Lnc-FSD2-31:1 in Pancreatic ductal adenocarcinoma promotes ATG7-mediated proliferation and fibrosis of cancer associated fibroblasts via exosome miR-4736

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Research Article

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Abstract

Background

Long non-coding RNA (lncRNA) is closely related to the occurrence of Pancreatic ductal adenocarcinoma (PDAC). Intercellular interactions between cancer associated fibroblasts (CAFs) and cancer cells influence malignant proliferation and chemotherapy resistance of PDAC in a variety of ways. We performed the global expression profiles of lncRNA between longevity and short survival PDAC patients' pancreatic tissues, to identify molecular mechanism of PDAC.

Method

Tissue microarray (TMA) was used to explore survival related lncRNA in PDAC. The localization of lncRNA was confirmed by RNA fluorescence in-situ hybridization (FISH) analysis. Functional assays were introduced to demonstrate the anti-tumor effects of lncRNA *in vitro* and *in vivo*. Exosomes and primary CAFs were isolated from PDAC cell lines and patient tissues. Bioinformatics and 3D co-culture model were used to find the downstream targets. KPC transgenic mouse model and human cytokine array were also applied to detect the mechanism of PDAC.

Results

We found that lnc-FSD2-31:1 was elevated significantly in longevity survival patients. lnc-FSD2-31:1 suppressed PDAC proliferation, invasion, and metastasis *in vitro* and *in vivo*. lnc-FSD2-31:1 also act as a competing endogenous RNA (ceRNA) to inhibit miR-4736 expression in PDAC cells. Exosome miR-4736 secreted by PDAC was transported to CAFs. We found miR-4736 inhibited the proliferation, autophagy and promoted the fibrosis of CAFs by binding to the 3'-untranslated region(3'-UTR) of ATG7. Under the stimulatory of miR-4736, CAFs particularly secreted IL-32α, IL-1β, IL-1ra, G-CSF, IL-2, IL-4. Furthermore, miR-4736 antagomir intraperitoneally injected can suppress tumor growth in KPC mice. Exosome miR-4736 of plasma might be a biomarker of PDAC.

Conclusions

lnc-FSD2-31:1 suppress PDAC progression and act as a competing endogenous RNA (ceRNA) to inhibit miR-4736 expression in PDAC cells. Exosome miR-4736 released by PDAC inhibit autophagy and promote fibrosis of CAFs via targeting ATG7. miR-4736 might be a promising biomarker and treatment target of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a malignant tumor with high mortality and short survival. The 5-year survival rate of PDAC is only 10% and death rates is increasing[1]. Most of PDAC patients were diagnosed too late[2, 3], surgical resection and chemotherapy have limited benefits[2, 4, 5]. Therefore, the mechanism of proliferation and metastasis of PDAC has become studied, as the functions of non-coding RNA were revealed, researches on non-coding RNA in PDAC have been gradually carried out.

Long non-coding RNA (lncRNA) is defined as non-coding RNA with the length longer than 200 nucleotides, which could not encode proteins[6]. However, lncRNA can modulate protein structure, influence chromatin splicing, stability and translation[6, 7]. lncRNA not only influence nearby genes, but also affect distant genes in different ways[6]. Currently, some studies proved that lncRNA drive important cancer phenotypes and pathways[8–11], such as drug resistance[8], invasion[12], immunosuppression[13], proliferation and metastasis[14] in PDAC. Based on the close relationship between lncRNA and PDAC, we explored the relevant mechanism of lncRNA in PDAC, and found that lncRNA regulated PDAC by microRNA (miRNA).

MicroRNA is defined as non-coding RNA that consist of 19–25 nucleotides in length[15]. MiRNA participated in progression of cancer, some miRNAs show as tumor inhibitors, whereas some act as oncogenic factors[16]. MiRNA can suppressed target RNA by constitute the RNA-induced silencing complex (RISC) and binding with Argonaute (AGO) family protein[15, 17]. MiRNA can regulate progression of cancers and predict some diseases[15, 18–22]. In PDAC, miRNA can be an independent predictor of short overall survival after surgery[23] or metastasis to lymph node[24]. In our research, miRNA endocytosed into exosomes and regulated CAFs, exosome miRNA might be a potential biomarker of PDAC.

Exosome is a kind of extracellular vesicle with the size from 40–150 nm released by cells[25, 26]. HSP70, TSG101, ALIX, CD9, CD63, CD81 are the marker of exosomes[26]. Exosomes can cargo proteins and nucleotides[26], which have been proved promoted[27–30] or suppressed tumorigenesis[31–33]. Exosomes can detected in varieties of body fluids, including blood, urine, bile, ascites and so on[34, 35], therefore, they can be used as the novel templates for tumor diagnostic[36–38]. Multiple functions of exosomes in PDAC have been reported[29, 39–41], we found that exosomes serve as a “bridge” between PDAC and CAFs, and CAFs are important component of the tumor microenvironment (TME).

The extracellular matrix (ECM) is the acellular component in organs and tissues, and the existence core of multicellular organisms[42]. The delicate and complex interactive regulatory networks of cells in ECM consist of the TME[42]. Tumors and non-malignant cells co-regulate ECM, abnormal ECM in TME can induced the initialization or progression of tumor[42, 43]. Non-malignant cells in tumor including cancer-associated fibroblasts (CAFs), immune cells, and endothelial cells[44][45]. The most characteristics of PDAC is that the stroma component volume up to 90%, in which CAFs plays an irreplaceable role[46]. CAFs can resist cell death, avoid immune destruction, induce angiogenesis, promote proliferation, active invasion and metastasis in PDAC[46, 47]. The signal interaction between PDAC and CAFs has been reported[48–50], coincidentally, our research revealed that PDAC regulated CAFs via exosome miRNA.

Autophagy is the process that cells components are degraded by lysosomes, the main functions of autophagy are cytoplasm elimination, metabolites generation, and material transportation from cytoplasm to lysosome[51]. Autophagy can affect physiological and pathological processes of cells, it is closely relate to neurodegenerative diseases, cancers, inflammatory and autoimmune diseases[51]. ATG7, as the key gene in autophagy, is the downstream of exosome miRNA in our research.

