Long Noncoding RNA DUXAP8 Promotes Pancreatic Carcinoma Cell Migration and Invasion Via Pathway by miR-448/WTAP/Fak Signaling Axis

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Objectives: Pancreatic carcinoma (PC) has become the fourth leading cause of cancer deaths. Long noncoding RNA DUXAP8 has also been reported to play a regulatory role in PC progression. However, its molecular mechanism in PC is not fully elucidated.

Methods: Quantitative real-time polymerase chain reaction was used to detect the levels of DUXAP8, microRNA (miR)-448, Wilms tumor 1-associating protein (WTAP), focal adhesion kinase (Fak), and matrix metalloproteinase 2/9. Western blotting was carried out to detect matrix metalloproteinase 2/9, WTAP, Fak, and p-Fak. The interaction between DUXAP8 and miR-448 as well as WTAP and miR-448 was validated by bioinformatics and dual-luciferase reporter assays. Transwell assay was used to analyze cell invasion and migration. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay was used to analyze cell proliferation.

Results: DUXAP8 was upregulated, whereas miR-448 was downregulated in PC tissue and cells. Meanwhile, DUXAP8 knockdown or miR-448 overexpression inhibited migration, invasion, and proliferation of PC cells. DUXAP8 directly targeted miR-448, and miR-448 directly bound to WTAP. Downregulation of miR-448 reversed the inhibition of migration and invasion of PC cells by DUXAP8 knockdown.

Conclusions: DUXAP8 sponges miR-448 to modulate migration, invasion, and proliferation of PC cells, indicating a novel mechanistic role of DUXAP8 in the regulation of PC progression.

Key Words: pancreatic carcinoma, lncRNA DUXAP8, miR-448, WTAP, Fak

Abbreviations: PC = pancreatic carcinoma, lncRNAs = long noncoding RNAs, ceRNA = competing endogenous RNAs, WTAP = Wilms tumor 1-associating protein, Fak = focal adhesion kinase, miRNAs = microRNAs, ATCC = American Type Culture Collection, PBS = phosphate-buffered saline, qPCR = quantitative polymerase chain reaction, MMP = matrix metalloproteinase

As one of the most deadly neoplasms, pancreatic carcinoma (PC) has become the fourth leading cause of cancer deaths worldwide. Because of lack of early diagnostic markers, abnormal metastasis, and high resistance to conventional cancer therapies, the prognosis for the patients remains extremely poor. Therefore, exploring the molecular mechanism of carcinogenesis and progression of PC as well as revealing the downstream signaling pathways to develop effective therapies for pancreatic cancers are urgently required.

Long noncoding RNAs (lncRNAs), a class of noncoding RNAs with length longer than 200 nucleotides, were involved in various biological processes, including cell cycle control, alternative splicing, cell differentiation, cancer metastasis, epigenetic regulation, and drug resistance. Their levels were also reported to be abnormally regulated in many cancers, such as non–small cell lung cancer, breast cancer, and PC. Recent studies have revealed that pseudogenes, a type of lncRNA, play critical roles in posttranscriptional or transcriptional regulation of gene expression and are related to diverse pathological settings. Among them, lncRNA DUXAP8 has also been shown to play a regulatory role in PC progression. DUXAP8 was highly expressed in human PC and associated with shorter overall survival of PC patients. It promotes growth of PC cells by epigenetically silencing CDKN1A and KLFL. However, the molecular mechanism of DUXAP8 in PC is still limited. Therefore, more effort should be paid to reveal its regulatory mechanism in depth.

MicroRNAs (miRNAs), endogenous RNAs of approximately 22 nucleotides, act as intrinsic mediators in various biological processes by inhibiting gene expression at the posttranscriptional level. Recent studies have shown that miRNAs are aberrantly expressed in many types of cancers and are involved in the regulation. Previous studies showed that miR-448 was associated with cancer progression of many types, including lung squamous cell carcinoma, breast cancer, and PC. For example, miR-448 can inhibit PC by targeting Rab2B, whereas it also suppresses metastasis of pancreatic ductal adenocarcinoma through targeting the JAK1/STAT3 pathway. Through bioinformatics analysis, we have predicted that there was a potential binding site of miR-448 on DUXAP8. However, the targeted relationship between them has not been reported. Thus, it is of much significance to investigate the regulatory mechanism between DUXAP8 and miR-448 on PC.

