miR-32-5p suppresses the proliferation and migration of pancreatic adenocarcinoma cells by targeting TLDC1

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Abstract. Pancreatic adenocarcinoma (PAAD) is one of the most fatal types of cancer in humans. However, the molecular mechanisms underlying the migration and invasion abilities of PAAD cells remain unclear. The aim of the present study was to explore the regulatory roles of micro RNA (miR)-32-5p in PAAD cells. miR-32-5p mimic and inhibitor were used to transfect the human PAAD AsPC-1 cell line to determine the role of miR-32-5p in cell proliferation and metastasis. The StarBase database predicted the binding of miR-32-5p to the target gene TBC/CysM-associated domain containing 1 (TLDC1). Further analyses were performed to assess miR-32-5p and TLDC1 expression levels in healthy and PAAD tissues, as well as the association between miR-32-5p or TLDC1 expression and the prognosis of patients with PAAD. The interaction between miR-32-5p and TLDC1 was verified using the dual-luciferase reporter assay. miR-32-5p expression levels were detected by reverse transcription-quantitative PCR and western blotting, respectively. The cell counting Kit-8 assay was utilised to assess cell proliferation, whereas the wound-healing and Transwell assays were conducted to assess cell migration and invasion, respectively. miR-32-5p expression levels were markedly lower in PAAD tissue compared with those in healthy tissue, and were significantly lower in PAAD cell lines compared with those in the human pancreatic duct cell line HPDE6, which corresponded with poor prognosis. miR-32-5p significantly inhibited the proliferation of PAAD cells and markedly reduced migration and invasion compared with the negative controls. miR-32-5p was shown to target TLDC1, with miR-32-5p expression in PAAD being negatively correlated with TLDC1 expression. High TLDC1 expression levels were associated with a poorer prognosis compared with low TLDC1 expression levels. Co-transfection of miR-32-5p mimic and pcDNA/TLDC1 demonstrated that TLDC1 significantly reversed miR-32-5p-mediated inhibition of proliferation, migration and invasion of PAAD cells. Overall, the present study demonstrated that miR-32-5p may serve as a tumor-suppressor gene by inhibiting the proliferation and migration and invasion of PAAD cells via the downregulation of TLDC1. Therefore, miR-32-5p may serve as a potential diagnostic or prognostic marker for PAAD.

Introduction

Pancreatic adenocarcinoma (PAAD) is a common gastrointestinal cancer associated with a high mortality rate, resulting in >227,000 deaths annually worldwide (1-3). The most common type of PAAD is pancreatic ductal adenocarcinoma, accounting for 80-90% of all pancreatic tumors (4). PAAD is among the most invasive types of carcinoma, and clinical diagnosis is often delayed due to a lack of known PAAD-specific symptoms (5). PAAD is therefore often diagnosed following metastasis and/or increases in cancer aggression, both of which greatly reduce the survival rate (6). The prognosis of PAAD is poor with a 1-year survival rate of <10% (2). However, a definitive diagnosis of PAAD is not currently possible due to a lack of reliable tumor markers, which are urgently needed for initial detection and intervention (5). Currently, chemotherapy is the only available option for the treatment of advanced end-stage or metastatic PAAD (5).

MicroRNAs (miRNAs/miRs) are small, endogenous, non-coding RNAs that range in length from 19-25 nucleotides (7). miRNA can degrade or inhibit translation of mRNA by partial or total binding of the 3'-untranslated region (UTR) of the target molecule (7). miRNAs are key regulatory factors in various types of tumors, functioning as tumor oncogenes or tumor suppressors to regulate cell proliferation, differentiation, metastasis and apoptosis (7,8). miR-32-5p, a member of the miR-32 family and located on chromosome band Xq26.2 (9), is involved in the regulation and development of numerous types of carcinoma. For example, Ye et al (10) reported that
miR-32-5p inhibits the migration, invasion and proliferation of colorectal carcinoma cells; Wang et al (11) demonstrated that miR-32-5p represses the migration and invasion of clear cell renal cell carcinoma cells, and is positively correlated with a good prognosis; and Liu et al (12) indicated that miR-32-5p is downregulated in cervical carcinoma tissues and inhibits the proliferation, migration and invasion of cervical carcinoma cells.