In this study, transcriptome sequencing was performed to explore the differential lncRNAs between longevity and short survival PDAC patients. lnc-FSD2-31:1 was found to suppress PDAC progression, promote autophagy and inhibit fibrosis of CAFs *in vivo* and *in vitro*. lnc-FSD2-31:1 act as a competing endogenous RNA (ceRNA) to inhibit miR-4736 expression in PDAC cells. Exosome miR-4736 secreted by PDAC was transported into CAFs and bind with 3'-UTR of ATG7. Our study highlights the hallmark of exosome miR-4736 from plasma and miR-4736 might be a novel therapeutic target in PDAC.

Methods

Patients and tissues

Tissues (102 PDAC and 40 adjacent normal) from PDAC patients who underwent pancreatic resection in the department of pancreatic and biliary (The first Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China) from January 2014 to June 2021. The clinical characteristics of patients was listed in the Supplement Table 1. Our experiments were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

Cell lines and culture

Human pancreatic duct epithelial (HPDE) cells were purchased from the ATCC. BxPC-3, PANC-1, CFPAC-1, SW1990 and PANC-02 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were used within 6 months after ordered and cultured in DMEM (Gibco, USA) RPMI 1640 (HyClone, USA) or with 10% FBS (Gibco, USA), 1% penicillin/streptomycin with 5% CO₂ at 37 °C.

Transduction

Full-length of lnc-FSD2-31:1 and ATG7 was synthesized and subcloned into a lentiviral expression vector. The lentiviruses with scrambled shRNA against lnc-FSD2-31:1 and ATG7 were constructed (GeneChem, Shanghai China). Both control vector and negative control were transfected into cells to produce lentivirus, selected by 2.5µg/ml puromycin (Sigma, USA) for 2 weeks. The target sequences of the lentiviruses and shRNA listed in Supplementary Table 1.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from cells and tissues were extracted by the AxyPrep Multisource Total RNA Miniprep Kit from Axygen (Corning, Jiangsu, China). The first-strand cDNA was synthesized by the Rever TraAce qPCR RT

Master Mix with gDNA Remover Kit (FSQ-301, FSQ-101, TOYOBO, Japan). qRT-PCR (SYBR Green Assay, Roche Diagnostics GmbH, USA) was conducted on 7500 FAST Real-Time PCR System (Applied Biosystems). The relative expression levels of LncRNA, miRNA and mRNA were calculated using the $2^{-\Delta\Delta T}$ method. GAPDH and U6 was used as an endogenous control. The primer sequences were listed in Supplementary Table 2 and purchased from Comate Bioscience (Institute of Biotechnology, Jilin, China) and RIBOBIO (Guangdong, China).

Hematoxylin and eosin staining (H&E) and Immunohistochemical (IHC) staining

Specimens(human and mice) were fixed in 10% buffered formalin for 24h, embedded in paraffin and sectioned (5mm), stained with H&E, and examined via microscopy($\times 10$, Nikon, Japan). The sections were de-paraffinized and rehydrated, followed by incubating in 3% hydrogen peroxide for 15 minutes, rinsing in water and blocking in 2.5% normal horse serum. The sections were incubated with antibodies and captured by microscopy($\times 20$, Nikon, Japan). The antibodies listed in the Supplementary Table 3. The total number of positive pixels were counted in five fields and calculated by image J.

Fluorescence in situ hybridization (FISH)

FISH was performed with the Fluorescent In Situ Hybridization Kit (RiboBio, Guangdong, China). The sections were fixed in 4% formaldehyde for 10 minutes, permeabilized in PBS within 0.5% Triton X-100 at 4 °C for 5 minutes, washed with PBS three times for 5 minutes, and prehybridized at 37 °C for 30 minutes. Then the hybridization probes of Inc-FSD2-31:1(RiboBio), miR-4736 (GeneBiogist, ShangHai, China), U6(RiboBio), and 18S(RiboBio) was used in the hybridization solution at 37°C overnight. The second day, the sections were washed and counterstained with DAPI, and imaged with a fluorescence microscope ($\times 20$, Nikon, Japan).

MiR-4736 mimic, miR-4736 inhibitor and their negative control (RiboBio, Guangdong, China) were transiently transfected into cells by Lipofectamine 2000(Life Technologies Limited Paisley, Grand Island, NY, USA).

Western blot assays

The proteins extracted from cells, exosomes, and RNA immunoprecipitation using RIPA buffer (Beyotime, China). Total protein (20-40 μ g) was subjected to 8%, 10%, 12% SDS-PAGE and transferred to NC membranes. The membranes were blocked with 5% skim milk and incubated with antibodies overnight at 4 °C. The second day, the membranes were washed and incubated with secondary antibodies (Santa CruzBiotech, USA). The bands were visualized with the Molecular Imager System (BIO-RAD, USA). The antibodies listed in the Supplementary Table 3.

Transmission electron microscope

CAFs were fixed in 2.5% glutaraldehyde, 0.1M orthophosphoric acid washed three times, and postfixes in 1% osmium tetroxide buffer, 0.1M orthophosphoric acid washed three times again, dehydrated with

ethanol and acetone, then embedded in spur resin, and cut into thin sections. The sections were stained by the saturated solution of uranyl acetate and lead citrate. Sections were examined at 80 kV using the JEOL 1200EX transmission electron microscope.

Immunofluorescence (IF)

The sections (tissues or cells) were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton X-100 for 20 minutes, then incubated with antibodies

for 2 hours, washed with PBS for three times, incubated with secondary antibodies for 1 hour. Finally, The sections counterstained with DAPI and detected by a fluorescence microscopy(×20, Nikon, Japan). The antibodies listed in the Supplementary Table 3.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) was performed with RNA Immunoprecipitation (RIP) Kit (Bes5101, BersinBio™, China) by the manufacturer's protocols. In summary, CAFs were collected and lysed in lysis buffer, and then incubated with magnetic beads coated with anti-AGO2(Abcam), anti-ATG7(Cell Signaling Technology), or anti-IgG antibody. Afterwards, the immunoprecipitated RNAs were extracted as described above. The co-precipitated RNAs were then detected by qRT-PCR.

