Role of interleukin-33 in the clinical pathogenesis of chronic apical periodontitis

Tana Gegen¹,2, Yanxia Zhu², Qinnuan Sun² and Benxiang Hou¹

Abstract
Objective: This study investigated interleukin (IL)-33 expression in chronic apical periodontitis (CAP) lesions and possible relationships with receptor activator of nuclear factor κ-B ligand (RANKL) and osteoprotegerin (OPG).
Methods: Inflammatory cell infiltration in CAP lesions and samples of healthy periapical tissue (n = 30 each) was evaluated by hematoxylin and eosin staining. IL-33, RANKL, and OPG expression levels were assessed by immunohistochemistry and real-time PCR. In CAP lesions alone, relationships between mRNA level of IL-33 and mRNA levels of both RANKL and OPG were analyzed by Spearman rank correlation.
Results: Histological analysis revealed a large number of inflammatory cells in CAP lesions, and immunohistochemistry revealed IL-33-positive cells. There were more IL-33- and RANKL-positive cells in CAP lesions than in healthy periapical tissue, whereas there were fewer OPG-positive cells in CAP lesions than in healthy periapical tissue. In CAP lesions alone, IL-33 mRNA level was negatively correlated with mRNA level of RANKL and positively correlated with mRNA level of OPG.
Conclusions: IL-33 is highly expressed in CAP lesions, where it is negatively correlated with RANKL and positively correlated with OPG expression. IL-33 may protect against bone resorption via RANKL suppression and OPG induction, and constitutes a potential target for CAP treatment.

Keywords
IL-33, chronic apical periodontitis, RANKL, osteoprotegerin, bone resorption, inflammation, endothelium, epithelium

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¹Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, Beijing, China
²Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

Corresponding author:
Benxiang Hou, Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, Beijing 100050, China.
Email: endohou@163.com
Introduction

Chronic apical periodontitis (CAP) is a common oral inflammatory disease resulting from caries, tooth fracture, iatrogenic causes, or factors that cause contamination and pulp necrosis. In periapical lesions, an initial short acute inflammatory response of varying intensity is accompanied by pain, tooth elevation, and tenderness to percussion. Tissue changes include hyperemia and neutrophil recruitment, which are generally limited to the periodontal ligament. With persistent inflammation, periapical tissue is infiltrated by macrophages, lymphocytes, and plasma cells, resulting in periapical bone resorption. The development of periapical lesions is closely associated with cytokine activity.

Interleukin (IL)-33 is a proinflammatory cytokine belonging to the IL-1 family that can activate mast cells, lymphocytes, and eosinophils to produce type 2 helper T cell (Th2)-associated cytokines. IL-33 as both an intracellular nuclear transcription factor and an extracellular cytokine. Notably, it has been detected in human gingival crevicular fluid and in patients with periodontitis, suggesting that it mediates the inflammatory response in periodontal disease. IL-33 is expressed in periapical granulomas and radicular cysts, and may be involved in periapical inflammation and tissue fibrosis.

CAP is characterized by the resorption of alveolar bone; in periapical lesions, this is triggered by the proliferation of immature osteoclast precursors and their differentiation into mature osteoclasts, which promotes the degradation of organic and inorganic bone components. Osteoclast differentiation is primarily regulated by receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG). RANKL—also known as osteoclast differentiation factor, OPG ligand, and tumor necrosis factor (TNF)-related activation-induced cytokine—is the most potent known inducer of osteoclastogenesis. The natural decoy receptor for RANKL is OPG (also known as osteoclastogenesis inhibitory factor). RANKL expression has been detected in human periapical granulomas and is involved in bone loss associated with periapical lesions. Although some studies have implicated IL-33 in CAP, few have examined the associations between IL-33 and RANKL, IL-33 and OPG, or IL-33 and clinical features of CAP. Moreover, the mechanism by which IL-33 contributes to CAP lesions has not yet been elucidated.

To address these questions, the present study investigated the role of IL-33 and its relationships with RANKL and OPG during the development of CAP. Our results may provide insight into the pathogenesis of CAP and the relationship between inflammation and bone resorption.