TBC/LysM-associated domain-containing 1 (TLDCl) is an evolutionarily conserved protein 1 (13). Bioinformatics databases were analyzed to gain insight into the molecular functions of TLDCl, also known through genomic and proteomic studies as KIAA1609 (14), LOC57707 (15), or mEAK-7 (mammalian EAK-7 or MTor associated protein, eak-7 homolog) (16). Independent reports reveal that TLDCl mRNA is overexpressed in diseases, such as hepatocellular carcinoma (17) and lymph node-positive breast cancer (18). TLDCl activates an alternative mTOR signaling pathway through S6K2 and 4E-BP1 to regulate cell proliferation and migration (13). miR-1911-3p targets mEAK-7 to suppress mTOR signaling in human lung cancer cells (19). It was hypothesized that miR-32-5p and TLDCl may serve an important role in the development of Paad.

Relatively few previous studies have investigated the role and underlying mechanisms of miR-32-5p in the pathogenesis of Paad. For example, to the best of our knowledge, only one previous study has reported that growth arrest specific 5 could positively regulate PTEN-induced tumor suppressor pathway via miR-32-5p, thus inhibiting the metastasis of pancreatic cancer (20). Therefore, the aim of the present study was to investigate the relationship between miR-32-5p and TLDCl, and the underlying mechanisms in the onset and progression of Paad.

Materials and methods

Cell culture. Human Paad cell lines (BxPC-3, AsPC-1 and PANC-1) were obtained from American Type Culture Collection. PANC-1 and AsPC-1 cells were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, whereas BxPC-3 cells were incubated in RPMI medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The human pancreatic HPDE6 cell line was purchased from Shanghai Gefeian Biotechnology Co., Ltd. and incubated in keratinocyte serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The human keratinocyte HPDE6 cell line was purchased from Shanghai Genechem Co., Ltd. AsPC-1 cells were seeded (5x10^4 cells/well) into 24 well plates. Subsequently, cells were co-transfected with the reporter plasmid (TLDCl 3'UTR construct; 500 ng) and miR-32-5p mimic or NC mimic (900 ng) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. Luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity. The co-transfection systems were as follows: miR-32-5p mimic + TLDCl-WT; NC-mimic + TLDCl-WT; miR-32-5p mimic + TLDCl-MUT; and NC-mimic + TLDCl-MUT.

Cell Counting Kit-8 (CCK-8) assay. AsPC-1 cells (4x10^4 cells/well) were seeded into a 96-well culture plate following incubation for 24, 48, 72 and 96 h at 37°C with the CCK-8 reagent (ImmunoWay Biotechnology Company) according to the manufacturer's protocol, cell proliferation was measured at a wavelength of 450 nm using a microplate reader.

Wound-healing assay. Transfected human AsPC-1 cells were seeded (2x10^4 cells/well) into the wells of 6-well plates and incubated in serum-free medium for ~24 h. At 100% confluence, a linear wound to the cell monolayer was made using the sterilized tip of a liquid pipette gun and the cells were cultured for 48 h at 37°C. Subsequently, the media was discarded and the plates were washed three times in PBS. Plates were imaged and the serum-free medium was added prior to incubation for an additional 24 h at 37°C. The plates were then imaged again after the 24-h incubation. Cells in randomly selected fields of view were counted using a light microscope (magnification, x100). The migratory ability of cells was indicated by gap closure. The assay was performed in triplicate.

