miR-23a promotes IKKα expression but suppresses ST7L expression to contribute to the malignancy of epithelial ovarian cancer cells

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Background: Dysregulation of microRNAs (miRNAs) has been found in human epithelial ovarian cancer (EOC). However, the role and mechanism of action of miR-23a in EOC remain unclear.

Methods: The roles of miR-23a, IKKα, and ST7L in EOC were determined by MTT, colony formation, wounding healing, transwell, flow cytometry, immunofluorescence, RT–qPCR, and western blotting experiments. miR-23a target genes were validated by EGFP reporter assays, RT–qPCR, and western blotting analysis.

Results: miR-23a is upregulated and promotes tumorigenic activity by facilitating the progress of cell cycle and EMT and repressing apoptosis in EOC cells. miR-23a enhances the expression of IKKα but suppresses the expression of ST7L by binding the 3′UTR of each transcript in EOC cells. The proliferation, migration, and invasion of EOC cells are increased by IKKα and inhibited by ST7L. Furthermore, miR-23a activates NF-κB by upregulating IKKα and WNT/MAPK pathway by downregulating ST7L.

Conclusions: miR-23a functions as an oncogene by targeting IKKα and ST7L, thus contributing to the malignancy of EOC cells.
Suppression of tumorigenicity 7 like (ST7L) was identified based on its similarity to the ST7 tumour-suppressor gene (Katoh, 2001). ST7L is downregulated in different cancers, including gastric cancer (Kirikoshi and Katoh, 2002) and glioma (Chen et al., 2013). However, the significance of ST7L in EOC and the underlying mechanism have not been elucidated.

In this study, we demonstrated that miR-23a may function as an oncogene by directly upregulating IKKα to promote NF-κB activation and by downregulating ST7L to activate the WNT/ MAPK pathway in EOC cells. Collectively, these findings may provide insight into tumorigenesis and a potential biomarker for EOC.

Materials and Methods

Cell lines and transfection. OVCAR3 and SKOV3 cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin in a humidified 5% (v/v) CO₂ atmosphere at 37 °C. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

Plasmid construction. For miR-23a expression vector (pri-miR-23a), 307 bp containing miR-23a encoded region was amplified from genomic DNA and cloned into the pcDNA3 vector at BamHI and EcoRI sites. The 2'-O-methyl-modified miR-23a antisense oligonucleotide (ASO-miR-23a) was chemically synthesised as an inhibitor of miR-23a. The 3'UTRs of IKKα and ST7L that contain the miR-23a-binding sites and mutant 3'UTR fragments with mutant miR-23a-binding sites were obtained by annealing double-strand DNA and inserting it into the pcDNA3/EgFP vector. The plSiencer/shRNA-IKKα (shR-IKKα) and plSiencer/shRNA-ST7L (shRNA-ST7L) plasmids expressing siRNA targeting IKKα or ST7L were constructed by annealing double-strand hairpin cDNA and inserting it into the plSiencer 2.1-U6 neo vector (Ambion, Austin, TX, USA). BamHI and EcoRI sites. The relevant control vector is plSiencer 2.1-U6 neo, which was named ‘plSiencer’ in short in paper. The full-length sequences of human ST7L cDNA (NM_017744.4) were obtained by RT–PCR and cloned into pcDNA3 at KpnI and EcoRI sites. The resulting plasmid was termed pcDNA3/ST7L. All primers for PCR amplification are provided in Supplementary Table S1.

Cell proliferation assay. OVCAR3 and SKOV3 cells were seeded in 96-well plates at 4000 cells per well 1 day prior to transfection. The cells were transfected with pri-miR-23a, ASO-miR-23a or the respective control. Cell viability at 48 and 72 h posttransfection was determined by MTT assay. The absorbance at 570 nm was measured using a Quant Universal Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Colonies-forming assay. For the colony-forming assays, the cells were counted at 24 h posttransfection and seeded into 12-well plates in triplicate at a density of 400 cells per well (OVCAR3 cells) or 300 cells per well (SKOV3 cells). Culture medium was replaced every 3 days. The cells were stained with crystal violet, and colonies containing >50 cells were counted.

