaling pathway.

Materials and methods

Patients and sampling. A total of 64 post-laminectomy patients with lumbar disc herniation were recruited for the present study, of which 40 patients developed epidural scars and the other 24 patients were without scars. Their lumbar disc tissues were sampled during the laminectomy and the removed tissues were collected, labeled and saved at -80°C for future use. The procedures were conducted at the Central Hospital of Zibo Mining Refco Group Ltd. (Zibo, China) and the mean age of the patients was 49 years old (ranging between 38 and 60 years) with a 50:50 sex ratio. The samples were collected from the patients between May 2016 and May 2017. Patients who had received chemotherapy or radiotherapy, or had other systemic disease were excluded. The present study was approved by the Ethics Committee of Central Hospital of Zibo Mining Refco Group Ltd. (Zibo, China). All participants gave written consent.

Cell culture. Samples of epidural scar tissues were taken and human primary epidural fibroblasts were isolated as previously described (16). The primary fibroblasts were obtained from the epidural scars using the enzymatic digestion method. A total of 5 mg obtained tissues were cut into small pieces and mixed with 3 ml 0.2% collagenase II. An equal volume of Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with fetal bovine serum (FBS) (both from Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to terminate the digestion. The mixture was subsequently centrifuged at 500 x g for 7 min at 37°C. The precipitate was subsequently resuspended in 2 ml medium, and centrifugation repeated, followed by further resuspension. The isolated cells were incubated in DMEM supplemented with 10% FBS, 100 µg/ml streptomycin and penicillin. Cells were incubated in a humidified incubator at 37°C and 5% CO₂.

BAMBI overexpression and transfection. The BAMBI overexpression vectors were obtained from Addgene, Inc. (Cambridge, MA, USA). The cDNA for BAMBI was amplified by polymerase chain reaction (PCR). The thermocycling conditions were set as the following: Denaturation for 60 sec at 95°C, annealing for 45 sec at 56°C and elongation for 60 sec at 72°C. The obtained BAMBI cDNA was subcloned into the pBABE-puro vector (Cell Biolabs, Inc., San Diego, CA, USA) to generate the overexpression vector of BAMBI, named pBABE-BAMBI, which was used at 1.2 µg/ml. Transfection was performed using Lipofectamine™ 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The empty pBABE-puro vector was used as a control. Cells (2x10⁵/well) were plated in a 6-well plate in DMEM supplemented with 10% FBS for 24 h to yield 70% confluency.

Small interfering (si)RNA-mediated BAMBI silencing and transfection. The cells were then transiently transfected with 30 nM BAMBI siRNA or nontargeting (random) siRNA (Ambion; Thermo Fisher Scientific, Inc.) using a Silencer™ siRNA Transfection kit II (Ambion; Thermo Fisher Scientific, Inc.) for 48 h. Scrambled siRNA was used as a control. Following transfection, total RNA was extracted for quantification of BAMBI gene expression, viable cells were counted with a hemocytometer and apoptotic analysis was conducted using a Cell Counting Kit-8 (CCK-8) assay and flow cytometry analysis. A light microscope (magnification, x100) was used to count viable cells. The sequences of the siRNA used in the present study were BAMBI siRNA (5’-AUAAGUCAUGUAUGGCAA-3’) and scrambled siRNA (5’-AAACCGUCUAUGGCAA-3’).

