

Original Research Article

Epimedin C prevents glucocorticoid-induced osteoporosis via balancing EphB4/EphrinB2 axis in a rodent model

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Abstract

Purpose: To study the effect of Epimedin C on glucocorticoid (GC)-induced osteoporosis in a rodent model as well as its mechanism of action via balancing EphB4/EphrinB2 axis.

Methods: Forty-eight C57BL/6 male mice were divided randomly into control, dexamethasone injection (DI), Epimedin C gavage (EG), and DI + EG groups. Micro-computed and hematoxylin-eosin staining was used to examine bone structure. The relationship between EphB4/EphrinB2 and osteoporosis was preliminarily verified by immunohistochemistry. Evidence of Epimedin C preventing the formation of GC-induced osteoclasts was determined by trap staining. Western blot analysis was conducted to determine the levels of EphrinB2, EphB4, Runx2, and LGR4 in mouse bone tissues.

Results: In the DI group, morphological examination showed bone loss. The use of immunohistochemistry and Western blot studies showed that EphB4 levels decreased and EphrinB2 levels increased in DI group mice ($p < 0.05$). The morphological parameters of osteoporosis improved in mice of DI + EG group compared to that in DI group mice, while EphB4 levels were elevated and EphrinB2 levels decreased in comparison with the values in the DI group mice ($p < 0.05$).

Conclusion: Epimedin C prevents bone loss by balancing the process of bone formation and remodeling bidirectionally. It affects bone metabolism by regulating protein levels of Runx2 and LGR4. However, the specific regulatory processes and targets of the anti-osteoporotic effect of Epimedin C regarding the EphB4/EphrinB2 signaling pathway still need to be confirmed.

Keywords: Epimedin C, glucocorticoid-induced osteoporosis (GIOP), EphB4/EphrinB2, Traditional Chinese medicine, Mouse model

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INTRODUCTION

The most frequent secondary osteoporosis is glucocorticoid-induced osteoporosis (GIOP), which is also the most pronounced GC's side effect; Compared to synthetic drugs, traditional

Chinese medicines (TCMs) have long been used to prevent and treat osteoporosis, a practice that has received growing researcher's attention due to fewer adverse events being reported during long-term use [1]. As an example, icariin, the flavonoid compound extracted from the kidney

drug *Epimedium*, could reduce the RANKL-induced embryo bone loss, promote bone anabolic metabolism and improve bone health, all of which contribute to a unique anti-osteoporosis advantage [2].

Icariin has been shown to promote the expression of both osteoprotegerin (*OPG*) mRNA and Runt-related transcription factor 2 (*Runx2*, as osteoblast marker gene) mRNA for stimulating osteoblast formation. However, it can also reduce RANKL-induced differentiation of osteoclasts for preventing bone loss [3]. Meanwhile, Epimedin C had similar effects on the differentiation and proliferation of osteoblast cell [4]. Epimedin C, another isolated compound, has shown better anti-osteoclastogenic activity than icariin, as shown in previous experiments [5]. The Wnt, RANK/RANKL/*OPG*, and EphB4/EphrinB2 signaling pathways share common key upstream and downstream factors and interact with each other [6], laying the foundation for the suggestion that Epimedin C exerts an anti-osteoporotic effect by affecting EphB4/EphrinB2 signal transduction. To further verify the relationship between anti-osteoporosis effect of Epimedin C and expression of EphB4/EphrinB2 with its downstream genes, *in vivo*, experiments were performed.

EXPERIMENTAL

Reagents and instruments

Epimedin C (batch no. 110642-44-9) was supplied by Cheng-Du PUSH Biological Technology (China). Dexamethasone (batch no. SD9530) was bought from Shang-Hai Heng-Fei Biotechnology Co. Ltd. Primary antibodies against EphB4 (bs-10659R), EphrinB2 (bs-6046R), LGR4 (bs-22163R), and Runx2 (bs-1134R) as well as goat anti-rabbit biotinylated secondary antibodies were purchased from Beijing Bioss Biological Technology (China).

Animals

C57BL/6 male mice (aged eight weeks, 20 ± 2.1 g) were obtained from the Experimental Animal Center in the Wuhan Hospital of Traditional Chinese and Western Medicine, with approval from the Medical Ethics Committee in The First Hospital of Wuhan (approval no. 2018/17). All animal-related experiments were performed in accordance with the corresponding guidelines for the care and use of experimental animals [7] and the mice were divided into four groups as follows: control, dexamethasone injection (DI), Epimedin C gavage (EG), and DI + EG groups.