Mammalian Wilms tumor 1-associating protein (WTAP) is a nuclear protein widely expressed in various tissues and plays an important role in the normal cellular and physiological processes. It can act as an oncogenic protein in many tumors.
Focal adhesion kinase (Fak) is a cytoplasmic protein tyrosine kinase that is overexpressed and activated in several solid cancers.\textsuperscript{22} Several studies confirmed a strong relationship between Fak and PC.\textsuperscript{6,23} Recently, WTAP was identified to promote metastasis by stabilizing Fak messenger RNA (mRNA) in PC.\textsuperscript{24} However, whether DUXAP8 regulates the WTAP/Fak axis to promote PC cell migration and invasion needs to be further elucidated.

In this study, we investigated the functional implication of DUXAP8 in PC, suggesting that DUXAP8 knockdown inhibited PC cell proliferation, migration, and invasion via the miR-448/WTAP axis. It is, for the first time, revealed that DUXAP8 regulated the expression of WTAP through sponging miR-448. Our findings provided the first clue showing the regulatory role of the DUXAP8/miR-448/WTAP/Fak pathway in PC cells, and DUXAP8 might be a potential therapeutic target for PC.

**MATERIALS AND METHODS**

**Sample Collection**

This study was approved by the ethics committee of The Xiangya Hospital of Central South University, Changsha, China. Briefly, 24 paired PC tissues and adjacent normal tissues were collected from patients undergoing surgical resection at Xiangya Hospital from 2017 to 2019. Samples were frozen and stored in liquid nitrogen immediately after excision for further use. Patients provided written informed consent.

**Cell Culture**

Human normal pancreatic cells HPDE6, human embryonic kidney cells HEK 293T cells, and pancreatic cancer cell lines BxPC-3 were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human pancreatic cancer cell lines PANC-1, AsPC-1, and Capan-1 were purchased from the American Type Culture Collection (ATCC, Manassas, Va.). The cells were grown in Dulbecco modified Eagle medium (PANC-1) or RPMI 1640 (HPDE6, BxPC-3, AsPC-1, Capan-1) medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1 \times 10^{-5}\text{unit/L penicillin, and 100 mg/L streptomycin (Invitrogen, Carlsbad, Calif)} at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}.

**Cell Transfection**

pGPH1 plasmid–expressing short hairpin RNA targeting DUXAP8 and its scrambled control short hairpin RNA–expressing pGPH1 plasmid, miR-448 mimics/inhibitor, and their scrambled control oligos were purchased from GenePharma (Shanghai, China). Cells were planted in 6-well plates for 24 hours, and then transfected with the indicated recombinant vectors, miR-448 mimics, or inhibitor with 40% to 60% confluence using Lipofectamine 3000 reagent (Invitrogen). The transfected cells were harvested for 48 hours after transfection.

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction**

The total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed with ImProm-II Reverse Transcription System (Promega, Madison, Wis.). The quantitative real-time polymerase chain reaction (PCR) was then performed with gene-specific primers and an SYBR Green quantitative PCR (qPCR) assay kit (TaKaRa, Beijing, China). The target gene expression level was calculated with the 2^{-ΔΔCt} method, which was normalized to GAPDH mRNA or U6 snRNA. All assays were performed in triplicate.

Western Blot Analysis

Cells were treated with an RIPA protein extraction reagent (Beyotime, Beijing, China) containing protease and phosphatase inhibitor (ThermoFisher Scientific, Waltham, Mass). After the protein concentration was determined by a BCA Protein Assay Reagent Kit (ThermoFisher Scientific), approximately 50 \mu g of the protein extract was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Sigma, St. Louis, Mo), and then incubated with specific primary antibodies at 4°C overnight. It was then washed with Tris-Buffered Saline and Tween 20 (TBST); horseradish peroxidase–labeled goat antimouse/rabbit immunoglobulin G (1:5,000; Sigma) was used as the secondary antibody. Quantity One software (Bio-Rad Laboratories, Inc., Hercules, Calif) was used to perform quantification of band intensity.

