miR-885-5p suppresses osteosarcoma proliferation, migration and invasion through regulation of β-catenin

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Received December 9, 2017; Accepted September 24, 2018

DOI: 10.3892/ol.2018.9768

Abstract. MicroRNAs (miRs) have been reported to serve key roles in cancer. To investigate the function of miR-885-5p in osteosarcoma, the expression levels of miR-885-5p were analyzed in 85 osteosarcoma tissue samples and adjacent non-cancerous tissue samples, using reverse transcription-quantitative polymerase chain reaction analysis. It was demonstrated that miR-885-5p was downregulated in osteosarcoma tissues and cell lines. Notably, the expression level of miR-885-5p was closely associated with tumor size, Tumor-Node-Metastasis stage and lymph node metastasis. Additionally, low expression levels of miR-885-5p also predicted a poor prognosis of osteosarcoma. To further decipher the roles of miR-885-5p in osteosarcoma, it was determined that β-catenin, a key component of the Wnt signaling pathway, was a target of miR-885-5p. Furthermore, several functional experiments, including a colony formation assay, CCK-8 assay, wound healing assay and Transwell invasion assay, revealed that miR-885-5p suppressed cell proliferation, migration and invasion through inhibition of β-catenin. The results of the present study provide a novel insight into the molecular roles of miR-885-5p in osteosarcoma.

Introduction

Osteosarcoma has become one of the most common types of bone cancer (1,2). The clinical treatment for osteosarcoma has little effect on amputated patients due to pulmonary metastases (3). Although surgery and neoadjuvant chemotherapy has improved the 5-year survival rate to ~60% (4), distant metastasis may occur due to poor responses to chemotherapy. Therefore, understanding the detailed mechanisms involved in osteosarcoma development is important.

MicroRNAs (miRs) are highly conserved, non-coding RNA molecules that serve central roles in multiple cancer-associated signaling pathways, including invasion and metastasis, through post-transcriptional regulation of genes (5-8). miRs are able to recognize the 3′-untranslated regions (3′-UTRs) of target genes and result in their degradation or destabilization through the RNA-induced silencing complex. miR-885-5p has been reported to be upregulated in pancreatic cancer and breast cancer (9,10). In breast cancer, miR-885 targets the 3′-UTR of E2F transcription factor 1 to downregulate its expression, thereby regulating the proliferation of human breast cancer MCF-7 cells (10). Previous work has demonstrated that miR-885-5p may be upregulated in osteosarcoma tissues (11); however, the function of miR-885-5p in osteosarcoma is yet to be elucidated.

The Wnt/β-catenin signaling pathway serves as a key oncogenic pathway in multiple cancer types, including osteosarcoma (12). β-catenin is a key component of the Wnt/β-catenin signaling pathway, and is re-localized from the cytosol to the nucleus upon Wnt ligand stimulation, thereby regulating gene expression (13). A number of previous studies have suggested that Wnt/β-catenin may regulate various downstream targets, including c-Myc proto-oncogene (c-myc), cyclin D1 and mitogen-activated protein kinase 8 (MAPK8), which regulate migration, cell proliferation and stemness (14-16). Notably, a previous study has demonstrated that miR-885 may interact with β-catenin in bovine semitendinosus and masseter muscles (17). However, whether miR-885-5p is able to regulate β-catenin in osteosarcoma cells requires further investigation.