Transwell assay. At 48 h post-transfection, AsPC-1 cells (5x10^4) were transferred to a Transwell chamber containing
Matrigel (BD Biosciences). Matrigel pre-coating was conducted for 30 min at 37°C. Prior to conducting the assay, the lower and upper chambers were filled with pre-warmed media for hydration. Cells were then digested and resuspended in serum-free media. The upper chamber was loaded with cells suspended in serum-free media and the lower chamber with media supplemented with 10% FBS. The assay was conducted for 48 h at 37°C. Following completion of the assay, the cells in the lower chamber were fixed for 30 min with 40% methanol at 25°C and then stained using 0.1% crystal violet for 20 min at 25°C. Stained cells were visualized using an inverted light microscope (Olympus Corporation; magnification, x100).

Western blotting. Total protein was extracted from AsPC-1 cells using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) containing a protease inhibitor cocktail (Roche Diagnostics) and quantified using a BCA kit (Beyotime Institute of Biotechnology,). Total protein (30 µg; 25 µl/well) was separated via 12% SDS-PAGE acrylamide gel. The separated proteins were then transferred onto a PVDF membrane, which was blocked with 5% skimmed milk in TBS containing 0.1% Tween-20 for 1 h at 25°C. The membranes were incubated overnight at 4°C with primary antibodies against TLDC1 (1:1,000; cat. no. aa 426-456; 4a Biotech co., ltd.) and β-actin (1:2,000; Cell Signaling Technology Inc.). Following the primary incubation, membranes were washed three times with TBST (0.05% Tween-20) and incubated at 25°C for 2 h with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G; 1:2,000; cat. no. ab6721; Abcam). The membranes were washed a further three times before visualization using ECL chemiluminescent detection system (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: Pre-denaturation at 95°C for 30 sec; denaturation at 95°C for 10 sec; annealing and extension at 60°C for 30 sec for 40 cycles. miR-32-5p expression levels were quantified using the 2^{-ΔΔCq} method (21) and normalized to the internal reference gene, U6. The primers used for RT-qPCR are shown in Table 1.

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

| Gene   | Sequence (5’ → 3’)           |
|--------|-------------------------------|
| miR-32-5p | F: TAT TGC ACA TTA CTA AGC CTT |
|         | R: GAA TAC CTC GGA CCC TGC    |
| U6     | F: GCT TCG GCA GCA CAT ATA CTA AAA |
|         | R: CGC TTC ACG AAT TTG CGT GTCAT |

miR, microRNA; F, forward; R, reverse.

Statistical analysis. All statistical analyses were performed using SPSS version 24.0 (IBM Corp.). Data are presented as the mean ± SD. The unpaired Student's t-test was used to compare the means between two groups, whereas one-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons. Kaplan-Meier survival curves were analyzed using the log-rank test to determine statistical significance. Pearson's correlation coefficient test was used to determine whether there was a linear correlation between miR-32-5p and TLDC1 expression in PAAD. All experiments were repeated three times independently. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-32-5p is lowly expressed in PAAD tissues and cells. The starBase database was used to assess the expression pattern of miR-32-5p in PAAD and to identify the relationship between

Figure 1. Expression levels of miR-32-5p in PAAD tissues and cells. (A) Expression of miR-32-5p in PAAD and healthy tissues was determined using the starBase database. (B) Relationship between miR-32-5p expression and the survival rate of patients with PAAD was determined using the starBase database. (C) Expression of miR-32-5p in HPDE6 cells and human PAAD cell lines (PANC-1, AsPC-1 and BxPC-3) detected by reverse transcription-quantitative PCR. ***P<0.001 vs. HPDE6. miR, microRNA; PAAD, pancreatic adenocarcinoma; RPM, reads per million.
miR-32-5p expression levels and PAAD prognosis. The results revealed that miR-32-5p expression was downregulated in the PAAD patient group compared with the healthy group (Fig. 1A; P<0.05). Low miR-32-5p expression was associated with a poor prognosis of patients with PAAD compared with high miR-32-5p expression (Fig. 1B; P=0.12). Furthermore, miR-32-5p expression was measured in HPDE6 and PAAD cell lines (AsPC-1, PANC-1 and BxPC-3). The RT-qPCR results demonstrated that miR-32-5p expression levels were significantly reduced in the PAAD cells compared with those in HPDE6 cells (Fig. 1C; P<0.001). These findings indicated that miR-32-5p might be involved in the development and progression of PAAD.