Prediction of miRNA targets. The hypothetical targets of miR-23a were predicted using TargetScan 7.0, PicTar, and miRanda.

RT-qPCR. First-strand cDNA was generated through reverse transcription of total cellular RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). SYBR Premix Ex Taq Kit (TaKaRa, Shiga, Japan) was used according to the manufacturer’s instructions, and RT–qPCR was performed and analyzed by the iQ5 Detection System (Bio-Rad, Hercules, CA, USA). All the primers (Supplementary Table S1) were purchased from AuGCT, Inc. (Beijing, China).

Western blotting analysis. Cell extracts were prepared with RIPA lysis buffer and analyzed by immunoblotting. Antibodies of ICAM-1, Vimentin, E-cadherin, wtnt2, Dvl1, MAP3K7, β-catenin, GAPDH, CENPA, ST7L, and IKKα were purchased from Tianjin Saier Biotech (Tianjin, China) and the secondary goat anti-rabbit antibody was obtained from Sigma-Aldrich (St Louis, MO, USA). LabWorks Image Acquisition and Analysis Software (UVP, Upland, CA, USA) were used to quantify band intensities.

Immunofluorescent staining. Cells transfected with the indicated plasmids were seeded in 14-well plates and then treated with 20 ng ml⁻¹ TNF-α for 4 h before immunofluorescent staining. The subsequent protocol was previously reported by Chen et al. (2004).

Wound-healing assay. Cells were cultured in 12-well plates. When cell confluence reached approximately 80%, scratches were generated using a 50-μl pipette tip. Non-adherent cells were removed by PBS washes. Wounded cultures were incubated in medium containing 5% FBS for 0, 48, and 72 h. Five fields of observation were randomly captured for each well.

Transwell assays. These assays were performed as described by Guo et al. (2015).

Cell cycle and apoptosis flow cytometry analyses. These analyses were conducted according to the protocol provided by KeyGen Biotech (http://www.keygentec.com.cn).

TPO/FOP flash reporter assays. To assay the transcriptional activity of Wnt pathway (Jin et al., 2013), cells were transiently transfected with a mixture of corresponding plasmids, pTOP/Flash, or pFOP/Flash and pRL-TK vectors as previously controls. Dual Luciferase Reporter Assay System was used to assay Firefly and Renilla Luciferase activity ratio.

Statistical analysis. Student’s t-test was used to compare differences between two groups. P<0.05 was considered statistically significant. *P<0.05; **P<0.01; ***P<0.001.
miR-23a is upregulated and promotes tumorigenic activity in EOC cells. miR-23a was reported to be upregulated in OC tissue (Nam et al., 2008). To determine the role of miR-23a in EOC cells, gain- and loss-of-function assays were performed in OVCAR3 and SKOV3 cells transfected with either pri-miR-23a or ASO-miR-23a. RT–qPCR showed that pri-miR-23a resulted in a 6–7-fold increase of miR-23a levels and that ASO-miR-23a reduced miR-23a levels by 60–70% relative to controls (Figure 1A). MTT assays showed that miR-23a promoted the viability of OVCAR3 and SKOV3 cells (Figure 1B). The colony-formation rates of OVCAR3 and SKOV3 cells were increased by pri-miR-23a and decreased by ASO-miR-23a (Figure 1C) and colony-formation rates of untreated OVCAR3 and SKOV3 cells as control group are shown in Supplementary Figure S1A. Cell cycle analysis showed that miR-23a overexpression caused an increase in OVCAR3 (Figure 1D) and SKOV3 (Supplementary Figure S1B) cells in S phase and a decrease in those in G1 phase. Furthermore, apoptosis analysis showed a lower apoptosis rate among OVCAR3 (Figure 1E) and SKOV3 (Supplementary Figure S1C) cells when miR-23a was overexpressed. Wound-healing assays revealed that EOC cells’ migration were significantly promoted by miR-23a but inhibited by ASO-miR-23a (Figure 1F). Consistently, transwell assay results showed that cells’ invasion were increased by pri-miR-23a and inhibited by ASO-miR-23a (Figure 1G). Taken together, these results indicate that miR-23a may have an oncogenic role in EOC cells.