Materials and methods

Collection of tissue samples

The study protocol was approved by the Ethics Committee of the Affiliated Hospital, Inner Mongolia Medical University. Tissue samples were collected from study subjects who had provided written, informed consent to participate in the study. Healthy periapical tissue samples were derived from healthy subjects undergoing permanent tooth extraction for orthodontic purposes. Periapical lesion samples were obtained from the teeth of patients who had been diagnosed with CAP, during apical surgery or extraction. The sizes of periapical lesions in all patients were determined by radiographic measurements. Inclusion criteria were as follows: 1) the presence of severe pain with percussion and/or palpation, but without throbbing around the tooth apex; 2) a lack of pulpal activity in response to electric pulp testing; 3) radiographic evidence of connected lesions with a transmission area of bone destruction around the apical area.
of the affected tooth and a clear perimeter without a dense white line; and 4) no clinical value in further treatment or repair and a determination by the clinician that the affected teeth should be removed. Exclusion criteria were as follows: 1) a history of root canal therapy or antibiotic therapy in the previous 2 months; 2) the presence of periodontal disease or periodontic/endodontic lesions; 3) a history of systemic diseases that could affect periodontal status; and 4) current pregnancy or ongoing lactation.

**Histological analysis**

All samples were immediately fixed in 4% paraformaldehyde for 48 hours, then embedded in paraffin and cut into 4-μm-thick serial sections. The sections were stained with hematoxylin and eosin (H&E) and analyzed by an experienced pathologist under a light microscope (BA200; Motic, Hong Kong) and divided into CAP and control (healthy subjects) groups based on histological features. The H&E-stained specimens were used for both histopathological and morphometric analyses.

**Immunohistochemistry**

Endogenous peroxidase activity in deparaffinized sections was neutralized by 30 minutes incubation in 0.3% hydrogen peroxide in methanol. Sections were then blocked with 5% normal serum (Maixin, Shenzhen, China); antigen retrieval was performed by heating sections at 90°C for 10 minutes, followed by washing in phosphate-buffered saline (PBS) to reduce non-specific binding. Sections were incubated overnight in dilution buffer (ABD-0030, Maixin) with antibodies against IL-33 (ab207737, 1:250 dilution; Abcam, Cambridge, UK), RANKL (12A668, 1:500 dilution; Novus Biologicals, Centennial, CO, USA), and OPG (98A1071, 1:500 dilution; Novus Biologicals). After the sections had been washed with PBS, they were incubated with biotinylated secondary antibody (Kit 5010, Maixin) for 45 minutes, then washed again with PBS; immunoreactivity was visualized by the addition of diaminobenzidine (DAB) solution (Maixin). As a negative control, PBS was used instead of the primary antibody. The sections were counterstained with hematoxylin and photographed under an optical microscope (Motic). Brown staining indicated a positive reaction.

Protein expression in the tissue was analyzed semi-quantitatively by two investigators at 200× magnification. The numbers of IL-33-, RANKL-, and OPG-positive cells were counted in five representative histological fields with positive expression of each marker at 400× magnification. The mean numbers of positive cells were calculated using ImageJ software (version 1.36, National Institutes of Health, Bethesda, MD, USA).

**Real-time (RT)-PCR analysis**

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer’s instructions, and digested with DNase (Tiangen Biotech, Beijing, China). RNA concentration and quality were evaluated using a NanoDrop ND-1000 system (NanoDrop Technologies, Wilmington, DE, USA); the A260/A280 of isolated RNA between 1.8 and 2.2. cDNA was synthesized from the RNA using the SuperScript III First-strand Synthesis System (Life Technologies), in accordance with the manufacturer’s protocol.

RT-PCR was performed using the Maxima SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA, USA) on a three-step real-time system (Applied Biosystems, Foster City, CA, USA). The sequences of the primers, which were designed based on gene sequences published in GenBank, are shown in Table 1. The specificities of the primers were independently tested prior to RT-PCR with positive and
negative (nuclease-free water) controls. \(\beta\)-actin served as a loading control to normalize gene expression. Relative expression levels were calculated using the \(2^{-\Delta\Delta CT}\) method.

