Long non-coding RNA BRM promotes proliferation and invasion of papillary thyroid carcinoma by regulating the microRNA-331-3p/SLC25A1 axis

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Abstract. Long non-coding RNA BRM (lncBRM) was first identified in liver cancer stem cells and was reported to promote multiple cancer types. However, the function of lncBRM in papillary thyroid carcinoma (PTC) remains unclear. The primary focus of the present study was to determine the biological role of lncBRM in PTC. Reverse transcription-quantitative PCR assays revealed that lncBRM was upregulated in PTC tissues and cells. Cell Counting Kit-8, Transwell invasion and colony-formation assays were performed to assess cell proliferation, invasion and migration, respectively. Furthermore, high expression of lncBRM was associated with poor overall survival time in patients with PTC. lncBRM knockout significantly suppressed cell proliferation, migration and invasion. lncBRM was predicted to bind to microRNA (miR)-331-3p and targets SLC25A1. Overexpression of miR-331-3p or inhibition of SLC25A1 resulted in significantly suppressed proliferation, migration and invasion of PTC cells. Rescue assays demonstrated that inhibition of miR-331-3p significantly abrogated the effects of lncBRM knockout on PTC cell proliferation, migration and invasion. In conclusion, the present study suggests that lncBRM promotes PTC by regulating miR-331-3p and targeting SLC25A1.

Introduction

In 2018, thyroid cancer was the most common thyroid malignancy, accounting for ~1% of all malignant tumors, and includes papillary, follicular, undifferentiated and medullary carcinomas (1). Thyroid cancer occurs mostly in young adults aged ~40 years, and the ratio men to women is 1.0:2.5 to 1.0:3.0 (2). Papillary thyroid carcinoma is typically exhibits lower malignancy, and better prognosis and is the most common type of thyroid cancer, accounting for 85-90% of cases (3). In recent years, the incidence of thyroid cancer has steadily increased (4). Most patients (~90%) with PTC can be effectively treated via surgical removal and have a good 5-year survival rate (5). However, a small proportion of patients with PTC have poor prognosis and survival rates (6). Therefore, it is necessary to illustrate the molecular mechanism underlying proliferation and invasion of thyroid cancer, in order to identify potential therapeutic targets (7).

Long non-coding RNA (lncRNAs) is a type of RNA >200 nucleotides in length, with little or no coding potential (8). Recently, lncRNAs were found to serve a pivotal role in several biological processes (9-11), such as embryo development (12,13), immunology (14,15) and cancer (16-18). Dysregulation of lncRNAs have been shown to be involved in tumorigenesis and tumor progression in PTC (19-21), suggesting the potential of lncRNAs as diagnostic markers or therapeutic agents for PTC (22,23). For example, lncRNA LINC00460 promotes carcinogenesis by sponging microRNA (miR)-613 in PTC and upregulating the expression of sphingosine kinase 2 (24). Additionally, lncRNA FOXD2-AS1 was found to be upregulated in PTC tissues and function as a competing endogenous RNA (ceRNA) to enhance the expression of KLK7, by sponging miR-485-5p (25).

lncBRM was first reported to be highly expressed in liver cancer stem cells and was shown to be associated with the progression of liver cancer, by interacting with BRM and regulating the YAP signaling pathway (26). Recently, lncBRM was proven to exhibit a pivotal role in ovarian cancer, through the upregulation of SOX4 and thereby facilitating proliferation, migration and invasion of ovarian cancer cells (27). Furthermore, lncBRM was shown to regulate the proliferation and invasion of colorectal cancer cells, by sponging miR-204-3p and upregulating translationally-controlled tumor protein 1 (TPT1) (28). However, the role of lncBRM in PTC remains unclear. In the present study, the expression patterns, biological functions and mechanisms of action of lncBRM in PTC progression were elucidated.
Materials and methods

Samples and cell lines. PTC samples and matched normal tissues were obtained from 90 patients (mean age, 63.5±5.1 years; age range, 37.5-72.4 years; 32 men and 58 women) with PTC undergoing surgery at The People's Hospital of Tong Liang District (Chongqing, China). The pathological diagnosis of all specimens was graded according to the classification of thyroid malignancy of the World Health Organization (2004) by two experienced pathologists (29). Among these patients, 41 cases were high-grade and 46 cases were low-grade, and 41 cases were non-metastasis and 44 cases were metastasis. All tissues were frozen in liquid nitrogen until further use. The study was approved by the Ethics Committee of The People's Hospital of Tong Liang District (permission no. 2015KT57). All patients provided written informed consent.

