Tropical Journal of Pharmaceutical Research December 2021; 20 (12): 2481-2487
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i12.4

Original Research Article

Downregulation of miR-34a represses insulin resistance and mediates glucose metabolism by upregulating IGF2 to activate PI3K/AKT signaling pathway

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Sent for review: 9 August 2021 *Revised accepted: 25 November 2021*

Abstract

Purpose: *To investigate the effects of miR-34a on insulin resistance and glucose metabolism in type 2 diabetes.*

Methods: *Human hepatocarcinoma (HepG2) cells were incubated with palmitic acid (PA) for the establishment of a cell model of insulin resistance. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), while insulin resistance was evaluated by glucose consumption. Expressions of miR-34a and glucose transporter 4 were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR), while western blotting was used to determine protein expressions of glucose transporter 4 and proteins involved in the downstream pathway. Glucose uptake was assessed by flow cytometry whereas the target gene of miR-34a was determined using a luciferase activity assay.*

Results: *PA treatment induced a decrease in cell viability in HepG2 cells, and promoted glucose production and miR-34a expression. Silencing of miR-34a conferred insulin sensitivity on PA-treated HepG2 cells. Palmitic acid treatment also reduced insulin-induced NBDG [2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] uptake in HepG2 cells, while knockdown of miR-34a attenuated a PA-induced decrease of 2-NBDG uptake in insulin-induced HepG2 cells. Knockdown of miR-34a promoted mRNA and protein expression of glucose transporter 4 in PA and insulin-induced HepG2 cells. MiRNA-34a directly bound to the 3´-UTR of insulin-like growth factor 2 (IGF2), and silencing of miR-34a attenuated the PA-induced decrease in IGF2 expression in HepG2 cells. Interference of miR-34a attenuated IGF2 silencing of the induced decrease in IGF2, glucose transporter 4, and AKT phosphorylation in PA-treated HepG2 cells.*

Conclusion: *Downregulation of miR-34a promotes glucose consumption and represses insulin resistance by upregulating IGF2 to activate AKT pathway, thus providing a potential target for the treatment of type 2 diabetes.*

Keywords: *MiR-34a, Insulin-like growth factor 2, Insulin resistance, Glucose metabolism*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Type 2 diabetes mellitus is a major health problem worldwide, and the main pathogenic indicators of type 2 diabetes mellitus are obesity, genetic factors, or reduced glucose tolerance [1]. Insulin resistance associated with abnormal glucose metabolism is the key feature of type 2 diabetes [2]. Amelioration of insulin resistance mediates glucose metabolism in the liver, thus mitigating type 2 diabetes [3].

MicroRNAs are involved in metabolic pathways, metabolic homeostasis, and organismal energy balance [4]. Through regulation of the insulin signaling pathway, miRNAs also play major roles in insulin resistance-associated type 2 diabetes [5]. MiR-34a regulates B-cell lymphopoiesis to protect against type 1 diabetes [6], and miR-34a is up-regulated in patients with type 2 diabetes through regulation of β-cell functionality and insulin resistance [7]. However, the role and mechanism of miR-34a regarding insulin resistance and glucose metabolism have not been reported.

The insulin-like growth factor (IGF) axis, particularly the IGF1 and IGF2 genes, is a candidate for correcting deficiencies in immunoregulation and impaired β cell viability and function [8]. IGFs have been regarded as novel prognostic biomarkers to improve the clinical diagnosis of type 1 diabetes [9]. In patients with type 2 diabetes mellitus, IGF2 levels were found to be up-regulated during liraglutide treatment through regulation of glucose metabolism [10]. MiR-34a has therefore been hypothesized to regulate insulin resistance and glucose metabolism through mediation of IGF2. In this study, the expression levels of miR-34a in palmitic acid (PA)-induced human hepatocarcinoma (HepG2) cells were first determined, and the effects of miR-34a on insulin resistance and glucose metabolism in PA-treated HepG2 cells were also evaluated. The miR-34a pathway and identification of downstream target genes may provide a potential therapeutic strategy for management of type 2 diabetes.

