The aim of the present study was to investigate the effect of the histone H3K9 demethylase inhibitor, IOX1, on the mechanism of hepatic fibrosis in TGF-β-induced human hepatic stellate LX-2 cells. Cellular proliferation, apoptosis, histone H3K9 dimethylation (H3K9me2), protein expression of extracellular matrix (ECM)-related proteins α-smooth muscle actin (SMA), type I collagen (Col I), MMP-1 and TIMP-1 were measured. H3K9me2 levels in the promoter region of ECM-related genes were detected by real-time cell analysis (RTCA), flow cytometry, western blotting and chromatin immunoprecipitation (ChIP) in LX-2 cells. IOX1 significantly inhibited cell proliferation and the IC_{50} of IOX1 was 100 µM in cells treated with IOX1 for 48 h. IOX1 significantly induced apoptosis in LX-2 cells in a concentration-dependent manner. In addition, different concentration of IOX1 increased the level of H3K9me2 and downregulated the expression of α-SMA, Col I, MMP-1 and TIMP-1 in TGF-β-induced LX-2 cells. ChIP measurements indicated that H3K9me2 levels in the promoter region of the corresponding genes were increased in TGF-β-induced LX-2 cells. IOX1 may elevate H3K9me2 in the promoter region of Col I, MMP-1, and TIMP-1 genes to regulate α-SMA, Col I, MMP-1 and TIMP-1 protein expression to induce cell apoptosis, inhibit LX-2 cell proliferation and oppose hepatic fibrotic activity.

Introduction

Lysine (K) methylation of histone (H) is a type of epigenetic modification. There are numerous sites on the lysine residue of histone 3 (H3) that may be methylated, including H3K4, H3K9 and H3K27. Methylation of histones have multiple effects on gene expression. For example, H3K4 dimethylation (H3K4me2) may trigger gene transcription; however, H3K9 dimethylation (H3K9me2) will inhibit gene transcription (1-3). Histone methylation status is controlled by a balance of histone lysine methyltransferases (HKMTs) and histone lysine demethylases (HKDMs).

Proliferation and activation of hepatic stellate cells (HSC), and balancing extracellular matrix (ECM) synthesis and metabolism have become research hotspots of hepatic fibrosis treatment (4,5). There have been several reports that modification of aberrant histone methylation status may inhibit the proliferation and activation of HSCs to prevent the development of hepatic fibrosis (6,7). Yang et al (6) found that the histone H3K27 methyltransferase inhibitor, DZNep, displays anti-hepatic fibrosis activity through alteration of the aberrant H3K27 methyl group in HSCs. Dong et al (7) demonstrated that knockout of lysine demethylase 4D (KDM4D) protein, a demethylase of H3K9 and H3K36, increased methylation levels of H3K9 and H3K36 in HSCs to decrease α-smooth muscle actin (SMA) and Col I expression, thereby inhibiting the proliferation and activation of HSC. The results of these studies indicated that reduction of histone methylation in activated HSCs by corresponding inhibitors or siRNA methods may inhibit HSC proliferation and activation. It has been demonstrated that the H3K9 methylation level was decreased during HSC activation (7); however, it is unknown whether modification of H3K9 methylation by histone H3K9 demethylase inhibitors affects HSC proliferation, activation and ECM synthesis, and metabolism in the activated HSCs.

Although numerous histone demethylase inhibitors have been developed, including GSK-J1 for H3K27, and CP2 for H3K9 and H3K36 (8,9), there are still no inhibitors of H3K9 demethylase available. 5-carboxy-8-hydroxyquinoline (IOX1)
is a potent 8-hydroxyquinoline-based histone demethylase inhibitor that is capable of chelation with Fe (II) in histone H3K9 demethylase active center to impede single electron transfer to block hydroxyl methylation, resulting in an increase of H3K9 methylation in cells (10). So far, few studies have reported the application of IOX1 to prevent and treat disease. Hu et al (11) have reported that following pre-treatment with IOX1, the percentage of cells in the G0/G1 phase was increased and the percentage of cells in the S phase was decreased in cells, indicating that IOX1 significantly inhibited the proliferation of vascular smooth muscle cells by slowing down the progression of the cell cycle from the G0/G1 to the S phase. IOX1 stimulation on angiotensin II (Ang II)-pretreated vascular smooth muscle cells resulted in the elevation of H3K9 methylation enrichment in the cyclin D1 gene promoter region, therefore inhibiting cyclin D1 gene expression. Since cyclin D1 is involved in cell proliferation, downregulation of cyclin D1 will lead to cellular cycle arrest at the G0/G1 phase. Therefore, it was hypothesized that IOX1 has anti-atherosclerotic activity based on the findings that IOX1 decreases proliferation of vascular smooth muscle cells via inhibition of cyclin D1 expression.

