Research paper

Scutellarin enhances osteoblast proliferation and function via NF-κB-mediated CXCR4 induction

Jingtao Wang, Baodong Zhao, Shimao Yang, Dashan Wang, Hao Xu, Minhua Teng

Keywords: Scutellarin, Osteoblast, Proliferation, NF-κB, CXCR4

In the current study, we aimed to examine the function of scutellarin on human osteoblast proliferation and osteogenic function. The results indicated that scutellarin enhanced osteoblast proliferation over a seven day period. This increase in cell proliferation was associated with corresponding increases in osteoblast activity, as measured by alkaline phosphatase (ALP) secretion, intracellular calcium ion influx, and calcium deposition. These anabolic effects were associated with C-X-C chemokine receptor type 4 (CXCR4) mRNA levels and protein induction. Knockdown of CXCR4 reversed the scutellarin-induced increases in cell proliferation, ALP activity, and calcium deposition. Furthermore, scutellarin increased p65 phosphorylation in a dose-dependent manner, which resulted in the increased binding of phosphorylated p65 to the CXCR4 gene promoter region, to increase CXCR4 protein expression. p65 phosphorylation inhibition resulted in a decrease in CXCR4 protein expression. A p65 inhibitor blocked scutellarin-induced increases in osteoblast proliferation and function. Moreover, in a rat model of estrogen-deficient osteoporosis, scutellarin restored ovariectomy-induced bone loss in mice. Taken together, both cellular and animal models support the novel findings that scutellarin increased osteoblast proliferation and function through NF-κB/p65-mediated CXCR4 induction.

1. Introduction

Osteoporosis, a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to an elevating risk of fracture, is estimated to affect >200 million people globally (Kling et al., 2014; Shen et al., 2017). The World Health Organization (WHO) projected an alarming three-fold rise in its prevalence over the next 50 years (Diem et al., 2017; Ensrud and Crandall, 2017). Age-related osteoporosis, characterized by losses within both the cortical and trabecular regions of bone, is thought to be primarily driven by a decline in the new formation of bone (Diem et al., 2017). This concept is confirmed by in vivo evidences that osteoblast proliferation and maturation are reduced, and associated with a concomitant decline in bone mass with increasing age (Lovato and Lewiecki, 2017). As such, anabolic therapeutics that can attenuate these processes would be beneficial.

Scutellarin is the major effective constituent of Erigeron breviscapus (Liu and Ho, 2017). Scutellarin has been used to treat a variety of diseases in the clinic (Hou et al., 2017). The features of scutellarin are that it is stably sourced, of high abundance within Erigeron breviscapus, high content, only lowly toxic, and its quality may be controlled (Han et al., 2017). Moreover, the pharmacological effects of scutellarin have been elucidated and identified. In animal studies, scutellarin has been reported to be neuroprotective in rat cerebral ischemia reperfusion (CIR) models via augmentation of the antioxidant defense capacity (Dong et al., 2016). In addition, scutellarin prevented Eustachian tube dysfunction (EtD) in diabetic rats and inhibited protein kinase C translocation in rat diabetic thoracic aortae (Du et al., 2015). However, the function of scutellarin on human osteoblast proliferation and osteogenic function are still unclear.

Abbreviations: ALP, alkaline phosphatase; CXCR4, C-X-C chemokine receptor type 4; WHO, World Health Organization; CIR, cerebral ischemia reperfusion; EtD, Eustachian tube dysfunction; PBS, phosphate buffered saline; CPC, cetylpyridinium chloride; DAPI, 4′,6-diamidino-2-phenylindole; RIPA, radio-immunoprecipitation assay; OVX, ovariectomy; IP, intraperitoneal; ChIP, chromatin immunoprecipitation; BMP2, bone morphogenetic protein 2; BMD, bone mineral density; MSCs, mesenchymal stem cells

⁎ Corresponding author at: Oral Implant Center, The Affiliated Hospital of Qingdao University, No. 59, Haier Road, Laoshan District, Qingdao, Shandong 266100, China.

E-mail address: minghuatengnht163.com (M. Teng).