Model establishment

Mice of DI and DI + EG groups were intramuscularly injected with 1 mL of dexamethasone (0.125 mg/kg) three times a week for four weeks, as previously described [7]. Mice of the control group and the DI group received injection with the same volume of saline. From the fifth week, mice of control group and the DI group were intragastrically administered with saline, while mice of the EG group and the DI + EG group were administered with Epimedin C (200 mg/kg body weight from a stock solution of 5 mg/mL) three times a week for four weeks [8,9]. After eight weeks, the mice were sacrificed and the right femur of the mice was isolated.

Micro-computed tomography assessment of bone microstructure

The femoral bone tissues were placed in a micro-CT scanner to obtain images of different parts of the same specimen. After the scan was completed, the femoral neck was selected as the region of interest (ROI). Comprehensive TeX Archive Network (CTAn) software was used to determine morphological parameters in the three-dimensional ROI, including the trabecular bone thickness (Tb. Th, mm), number (Tb. N, 1/mm) and spacing (Tb.Sp, mm), as well as the bone mineral content (BMC), and the tissue mineral density (TMD).

Bone structure examination by hematoxylin-eosin (H & E) staining

After eight weeks, the right distal femur tissues of the mice were taken and placed in a PBS solution with 4 % of paraformaldehyde. Following decalcification with 10 % EDTA for 4 weeks, tissues were embedded in paraffin and sliced, which were then stained using hematoxylin-eosin for observation of their pathological changes under an inverted microscope. Following this, pictures of the tissues were taken and recorded, and the software Image J software (1.52a, USA) was performed for image analysis and determining the percentage of trabecular bone area.

Immunohistochemical staining

Mouse femurs were prepared into 4 μ m sections following decalcification, which was fixed in 10 % neutral buffered formalin for 10 min at ambient temperature, and washed subsequently with PBS and blocked in methanol at room temperature for another 10 min. After being rinsed twice with pre-chilled PBST for five minutes each time, sections

were blocked with 10 % of bovine serum under ambient temperature for one hour. After removing the blocking solution, slices were incubated at 37 °C overnight using primary antibodies diluted with the blocking buffer (1:1000 for rabbit anti-EphB4 and rabbit anti-EphrinB2). After being washed with PBST and incubated with the secondary antibody at 37 °C for one hour, the sections were rinsed with PBS-Tween-20 three times with 5 min for each time, together with the addition of DAB for color development. Afterward, the sections were rinsed again with pre-chilled PBST 3 times each for 5 min duration, washed with double distilled water, re-stained with hematoxylin, dehydrated, and sealed. Pictures were taken, and Image J software was used for analyzing the integrated optical density (IOD) of the target proteins.

Construction of CRISPR- EphB4

An intron region of EphB4 (ID: 13846) was selected as targeting site in CRISPR system construction, the plasmid containing Cas9 1.1 were purchased from Addgene (#71814), gRNA sequence GTTCTCAGCCCCAGGAGGCA NGG was selected as a final version in online tool support by Snapgene and was clone into the plasmid eSpCas9 (1.1). The construct was used in cell transfection experiment by electric shock method described by Bio-Rad (Bulletin-5904).

TRAP activity assay

The mice femur slices were dewaxed and put into incubation fluid for 50 min. After being rinsed in distilled water, sections were stained with hematoxylin and then washed again. A picture of the sections was taken. NDP software (NDP, view 2, Japan) was used for choosing three ROIs and calculating the mean number of osteoclasts in bone tissues.

Western blot analysis

Right, femur specimens of mice from each group were placed in a homogenizer and total protein lysate was added, after which the protein concentration was measured under 4 °C and centrifugation at 12,000 rpm. The concentration of protein was analyzed using the BCA approach. Protein was loaded at 45 µg per lane, and 5-fold of buffer was added under 100 °C in three min, after which 12 % SDS-page electrophoresis was performed, 100 v constant pressure wet transfer for 1.5 h. Protein was then separated through electrophoresis and transferred onto PVDF membranes, which were then placed in Tris-buffered saline containing 0.1 % of Triton X-100 (TBST) and 5 % of skimmed

milk under ambient temperature for two hours. Following that, incubating the membrane with the primary antibodies {β-actin (1:8000), EphB4 (1:500), EphrinB2 (1:500), Runx2 (1:500), and LGR4 (1:500)} overnight at 4 °C. After washing with TBST three times, incubating the membrane with secondary antibodies at ambient temperature for one hour. The software Image J was adopted for determining the band density.