EdU-5-Ethynyl-2'- deoxyuridine assay

Cell proliferation experiments were performed with EdU assay kit (C10310-1/2/3, RiboBio, China). In summary, the cells were treated with 10 mmol/L EdU for 2 hours at 37 °C, fixed with 4% paraformaldehyde for 30 minutes, incubated with 2 mg/ml of glycine for 5 min, washed with PBS, treated with 0.5% Triton X-100 for 10 minutes, added 100μL 1X Apollo, washed with PBS again, Next, the cells were observed with by a fluorescence microscopy(×20, Nikon, Japan).

Wound healing assay

The monolayer cells cultured in 6-well plates and pretreated with mitomycin C (10μg/mL) for 2 h, scraped by a 200-μL pipette tip to produce linear regions, incubated in serum-free medium. Acquired the images at 0 h and 24 h by a microscopy(×10, Nikon, Japan).

Transwell assay

Transwell assay using 8μm pore size Falcon® inserts coated with or without Matrigel (BD Biosciences, USA), The cells were plated in the upper part and 500μl medium containing 10% FBS filled lower part. Incubating cells for 24h and removed the cells on the top surface of the filter after 48h. Cell count and photographs taken were performed under a microscopy (×20, Nikon, Japan).

Cell Counting Kit-8 (CCK-8) arrays

The cells from different groups were distributed into 96-well plates (100 μ L/well; 3000 cells/well) and incubated at 37°C with 5% CO₂. At the time points of 12, 24, 36, 48 and 60 hours of incubation, CCK-8 solution (10 μ L/well) was incubated with each well for 2 hours. The absorbance was measured at 450 nm (ELx808, BioTek, USA).

Dual Luciferase reporter assay

Lnc-FSD2-31:1 were divided into 4 fragments (including lnc-FSD2-31:1-WT 1-4) and lnc-FSD2-31:1-MUT (5), the fragments were cloned into the pGL3.10 vector, co-transfected with miR-4736 NC or miR-4736 mimic into PANC-1. The wild-type and two mutated binding sites of ATG7 were cloned into the pGL3.10 vector, and co-transfected with miR-4736 NC or miR-4736 mimic into 293T cells. After 48h, the luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega) and normalized using Renilla luciferase activity. The pGL3.10 plasmids were designed and purchased from GenScript (Nanjing, China). The sequences were listed in the Supplementary Information.

Human Cytokine Arrays

The supernatants from different CAFs panels were collected and remove other particulates by 0.2 μ m filter, then added into the human cytokine array panel A (ARY005, R&D system, USA), all procedures we performed followed the manufacturer's instructions. In briefly, Mixed supernatants, Buffer and Antibody Cocktail, incubated for one hour at room temperature, added into the blocked membranes, incubated overnight at 2-8 °C. Washed membranes and incubated with diluted Streptavidin-HRP at room temperature for 30 minutes, finally, added Chemi Reagent Mix and exposed membranes to X-ray film for 1-10 minutes.

Isolated and cultured primary CAFs

Collected and minced human PDAC fresh tissues, digested with enzymes (0.1mg/ml Hyaluronidase, 0.1mg/ml Dnase, 2mg/ml Collagenase), After the filtration and centrifugation, cells were plated on Poly-L-Lysine coated plates in DMEM/F-12+HEPES (Gibco, USA) with 20% FBS(Gibco, USA). The remaining minced tissues were cultured in DMEM/F-12+HEPES (Gibco, USA) with 20% FBS(Gibco, USA) directly. Cells were screened by differential adherent, identified by IF and flow cytometry.

Orthotopic xenograft models, Treatment of KPC transgenic mouse

The experiments protocol was approved by the Institutional Review Board of the First Affiliated Hospital of Harbin Medical University. Female BALB/c Nude mice of 6 weeks were purchased from Charles River company (Beijing). PANC-1 cells expressed with different level of lnc-FSD2-31:1 and CAFs expressed with different level of ATG7 by lentiviral transfection, Mixed at a 3:1 ratio. Anesthetized mice with intra-peritoneal injections of 2.5% 2,2,2-Tribromoethanol (Sigma-Aldrich, Shanghai), Mixed cells (10⁶cells/mouse) were orthotopically injected into the pancreas of nude mice. The mice were imaged weekly using the NightOWL II LB983 In vivo imaging system (BERTHOLD TECHNOLOGIES GmbH & Co.

KG, Germany). After 5 weeks, all mice were euthanized, recorded the weight of tumors and the number of abdominal metastases, all the specimens were fixed in 4% formaldehyde.

The C57BL/6-*Trp53em4(R172H)Krasem4(LSL-G12D)*Tg(Pdx1-cre)Smoc male mice of 8 weeks were purchased from Model Organisms (Shanghai). Intraperitoneally injected 50nmol miR-4736 antagomir/NC (RiboBio, Guangdong, China) into mice twice a week (until first dead), observed mice by MRI scan every two weeks. Except dead mice, the remaining mice were euthanized after 80 days treatments. Recorded the volume of tumors and fixed in 4% formaldehyde.

Flow cytometry analysis

The primary CAFs were digested by 0.25% of trypsin and blocked with 10%FBS, incubated with anti-FAP antibodies (Invitrogen,USA) for 1h, washed with PBS, incubated with IC Fixation Buffer (eBioscience, USA) for 1h, washed with PBS, incubated with anti- α -SMA antibodies (Abcam, Cambridge, UK) for 1h, washed with PBS, incubated with Mouse Anti-Rabbit IgM/APC antibody (Bioss, Beijing) and Rabbit Anti-Mouse IgG-Fc/PE antibody (Bioss, Beijing) for 1h, washed with PBS, analyzed through flow cytometer (NovoCyte 3110, Agilent, USA).

Exosome isolation and identification

PDAC cells were cultured 10 cm plates with 10%FBS (exosome removed). exosomes were isolated from PDAC cells supernatant by ultracentrifugation. Discard the cell debris after culture medium centrifugation at $300 \times g$, 2,000g and $10,000 \times g$, then the supernatant was centrifuged at $110,000 \times g$ for 70 min (all steps were performed at 4 °C), the pellet containing the exosome was collected and resuspended in PBS. Exosomes were observed by transmission electron microscopy (TEM) (JEOL 1200EX, Japan), quantified by western blot (the exosomes characteristic proteins Alix, CD63, and TS101) and Nano-Sight NS300 (Malvern Instruments Ltd, UK).