METHODS

Cell culture, treatment, and transfection

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco BRL) in a 37°C incubator. Cultured HepG2 cells at 85% confluency were incubated with 0, 0.1, 0.25, 0.5, or 1 mM PA (Sigma-Aldrich, St. Louis, MO, USA) for 16 h. For cell transfection, HepG2 cells under 0.5 mM PA treatment were transfected with siRNA targeting IGF2 (200 nM; RiboBio, Guangzhou, China), antagomiR-NC, or antagomiR-34a (100 nM; RiboBio), or co-transfected with siIGF2 and antagomiR-34a with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Cell viability

HepG2 cells were plated on 96-well plates and incubated with 0, 0.1, 0.25, 0.5 or 1 mM PA (Sigma-Aldrich) for 16 h. Then, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) solution (0.5 mg/mL, Sigma-Aldrich) was added to each well, and incubated another 3 h. Lysis buffer (10% SDS in 0.01 M HCl) was added to each well to dissolve the formazan crystals, and the absorbance at 570 nm was measured using a spectrophotometer (Thermo Fisher Scientific).

Glucose production

HepG2 cells in the presence of different concentrations of PA were cultured in 6-well plates for 16 h, and then washed with phosphate-buffered saline (Sigma-Aldrich) before incubation in phenol red, glucose-free DMEM with 20 mM sodium lactate, and 2 mM sodium pyruvate for 5 h. Insulin (100 nM, Sigma-Aldrich) was added to the wells at 4 h after treatment with glucose-free DMEM. The glucose content was determined using a glucose assay kit (BioVision, Milpitas, CA, USA).

Glucose consumption

HepG2 cells with or without antagomiR-NC or antagomiR-34a transfection were cultured in 96-well plates for 24 h, and then exposed to DMEM containing 0.5 mM PA and 4.5 g/L glucose for 16 h. The cells were then incubated with or without 100 nM insulin for another 1 h before determination of glucose content in the cultured medium using an assay kit (BioVision). Glucose consumption was determined as the glucose concentration between blank wells and test wells.

Glucose uptake

HepG2 cells with or without antagomiR-NC or antagomiR-34a transfection were cultured in 12-well plates and then exposed to DMEM medium containing 0.5 mM PA for 16 h. The medium was exchanged to glucose-free DMEM containing 100 nM insulin, and the cells were cultured in the medium for 10 min before incubation with 60 mM 2-NBDG (a fluorescent D-glucose analogue;

Sigma-Aldrich) for 1 h. The fluorescence was then determined using FACScalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Dual luciferase reporter assay

The 3´-untranslated (3'-UTR) region of IGF2 that contains the predicted miR-34a binding site was cloned into the pmirGLO vector (Promega, Madison, WI, USA) and named pmirGLO-IGF2-wt. The mutated sequence of the IGF2 3´-UTR region that no longer has the predicted miR-34a binding site was also cloned into the pmirGLO vector (Promega) and named pmirGLO-IGF2-mut. The HepG2 cells were co-transfected with pmirGLO-IGF2-wt or pmirGLO-IGF2-mut and the NC mimic or miR-34a mimic using Lipofectamine 2000. A dual luciferase reporter assay system (Promega) was used to determine the luciferase activity.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs or the miRNAs were isolated from treated cells using TRIzol (Thermo Fisher Scientific) or an miRcute miRNA isolation kit (Tiangen, Beijing, China), respectively. The RNAs were then reverse-transcribed into cDNAs using a reverse transcription reagent kit (TaKaRa, Dalian, China). The expressions of miR-34a, IGF2, and glucose transporter 4 were detected using SYBR Green Master (TaKaRa) with U6 or GAPDH as the endogenous controls. The primer sequences used are shown in Table 1.