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In the current study, we aimed to identify the bone formation properties of scutellarin and to establish its mechanism of action using a human osteoblast model. We described the novel finding that scutellarin enhances osteoblast function by increasing C-X-C chemokine receptor type 4 (CXCR4) expression, through NF-κB/p65 signaling pathway activation.

2. Material and methods

2.1. Cell culture

Cryopreserved Clonetics™ primary human osteoblasts from a normal female donor were purchased from Lonza (Maryland, USA) and maintained in Osteoblast media (Lonza, USA). Culture media were replenished every 48–72 h and cells between passages 3 and 6 were used. Scutellarin was obtained from Sigma (St Louis, MO, USA).

2.2. Cell proliferation assay

Following 14 days of culture in complete growth medium with scutellarin, osteoblast proliferation was measured using a CellTiter 96 AQueous one solution cell proliferation assay (Promega, USA) based on the manufacturer’s instructions.

2.3. Ponceau S assay

Following culturing at the indicated scutellarin concentrations and time points, osteoblasts were fixed for 30 min on ice with 100% methanol. The cells were subsequently washed twice with phosphate buffered saline (PBS), and stained with 1% Ponceau S solution for 10 min. Any excess solution was removed and the stained areas were extracted with PBS. The extracted colorimetric solution was read with a microplate reader at an absorbance wavelength of 540 nm.

2.4. Alkaline phosphatase (ALP) assay

Following 14 days of culture in the osteogenic differentiation medium with scutellarin, osteoblasts were washed with PBS and subsequently fixed for 30 min on ice with 100% methanol. The cells were subsequently washed twice with phosphate buffered saline (PBS), and stained with 1% Ponceau S solution for 10 min. Any excess solution was removed and the stained areas were extracted with PBS. The extracted colorimetric solution was read with a microplate reader at an absorbance wavelength of 540 nm. Total ALP was normalized against total protein to give ALP activity per unit cell.

ALP staining was performed using an ALP staining kit (Sigma, St Louis, MO, USA) according to the manufacturer’s suggested protocols. Alizarin Red staining was performed to detect matrix mineralization with 2% Alizarin Red S (ARS; Sigma, St. Louis, MO, USA), pH 4.2, for 10 min at room temperature. Each experiment was repeated in triplicate.

2.5. Intracellular calcium assay

Osteoblasts were cultured in complete growth medium containing scutellarin for 5 days before incubation with Fluo-4 AM (Thermo Fisher Scientific, USA) according to manufacturer’s instructions; followed by 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA). Fluorescence was measured by confocal microscopy.

2.6. Calcium deposition assay

Following 21 days of culturing in complete growth medium with scutellarin, osteoblasts were fixed for 30 min on ice with 100% methanol. Alizarin red S solution (2%) was used to stain deposited calcium as previously described, and was normalized to total protein, to give calcium deposition per unit cell.

2.7. Reverse transcription and quantitative RT–PCR

Osteoblasts were cultured in their respective conditions and total RNA was extracted using TRIZol® (Thermo Fisher Scientific, USA) according to manufacturer’s instructions (Tong et al., 2017a, 2017b, 2017c). mRNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, USA). qRT-PCR was performed using 1 μl cDNA with TaqMan® Gene Expression Assays Probes (Applied Biosystems, USA). Each sample was run in triplicate and relative gene expression 2^−ΔΔCt was determined. The primers are list as follows: OC, Forward: 5′-CTGACCTCACAGATGCCAA-3′, Reverse: 5′-GTTGATAGTCTGACACAA-3′; Cdx1, Forward: 5′-TCTTTGTCTCTGGGCTTGTGC-3′; CXCR4, Forward: 5′-TCAGTGTGGCTGACCTCTCTTT-3′; Reverse: 5′-CTTGGCCTTTGACTTGTTGT-3′; β-actin, Forward: 5′-TGAGGAGGAAATCGTGAGTG-3′, Reverse: 5′-TGCTTGATCCACACTGTC-3′.

2.8. Western blotting

Osteoblasts were lysed with radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, USA), and quantified using a BioRad Bradford protein assay (Bio-Rad Laboratories, USA). Immunoblot analysis of total cell lysate was performed as per standard protocol (Tong et al., 2017a, 2017b, 2017c). Primary antibodies used were CXCR4 (Abcam, USA), lamin A/C, p-p65 (Tyr 705), p65 (Cell Signaling, USA), and β-actin (Sigma-Aldrich, USA).

