Original Paper

ALKBH5 Inhibits Pancreatic Cancer Motility by Decreasing Long Non-Coding RNA KCNK15-AS1 Methylation

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Key Words

Long non-coding RNA • Pancreatic cancer • KCNK15-AS1 • ALKBH5 • m⁶A

Abstract

Background/Aims: Mounting evidence suggests that epitranscriptional modifications regulate multiple cellular processes. N⁶-Methyladenosine (m⁶A), the most abundant reversible methylation of mRNA, has critical roles in cancer pathogenesis. However, the mechanisms and functions of long non-coding RNA (lncRNA) methylation remain unclear. Pancreatic cancer resulted in 411,600 deaths globally in 2015. By the time of pancreatic cancer diagnosis, metastasis has often occurred in other parts of the body. The present study sought to investigate lncRNA m⁶A modification and its roles in pancreatic cancer. **Methods:** Differential expression between cancer cells and matched normal cells was evaluated to identify candidate lncRNAs. The lncRNA *KCNK15-AS1* was detected in cancer tissues and various pancreatic cells using RT-qPCR. *KCNK15-AS1* was transfected into cells to explore its role in migration and invasion. Then, m⁶A RNA immunoprecipitation was performed to detect methylated *KCNK15-AS1* in tissues and cells. Epithelial-mesenchymal transition (EMT) markers were used to evaluate *KCNK15-AS1*-mediated EMT processes. **Results:** *KCNK15-AS1* was downregulated in pancreatic cancer tissues compared with paired adjacent normal tissues. *KCNK15-AS1* inhibited migration and invasion in MIA PaCa-2 and BxPC-3 cells. Furthermore, total RNA methylation in cancer cells was significantly enriched relative to that in immortalized human pancreatic duct epithelial (HPDE6-C7) cells. In addition, the m⁶A eraser ALKBH5 was downregulated in cancer cells, which can demethylate *KCNK15-AS1* and regulate *KCNK15-AS1*-mediated cell motility. **Conclusion:** Our results have revealed a novel mechanism by which ALKBH5 inhibits pancreatic cancer motility by demethylating lncRNA *KCNK15-AS1*, identifying a potential therapeutic target for pancreatic cancer.

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Introduction

Pancreatic cancer is one of the most aggressive types of cancer and is the sixth leading cause of cancer-related deaths in China [1] and the seventh worldwide [2], respectively. It causes 330,000 deaths annually and constitutes a notable global health problem [2]. Pancreatic cancer has an extremely poor prognosis, with a median survival of 5 to 8 months (less than 10% survival rate). Pancreatic cancer develops with variable symptoms, local invasiveness, or metastases to distant sites in its early stages [3]. Accordingly, it is important to investigate the mechanisms that contribute to metastasis in pancreatic cancer.

Long non-coding RNAs (lncRNAs) are non-coding RNAs comprising more than 200 nucleotides that lack protein coding capacity. Mounting evidence has demonstrated that lncRNAs play important roles in cancer cell proliferation, apoptosis, and metastasis. Although *HOTAIR* [4], *HOTTIP* [5], and *MALAT1* [6] have been reported in pancreatic cancer studies, the mechanisms by which lncRNAs regulate pancreatic cancer require further exploration.

More than 100 RNA modifications have been reported [7], but there are few reports on lncRNA modifications. *N*⁶-methyladenosine (*m*⁶A) modification is the most common internal modification in eukaryotic mRNAs [8]. Two recently identified mammalian RNA demethylases, the fat mass and obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5), have demonstrated that the *m*⁶A methylation modification is a dynamic and reversible process. However, the mechanism by which FTO and ALKBH5 regulate lncRNA methylation requires further investigation.

In this study, we demonstrate that the lncRNA *KCNK15-AS1* is significantly downregulated in pancreatic cancer, revealing its inhibitory functions on cell migration and invasion. Additionally, the *m*⁶A eraser ALKBH5 was found to be downregulated in cancer tissues, where it could demethylate *KCNK15-AS1* and regulate *KCNK15-AS1* expression. ALKBH5 was also found to be involved in *KCNK15-AS1*-mediated cell migration and invasion. In the future, ALKBH5-*KCNK15-AS1* might serve as a therapeutic target for patients with pancreatic cancer.