**Cell Proliferation Analysis**

A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma) was used to test cell viability according to the manufacturer’s instructions. Cells grew and were transfected in 96-well plates. We recorded cell proliferation every 24 hours after transfection of cells. Experiments were performed in triplicate.

Transwell Migration and Invasion Assay

Migration assay was performed with a 24-well transwell (Corning, Corning, NY). Cells were applied to the upper chamber containing RPMI without serum, and the cells migrated to the underside of the filter in 6 hours. Then cells on the lower surface of the filter were fixed and stained with 1% crystal violet solution and observed under a microscope (Nikon, Tokyo, Japan). For invasion assay, growth factor–reduced Matrigel-coated 24-well transwell was used instead (BD Biosciences, San Jose, Calif).

**Dual-Luciferase Reporter Assay**

The wild-type (WT) and mutant (MUT) forms of human WTAP 3′-untranslated region fragment and DUXAP8 gene containing putative binding sites for miR-448 were synthesized into pGL3-control vector by Genechem (Shanghai, China). Vectors and miR-448 mimics were cotransfected into cells with the help of Lipofectamine 3000 (Invitrogen). After 48 hours, the dual-luciferase reporter assay system (Promega) was used to determine the relative luciferase activity.

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Statistical Analyses

Data are presented as mean (standard deviation), and all experiments were repeated at least 3 times. Significance for comparisons between 2 independent samples were determined by Student t test. One-way analysis of variance with Tukey post hoc test was used for comparison among 3 or more groups. The statistical differences were calculated by GraphPad prism 7 software (GraphPad Software, Inc, La Jolla, Calif). Statistical significance was observed when \( P < 0.05 \).

RESULTS

LncRNA DUXAP8 and miR-448 Were Dysregulated in Pancreatic Cancer

First, we detected the expression level of DUXAP8 in 24 paired PC and corresponding adjacent pancreatic tissue samples to explore the potential role of DUXAP8 and miR-448. Figure 1A shows that DUXAP8 was significantly upregulated in tumor tissues. At the same time, the expression of miR-448 was downregulated in tumor tissues (Fig. 1B). We also measured the expression level of DUXAP8 in 4 human PC lines, including PANC-1, AsPC-1, BxPC-3, Capan-1, and the normal human pancreatic epithelial cell HPDE6. Figure 1C shows that the expression levels of DUXAP8 in PC cells were significantly increased compared with HPDE6 cells, whereas miR-448 was downregulated in PC cells. Specifically, we chose PANC-1, which had the highest DUXAP8 expression, and AsPC-1, which had the lowest DUXAP8 expression, among those 4 PC cells for further investigation.

DUXAP8 Facilitates PC Cell Proliferation, Migration, and Invasion

To further identify the impact of DUXAP8 on PC cell proliferation, migration, and invasion, short hairpin (sh)-DUXAP8 was transfected to PANC-1 and AsPC-1 cells for knocking down DUXAP8. Figure 2A shows that DUXAP8 was successfully knocked down by sh-DUXAP8. Cell proliferation assay revealed that DUXAP8 knockdown inhibited the cell proliferation ability (Fig. 2B). Moreover, transwell assay showed that DUXAP8 knockdown decreased cell migration (Figs. 2C, D) and invasion (Figs. 2E, F). Besides, both mRNA (Fig. 2G) and protein (Figs. 2H, I) levels of MMP2 and MMP9 were downregulated by DUXAP8 knockdown. Taken together, these results indicated that DUXAP8 was involved in the regulation of pancreatic cancer cell proliferation, migration, and invasion.