miR-32-5p inhibits the proliferation, migration and invasion of PAAD cells. To assess the effect of miR-32-5p overexpression and knockdown, the AsPC-1 cell line was selected due to moderate miR-32-5p expression levels. To determine whether miR-32-5p was associated with the proliferation, migration and invasion of PAAD cells, functional studies of AsPC-1 cells were performed. The results demonstrated that miR-32-5p mimic caused a significant increase in miR-32-5p mRNA expression compared with NC mimic, whereas miR-32-5p inhibitor significantly reduced miR-31-5p expression compared with NC inhibitor. This indicated the successful establishment of miR-32-5p overexpression and knockdown in AsPC-1 cells (Fig. 2A; both P<0.001). The CCK-8 assay results revealed that miR-32-5p mimic significantly impaired the proliferation of AsPC-1 compared with NC mimic (Fig. 2B; P<0.001 at 48 h; P<0.01 at 72 h; P<0.05 at 96 h). The metastatic potential of AsPC-1 cells was measured using wound-healing and Transwell assays. Cell migration was significantly impaired in miR-32-5p mimic-transfected cells compared with NC mimic-transfected cells (Fig. 2C; P<0.01). miR-32-5p knockdown significantly increased cell proliferation (Fig. 2B; P<0.01 at 72 h; P<0.001 at 96 h), and significantly increased cell migration (Fig. 2C; P<0.001) and invasion compared with NC inhibitor (Fig. 2D; P<0.001).

Figure 2. miR-32-5p inhibits the proliferation, migration and invasion of pancreatic adenocarcinoma cells. (A) miR-32-5p expression levels were detected at 72 h post-transfection using reverse transcription-quantitative PCR. (B) Effect of miR-32-5p expression on AsPC-1 cell proliferation was determined using the Cell Counting Kit-8 assay. (C) AsPC-1 cell migration was evaluated using the wound-healing assay. (D) AsPC-1 cell invasion was determined using the Transwell assay. *P<0.05, **P<0.01 and ***P<0.001 vs. NC mimic; ##P<0.01 and ###P<0.001 vs. NC inhibitor. miR, microRNA; NC, negative control; TLDC1, TBC/LysM-associated domain containing 1; OD, optical density.
Collectively, these findings demonstrated that miR-32-5p inhibited the proliferation and metastatic potential of PAAD cells.

**TLDC1 is a target of miR-32-5p.** To elucidate the downstream molecular mechanisms of miR-32-5p in the proliferation, migration and invasion of PAAD cells, the starBase database was searched to identify the target sequence of miR-32-5p. The results demonstrated that miR-32-5p targeted the 3’-uTr of Tldc1 (Fig. 3a). The dual-luciferase reporter assay was performed to confirm the interaction between miR-32-5p and Tldc1. The results demonstrated that luciferase activity was significantly reduced in cells co-transfected with TLDC1-WT and miR-32-5p mimic compared with those co-transfected with nc mimic (P<0.001). No significant difference in luciferase activity was observed in the MUT TLDC1 co-transfection system (Fig. 3B; P>0.05). Moreover, TLDC1 protein expression levels were significantly reduced in cells transfected with miR-32-5p mimic compared with the NC mimic group, whereas miR-32-5p inhibitor significantly increased the protein expression levels of TLDC1 compared with the NC inhibitor group (Fig. 3C; both P<0.05). These results demonstrated that miR-32-5p could both target and regulate TLDC1 in PAAD cells, which was further confirmed by bioinformatics analyses. The starBase database demonstrated that TLDC1 expression was increased in PAAD cells compared with the NC group (Fig. 3D; P<0.12). Furthermore, TLDC1 expression levels were negatively correlated with miR-32-5p in PAAD tissues (Fig. 3E; P<0.05). High TLDC1 expression levels were significantly associated with a better prognosis of patients with PAAD compared with lower TLDC1 expression levels (Fig. 3F; P<0.05). Overall, these results indicated that TLDC1 may serve a regulatory role in the proliferation, migration and invasion of PAAD cells via miR-32-5p.

