MicroRNA-1252-5p, regulated by Myb, inhibits invasion and epithelial-mesenchymal transition of pancreatic cancer cells by targeting NEDD9

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Abstract

**Background:** MicroRNAs (miRNAs) are known to be involved in the development and progression of pancreatic cancer (PAC). The expression level and role of miR-1252-5p in PAC remain unclear.

**Methods:** qRT-PCR and in situ hybridization were used to detect miR-1252-5p expression in PAC cells and tissues. Associations between miR-1252-5p expression and clinical characteristics or overall survival (OS) were assessed based on 102 patients with PAC who underwent surgical resection. Gain and loss of function of miR-1252-5p was studied in the PAC cell lines, Panc-1 and BxPC 3 *in vitro* and *in vivo*. The direct targets of miR-1252-5p were analyzed using public databases and a dual-luciferase reporter assay.

**Results:** The expression levels of miR-1252-5p are downregulated in PAC cell lines and tissue samples compared to control, and its expression is negatively associated with adverse clinical features and poor prognosis. *In vitro* and *in vivo* experiments show that miR-1252-5p overexpression inhibits the proliferation, migration, invasion and epithelial-mesenchymal transition of PAC cells, whereas miR-1252-5p knockdown enhances these biological behaviors. In addition, miR-1252-5p negatively regulates neural precursor cell expressed, developmentally downregulated 9 (NEDD9) by directly binding its 3'-UTR. NEDD9 restoration at least partially abolishes this effect of miR-1252-5p in PAC cells. Further mechanistic study revealed that the SRC/STAT3 pathway is involved in miR-1252-5p/NEDD9 mediation of biological behaviors in PAC. We also verified that Myb inhibited miR-1252-5p by directly binding at its promoter.

**Conclusion:** MiR-1252-5p may act as a tumor-suppressing miRNA in PAC and may be a potential therapeutic target for PAC patients.

Introduction

In the United States, pancreatic cancer (PAC) is the fourth most common cause of cancer-related death, with an estimated 40,560 deaths in 2015. ¹ Despite remarkable advances in diagnosis and therapy, including novel chemotherapeutic drugs and targeted therapy, the long-term survival of patients with PAC remains unsatisfactory due to high rates of distant metastasis.² It is therefore essential to study the underlying mechanisms of PAC aggressiveness and invasion, which may yield novel therapeutic targets.

MicroRNAs (miRNAs) are a group of endogenous evolutionarily conserved non-coding small RNAs, that can regulate the expression of target mRNA via post-transcriptionally binding its 3'-untranslated region (UTR).³ It has been demonstrated that aberrantly expressed miRNAs contribute to cancer growth, apoptosis, distant metastasis, and drug-resistance in PAC.⁴,⁵ In humans, miR-1252-5p, previously named miR-1252, is located on 12q21.2, and has been reported to be involved in the progression of various cancer types, including non-small cell lung cancer (NSCLC)⁶,⁷ and head and neck cancers.⁸ For example, Tian *et al.* reported that circABCB10 acted as an oncogene in NSCLC by sponging miR-1252 to enhance Fork head box 2 (FOX2) synthase expression and subsequently facilitate NSCLC aggressiveness.⁶
However, the expression, biological roles and the underlying molecular mechanisms of miR-1252-5p in PAC remain to be fully characterized.

It is well established that epithelial-to-mesenchymal transition (EMT) is linked to the proliferation and invasion of various cancer types, including PAC. During EMT, epithelial cells are transformed to have an invasive mesenchymal phenotype, and exhibit changes in expression of EMT markers: E-cadherin (E-cad) is downregulated and Vimentin (Vim) and N-cadherin (N-cad) are upregulated. Additional transcription factors, including Twist, the Snail/Slug family and ZEB1/ZEB2, act as molecular switches in the EMT process. EMT-regulating miRNAs, including miR-200c and miR-21, have been increasingly reported in association with cancer. Mostly, miRNAs negatively regulate EMT, with only a small number of them (such as miR-21) inducing EMT. However, the association between miR-1252-5p and EMT in PAC has not been investigated.

In this study, we show for the first time that miR-1252-5p expression is suppressed in primary PAC tumor tissues (TT) compared with matching non-cancerous adjacent tissues (NAT). Downregulation of miR-1252-5p has potential as a biomarker of the poor prognosis for patients with PAC. In human PAC cell lines, miR-1252-5p is shown to target the 3′-UTR of the mRNA of neural precursor cell expressed, developmentally downregulated 9 (NEDD9). Studies have observed that NEDD9 is up-regulated and may promote the invasion and metastasis of many types of human malignancy, including PAC. We also demonstrate that the SRC/STAT3 pathway may be involved in the biological function of miR-1252-5p targeting of NEDD9 in PAC.