Based on these findings, the present study measured the activity of IOX1 on cellular proliferation, apoptosis and production of α-SMA, Col I, and its ECM metabolism-related enzymes, effects of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases 1 (TIMP-1) protein expression via H3K9me2 modification in HSC cells. In addition, chromatin immunoprecipitation (ChIP) was performed to investigate the effects of IOX1 on H3K9me2 methylation of promoters and downregulation of α-SMA, Col I, MMP-1 and TIMP-1 gene in HSC-LX-2 cells.

Materials and methods

Cell line and materials. The LX-2 cell line was purchased from the Cell Bank of Culture of the Chinese Academy of Sciences. IOX1 and dimethyl sulfoxide were obtained from MilliporeSigma. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc.). Trypsin and rainbow marker were purchased from Beijing Solabao Information Technology Co., Ltd. Cell lysis buffer, protease inhibitors and bicinchoninic acid assay (BCA) protein quantification kit were from Shanghai Biyuntian Sciences. IOX1 and dimethyl sulfoxide were obtained from MilliporeSigma. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc.). Trypsin and rainbow marker were purchased from Beijing Solabao Information Technology Co., Ltd. Cell lysis buffer, protease inhibitors and bicinchoninic acid assay (BCA) protein quantification kit were from Shanghai Biyuntian Sciences. IOX1 and dimethyl sulfoxide were obtained from MilliporeSigma. Dulbecco's modified Eagle's medium (DMEM), according to the manufacturer's instructions. LX-2 cells in a 10-cm culture dish were fixed in 3 ml

Cell culture and treatment. LX-2 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were pretreated with 5 ng/ml TGF-β (PeproTech China) for 48 h, and then the cells were harvested for subsequent experiments.

Cellular proliferation assay. The effect of different IOX1 concentrations on LX-2 cell proliferation was detected by Real-time Cell Analysis (ACEA Biosciences, Inc.). The RTCA

ChIP. ChIP assay was performed using a ChIP kit (MilliporeSigma), according to the manufacturer's instructions. LX-2 cells in a 10-cm culture dish were fixed in 3 ml

Morphological alteration of the LX-2 cells observed by inverted microscopy. LX-2 cells in the logarithmic growth phase were randomly divided into the control group (not treated with TGF-β) and IOX1 treatment groups (0, 100, 200 and 300 µM, respectively). After 48 h, the morphological changes of the IOX1-treated cells were observed under a conventional light microscope (×100).

Flow cytometry. LX-2 cells (1x10⁴ in an uncoated 10-cm culture dish) were treated with 0, 100, 200 and 300 µM IOX1 at 37°C, respectively. Untreated cells were used as negative controls. After 48 h, the cells were collected by trypsin digestion without EDTA. A total of 1x10⁶ cells in each condition were prepared as a suspension in 200 µl Annexin-VFITC binding solution and propidium iodide staining solution. The cellular suspension was kept on ice, away from light for 20 min and was then analyzed using a flow cytometer.

Western blotting. LX-2 cells were collected, lysed with lysis buffer, kept on ice for 10 min, sonicated and centrifuged at 2,000 x g for 20 min at 4°C. The protein concentration of the supernatant was assayed using a BCA protein quantification kit. A total of 40 μg the sample was loaded onto an SDS-PAGE gel (10%), electrophoresed, transferred onto a PVDF membrane, blocked with 5% skimmed milk in TBS-0.1% Tween-20 buffer (TBST) at room temperature for 90 min, and incubated with the primary antibodies at 4°C overnight: Anti-α-SMA (cat. no. 5694; 1:1,500), Coll (cat. no. 34710; 1:1,500), MMP-1 (cat. no. 134184; 1:1,500), TIMP-1 (cat. no. 219226; 1:1,500), anti-H3K9 (cat. no. 1220; 1:500), anti-β-actin (cat. no. 8226; 1:1,500), TIMP-1 (cat. no. 1791; 1:1,000; all from Abcam). The chromatin immunoprecipitation (ChIP) kit was purchased from MilliporeSigma (cat. no. 17-371).