**Statistical analysis**

Data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS software (version 21.0, IBM Corp., Armonk, NY, USA). The numbers of IL-33-, RANKL-, and OPG-positive cells were counted for each group; these data were analyzed with the Mann–Whitney U test. In analysis of the CAP group alone, the relationships between IL-33 and RANKL mRNA expression levels, and between IL-33 and OPG mRNA expression levels, were assessed with the Spearman rank correlation test using Prism software (version 5, GraphPad, La Jolla, CA, USA). Differences with \(P < 0.05\) were considered to be statistically significant.

**Results**

**Characteristics of patients and healthy subjects**

Thirty periapical lesion samples were obtained from the teeth of patients who were diagnosed with CAP during apical surgery or extraction. Thirty healthy periapical tissue samples were derived from healthy subjects undergoing permanent tooth extraction for orthodontic purposes. Detailed characteristics of CAP patients are listed in Table 2.

**Analysis of inflammation in CAP**

CAP can occur in any tooth, including incisors, premolars, and molars. X-rays revealed a low-density shadow around the periapical area of affected teeth, along with a clear perimeter without a dense white line (Figure 1a). The mean diameter of periapical lesions was 2.88 ± 1.04 mm (\(P < 0.001\)) (Figure 1b). Histological analysis by H&E staining showed a large number of inflammatory cells—primarily lymphocytes, plasma cells, and neutrophils in periapical lesions, along with fibroblasts and newly formed capillaries (Figure 1c). Semi-quantitative analysis indicated that more inflammatory cells were present in periapical lesions than in healthy periapical tissue (\(P < 0.05\)) (Figure 1d). These results indicated that inflammation was present in the surrounding the periapical area.

**Involvement of IL-33 in CAP**

Immunohistochemical analysis revealed that IL-33-positive cells were present in

| Site   | Primer sequence (5′–3′)                        | Annealing temperature (°C) | Elongation time (s) | PCR product size (bp) |
|--------|------------------------------------------------|----------------------------|---------------------|-----------------------|
| IL-33  | 5′-AGAGCCCTAGATGAGACACCGAAT-3′ (F)             | 60                         | 60                  | 90                    |
|        | 5′-GCCAGGGTCAGAAGGGATG-3′ (R)                  |                            |                     |                       |
| RANKL  | 5′-CACAGCACATCAGAGCAGAAGA-3′ (F)               | 60                         | 60                  | 146                   |
|        | 5′-GGACAGACTCACTTATGGGAACC-3′ (R)              |                            |                     |                       |
| OPG    | 5′-AGCTGCAGTACGTCAAGCAGGGA-3′ (F)              | 60                         | 60                  | 164                   |
|        | 5′-TTGCAAAACTGTATTGCCTCCTGG-3′ (R)             |                            |                     |                       |
| ATCB   | 5′-CATCGGACACGGCATCGCA-3′ (F)                  | 60                         | 60                  | 211                   |
|        | 5′-TAGCAGACCTGGATAGCAAC-3′ (R)                 |                            |                     |                       |

ATCB, \(\beta\)-actin; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor \(\kappa\)-B ligand.
periapical lesions (Figure 2a; controls for comparison are shown in Figure 2b). There were more IL-33-positive cells in CAP lesions than in healthy periapical tissue (P < 0.05). Various cell types were positive for IL-33, including endothelial cells (Figure 2c), epithelial cells (Figure 2d), and inflammatory cells (P < 0.05) (Figure 2e). Similar results were obtained by RT-PCR: IL-33 mRNA expression was higher in CAP lesions than in healthy periapical tissue (P < 0.05) (Figure 2f). These results suggested that IL-33 plays an important role in the pathogenesis of CAP.