Reverse transcription-quantitative (RT-q)PCR assay. Total RNA was isolated from transfected cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and treated with DNasel (Promega Corporation, Madison, WI, USA). RT was performed by using the Multiscribe RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and random hexamers or oligo(dT). The RT conditions were 10 min at 25°C, 30 min at 48°C and a final step of 5 min at 95°C. qPCR was performed with SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Biotechnology, Dalian, China) in the ABI7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR thermocycling conditions were as follows: 95°C for 30 sec, 1 cycle; 94°C for 15 sec, 55°C for 30 sec, 30 cycles. The expression of genes in all groups was calculated using the 2⁻ΔΔCq method (17). All experiments were repeated three times independently. The primers used were as follows: mir-519d-3p forward, (5’-GcG Gaa aaG TGc TTa caG TG-3’); mir-519d-3p reverse, (5’-aTc caG TGc aGG GTc TTa caG TG-3’); u6 forward, (5’ -GcG Gaa aaG TGc TTa caG TG-3’); u6 reverse, (5’-ATCCAGTCAGGGTC CGAGG-3’); Bambi overexpression vector, (5’-AAAAGGAGAGAGGTGGTCTTCCTTTAGTG-3’); and anti-Bambi siRNA (5’-ATCCAGTCAGGGTC CGAGG-3’).

Transfection of mimic and inhibitor. miR-519d-3p mimic (5’-CAA AGTGGCCTCCCTTTTAGTG-3’) and antago-miR-519d-3p (5’-GGTTGACAGGAGAATACCTC-3’) were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), and respectively compounded into concentrations of 20, 40 and 80 nM and 10, 20 and 40 nM using RNAse-free water (Takara Biotechnology, Dalian, China), as well as NC mimic and antago-NC. One day before transfection, cultured cells were plated in 96-well plate, trypsin digesting and counting at a density of 1x10⁴. Transfection was performed at 90% yield using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) for 24 h.

CCK-8 assay. The cells pretreated with different concentrations of miR-519d-3p mimic (20, 40 or 80 nM) and antago-miR-519d-3p (10, 20 or 40 nM) or BAMBI vector or BAMBI siRNA as described previously were used for the CCK-8 assay. Cells (2x10⁵ well) were plated in a 96-well plate and proliferation was determined using a CCK-8 kit (Vazyme, Piscataway, NJ, USA) according to the manufacturer’s protocol, and subsequently detected the absorbance at 450 nm wavelength.

Flow cytometry. Flow cytometry was performed to observe the numbers of living cells. Cells (2x10⁵/ml) cultured as above were trypsinized and washed with PBS. This pellet was...
resuspended in 70% ethanol and kept at -20°C until staining. The samples were then treated with 1 µl RNase for 30 min at room temperature and stained with 0.5 ml propidium iodide (PI/Annexin V Cell Apoptosis Detection kit; Sigma-Aldrich; Merck KGaA) for 1 h at 4°C. Following the incubation, the cells were washed one time and then analyzed using a flow cytometer (Accuri C6; BD Biosciences, San Jose, CA, USA).

**ELISA assay.** Tumor necrosis factor (TNF)-α and IL-1 production in cell supernatants was measured using the Rat Betacellulin ELISA kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China; cat. no. EK1297) according to the manufacturer's protocol.

**Western blotting.** The protein used for western blotting was extracted using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Guangzhou, China) supplemented with protease inhibitors (Beyotime Institute of Biotechnology). The supernatant was collected and the protein concentration was measured using a bicinchoninic acid commercial kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (25 µg) were separated on 12-15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk buffer overnight at 4°C and were incubated with primary antibodies of 1:500 dilution, including those against human phosphorylated (p) mothers against decapentaplegic homolog (Smad)2/3 (cat. no. 5678) and α-smooth muscle actin (α-SMA; cat. no. 68463) from Cell Signaling Technology, Inc. (Danvers, MA, USA), and fibronectin (FN; cat. no. ab158459), col I (cat. no. ab90395) and GAPDH (cat. no. KC-5G4; 1:800; KangChen Bio-tech., Ltd., Shanghai, China), at 4°C overnight. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. ab205718; 1:1,000; Abcam) for 1 h at room temperature and then detected using a Gel imaging system (Thermo Fisher Scientific, Inc.). ImageJ software (v1.8.0; National Institutes of Health, Bethesda, MD, USA) was used to analyze the protein band intensities.

