microRNA-329 reduces bone cancer pain through the LPAR1-dependent LPAR1/ERK signal transduction pathway in mice

Xian-Ping Wu, Yan-Ping Yang, Rui-Xuan She, Zu-Min Xing, Han-Wen Chen and Yi-Wen Zhang

Abstract
Background: Bone cancer pain (BCP) is a common symptom occurring among patients with cancer and has a detrimental effect on their quality of life. Growing evidence has implicated microRNA-329 (miR-329) in the progression of bone diseases. In the present study, we aimed to elucidate the potential effects of miR-329 on BCP in a BCP mouse model via binding to lysophosphatidic acid receptor 1 (LPAR1) through the LPAR1/extracellular signal-regulated kinase (ERK) signaling pathway.

Methods: Initially, a BCP mouse model was established via injection of $4 \times 10^4$ murine breast tumor (4T1 cell) cells (4 μl). The interaction between miR-329 and LPAR1 was identified using a bioinformatics website and dual luciferase reporter gene assay. The modeled mice were subsequently treated with miR-329 mimic, LPAR1 shRNA, or both, in order to examine the effect of miR-329 on the paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) of mice, the expression of LPAR1/ERK signaling pathway-related genes.

Results: The positive expression rate of LPAR1 protein and extent of ERK1/2 phosphorylation were increased in BCP mouse models. LPAR1 is a target gene of miR-329, which can inhibit the expression of LPAR1. In response to miR-329 overexpression and LPAR1 silencing, BCP mice showed increased PWT and PWL, along with decreased LPAR1 expression and ratio of p-ERK/ERK.

Conclusions: Altogether, the results obtained indicated that miR-329 can potentially alleviate BCP in mice via the inhibition of LPAR1 and blockade of the LPAR1/ERK signaling pathway, highlighting that upregulation of miR-329 could serve as a therapeutic target for BCP treatment.

Keywords: bone cancer pain, LPAR1, LPAR1/ERK signaling pathway, microRNA-329, mouse model

Received: 13 January 2019; accepted in revised form: 19 August 2019.

Introduction
Bone cancer pain (BCP) is a frequently occurring clinical complication of bone metastasis, and can result in a notable reduction in quality of life.\textsuperscript{1} Due to the high possibility of metastasis to the bone, there is a high prevalence of BCP in patients with advanced breast, lung, and prostate cancers.\textsuperscript{2} Reports have highlighted a link between metastases and symptoms of bone cancer, including pain, hypercalcemia, anemia, elevated susceptibility to infection, skeletal fractures, compression of the spinal cord, and spinal instability.\textsuperscript{3} The current therapeutic approaches to BCP have been found to have unsatisfactory outcomes and undesirable side effects.\textsuperscript{4} Thus, there is an urgent need to find newer and more accurate prediction tools to provide better diagnoses and prognosis for BCP. MicroRNAs (miRNAs or miRs) are presently an area of interest in research due to the multiple associations they have with various cancers. miRNAs are small noncoding RNA molecules that have been demonstrated to play central
roles in the occurrence and progression of bone cancer.5

Previous studies have revealed that several miRNAs, including miR-124, miR-132, and miR-326, possess various functions involved in the progression of BCP.6–8 miR-329, particularly, has been found to play a role as a tumor suppressor in the pathogenesis and progression of bone cancer.9 Furthermore, miR-329, along with peroxisome proliferator-activated receptor γ (PPARγ), could potentially serve as an intracellular receptor for lysophosphatidic acid (LPA). After reviewing previous literature and studies, we drew the hypothesis that miR-329 may target the lysophosphatidic acid receptor 1 (LPAR1) gene.10,11 LPAR1 is a bioactive lipid involved in the development of BCP through its regulation of the P2X3 receptor.12 Furthermore, upregulation in LPA has been linked with an elevated risk of metastasis and pain development in bone cancer.13 LPA-dependent cell migration has been achieved previously through the LPAR3-Gi-extracellular signal-regulated kinase (ERK)-pathway, independently of LPAR1.14 The silencing of LPAR1 inhibits cell migration and prevents ERK1/2 phosphorylation from occurring following spinal cord injury in rats; therefore, LPAR1 ligation activates the ERK1/2 signaling pathway in LPA-induced olfactory ensheathing cell chemotactic migration.15 Another study has suggested that the ERK signaling pathway is activated in different cell types in the rat spinal cord of BCP model.16 The ERK signaling pathway has also been found to be involved in the modulation of BCP, indicating that the ERK signaling pathway could potentially provide a new therapeutic approach for BCP patients.17 Based on the aforementioned, it can be hypothesized that miR-329 may affect the development of BCP involving the regulation of LPAR1, and the LPAR1/ERK signaling pathway. Thus, the current study aims to investigate the mechanism by which miR-329/LPAR1/ERK axis participates in the regulation of BCP.