The normal human thyroid follicular epithelial cell line Nthy-ori 3-1, and human thyroid cancer cell lines TPC-1 and SW1736, were obtained from the American Type Culture Collection. All cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 6 h, successfully transfected cells were confirmed by reverse transcription-quantitative PCR (RT-qPCR) analysis and cultured in six-well plates using RPMI-1640 medium containing 10% FBS for two days at 37˚C and 5% CO₂. CCK-8 solution (10 µl; Beyotime Institute of Biotechnology) was added into each well for 4 h at 37˚C. Cell counting Kit‑8 (CCK‑8) assays. TPC-1 and SW1736 cells (1x10⁴) were seeded in a 96-well plate and cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C and 5% CO₂. CCK-8 solution (10 µl; Beyotime Institute of Biotechnology) was added into each well for 4 h at 37˚C. Cell proliferation was assessed 24, 48 and 72 h post-transfection. The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.).

Table I. Associations between lncBRM expression and clinicopathological features in 90 patients with papillary thyroid carcinoma.

| Variables                  | Low (n=45) | High (n=45) | P-value |
|----------------------------|------------|-------------|---------|
| Age, years                 |            |             |         |
| >60                        | 23         | 18          | 0.397   |
| ≤60                        | 22         | 27          |         |
| Sex                        |            |             |         |
| Male                       | 21         | 23          | 0.833   |
| Female                     | 24         | 22          |         |
| TNM stage                  |            |             |         |
| I-II                       | 30          | 19          | 0.033   |
| III-IV                     | 15          | 26          |         |
| Lymph node metastasis      |            |             |         |
| Yes                        | 16          | 28          | 0.020   |
| No                         | 29          | 17          |         |

TNM, Tumor-Node-Metastasis.