Statistical Analysis

Prism 7.04 (Graph Pad) and ImageJ software were applied to perform statistical and pictural analyses. Data were expressed as mean $\pm$ standard deviation. Kaplan–Meier survival analysis were used to evaluate overall survival. the comparison between two groups were analyzed using Student t-tests. Differences were considered significant when $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$; and non-significant when $P > 0.05$.

Results

Lnc-FSD2-31:1 overexpression correlated with longevity survival

To gain insight into the mechanism of lncRNA in PDAC, we explored the expression profiles of lncRNA between 3 longevity (> 5 years) and 3 short survival (< 6 months) patients' tissues of PDAC (Fig. 1a and 1b). There were 4,515 differentially expressed lncRNAs, sequenced the reciprocal of the down-regulated genes with the up-regulated genes. We verified top 10 lncRNAs expression level in 89 PDAC and 40 cancer adjacent tissues via Quantitative Realtime Polymerase Chain Reaction (qRT-PCR) (Fig. 1c, Supplement Fig. 1a and Table 1). Only lnc-FSD2-31:1 was found to be significantly elevated in longevity survival PDAC patients (Fig. 1c). Since lnc-FSD2-31:1 has not been studied, its non-coding properties were confirmed by coding-potential analysis[52, 53] (Supplement Fig. 1b and 1c). Moreover, the lnc-FSD2-31:1 expression in Human Pancreatic Ductal Epithelial (HPDE) cells was higher than four PDAC cell lines (BxPC-3, PANC-1, CFPAC-1 and SW1990) (Fig. 1e). Overall survival was statistically longer in high expression lnc-FSD2-31:1 cohort than in the low expression cohort (Fig. 1f). To sum up, lnc-FSD2-31:1 is highly expressed in longevity survival PDAC patients.

lnc-FSD2-31:1 inhibits tumor proliferation and metastasis in PDAC

RNA fluorescence in-situ hybridization (FISH) analysis confirmed that lnc-FSD2-31:1 located in the nucleus and cytoplasm (Fig. 2a and 2b). The results showed that HPDE cells and 4 PDAC cell lines were free from mycoplasma contamination (Supplementary Fig. 2a). According to the different expression levels of lnc-FSD2-31:1 in four tumor cells, lnc-FSD2-31:1 was effectively silenced in BxPC-3 and PANC-1, overexpressed in CFPAC-1 and SW1990 respectively through lentivirus transfection (Supplementary Fig. 2b-e). The proliferative capacity was determined by EdU assays, lnc-FSD2-31:1 silenced can increased proliferation capability in BxPC-3 and PANC-1 (Fig. 2c-e), lnc-FSD2-31:1 overexpression suppressed proliferation capability in CFPAC-1 and SW1990 (Fig. 2f-h). Cell Counting Kit-8 (CCK-8) assays also demonstrated that lnc-FSD2-31:1 silencing resulted in significantly increased BxPC-3 and PANC-1 proliferation (Fig. 2i and 2j), conversely, overexpressed lnc-FSD2-31:1 reduced CFPAC-1 and SW1990 proliferation (Fig. 2k-l). The migration and invasion capacities were assessed by wound healing and Transwell array, lnc-FSD2-31:1 silenced promoted BxPC-3 and PANC-1 migration and invasion (Fig. 2m and 2n, Supplementary Fig. 2f-h), overexpression lnc-FSD2-31:1 inhibited CFPAC-1 and SW1990 migration and invasion (Fig. 2o and 2p, Supplementary Fig. 2i-k). Immunohistochemical (IHC) staining performed that the Ki67 index from high lnc-FSD2-31:1 expression cohort was significantly decreased (Fig. 2q, Supplement Fig. 2l)[54–57]. These results demonstrate that lnc-FSD2-31:1 acts as a tumor suppressor gene that inhibits PDAC proliferation, invasion, and metastasis *in vitro*.

lnc-FSD2-31:1 in tumor cells regulate CAFs through exosomes

KEGG pathway analysis revealed that the longevity group had more signal transport genes enrichment compared with short survival group (Fig. 3a). There are various signaling transport ways in tumor, exosome is well known as a common intercellular signal transport way, that regulate tumorigenesis and progression of tumor[26–33]. To explore distribution of exosomes released by PDAC cells in vivo, we

simulated the release of exosomes and detected their localization in orthotopic xenograft model. First, we isolated exosomes by ultracentrifugation from PDAC (BxPC-3, PANC-1, CFPAC-1 and SW1990) cells, which observed under the transmission electron microscopy (TEM) (Fig. 3b). The markers of exosomes such as Alix, CD63, and TS101[25] were detected by western blot (WB) (Fig. 3c). The exosomes size and concentration were detected by Nanoparticle tracking analysis (Fig. 3d). Next, exosomes were incubated with the green fluorescence-stained PKH67, and then injected into xenograft mouse through tail vein (Supplementary Fig. 3a and 3b), after 4 hours later, we observed that exosomes were distribute in the pancreatic stroma (Fig. 3e). We performed co-localization staining for exosomes and alpha-smooth muscle actin (α -SMA) protein in sections, the results proved that co-localization existed between exosomes and α -SMA (Fig. 3f), which is a classical markers of CAFs[58]. Therefore, CAFs, as skeletal components in pancreatic stroma, might be the target of exosomes. In order to explore the function of CAFs more accurately and stably, we isolated primary CAFs from patient tissue (Fig. 3g and 3h). We performed immunofluorescence (IF) with α -SMA, pan-cytokeratin and CD-31 antibodies to identify isolated CAFs, the results showed that CAFs were only positive for α -SMA (Fig. 3i). The flow cytometry result manifested that 97.31% of the cells were simultaneous positive for Fibroblast activation protein α (FAP) and α -SMA(Fig. 3j), which are markers of CAFs [59]. These evidences confirmed the accuracy and purity of primary CAFs. Ultimately, 3D co-culture system was established to explore the relationship between exosomes with CAFs, and the role of lnc-FSD2-31:1 of PDAC in ECM (Fig. 3k). After 48h cultured, the IF results revealed that CAFs were significantly activated in lnc-FSD2-31:1 silenced group. Interestingly, this effect was blocked by the exosome inhibitor GW4869. lnc-FSD2-31:1 overexpressed group led to the opposite results and blocked by GW4869 too (Fig. 3l and 3m). lnc-FSD2-31:1 silenced group increased FAP, collagen 1, Fibronectin protein levels of CAFs, and lnc-FSD2-31:1 overexpressed group decreased these proteins levels, the effects were attenuated by GW4869(Fig. 3n and 3o, Supplementary Fig. 3c and 3d). In addition, CCK-8 and EdU assays showed that lnc-FSD2-31:1 silenced PANC-1 promoted CAFs proliferation via tumor organoid-conditioned media (CM), the effects also blocked by GW4869, lnc-FSD2-31:1 overexpressed PANC-1 displayed the contrary results (Fig. 3p-u). In conclusion, our exploration proves that lnc-FSD2-31:1 in PDAC cells can regulate the fibrosis and proliferation of CAFs through exosomes.