Cell membrane treatment. LX-2 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were pretreated with 5 ng/ml TGF-β (PeproTech China) for 48 h, and then the cells were harvested for subsequent experiments.

Cellular proliferation assay. The effect of different IOX1 concentrations on LX-2 cell proliferation was detected by Real-time Cell Analysis (ACEA Biosciences, Inc.). The RTCA cellular functional analysis system detects the electrical impedance of adherent cells through the electrode at the bottom of the EPlate, which reflects the number, viability, morphology and adherence of the cells by using the cell index (CI) as an index. Cells in the logarithmic growth phase were seeded at a density of 5x10⁵ cells per well. Following a 10 h synchronization with DMEM (Gibco; Thermo Fisher Scientific, Inc.), the cells were treated with DMSO (MilliporeSigma) or IOX1 at a final concentration of 50, 100, 200 and 400 µM at 37°C, respectively. Untreated cells were used as controls. The effect of IOX1 on LX-2 cell proliferation was observed and recorded once every 15 min for 72 h. Each assay condition was performed in triplicate. RTCA software (xCELLigence DP System; Agilent Technologies, Inc.) calculated corresponding CI values. The concentration of IOX1 administered in the subsequent experiments was determined based on the results of the RTCA experiment.
4% formaldehyde at room temperature for 10 min, collected in a tube, washed twice with PBS, suspended in 1 ml PBS containing 5 μl protease inhibitors, and sonicated to break the DNA at 200-1,000 bp (the DNA size was checked by agarose gel electrophoresis). Subsequently, antibodies (anti-H3K9me2) were added and the mixtures were shaken mildly overnight at 4°C, followed by addition of 60 μl Protein G Agarose, washing with ChIP buffer, addition of 8 μl 5M NaCl, mixing and cross-linking overnight at 65°C. The DNA of the IP sample was isolated. ChIP-enriched samples were analyzed in triplicate by quantitative PCR (qPCR) using promoter primers of α-SMA, Col I, MMP-1 and TIMP-1. The primer sequences were as follows: Col I forward, 5'-GGCGAGAGTGTAACAA GG-3' and reverse, 5'-GCCAAGGTCTCCAGGACAC-3'; α-SMA forward, 5'-CCGAATGCAGAAGGAGATCA-3' and reverse, 5'-GGGAGAGAGGGCCAGAT-3'; TIMP-1 forward, 5'-TGCAAGATGGACTTGCAC-3' and reverse, 5'-GATTCCAAGGACAGACAG-3'; and MMP-1 forward, 5'‑GAGTCCTAGTTGTTGCTCAG-3' and reverse, 5'-TCAATGCGATTCACAC-3'. qPCR was performed using SYBR® Green mix from Thermo Fisher Scientific, Inc. The reaction conditions included an initial pre-denaturation step at 94°C for 10 min followed by 50 cycles at 94°C for 20 sec and 60°C for 1 min. Data were analyzed using the 2^(-ΔΔCt) method and normalized to input samples (12). ΔCq (normalized ChIP)=Ct(ChIP)−[Ct(Input)-6.64].

Statistical analysis. All data were analyzed using SPSS statistical software (version 20.0; IBM Corp.) and expressed as the mean ± standard deviation. One-way analysis of variance was used for comparison between groups. Half maximal inhibitory concentrations (IC_{50}) were automatically calculated using the xCELLigence system of RTCA. The least significant difference test was used for comparison between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity of IOX1 on LX-2 cells. The cytotoxicity of IOX1 on LX-2 cells was analyzed using RTCA. LX-2 cells were treated with different concentrations of IOX1. Live cells were counted by RTCA for 72 h. Compared with the normal control group, cytotoxic effects of IOX1 were observed after 20 h, which was more notable in cells treated with higher concentrations of IOX1.