DUXAP8 Interacts With miR-448 and Regulates WTAP/Fak Signaling Pathway

To further investigate the relationship between DUXAP8 and miR-448, we detected the expression of miR-448 after DUXAP8 knockdown, and as shown in Figure 3A, miR-448 was upregulated when DUXAP8 was knocked down. At the same time, both the mRNA (Fig. 3B) and protein (Figs. 3C, D) levels of WTAP and Fak were decreased after DUXAP8 knockdown. Furthermore, we also predicted potential binding sites between DUXAP8 and miR-448 by bioinformatics analysis. Figure 3E shows the direct binding site between DUXAP8 and miR-448. Moreover, luciferase reporters of WT-DUXAP8 and its mutated form (MUT-DUXAP8) were constructed. The results demonstrated that overexpression of miR-448 significantly decreased the luciferase activity of WT-DUXAP8 in HEK 293T cell (Fig. 3F). No effect was observed on the mutated form (Fig. 3F), indicating a direct interaction between DUXAP8 and miR-448. Taken together, these results indicated that DUXAP8 could sponge miR-448 and regulate the WTAP/Fak signaling pathway.

FIGURE 1. The expression of lncRNA DUXAP8 and miR-448 in pancreatic cancer tissue and cells. A, DUXAP8 expression in pair samples of PC and adjacent normal tissues by qPCR (n = 24). B, Relative expression of miR-448 in pair samples of PC and adjacent normal tissues by qPCR (n = 24). C, DUXAP8 expression in normal HPDE6-C7 cells and 4 types of PC cells (PANC-1, AsPC-1, BxPC-3, Capan-1) by qPCR. D, Relative expression of miR-448 in normal HPDE6-C7 cells and 4 types of PC cells (PANC-1, AsPC-1, BxPC-3, Capan-1) by qPCR. *P < 0.05, **P < 0.01, ***P < 0.001. © 2021 The Author(s). Published by Wolters Kluwer Health, Inc. www.pancreasjournal.com | 319
FIGURE 2. DUXAP8 knockdown inhibits PC cell proliferation and migration. A, DUXAP8 expression in PANC-1 and AsPC-1 cells after transfected with sh-DUXAP8 and sh-NC. B, Cell proliferation in PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC was detected by the MTT assay. C and D, The migration ability of PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC was measured by transwell assays. E and F, The invasion ability of PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC was measured by transwell assays. Wells were repeated in triplicate, and the invaded/migrated cells were quantified per field of view and statistically analyzed. G, MMP-2 and MMP-9 mRNA expression levels in PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC by qPCR. H and I, Protein level of MMP-2 and MMP-9 expression in PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC by Western blot. *P < 0.05, **P < 0.01, ***P < 0.001.
miR-448 Inhibits PC Cell Proliferation and Migration

We further identify the impact of miR-448 on cell proliferation and migration by transfecting miR-448 mimics or NC mimics in PANC-1 and AsPC-1 cells. Figure 4A shows that miR-448 was successfully upregulated by miR-448 mimics. The MTT assay revealed that miR-448 could inhibit the cell proliferation of those 2 cell lines (Fig. 4B). Moreover, transwell assay showed that miR-448 overexpression decreased cell migration (Figs. 4C, D) and invasion (Figs. 4E, F). Meanwhile, both mRNA (Fig. 4G) and protein (Figs. 4H, I) levels of MMP2 and MMP9 were downregulated by miR-448 overexpression. Collectively, these results indicated that miR-448 was also involved in the regulation of pancreatic cancer cell proliferation, migration, and invasion.

miR-448 Regulates Fak Signaling by Targeting WTAP

To reveal the interaction between miR-448 and the WTAP/Fak pathway, WTAP and Fak expression in PANC-1 and AsPC-1 cells was detected after miR-448 overexpression. Figure 5A shows that