Although previous work reported that miR-885-5p may be upregulated in osteosarcoma tissues (11), the detailed mechanism of action of miR-885-5p remains unknown. The aim of the present study was to verify whether miR-885-5p displays abnormal expression levels in osteosarcoma and to further decipher the molecular mechanism of miR-885-5p in osteosarcoma. In the present study, it was demonstrated that miR-885-5p was downregulated in osteosarcoma tissues and cell lines. miR-885-5p expression levels were closely associated with tumor size, Tumor-Node-Metastasis (TNM) stage and lymph node metastasis, in addition to the prognosis of osteosarcoma. Furthermore, β-catenin, a key component of the Wnt signaling pathway, was demonstrated to be a target of miR-885-5p. Additionally, miR-885-5p suppressed cell proliferation, migration and invasion through regulation of β-catenin. These results suggested that miR-885-5p may serve key roles in osteosarcoma development, and may be a novel target for molecular therapy.
Materials and methods

Human tissue samples. Eighty-five pairs of osteosarcoma tissue samples and adjacent non-cancerous tissue samples were collected from patients who underwent osteosarcoma without preoperative systemic therapy at Jingjiang Hospital of Chinese Medicine (Jingjiang, China) between April 2012 and October 2016. Following surgical removal, all tissue samples were immediately frozen in liquid nitrogen. All patients gave consent and all human tissue experiments were approved by the Ethics Committee of Jingjiang Hospital of Chinese Medicine.

Cell lines and cell culture. Human osteosarcoma MG-63 and U2OS cells, and the osteoblast cell line hFOB1.19, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin. All cells were maintained at 37˚C in a humidified incubator with 5% CO₂.

Cell transfection. The empty vector (pcDNA3.1) and the plasmids containing β-catenin were purchased from YouBio (Hunan, China). miR-885-5p mimics and inhibitors were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A total of 5x10⁵ cells were transfected with 2.5 µg plasmid or mimic control (Mi-C), inhibitor-control (In-C) or miR-885-5p mimics/inhibitors using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The primer sequences were as follows: miR-885-5p mimic forward, 5'-UCCAUUACACUA CCCUGCCUCU-3' and reverse, 5'-AGGCGGGUGUGUAUGGAUU-3'; mimic control forward, 5'-UUUCCCGGAA CGUGUCAGGT-3' and reverse, 5'-ACGUGACAGCU CCGAGATT-3'; miR-885-5p inhibitor, 5'-AGGGCGAGG UAGUGUAUGGA-3'; inhibitor control, 5'-CAGUCUUUU GUGUGUACAA-3'. Following transfection for 48 h, the cells were harvested and used for further experiments.

Luciferase reporter assay. The β-catenin 3'-UTR sequences were subcloned into the pGL3-basic luciferase reporter vector (Promega Corporation, Madison, WI, USA). A total of ~1.0x10⁵ MG-63 and U2OS cells were seeded into 12-well plates and co-transfected with β-catenin 3'-UTR, Renilla and pre-NC or miR-21 mimics using Lipofectamine® 2000. Following transfection for 24 h, the luciferase reporter assay was performed using the Dual-Luciferase Reporter kit (Promega Corporation), according to the manufacturer's protocol. The relative luciferase activity was normalized to Renilla luciferase activity. Each independent experiment was performed at least three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissue samples and cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 2 µg RNA was used as a template to produce cDNA using the QuantScript RT kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. The reverse transcription conditions were as follows: 5 min at 25˚C, 30 min at 42˚C and 5 min at 85˚C. SYBR Green (Roche Diagnostics GmbH, Mannheim, Germany) was used to perform a qPCR assay using an ABI PRISM 7500 sequence detection system. The qPCR conditions were as follows: 5 min at 98˚C, followed by 33 cycles of denaturation at 98˚C for 30 sec, annealing at 56˚C for 30 sec and extension at 72˚C for 30 sec. The relative expression of mRNA was calculated by normalizing target gene expression to that of the internal control, using the 2¬ΔΔCq formula (18). Each independent experiment was performed at least three times. The primer sequences were as follows: c-myc forward, 5'-GATTCT CTGCTCTCCTCGAC-3' and reverse, 5'-TTCTCTCCTATC TTCTTTGGTC-3'; mmP7 forward, 5'-CAGAGCTTACCG CATATTACAG-3' and reverse, 5'-GTTATATCCTAGAC TGCTACC-3'; survivin forward, 5'-CCCATCTCCTACATTC AAGAAGTCG-3' and reverse, 5'-TCTTTGAAGCGAGAAGA AACG-3'; β-catenin forward, 5'-GCTGGAAATCTTTCTCA CTC-3' and reverse, 5'-TCCACAGTGCCTTTTACG-3'; GAPDH forward, 5'-TGAACATTCAAGAGGTTG-3' and reverse, 5'-TTTACTTCTTGAAGGCCATG-3'.