lnc-FSD2-31:1 in PDAC cells affect ATG7 levels of CAFs through exosomes

To explore the downstream of lnc-FSD2-31:1, we performed RNA sequencing (RNAseq) analysis of 3 pairs CAFs cultured by exosomes from PANC-1 with different lnc-FSD2-31:1 expression, there are 93 mRNAs expressed differentially. Sequenced the genes and considered p-value, ATG7 mRNA was the most differentially expressed (Fig. 4a), ATG7 is a key autophagic protein[51], our team have reported relationship between PDAC and autophagy[60]. Meanwhile, KEGG pathway analysis revealed that the lnc-FSD2-31:1 control group had more autophagy genes enrichment compared with lnc-FSD2-31:1 silenced group (Fig. 4b). We divided exosomes into 4 groups based on different lnc-FSD2-31:1 level in PDAC, exosomes 1 (lnc-FSD2-31:1 control), exosomes 2(lnc-FSD2-31:1 silenced), exosomes 3(vector), exosome 4(lnc-FSD2-31:1 over expression). CAFs were cultured with exosomes 1-4 respectively, after 24 h, qRT-

PCR conformed that ATG7 mRNA expression in CAFs was positively correlated Inc-FSD2-31:1 level in PDAC (Supplement Fig. 4a). To verify whether Inc-FSD2-31:1 is related to autophagy of CAFs. We extracted protein of CAFs cultured by exosomes 1–4, LC3 (micro-tubule-associated protein 1 light chain 3) related to autophagosome formation and p62 represented degradation of autophagosomes[51, 61], intriguingly, contrast to exosomes 1, exosomes 2 could reduce ATG7 protein, LC3B II/I protein, and increased p62 protein, On the contrary, for exosomes 3, exosomes 4 could increase ATG7 protein, LC3B II/I protein, and reduced p62 protein (Fig. 4c, Supplementary Fig. 4b). To assess the co-effects of Inc-FSD2-31:1 and ATG7 *in vivo*, orthotopic xenograft models were performed (Fig. 4d and 4e). ATG7 was effectively knockdown and overexpressed by lentivirus transfection (Supplementary Fig. 4c and 4d). In the first model, we co-injected Inc-FSD2-31:1 NC or OE PANC-1 cells with ATG7 NC# or KD CAFs. In the second model, we co-injected Inc-FSD2-31:1 NC# or KD PANC-1 with ATG7 NC or OE CAFs. At a 3:1 ratio of PANC-1 and CAFs orthotopic injection, we observed the tumor growth weekly and recorded end of week 5 (Fig. 4f-h). Compared to PDAC NC + CAF NC# group, the tumor of PDAC OE + CAF NC# group increased relatively slowly (Fig. 4f). High Inc-FSD2-31:1 expression in PDAC inhibited the tumor proliferation, that the weight of tumors was smaller and the number of abdominal metastases was lower (Fig. 4h-k). However, when ATG7 was knockdown in CAFs, the inhibition effects were disappeared (Fig. 4g-j). Compared to PDAC NC#+CAF NC group, the group of PDAC KD + CAF NC increased relatively faster (Fig. 4f). Low Inc-FSD2-31:1 expression in PDAC promoted the tumor proliferation, the weight of tumors was higher and the number of abdominal metastases was much more (Fig. 4g-j), in addition, 2 of 6 model developed liver metastases in PDAC KD + CAF NC group (Fig. 4k). Nevertheless, when ATG7 was overexpressed in CAFs, the promotion effects were disappeared (Fig. 4g-j). IHC analysis was performed for the orthotopic tumor specimens. Compared to PDAC NC + CAF NC# group, the group of PDAC OE + CAF NC# decreased levels of Ki67, α -SMA, collagen 1, Fibronectin and p62, increased levels of ATG7 and LC3, when ATG7 was knockdown in CAFs, the trends were fade (Fig. 4l, Supplement Fig. 4e-k). Moreover, compared to PDAC NC#+CAF NC group, the group of PDAC KD + CAF NC increased levels Ki67, α -SMA, collagen 1, Fibronectin and p62, decreased levels of ATG7 and LC3, when ATG7 was overexpressed in CAFs, the trends were also fade (Fig. 4m, Supplement 4d-j). Collectively, these data suggested that Inc-FSD2-31:1 in PDAC affect ATG7 expression in CAFs through exosomes, The Inc-FSD2-31:1 level in PDAC can influence tumor proliferation, fibrosis, and autophagy levels in orthotopic xenograft models, and this effect would be significantly weakened by ATG7 levels in CAFs, indicating that ATG7 in CAFs is the downstream target of Inc-FSD2-31:1 in PDAC.

MiR-4736 is the direct downstream target of Inc-FSD2-31:1 in PDAC

To explore whether Inc-FSD2-31:1 target CAFs through exosomes, we detected the expression of Inc-FSD2-31:1 in exosomes isolated from PDAC, surprisingly, qRT-PCR showed that there was rarely Inc-FSD2-31:1 (Fig. 5a). The result indicated that Inc-FSD2-31:1 in PDAC might transcellular regulate CAFs by others in exosomes. Evidences suggested that miRNA is the most abundant in exosomes[15]. Consequently, we attempted to find the “bridge” that connect Inc-FSD2-31:1 and ATG7, the IncRNASNP2 and Targetscan database was employed and predicted that 17 miRNAs could bind to Inc-FSD2-31:1 and