Statistical analysis

Experimental distributed data were shown as mean ± standard deviation (SD). The software, SPSS 20.0 package (USA), was applied for statistical analyses, with Dunnett's tests and one-way analysis of variance (ANOVA) method adopted for multiple comparisons between groups. GraphPad Prism 8 (GraphPad Software, Inc., USA) was used to analyze all statistics, and $p < 0.05$ was considered statistically significant.

RESULTS

Epimedin C maintains bone structure

Epimedin C improved the parameters of trabecular bones in GIOP. Micro-CT technique was utilized for quantitative histomorphometry analysis of the longitudinal cross-sections collected from morphological observations of the right femoral head. Three-dimensional (3D) reconstructions to sagittal view of micro-CT scanning showed that the trabecular network appeared to be porous in DI group (Figure 1 A), while the Tb. N, Tb. Th, BMC, and TMD of the DI group significantly decreased, and the Tb.Sp increased ($p < 0.05$; Figure 1 B). In contrast, bone loss activity was greatly improved in the DI + EG group; the trabecular bone recovered to a certain degree, the density increased, and the bone trabecula was more regular (Figure 1 B). Compared to the DI group, the Tb.N, Tb. Th, BMC, and TMD in the DI + EG group were unregulated while the Tb.Sp was downregulated ($p < 0.05$). Following administration of Epimedin C, in the EG group, the number of bone trabecula increased while bone trabecula was more densely concatenated, and the Tb. N, Tb. Th, BMC, and TMD were upregulated while the Tb.Sp decreased in comparison with the corresponding control group values ($p > 0.05$, Figure 1 B). Histological analysis further confirmed that Epimedin C had an anti-osteoporotic effect on glucocorticoid-induced osteoporosis *in vivo*. The area of bone trabeculae also evaluated by HE staining reveals remarkable recovery (Figure 1 C).

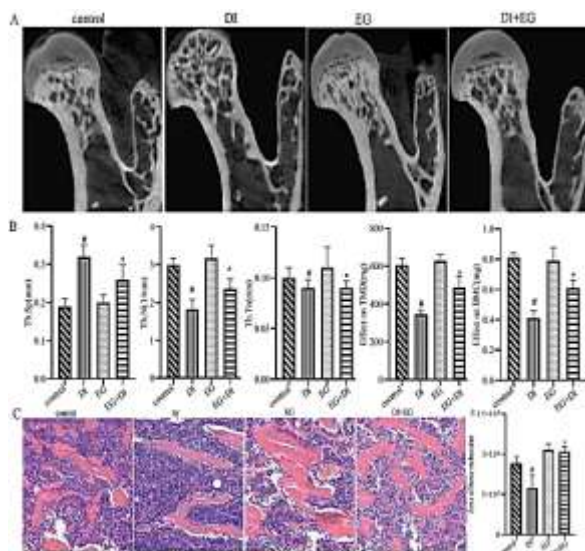


Figure 1: Micro-CT imaging assay, calculation of Bone parameters and relative area of trabeculae (A) Micro-CT image of mice femur in the control group, DI group, EG group, DI + EG group. (B) Relative bone parameters such as Tb.N, Tb. Th, BMC, and TMD were expressed in four groups of mice. * $P < 0.05$ versus the control group, and # $p < 0.05$ versus the DI group. (C) Area of bone trabeculae in the mice of four groups; HE staining ($\times 200$). * $P < 0.05$ versus the control group, and # $p < 0.05$ versus the DI group