miR-23a upregulates IKKα expression but downregulates ST7L expression by binding their 3’ UTRs in EOC cells. Target prediction using bioinformatics methods suggested hundreds of candidate targets for miR-23a; we chose IKKα and ST7L as putative targets for further study after considering available functional knowledge. To validate whether IKKα and ST7L are directly targeted by miR-23a, we performed an EGFP reporter assay using EGFP reporter vectors containing either the wild-type 3’ UTR or a mutant 3’ UTR with a mutation in the complementary seed sequence of IKKα and ST7L (Figure 2A). In OVCAR3 and SKOV3 cells, pri-miR-23a significantly enhanced the fluorescence intensity of pcDNA3/EGFP-IKKα 3’ UTR (Figure 2B), whereas overexpression of miR-23a significantly decreased the intensity of pcDNA3/EGFP-ST7L 3’ UTR (Figure 2D). However, the intensity with the mutant 3’ UTR of IKKα or ST7L was not influenced by either overexpression or inhibition of miR-23a (Figure 2C and E). These results suggest that miR-23a directly target IKKα and ST7L but has opposite effects on the two targets.

Next we further determined the effect of miR-23a on endogenous IKKα and ST7L expression. RT–qPCR showed that IKKα mRNA levels were increased but that ST7L mRNA levels were decreased in OVCAR3 and SKOV3 cells transfected with pri-miR-23a (Figure 2F). Furthermore, western blotting assays showed a similar relationship: miR-23a increased IKKα expression but decreased ST7L expression (Figure 2G, Supplementary Figure S2). These results indicate that miR-23a promotes IKKα expression but suppresses ST7L expression at both the mRNA and protein levels in EOC cells.

IKKα promotes EOC cell growth, migration, and invasion. An abundance of data indicates that IKKα functions as an oncogene and has an important role in activating the NF-κB pathway (Anest et al., 2003; Alameda et al., 2016). To determine the effect of IKKα on cell proliferation, migration, and invasion in EOC, we depleted its expression by a specific shRNA. RT–qPCR and western blotting assays validated the efficiency of shR-IKKα (Figure 3A and B). Next MTT and colony-formation assays were used to examine the influence of shR-IKKα on OVCAR3 and SKOV3 cells’ proliferation. Knockdown of IKKα reduced cell viability (Figure 3C) and relative colony-formation rate (Figure 3D). Cellular apoptosis assays showed that shR-IKKα increased the apoptosis of OVCAR3 and SKOV3 cells (Figure 3E), but no difference of cell cycle analysis was shown in Supplementary Figure S3A.

Wound-healing assays showed that IKKα knockdown significantly impeded OVCAR3 and SKOV3 cells’ migration (Figure 3F). Transwell assays indicated that IKKα knockdown markedly decreased OVCAR3 and SKOV3 cells’ invasion (Figure 3G). These results indicate that IKKα promotes the malignant phenotype of EOC cells, which is similar to the effects of miR-23a.

ST7L functions as a tumour suppressor in EOC cells. To determine the impact of ST7L on the phenotype of EOC cells, we constructed a ST7L overexpression vector (pcDNA3/ST7L) and a knockdown plasmid (shR-ST7L) (Figure 4A and B) and conducted functional assays. At 48 and 72h posttransfection, OVCAR3 and SKOV3 cells viability were decreased by overexpression of ST7L and increased by ST7L knockdown (Figure 4C). In addition, negative colony-formation rate was induced by shR-ST7L and decreased by pcDNA3/ST7L compared with the control groups (Figure 4D). Furthermore, cell cycle analysis showed that ST7L overexpression increased the number of cells in G1 phase and decreased the number in S phase (Figures 4E, Supplementary Figure S3B), but ST7L was not observed to significantly influence cell apoptosis (Supplementary Figure S4). To determine whether ST7L influences the migration and invasion of EOC cells, migration (wound healing) and invasion (transwell) assays were performed in OVCAR3 and SKOV3 cells. As expected, overexpression of ST7L significantly suppressed OVCAR3 and SKOV3 cells’ migration and invasion, whereas shR-ST7L increased the migration and invasion of these cells (Figure 4F and G).