Materials and methods

Ethics statement

All animal experiments were performed in strict accordance with the guidelines of the International Association for the Study of Pain. Approval for the study was provided by the Ethics Committee of Laboratory Animal Use of Shunde Hospital of Guangzhou University of Chinese Medicine (Approval Number 201803002).

Model establishment

A total of 78 female C3H/HeJ mice (weight: 18–25 g) were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). All purchased mice were housed in individual cages at 23 ± 1°C with 12 h day/night cycle, and allowed free access to food and water. The experiment was conducted following a 1-week adaptation. Then, 64 mice were anesthetized with 3% pentobarbital sodium (H31020240; Shanghai New Asia Pharmaceutical Co., Shanghai, China), followed by disinfection and shaving of the left hindlimbs. Frontal incisions to the left tibia were made to expose the tibia (approximately 1 cm in length), and a pinhole was made. A 5 μl microinjector was used to inject 4 × 10⁴ murine breast tumor (4T1 cell) cells (4 μl; CoBioer Biosciences Co., Ltd., Nanjing, Jiangsu, China) to the tibial bone marrow cavity. After injection, the pinhole was immediately sealed with bone wax, followed by skin closure. Gentamicin was injected into the wounds of the mice to prevent infection. If the mice could not walk due to pain in the left hindlimb, the mouse model of BCP was regarded as a success.18 The mice in the sham group were injected with normal saline without 4T1 cells at the same site.19

Catheterization of the spinal subarachnoid space and animal grouping

A total of 14 mice undergoing sham operation and 64 successfully modeled mice were anesthetized through intraperitoneal injection of 0.6% pentobarbital sodium (0.05 ml/kg), followed by catheterization of the spinal subarachnoid space. The mice were then fixed in the prone position, and the L6-S1 interspinous space was used as the center for disinfection. Next, a 1-cm longitudinal incision was made to expose the interspinous space. A PE-10 catheter with a built-in guide wire was placed into the spinal canal; a sense of loss and the presence of a jitter on the tail indicated the successful insertion of the catheter into the spinal canal. The guide wire was then extracted, and a PE-10 catheter was slowly placed into the subarachnoid space. At this time, overflow of clear cerebrospinal fluid was observed. The depth of the insertion was 3 cm, and the proximal catheter was sutured and fixed. A subdural tunnel was punctured under the skin with an epidural needle.
to guide the lateral end of the catheter to the back of the neck with an exposing distance of 2 cm. The skin was then sutured, and the catheter was sealed with the use of a hot melt. After surgery, the mice were administered food in single cages. The activities of the mice were observed following a 24-h period of the mice staying awake. If paralysis, lameness, or abnormal behavior were present, the mice were removed from the study. Finally, the mice were treated with an intrathecal injection of 2% lidocaine (20 μl), with no paralysis of the lower limb observed within a period of 30 s. The specific grouping is shown in Table 1.

X-ray scanning
On the days 7 and 21, two mice from the sham group and two mice from the BCP group were randomly selected. Next, X-ray scanning was conducted to scan the modeled hindlimb and contralateral hindlimb of the BCP mouse models. Following the intraperitoneal administration of pentobarbital sodium (0.6%, 0.05 ml/kg) for anesthetic purposes, the mice were placed in front of the X-ray scanner (Kodak, Italy), and exposed to X-rays at 30 kV for 1 min. Radiographs were acquired, and the films were developed using a developer (CAS No. 7757-83-7; Hefei Jiankun Chemical Co., Hefei, Anhui, China). Subsequently, the extent of tumor-induced bone destruction was assessed. The image of neoplastic bones was scored on a five-point scale: 0 point: normal bone structure without bone destruction; 1 point: small and few radioactive bone defect lesions appeared near the injection site (the number of lesions less than or equal to 3); 2 points: medullary bone defects and a large number of radioactive lesions (the number of lesions more than 3); 3 points: medullary bone loss and slight cortical bone damage; 4 points: complete unilateral cortical bone defects; 5 points: cortical bone loss and displaced fractures appeared on both sides of the bone.18