FIGURE 3. DUXAP8 could directly target miR-448 and regulate the WTAP/Fak signaling pathway. A, Relative expression of miR-448 in PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC by qPCR. B, Relative mRNA expression of WTAP, Fak, and p-Fak in PANC-1 and AsPC-1 cells transfected with sh-DUXAP8 and sh-NC by Western blot. C, The predicted binding site between DUXAP and miR-448. D, The luciferase activity of WT-DUXAP and MUT-DUXAP in HEK 293T cells transfected with miR-448 mimics or NC mimics.
FIGURE 4. miR-448 overexpression decreases PC cell proliferation and migration. A, Relative expression of miR-448 in PANC-1 and AsPC-1 cells transfected with miR-448 mimics or NC mimics. B, Cell proliferation in PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics was detected by the MTT assay. C and D, The migration ability of PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics by transwell assays. E and F, The invasion ability of PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics by transwell assays. Wells were repeated in triplicate, and the invaded/migrated cells were quantified per field of view and statistically analyzed. G, Relative mRNA expression of MMP-2 and MMP-9 in PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics. H and I, Protein level of MMP-2 and MMP-9 expression in PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics. *P < 0.05, **P < 0.01, ***P < 0.001.
both WTAP and Fak mRNA were downregulated by miR-448 overexpression. Meanwhile, WTAP and Fak protein as well as phosphorylated Fak were decreased after miR-448 overexpression (Figs. 5B, C), indicating that WTAP and Fak could be the downstream molecule of miR-448. To further validate whether WTAP was the target of miR-448, bioinformatics analysis was carried out and predicted the putative binding site of miR-448 on WTAP (Fig. 5D). Based on this, luciferase reporters of WTAP 3′-untranslated region (WT-WTAP) and its mutated form without miR-448 binding site (MUT-WTAP) were constructed. Figure 5E shows that overexpression of miR-448 significantly decreased the luciferase activity of WT-WTAP but not MUT-WTAP in HEK 293T cells. Taken together, these results indicated that WTAP was the target of miR-448.

**FIGURE 5.** miR-448 regulates Fak signaling by targeting WTAP. A, WTAP and Fak mRNA expression in PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics. B and C, Protein level of WTAP, Fak, and p-Fak in PANC-1 and AsPC-1 cells transfected with miR-448 mimics and NC mimics. D, The predicted binding site between WTAP and miR-448. E, The luciferase activity of WT-WTAP and MUT-WTAP in PANC-1 and AsPC-1 cells transfected with miR-448 mimics or NC mimics. *P < 0.05, **P < 0.01, ***P < 0.001.
Abnormal expression of lncRNA DUXAP8 has been proven to be closely related to multiple cancer progression, such as hepatocellular carcinoma, renal cell carcinoma, bladder cancer, and non–small cell lung cancer. A previous study also showed that DUXAP8 could promote PC cell growth epigenetically. In the present study, elevated DUXAP8 expression was demonstrated in PC tumor tissue and cancer cells. Furthermore, we proved that DUXAP8 could promote PC cell migration and invasion.
This is the first report that DUXAP8 might be a regulator of PC metastasis and thus a target for treatment of malignant pancreatic cancers.

Accumulating studies suggested that lncRNAs could function as miRNA sponges to sequester miRNAs away and thus upregulate their downstream target genes. This competing endogenous RNA (ceRNA) mechanism has been reported to be involved in the development of multiple cancers. However, only 2 lncRNAs, NORAD1 and Linc00511, have been found to play a regulatory role as ceRNA in PC. In this study, we demonstrated that DUXAP8 can directly bind with miR-448 through bioinformatics and luciferase assay, and we further showed that DUXAP8 regulated the invasion and migration of pancreatic cancer as a sponge of miR-448. As far as we know, we provided the first clue that DUXAP8 was the ceRNA of miR-448. In addition, miR-448 was reported to play critical roles in tumorigenesis and progression of multiple cancers including PC. Although WTAP could promote metastasis and chemoresistance to gemcitabine by stabilizing Fak mRNA in PC, however, there were no reports to explore whether there was a regulatory relationship between the 2 of them. Here, WTAP was revealed to be the target of miR-448 in PC, and miR-448 could inhibit the invasion and migration of PC cells by downregulating WTAP. Thus far, we are the first to validate the interaction between miR-448 and WTAP and demonstrate that miR-448 could inhibit PC cell invasion and migration through the WTAP/Fak signaling pathway.

To deeply understand the complex mechanism of DUXAP8 regulating PC cell invasion and migration through the miR-448/WTAP axis, there is still much to be done in the future. Because there is more complex situation in the animal body than cellular models, whether this mechanism could be applied to animal models, whether this mechanism could be applied to an animal model is uncertain. Thus, we should further test this mechanism in mice models and prove its potential of being therapeutic targets.

In conclusion, our study indicated that DUXAP8 could promote the invasion and migration of PC via the miR-448/WTAP axis, suggesting an oncogene role of DUXAP8 in PC.

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