A cellular viability curve of LX-2 cells and the IOX1 concentrations. (A) LX-2 cells were treated with 0 (red), 50 (green), 100 (dark blue), 200 (pink) and 400 (light blue) μM IOX1 for 72 h. (B) The IC_{50} curve of IOX1 on the LX-2 cells at 48 h. RTCA, real-time cell analysis; IC_{50}, half maximal inhibitory concentrations.

Figure 1. Cytotoxicity of IOX1 on the proliferation of the LX-2 cells by RTCA. (A) LX-2 cells were treated with 0 (red), 50 (green), 100 (dark blue), 200 (pink) and 400 (light blue) μM IOX1 for 72 h. (B) The IC_{50} curve of IOX1 on the LX-2 cells at 48 h. RTCA, real-time cell analysis; IC_{50}, half maximal inhibitory concentrations.

IOX1 treatment induces alterations in LX-2 cellular morphology at 48 h. In parallel with increasing concentrations and prolonged treatment duration of IOX1, LX-2 cells became brighter and some were necrosed, demonstrating a concentration- and time-dependent cytotoxicity (Fig. 2).

IOX1 treatment induces apoptosis of the LX-2 cells. Using flow cytometry, cellular apoptosis of LX-2 cells treated with different concentrations of IOX1 was observed (Fig. 3). The apoptotic rate of cells treated with 100, 200 or 300 μM IOX1 was significantly higher than that in the control group (P<0.05).

IOX1 treatment increases H3K9me2, but decreases α-SMA, Col I, MMP-1 and TIMP-1 protein levels in TGF-β-induced LX-2 cells. To inspect the anti-fibrotic activity and mechanism of IOX1, H3K9me2 and protein expression of fibrosis-related factors (α-SMA, Col I, MMP-1 and TIMP1) were measured by western blotting in a cellular fibrosis model and in IOX1 treated cells. Compared with controls, H3K9me2 levels were significantly increased in the 100, 200 or 300 μM IOX1-treated groups. By contrast, protein expression of α-SMA, Col I, MMP-1 and TIMP1 were significantly decreased in the TGF-β-induced LX-2 cells treated with 300 μM IOX1 compared with controls (P<0.05). The levels of Col I, MMP-1 and TIMP1 were decreased in cells treated with 100-200 μM IOX1 compared with controls (P<0.05; Fig. 4).

IOX1 induces H3K9 methylation at promotor regions of fibrotic factors. To investigate the molecular mechanism of the anti-fibrotic activity of IOX1, ChIP was performed to observe whether IOX1 treatment may increase H3K9me2 levels at the promotor region of the fibrotic factors (α-SMA, Col I, MMP-1 and TIMP1). Cell lysates of LX-2 cells treated with 200 μM IOX1 for 48 h was immunoprecipitated by H3K9me2 antibody.
Total RNA of the immunoprecipitates was then extracted, reversed transcribed into cDNA, and then underwent qPCR with corresponding promotor region primers of α-SMA, Col I, MMP-1 or TIMP1, respectively. Compared with controls, H3K9me2 levels at promotor regions of MMP-1, TIMP-1 and Col I were significantly increased in IOX1-treated cells (P<0.05). However, the H3K9me2 level at the promotor region of α-SMA were not significantly different from the control group (P>0.05; Fig. 5). Combined with our previous findings in Fig. 4, this suggested that IOX1 increases H3K9me2 at
the promotor regions of MMP-1, TIMP-1 and Col I, and may attenuate cellular fibrosis of LX-2 induced by TGF-β.

**Discussion**

HSCs located in the space of Disse between hepatocytes and sinusoidal endothelial cells, the principal function of which is to store and metabolize vitamin A in its physiological state. Therefore, they are referred to as lipid storage cells. Under pathological conditions, HSCs lose their lipid phenotype and begin to synthesize ECM. The synthetic speed of ECM is more than that of the degradation, resulting in a large deposition of ECM in the cells. This process is known as activation of the HSC. Therefore, activation of HSC is a key process during the development of hepatic fibrosis (13,14).