**TLDC1 reverses the suppressive effect of miR-32-5p on the proliferation, migration and invasion of PAAD cells.** To explore the effect of TLDC1 on the proliferation, migration and invasion of PAAD cells mediated by miR-32-5p, simultaneous overexpression of TLDC1 and miR-32-5p was induced. The results demonstrated that TLDC1 protein expression levels were significantly increased in cells transfected with NC + pcDNA/TLDC1 compared with those transfected with pcDNA/control, indicating that TLDC1 was successfully overexpressed (Fig. 4A; all P<0.001). Transfection with miR-32-5p mimic significantly reduced TLDC1 protein expression levels compared with the
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However, co-transfection with miR-32-5p and TLDC1 significantly reversed the decrease in TLDC1 protein expression levels induced by miR-32-5p compared with miR-32-5p + pcDNA/control (Fig. 4B; P<0.01). Compared with the NC + pcDNA/control group, miR-32-5p overexpression was also shown to significantly impair the proliferation of AsPC-1 cells, whereas TLDC1 overexpression significantly reversed miR-32-5p mimic-mediated effects (Fig. 4C; both P<0.001). The wound-healing assay results showed that cell migration was significantly decreased in the miR-32-5p mimic + pcDNA/control group compared with that in the NC mimic + pcDNA/control group (P<0.01), and TLDC1 overexpression significantly reversed miR-32-5p mimic-mediated inhibition of migration (Fig. 4D; P<0.01). Furthermore, the Transwell assay results indicated miR-32-5p overexpression significantly decreased the number of invasive cells compared with the NC mimic + pcDNA/control group (P<0.001), and TLDC1 overexpression partially restored this decrease (Fig. 4E; P<0.001). In summary, these data suggested that TLDC1 was a target of miR-32-5p and reversed
miR-32-5p-mediated inhibition of the proliferation, migration and invasion of PAAD cells.

Discussion

PAAD is characterized by poor prognosis and resistance to therapy, which explains the relatively high mortality rate compared with other types of cancer (22-24). At present, the treatment options for PAAD are very limited, with surgical resection of the tumor remaining the most effective (25-27). However, for the majority of patients with PAAD, surgery is not a viable option due to a high chance of a relapse occurring (23,28,29). Therapeutic prospects for PAAD remain poor even with improved treatment strategies because of the high risk of metastasis (23,30,31). Due to the complicated configuration of the adjoining anatomy of the pancreas, whereby the pancreas is in contact with numerous neighboring structures, pancreatic cancer cells tend to also aggressively attack the adjacent tissues (24,25,30,32). Therefore, it is essential to identify the mechanisms underlying the proliferation and metastasis of PAAD.

Previous studies have determined that multiple miRNAs can influence the occurrence and progression of PAAD by modulating the expression of various target genes. Zhang et al (33) reported that miR-135b-5p contributed to the epithelial-mesenchymal transition, migration and invasion of PAAD cells by targeting the nuclear receptor protein, nuclear receptor subfamily 3 group C member 2. Furthermore, Daniel et al (34) revealed that miR-32-5p is downregulated in prostate cancer tissues, whereas Wang et al (35) reported that miR-32-5p is lowly expressed in triple-negative breast cancer. Collectively, these previous reports suggest that miR-32-5p regulates the development of various types of cancer to a certain degree. Hence, the aim of the present study was to investigate the role, and identify the target genes and physiological functions of miR-32-5p in PAAD. The present study demonstrated that miR-32-5p expression levels were markedly reduced in PAAD tissues and cell lines compared with healthy tissues and healthy human pancreatic cells. Moreover, elevated miR-32-5p expression levels were associated with a better prognosis of patients with PAAD compared with those with lower miR-32-5p expression levels, which indicated that miR-32-5p may display a tumor-suppressive effect in the onset and progression of PAAD.