Western blotting

Proteins were extracted from treated cells using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). Protein concentrations were determined using an acid protein kit (Thermo Fisher Scientific) and then separated using SDS-PAGE. Following electroblotting onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) and blocking with 5% bovine serum albumin, the membranes were incubated overnight with the following primary antibodies: anti-IGF2 (1:2,000, Cell Signaling Technology, Beverly, MA, USA), anti-glucose transporter 4 (1:2,500, Cell Signaling Technology), anti-AKT and anti-p-AKT (1:3,000, Cell Signaling Technology), and anti-β-actin (1:3,500, Cell Signaling Technology). Horseradish peroxidase-labeled secondary antibody (1:5,000; Cell Signaling Technology) was used to treat the membranes as a secondary antibody, and enhanced chemiluminescence (KeyGen, Nanjin, China) was used to visualize the immunoreactivities of the bands in membranes.

Statistical analysis

Data are expressed as the mean ± standard error of the mean, and statistical analysis was conducted using one-way analysis of variance or Student's *t*-test, using Prism software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Upregulation of miR-34a in PA-treated HepG2 cells

To establish a type 2 diabetes-like cellular model, HepG2 cells were incubated with PA. The MTT assay showed that PA treatment decreased the cell viability of HepG2 cells in a dose-dependent manner (Figure 1 A). Glucose production in HepG2 cells post-insulin treatment (100 nM) was reduced when compared to the control (Figure 1 B). Insulin resistance was induced in HepG2 through increased glucose production induced by PA treatment, even in the presence of 100 nM insulin (Figure 1 B). MiR-34a expression was up-regulated in HepG2 cells post-PA induction in a dose-dependent manner (Figure 1C), suggesting that miR-34a might participate in the regulation of type 2 diabetes.

Table 1: Primers used in PCR

ID	Sequence (5´- 3´)
GAPDH F	TGCACCACCAACTGCTTAGC
GAPDH R	GGCATGGACTGTGGTCATGAG
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
IGF2 F	GTGGGTGTGGTTAAGCTGCAA
IGF2 R	GTCCGAACAGACAAACTGAA
glucose transporter 4 F	ACATACCTGACAGGGCAAGG
glucose transporter 4 R	CGCCCTTAGTTGGTCAGAAG
miR-34a F	CCCGTTGGCAGTGTCTTAGCT
miR-34a R	GTGCAGGGTCCGAGGT

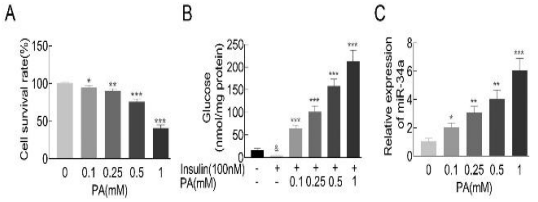

Figure 1: Up-regulation of miR-34a in palmitic acid (PA) treated human hepatocarcinoma (HepG2) cells. (A) PA treatment decreased the cell viability of HepG2 cells in a dose-dependent manner. (B) PA treatment increased glucose production in HepG2 cells post 100 nM insulin induction in a dose-dependent manner. (C) PA treatment increased expression of miR-34a in HepG2 cells in a dose-dependent manner. $^{*}P < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Knockdown of miR-34a conferred insulin sensitivity on PA-treated HepG2 cells

To investigate the role of miR-34a in type 2 diabetes, HepG2 cells were transfected with antagomiR-34a and then incubated with 0.5 mM PA. The PA-induced increase in miR-34a expression in HepG2 cells was down-regulated by antagomiR-34a (Figure 2 A). Palmitic acid induced insulin resistance in HepG2 cells by down-regulation of glucose consumption was induced by insulin treatment (Figure 2 B). Knockdown of miR-34a ameliorated insulin resistance in PA-treated HepG2 cells by up-regulation of glucose consumption (Figure 2 B), showing that knockdown of miR-34a conferred insulin sensitivity in PA-treated HepG2 cells.

