

Original Research Article

MicroRNA-595 promotes osteogenic differentiation of bone marrow mesenchymal stem cells by targeting HMGA2

Bingjun Gao^{1*}, Yarong Wu², Lijian Zhou¹, Xin Chen¹

¹Department of Osteology, ²Department of Hematology, The People's Hospital of Danyang; Affiliated Danyang Hospital of Nantong University, Danyang 212300, Jiangsu Province, China

*For correspondence: **Email:** bingjun_g@163.com; **Tel:** +86-51186553701

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Abstract

Purpose: To investigate the effect of miR-595 on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs).

Methods: Human BMSCs were osteogenically differentiated, and protein expression of alkaline phosphatase (ALP), osteocalcin (OCN), and Runt-related transcription factor 2 (RUNX2) were evaluated by western blot. Expression of miR-595 was measured by quantitative reverse transcription (qRT-PCR). The effect of miR-595 on viability of BMSCs was determined by MTT assay. Osteogenic differentiation of BMSCs was assessed by ALP and Alizarin red S (ARS) staining. The target gene of miR-595 was predicted by TargetScan analysis and validated by luciferase activity assay.

Results: MiR-595 expression was higher in osteogenically differentiated BMSCs than in undifferentiated BMSCs ($p < 0.01$). Osteogenic ALP, OCN, and RUNX2 were also upregulated ($p < 0.01$). MiR-595 expression increased the viability of BMSCs, mineralized bone matrix formation, and ALP activity. High mobility group AT-hook 2 (HMGA2) expression was lower in osteogenically differentiated BMSCs and was found to be a target of miR-595. Overexpression of HMGA2 attenuated the miR-595-induced increase in cell viability, ALP activity, mineralized bone matrix formation, and osteogenic gene expression in BMSCs.

Conclusion: The miR-595/HMGA2 axis is involved in osteogenic differentiation of BMSCs suggesting that it is a promising therapeutic target for osteoporosis.

Keywords: MiR-595, HMGA2, Osteogenic differentiation, BMSCs, Osteoporosis

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INTRODUCTION

Osteoporosis is the most common systemic bone disease and involves bone deterioration and loss of bone mass resulting in a high risk of bone fractures [1]. Bone marrow mesenchymal stem cells (BMSCs) differentiate into osteoblasts in bone tissue and function in bone formation and

resorption during bone remodeling [2]. In recent years, it has been shown that decreased osteogenic and increased lipogenic differentiation of BMSCs is the fundamental cause of osteoporosis [3]. Stem cell therapies for osteoporosis ameliorate bone resorption by enhancing osteogenic differentiation of BMSCs [4]. Therefore, targeting regulators of osteogenic

differentiation of BMSCs is a promising strategy for treating osteoporosis.

MicroRNAs (miRNAs) have been shown to regulate osteogenic differentiation of BMSCs by modulating osteogenic gene expression [5]. Therefore, osteoblast-associated miRNAs may contribute to the progression of osteoporosis [6]. MiR-595 expression was shown to be significantly lower in the serum of osteoporosis patients when compared to healthy subjects [7]. However, the role of miR-595 in osteogenic differentiation of BMSCs remains elusive.

In this study, the miR-595 expression level and its effect on osteogenic differentiation of BMSCs were investigated. TargetScan analysis showed that miR-595 targets high mobility group AT-hook 2 (HMGA2); thus, the effect of the miR-595/HMGA2 axis on osteogenic differentiation of BMSCs was explored.

EXPERIMENTAL

Culture and osteogenic differentiation of BMSCs

Human BMSCs were obtained from the European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO, USA) and were cultured in low-sugar DMEM containing 10 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). For osteogenic differentiation, BMSCs were cultured in this growth medium for 48 h, the growth medium was replaced with osteogenic induction medium (DMEM containing 100 nM dexamethasone, 10 mM β -glycerophosphate, and 200 μ M ascorbic acid), and cells were cultured for 14 d at 37 °C until 70–80 % confluence was reached.