2.9. CXCR4 and p65 silencing

Osteoblasts were transfected with Stealth RNAi™ siRNA human CXCR4 and p65 small interfering RNA (siRNA) (Thermo Fisher Scientific, USA) with Lipofectamine 2000 in Opti-MEM. The used negative control was the Stealth RNAi™ siRNA Negative control. Transfection and conditioned media were refreshed every 48–72 h.

2.10. ChIP assay

ChIP assays were performed with a SimpleChIP® Plus Enzymatic Chromatin IP kit (Cell Signaling, USA), following 7 days of culture with scutellarin. Antibodies used were p65 (Cell Signaling, USA). Primers for the binding sites of the CXCR4 promoter were purchased from Integrated DNA Technologies (CA, USA). Binding sites: forward 5′-ACCGAGACCTGGTCAAG-3′, reverse 5′-ATCCAGCTGAAACCA-3′.

2.11. CXCR4 promoter luciferase reporter assay

Osteoblasts were transfected with plasmids containing a Renilla luciferase coding sequence under the control of the human CXCR4 promoter region with or without the binding sites (GeneArt Thermo Fisher Scientific, USA) with Lipofectamine 2000 in Opti-MEM. Firefly luciferase expression plasmids (Thermo Fisher Scientific, USA) were simultaneously co-transfected to serve as a transfection control. Osteoblasts were subsequently treated with scutellarin for 12 h, and luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega, USA).

2.12. Ovariectomized rat model

In brief, bilateral ovariectomy (OVX) was performed on 12-week-old female Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China). The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Oral Implant Center, the Affiliated Hospital of Qingdao University. At four weeks after OVX induction, the OVX rats were treated with the vehicle (0.9% saline) (OVX-Vehicle) or scutellarin (40 mg/kg/day) (Scutellarin) for
eight weeks by intraperitoneal (IP) injection. Sham-operated rats were also administered with the vehicle for comparison (Sham-Vehicle). Femurs were scanned and analyzed by a Quantum GX microCT imaging system (PerkinElmer).

2.13. Statistical analyses

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using GraphPad Prism V software. Statistical differences were determined using Student's t-test or one-way ANOVA. *P < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of scutellarin on human osteoblast function

To determine whether scutellarin (Fig. 1A) has an effect on cell proliferation, we cultured osteoblasts in complete growth medium containing increasing concentrations of scutellarin. After 14 days of culturing, scutellarin increased cell proliferation in a dose-dependent manner, as measured by MTS assay (Fig. 1B) and total protein content analysis (Fig. 1C). At the maximal dose of scutellarin, osteoblast proliferation and total protein content were increased.

To investigate osteoblast function, we measured the changes in ALP levels, calcium influx, and calcium deposition, following exposure to scutellarin. Osteocalcin (OC) and type I collagen (Col1A1) mRNA levels were increased after treatment (Fig. 1D). Scutellarin increased ALP secretion per unit cell in a dose-dependent manner, with a maximum increase observed with scutellarin (Fig. 1E). Additionally, scutellarin dose-dependently increased levels of free intracellular calcium ions per
Fig. 2. Effects of scutellarin on osteoblast CXCR4 expression. (A) Osteoblasts were exposed to 10 μM scutellarin. mRNA was harvested at the indicated time points, and changes in CXCR4 levels were measured. (B) Effects on CXCR4 mRNA expression after 24 h of treatment with the indicated concentration of scutellarin. Each data point is the mean of three independent experiments and expressed as fold change (± SD) relative to untreated controls. (C) Osteoblasts were treated with indicated doses of scutellarin, and CXCR4 protein levels were analyzed by western blotting.