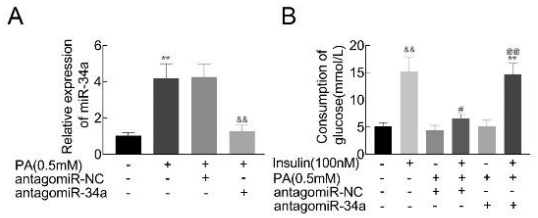

Figure 2: Knockdown of miR-34a conferred insulin sensitivity in palmitic acid (PA) treated human hepatocarcinoma (HepG2) cells. (A) The PA-induced increase of miR-34a expression in HepG2 was down-regulated by transfection with antagomiR-34a. (B) The PA-induced reduction in glucose consumption in HepG2 cells was induced by insulin, and knockdown of miR-34a decreased insulin resistance in PA-treated HepG2 cells through an increase in glucose consumption. $^{\#}P < 0.05$; ** vs $^{\&\&}p < 0.01$

Knockdown of miR-34a promoted glucose metabolism in PA-treated HepG2 cells

To assess the role of miR-34a on glucose metabolism, glucose uptake using analysis of 2-NBDG was determined by flow cytometry. PA treatment decreased 2-NBDG fluorescence in HepG2 cells under insulin induction (Figure 3A), while knockdown of miR-34a increased the fluorescence (Figure 3 A), indicating that knockdown of miR-34a induced glucose uptake in PA-treated HepG2 cells. Moreover, mRNA (Figure 3 B) and protein levels (Figure 3 C) of glucose transporter 4 were up-regulated by transfection with antagomiR-34a in HepG2 cells, with or without insulin treatment.

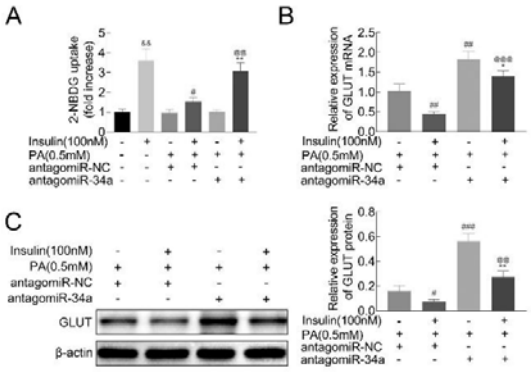

Figure 3: Knockdown of miR-34a promoted glucose metabolism in palmitic acid (PA)-treated human hepatocarcinoma (HepG2) cells. (A) Insulin treatment increased 2-NBDG fluorescence in HepG2 cells, while PA treatment decreased fluorescence. Knockdown of miR-34a decreased the insulin-induced increase of 2-NBDG fluorescence. (B) The mRNA of glucose transporter 4 was up-regulated by transfection with antagomiR-34a in PA-treated HepG2 cells with or without insulin treatment. (C) The protein level of glucose transporter 4 was up-regulated by transfection with antagomiR-34a in PA-treated HepG2, with or without insulin treatment. $^{*\#}P < 0.05$, $^{**,\ \&\&}$ $^{@@}p < 0.01$; $^{@@@}p < 0.001$

MiR-34a bound to IGF2

IGF2 was predicted as a target gene of miR-34a (Figure 4 A). Transfection with the miR-34a mimic reduced the luciferase activity of pmirGLO-IGF2-wt (Figure 4B), while it showed no significant effect on pmirGLO-IGF2-mut (Figure 4 B). Palmitic acid treatment decreased mRNA (Figure 4 C) and protein (Figure 4D) expression of IGF2 in HepG2 cells, while knockdown of miR-34a decreased the PA-induced decrease in IGF2 (Figure 4 C and D), showing that miR-34a directly bound to *IGF2* and negatively regulated the expression of IGF2 in HepG2 cells.

Knockdown of miR-34a promoted activation of the AKT pathway by up-regulation of IGF2

To identify the downstream pathway in miR-34a-mediated insulin resistance and glucose metabolism, western blot analysis was

performed, which showed that PA treatment induced down-regulation of AKT phosphorylation in HepG2 cells (Figure 5), while knockdown promoted phosphorylation of AKT in PA-treated HepG2 cells (Figure 5). Silencing of IGF2 decreased protein expressions of IGF2 and glucose transporter 4 and decreased AKT phosphorylation in PA-treated HepG2 cells (Figure 5). However, knockdown of miR-34a decreased IGF2 silencing-induced decreased IGF2, glucose transporter 4, and AKT phosphorylation in PA-treated HepG2 cells (Figure 5). Together, these results indicated that knockdown of miR-34a promoted activation of the AKT pathway by up-regulation of IGF2 in PA-treated HepG2 cells.