Cell transfection and viability assays

A mimic and inhibitor of miR-595 (50 nM) and pcDNA-HMGA2 (300 μ g) were purchased from RiboBio (Guangzhou, China). BMSCs were transfected with pcDNA-HMGA2, miRNA mimic, or the miRNA inhibitor via Lipofectamine 2000 (Invitrogen), and the transfected cells were subjected to osteogenic induction.

To evaluate cell viability, transfected BMSCs were seeded and cultured for 0, 24, 48, or 72 h. Then, MTT solution (20 μ L, 5 mg/mL; Sigma-Aldrich) was added to the cells and they were incubated for another 4 h. Absorbance at 490 nm was measured using a microplate spectrophotometer (ELx800, Bio-TEK, Winooski, VT, USA).

Alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining

BMSCs were cultured in osteogenic medium for 7 d and then fixed with 4 % paraformaldehyde for ALP staining (Beyotime; Shanghai, China). Absorbance at 405 nm was measured using a microplate spectrophotometer (ELx800). For ARS staining, BMSCs were cultured in osteogenic medium for two weeks, fixed with 4 % paraformaldehyde, and stained with 40 mM ARS staining solution (Sigma-Aldrich). Cells were observed and photographed by microscopy (Olympus, Tokyo, Japan).

Bioinformatic analysis and luciferase reporter assay

TargetScan (http://www.targetscan.org/vert_72/) analysis was used to predict the target of miR-595. The 3' UTR of HMGA2 and the mutant 3' UTR of HMGA2 were cloned separately into the psiCHECK2 vector (Promega, Madison, WI, USA) to generate HMGA2-WT or HMGA2-MUT vectors, respectively. HEK-293 cells were co-transfected with HMGA2-WT and the miR-595 mimic or miR-NC, or co-transfected with HMGA2-MUT and the miR-595 mimic or miR-NC via Lipofectamine 2000. Two days post-transfection, luciferase activity was measured using the Dual-Luciferase Reporter detection System (Promega).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

BMSCs were cultured for 3, 7, or 14 d and then lysed, and RNA was extracted using TRIzol (Invitrogen). The isolated RNAs were synthesized into cDNAs. SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) was used for qRT-PCR analysis of ALP, OCN, Runx-2, and HMGA2, and the TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR analysis of miR-595. GAPDH and U6 were used as internal controls. The primers used are listed in Table 1.

Western blot assay

The ProteoPrep Total Extraction Sample Kit (Sigma-Aldrich) was used to extract proteins from cultured cells. Proteins were separated by SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). PVDF membranes were blocked in 5 % bovine serum albumin and incubated with primary antibodies against HMGA2 (1:2000; Abcam;

Table 1: Primers used for qRT-PCR

Item	Sequence (5'- 3')
GAPDH Forward	GCACCGTCAAGGCTGAGAAC
GAPDH Reverse	TGGTGAAGACGCCAGTGGA
ALP Forward	AACATCAGGGACATTGACGTG
ALP Reverse	GTATCTCGGTTTGAAGCTCT
miR-595 Forward	TGCGGGAAGTGTGCCGTGGTGT
miR-595 Reverse	GTCGTATCCAGTGCAGGGTCCG
OCN Forward	AGCCACCGAGACACCATGAGA
OCN Reverse	GGCTGCACCTTTGCTGGACT
Runx-2 Forward	CTTCGTCAGCGTCCTATCAGTTC
Runx-2 Reverse	CAGCGTCAACACCATCATTCTG
HMGA2 Forward	GGCTCAGATTGAGAACAGC
HMGA2 Reverse	GCTTCAACGGCAAAGTTCTC
U6 Forward	GCTTCGGCAGCACATATACT
U6 Reverse	GGTGCAGGGTCCGAGGTAT

Cambridge, MA, USA), ALP (1:2000; Abcam), OCN (1:2500; Abcam), Runx-2 (1:2500; Abcam), and β -actin (1:3000; Abcam). Then, membranes were incubated with HRP-labeled secondary antibody (Abcam). Signals were detected using the BeyoECL Plus Kit (Beyotime, Shanghai, China) and analyzed with Gel Pro Analyzer Software 4.0 (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Experiments were performed in triplicate at least, and data were expressed as mean \pm standard deviation. GraphPad Prism was used for statistical analysis. Statistical differences between groups were analyzed by the Student's *t*-test and one-way ANOVA followed by a Tukey or Kruskal-Wallis post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