Materials and Methods

Clinical samples

Fresh frozen pancreatic cancer tissues and their adjacent normal tissues were obtained from the First Affiliated Hospital of Nanjing Medical University. The samples were obtained within 10 min after tumor excision and immediately stored at -80°C until use in experiments. Written informed consent was obtained from all patients, and this study was approved by the Ethics Committee of the hospital (2017-SR-171).

Microarray analysis

Total RNA was extracted from three pancreatic cancer tissues and paired adjacent normal tissues, and the expression profile of 30,584 human lncRNAs was determined using Arraystar Human lncRNA Microarray V3.0. Volcano plot filtering, set at a threshold of ≥2.0-fold, was used to screen for lncRNAs that exhibited significantly different (*P* < 0.05; unpaired *t*-test) expression levels in the two study groups.

Cell culture and cell transfection

Human pancreatic cancer cell lines (MIA-PaCa-2 and BxPC-3) and human embryonic kidney cells (293T) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and the immortal human pancreatic duct epithelial cell line (HPDE6-C7), which is considered a normal pancreatic cell line, was obtained from the Pancreas Institute, Nanjing Medical University. HPDE6-C7, MIA-PaCa-2, and BxPC-3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. All cell lines were cultured in an atmosphere of 5% CO₂ and 99% relative humidity at 37°C. Lipofectamine 2000™ was applied to conduct transfections in accordance with the manufacturer's guidelines (Invitrogen, Carlsbad, CA).

RNA isolation and reverse transcription

Total RNA was isolated from cells and tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The NanoDrop ND2000 (Thermo Scientific Inc., Waltham, MA) was used to determine RNA purity and to quantify RNA concentration. A First Strand cDNA Synthesis Kit (TaKaRa, Beijing, People's Republic of China) was used to perform reverse transcription.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) primers were obtained from GenScript (Nanjing, People's Republic of China), and the sequences are listed in Table 1. According to the manufacturer's instructions, RT-qPCR was performed using the SYBR Prime-Script RT-PCR Kit (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China) with a Roche LightCycler 96 fluorescent quantitative PCR system (Roche, Mannheim, Germany). The reaction started at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. Actin was used as the internal control to normalize qPCR results. Relative gene expression levels were measured using cycle threshold (CT) values in the $\Delta\Delta CT$ calculation [4].

Cell migration and invasion assay

Transwell assays were conducted with an 8- μ m pore size insert with or without Matrigel Invasion Chambers (Millipore Corporation, Burlington, MA) to evaluate the properties of migration or invasion of pancreatic cells. Then, 48 h post-transfection, single-cell suspensions were seeded into the upper chamber, and the lower chamber was filled with 500 μ L of Dulbecco's Modified Eagle's Medium containing 10% FBS. After the indicated incubation time, the non-migrating or invading cells were wiped from the upper surface of the membranes, and cells were stained with 2% crystal violet solution after fixation in methanol. Images of migrated cells in each well were captured and counted in random fields using a microscope. Each experiment was conducted in triplicate.

Western blotting and antibodies

Total protein (60 μ g) was subjected to SDS-PAGE and transferred to a 0.45- μ m PVDF membrane (Millipore Corporation). Antibodies against ALKBH5 (Abcam, Cambridge, MA; 1:100) and actin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000) were used. Membranes were incubated overnight at 4°C with primary antibodies diluted 1:1, 000 to 1:10, 000 according to the manufacturers' instructions, and proteins were detected with a peroxide LumiGLO reagent (Cell Signaling Technology, Danvers, MA).

RNA immunoprecipitation

RNA immunoprecipitation (RIP) experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore Corporation). Antibodies against m⁶A (Abcam) or control proteins were diluted to 1:50. Total RNA (input control) and precipitation with the isotype control (IgG) for each antibody were assayed simultaneously. The co-precipitated RNAs were detected by qRT-PCR.