Because ST7L significantly decreased the migration and invasion of EOC cells, we tested whether ST7L affected the expression of key molecular markers of EMT, including E-cadherin, ICAM-1, and Vimentin. Western blotting analysis showed that overexpression of ST7L increased E-cadherin expression but decreased ICAM-1 and Vimentin expression in EOC cells. In contrast, miR-23a decreased E-cadherin protein levels but increased ICAM-1 and Vimentin protein levels in EOC cells (Figure 4H, Supplementary Figure S5). Taken together, these results indicate that ST7L suppresses malignant behaviour by repressing cell cycle and EMT.

Knockdown of IKKα and overexpression of ST7L partly rescue the tumorigenic phenotype induced by miR-23a in EOC cells. To demonstrate that miR-23a may function as an oncogene by directly regulating IKKα and ST7L, we performed rescue versions of the proliferation, migration, and invasion experiments. As shown in Figure 5A–D, knockdown of IKKα partially rescued the phenotypes induced by miR-23a. Similarly, overexpression of ST7L partly rescued the malignant behaviour of EOC cells (Figure 5E–H).

miR-23a positively regulates the NF-κB and WNT/MAPK pathways in EOC cells. Given that IKKα is essential for the NF-κB pathway, we next investigated the possibility that miR-23a affects the activation of NF-κB signaling pathway. The localization of NF-κB in OVCAR3 cells was examined by immunofluorescence after transfection with pri-miR-23a or ASO-miR-23a and pcDNA3/IKKα or cotransfection with pri-miR-23a and shR-IKKα. The nuclear distribution of NF-κB (p50) was increased in pri-miR-23a-transfected cells and decreased in ASO-miR-23a-transfected cells, which were treated with a low concentration of TNF-α (Figure 6A). Overexpression of IKKα increased the nuclear distribution of p50, and knockdown of IKKα partly rescued the effects of miR-23a (Figure 6B). We also demonstrated that the nuclear protein level of p50 was increased in cells transfected with

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miR-23a regulates the IKKα-NF-κB and ST7L-WNT/MAPK pathways
miR-23a regulates the IKK\(\alpha\)-NF-\(\kappa\)B and ST7L-WNT/MAPK pathways

Figure 1. miR-23a promotes the tumorigenic behaviour of EOC cells. (A) Relative level of miR-23a after transfection with pri-miR-23a or ASO-miR-23a. (B) Cell viability was determined at 48 h and 72 h posttransfection by MTT assays after transfection with pri-miR-23a or ASO-miR-23a. (C) In vitro cell growth was also assessed by colony-formation assays; relative colony-formation rate are shown. (D) Cell cycle assays showed that miR-23a increased the number of OVCAR3 cells in S phase. (E) miR-23a suppressed the apoptosis of OVCAR3 cells. (F) Wound-healing assays were used to measure migration at 0, 48, and 72 h; and the quantitative pattern are shown. (G) Transwell assays showed the invasion tendency. All of the experiments were repeated three times. *P<0.05; **P<0.01; ***P<0.001.

pri-miR-23a was decreased in cells transfected with shR-IKK\(\alpha\), and the cytoplasmic protein levels of p50 changed were shown, respectively (Figure 6C and D). To examine whether miR-23a influenced the activation of NF-\(\kappa\)B signaling pathway, we analyzed the expression level of the NF-\(\kappa\)B target gene IL-6 and CCL19 (Hayden and Ghosh, 2008) under alteration of miR-23a level in OVCAR3 and SKOV3 cell lines. RT–qPCR analysis showed that pri-miR-23a significantly increased the mRNA levels of IL-6 and CCL19 in OVCAR3 and SKOV3 cells (Supplementary Figure S6).