Fig. 3. Effects of CXCR4 on scutellarin-mediated osteoblast proliferation and function. (A) Effect of two siRNA CXCR4 sequences on CXCR4 protein expression. (B) Cell proliferation following CXCR4 knockdown. (C) mRNA level of OC and Col1A1 following CXCL4 knockdown. (D) Alkaline phosphatase (ALP) activity following CXCR4 knockdown. (E) Calcium deposition following CXCR4 knockdown. Each data point is the mean of three independent experiments and expressed as fold change (± SD) relative to untreated controls. **P < 0.01, ***P < 0.001.
unit cell, with the maximal increase observed with scutellarin relative to untreated controls (Fig. 1F). Furthermore, scutellarin also increased normalized calcium deposition relative to untreated controls (Fig. 1G). ALP staining and alizarin red staining were used to confirm the effect of Scutellarin on osteoblast differentiation (Fig. 1H). On aggregate, these results suggested that scutellarin can dose-dependently up-regulate osteoblast proliferation and function.

3.2. Scutellarin up-regulated CXCR4 expression in osteoblasts

It had been reported that bone mass and strength can be improved within a glucocorticoid-induced osteoporosis murine model by the transplantation of mesenchymal stem cells (MSCs) (Mitchell and Lyles, 1990; Klein, 2015). However, these bone-enhancing effects of the MSCs are abrogated when CXCR4 is deleted, suggesting the importance of CXCR4 in bone formation. Additionally, CXCR4 knockdown in an embryonic mouse model has recently been reported to disrupt postnatal bone formation. We hypothesized that the bone enhancing effects of scutellarin may be affected through the CXCR4 signaling pathways. We investigated the effects of scutellarin treatment on CXCR4 mRNA and protein expression. Interestingly, our findings showed that scutellarin had a strong effect on CXCR4 mRNA expression in a time- and dose-dependent manner (Fig. 2A). In parallel, CXCR4 protein levels were also dose-dependently increased by scutellarin (Fig. 2B). These findings suggest that CXCR4 expression was likely stimulated by scutellarin, and that the CXCR4 pathway may mediate scutellarin effects on osteoblast proliferation and differentiation.

3.3. Effects of CXCR4 knockdown on osteoblast proliferation and function

To further delineate the role of CXCR4 on scutellarin-induced osteoblast function, we established a CXCR4 knockdown model by transfecting osteoblasts with two different sequences of siRNA. As expected, western blotting analyses showed that scutellarin up-regulated CXCR4 protein expression in osteoblasts transfected with the scrambled sequence (Fig. 3A). Both siCXCR4 sequences successfully reduced CXCR4 protein expression to almost undetectable levels relative to the scrambled sequence (Fig. 3A). Of note, scutellarin treatment did not overcome the effects of the siRNA transfection on osteoblast CXCR4 expression. Using this CXCR4 knockdown model, we measured changes in osteoblast proliferation and function in the presence and the absence of scutellarin. With the scrambled control sequence, scutellarin induced a ~40% increase in osteoblast proliferation (Fig. 3B). In contrast to the scrambled controls, the presence of scutellarin did not attenuate the suppressive effects of CXCR4 knockdown on osteoblast proliferation, indicating that CXCR4 may play a role in mediating scutellarin effects on osteoblast proliferation (Fig. 3B). Furthermore, compared to scrambled control, knockdown of CXCR4 blocked scutellarin induced OC and Col1A1 mRNA upregulation in osteoblast (Fig. 3C).

In line with its effects on proliferation, CXCR4 knockdown also resulted in a decrease in ALP activity (Fig. 3D). Additionally, the previously observed scutellarin-induced elevations in ALP levels were no longer detected following CXCR4 knockdown. In addition, we also observed a similar pattern of change with respect to calcium deposition (Fig. 3E). Taken together, these results suggested that scutellarin-induced increases in osteoblast proliferation and function are likely
mediated by CXCR4. Following this, we next investigated the molecular mechanism involved in the regulation of CXCR4 by scutellarin.