Western blotting. Total protein was extracted from cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and the protein concentration was measured using a bicinchoninic acid assay. A total of ~50 µg protein/lane was separated by SDS-PAGE on a 10% gel, and subsequently transferred onto a nitrocellulose filter membrane. The membranes were blocked with 5% skimmed milk at room temperature for 1 h, and incubated with primary antibodies overnight at 4˚C. Following washing with PBS containing 0.1% Tween-20 (PBST) three times at room temperature, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were washed with PBST three times at room temperature, and the specific bands were visualized via chemiluminescence using an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA). The antibodies used were as follows: Anti-β-catenin (1:2,000; cat. no. ab32572), anti-β-actin (1:4,000; cat. no. ab8227), HRP-conjugated anti-rabbit antibody (1:5,000; cat. no. ab6721) and HRP-conjugated anti-mouse antibody (1:5,000; cat. no. ab6789) (all Abcam, Cambridge, UK).

Target prediction. Putative target genes of miR-885-5p were predicted by bioinformatics analysis using the MirTarget tool on the miRDB database (http://www.mirdb.org).

Wound healing assay. Wound healing assays were performed to investigate the effect of miR-885-5p on cell migration. In brief, ~5x10⁵ transfected MG-63 or U2OS cells were seeded into 6-well plates. Once the cells had reached 90-100% confluence, a 20 µl pipette tip was used to scratch a line into the cell monolayer. Detached cells were removed by washing with PBS, and the cells were maintained in serum-free DMEM at 37˚C. The relative distance of cell migration was measured via the width of the wound at 0 and 48 h following the scratch, under a light microscope (magnification, x20). Each independent experiment was performed at least 3 times.

Transwell invasion assay. Transwell invasion assays were performed to investigate the effect of miR-885-5p on cell
invasion. In brief, Transwell chambers were coated with 80 µl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and maintained at 37°C for 30 min. A total of ~2x10^5 transfected MG-63 or U2OS cells in 300 µl serum-free medium were seeded into the upper chamber. The lower chamber was filled with 500 µl medium containing 10% FBS. Following incubation for 24 h, cells were stained with 0.5% crystal violet at room temperature for 10 min and the cells on the surface of upper chamber were removed gently using a cotton swab. The invaded cells were counted under a light microscope (magnification, x20), in 5 random fields. Each independent experiment was performed at least 3 times.

Colony formation assay. Colony formation assays were performed in order to determine the effect of miR-885-5p on cell proliferation. Briefly, ~3x10^4 transfected MG-63 or U2OS cells in serum-free DMEM were seeded into 6-well plates. At 14 days later, the colonies were fixed with 4% paraformaldehyde at room temperature for 10 min and stained with 0.5% crystal violet at room temperature for 10 min. The number of colonies (>50 cells) was counted under a light microscope (magnification, x20). Each independent experiment was performed at least 3 times.

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assays was performed in order to determine the effect of miR-885-5p on cell proliferation. Briefly, ~3x10^4 transfected MG-63 or U2OS cells were seeded into 96-well plates and cultured in 200 µl DMEM supplemented with 10% FBS. A total of 20 µl CCK-8 reagent (Beyotime Institute of Biotechnology) was added at 0, 24, 48 and 72 h after seeding. Following incubation at 37°C for 1 h, the absorbance of each well was measured at 450 nm using a microplate reader. Each independent experiment was performed at least three times.