Hematoxylin-eosin staining
After X-ray scanning, the mice were euthanized and their respective left hindlimbs were collected. Subsequently, hair removal was conducted with the use of 8% sodium sulfide solution. The left hindlimbs were subsequently fixed in 10% formaldehyde solution for 24 h and then decalcified. Next, the left hindlimbs underwent conventional dehydration, paraffin embedding, and slicing. The sections were then washed with double-distilled water for 1 min, followed by staining with hematoxylin-eosin (HE) for 1 min, color separation with 1% hydrochloric acid for 5 s, and washing under running water for 30 min. The results from the staining were observed under a microscope. Finally, tumor growth and bone destruction were observed and photographed.
with the use of an optical microscope (XSP-36; Boshida Optical Instrument Co., Shenzhen, Guangdong, China).

**Extraction of mouse spinal cord in vivo**

On day 21, 10 mice each were randomly selected from the sham group and the BCP group. The mice were anesthetized through the intraperitoneal injection of pentobarbital sodium (0.6%, 0.05 mg/kg). When the mice were deeply anesthetized, they were euthanized and the skin on their back and muscles was separated to expose the thoracic and lumbar spine. This was when timing began; timing was stopped when the spinal cord was extracted and the dura mater spinalis had been stripped. The mice were then provided with oxygen, and laid flat, with a hot water bag used to provide warmth. The rongeur was used to bite the spinous process of the thoracic vertebra and expose the inter-laminar space. Bone scissors were then carefully inserted into the interlaminar space, and the vertebral arch was cut off one by one to remove the vertebral lamina, after which the intumescentia lumbalis of the spinal cord was exposed. Ophthalmic scissors were used to transect the spinal cord in the upper intumescentia lumbalis, and ophthalmic forceps were used to clamp the dura mater spinalis. The broken end of the intumescentia lumbalis of the spinal cord was gently lifted, and the connecting nerve roots were cut off on both sides of the spinal cord using ophthalmic scissors. Once the end of the intumescentia lumbalis had been cut off, the spinal cord was extracted using ophthalmic forceps, and placed in a culture dish [containing 0.1 mol/l precooled phosphate buffered saline (PBS)]. The mice were then decapitated and the dura mater spinalis was stripped. Following extraction, the samples were stored in liquid nitrogen for subsequent experiments.

**Immunohistochemistry**

The spinal cord intumescentia lumbalis segment and dorsal root ganglion were extracted from the samples described above. The tissues were fixed, embedded, cut into sections, and washed with PBS. Next, the sections were immersed in 3% hydrogen peroxide (H₂O₂) at 37°C for 10 min. After incubation was carried out in blocking solution at 37°C for 2 h, the sections were incubated with rabbit anti-mouse p-ERK1/2 (1: 400; # 4370; Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-mouse LPAR1 (1: 50; ab23698; Abcam, Inc., Cambridge, UK) at 4°C overnight, followed by three washes with 0.01 M PBS (5 min each). Afterwards, the sections underwent incubation with biotinylated goat anti-rabbit (1: 200) at 37°C for 1 h, followed by staining with 0.05% diaminobenzidine (DAB; Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China). The reaction was then terminated using Tris-buffered saline (TBS). The sections were dehydrated using gradient alcohol, cleared with xylene, and mounted with neutral balsam. Finally, the samples were photographed under an optical microscope (XSP-36; Boshida Optical Instrument Co., Shenzhen, China). Finally, 10 visual fields were randomly selected to calculate the percentage of positive cells, with 100 cells counted in each field. The staining degree was classified as follows: no color (negative), light yellow (weakly positive), brown yellow (moderately positive), and brown (strongly positive).