### 3.4. Scutellarin enhanced p65 phosphorylation and the subsequent binding to the CXCR4 promoter

Next, we set out to investigate the effects of scutellarin on p65 phosphorylation and its relationship to CXCR4 protein expression in the osteoblast. Indeed, scutellarin dose-dependently increased S536 phosphorylation of p65 in the osteoblasts (Fig. 4A). Knockdown of p65 by siRNA abrogated CXCR4 induction by scutellarin in osteoblasts (Fig. 4B). Following scutellarin treatment, nuclear fractionation was used to detect p65 translocation (Fig. 4C). Moreover, NF-κB inhibitor BAY 11-7082 blocked p65 nuclear translocation (Fig. 4C). In addition, NF-κB inactivation by BAY 11-7082 suppressed CXCR4 expression and p65 phosphorylation, which was induced by scutellarin (Fig. 4C), indicating that CXCR4 expression by scutellarin is modulated by the nuclear translocation of p65. Next, we sought to determine whether p65 could directly promote CXCR4 transcription activity. It was found that p65 was introduced into the genomic region containing the κB sites following scutellarin treatment by chromatin immunoprecipitation (ChIP) (Fig. 4D). The above data suggested that scutellarin induces p65 to directly bind to the CXCR4 promoter to drive its transcriptional activation.

### 3.5. p65 inactivation blocked scutellarin-induced osteoblast function

To confirm our findings thus far, we explored whether the inhibition of p65 phosphorylation could impair the previously observed scutellarin-induced upregulation of osteoblast proliferation and function. Inhibition of p65 phosphorylation with BAY 11-7082 resulted in a decline in osteoblast proliferation (Fig. 5A). While scutellarin treatment increased proliferation, the presence of BAY 11-7082 abrogated this effect (Fig. 5A). Similarly, inhibition of p65 phosphorylation attenuated the effects of scutellarin on osteoblast ALP activity (Fig. 5B) and calcium deposition (Fig. 5C). These results suggested that the previously observed scutellarin-induced increases in ALP levels and calcium deposition might be mediated, in part, by p65 phosphorylation.

### 3.6. Scutellarin increased bone microarchitecture in an OVX rat model

Three-dimensional images of femoral metaphyses generated by Micro CT showed differences in trabecular micro-architecture between OVX and scutellarin treatment group (Fig. 6A). As shown in Fig. 6B–D, OVX decreased bone mineral density (BMD), bone mineral content (BMC) and trabecular thickness compared to values obtained for the sham group. By contrast, trabecular space was increased post OVX treatment. All of these OVX effects were reversed by scutellarin treatment.

### 4. Discussion

In this study, we showed that scutellarin dose-dependently enhances cell proliferation, ALP activity, and calcium deposition in human osteoblasts. The anabolic effects of scutellarin on osteoblast function were associated with corresponding increases in CXCR4 mRNA and protein expression levels. Conversely, knockdown of CXCR4 expression abrogated these scutellarin-induced effects. The anabolic actions of scutellarin are associated with the phosphorylation of the transcription factor p65. ChIP experiments indicated that phosphorylated p65 binds to the promoter of the CXCR4 to activate its transcription. The specific inhibition of p65 phosphorylation abrogated the increase in CXCR4 protein expression observed previously. Additionally, using an OVX rat osteoporosis model, we demonstrated that the administration of scutellarin at least partially prevented the development of osteoporosis. Prevention of osteoporosis by scutellarin is associated with increases in osteoblast number and CXCR4 expression. On aggregate, these cellular and animal data indicate that scutellarin stimulated p65 phosphorylation to increase CXCR4 expression, leading to increased osteoblast proliferation and anabolic function. Our results imply that CXCR4...
signaling may be a potential therapeutic target for the development of anabolic anti-osteoporotic medications.

The G-protein coupled receptor, CXCR4, is involved in a variety of physiological and pathological events including blood homeostasis, immune responses, and bone remodeling (Domanska et al., 2013; Jacobson and Weiss, 2013; Karpova and Bonig, 2015; Zhao et al., 2015). CXCR4 activation also enhances MSC migration by regulating cellular adhesion and cytoskeletal changes, primarily via the phosphorylation of focal adhesion kinases (Yang et al., 2015; Liu et al., 2016). This increase in MSC motility is important for bone formation, as MSCs need to migrate to active bone remodeling sites before it can form new bone (Li et al., 2017a, 2017b). Conversely, conditional inactivation of CXCR4 in osteogenic cells in a murine model led to the development of an osteopenic skeleton characterized by a reduction in cortical bone mass and trabecula (Zhu et al., 2011). Overexpression of CXCR4 through adenovirus transfection in MSC-like cells can result in the homing of these stem cells to the bone, resulting in an increase in bone mass and strength in a glucocorticoid-induced osteoporosis murine model (Chen et al., 2013). CXCR4 expression and signaling in osteoblasts may also have a wider role, with evidence suggesting that it may also modulate MSC and osteoclast precursors’ proliferation, maturation, and migration (Christopher et al., 2009; Drynda et al., 2017). However, the mechanism by which CXCR4 brings about these anabolic functions remains unclear.