Statistics. SPSS software version 21.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Data are presented as the mean ± standard deviation. The Kaplan-Meier method followed by the log-rank test was used to analyze the association between miR-885-5p expression levels and survival rate. The significance of the differences between two groups was analyzed by Student's t-test and the difference between multiple groups was analyzed by one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-885-5p predicts a poorer outcome in patients with osteosarcoma. To identify the function of miR-885-5p in osteosarcoma, the expression levels of miR-885-5p were analyzed in 85 osteosarcoma samples and adjacent non-cancerous tissue samples using RT-qPCR. As presented in Fig. 1A, the expression levels of miR-885-5p were reduced compared with adjacent non-cancerous tissues using RT-qPCR. As presented in Fig. 1A, the expression levels of miR-885-5p in osteosarcoma tissue samples were reduced compared with adjacent non-cancerous tissues. The expression levels of miR-885-5p in osteosarcoma cell lines, including MG-63 and U2OS, were also determined. The osteoblast cell line hFOB1.19 was used as a control group. The results revealed that miR-885-5p was significantly downregulated in MG-63 and U2OS cells, compared with hFOB1.19 cells (Fig. 1B). To further decipher the role of miR-885-5p in osteosarcoma, the association between miR-885-5p expression levels and patient clinical information was analyzed. The results suggested that miR-885-5p expression was negatively associated with tumor size, TNM stage and lymph node metastasis (Table I). Additionally, Kaplan-Meier survival analysis followed by the log-rank test suggested that patients with low levels of

<table>
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<th>No. of patients (n=85)</th>
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<td>≥18</td>
<td>39</td>
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miR, microRNA; TNM, Tumor-Node-Metastasis.
miR-885-5p had a poorer overall survival compared with those with high miR-885-5p expression (Fig. 1C). The results of the present study indicated the importance of miR-885-5p in osteosarcoma.

miR-885-5p targets β-catenin in osteosarcoma cells. Previous studies have reported that miRNAs may suppress protein expression by directly binding to RNA. The miRDB database was used to discover the potential targets of miR-885-5p, and it was suggested that β-catenin may be a candidate target. To further verify whether miR-885-5p may regulate β-catenin expression levels, MG-63 and U2OS cells were transfected with miR-885-5p mimics or miR-885-5p inhibitors. The expression levels of miR-885-5p were detected by RT-qPCR, which demonstrated that miR-885-5p was markedly upregulated or downregulated following transfection with miR-885-5p mimics or miR-885-5p inhibitors, respectively (Fig. 2A). Subsequently, the expression levels of β-catenin were determined by RT-qPCR and western blotting. The results of the RT-qPCR experiments demonstrated that the ectopic expression of miR-885-5p in MG-63 and U2OS cells resulted in the downregulation of β-catenin expression, whereas inhibition of miR-885-5p led to increased expression levels of β-catenin (Fig. 2B). Additionally, the protein expression levels of β-catenin were also reduced following increased expression of miR-885-5p in MG-63 and U2OS cells, and were upregulated following the inhibition of miR-885-5p (Fig. 2C). To further detect the interaction between miR-885-5p and β-catenin, luciferase reporter assays were performed. The relative luciferase activity was decreased upon co-transfection with miR-885-5p mimics and the β-catenin-3'-UTR plasmid in MG-63 and U2OS cells (Fig. 2D). Subsequently, the expression levels of three Wnt/β-catenin target genes, c-myc, matrix metalloproteinase 7 (MMP7) and survivin, were detected following miR-885-5p overexpression. As presented in Fig. 2E, ectopic expression of miR-885-5p led to decreased expression levels of c-myc, MMP7 and survivin, compared with control groups; however, knockdown of miR-885-5p led to elevated expression levels of c-myc, MMP7 and survivin, compared with the control groups (Fig. 2E). These results demonstrated that β-catenin may be the target of miR-885-5p in osteosarcoma.