Confocal microscopy

Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min and permeabilized with 0.1% Triton-X and 1% BSA in PBS for 30 min at room temperature. DNA was stained using 4',6-diamidino-2-phenylindole (DAPI). *KCNK15-AS1* antisense probe hybridization was performed at 37°C for about 16 h to detect *KCNK15-AS1*. The sequences of probes are listed in Table 2. Imaging was performed using a Zeiss Axiovert 200M laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Table 1. Primers used for RT-qPCR

Primer	Sequence
ALKBH5	F: 5'-TGAGCACAGTCACGCTTCCC-3'
	R: 5'-TCCGTGTCTTCTTTAGCGACTC-3'
KCNK15-AS1	F: 5'-TAAAGCCATCACCAGGACAAC-3'
	R: 5'-ATACATTTGGCTCCCATGGCTA-3'
β -actin	F: 5'-GTGGCCGAGGACTTTGATTG-3'
	R: 5'-CCTGTAACAACGCATCTCATATT-3'

Statistical analyses

Values are presented as means $\pm$ SD. Results were analyzed using unpaired Student's *t*-tests. Values of $P < 0.05$ were considered statistically significant.

Results

lncRNA KCNK15-AS1 is downregulated in pancreatic cancer tissues and cells

To acquire the transcriptional profile of lncRNAs in pancreatic cancer, paired pancreatic cancer tissues and normal tissues ($n = 3$) were analyzed using ArrayStar lncRNA microarrays. We identified seven candidate lncRNAs (Fig. 1A) that were deregulated significantly between cancer and paired normal tissues. Some of these lncRNAs have previously been investigated in other cancer types, but no published studies have focused on *KCNK15-AS1*. We examined *KCNK15-AS1* expression in 69 paired clinical pancreatic cancer and normal tissues (Fig. 1B). *KCNK15-AS1* was downregulated in cancer tissues compared with paired normal tissues. Then, we compared the *KCNK15-AS1* expression levels in normal pancreatic ductal epithelial cells (HPDE6-C7) and pancreatic cancer cells (BxPC-3, MIA-PaCa-2, AsPC-1, and PANC-1). BxPC-3 and MIA-PaCa-2 had the lowest *KCNK15-AS1* expression levels among all cells (Fig. 1C). Thus, we used BxPC-3 and MIA-PaCa-2 to investigate the role of *KCNK15-AS1* in pancreatic cancer pathogenesis.

KCNK15-AS1 inhibits cell migration and invasion

To investigate the regulatory function of *KCNK15-AS1*, we overexpressed *KCNK15-AS1* in BxPC-3 and MIA-PaCa-2 cells (Fig. 2A). Because metastasis is the most malignant feature of pancreatic cancer, we first evaluated cell motility after inducing *KCNK15-AS1*. The results showed that overexpression of *KCNK15-AS1* in MIA-PaCa-2, resulted in significantly reduced migrating and invading cells ($P < 0.001$) (Fig. 2B and C). Similar results were observed for BxPC-3 cells (Fig. 2D and E).

Table 2. Probes used for RNA-FISH

Probe	Sequence
KCNK15-AS1 probe 1	GCTGCCTTGGGATGCGATGAGTT
KCNK15-AS1 probe 2	TGTATAAGCCTCCCTAACAGAAGCCCA
KCNK15-AS1 probe 3	TCTGGTGGCCACAGTTCTGGAGG

Fig. 1. *KCNK15-AS1* is downregulated in pancreatic cancer tissues and cells. (A) Representative result of pancreatic cancer lncRNA microarray analysis. N, Normal tissue; Ca, cancer tissue. (B) In total, 69 paired pancreatic cancer tissues and normal tissues shows significantly decreased *KCNK15-AS1* in cancer tissues. (C) Evaluation of *KCNK15-AS1* in different pancreatic cells. * $P < 0.05$, ** $P < 0.01$. lncRNA, long non-coding RNA.