MiR-595 expression increased during osteogenic differentiation of BMSCs

To investigate the relationship between miR-595 and osteoporosis, miR-595 expression in BMSCs post-osteogenic differentiation (d 0, 3, 7 and 14) was evaluated by qRT-PCR analysis. MiR-595 expression increased during osteogenic differentiation in a time-dependent manner (Figure 1A). Both mRNA (Figure 1A) and protein (Figure 1B) expression of osteogenic genes, including ALP, OCN, and Runx-2, were higher in osteogenic differentiated BMSCs than in undifferentiated BMSCs, suggesting that miR-595 may be involved in osteogenic differentiation of BMSCs.

MiR-595 promoted proliferation of BMSCs

To assess the effect of miR-595 on amplification of BMSCs, BMSCs were transfected with a miR-

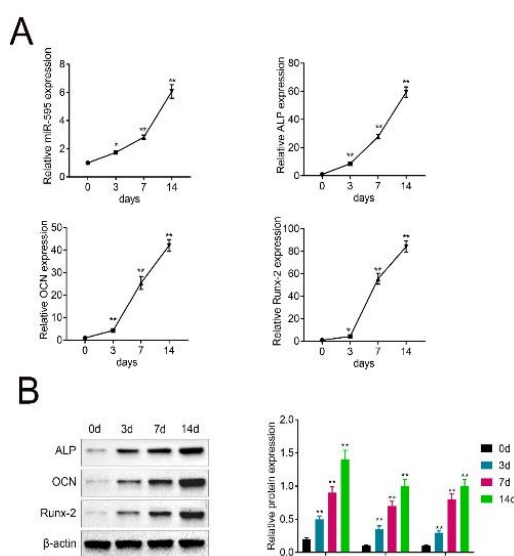


Figure 1: Increased miR-595 expression during osteogenic differentiation of BMSCs. (A) miR-595, ALP, OCN, and Runx-2 mRNA expression increased on d 3, 7, and 14 of BMSC differentiation when compared to undifferentiated BMSCs on d 0. mRNA expression was measured by qRT-PCR. (B) miR-595, ALP, OCN, and Runx-2 protein expression increased on d 3, 7, and 14 of BMSC differentiation when compared to undifferentiated BMSCs on d 0. Protein expression was evaluated by western blot. $n = 3$. ** $p < 0.01$

595 mimic or inhibitor. MiR-595 expression was higher in BMSCs transfected with the miR-595 mimic when compared with negative controls, and miR-595 expression was lower in BMSCs transfected with the miR-595 inhibitor when compared with negative controls (Figure 2 A). A functional assay showed that transfection with the miR-595 mimic increased proliferation of BMSCs (Figure 2 B), and transfection with the miR-595 inhibitor decreased amplification of BMSCs (Figure 2 B), suggesting that miR-595 has a proliferative effect on BMSCs. Moreover,

overexpression of miR-595 promoted amplification of BMSCs in non-differentiation media (Figure 3 A) but did not affect ALP or Runx-2 protein expression (Figure 3 B).

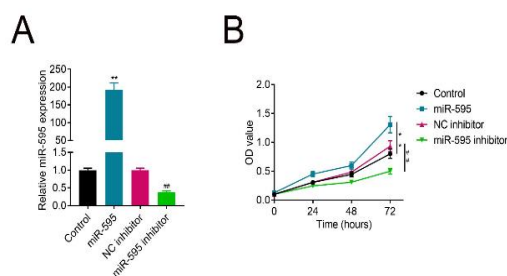


Figure 2: MiR-595 promoted amplification of BMSCs. (A) miR-595 expression in undifferentiated BMSCs was upregulated upon transfection with the miR-595 mimic and downregulated upon transfection with the miR-595 inhibitor 2 d post-transfection. $n = 3$. (B) Transfection with the miR-595 mimic increased amplification of BMSCs, and transfection with the miR-595 inhibitor decreased amplification of BMSCs 2 d post-transfection, $n = 3$ **, ## $p < 0.01$