miR-885-5p inhibits osteosarcoma cell proliferation through regulation of β-catenin. As presented in Table I, it was reported that miR-885-5p expression was negatively associated with tumor size. Consequently, the effect of miR-885-5p on osteosarcoma cell proliferation was investigated using colony formation and CCK-8 assays. The results of the colony
formation assay indicated that the number of colonies was decreased following the elevated expression of miR-885-5p in MG-63 and U2OS cells, an effect that may be offset by simultaneous ectopic expression of β-catenin; however, the number of colonies was increased when cells were transfected with miR-885-5p inhibitor (Fig. 3A). Furthermore, the results of the CCK-8 assay demonstrated that MG-63 cells with miR-885-5p overexpression grew more slowly compared with those transfected with Mi-C, an effect that was offset by the simultaneous ectopic expression of β-catenin (Fig. 3B). By contrast, MG-63 cells transfected with an miR-885-5p inhibitor grew more quickly compared with those transfected with an In-C (Fig. 3B). Similar results were observed in U2OS cells (Fig. 3B). These data collectively suggested that miR-885-5p

Figure 2. miR-885-5p targets β-catenin in osteosarcoma cells. (A) MG-63 and U2OS cells were transfected with Mi-C, miR-885-5p mimics, In-C or miR-885-5p inhibitor. Following transfection for 48 h, expression levels of miR-885-5p were determined by RT-qPCR. (B) MG-63 and U2OS cells were transfected with Mi-C, miR-885-5p mimics, In-C or miR-885-5p inhibitor. After transfection for 48 h, the expression of β-catenin was determined by RT-qPCR and (C) western blotting. (D) MG-63 and U2OS cells were co-transfected with β-catenin, Renilla and control mimic or mimics. Following transfection for 24 h, luciferase reporter assay was performed. (E) MG-63 and U2OS cells were transfected with Mi-C, miR-885-5p mimics, In-C or miR-885-5p inhibitor. Following transfection for 48 h, RT-qPCR analysis was performed. *P<0.05 vs. Mi-C; †P<0.05 vs. In-c. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Mi-c, mimic-control; In-c, inhibitor-control; mmP7, matrix metalloproteinase 7; c-myc, c-Myc proto-oncogene.
may inhibit osteosarcoma cell proliferation via regulation of β-catenin.

**miR-885-5p suppresses the migratory and invasive capacities of osteosarcoma cells.** Subsequently, the effect of miR-885-5p on cell migration and invasion was investigated using a wound healing assay and a Transwell invasion assay, respectively. As presented in Fig. 4A, the migratory capacities of the MG-63 and U2OS cells were markedly downregulated following the elevated expression of miR-885-5p, an effect that was offset by simultaneous ectopic expression of β-catenin (Fig. 4A). However, the migratory capacities of the MG-63 and U2OS cells were notably enhanced when transfected with miR-885-5p inhibitors (Fig. 4A). Furthermore, the effect of miR-885-5p
Figure 4. miR-885-5p suppresses the migratory and invasive capacities of osteosarcoma cells. (A) MG-63 and U2OS cells were transfected with Mi-c, miR-885-5p mimics or co-transfected with miR-885-5p mimics and pcDNA3.1/β-catenin; or In-c or miR-885-5p inhibitor, respectively. A wound healing assay was performed to determine the effect of miR-885-5p on cell migration. (B) MG-63 and U2OS cells were transfected with Mi-c, miR-885-5p mimics or co-transfected with miR-885-5p mimics and pcDNA3.1/β-catenin; or In-c or miR-885-5p inhibitor, respectively. Transwell invasion assay was performed to determine the effect of miR-885-5p on cell invasion. *P<0.05 vs. Mi-c; **P<0.05 vs. In-c. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Mi-c, mimic-control; In-c, inhibitor-control.
on the invasion of osteosarcoma was evaluated by Transwell invasion assay. The invasive ability of MG-63 and U2OS cells exhibited a similar cell migration trend; the invasive capacities of the MG-63 and U2OS cells were significantly downregulated following the elevated expression of miR-885-5p, an effect that was offset by the simultaneous ectopic expression of β-catenin (Fig. 4B). However, the invasive capacities of the MG-63 and U2OS cells were notably upregulated when transfected with miR-885-5p inhibitors (Fig. 4B). These results suggested that miR-885-5p suppressed the migratory and invasive capacities of osteosarcoma cells.