**Modulation of bone resorption in CAP by IL-33 through suppression of RANKL**

Immunohistochemical analysis revealed that more RANKL-positive cells were present in periapical lesions than in healthy periapical tissue (Figure 3a; controls for comparison are shown in Figure 3b). Most of these cells were inflammatory cells (P < 0.05); no

| Table 2. Characteristics of the study population. |
|-----------------------------------------------|
| Characteristic                             | Group             |
|                                            | CON (n = 30) | CAP (n = 30)  |
| Age (years) (mean ± SD)                    | 21.77 ± 4.93 | 45.20 ± 1.98 |
| Sex                                         |                |
| Male                                       | 10 (33.34%) | 14 (46.67%)  |
| Female                                     | 20 (66.66%) | 16 (53.33%)  |
| Oral health condition                      |                |
| Good                                       | 17 (56.67%) | 10 (33.33%)  |
| Poor                                       | 13 (43.33%) | 20 (66.67%)  |
| Clinical symptoms                          |                |
| Present                                    | 0            | 30            |
| Absent                                     | 30           | 0             |
| Tooth location                             |                |
| Incisors                                   | 3 (10%)      | 6 (20%)       |
| Premolars                                  | 23 (76.67%)  | 7 (23.33%)    |
| Molars                                     | 4 (13.33%)   | 17 (56.67%)   |
| Tooth looseness                            |                |
| None                                       | 30           | 7             |
| I                                          | 0            | 20            |
| II                                         | 0            | 3             |
| III                                        | 0            | 0             |
| Percussion                                 |                |
| (−)                                        | 30           | 0             |
| (+)/(++)/(+++)/(++++)                      | 0            | 30            |
| Electrical activity of dental pulp         |                |
| Viable                                     | 30           | 0             |
| Nonviable                                  | 0            | 30            |
| Diameter of periapical lesion (mm) (mean ± SEM) | 0             | 2.88 ± 1.04 |

Data are shown as n (%) unless otherwise indicated.
CAP, chronic apical periodontitis; CON, control; SD, standard deviation.
changes in RANKL expression were observed in endothelial and epithelial cells (Figure 3c, d). RANKL mRNA expression was higher in CAP lesions than in healthy periapical tissue (P < 0.05) (Figure 3e). In addition, in analysis of the CAP lesions alone, RANKL mRNA expression was negatively correlated with the mRNA expression of IL-33 (Figure 3f).

**Inhibition of bone resorption in CAP by IL-33 through induction of OPG**

Immunohistochemical analysis revealed that OPG was expressed around endothelial and epithelial cells (Figure 4a; controls for comparison are shown in Figure 4b), and that OPG was expressed at lower levels in CAP lesions than in healthy periapical tissue (P < 0.05) (Figure 4c, d). OPG immunopositivity was observed in inflammatory cells of periapical lesions, but expression was reduced in CAP lesions compared with healthy periapical tissue (P < 0.05) (Figure 4e). Moreover, in analysis of the CAP lesions alone, IL-33 and OPG mRNA expression levels were positively correlated (Figure 4f). Therefore, IL-33 may modulate bone resorption in CAP through inhibition of osteoclast formation.
Figure 2. IL-33 was upregulated in chronic apical periodontitis. (a) Representative photomicrographs of immunohistochemical staining for IL-33 in periapical lesions. Positive staining was present around the endothelium and epithelium; IL-33-positive cells included endothelial, epithelial, and inflammatory cells. Original magnification, 400×; scale bar = 50 μm. (b) Positive control: a large amount of IL-33 positive expression was present in human tonsil tissue, and its primary staining site was in the nucleus. Negative control: IL-33 antibody staining was replaced by phosphate-buffered saline, and no staining was observed. Original magnification, 400×; scale bar = 50 μm. (c) IL-33-positive expression in endothelial cells was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (d) IL-33-positive expression in epithelial cells was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (e) IL-33-positive expression in inflammatory cells was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (f) IL-33 mRNA expression was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). All data are shown as mean ± standard deviation. Relative changes in gene expression were calculated by the \(2^{-\Delta\Delta CT} \) method, and were compared using the Mann–Whitney U test. ***P < 0.001; ****P < 0.0001.