Plasmids and transfections. TPC-1 and SW1736 cells were seeded into six-well plates at a density of 1x10⁵ cells/well, and cultured overnight until they reach 70–80% confluence. miR-331-3p mimic, empty vector control, miR-331-3p inhibitor were used at 50 nM. The short hairpin (sh)RNA specifically targeting lncBRM, SLC25A1 and scrambled negative control shRNA were provided by GenePharma Co. Ltd and used at 1 mg/ml. The coding sequence of SLC25A1 was constructed into pMy vectors (Addgene, Inc.) to overexpress SLC25A1 that was used at 1 mg/ml. The empty control, which served as a negative control, was also used at 1 mg/ml. Plasmids were transfected into the cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 6 h, successfully transfected cells were confirmed by reverse transcription-quantitative PCR (RT-qPCR) analysis and cultured in six-well plates using RPMI-1640 medium containing 10% FBS for two days at 37˚C and 5% CO₂ to expand for subsequent experiments. The reaction was performed at 42˚C for 1 h, and the enzyme was subsequently inactivated at 85˚C for 5 min. qPCR was performed using SYBR Green PCR Master mix reagents (Takara Bio, Inc.) in a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). miR-331-3p expression was determined using SYBR Premix Ex Taq II (GeneCopoeia, Inc.) according to the following conditions: 10 min of pre-denaturation at 95˚C, followed by 40 cycles of 10 sec denaturation at 95˚C, 20 sec annealing at 60˚C and 30 sec extension at 72˚C. SLC25A1 mRNA expression was measured using a SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following procedures: 10 min pre-denaturation at 95˚C, followed by 36 cycles of 10 sec denaturation at 95˚C, 20 sec annealing at 60˚C and 34 sec extension at 72˚C. 18S was used as internal reference for miR-331-3p and SLC25A1 mRNA expression. Relative expression of miR-6852 and LEF1 mRNA was calculated using 2-ΔΔCq method (30). The sequences of the primers used were as follows: miR-331-3p mimics, 5’-GCCCCUCGUCCUACCCUAGAA-3’; and miR-331-3p inhibitor, 5’-TTCTUGUTUGGCUCUGG GGC-3’. pMIR-SLC25A1-3’UTR (wild type or mutant) or pMIR-lncBRM (wild type or mutant) and miR-331-3p mimics were transfected into TPC-1 or SW1736 cell lines along with pRL-TK vectors (Promega Corporation). Following culture for 24 h, luciferase activity was measured using a dual Glo™ reader (BioTek Instruments, Inc.) according to the manufacturer’s protocol. The reaction was performed at 42˚C for 1 h, and the enzyme was subsequently inactivated at 85˚C for 5 min. qPCR was performed using SYBR Green PCR Master mix reagents (Takara Bio, Inc.) in a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). miR-331-3p expression was determined using SYBR Premix Ex Taq II (GeneCopoeia, Inc.) according to the following conditions: 10 min of pre-denaturation at 95˚C, followed by 40 cycles of 10 sec denaturation at 95˚C, 20 sec annealing at 60˚C and 30 sec extension at 72˚C. SLC25A1 mRNA expression was measured using a SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following procedures: 10 min pre-denaturation at 95˚C, followed by 36 cycles of 10 sec denaturation at 95˚C, 20 sec annealing at 60˚C and 34 sec extension at 72˚C. 18S was used as internal reference for miR-331-3p and SLC25A1 mRNA expression. Relative expression of miR-6852 and LEF1 mRNA was calculated using 2-ΔΔCq method (30). The sequences of the primers used were as follows: SLC25A1 forward, 5’-CCG TCGGTTTAGAATGTTCG-3’; and reverse, 5’-TAAACC CGGAGAAGAACCTCCT-3’. pMIR-lncBRM forward, 5’-GGCTAA GAGGCCAGGAGAG-3’ and reverse, 5’-TTCTACTT CGGCCCAATGGC-3’; and 18S forward, 5’-CAGCCACCC GAGATTGAGCA-3’ and reverse, 5’-TAGTAGCGACGGGCG GTGTG-3’.

RNA preparation and RT-qPCR. Total RNA was isolated from tissues or cells using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. Total RNA was subsequently reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Promega Corporation) according to the manufacturer’s protocol. The sequences of the primers used were as follows: miR-331-3p mimics, 5’-GCCUCGCUCCUACCCUAGAA-3’; and miR-331-3p inhibitor, 5’-TTCTUGUTUGGCUCUGG GGC-3’. pMIR-SLC25A1-3’UTR (wild type or mutant) or pMIR-lncBRM (wild type or mutant) and miR-331-3p mimics were transfected into TPC-1 or SW1736 cell lines along with pRL-TK vectors (Promega Corporation). Following culture for 24 h, luciferase activity was measured using a dual Glo™ luciferase assay system (Promega Corporation) according to the manufacturer’s protocols and normalized to Renilla luciferase activity.

Cell counting Kit-8 (CCK-8) assays. TPC-1 and SW1736 cells (1x10⁴) were seeded in a 96-well plate and cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C and 5% CO₂. CCK-8 solution (10 µl; Beyotime Institute of Biotechnology) was added into each well for 4 h at 37˚C. Cell proliferation was assessed 24, 48 and 72 h post-transfection. The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.).
Colony-formation assays. Cells in the logarithmic growth phase were treated with 0.25% trypsin to give single-cell suspensions. Each group was inoculated at a density of ~1,000 cells/well in the culture medium. The culture was terminated when colonies were visible, after 2-3 weeks. The supernatant was discarded and the colonies were fixed at room temperature with 4% paraformaldehyde for 15 min and stained with 0.5% Giemsa for 30 min at room temperature. The number of colonies were examined under a light microscope (magnification, ×100).