**Double-labeling immunofluorescence**

The spinal cord intumescentia lumbalis segment and dorsal root ganglion were extracted from the samples and fixed in 4% paraformaldehyde, followed by dehydration and cutting into coronal sections. Next, 3–5 drops of Triton X 100 were added, and the sections were blocked using 10% goat serum at room temperature for 1 h. The sections were then incubated with primary antibody rabbit anti-mouse p-ERK1/2 (1: 400; # 4370; Cell Signaling Technology, Beverly, MA, USA) and OX-42 (1: 200; lot 42210A; Cedarlane Laboratories, Hornby, Ontario, Canada) with the avoidance of light at 4°C overnight. Further incubation was carried out with a mixture of Alexa Fluor488 (1: 500) and Alexa Flour555 (1: 200) without light exposure at room temperature for 2 h. Thereafter, the sections were mounted using anti-fade mounting medium devoid of light, observed under a fluorescence microscope, and photographed using an Olympus camera (C7070wz) under an upright fluorescence microscope (Olympus-OE41217). Four fields were intercepted in the superficial layer of the left dorsal horn of the spinal cord of each section. Images of the sections were analyzed using Image-Pro Plus 6.0 software. The number of positive cells of the extent of ERK1/2 phosphorylation was calculated in each visual field, and the average value of the four sections was regarded as the average value of the extent of ERK1/2 phosphorylation in the dorsal horn of the spinal cord.
Dual luciferase reporter gene assay
The target relationship between miR-329 and the LPAR1 gene was predicted using bioinformatics prediction website (http://www.microRNA.org) and verified using dual luciferase reporter gene assay. The LPAR1-3’-untranslated region (3’UTR) gene segment was artificially synthesized and inserted into the pMIR-reporter through SpeI and HindIII endonuclease sites. The mutation sites of complementary sequence of seed sequence were designed in wild-type (Wt) LPAR1. After restriction endonuclease treatment, the target segment was inserted into the plasmid pMIR-reporter using T4 DNA ligase. The correctly sequenced luciferase reporter plasmids, including Wt and mutated (Mut), were cotransfected with a miR-329 mimic into HEK-293T cells. Following 48 h of transfection, the cells were harvested and lysed, and luciferase activity was determined using a luciferase assay kit (GM-040501A; Genomeditech Co., Shanghai, China).

Reverse transcription-quantitative polymerase chain reaction
Total RNA was extracted from spinal cord tissues using the Trizol one-step method (15596026, Invitrogen, Carlsbad, CA, USA). The purity and concentration of RNA were measured using ultraviolet (UV) spectrophotometry. The extracted RNA was reverse-transcribed into complementary DNA (cDNA) according to the instructions provided with the Primescript RT reagent kit (RR047A; TaKaRa, Tokyo, Japan). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using a real-time fluorescence quantitative PCR system (ABI 7500, ABI, Foster City, CA, USA) according to the instructions provided with the SYBR® Premix Ex Taq™ II Kit (RR420A, TaKaRa, Tokyo, Japan). The RT-qPCR conditions were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. Primers (Table 2) were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). The Ct values of each well were recorded. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal references. The relative expression was analyzed using the 2–∆∆Ct method, where ∆∆Ct was calculated as follows:

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\Delta\Delta Ct = (\text{average Ct value of the target gene in the experimental group} - \text{average Ct value of the housekeeping gene in the experimental group}) - (\text{average Ct value of the target gene in the control group} - \text{average Ct value of the housekeeping gene in the control group}).
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Western blot analysis
The spinal cord tissues were added to liquid nitrogen, then ground and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (P1004, Wuhan Biobao Biotech Co., Wuhan, Hubei, China) containing protease inhibitors and phosphatase inhibitors. Subsequently, protein was extracted from the spinal cord tissues in each group. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Inc., Rockford, IL, USA). Next,
protein was separated with the use of a 4% spacer gel and 10% separation gel, after which it was transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% skimmed milk powder for 2 h. After being washed with PBS-Tween-20 (PBST), the membranes were incubated overnight with the following primary rabbit anti-mouse antibodies: LPAR1 (1: 500, ab23698; Abcam, Inc., Cambridge, UK), ERK1/2 (1: 1000, ab17942; Abcam, Inc., Cambridge, UK), p-ERK1/2 (1: 2000, #4370; Cell Signaling Technology, Beverly, MA, USA), and GAPDH (1: 2500, ab9485; Abcam, Inc., Cambridge, UK), followed by three washes with PBST (10 min each). Next, further incubation was carried out with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (ab191866; Abcam, Inc., Cambridge, UK) at room temperature for 2 h. After three washes with TBS-Tween-20 (TBST) (10 min each), an enhanced chemiluminescence (ECL) reagent (Invitrogen, Carlsbad, CA, USA) was used to visualize the immunocomplexes on the membrane. Band intensities were quantified using Bio-Rad imaging system. Relative protein expression was taken as the ratio of the gray value of the target band to GAPDH. Each experiment was conducted in triplicate.