ALKBH5 demethylates KCNK15-AS1 and inhibits cell migration and invasion

Since mRNA m⁶A modification has been well studied, we considered whether *KCNK15-AS1* could be demethylated by ALKBH5. Therefore, we first evaluated *KCNK15-AS1* m⁶A enrichment in HPDE6-C7, MIA-PaCa-2, and BxPC-3 cells. *KCNK15-AS1* m⁶A enrichment was highest in MIA-PaCa-2 and BxPC-3 cells (Fig. 3A). ALKBH5 is a well-characterized RNA m⁶A demethylase; accordingly, we examined ALKBH5 expression levels in HPDE6-C7, MIA-PaCa-2, and BxPC-3 cells. The western blots indicated that ALKBH5 is downregulated in MIA-PaCa-2 and BxPC-3 cells (Fig. 3B). Confocal laser scanning microscopy revealed that the m⁶A signal colocalized with *KCNK15-AS1* (Fig. 3C), indicating that *KCNK15-AS1* undergoes m⁶A modification.

To investigate whether *KCNK15-AS1* is regulated by ALKBH5, we overexpressed ALKBH5 in BxPC-3 and MIA-PaCa-2 cells (Fig. 4A). As

Fig. 2. KCNK15-AS1 inhibits pancreatic cell migration and invasion. (A) RT-qPCR shows successful KCNK15-AS1 overexpression in MIA-PaCa-2 and BxPC-3 cells. (B) KCNK15-AS1 inhibits MIA-PaCa-2 migration and invasion (200× magnification). (C) Quantification of the results in (B). (D) KCNK15-AS1 inhibits BxPC-3 migration and invasion (200× magnification). (E) Quantification of the results in (D). *** $P < 0.001$. RT-qPCR, quantitative reverse-transcription PCR.

Fig. 3. KCNK15-AS1 undergoes m⁶A modification. (A) RT-qPCR followed by RNA immunoprecipitation shows that KCNK15-AS1 undergoes m⁶A modification in various pancreatic cells. (B) Western blotting reveal ALKBH5 expression in various pancreatic cells. (C) Confocal microscopy indicates that ALKBH5 interacts with KCNK15-AS1. RT-qPCR, quantitative reverse-transcription PCR.

Fig. 4. Influence of ALKBH5 overexpression on KCN15-AS1-mediated cell migration and invasion. (A) Western blotting reveal that successful ALKBH5 overexpression. (B) ALKBH5 decreases KCN15-AS1 methylation. (C) ALKBH5 decreases KCN15-AS1 expression in MIA-PaCa-2 and BxPC-3 cells. (D) ALKBH5 and KCN15-AS1 are co-expressed in pancreatic tissues. (E) ALKBH5 inhibits cell migration and invasion in MIA-PaCa-2 cells (200 $\times$ magnification). (F) Quantification of the results in (E). (G) ALKBH5 inhibits cell migration and invasion in BxPC-3 cells (200 $\times$ magnification). (H) Quantification of the results in (G). *** $P < 0.001$.

expected, the *KCNK15-AS1* m⁶A modification decreased after ALKBH5 overexpression (Fig. 4B). Based on RT-qPCR, *KCNK15-AS1* expression was elevated when ALKBH5 was overexpressed in MIA-PaCa-2 and BxPC-3 cells (Fig. 4C). We detected a linear correlation

between *ALKBH5* and *KCNK15-AS1* expression in pancreatic cancer tissues (Fig. 4D). Moreover, when *ALKBH5* was overexpressed, cell migration (Fig. 4E and F) and cell invasion (Fig. 4G and H) both decreased significantly.

Similarly, when silencing *ALKBH5* using siRNAs in BxPC-3 and MIA-PaCa-2 cells (Fig. 5A), resulted in increased *KCNK15-AS1* m⁶A enrichment (Fig. 5B), while the expression level of *KCNK15-AS1* decreased (Fig. 5C). Additionally, cell migration (Fig. 5D and E) and cell invasion (Fig. 5F and G) increased significantly after *ALKBH5* silencing.