Bioinformatics analysis. miR-331-3p was predicted as a potential lncBRM target by using miRDB tool (http://mirdb.org/miRDB/index.html). miRDB is an online database for miRNA target prediction and functional annotations. All the targets in miRDB were predicted by a bioinformatics tool, MirTarget, which was developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments. SLC25A1 was predicted as a potential miR-331-3p target by using TargetScan7 tool (http://www.targetscan.org/vert_72/). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA.

Transwell experiments. Two days after transfection, TPC-1 and SW1736 cells were prepared as single cell suspensions (1x10⁵ cells/ml) in serum-free RPMI-1640. Transwell chambers (8 mm pore; EMD Millipore) were inserted into 24-well plates containing 600 µl RPMI-1640 supplemented with 10% FBS in the lower chamber. A 100 µl cell suspension containing 1x10⁴ cell was added into the upper chamber. Cells were cultured for 48 h at 37°C. Non-migrated cells were scraped off and migrated cells were fixed using 4% formaldehyde for 30 min at room temperature, stained using 0.5% crystal violet for 30 min at room temperature and counted under a light microscope (magnification, ×200). The aforementioned procedure was also used to detect cell invasion; however, for the cell invasion assay, Transwell were pre-coated with 100 µl Matrigel (1 mg/ml; BD Biosciences) for 30 min at 37°C, prior to cell seeding.

Western blot analysis. Total proteins in each cell sample were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology). Proteins were quantified using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.), and 40 µg protein/lane were separated by 10% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride membranes. Membranes were subsequently incubated with 5% skimmed milk for 1 h at room temperature. TBS supplemented with 0.1% Tween 20 (TBST) was used to wash the membrane. Membranes were incubated with primary antibodies against SLC25A1 (1:2,000; cat. no. 15235-1-AP; ProteinTech Group, Inc.) and β-actin (1:5,000; cat. no. 60008-1-Ig; ProteinTech Group, Inc.) for 12 h at 4°C. Membranes were then incubated with goat anti-mouse (cat. no. 70-GAM007) and goat anti-rabbit (cat. no. 70-GAR007) secondary antibodies [1:5,000; Multisciences (Lianke) Biotech Co., Ltd.] for 2 h at room temperature. Membranes were washed with three times TBST for 15 min. Protein expression was monitored using the Pierce™ ECL Western Blotting Substrate (Beijing Solarbio Science & Technology Co., Ltd.) and quantified using Quantity One software v4.62 (Bio-Rad Laboratories Inc.).

Statistical analysis. GraphPad Prism version 6 (GraphPad Software, Inc.) was used to analyze the data. All results are expressed as the mean ± standard deviation. A Student's t-test was used to analyze the differences between two groups. A
one-way ANOVA with a pos-hoc Tukey's test was used for multiple comparisons. Kaplan-Meier analysis followed by a log-rank test was used to analyze survival rate. The association between lncBRM expression and clinical features listed in Table I was analyzed using a χ² test. P<0.05 was considered to indicate a statistically significant difference.

Results

lncBRM is upregulated in PTC tissues and cell lines. In order to determine the role of lncBRM in PTC progression, RT-qPCR was performed, which revealed increased expression of lncBRM in PTC tissues compared with the adjacent normal tissue (P<0.05; Fig. 1A). Consistently, lncBRM was also upregulated in the PTC cell lines TPC-1 and SW1736, compared with the normal thyroid Nthy-ori 3-1 cell line (both P<0.05; Fig. 1B). To further examine the prognostic significance of lncBRM, overall survival time was analyzed. Based on the median level of lncBRM expression, the 90 tissues were divided into two groups, lncBRM high and lncBRM low expression groups. Higher expression of lncBRM was associated with poor overall survival time in patients with PTC (P<0.05; Fig. 1C). Moreover, high expression of lncBRM was associated with metastasis and advanced stages of PTC (both P<0.05; Fig. 1D and E). The potential association between lncBRM expression and the clinicopathological features of patients with PTC was also explored. High lncBRM expression was associated with late Tumor-Node-Metastasis (P=0.033) stages and lymph node metastasis (P=0.02; Table I). Overall, lncBRM was upregulated in PTC and was associated with a poor prognosis.