Von Frey hair test and Hargreaves test
The von Frey hair test was applied to determine the paw withdrawal threshold (PWT) induced by mechanical stimulation in mice. Different amounts of hair pressure (in grams) were applied to the center of the mouse paw to induce a paw withdrawal response, and the minimum paw pressure (in grams) that induced paw withdrawal was defined as the PWT. The mice were then placed in the test environment 0.5 h in advance. The radiant heat was applied with main focus area placed on the plantar surface of each paw, and the time taken for the mouse to withdraw the paw was recorded by the stimulator. The upper limit of each stimulus was set as 15 s, with the test interval lasting a minimum of 10 min to prevent substantial damage to the mice. The PWL of mice was determined by the mean time of the paw withdrawal response of three tests.

Statistical analysis
Statistical analysis was performed using SPSS 21.0 software (IBM Corp Armonk, NY, USA). The measurement data were presented as mean ± standard deviation (SD). Data among multiple groups were compared using one-way analysis of variance (ANOVA), while data between two groups was compared using t-test. A value of \( p < 0.05 \) was considered as statistically significant.

Results
The mouse model of BCP is successfully established
X-ray scanning and HE staining were performed to detect whether the BCP mouse models had been successfully established. The X-ray results are shown in Figure 1. There was no obvious bone destruction in the left tibia of mice in the sham group. The cortical bone was continuous, and the border was smooth and clear, with no obvious abnormality observed in the bone marrow cavity. The X-ray of mice in the sham group was identified with the help of X-ray performed on normal bone tissues. Thus, the score of the sham group was 0 point. In the BCP group, the left tibia progressively developed distinct bone destruction along with changes to the modeling time, with uneven bone mineral density. Due to the increase in density as well as the low-density defect, the left tibia appeared as a cloud flocculent, the cortical destruction was discontinuous, and there was a positive correlation between bone destruction and the intensity of von Frey hair that induced an effective response at least three times.
On day 7, the middle and upper parts of the left tibia presented with obvious osteoporosis, while the cortical bone was invaded and thinned, and the structure of the bone was not clear; thus, the score of the BCP group on day 7 was 3 points. On day 21, the left tibia bone in the BCP group was found to be severely damaged, with a completely absent cortical bone, enlargement in the extent of the lesion, swelling in the surrounding tissues, and pathological fractures in certain sections of the tibia; thus, the score of the BCP group on day 21 was 4 points.

The results of HE staining analysis revealed that the bone marrow cells in the mouse tibial bone marrow cavity had grown normally, with complete, regular arrangement of trabecular bone, and no changes appeared in the bone structure in the sham group. In the BCP group, the mouse tibial bone marrow cavity was observed to be filled with tumor cells, and there was a significant damage in both the cortical bone and bone marrow, and the trabecular structure was destroyed. On day 7, the mouse tibial bone marrow cavity was almost filled with tumor cells, and degeneration and necrosis of tumor cells appeared in the central part. However, in the marginal part of the bone marrow cavity, tumor cells were mostly active, and the tumor had grown outwards, with most of the trabeculae and cortical bone destroyed. On day 21, the mouse tibial bone marrow cavity was filled with tumor cells, and most of the tumor cells in the central part were necrotic. In the marginal part of the bone marrow cavity, tumor cells were mostly active; the tumor growth was outwards and invaded the cortical bone, with the trabeculae and cortical bone completely destroyed. All the aforementioned results suggested that the mouse model of BCP was successfully established.

**Figure 1.** X-ray scanning and HE staining indicate the successful establishment of mouse models of BCP. Yellow arrows indicate normal trabecular bone structure and the black arrow indicates the destroyed trabecular bone structure by cancer cells on day 7. On day 21, almost all trabecular bone structures were destroyed.