CAP, chronic apical periodontitis; CON, control; IL, interleukin; S-1, sample 1; S-2, sample 2.
Discussion
CAP is an oral infectious disease that is characterized by pathogenic bone resorption around the apical region. Various cytokines (e.g., TNF-α, interferon-γ, IL-17A, and IL-21) play key roles in the pathogenesis of periapical lesions; IL-6 and IL-23 enhance the potentially destructive role of Th17 cells in these lesions. In contrast, some cytokines (e.g., IL-10, IL-9, IL-4, and IL-22) may exert protective effects. IL-10 was previously detected in periapical lesions, where it inhibited inflammatory cell influx and bone resorption. IL-4 is a prototypical Th2 cytokine that upregulates OPG levels and suppresses pro-inflammatory responses. In addition, IL-33—a recently identified member of the IL-1 cytokine family—transmits an alarm signal upon cell

Figure 3. IL-33 was negatively correlated with RANKL in chronic apical periodontitis. (a) Representative photomicrographs of immunohistochemical staining for RANKL in periapical lesions. RANKL-positive cells were primarily inflammatory cells of periapical lesions. Original magnification, 400×; scale bar = 50 μm. (b) Positive control: a large amount of RANKL-positive expression was observed in human lymph node tissue. Negative control: RANKL antibody staining was replaced by phosphate-buffered saline, and no staining was observed. Original magnification, 400×; scale bar = 50 μm. (c, d) RANKL-positive expression in inflammatory cells was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). However, RANKL-positive expression in endothelial and epithelial cells was not significantly different between periapical lesions from patients with CAP and healthy periapical tissues from subjects in the CON group. (e) RANKL mRNA expression was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (f) IL-33 mRNA expression was negatively correlated with RANKL mRNA expression in periapical lesions from patients with CAP. All data are presented as mean ± standard deviation. Relative changes in gene expression were calculated by the 2^{-ΔΔCt} method, and were compared using the Mann–Whitney U test. ***P < 0.001; ****P < 0.0001.
CAP, chronic apical periodontitis; CON, control; RANKL, receptor activator of nuclear factor κ-B ligand; S-1, sample 1; S-2, sample 2.
injury or tissue damage, which activates immune cells that express its receptor, ST2.14 IL-33 is also associated with Th2 responses and exerts similar bone-protective effects, such as inhibition of osteoclast differentiation.15

In the present study, we demonstrated that IL-33 is involved in CAP, which is consistent with the findings in previous reports.4 Immunohistochemical analysis of periapical tissue showed that multiple cell types express IL-33, including inflammatory, endothelial, and epithelial cells; this is consistent with the recent observation that IL-33 is expressed in inflammatory cells and fibroblasts in periapical granulomas and radicular cysts.16 IL-33 has also been detected in bone tissue—particularly in bone marrow—in the inflammatory state, but not in normal bone homeostasis.17

Figure 4. IL-33 was positively correlated with OPG in chronic apical periodontitis. (a) Representative photomicrographs of immunohistochemical staining for OPG in periapical lesions. Positive staining was present around the endothelium and epithelium; OPG-positive cells primarily included endothelial and epithelial cells in healthy periapical tissues. (b) Positive control: a large amount of OPG-positive expression was present in human kidney tissue. Negative control: OPG antibody staining was replaced with phosphate-buffered saline, and no staining was observed. Original magnification, 400 ×; scale bar = 50 μm. (c, d) OPG-positive expression in endothelial and epithelial cells was significantly lower in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). However, OPG-positive expression in inflammatory cells was significantly higher in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (e) OPG mRNA expression was significantly lower in periapical lesions from patients with CAP than in healthy periapical tissues from subjects in the CON group (P < 0.05). (f) IL-33 mRNA expression was positively correlated with OPG mRNA expression in periapical lesions from patients with CAP. All data are shown as mean ± standard deviation. Relative changes in gene expression were calculated by the 2^−ΔΔCt method, and were compared using the Mann–Whitney U test. ***P < 0.001; ****P < 0.0001.