ST7L is an inhibitor of the WNT pathway (Chen et al, 2013); to investigate the influence of miR-23a on the WNT signaling pathway, western blotting assays were utilised to detect downstream effectors of WNT signaling in cells transfected with miR-23a or ST7L. In both OVCAR3 and SKOV3 cells, the expression of wnt2, Dvl1, MAP3K7, and \(\beta\)-catenin, downstream effectors of the WNT/MAPK pathway, were reduced by miR-23a depletion and significantly increased by miR-23a restoration. Simultaneously overexpression of ST7L reversed the impact of miR-23a on wnt2, Dvl1, MAP3K7, and \(\beta\)-catenin expression (Figure 6E and F, Supplementary Figure S5). And TOP/FOP luciferase reporter assays were also performed, which is a common WNT pathway activation reporter assay (Gerard et al, 2011; Jin et al, 2013); the results showed that miR-23a
miR-23a regulates the IKKα-NF-κB and ST7L-WNT/MAPK pathways

Figure 2. miR-23a upregulates IKKα expression but suppresses ST7L expression by targeting their 3’UTRs in EOC cells. (A) The predicted miR-23a-binding sites in IKKα (labelled as CHUK) and ST7L mRNA using TargetScan7.0 are shown. (B) OVCAR3 and SKOV3 cells were cotransfected with wild-type pcDNA3/EGFP-IKKα 3’UTR or (C) 3’UTR-mut and ASO-miR-23a or ASO-miR-23a. (D) OVCAR3 and SKOV3 cells were cotransfected with pcDNA3/EGFP-ST7L 3’UTR or (E) 3’UTR-mut and pri-miR-23a or ASO-miR-23a. (F and G) Reverse transcription–quantitative PCR and western blotting showed IKKα and ST7L mRNA and protein levels after transfection with pri-miR-23a or ASO-miR-23a. All of the experiments were repeated three times. **P<0.01; ***P<0.001; NS, not significant.

Figure 3. Knockdown of IKKα inhibits EOC cell growth, migration, and invasion. (A and B) Reverse transcription–quantitative PCR and western blotting showed that knockdown of IKKα is efficient. (C) MTT assays showed that transfection with shR-IKKα suppresses cellular viability. (D) Colony-formation rate in OVCAR3 and SKOV3 cells transfected with shR-IKKα were lower compared with the control groups. (E) Knockdown of IKKα promoted the apoptosis of OVCAR3 cells. (F) Wound-healing assays showed that knockdown of IKKα suppresses OVCAR3 and SKOV3 cells’ migration at 0, 48, and 72 h. (G) Transwell assays showed that knockdown of IKKα suppresses cells’ invasion. All of the experiments were repeated three times. **P<0.01; ***P<0.001.
enhanced TOP/FOP flash ratio but ST7L weakened it (Figure 6G).

The results indicate that miR-23a modulates the NF-κB and WNT pathways in a IKKα- and ST7L-dependent manner, respectively in EOC cells.

**DISCUSSION**

miR-23a is organised in cluster with miR-27a and miR-24-2 and it has been reported that the three miRNAs derive from a single primary transcript. It was also reported that miR-23a and miR-27a are upregulated in serious OC and that they share common predicted targets belonging to WNT signaling pathway (Chhabra et al, 2010). The miR-23a–miR-27a–miR-24-2 cluster may coordinately regulate several processes, and a previous report also demonstrated that miR-23a was upregulated in OC (Nam et al, 2008). In the present study, we found that upregulation of miR-23a increased cell viability, growth, migration, and invasion by accelerating the G1/S transition and by inhibiting the apoptosis of OC cell lines. In addition, miR-23a activates NF-κB pathway by upregulating IKKα and WNT/MAPK pathway by downregulating ST7L and promotes EMT process. These results indicate that miR-23a might function as an oncogene in OC.

ST7L functions as a tumour suppressor in many cancers (Riker et al, 2008; Chen et al, 2013). However, the role of ST7L in EOC cells was unknown. Our results demonstrated that ST7L suppressed cell proliferation, migration, and invasion, thus functioning as a tumour suppressor in EOC cells, and miR-23a downregulated ST7L expression to repress its function as tumour suppressor.