**Vector construction and luciferase reporter assays.** The miR-519d-3p targets were predicted by TargetScan (https://www.targetscan.org/vert_71/) and rnahybrid (https://bibiserv.cbi.univie.ac.at/rnahybrid/). The miR-519d-3p targets were predicted by TargetScan (https://www.targetscan.org/vert_71/) and rnahybrid (https://bibiserv.cbi.univie.ac.at/rnahybrid/).

**Statistical analysis.** All experiments were repeated three times. The results of multiple experiments are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 19.0 statistical software (IBM, Corp., Armonk, NY, USA). The P-values were calculated using one-way analysis of variance with Bonferroni’s correction. The association of mir-519d-3p and BaMBi mRNA was evaluated using the online bioinformatics tools TargetScan (http://www.targetscan.org/vert_71/) and RNAhybrid (https://bibiserv.cbi.univie.ac.at/rnahybrid/).

**Results**

miR-519d-3p is upregulated in patients developing epidural scars post laminectomy and levels are positively associated with mass of the scar. As previously mentioned, following laminectomy, the removed lumbar disc tissues were collected, labeled and preserved at -80°C. A total of two years later, an investigation was undertaken to confirm whether each patient had an epidural scar and whether there had been a
relapse of lumbar disc herniation. A total of 24 scar-free patients and 40 patients who developed an epidural scar were randomly selected. The basic information and clinicopathological parameters of the patients were respectively listed in Tables I and II. Expression of miR-519d-3p in their lumbar disc tissues sampled previously was detected using RT-qPCR analysis and the results demonstrated that miR-519d-3p was significantly upregulated in patients who had developed an epidural scar, compared with scar-free patients (P<0.01; Fig. 1A). Furthermore, miR‑519d‑3p level was positively associated with the mass of scar by Pearson's correlation coefficient (P<0.0001, r²=0.9835; Fig. 1B). These results suggested that lumbar disc miR-519d-3p expression is associated with epidural scar formation.

miR‑519d‑3p promotes fibroblast proliferation, col I accumulation and inflammation. In order to investigate the role of miR-519d-3p in scar formation in vitro, epidural scar samples were taken from the patients and primary fibroblasts were isolated. Then, fibroblasts were transfected with miR‑519d‑3p mimic at the concentrations of 20, 40 and 80 nM and miR-519d-3p inhibitor at the concentrations of 10, 20 and 40 nM, respectively. Following incubation for 24 and 48 h, cell proliferation was analyzed with a CCK-8 assay and flow cytometry. It was demonstrated that 20 nM mimic transfection significantly increased the expression of miR‑519d‑3p, while 40 and 80 nM mimic further increased its level (Fig. 2A). Analysis demonstrated that upregulation of miR‑519d‑3p significantly increased cell viability and proliferation (P<0.05; Fig. 2B and C). The concentration of inflammatory cytokines, TNF-α and IL‑1, were additionally increased significantly (P<0.05; Fig. 2D). The expression levels of FN, α-SMA and col I proteins, which were connected with fibroblast formation, were determined by western blotting. The data demonstrated that miR-519d-3p overexpression resulted in a significant increase in the expression levels of FN, α-SMA and col I (P<0.05; Fig. 2E). However, the miR-519d-3p inhibitor significantly decreased the expression of miR‑519d‑3p (Fig. 3A). Furthermore, inhibiting miR-519d-3p restrained cell viability, proliferation and TNF-α and IL-1 secretion (P<0.05; Fig. 3B-D). However, the protein levels of FN, α-SMA and col I were decreased significantly, following inhibition of miR‑519d‑3p (P<0.05; Fig. 3E). These data indicated that miR-519d-3p controlled fibroblast proliferation, TNF-α and IL-1 secretion, and protein levels of FN, α-SMA and col I.

BAMBI is a negatively regulated target of miR‑519d. Using bioinformatics tools BAMBI was identified as a putative target of miR-519d-3p (Fig. 4A). Furthermore, by analyzing the BAMBI protein level and mass of scar tissue, the BAMBI protein level was demonstrated to be inversely associated with the mass of scar tissue in patients, as exhibited in Fig. 4B. In order to validate the association, luciferase reporter vectors were constructed containing wt or mut potential binding sequences

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<sup>a</sup>P<0.01 and <sup>b</sup>P<0.05.