KCNK15-AS1 and ALKBH5 inhibit epithelial–mesenchymal transition

Epithelial–mesenchymal transition (EMT) is associated with cell migration and invasion. We next examined EMT markers in *KCNK15-AS1*- and *ALKBH5*-overexpressing cells. The epithelial marker E-cadherin was increased and the mesenchymal markers (E-cadherin and Vimentin) were decreased in *KCNK15-AS1*-overexpressing cells (Fig. 6A). These results indicate that *KCNK15-AS1* inhibits EMT. Similar results were obtained in *ALKBH5* overexpression (Fig. 6B) or knockdown (Fig. 6C) cells.

Fig. 5. Influence of *ALKBH5* knockdown on *KCNK15-AS1*-mediated cell migration and invasion. (A) Western blots reveal that *ALKBH5* was knocked down successfully. (B) *KCNK15-AS1* methylation is increased after *ALKBH5* knockdown. (C) Knocking down *ALKBH5* decreases *KCNK15-AS1* in MIA-PaCa-2 and BxPC-3 cells. (D) Knocking down *ALKBH5* promotes cell migration and invasion in MIA-PaCa-2 cells (200× magnification). (E) Quantification of the results in (D). (F) Knocking down *ALKBH5* promotes cell migration and invasion in BxPC-3 cells (200× magnification). (G) Quantification of the results in (F). ** $P < 0.01$, *** $P < 0.001$.

Discussion

LncRNAs regulate different aspect of cellular processes including metastasis of cancer cells. Cytoplasmic and nuclear lncRNAs function differently according to the cellular distribution of lncRNAs [9-11]. Cytoplasmic lncRNAs often regulate protein biosynthesis [12], while nuclear lncRNAs tend to affect mRNA transcription and/or alternative splicing [13, 14]. However, the role of nuclear lncRNAs in epitranscriptional modification remains unclear. In this study, we identified *KCNK15-AS1*, a lncRNA significantly decreased in pancreatic cancer tissues and inhibited cell migration and invasion.

Recent studies have shown that the m⁶A mRNA modification is important for the stability mRNA [15], and m⁶A methylation is a post-transcriptional modification of RNA occurring in the nucleus [16]. RNA m⁶A methylation is reversible and can be demethylated by ALKBH5 or FTO [17, 18]. The m⁶A mRNA modification has been studied extensively; however, the importance of lncRNA methylation has remained elusive [19]. We found that *KCNK15-AS1* underwent m⁶A methylation in pancreatic cells HPDE6-C7, MIA-PaCa-2, and BxPC-3. *KCNK15-AS1* could be demethylated by ALKBH5. Moreover, *KCNK15-AS1* m⁶A methylation was negatively correlated with its expression level.

Although ALKBH5 is known to regulate m⁶A modification [20], the functions of ALKBH5 in pancreatic cells remain unclear. To address this, we overexpressed ALKBH5 in MIA-PaCa-2 and BxPC-3 cells, demonstrating that ALKBH5 could inhibit cell migration and invasion.

In this study, we found a significant decrease in the expression of lncRNA *KCNK15-AS1* in pancreatic cancer tissues. This exerted an inhibitory effect on cell motility. Furthermore, *KCNK15-AS1* was demethylated by ALKBH5 and was negatively correlated with m⁶A modification (Fig. 6D). It is possible that m⁶A could affect the stability of *KCNK15-AS1* based on RNA m⁶A analyses, but the mechanism remains unclear. We also report the novel observation that lncRNA can undergo methylation. We will investigate the potential clinical importance of lncRNA methylation and modifiers of their methylation in future research. Our data could be further developed as a valuable therapeutic strategy for pancreatic cancer prevention and control.

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Fig. 6. Inhibitory effects of KCN15-AS1 and ALKBH5 on epithelial-mesenchymal transition (EMT) and a schematic overview of this work. (A) KCN15-AS1 inhibits MIA-PaCa-2 and BxPC-3 EMT. (B) ALKBH5 overexpression inhibits MIA-PaCa-2 and BxPC-3 EMT. (C) ALKBH5 knockdown promotes MIA-PaCa-2 and BxPC-3 EMT. (D) ALKBH5 inhibits cell motility by demethylating and increasing expression of KCN15-AS1.

Disclosure Statement

The authors declare to have no competing interests.

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