ATG7 simultaneously (Fig. 5b). We detected the expression levels of 5 miRNAs in exosomes from different Inc-FSD2-31:1 expression PDAC, it showed that only miR-4736 was negatively correlated with Inc-FSD2-31:1 (Fig. 5c). The images of dual RNA-FISH confirmed co-localization of Inc-FSD2-31:1 and miR-4736 in PANC-1 (Fig. 5d). Subsequently, the miR-4736 mimic and inhibitor were introduced (Supplement Fig. 5a and 5b). The RNA immunoprecipitation (RIP) assay in PANC-1 showed that Inc-FSD2-31:1 and miR-4736 were enriched dramatically in anti-AGO2 pellets than in anti-IgG pellets (Fig. 5e), Inc-FSD2-31:1 expression in anti-AGO2 pellets increased/decreased from PANC-1 that transfected with miR-4736 mimic/inhibitor (Fig. 5f), it indicating that Inc-FSD2-31:1 act as a ceRNA to sponge miR-4736 in PDAC cells. LncRNASNP2 and Rtips were employed to identify the potential binding sites (Fig. 5g and 5h). We designed 5 gene fragments of Inc-FSD2-31:1, including Inc-FSD2-31:1-WT (1–4) and Inc-FSD2-31:1-MUT (5), co-transfected the dual luciferase reporter plasmids with miR-4736 NC or miR-4736 mimic into PANC-1 (Fig. 5i). The results manifested that the miR-4736 mimic lowered the luciferase activity of pGL3-Inc-FSD2-31:1-WT2 but not the others (Fig. 5j). Overall, we proved that Inc-FSD2-31:1 act as a ceRNA to directly inhibit miR-4736 expression in PDAC cells.

MiR-4736 directly target ATG7 to regulate the fibrosis and autophagy of CAFs

To further explore the relationship between miR-4736 and ATG7 in CAFs, we transfected miR-4736 mimic or inhibitor into CAFs, the results showed that ATG7 mRNA were negatively expressed (Supplement Fig. 6a). The FISH-IF displayed that miR-4736 and ATG7 proteins were co-located in CAFs (Fig. 6a). The RIP assay in CAFs revealed that miR-4736 and ATG7 were enriched obviously in anti-AGO2 pellets than in anti-IgG pellets (Fig. 6b), and miR-4736 was enriched in anti-ATG7 pellets than in anti-IgG pellets (Fig. 6c), means that miR-4736 could bind with 3'-UTR of ATG7 mRNA to directly inhibit expression at the post-transcriptional level. Targetscan and Rtips were employed and predicted two potential binding sites in ATG7 mRNA, which bind to miR-4736 (Fig. 6d). The wild-type and two mutated binding sites were cloned into the dual luciferase reporter plasmids, and co-transfected with miR-4736 NC or miR-4736 mimic into CAF cells. The results showed that the miR-4736 mimic lowered the luciferase activity of pGL3-ATG7 WT and pGL3-ATG7 MUT2, other than the pGL3-ATG7 MUT1 group, it suggested that only the prediction site 1 of ATG7 can bind to miR-4736 (Fig. 6e). Subsequently, the siRNA against ATG7 and an ATG7 overexpression plasmid were established (Supplement Fig. 6b-e). In western blot, the miR-4736 inhibitor reduced fibrosis of CAFs, with lower protein expression of Fibronectin, collagen 1, FAP and α -SMA, simultaneously, it increased autophagy levels, with a higher of ATG7, LC3B II/I and lower p62 protein expression, however, ATG7 siRNA would rescue these effects (Fig. 6f and 6g). Consistently, the miR-4736 mimic and ATG7 overexpression plasmid showed the opposite results (Fig. 6h and 6i). Next, we performed IF double staining to observed the dynamic changes of fibrosis and autophagy, the miR-4736 inhibitor result in attenuation of α -SMA, collagen 1, Fibronectin signing and enhancement of ATG7 signing, and ATG7 siRNA could abolish those function (Fig. 6j). The miR-4736 mimic also showed the opposite results (Fig. 6k). In CCK-8 arrays, miR-4736 inhibitor decreased proliferation of CAFs, ATG7 siRNA rescued the effect (Supplement Fig. 6f), whereas miR-4736 mimic increased the proliferation, ATG7 overexpression plasmid rescued the effect (Supplement Fig. 6g). To further confirm the effect of miR-

4736 on CAFs autophagy, we observed the account of autophagosomes in CAF cytoplasm by TEM (Fig. 6l). It shown that the number of autophagosomes was increased after transfected with miR-4736 inhibitor and decreased after transfected with miR-4736 mimic. Under the signal of exosome miR-4736-ATG7 axis stimulus, we explored whether CAFs can affect PDAC by human cytokine array. We divided CAFs into two panels, one panel transfected miR-4736 mimic or miR-4736 NC, another panel co-transfected miR-4736 mimic/NC with ATG7 plasmid. After 48h cultured, the supernatant was collected for cytokine array. The results revealed that miR-4736 mimic panel released more IL-32 α , IL-1 β , IL-1ra, G-CSF, IL-2, IL-4, sICAM-1 cytokine, interestingly, when co-transfected with ATG7 plasmid, the trend was significantly abrogated except sICAM-1 (Fig. 6m and 6n), meanwhile, these cytokines have been reported that can regulate PDAC[62–68]. Taken together, our research found Inc-FSD2-31:1 (PDAC) - miR-4736(exosome) - ATG7(CAFs) axis can regulate the fibrosis and autophagy functions of CAFs, whereafter, CAFs released cytokines to affect PDAC.