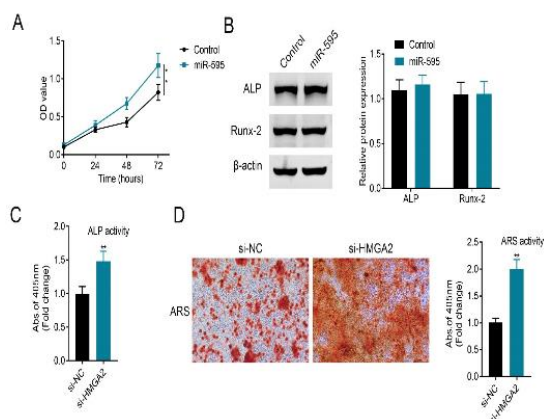


Figure 3: (A) Transfection with the miR-595 mimic increased proliferation of BMSCs cultured in undifferentiated media. (B) Transfection with the miR-595 mimic did not affect ALP and Runx-2 protein expression in BMSCs cultured in undifferentiated media. (C) Transfection with siHMGA2 increased ALP activity of BMSCs cultured in osteogenic medium for 7 d. (D) Transfection with siHMGA2 increased mineralized bone matrix formation of BMSCs cultured in osteogenic medium for 7 d. $n = 3$, ** $p < 0.05$

MiR-595 promoted osteogenic differentiation of BMSCs

To assess the effect of miR-595 on osteogenesis, BMSCs were transfected with a miR-595 mimic or inhibitor, cultured for 2 d, and then stained for ALP and ARS. Increased ALP activity was observed in osteogenic BMSCs transfected with the miR-595 mimic (Figure 4 A), and decreased ALP activity was observed in

BMSCs transfected with the miR-595 inhibitor (Figure 4 A). Moreover, osteogenically differentiated BMSCs transfected with the miR-595 mimic showed increased mineralized bone matrix formation compared to BMSCs transfected with the negative control (control, Figure 4 B). However, BMSCs transfected with the miR-595 inhibitor showed decreased mineralized bone matrix formation compared to BMSCs transfected with the negative control (NC) inhibitor (Figure 4 B). These data suggest that miR-595 promotes osteogenic differentiation of BMSCs.

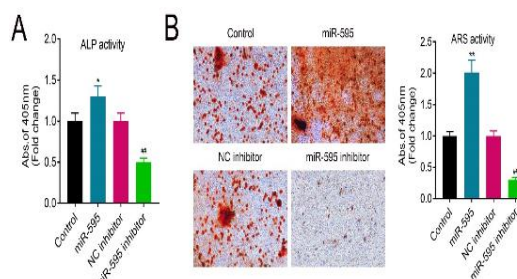


Figure 4: MiR-595 promoted osteogenic differentiation of BMSCs. (A) Osteogenically differentiated BMSCs transfected with the miR-595 mimic showed increased ALP activity 2 d post-transfection, and BMSCs transfected with the miR-595 inhibitor decreased ALP activity. ALP activity was measured by ALP staining, $n = 3$. (B) Mineralized bone matrix formation increased in osteogenically differentiated BMSCs transfected with the miR-595 mimic 2 d post-transfection, and mineralized bone matrix formation decreased in BMSCs transfected with the miR-595 inhibitor. Mineralized bone matrix was detected by ARS staining. $n = 3$, * $p < 0.05$, ## $p < 0.01$

MiR-595 binds HMGA2 mRNA

Binding between miR-595 and HMGA2 was predicted by TargetScan (http://www.targets can.org/vert_72/) analysis (Figure 5 A). Luciferase activity of HMGA2-WT in HEK-293 cells decreased upon transfection with the miR-595 mimic (Figure 5 B); however, luciferase activity of HMGA2-MUT remained unchanged upon transfection with the miR-595 mimic (Figure 5 B), demonstrating that miR-595 binds HMGA2. Additionally, HMGA2 expression decreased in BMSCs transfected with miR-595 (Figure 5 C and D), and HMGA2 increased in BMSCs transfected with the miR-595 inhibitor (Figure 5 C and D). HMGA2 expression decreased during osteogenic differentiation of BMSCs in a time-dependent manner (Figure 5 E and F). Moreover, silencing of HMGA2 increased ALP activity of BMSCs (Figure 3 C) and increased mineralized bone matrix formation (Figure 3 D), suggesting that HMGA2 is involved in osteogenic differentiation of BMSCs.