Epimedin C exhibits anti-osteoporotic effect for EphB4/EphrinB2 expression

Based on HE staining and Micro-CT results, a model for mice with GIOP was successfully established. The anti-osteoporosis effect of Epimedin C had previously been confirmed by morphometric measurements, but its relationship with the EphB4/EphrinB2 signaling pathway remained unknown. The expression of EphB4/EphrinB2 in bone cells using immunohistochemistry, in which the cytoplasm, membrane, and surrounding matrix of target cells were stained brown was observed (Figure 2 A). Expression of EphrinB2 in the DI group mice was upgraded than that in the control group mice, whereas expression of EphB4 in the DI group mice was downgraded than that in the control group ones ($p < 0.05$; Figure 2 B and C). Concerning the EG group, expression of EphB4 and EphrinB2 increased ($p < 0.05$, Figure 2). Besides, expression of EphrinB2 in the DI + EG group decreased than in the DI group, and expression of EphB4 in the DI + EG group mice increased than that in the DI group mice ($p < 0.05$; Figure 2). Hence, Epimedin C exerted an anti-osteoporotic effect by increasing expression of EphB4 and decreasing expression of EphrinB2.

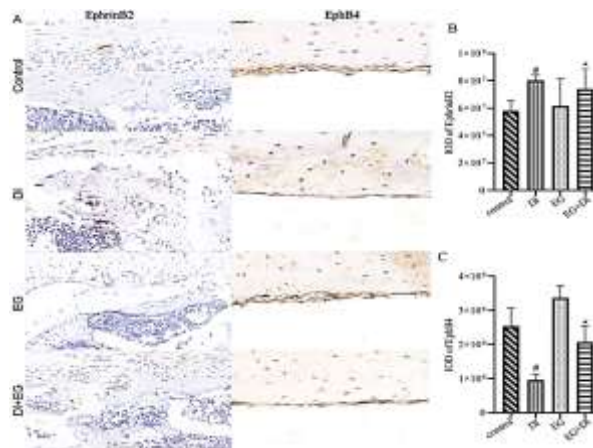


Figure 2: Immunohistochemistry staining of EphB4/EphrinB2 ($\times 400$). (A) The cytoplasm, membrane and surrounding matrix of target cells were stained brown in immunohistochemistry. (B)(C) Expression of EphrinB2 and EphB4 in the four group mice. * $P < 0.05$ versus control group; # $p < 0.05$ versus DI group

Epimedin C inhibits the formation of GIOP-induced osteoclasts

The osteoclasts in bone tissue appeared pink while the cell nucleus appeared blue (Figure 3 A). The osteoclasts increased significantly in DI group mice than that in control group mice ($p < 0.05$; Figure 3 B). In comparison with the control group, Epimedin C reduced the number of osteoclasts in the DI + EG group mice ($p < 0.05$; Figure 3 B). No difference in osteoclasts was observed between the EG and the control groups ($p > 0.05$; Figure 3 B).

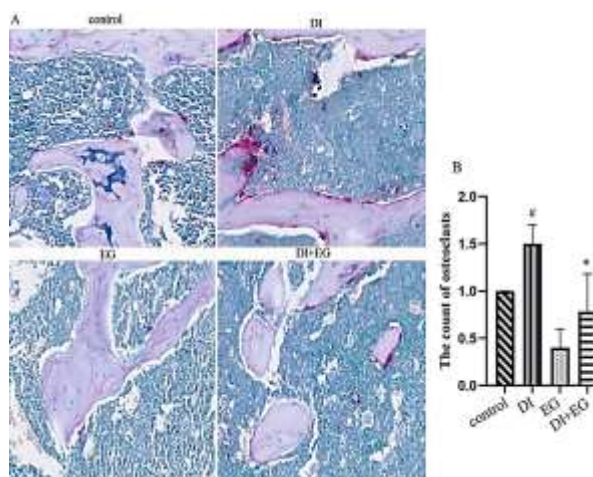


Figure 3: TRAP staining of osteoclasts ($\times 400$), (A) The osteoclasts appeared pink and cell nucleus appeared blue, (B) Number of osteoclasts in the four group mice. * $P < 0.05$ versus the control group; # $p < 0.05$ versus DI group. Relative content of osteoclasts in group DI, ED, and DI + EG, versus control group

Epimedin C affects bone metabolism-related protein expressions

Based on immunohistochemical results, the molecular mechanisms of anti-osteoporosis influence of Epimedin C concerning the EphB4/EphrinB2 signaling pathway at the protein level, was further analyzed using Western Blot. The mechanism of Epimedin C was shown to relate to expressions of EphB4, EphrinB2, Runx2, and LGR4. Expressions of EphB4 (Figure 4 B), Runx2 (Figure 4 E), and LGR4 (Figure 4 D) in DI group decreased, while expression of EphrinB2 increased ($p < 0.05$; Figure 4 A). For DI + EG group, Epimedin C downregulated levels of EphrinB2 and significantly upregulated expressions of EphB4, Runx2, and Grb4 ($p < 0.05$, Figure 4 A, B, D and E). EphB4, Runx2, and LGR4 were upregulated, while expressions of EphrinB2 in EG group decreased ($p < 0.05$; Figure 4 A and B).