MiR-4736 might be a potential therapeutic and predictive target of PDAC

Due to the broad and critical roles of miRNA, the treatment research on the application of miRNA is gradually carried out[69]. Antagomir miRNA can prevents the complementary pairing of miRNA, it is regarded as a precise and effective targeted therapy[70–72]. We demonstrated that the presence of miR-4736 in mouse pancreatic cancer cell lines PANC-02 and KPC (LSL-Kras^{G12D/+}, LSL-Trp53^{R172H/+}, Pdx-1-Cre) transgenic mouse pancreatic cancer tissue via FISH and qRT-PCR (Fig. 7a, supplement Fig. 7a-c). To explore the therapeutic effect of miR-4736 in vivo, we intraperitoneally injected miR-4736 antagomir into KPC (LSL-Kras^{G12D/+}, LSL-Trp53^{R172H/+}, Pdx-1-Cre) transgenic mouse. Twelve KPC mice were equally divided into experimental group and control group, which intraperitoneally injected 50nmol miR-4736 antagomir/NC twice a week (until first dead), observed by MRI scan every two weeks. At the 58th, 66th, 71th, 81th day after first treatment, four control KPC mice were dead, at the 73th day, one experimental mouse dead, at the 84th, the remaining seven mice were sacrificed (Fig. 7b). The miR-4736 antagomir treated animals showed stagnancy in tumor growth (Fig. 7c and 7d). It was significantly decreased in harvested tumor volume in the experimental group receiving miR-4736 antagomir treatment (Fig. 7c-f). HE sections of pancreatic tumors showed significant decrease of tumor burden in the experimental group (Fig. 7g and 7h). Contrast to the control group, IHC images displayed that the experimental group decreased levels of Ki67, p62, α -SMA, collagen 1 and Fibronectin, increased levels of ATG7 and LC3B II/I obviously (Fig. 7i and 7j). In addition, we isolated exosomes from plasma of 21 nonmalignant patients and 15 PDAC patients, and qRT-PCR confirmed that the plasma exosome miR-4736 from PDAC patients were obviously higher than nonmalignant patients (Fig. 7k). Intriguingly, there is no significantly different in urine exosome miR-4736 between them (Fig. 7l). Collectively, the evidences suggest that miR-4736 might be a potential therapeutic and predictive target of PDAC.

Discussion

In this study, we have identified a novel long noncoding RNA, lnc-FSD2-31:1, which aberrant overexpression is corresponding with longevity survival PDAC patients. lnc-FSD2-31:1 was proved that reduced the PDAC burden, in addition, it acts as a ceRNA to inhibit miR-4736 expression in PDAC cells. Exosomes miR-4736 were secreted by PDAC cells and endocytosed into CAFs, miR-4736 suppressed ATG7 protein expression by binding to the 3'UTR region of ATG7 in CAFs. Further experiments demonstrated that miR-4736 inhibited the autophagy, promoted the proliferation and fibrosis of CAFs, even more, under the stimulated of these pathway, CAFs secreted IL-32 α , IL-1 β , IL-1ra, G-CSF, IL-2, IL-4 particularly, it may indicate that CAFs impact the progression of PDAC in turn. In addition, miR-4736 antagomir intraperitoneally injected could suppress tumor progression in KPC mice, and exosomes miR-4736 expressed extraordinary highly in plasma of PDAC patients, the data means that miR-4736 might be a potential therapeutic and predictive target of PDAC (Fig. 8a).

lnc RNA is an important regulator of PDAC[11, 14, 73, 74], our previous studies have found that lnc-MALAT1 promotes PDAC proliferation and metastasis[14]. In this study, we identified a novel lnc RNA lnc-FSD2-31:1 through RNAseq, we did not find any report and even genetic sequence in NCBI, its sequence was just searched on lncipedia database, we proved that lnc-FSD2-31:1 do not have coding capability. lnc-FSD2-31:1 is positively correlated with longevity survive of PDAC by tissue validation. *In vitro* and *in vivo* experiments, it was found that lnc-FSD2-31:1 can inhibit proliferation, metastasis, and invasion functions of tumor cells. Based on the KEGG analysis, we adopted the 3D co-culture system and orthotopic xenograft model to prove that lnc-FSD2-31:1 in PDAC cells can regulate the fibrosis and proliferation of CAFs through exosomes.

CAF, as an important cell in pancreatic TME, has been extensively studied. In the early time, CAFs have been considered a tumorigenic cell[75–77], but targeted therapies that inhibit CAFs have failed in PDAC[78], it remind us that CAFs may be more complex than we thought before. Elyada, E. et al conducted single-cell RNA sequencing in human PDAC tumor, and heterogeneity of CAFs were confirmed[79]. The heterogeneity of CAFs are mainly affected by PDAC signals, Biffi, G. et al showed that IL1 signals from PDAC induced iCAFs and TGF β induced myCAFs[68]. In our research, it was found that lnc-FSD2-31:1 level change in PDAC can alter ATG7 expression in CAFs, while it is known that ATG7 protein plays an important role in autophagy regulation[80]. *In vivo* experiments, increased ATG7 expression in CAFs inhibit tumor proliferation, while reduced ATG7 expression can accelerate tumor proliferation. It reflected that CAFs is heterogeneous under the change of environment. In addition, the mechanism of CAF fibrosis and autophagy negative modulation is still unclear, Autophagy may accelerate the reduction of the numbers of CAFs, and then reduce the level of fibrosis, but there may also be a complex regulatory mechanism between them, so it requires further research. We also proved that lnc-FSD2-31:1 from PDAC can affected the proliferation, autophagy, and fibrosis function of CAFs through exosomes. Currently, there is no study on the regulation of CAF by PDAC through lncRNA, it showed that our research is innovative. Interestingly, under the signals from PDAC, CAFs particularly released IL-32 α , IL-1 β , IL-1ra, G-CSF, IL-2, IL-4, which have been reported that influence the PDAC cells[62–66]. Therefore, our studies prove that PDAC can regulate CAFs, and CAFs may also have an impact on

PDAC, but more research are needed on whether or how the synergistic/countervailing effects worked between them.

ATG7 in CAFs is the target of exosomes in our study. The ATG gene family was found to mediate the formation of membrane structures that transport cytoplasmic contents to lysosomal degradation, they are the key genes for autophagy[81]. Autophagy was traditionally believed that positively correlated with the occurrence and development of cancer[82]. Hydroxychloroquine and chloroquine of autophagy inhibitor have been used for cancer treatment[82, 83], including PDAC[80, 82–84]. However, some studies found that inhibition of autophagy significantly accelerated tumor formation in the PDAC mouse model[85, 86]. At present, most studies focused on the autophagy in tumor cells, however, pancreatic tumors differ from other tumors in that they are rich of stromal cells, thus, the autophagy of CAFs have also aroused people's attention. Sousa, C. et al report that the autophagy in CAFs, which promote pancreatic tumors growth[87], Endo, S. et al also found that autophagy is required for PSC activation and is associated with the progression of PDAC[88]. Strangely, we found the contradictory results that CAFs autophagy was negatively correlated with fibrosis, under the signal from PDAC. In orthotopic xenograft models of PDAC, overexpressed lnc-FSD2-31:1 in tumor cells plays a cancer suppressor, but this effect is blocked when ATG7 inhibited in CAFs, ATG7 also suppress PDAC progression independently (Fig. 4f-h). We suspected that CAFs may regulate their fibrosis level by reducing their number via autophagy, thus triggering the effect of inhibiting tumor proliferation, however, the heterogeneity of CAFs cannot be excluded, the regulatory relationship between them needs to be further explored.