Discussion

miRNAs have been reported to be dysregulated in a number of cancer types and have been suggested to serve crucial roles in tumors by activating oncogenes or suppressing tumor suppressor genes (5,19,20). Although previous work demonstrated that miR-885-5p may be upregulated in osteosarcoma tissues (11), the detailed mechanism of action of miR-885-5p remains unknown. The present study reported that miR-885-5p was downregulated in osteosarcoma tissues and cell lines. The results of the present study are contrary to those of previous studies, which reported that miR-885-5p was upregulated in pancreatic cancer and breast cancer (9,10). This suggests that miR-885-5p may serve alternative functions in different parts of the human body or in different cancer types. Furthermore, although a previous report indicated that miR-885-5p was upregulated in osteosarcoma (11), the number of tissue samples used was not sufficient. The present study detected the expression levels of miR-885-5p in osteosarcoma cell lines, demonstrating that miR-885-5p was downregulated in osteosarcoma tissues and cell lines.

The Wnt/β-catenin signaling pathway has been reported to promote cell survival and mobility. Furthermore, activation of the Wnt/β-catenin signaling pathway improves resistance to drug-induced apoptosis. Previous reports indicated that β-catenin inhibition is able to prevent chemoresistance by suppressing O6-methylguanine-DNA methyltransferase activation (21). Additionally, miR-320 is able to suppress β-catenin expression in prostate cancer (22). Ma et al (23) demonstrated that the Wnt/β-catenin and neurogenic locus notch homolog protein signaling pathways may regulate the sensitivity of osteosarcoma cells to chemotherapy. The present study demonstrated that β-catenin may be a target of miR-885-5p. Additionally, the downstream targets of the Wnt/β-catenin signaling pathway, including c-myc, mmP7 and survivin, were also regulated by miR-885-5p. miR-885-5p may regulate the proliferation, migration and invasion of cancer cells by targeting β-catenin. However, whether miR-885-5p is able to suppress osteosarcoma cancer cell proliferation and metastasis in vivo requires further investigation.

It has been hypothesized that overexpression of miR-885-5p in osteosarcoma may suppress tumor development through inhibition of the Wnt/β-catenin signaling pathway. Furthermore, miR-885-5p has been reported to target cyclin-dependent kinase 2 and minichromosome maintenance complex component 5, activate cellular tumor antigen p53, and inhibit neuroblastoma proliferation and survival (24). Although previous work indicated that miR-885-5p was significantly upregulated in liver metastasis and colorectal cancer, and activated epithelial-to-mesenchymal transition (25), it was hypothesized that miR-885-5p may interact with alternative proteins and serve different functions in liver metastasis and colorectal cancer.

In summary, the present study revealed that miR-885-5p was downregulated in osteosarcoma tissues and cell lines, and downregulation of miR-885-5p was closely associated with tumor size, TNM stage and lymph node metastasis. Furthermore, downregulation of miR-885-5p predicted a poor prognosis. In osteosarcoma cells, miR-885-5p suppressed cell proliferation, migration and invasion through inhibition of β-catenin, a key component of the Wnt signaling pathway. Thus, miR-885-5p may be a potential therapeutic target for the treatment of osteosarcoma.

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

YL and GCH conceived and designed the work. YL, GCH, ZLB, and WQT constructed expression plasmids, prepared proteins and performed experiments. YL, GCH, ZLB, and WQT analyzed the data. ZLB, YL and GCH wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided their consent and all human tissue experiments were approved by the Ethics Committee of Jingjiang Hospital of Chinese Medicine (Jingjiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