lncBRM knockout inhibits the progression of PTC cells. In order to elucidate the mechanism by which lncBRM regulates PTC progression, TPC-1 and SW1736 cells were transfected with shRNA targeting lncBRM and expression of lncBRM was significantly decreased in the transfected cells (both P<0.05; Fig. 2A). A CCK-8 assay was performed to examine the proliferation of shcontrol or shlncBRM TPC-1 and SW1736
cells. lncBRM knockout resulted in significantly decreased proliferation of TPC-1 and SW1736 cells after 48 and 72 h (both P<0.05; Fig. 2B). Transwell assays revealed significantly decreased invasion and migration of TPC-1 cells and SW1736 cells following lncBRM knockout (all P<0.05; Fig. 2C). Furthermore, colony-formation assays revealed that lncBRM promoted the proliferation ability of TPC-1 and SW1736 cells (P<0.05; Fig. 2D).

**lncBRM negatively regulates miR-331-3p expression in PTC.** Bioinformatics analysis was conducted in order to elucidate the mechanism by which lncBRM promotes PTC progression. lncBRM formed complementary base pairing with miR-331-3p (Fig. 3A). To verify whether lncBRM binds to miR-331-3p directly, a luciferase reporter assay was performed. The luciferase activity was significantly decreased following co-transfection with WT-lncBRM and miR-331-3p mimics (P<0.05; Fig. 3B). However, luciferase activity remained unchanged in cells co-transfected with WT-lncBRM and miR-331-3p mimics (Fig. 3B). Moreover, the expression of miR-331-3p was increased following lncBRM knockout compared with the control cells (P<0.05; Fig. 3C). In order to investigate the association between miR-331-3p and lncBRM, cells were transfected with miR-331-3p mimics or inhibitor plasmids and transfection efficiency was confirmed (Fig. 3D). Transfection of miR-331-3p mimic significantly inhibited the expression of lncBRM in TPC-1 cells, whereas transfection with miR-331-3p inhibitor increased lncBRM expression compared with control (both P<0.05; Fig. 3E). Consistently, CCK-8 assays showed proliferation was decreased after lncBRM knockout in TPC-1 and SW1736 cells compared with control after 48 and 72 h (both P<0.05). However, the decreased proliferation caused by lncBRM knockout was reversed by inhibition of miR-331-3p in TPC-1 and SW1736 cells (Fig. 3F). Overall, these data suggest that lncBRM can bind to miR-331-3p and regulate TPC-1 cell proliferation.

**lncBRM regulates SLC25A1 expression through miR-331-3p.** A TargetScan7 analysis was performed to identify potential genes that are regulated by miR-331-3p, and SLC25A1 was identified as a potential candidate gene. There was a potential binding site at SLC25A1-3'UTR with miR-331-3p (Fig. 4A). The role of SLC25A1 in PTC progression was explored in the present study. Firstly, ectopic expression of miR-331-3p or lncBRM knockout resulted in significantly decreased luciferase activity of wild type (P<0.05; Fig. 4B) but not mutant SLC25A1-3'UTR. Inhibition of miR-331-3p restored the decreased luciferase activity caused by lncBRM knockout.
Ectopic expression of miR-331-3p resulted in decreased SLC25A1 expression (P<0.05), whereas miR-331-3p inhibitor promoted SLC25A1 expression (P<0.05; Fig. 4C). Furthermore, overexpression of miR-331-3p or knockout of lncBRM resulted in decreased SLC25A1 expression, whereas inhibiting miR-331-3p restored this phenotype (all P<0.05; Fig. 4D and E). These data suggest that lncBRM regulates SLC25A1 expression by targeting miR-331-3p.