DISCUSSION

MiRNAs modulate insulin secretion, insulin signaling pathways, glucose transport, and participate in the pathogenesis of insulin resistance, glucose metabolism, and type 2 diabetes [5]. Therefore, miRNAs are regarded as promising diagnostic, prognostic, and therapeutic targets for the treatment of type 2 diabetes [6]. Because miR-34a has been shown to protect against type 1 diabetes [6] and was up-regulated in patients with type 2 diabetes [7], miR-34a might regulate insulin resistance and glucose metabolism.

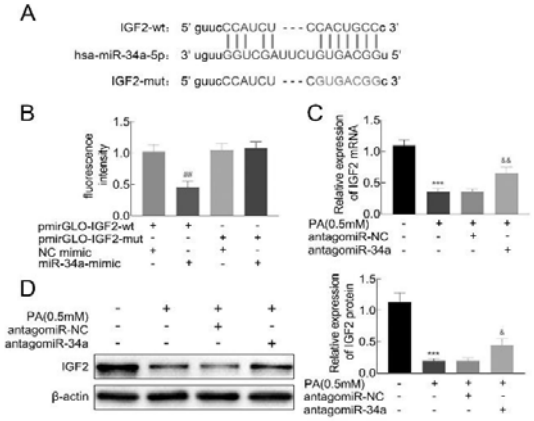

Figure 4: MiR-34a bound to *IGF2*. (A) The potential binding site between miR-34a and IGF2. (B) Transfection with the miR-34a mimic reduced the luciferase activity of pmirGLO-IGF2-wt, while it had no significant effect on pmirGLO-IGF2-mut. (C) Palmitic acid (PA) treatment decreased the mRNA expression of IGF2 in HepG2 cells, while knockdown of miR-34a decreased the PA-induced decrease of IGF2. (D) PA treatment decreased the protein expression of IGF2 in HepG2 cells, while knockdown of miR-34a decreased the PA-induced decrease of IGF2. $^{\&}P < 0.05$, $^{\#\#,\&\&}p < 0.01$, $^{***}p < 0.001$

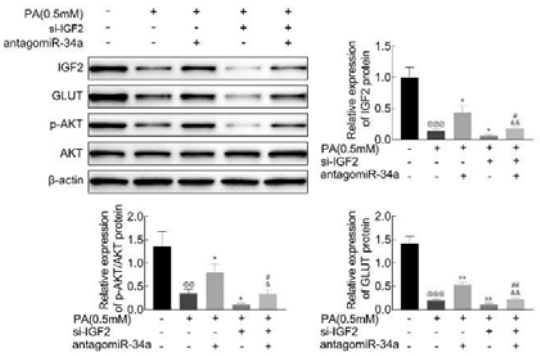

Figure 5: Knockdown of miR-34a promoted activation of the AKT pathway by up-regulation of IGF2. Palmitic acid (PA) treatment induced down-regulation of AKT phosphorylation in HepG2 cells, and silencing of IGF2 decreased protein expression of IGF2 and glucose transporter 4 and AKT phosphorylation in PA-treated HepG2 cells. Knockdown of miR-34a attenuated IGF2 silencing-induced decreases of IGF2, glucose transporter 4, and AKT phosphorylation in PA-treated HepG2 cells. $^{*,\&,\#}P < 0.05$; $^{**,\&\&,\#\#,@@}p < 0.01$; $^{@@@}p < 0.001$