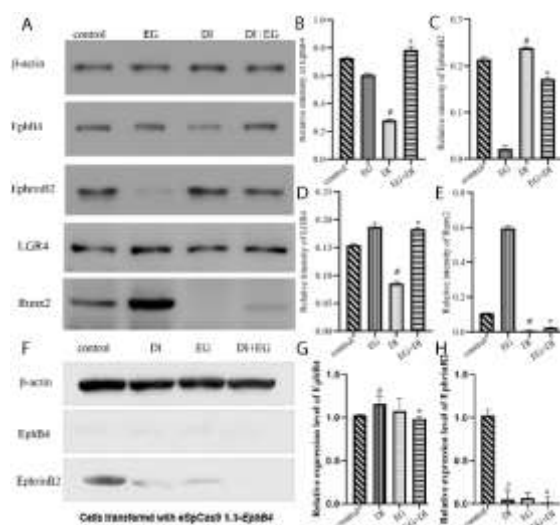


Figure 4: Western blotting results for EphB4, EphrinB2, Runx2, and LGR4. (A) Expressions of EphB4, Runx2, and LGR4 decreased in the DI group, while expression of EphrinB2 increased. In DI + EG group, Epimedin C downregulated the levels of EphrinB2 and EphB4, and expressions of EphB4, Runx2, and Grb4 (B-E) were upregulated. $\#P < 0.05$ versus the control group, and $*p < 0.05$ versus the DI group. (F) CRISPR Cas9 drives downregulation of EphB4 and the expression of it and EphrinB2 were confirmed by immuno-blot, relative expression level was calculated as shown in G ($p < 0.05$).

Treatment with Epimedin C inhibited the decline in expressions of EphB4, Runx2, and LGR4, and downregulated the protein level of EphrinB2. These results showed that bone metabolism-related proteins were also affected significantly, and correlated with Epimedin C involved antagonistic behavior against DI-induced OP. To ascertain that EphB4/EphrinB2 Axis, CRISPR

Cas9 system was operated on rodent model, the Cas9 1.1 drive gene editing (targeting intron of EphB4) was confirmed by immune-blot results. The antagonistic relationship was visualized by probe EphrinB2 protein in isolated tissues, as shown in Figure 4 F and G. Expression of EphrinB2 was influenced by downregulated EphB4 which verified the cross-link relationship between them.

DISCUSSION

It is recognized that GIOP is a public health problem due to related levels of disability and mortality. Medication for GIOP can lead to hypocalcemia, severe renal impairment, osteonecrosis, and atypical femoral fractures following long-term medication [10]. Icariin, an anti-osteoporotic agent with strong osteogenic activity, induces osteogenic differentiation and prevents bone resorption by activating the signaling pathway of Wnt/ β -catenin [11]. With a total flavonoid content of more than 50 % in Herba *Epimedii*, Epimedin A, B, C, and icariin are considered major bioactive components with the ability to strengthen the osteoblast cell proliferation, as well as having a potential effect on osteoporosis [12].

In a previous study, Epimedin C was observed to be more effective than icariin in the osteoporosis zebrafish model at low concentrations [13]. While it has been shown in previous studies that Epimedin C has advantages over icariin for preventing GIOP, icariin promotes osteogenic differentiation more strongly than Epimedin C. This study suspected that in the GIOP model, Epimedin C may have been inclined to regulate osteoclastic differentiation for hindering the bone loss process. However, there are few reports about the antiosteoporosis mechanism of Epimedin C that affects the bone metabolism of glucocorticoid-induced osteoporosis.