CAP, chronic apical periodontitis; CON, control; OPG, osteoprotegerin; S-1, sample 1; S-2, sample 2.
Furthermore, IL-33 is present in human gingival crevicular fluid\textsuperscript{18–20} and is involved in the development of human chronic periapical lesions along with RANKL, OPG, TNF-$\alpha$, and cathepsin K.\textsuperscript{4} IL-33 is presumed to function as a proinflammatory cytokine that promotes bone loss in periapical lesions, due to its concomitant expression with known contributing factors. However, despite the reported anti-osteoclastogenic activity of IL-33, its similar expression levels in both inactive and active lesions suggests that it may not be a major determinant of periapical lesion inactivity.\textsuperscript{8} Pro- and anti-inflammatory factors regulate the stable or progressive nature of periapical granulomas by modulating the balance of RANKL and OPG;\textsuperscript{8} expression of both proteins in these lesions suggests their involvement in bone metabolism.\textsuperscript{21} However, the relationships between IL-33 and RANKL, as well as IL-33 and OPG, remain unclear.

Here, we found that, in analysis of CAP lesions alone, IL-33 expression was negatively correlated with that of RANKL and positively correlated with that of OPG in periapical lesions. The resorption of periapical bone is caused by an imbalance between osteoblast and osteoclast activity. Osteoclasts are large multi-nucleated cells formed by the fusion of precursor cells of the monocyte-macrophage lineage; their differentiation and activity largely depend on RANKL and OPG.\textsuperscript{22} Moreover, activation of the RANK receptor by RANKL induces the differentiation of precursor cells into osteoclasts.\textsuperscript{22} OPG functions as a soluble decoy-like factor for RANKL that inhibits osteoclast formation and (consequently) bone resorption.\textsuperscript{22} Thus far, there remains controversy regarding the role of IL-33 in bone formation or bone resorption. In a rat model of periodontitis, IL-33 expression was upregulated, concurrently with that of RANKL.\textsuperscript{23} IL-33 is reportedly overexpressed in the gingiva of patients with chronic periodontitis and functions as a trigger factor for the recruitment of B and T lymphocytes that express RANKL.\textsuperscript{24} One study showed that the interaction between IL-33 and RANKL can promote severe alveolar bone loss related to periodontal disease through differentiation of osteoblastic cells. Furthermore, both TNF-$\alpha$ and IL-33 were shown to mediate bone loss in a RANKL-independent manner and induce the expression of RANKL, concomitantly with other proinflammatory cytokines.\textsuperscript{25} However, some studies have shown that IL-33 can block osteoclast formation in the presence of exogenous RANKL;\textsuperscript{26} notably, the inhibition of osteoclastogenesis by IL-33 has been demonstrated both in vivo and in vitro.\textsuperscript{9,10} Our current experimental results demonstrate that IL-33 inhibits osteoclast formation and induces osteoblast differentiation through regulation of the expression levels of RANKL and OPG.

Thus, the synergistic effects of RANKL and proinflammatory cytokine signaling induced in periapical areas in response to bacterial stimuli may be responsible for the progression of periapical lesions.\textsuperscript{5} However, further studies are needed to identify the components that mediate periapical bone destruction, as well as their specific functions. The mechanism by which IL-33 participates in bone resorption in CAP remains unclear; further studies are needed to determine the role of IL-33 in the development and outcome of bone resorption in CAP, both in vitro and in vivo. In summary, IL-33 could contribute to the inhibition of bone resorption in CAP by promoting the expression of OPG and inhibiting the expression of RANKL. IL-33 may be a useful target for treatment of CAP in the future.

**Conclusions**

This study demonstrated that IL-33 is highly expressed in periapical lesions. In analysis of
CAP lesions alone, mRNA expression of IL-33 was negatively correlated with that of RANKL and positively correlated with that of OPG. Based on these findings, we speculate that IL-33 protects against bone resorption in CAP via suppression of RANKL and induction of OPG, and can thus serve as a therapeutic target in patients with CAP.

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Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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ORCID iD
Benxiang Hou https://orcid.org/0000-0003-3048-538X

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