Figure 1. Expression of miR-519d-3p is increased in patients with epidural scars following laminectomy. (A) Comparison of the miR-519d-3p levels between patients who developed epidural scars and those who did not. (B) Proportion of miR-519d-3p/total scar mass. Following laminectomy, 24 patients without scars and 40 patients who developed an epidural scar were randomly selected. Expression of miR-519d-3p in the lumbar disc tissues samples was detected using reverse transcription-quantitative polymerase chain reaction. Control, non-scarred patients; miR, microRNA; scar, scarred patients.
in the BAMBI 3'-UTR. The wild-type BAMBI (BAMBI-wt) and mutant BAMBI (BAMBI-mut) vectors were co-transfected with miR-519d-3p mimic, into the primary fibroblasts. The results in Fig. 4C revealed that luciferase activity in BAMBI-wt and miR-519d-3p mimic co-transfected cells was significantly reduced, compared with the NC mimic group (P<0.05), whereas, cells co-transfected with BAMBI-mut and miR-519d-3p mimic demonstrated similar fluorescence intensity compared with the NC mimic group with no significant difference. BAMBI-mut has four different bases with BAMBI-wt; CUUU alters to AAAA (Fig. 4D). In primary fibroblasts, miR-519d-3p mimic transfection caused a significant reduction in BAMBI protein expression, however p-smad2/3 were significantly increased (P<0.05; Fig. 4E). However, antago-miR-519d-3p transfection significantly upregulated BAMBI protein expression level and significantly inhibited smad2/3 phosphorylation (P<0.05; Fig. 4E). These data demonstrated that BAMBI is a target of miR-519d-3p and miR-519d-3p negatively regulates BAMBI expression and fibrosis.

BAMBI negatively regulates epidural scar formation. In order to further clarify the mechanism of miR-519d-3p, the role of BAMBI in scar formation was investigated. The pcDNA-BAMBI vector or BAMBI siRNA were transfected into the primary fibroblasts. Then, the effect of BAMBI on the functions of fibroblasts, including cell proliferation, secretion of inflammatory cytokines and associated protein expression levels were investigated. The results demonstrated that pcDNA-BAMBI transfection
significantly inhibited cell viability and proliferation (P<0.05; Fig. 5A and B), TNF-α and IL-1 secretion (P<0.05; Fig. 5C) and significantly reduced fibrosis associated protein levels, including FN, α-SMA, col I, p-smad2/3 (P<0.01; Fig. 5D). Conversely, transfection with the BAMBI siRNA increased cell viability and proliferation, TNF-α and IL-1 secretion, and enhanced fibrosis associated protein levels, suggesting an increased degree of fibrosis. These data indicated that BAMBI possessed a suppressive effect on scar formation.

Discussion

Lumbar laminectomy is one of the most common treatments for lumbar disc herniation and other lumbar disorders with serious complications (19,20), including failed back surgery syndrome (21,22), mainly caused by EF. EF causes spinal epidural adhesion and scarring (23). EF is demonstrated in the epidural space and contributes greatly to postoperative pain and recurrent lumbar disc herniation (24). Extensive EF often results in negative effects on patients. Unsatisfactory clinical outcomes include radiculopathy, persistent low back pain and disability (25). Various materials or drugs have been used to inhibit formation of epidural fibrosis and reduce the compressive effect on neural structures (26,27).