**Figure 4.** ST7L functions as a tumour suppressor. (A and B) Reverse transcription–quantitative PCR and western blotting showed that overexpression and knockdown of ST7L are efficient. (C) MTT assays showed that overexpression of ST7L inhibited cell viability and knockdown of ST7L increased cell viability. (D) Colony-formation rate were lower after transfection with pcDNA3/ST7L and higher after transfection with shR-ST7L compared with the control groups. (E) The cell cycle profiles after treatment with pcDNA3/ST7L or shR-ST7L were detected by flow cytometry. (F) Wound-healing assays showed that migration was suppressed by overexpression of ST7L and promoted by knockdown of ST7L. (G) Transwell assays showed that overexpression of ST7L inhibited invasion. (H) Western blotting showed the protein levels of ICAM-1, Vimentin, and E-cadherin after transfection with pri-miR-23a or ASO-miR-23a and pcDNA3/ST7L or shR-ST7L. All of the experiments were repeated three times. *P<0.05; **P<0.01; ***P<0.001.
miR-23a regulates the IKKα-NF-κB and ST7L-WNT/MAPK pathways

Figure 5. Knockdown of IKKα and overexpression of ST7L partly rescue the tumour phenotype induced by miR-23a. Cotransfection with pri-miR-23a and shR-IKKα showed that IKKα knockdown partly rescued cell (A and B) proliferation, (C) migration, and (D) invasion. Cotransfection with pri-miR-23a and pcDNA3/ST7L showed that overexpression of ST7L partly rescued cell (E and F) proliferation, (G) migration, and (H) invasion. All of the experiments were repeated three times. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Although it is well known that miRNAs target the 3'UTR of mRNAs and lead to mRNA decay or translation suppression, recent studies have revealed that miRNAs can result in posttranscriptional activation of target genes (Vasudevan, 2012). However, the detailed mechanism is poorly understood. In our study, the 3'UTR of IKKα was predicted to contain miR-23a-binding sites, and the EGFP report assay indicated that miR-23a targeted the 3'UTR and activated the reporter expression. IKKα has been reported to be upregulated in OC (Anunziata et al, 2010); we also demonstrated that miR-23a significantly upregulated the expression of IKKα, and this upregulation by miR-23a was accompanied by an increase in classic NF-κB signaling pathway activity in EOC cells, as evidenced by the elevated miRNA levels of IL-6 and CCL19, which are the reported targets of NF-κB (Dejardin et al, 2002; Alberti et al, 2012). Taken together, our results suggest that miR-23a may enhance NF-κB activity by upregulating IKKα and demonstrate the increased level of IKKα upon miR-23a ectopic expression. As a result, we provide a new example of translation upregulation by miRNAs.

Collectively, our results reveal an oncogenic function of miR-23a in EOC cells. miR-23a upregulated the IKKα expression by binding the 3'UTR and thus increase the activity of the NF-κB signaling pathway; miR-23a downregulated the ST7L expression by binding the 3'UTR and activated the WNT/MAPK pathway (Figure 6H). These findings may help us better understand the molecular mechanism of ovarian carcinogenesis and may have potentially diagnostic and therapeutic value in the future.
Figure 6. miR-23a positively regulates the NF-κB and WNT/MAPK pathways. (A–D) OVCAR3 cells were transfected with the indicated combinations of pri-miR-23a, ASO-miR-23a, pcDNA3/IKKα, pri-miR-23a, and shR-IKKα. After 48 h, cells were harvested, and p50 was detected by immunofluorescent staining and western blotting. n-p50: nuclear p50, the control group was CENPA; c-p50: cytoplasmic p50, the control group was GAPDH (glyceraldehyde 3-phosphate dehydrogenase). (E) Western blotting were used to measure the protein levels after transfection with pri-miR-23a or ASO-miR-23a. (F) Western blotting showed the protein levels in cells treated with pcDNA3/ST7L or shR-ST7L. (G) OVCAR3 and SKOV3 cells were cotransfected with pTOP/flash or pFOP/flash, pcDNA3, pri-miR-23a, pcDNA3/ST7L, or pri-miR-23a and pcDNA3/ST7L, respectively, after which luciferase reporter assays were performed. (H) The model by which miR-23a upregulates IKKα and downregulates ST7L to activate NF-κB and WNT/MAPK pathways to promote tumour malignancy. All of the experiments were repeated three times. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HT conceived and supervised the project. ZY, XLW, and RB performed the experiments. ML, WYL, and XL analyzed the data. ZY, XLW, RB, and HT wrote the manuscript. All the authors reviewed the manuscript.

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