Based on the differential expression of miR-32-5p in PAAD, it was speculated that miR-32-5p may be associated with the proliferation, migration and invasion of PAAD. The present study demonstrated that overexpression of miR-32-5p significantly suppressed the proliferation and markedly decreased the migration and invasion of PAAD cells. Collectively, these results suggested that miR-32-5p had an antitumor effect in PAAD cells, serving an oncogenic role in the progression of PAAD. Therefore, miR-32-5p may have the potential to function as a diagnostic biomarker and therapeutic target for PAAD.

Various miRNAs have been shown to serve roles in tumor development and progression by mediating cell proliferation and metastasis. miR-337-3p has been reported to regulate the proliferation, invasion, migration and apoptosis of cervical cancer cells by targeting Ras-related protein Rap1A expression (36), whereas miR-124 was found to regulate metastasis and the epithelial-mesenchymal transition in triple-negative breast cancer by targeting zinc finger E-box-binding homeobox 2 protein expression (37). Furthermore, miR-335 was demonstrated to suppress the proliferation of lung cancer cells by targeting transformer-2 protein homolog β expression (38). Further identification of miR-32-5p target genes will help to further elucidate the role of miR-32-5p in the pathogenesis of PAAD. miRNAs generally bind to the 3'-UTR of target mRNAs (39). In the present study, the starBase database was searched to predict the direct target gene of miR-32-5p in PAAD, which identified TLDC1. Furthermore, these results demonstrated that TLDC1 expression was relatively higher in PAAD tissue compared with that in healthy tissue and negatively correlated with miR-32-5p expression in patients with PAAD. Patients with lower TLDC1 protein expression levels exhibited a better prognosis compared with those displaying higher TLDC1 protein expression levels. The present study therefore indicated that TLDC1 may serve a negative regulatory function in the development of PAAD induced by miR-32-5p.

miR-32-5p serves a key biological role in the tumorigenesis of various types of cancer by regulating multiple target genes. In cervical cancer, homeobox B8 is reported to reverse the proliferation, invasion and migration of cells inhibited by miR-32-5p (12). In colorectal cancer, the transducer of ERBB2-1 protein was found to reverse the inhibitory effect of miR-32-5p on cell sensitization and the promoting effect of miR-32-5p on cell migration and invasion (7). In ovarian cancer, SMG1 nonsense mediated mRNA decay-associated PI3K-related kinase was shown to reduce the promoting effect of miR-32-5p on cell proliferation and motility (9). The present study demonstrated that miR-32-5p suppressed the proliferation, migration and invasion of PAAD cells, which was rescued by TLDCC1 overexpression.

The present study revealed that miR-32-5p was lowly expressed in PAAD. miR-32-5p was shown to negatively target TLDC1, inhibiting the proliferation, migration, and invasion of PAAD cells. As a tumor suppressor gene, miR-32-5p may serve as a promising biomarker for the diagnosis and prognosis of PAAD. However the present study had a number of limitations. For example, the results were not verified using animal models or other PAAD cells. Moreover, the upstream regulation of miR-32-5p and the pathways involved in TLDC1 requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PY and CT designed the study and wrote the manuscript with contributions from all authors. All authors participated in
performing the experiments. BC, PL, JS, GX, ZW, and GZ were responsible for data acquisition and the interpretation of data. YH, WY and GW were responsible for statistical analysis, the literature search and revising the article critically for important intellectual content. All authors read and approved the final manuscript. PY, CT and GZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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