BCP, bone cancer pain; HE, hematoxylin-eosin.
Higher LPAR1 expression and extent of ERK1/2 phosphorylation are identified in BCP

The positive expression rate and localization of LPAR1 protein and extent of ERK1/2 phosphorylation were detected using immunohistochemistry and double-labeling immunofluorescence. The results from the immunohistochemistry (Figure 2a) revealed that, in spinal cord tissues, LPAR1 protein was located on the plasma membrane of the surface of spinal cord dorsal horn microglia, while p-ERK1/2 was located in the cytoplasm. Compared with the sham group, the positive expression rate of LPAR1 protein and extent of ERK1/2 phosphorylation increased from 5.3% and 6.7% to 76.8% and 61.9%, respectively, on day 21 in the BCP group (Figure 2b). The results from double-labeling immunofluorescence (Figure 2c, d) showed that p-ERK1/2 was expressed in the microglia in the BCP group.

In comparison with the sham group, the extent of...
ERK1/2 phosphorylation increased from 6.0% to 37.2% on day 21 in the BCP group.

**LPAR1 is a target gene of miR-329**

Based on the results obtained from the bioinformatics website (http://www.microrna.org), LPAR1 was found to be the target gene of miR-326 (Figure 3a). To confirm that LPAR1 is a direct target gene of miR-329, luciferase reporter vector recombinant plasmids Wt-miR-329/LPAR1 and Mut-miR-329/LPAR1 with inserted Wt and Mut LPAR1 3’-UTR sequences, respectively, were constructed. The results from the dual luciferase reporter gene assay (Figure 3b) verified that, compared with the negative control (NC) group, there was a decrease in the luciferase activity of LPAR1-Wt by approximately 50% in the miR-329 mimic group (p<0.05); however, no significant difference was observed in the luciferase activity of LPAR1-Mut (p > 0.05). These results indicated that miR-329 could specifically bind to LPAR1.

**Upregulated miR-329 expression reduces pain threshold in mice with BCP**

Next, PWT and PWL were assessed in mice by use of the von Frey hair test and Hargreaves test, respectively (Figure 4). At 1–2 weeks after cancer cell inoculation, evident thermal hyperalgesia was observed in the hind limbs of mice in the remaining six groups in comparison with that of the sham group (p < 0.05). Compared with heat hyperalgesia, the mechanical-induced tactile allodynia appeared earlier in tumor mice, with clear tingling pain observed during the week of inoculation of cancer cells. There was no significant difference detected in the response threshold of the contralateral hind limbs to thermal stimulation and mechanical stimulation in tumor mice at each time point (p > 0.05). Compared with the sham group, there was no difference in relation to the pain threshold of mice in each group on day 3 (p > 0.05). The mouse pain thresholds among each group decreased to 25–80% between days 7 and 21 (p < 0.05). No significant difference was found regarding the pain threshold exhibited between the NC and BCP groups (p > 0.05). Compared with the BCP group, the pain thresholds of the miR-329 mimic, LPAR1 shRNA, and miR-329 mimic + LPAR1 shRNA groups increased to 25–50%, whereas those of the miR-329 mimic + LPAR1 cDNA group decreased to 15–50% (p < 0.05). The results suggested that overexpressed miR-329 can reduce the pain threshold in mice with BCP.

**miR-329 has analgesic effects on mice with BCP through the LPAR1-mediated LPAR1/ERK signaling pathway**

RT-qPCR and western blot analysis were conducted to measure the expression of miR-329, LPAR1, and ERK in transfected cells in vivo in mice with BCP in order to investigate their function and mechanism of action. As shown in

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**Figure 3.** LPAR1 is a target gene of miR-329: (a) predicted binding sites for miR-329 in LPAR1-3’UTR; (b) luciferase activity of cells transfected with LPAR1-3’UTR-Wt and LPAR1-3’UTR-Mut; *p < 0.05 versus the NC group. The experiment was repeated three times independently.