miRs comprise a broad class of small non-coding RNAs that control the expression of complementary target messenger RNAs. Notable features of miR include their redundancy with respect to their target binding sequences in the 3'-UTR of mRNA and their relatively small total number, which is speculated to range from 500 to 1,000 (10). Using various computational and experimental approaches, hundreds of miRs have been identified in numerous animal species.
Dysregulation of miRs by several mechanisms has been described in various disease states (7,8). As demonstrated in previous studies, miR-519d-3p is an extracellular matrix and circulating miR that is abnormally expressed in fibrotic tissue and serum (28,29). miR-519d-3p has been proposed as a potential pro-fibrotic miR during heart regeneration and has been revealed to be positively associated with the degree of hepatic fibrosis in patients (28,30). Recent years have witnessed an increase in the number of studies on the dysregulation and role of miRs. Evidence has also indicated that miR-519d-3p serves an inhibitory role in the invasion and migration of trophoblast cells (31). miR-519d-3p has been identified to be increased and its level was proportional to the mass of scar in the present study. To further investigate miR-519d-3p in EF and scar formation, the regulated function of miR-519d-3p in fibroblasts was studied. Abnormal expression of miR-519d-3p affected fibroblast proliferation, secretion of inflammatory cytokines, and protein expression, which were associated with fibrosis. To the best of the authors' knowledge, this is the first report investigating miR-519d-3p and its regulation of spinal EF and scar formation.

Using the bioinformatics software programs RNAhybrid and TargetScan, BAMBI was identified as a putative target of miR-519d-3p. BAMBI is a protein, which in humans is encoded by the BAMBI gene. BAMBI is regarded as a pseudo-receptor of TGF-β, for they share a common transmembrane glycoprotein responsible for signal receiving (32,33), however BAMBI lacks the intracellular serine/threonine kinase domain required for signal transduction (34). In addition, studies also revealed that BAMBI restrains TGF-β signaling and serves a negative role in fibrosis of the liver and several other tissues (13,35). It has previously been noted that BAMBI has a negative impact on the growth of human keloid cells (13). However, studies on the role of BAMBI in epidural fibrosis and related diseases, particularly in fibroblasts and scarring, have not yet been reported. In the present study, it was
demonstrated that expression of the BAMBI protein was decreased in lumbar disc tissues from patients and its level was inversely proportional to mass of scar. It was demonstrated in the present study that miR-519d-3p directly binds to the 3'-UTR of BAMBI. miR-519d-3p overexpression in vitro indicated that miR-519d-3p significantly inhibited BAMBI expression, however phosphorylation of smad2/3 and TGF-β were increased. Inhibition of miR-519d-3p had the opposite effect on BAMBI expression and smad2/3 phosphorylation. The targeting association between miR-519d-3p and BAMBI is a novel finding of the present study.

Following a thorough review of the literature and the aforementioned experimental results, the authors hypothesize that miR-519d-3p may regulate postoperative epidural scar formation via BAMBI. This conjecture was verified and the results demonstrated that upregulation of the BAMBI level reduced cell proliferation, inflammatory cytokine secretion, and decreased fibrosis associated protein expression, suggesting a reduced degree of fibrosis. Conversely, reducing BAMBI increased cell proliferation, TNF-α and IL-1 secretion, and enhanced fibrosis associated protein levels. BAMBI is postulated to modulate TGF-β/Smad signaling. Activation of the TGF-β/Smad signaling pathway mediates postoperative epidural scar formation (36,37). The results of the present study demonstrated that BAMBI inhibited smad2/3 phosphorylation, suggesting depression of TGF-β/Smad signaling and inhibition of epidural scar formation.

In conclusion, the role and mechanism of miR-519d-3p and BAMBI in suppressing postoperative epidural scar formation was examined. miR-519d-3p was a direct negative regulator of BAMBI and served a crucial role in regulating cell proliferation, inflammatory factors TNF-α, and IL-1 and fibrosis-associated protein expression. The results raise the possibility of using miR-519d-3p as a potential therapeutic agent to suppress the TGFβ/Smad mediated postoperative epidural scar formation.

Acknowledgements

Not applicable.

Funding

No funding was received.

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

LY analyzed the data and prepared the manuscript. QG and FL performed the experiments. MG was involved in data interpretation and the critical revision of the manuscript for important intellectual content. DZ designed the study.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Central Hospital of Zibo Mining Refco Group Ltd. (Zibo, China). All participants gave written consent.
Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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