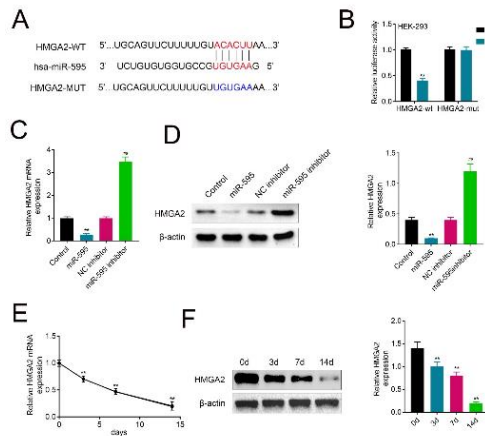


Figure 5: MiR-595 binds HMGA2 mRNA. (A) The potential binding interaction between miR-595 and HMGA2. (B) The luciferase activity of HMGA2-wt in HEK-293 cells decreased upon transfection with the miR-595 mimic, but the luciferase activity of HMGA2-mut remained unchanged upon transfection with the miR-595 mimic. Luciferase activity was measured using the dual luciferase activity assay, $n = 3$. (C) HMGA2 mRNA expression decreased in BMSCs transfected with the miR-595 mimic 2 d post-transfection, and HMGA2 mRNA expression increased in BMSCs transfected with the miR-595 inhibitor. mRNA expression was measured by qRT-PCR, $n = 3$. (D) HMGA2 protein expression decreased in BMSCs transfected with the miR-595 mimic 2 d post-transfection, and HMGA2 protein expression increased in BMSCs transfected with the miR-595 inhibitor. Protein expression was evaluated by western blot, $n = 3$. (E) HMGA2 mRNA expression in BMSCs cultured in osteogenic medium was downregulated on d 5, 10, and 15 when compared to undifferentiated BMSCs on d 0. mRNA expression was measured by qRT-PCR, $n = 3$. (F) Downregulation of HMGA2 protein expression in BMSCs cultured in osteogenic medium was downregulated on d 5, 10, and 15 when compared to undifferentiated BMSCs on d 0. Protein expression was evaluated by western blot, $n = 3$, $## p < 0.01$

HMGA2 expression attenuated osteogenic differentiation of BMSCs induced by miR-595

To assess the role of the miR-595/HMGA2 signaling axis on osteoporosis, BMSCs transfected with miR-595 mimic and pcDNA-HMGA2 were subjected to ALP and ARS staining. Transfection with pcDNA-HMGA2 attenuated the miR-595-induced proliferation of BMSCs (Figure 6 A). HMGA2 overexpression reversed the effects of miR-595 overexpression on ALP activity (Figure 6 B) and mineralized bone matrix formation (Figure 6 C) in osteogenically differentiated BMSCs. Moreover, HMGA2 expression reversed the effect of miR-595 expression on ALP, OCN, and Runx-2 expression during osteogenic differentiation of

BMSCs (Figure 6 D). These data demonstrated that the miR-595-induced osteogenic differentiation of BMSCs is negated by HMGA expression.