TGF-β is currently known as the strongest cytokine that promotes hepatic fibrosis, which activates HSC through the TGF-β-Smad signaling pathway to increase ECM synthesis in HSCs and alters ECM metabolism, thereby promoting liver fibrosis development (15,16). In the present study, the human activated HSC LX-2 cell line was used as the research object. To mimic the activation of HSC, LX-2 cells were stimulated with TGF-β. With this cellular model, the effect of IOX1 on H3K9 methylation and ECM metabolism was investigated. Histone demethylase inhibitors include IOX1, JIB-04, GSK and ML324. Among these, GSK specifically inhibits the activity of KDM6, ML324 specifically inhibits the activity of KDM4, while IOX1 and JIB-04 may inhibit the activity of KDM3 in addition to the activity of KDM4 and KDM6. The only substrate of KDM3 is H3K9me, and KDM3 may demethylate H3K9me. In the present study, IOX1 was selected as an inhibitor of KDM3 as IOX1 treatment may inhibit the proliferation of cardiomyocytes in angiotensin-pretreated cardiomyocytes and IOX1 may inhibit cell cycle-associated proteins through upregulation of H3K9me levels (11).

A 48-h intervention with IOX1 demonstrated that the IC50 of IOX1 on the LX-2 cells was 100 µM. These data suggested that the IC50 of IOX1 on the LX-2 cells was 100 µM. These data suggested that the IC50 of IOX1 is higher than that of other histone demethylase inhibitors. The IC50 of the H3K27 demethylase inhibitor, GSK, and the ML324 inhibitor, JMJD2, are 9.9 nM and 0.2 µM, respectively (17). We hypothesized that IOX1 contains a hydrophilic hydroxyl group (18), which may have a different polarity to the other histone demethylase inhibitors, resulting in lower cellular permeability and thus a relatively large IC50. Based on these data, 100, 200, 300 µM IOX1 were selected as the conditions for the subsequent experiments. Further observations revealed that at 100 µM or more IOX1 may significantly improve H3K9me levels in LX-2 cells. The level of H3K9me was significantly increased with increasing doses of IOX1, suggesting that 100 µM or more IOX1 may upregulate the H3K9 dimethyl group through inhibition of the activity or expression of H3K9 demethylase.

Cellular apoptosis analysis by flow cytometry demonstrated that IOX1 treatment over 100 µM for 48 h significantly promoted cellular apoptosis of the TGF-β-induced LX-2 cells and enhanced
the apoptotic rate of the cells in a dose-dependent manner. This finding suggested that IOX1 induced cellular morphological alterations and that death of the LX-2 cells may occur through an apoptotic mechanism. Indeed, a large number of studies have confirmed that decreasing activated HSCs or promoting the apoptosis of the activated HSCs may effectively restrain the development of liver fibrosis (19). It has been demonstrated that drugs that promote HSC apoptosis reveal anti-fibrosis activity (20). Liu et al. (20) demonstrated that tanshinone extracted from Salvia miltiorrhiza Bunge revealed a potential anti-fibrotic activity through regulation of the ERK-Bax-Caspase pathway to promote HSC apoptosis. Therefore, based on these results and those of the present study, we hypothesized that IOX1 may exert anti-hepatic fibrosis via promoting the apoptosis of HSC cells through the regulation of H3K9 methylation.

Morphological and behavioral alterations (including cellular mobility and contractility) of HSCs during fibrosis recruit cytoskeletal proteins, including α-SMA, which serve an important role in this morphological change (21,22). Therefore, the expression of α-SMA is considered a marker of HSC activation (23,24). In addition, activated HSCs express a large number of ECMs, the most abundant being Col I (25,26). Therefore, repression of HSC activation through a decrease in ECM synthesis, particularly Col I, is an effective strategy for treating liver fibrosis. In the present study, western blot analysis indicated that IOX1 above 100 µM significantly downregulated the expression of Col I in LX-2 cells, but 100-200 µM IOX1 did not alter α-SMA levels. However, 300 µM IOX1 significantly decreased α-SMA protein expression, suggesting that IOX1 may inhibit the synthesis of Col I and α-SMA. Further analysis using ChIP assays demonstrated that H3K9me2 expression in the promoter region of the Col I was significantly increased in LX-2 cells treated with 200 µM IOX1 for 48 h. This indicated that IOX1 may decrease Col I gene expression via elevation of the H3K9me2 level at promoter region. However, no notable alteration of H3K9me2 was observed in the α-SMA promoter region in this experiment. Perugorria et al. (27) reported that the expression of α-SMA gene in activated HSCs was controlled by H3K4me3. Therefore, we hypothesized that IOX1 has no direct regulatory effect on the expression of α-SMA gene.