To determine whether lncBRM exerts its biological effects through miR-331-3p and SLC25A1, rescue assays were performed by overexpressing of SLC25A1 or inhibiting SLC25A1 in lncBRM knockout TPC-1 and SW1736 cells (Fig. 4F). Transwell assays demonstrated that overexpression of miR-331-3p and knockout of lncBRM or SLC25A1 resulted in significantly decreased migration of cells (all P<0.05). Inhibition of miR-331-3p or overexpression of SLC25A1 in
IncBRM-depleted TPC-1 and SW1736 cells significantly restored cell migration abilities (Fig. 4G and H).

Discussion

Thyroid cancer is one of the most prevalent endocrine tumors and the incidence rate is increasing steadily. Therefore, it is important to illustrate the mechanisms of thyroid cancer (31). In recent years, IncRNAs have been shown to serve vital roles in many biological processes (32). Moreover, several studies revealed the roles of IncRNAs in the regulation of PTC progression. For example, Xia et al (33) reported that IncRNA HOXA-AS2 was upregulated in PTC tissues and promoted migration and invasion of PTC cells by regulating the miR-520c-3p/S100A4 pathway. Wang et al (34) demonstrated that LINC01186 suppressed proliferation and invasion of PTC cells by decreasing the expression of YAP1 and increasing LATS1 expression.

IncBRM was found to play vital roles in some cancer types. Xi et al (27) found that IncBRM facilitated ovarian cancer cell proliferation, migration and invasion via SOX4 upregulation. Li et al (28) reported that IncBRM can sponge miR-204-3p and target TPT1 to promote colorectal cancer cell migration, invasion and proliferation. However, the role of IncBRM in thyroid cancer remains unclear. In the present study, IncBRM was determined to be upregulated in PTC tissues and cell lines. IncBRM knockout decreased TPC-1 cell proliferation, migration and invasion. Furthermore, bioinformatics analysis identified miR-331-3p as a potential target of IncBRM.

Solute carrier (SLC) members are a type of membrane transport proteins (35). SLC25A1 is a mitochondrial carrier, which promotes the flux of citrate/isocitrate across the mitochondria (35). Previously, SLC25A1 was reported to play a vital role in promoting the mitochondrial pool of citrate and redox balance and therefore promoting self-renewal abilities of cancer stem cells in non-small cell lung cancer (36). In addition, SLC25A1 is required for the conversion of glucose into acetyl-CoA for fatty acid synthesis and is increased by PGC1α to promote tumor growth in liver and colon cancer (37). However, the role of SLC25A1 in thyroid cancer remains unclear. In the present study, IncBRM was demonstrated to sponge miR-331-3p and target SLC25A1. The knockout of SLC25A1 resulted in decreased TPC-1 cell migration and invasion, which was rescued by the overexpression of SLC25A1. Thus, SLC25A1 is involved in PTC progression. However, the role of SLC25A1 in other cancer types remains to be explored.

Despite several studies demonstrating the role of IncRNAs in promoting or inhibiting cancer progression (38-40), there are still unanswered questions. For example, most IncRNAs have no coding potential, however, some may regulate cancer progression by coding for small peptides. The ceRNA hypothesis suggests that most IncRNAs are implicated in the pathogenesis of cancer, by serving as miRNA sponges to modulate the expression of miRNA target genes (41). However, whether other mechanisms exist, requires further exploration.

In conclusion, the present study demonstrated increased expression of IncBRM in PTC tissues and cell lines. Bioinformatics analysis predicts IncBRM as a sponge for miR-331-3p that target SLC25A1. Overexpression of IncBRM promotes PTC cells proliferation, migration and invasion, which were rescued by ectopic expression of miR-331-3p or SLC25A1 knockout.

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Availability of data and materials

All data generated or analyzed during this study are included in the published article.

Authors' contributions

SL and XH designed the study, analyzed and interpreted the data, and wrote the manuscript. DZ, LC and SG performed some experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the protocol for this study was approved by the Institutional Ethics Committee of The People's Hospital of Tong Liang District and all enrolled patients signed a written informed consent document.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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