Interestingly, p65 phosphorylation can regulate CXCR4 expression in mouse embryonic fibroblast cells (Zhi et al., 2014; Zhi et al., 2015). Our data extends this further by demonstrating the specific binding of phosphorylated p65 to the CXCR4 promoter region in human osteoblasts to activate this transcription. Additionally, this information led us to propose that scutellarin-induced p65 phosphorylation may mechanistically increase CXCR4 expression to stimulate osteoblast proliferation and function. This new data adds to the present understanding of the effects of CXCR4 on bone health, wherein the beneficial effects of CXCR4 were exerted through neovascularization, by stimulating the homing of hematopoietic endothelial progenitor cells to the site of bone repair. Furthermore, it has been demonstrated that CXCR4 signaling is dependent on bone morphogenetic protein 2 (BMP2) activation, determining the fate of perivascular cells, converting them into pericytes, before conversation into osteoblasts and osteocytes, which eventually integrate into newly forming bones (Guang et al., 2013; Li et al., 2017a, 2017b). Our data indicates another mechanism through which CXCR4 signaling can induce bone osteoblastic function. These findings are supported by the observation that conditional inactivation of p65 in the osteoblasts of mice results in a reduction of both bone mineral density (BMD) and bone strength. Taken together, these data support the view that the p65 signaling pathway in osteoblasts is vital
for bone formation. A challenge for the future would be to determine the mechanism whereby scutellarin regulates p65 phosphorylation.

Our results have shown that scutellarin can retard the development of osteoporosis in OVX rats. Thus, both cellular and animal models indicate that scutellarin has an anabolic effect. Taken together, these findings suggest that scutellarin may have a dual mode of action, having both anabolic and anti-resorptive actions for the treatment of osteoporosis. The mechanism of action of scutellarin, which involves the targeting of both osteoblasts and osteoclasts, is unique amongst osteoporotic drugs.

The pathogenesis of age-related osteoporosis, and the resulting reduction of BMD at the cortical and trabecular regions, has been recognized as primarily driven by declining osteoblast numbers and function, putatively by increasing p65 phosphorylation, resulting in the hypothesis that scutellarin up-regulates osteoblast proliferation and differentiation potency of mouse bone marrow mesenchymal stromal stem cells. Int. J. Biochem. Cell Biol. 45 (8), 1813–1820.


In conclusion, this present study provides new data to support the hypothesis that scutellarin up-regulates osteoblast proliferation and function, putatively by increasing p65 phosphorylation, resulting in the up-regulation of CXCR4 expression. Additionally, our findings also suggest the possibility for CXCR4 to be a therapeutic target for the treatment of osteoporosis. These findings provide a strong impetus for further animal and human clinical studies to be conducted to confirm the therapeutic potential of scutellarin for osteoporosis.

Conflicts of interest

The authors declare no conflict of interest and all authors have agreed on the submission.

Founding

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Authors’ contributions

In this work, Jingtao Wang and Minhua Teng conceived the study and designed the experiments. Jingtao Wang, Baodong Zhao, Shimaoy Yang, Dashan Wang and Hao Xu contributed to the data collection, performed the data analysis and interpreted the results. Jingtao Wang wrote the manuscript; Minhua Teng contributed to the critical revision of article. All authors read and approved the final manuscript.

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In this work, Jingtao Wang and Minhua Teng conceived the study and designed the experiments. Jingtao Wang, Baodong Zhao, Shimaoy Yang, Dashan Wang and Hao Xu contributed to the data collection, performed the data analysis and interpreted the results. Jingtao Wang wrote the manuscript; Minhua Teng contributed to the critical revision of article. All authors read and approved the final manuscript.

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