The increase in ECM accumulation during HSC activation is not the only factor in ECM deposition. Indeed, ECM degradation serves a more critical role in the development of liver fibrosis (28,29). MMP-1 is a metalloproteinase that degrades collagen-based ECM, whose activity is inhibited by TIMP-1 (30,31). During hepatic fibrosis, the expression of MMP-1 is downregulated, resulting in decreased ECM degradation. An increase in TIMP-1 expression inhibits the activity of MMP-1, leading to an imbalance of ECM synthesis and metabolism, which ultimately leads to the development of hepatic fibrosis (32). Therefore, a correction of this imbalance between MMP-1 and TIMP-1 to promote degradation of ECM may effectively reverse the occurrence and development of hepatic fibrosis. The results of the present study demonstrated that the MMP-1 protein expression level was higher in the TGF-β stimulated cells, but quickly declined in the TGF-β-induced LX-2 cells stimulated with 100-300 µM IOX1 for 48 h. In our previous experiments, it was found that MMP-1, TIMP-1 and the ratio of TIMP-1/MMP-1 were upregulated during hepatic fibrosis, resulting in increased ECM deposition, thereby promoting the occurrence and development of hepatic fibrosis (33). Based on these results and those of our previous study, we hypothesized that the increase of MMP-1 in the TGF-β-stimulated cells was due to the upregulation of Col I expression, while at the concentration of 100-300 µM IOX1, the expression of Col I in the cells is downregulated, at which time the cells no longer need to produce a large amount of MMP-1 to degrade ECM, resulting in the decreased expression of MMP-1.

Wang et al. (34) reported that expression of histone H3K4 demethylase and retinal-binding protein (RBP2) were increased in the livers of patients with cirrhosis, compared with normal healthy controls. Treatment with RBP2 siRNA in the TGF-β-induced HSC LX-2 cell line increased the H3K4 methylation level and downregulated α-SMA and vimentin, thereby inhibiting the proliferation and activation of HSCs. The results of the present study demonstrated that the TIMP-1 protein expression level was markedly downregulated with IOX1 treatment. Further analysis with H3K9me2 ChIP suggested that the level of H3K9me2 enrichment in the promoter region of MMP-1 and TIMP-1 genes was increased after 48-h stimulation with 300 µM IOX1 in the LX-2 cells. As a demethylase inhibitor, IOX1 may upregulate H3K9 methylation, thereby inhibiting gene transcription; therefore, the expression level of MMP-1 is lower than the baseline. However, protein expression of MMP-1 does not equate to its activity. TIMP-1, as an MMP-1 inhibitory protein, may inhibit MMP-1 activity. A total of 300 µM IOX1 treatment downregulated TIMP-1 levels and the inhibitory effect on MMP-1 activity was weakened. These data demonstrated that IOX1 may inhibit the expression of TIMP-1 protein through H3K9me2, thereby decreasing the inhibition of MMP-1 activity by TIMP-1 to improve the degradation of extracellular matrix proteins by MMP-1.

The histone demethylase inhibitor IOX1 not only inhibited LX-2 cell proliferation and activation, and promoted LX-2 apoptosis, but also upregulated H3K9me2 levels in the promoter region of Col I, MMP-1 and TIMP-1 genes. These alterations may decrease the synthesis of Col I and increase the degradation of ECM through activation of ECM metabolic enzymes, thereby exerting its anti-hepatic fibrotic activity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QY and XY conceived and designed the experiments. TT performed the experiments. RJX and BH analyzed the data.
KZD was responsible for data acquisition. TT and QY confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study protocol was reviewed and approved by the Ethics Committee of Guizhou medical university (Guiyang, China; approval no. 1900005).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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