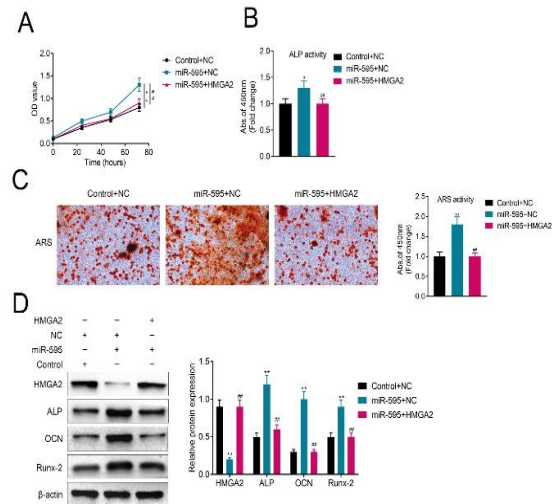


Figure 6: HMGA2 expression attenuated osteogenic differentiation of BMSCs induced by miR-595. (A) Increased HMGA2 expression attenuated the effect of miR-595 overexpression on proliferation of BMSCs cultured in osteogenic medium over time. (B) Increased HMGA2 expression attenuated the effect of miR-595 overexpression on ALP activity in osteogenically differentiated BMSCs. (C) Increased HMGA2 expression attenuated the effect of miR-595 overexpression on mineralized bone matrix formation in osteogenically differentiated BMSCs. (D) Increased HMGA2 expression attenuated the effect of miR-595 overexpression on ALP, OCN, and Runx-2 protein expression in osteogenically differentiated BMSCs. $n = 3$, $*$, $** p < 0.05$; $### p < 0.01$

DISCUSSION

BMSCs originate from bone marrow and can differentiate into bone, adipose tissue, muscle, and cartilage; thus, they have multipotential differentiation capacity and great potential for regenerative therapies [4]. BMSCs recruited from neighboring microenvironments can differentiate into osteoblasts during bone formation [3]. MiRNAs that regulate expression of osteogenic genes and participate in osteogenic differentiation of BMSCs are potential therapeutic targets for osteoporosis [6]. The effect of miR-595 on osteogenic differentiation of BMSCs was investigated.

ALP, OCN, and Runx-2 are osteoporotic genes that have been shown to be upregulated during osteogenic differentiation of BMSCs [8]. This study confirmed upregulation of ALP, OCN, and Runx-2 in BMSCs cultured in osteogenic medium. Significant upregulation of miR-595 in

osteogenically differentiated BMSCs relative to undifferentiated BMSCs indicated that miR-595 may be involved in osteogenic differentiation of BMSCs. Functional assays demonstrated that miR-595 expression increased proliferation of BMSCs, promoted mineralization of the extracellular matrix, and increased ALP activity. In addition, miR-595 inhibition decreased ALP activity and extracellular matrix mineralization confirming that miR-595 promotes osteogenic differentiation of BMSCs.

This study also demonstrated that HMGA2 expression is reduced in osteogenically differentiated BMSCs when compared to undifferentiated BMSCs; thus, HMGA2 may be the target of miR-595. HMGA2 polymorphisms have been associated with bone mineral density [9]. HMGA2 has been shown to function in let 7-mediated osteogenic differentiation of MSCs [10] and to suppress osteogenic differentiation of stem cells [11].

In this study, HMGA2 expression reversed the promotional effects of miR-595 on amplification of BMSCs, mineralization of the extracellular matrix, ALP activity, and expression of ALP, OCN, and Runx-2, suggesting that miR-595 promotes osteogenic differentiation of BMSCs by negatively regulating HMGA2. However, there are controversial viewpoints on the effect of HMGA2 on osteogenic differentiation. For example, miR-98-5p prevented osteogenic differentiation through downregulation of HMGA2 expression [12], suggesting that HMGA2 plays a positive role in osteoblastic differentiation. It has also been shown that HMGA2 functions as a transcriptional factor to modulate ataxia-telangiectasia-mutated-and-Rad3-related kinase/checkpoint kinase 1 [13], ten–eleven translocation 1/Homeobox A9 [14], and Notch, TGF β /Smad, and TGF β /ERK pathways [15]. Further, the HMGA2-mediated JNK pathway has been implicated in miR-497-5p-mediated suppression of osteogenic differentiation [16]. Thus, the downstream pathways involved in miR-595/HMGA2-mediated osteogenic differentiation should be explored in future studies.

CONCLUSION

The findings of this study show that miR-595 expression is upregulated during osteogenic differentiation of BMSCs. Inhibition of miR-595 lowers expression of osteogenic genes, reduces amplification of BMSCs, suppresses mineralization of the extracellular matrix, and reduces ALP activity through upregulation of HMGA2. Therefore, miR-595 promotes

osteoporosis and could be targeted to treat osteoporosis.

DECLARATIONS

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. Bingjun Gao designed the study and supervised the data collection. Yarong Wu analyzed and interpreted the data. Lijian Zhou and Xin Chen prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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