LARP1, lysophosphatidic acid receptor 1; NC, negative control; 3’UTR, 3’-untranslated region.
Figure 5(a), compared with the sham group, miR-329 expression decreased to 29%, 31%, and 26% in the BCP, NC, and LPAR1 shRNA groups, but increased to approximately 50% in the miR-329 mimic, miR-329 mimic + LPAR1-cDNA, and miR-329 mimic + LPAR1 shRNA groups (all \( p < 0.05 \)). However, no significant difference was observed in miR-329 expression among the BCP, NC, and LPAR1 shRNA groups (\( p > 0.05 \)). Additionally, no remarkable difference was found in miR-329 expression among the miR-329-mimic, miR-329-mimic + LPAR1-cDNA, and miR-329 mimic + LPAR1 shRNA groups (\( p > 0.05 \)). Compared with the BCP group, miR-329 expression increased 1.1-fold in the miR-329-mimic, miR-329-mimic + LPAR1-cDNA, and miR-329 mimic + LPAR1 shRNA groups (\( p < 0.05 \)). As shown in Figure 5(b), compared with the sham group, the expression of LPAR1 increased to about 50% in the BCP, NC, and miR-329-mimic + LPAR1-cDNA groups, while it decreased to 53%, 55%, and 62% in the LPAR1 shRNA, miR-329-mimic, and miR-329 mimic + LPAR1 shRNA groups (\( p < 0.05 \)). No significant difference in LPAR1 expression was detected between the BCP group and the NC group (\( p > 0.05 \)). There was no significant difference in the expression of LPAR1 between the miR-329 mimic + LPAR1 shRNA group and the BCP group (\( p > 0.05 \)). Compared with the BCP group, the expression of LPAR1 in the miR-329 mimic + LPAR1-cDNA group increased to 21% (\( p < 0.05 \)). As shown in Figure 5(c, d), compared with the sham group, expression of LPAR1 and ratio of p-ERK/ERK increased to 50–80% in the BCP, NC, and LPAR1 shRNA groups while the
miR-329-mimic, miR-329-mimic + LPAR1-cDNA, and miR-329 mimic + LPAR1 shRNA groups presented with no marked difference in the expression of LPAR1 and ratio of p-ERK/ERK (all $p > 0.05$). Compared with the BCP group, the LPAR1 expression and ratio of p-ERK/ERK increased to approximately 75% in the miR-329-mimic + LPAR1-cDNA group. These findings suggest that upregulation in miR-329 might have analgesic effects on BCP mice by downregulating LPAR1 expression and inhibiting the LPAR1/ERK signaling pathway.
Discussion
Recent studies have demonstrated that miRNAs play a functional role in the regulation of various pathological aspects of bone diseases by silencing posttranscriptional gene expression. An example of such includes the ability of miR-329 in suppressing proliferation, invasion, and migration abilities of osteosarcoma cells through Rab10 downregulation. In the present study, we aimed to provide further insight into the underlying mechanism by which miR-329 influences pain induced by bone cancer, and whether the effects involves LPAR1 and the LPAR1/ERK signaling pathway. Our findings demonstrate that miR-329 could potentially relieve BCP pain in mice by blocking the LPAR1/ERK signaling pathway through LPAR1 suppression.

Initially, our results revealed that the positive expression rate of LPAR1 and p-ERK1/2 proteins increased in mice with BCP. Consistently, the number of LPAR1-positive dorsal root ganglion neurons was significantly greater in rats with bone cancer than in control rats. Immunohistochemistry staining revealed a high localization of LPA in isolated dorsal root ganglion neurons in BCP rats. The activation of the Akt/ERK signaling pathway has been confirmed in BCP mice. The expression of p-ERK1/2 in the L6-S2 dorsal spinal cord evidently increased in E2 with comorbid rats.

Moreover, the findings from behavioral tests revealed that overexpressed miR-329 can result in the reduction of pain threshold in mice with BCP, as indicated by the increase in PWT and PWL seen in mice following treatment with miR-329 mimic. BCP mice presented with a lower PWT and PWL score compared with the sham mice. miR-200b and miR-429 overexpression resulted in the alleviation of neuropathic pain by elevating PWT and PWL in chronic constriction injury (CCI) rats. Moreover, overexpression of miR-16 has been reported to significantly decrease pain threshold.

In conclusion, our results provide further insights into the underlying mechanism by which analgesic effects are achieved through the upregulation of miR-329 expression in BCP through inhibition of the LPAR1/ERK signaling pathway by binding to LPAR1. Thus, miR-329 might be a promising potential therapeutic target for BCP. Although our findings present a significant proposition for the therapeutic development in response to BCP, further studies are required to elucidate the therapeutic strategies.
specific mechanism of miR-329 in BCP and to explore the clinical diagnostic value for BCP based on different cell types and miR-329 antagonim experiments.

Acknowledgments
Xian-Ping Wu, Yan-Ping Yang, Rui-Xuan She contributed equally and are regarded as joint first authors. We would like to express our sincere appreciation to the reviewers for their helpful comments on this article.

Funding
The author(s) received no financial support for the research, authorship, and publication of this article.

Conflict of interest statement
The authors declare that there is no conflict of interest.

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