We found lnc-FSD2-31:1 in PDAC can regulate autophagy and fibrosis of CAFs via exosomes, which act as a “bridge” between PDAC cells and TME. Exosomes can play a crucial role in intercellular transportation and signal transduction[25, 26], they influence the initiation, progression and treatment of cancer[89]. Recently, Stefanius, K. et al report that exosomes from PDAC initiate cell transformation by stimulating NIH/3T3 recipient cells[90]. Xie, Z. et al found the exosome CD44v6/C1QBP derived from PDAC formulate the hepatic metastasis microenvironment[40]. As an important carrier, the functions of exosomes are realized through their contents, and miRNA is one of the most abundant cargo[91]. MiRNA has been discovered less than three decades[92], but its structures, mechanisms, and functions have been understood[15], miRNA plays an important role in the whole process of tumor, including promoting or suppressing cancer[15]. In our research, we identified that miR-4736 was endocytosed by exosomes in PDAC and target ATG7 of CAFs, which could inhibit CAFs autophagy and promote CAF fibrosis. Based on the important role of miRNA, the treatment on the application of miRNA is gradually carried out. MiRNA has its unique advantages, when used to therapy. Firstly, miRNA is a naturally existing molecules structure in body, unlike artificial chemical, secondly, a miRNA can target multiple genes via one pathway, resulting in a broader effects[69], beside, it also has good bioavailability, solubility, and metabolic stability in body[93]. Now, there are three miRNA mimic or anti-miRNA clinical research on phase II or III (NCT02826525, NCT03601052, NCT0120042). In our experiment, we intraperitoneally injected miR-4736 antagomir into KPC mice, and identified anti-miR-4736 may be a choice of treatment strategy for pancreatic cancer. However, there are also disadvantages of miRNA therapy. Firstly, human immune system can recognizes the miRNA by pathogen-associated molecular pattern, the miRNA immunogenicity

may induce immune protection of the body, secondly, miRNA may have side effects for targeting multiple genes, beside, unexpected on-target effects and off-target effects may exist[69, 94]. Coincidentally, exosomes, like a biomedical drug capsule, can compensate for disadvantages of miRNA therapy. Exosomes possess low immunogenicity, excellent cellular uptake mechanism, stability, pharmacokinetics, better biocompatibility and targetability[8, 95]. Poggio, M. et al certified that blockade of exosome PD-L1 prolongs survival of mouse by promoting anti-tumor immunity[96]. Zhou, W. et al designed that used exosomes from immunogenically dying PDAC cells for vaccination against PDAC[97]. Imaginatively, Sancho-Albero M. et al developed anticancer drug Panobinostat, it was a bio-artificial device that loaded Pd catalysts nanosheets into cancer-derived exosomes, and showed priority access to progenitor cells[98]. Moreover, exosomes can be detected in all kinds of body fluids, so they have great potential and advantages in disease diagnosis[89]. Meanwhile, miRNA can used as an independent predictor, such as short overall survival after surgery[23] and metastasis to lymph node[24]. In our study, exosomes miR-4736 expression is significantly elevated in blood plasma of PDAC patients, which might be a potential biomarker for diagnosing PDAC. Pang, Y. et al have reported Dual-SERS biosensor to detect early pancreatic cancer by one-step test of exosome miRNA in blood[99]. Li, S et al also invented a sandwich-based evanescent wave fluorescent biosensor for exosome detection simply and rapidly[100]. From the current research progression, the detection of exosome in the future may become more conveniently, effectively, fast, and practically.

Conclusions

We identified a novel LncRNA Inc-FSD2-31:1 that can suppress PDAC progression and acts as a ceRNA to inhibit miR-4736 expression in PDAC cells. Exosomes miR-4736 are transport in CAFs to abrogate ATG7 protein expression, that inhibit autophagy, promote proliferation and fibrosis of CAFs. Meanwhile, CAFs particularly release cytokines under miR4736 stimulatory. Our findings highlight plasma exosomes miR-4736 might be a potential biomarker of PDAC and miR-4736 antagomir intraperitoneally injected could suppress tumor growth in KPC mice, it may provide a new therapeutic target for PDAC.

Abbreviations

PDAC
pancreatic ductal adenocarcinoma
lncRNA
Long non-coding RNA
FISH
RNA fluorescence in-situ hybridization
IF
immunofluorescence
RIP
RNA immunoprecipitation

CAFs
cancer-associated fibroblasts
3'-UTR
3'-untranslated region
miRNAs
MicroRNAs
RISC
RNA-induced silencing complex
AGO
Argonaute
TME
tumor microenvironment
ECM
extracellular matrix
qRT-PCR
Quantitative Realtime Polymerase Chain Reaction
HPDE
Human Pancreatic Ductal Epithelial
IHC
Immunohistochemical
CCK-8
Cell Counting Kit-8
KEGG
Kyoto Encyclopedia of Genes and Genomes enrichment analysis
TEM
transmission electron microscopy
WB
western blot
 α -SMA
alpha-smooth muscle actin
FAP
Fibroblast activation protein
CM
organoid-conditioned media
RNAseq
RNA sequencing
KPC
LSL-Kras^{G12D/+}, LSL-Trp53^{R172H/+}, Pdx-1-Cre
PSC
pancreatic stellate cells.

Declarations

Ethics approval and consent to participate

PDAC and normal pancreatic tissues were acquired from patients undergoing a surgical procedure at The First Affiliated Hospital of Harbin Medical University (Harbin, China). Patient consent was obtained prior to the initiation of the study.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

XG and LL conceived of the presented idea and researched the background of the study. YL, JH, ZZ, CC, TZ, WY, YZ, LL, YX, GL, and DL prepared the figures and tables. XB, GW, HC, BS, and HC wrote the manuscript. All the authors read and approved the final manuscript.

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Tables

Table 1 is available in the Supplemental Files section.

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