PA, a common saturated free fatty acid, is widely used to induce insulin resistance in HepG2 cells, which is regarded as a type 2 diabetes-like cellular model [11]. Suppression of glycogen synthesis and promotion of gluconeogenesis induced by PA reduces insulin-dependent glucose uptake [11]. The results of the present study showed that PA treatment induced decreased cell viability in HepG2 cells and increased glucose production in HepG2 cells post insulin induction. Moreover, insulin-induced up-regulation of glucose uptake was repressed by PA treatment, suggesting the successful establishment of type 2 diabetes-like cellular model. A previous study showed that miR-34a was up-regulated in patients with type 2 diabetes [7]. A significant up-regulation of miR-34a was confirmed in PA-treated HepG2 cells in this study. Antagonism of miR-34a attenuated ceramide accumulation-induced loss of insulin sensitivity by modulation of glucose transporter 4 localization in aging skeletal muscle [12]. The results of the present study showed that knockdown of miR-34a conferred insulin sensitivity in PA-treated HepG2 cells and decreased glucose uptake by up-regulation of glucose transporter 4. Therefore, knockdown of miR-34a might ameliorate development of type 2 diabetes through mitigation of insulin resistance and modulation of glucose metabolism.

With the ability to regulate the mass and function of β-cells, IGF2 has been implicated in the pathogenesis of insulin resistance [13]. Moreover, IGF2 regulates bone growth by modulation of glucose metabolism in

chondrocytes [14]. Up-regulation of muscle glucose uptake driven by IGF2 has been shown to be related to the hypoglycemic effect in type 2 diabetes [15]. The results of the present study showed that miR-34a bound to the 3´-UTR of IGF2, and knockdown of miR-34a decreased the PA-induced decrease of IGF2 in HepG2 cells. Therefore, miR-34a might regulate insulin resistance and glucose metabolism by targeting IGF2. However, the role of IGF2 in insulin resistance and glucose metabolism in PA-treated HepG2 needs to be further investigated.

During normal insulin signaling, binding to insulin induces dimerization and autophosphorylation of insulin receptors, thus recruiting PI3K to trigger activation of AKT and glucose transporter 4 translocation, which increases glucose metabolism [17]. However, ceramides, the lipotoxic metabolites of free fatty acid-impaired insulin signaling, suppress the activation of AKT [17], and activation of PI3K/AKT alleviates insulin resistance [17]. Moreover, knockdown of miR-34a alleviates ceramide accumulation in aging skeletal muscle [12]. The results of the present study indicated that knockdown of miR-34a attenuated PA-induced decreases in glucose transporter 4 and p-AKT in HepG2 cells, suggesting that down-regulation of miR-34a repressed insulin resistance and mediated glucose metabolism by activation of the PI3K/AKT signaling pathway. Moreover, IGF2 has been shown to promote phosphorylation of AKT [18]. Silencing of IGF2 in the present study aggravated the PA-induced decrease of glucose transporter 4 and p-AKT in HepG2 cells, and knockdown of miR-34a attenuated IGF2 silencing-induced decreases of glucose transporter 4 and p-AKT in PA-treated HepG2 cells, suggesting that miR-34a might participate in the progression of type 2 diabetes through regulation of the IGF2/AKT pathway.

CONCLUSION

The findings of this study demonstrate the protective effect of miR-34a silencing in a PA-treated HepG2 cell model. The results could identify a target for the treatment of type 2 diabetes. However, the *in vivo* role of miR-34a during insulin resistance and glucose metabolism in diabetic mice needs to be further investigated.

DECLARATIONS

Acknowledgement

This work was supported by the Natural Science Foundation of Xinjiang Uygur Autonomous Region (Grant no. 2020D01A20).

Competing interests

There are no conflicts of interest to disclose with regard to this work.

Availability of data and materials

All data generated or analyzed during this study are included in this report.

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. Bohuan Duan and Yanjun Wang designed the study and supervised the data collection. Jiwen Liao analyzed and interpreted the data. Aizatiguli·Kadeer, Jia Wang, and Palidan·Wubuer prepared the manuscript for publication and reviewed the manuscript draft. All authors read and approved the final manuscript.

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