In the mechanism of GIOP, glucocorticoids could upregulate expressions of RANKL, CSF, and osteoprotegerin, and induce the apoptosis of mature osteoblasts and osteocytes by downregulating expressions of osteoblast collagen gene and type I collagen [14]. It is well known that the interaction of EphB4/EphrinB2 is involved in the balance of bone formation and adsorption during bone homeostasis [15]. It has been proposed that treatment with ephrinB2-FC followed by activation of EphB4 in mesenchymal stem cells may increase the expression of POSTN, which could interact with Wnt signaling pathway to promote osteogenic differentiation [16]. The LGR4 is the osteoclastic target, which is related to RANKL expression, while authors of

another study suggested that LGR4 could promote BMSC migration, stimulate osteoblastic, adipogenic, and myogenic differentiation as well as promote fracture healing [17]. Therefore, protein expressions of Runx2, EphB4, EphrinB2, and LGR4 have been used as protein markers to determine the level of bone metabolism in this research.

This study confirmed the mechanism of the anti-osteoporosis effect of Epimedin C in that it significantly increased expressions of EphB4 and Runx2 at the protein level for promoting osteoblast proliferation and differentiation, upregulated LGR4 expression for competitively inhibiting osteoclast differentiation, and decreased EphrinB2 expression, thereby preventing osteoclastic differentiation. Epimedin C did not only bidirectionally regulate bone metabolism by interfering with EphB4/EphrinB2 signaling but also affected Runx2 expression in Wnt signal pathway and LGR4 in OPG/RANK/RANKL pathway, exerting an anti-osteoporosis effect. Based on the results of this study, it is clear that when treated with dexamethasone, bone loss is accompanied by a change of morphological parameters. Micro-computed tomography was utilized to detect early changes in bone sensitivity for predicting fracture risk and assessing the anti-osteoporotic effect of the agents used. Compared with the control group, the Tb.Th, Tb.N, TMD, and BMC decreased, while Tb.Sp increased significantly in DI group.

TRAP was an S protein marker specifically expressed in osteoclast reported by other articles [18] that it is well enough to be a candidate marker in this study. From the result of trap staining, Epimedin C may inhibit the formation of GIOP-induced osteoclasts. Additionally, expressions of EphB4 reduced and EphrinB2 increased in the DI group. Epimedin C decreased levels of EphrinB2 and enhanced expressions of EphB4, Runx2, and Grb4 in the DI + EG group. Epimedin C could regulate expressions of Runx2 and EphB4 for osteogenesis and expressions of EphrinB2 and Grb4 for osteoclast in the mouse model of GIOP. It is possible that Epimedin C can prevent osteoporosis by regulating the expression of EphB4/EphrinB2 bidirectionally. It is worth noting that without dexamethasone, bone morphological parameters were improved, while expression of EphB4 was slightly decreased and expression of EphrinB2 was significantly increased in the EG group mice.

Considering the balancing of bone metabolism, Epimedin C may incline to prevent the GIOP by influencing the process of osteoclastic

differentiation, although this requires further verification. The existence of EphB4/EphrinB2 axis in the rodent model was verified by immunoblot by targeting two proteins in tissues, CRISPR Cas9 drive EphB4 expression was significantly decreased that influenced the EphrinB2 level at the same time, which means the cross-link were detected in systems. When Epimedin C acted as an effector in DI model, the EphB4/EphrinB2 Axis still work and showed the final result, exhibits that Epimedin C could be a potential therapeutic agent in IG model to antagonize against the dexamethasone-induced OP.

CONCLUSION

The findings of this study suggest that in a model of mice with GIOP, Epimedin C significantly inhibits glucocorticoid-induced osteoporosis, via a mechanism involving the bidirectional regulation of EphB4/EphrinB2 expression. Although the specific regulatory processes and targets of the anti-osteoporotic effect of Epimedin C concerning EphB4/EphrinB2 signaling pathway have not been entirely confirmed in this study, further studies will be conducted to provide new ideas. Furthermore, Epimedin C suppresses or reverses lipopolysaccharide-induced proliferation and mineralization of osteoblasts by regulating BMP-2/Runx2 signaling pathway. Therefore, Epimedin C has a potential application as anti-osteoporosis therapy.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The data sets generated and analyzed during the present study are included in this published article. Further details are available for non-

commercial purposes from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ming Huang contributed substantially to the study design. Ling Qiu and Quan-wei Yang equally contributed to this work, and they are co-first author. Ling Qiu and Ming Huang drafted the manuscript, and Jing Feng and Ying Wang critically revised it. All authors approved the final version submitted for publication.

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