**Materials And Methods**

2.1. Ethics and Reagents

The study protocol was performed according to the principles of the Declaration of Helsinki and the ethics committee of the Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu Province, China. Informed consent was obtained from all patients.

The following primary antibodies were purchased from Abcam (Cambridge, England): anti-E-cad, anti-Vim, anti-Snail, anti-N-cad, anti-Twist, anti-PCNA, anti-Bax, anti-Bcl-2, anti-ZEB1, anti-NEDD9 and anti-GAPDH. The following primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA): anti-t-SRC, anti-p-SRC, anti-t-STAT3, anti-p-STAT3. PP1, an inhibitor of SRC, was purchased from Selleck Chemicals (Houston, TX, USA).

2.2 Study population and follow-up

PAC tumor tissue samples and matched NATs were collected from 102 patients who underwent surgery in the Department of Hepatobiliary Surgery at the Affiliated Hospital of Jiangnan University (Wuxi, Jiangsu Province, China) between January 2009 and December 2013. Primary carcinomas were assessed according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.
None of the patients had received neoadjuvant therapy before surgical resection.

Vital status data were available from municipal registration offices or obtained by calling the patients or their next of kin. Overall survival (OS) was defined as the time from the date of surgery to the time of last follow-up or death from any cause. Censoring occurred if patients remained alive at last follow-up or were lost to follow-up.

2.3 Tissue microarray, in situ hybridization, immunohistochemistry and scoring

TTs and NATs from 102 patients were formalin-fixed, paraffin-embedded (FFPE) and used to construct a tissue microarray, using an automated tissue arrayer (Beecher Instruments, Sun Prairie, Wisconsin) that punched duplicates from all samples. For in situ hybridization, slides were hybridized overnight at 60°C with a miRCURY DIG-labelled locked nucleic acid (LNA)-based probe specific for miR-1252-5p, according to the manufacturer's protocol (Exiqon, Vedbaek, Denmark). For immunohistochemistry, these slides were incubated with antibodies against E-cad and Vim using the DAKO Envision system (DAKO, Carpinteria, California), as described previously.

The slides were scored for staining intensity according to a semi-quantitative scoring system (0, 1, 2 and 3 points scored for none, weak, intermediate and strong staining, respectively) and percentage of positive cells (0, 1, 2, and 3 scored for <10%, 10% to 50% and >50% positive tumor cells, respectively). Thus, the expression of miR-1252-5p, E-cad and Vim were scored overall as 0, 1, 2, 3, 4, 6, or 9. Expression was defined as “low” if the final score was < 4 points and as “high” if it was > 4 points. Scoring was performed by two independent pathologists who were blinded to the clinicopathological characteristics of the samples. Kappa statistics were employed as a measurement of agreement between the two pathologists.

2.4. Cell culture

A total of 5 human PAC cell lines, ASPC-1, CAPAN-2, Panc-1, SW1990 and BxPC-3, and a normal human pancreatic epithelium cell line (HPNE) were purchased from the American Type Culture Collection. Cells were cultured in standard Dulbecco’s modified Eagle’s medium (DMEM, Corning, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin and grown in a humidified incubator with 5% CO₂ at 37°C.

2.5. Cell transfection

miR-1252-5p inhibitor, mimic, and their negative controls (NC), anti-miR-NC and miR-NC, were designed and synthesized by GenePharma (Shanghai, China). The overexpression plasmid for NEDD9 (pCDNA3.1-NEDD9), specific siRNA for NEDD9 (ShNEDD9), and the corresponding negative controls (empty vector [EV] and sh-NC) were synthesized by GenePharma (Shanghai, China). At 30-50% confluence, these materials were transfected to cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol.
2.6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Additional fresh TT and NAT samples were available from 18 patients with PAC. All tissues were confirmed by pathological analysis and immediately frozen in liquid nitrogen and stored at -80°C. Using TRizol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from TTs, NATs and PAC cell lines, according to the manufacturer's instructions. qRT-PCR was performed using a SYBR® Premix Ex Taq kit (Takara Bio Inc., Shiga, Japan) and a 96-well real time PCR system (Bio-Rad Inc., Hercules, CA). GAPDH or U6 snRNA was used as a loading control. The primers for miR-1252-5p were purchased from Sangon (Inc, Shanghai, China), and the details of the primers for GAPDH, NEDD9, E-cad, N-cad, ZEB1, Twist, Snail and Vim are listed in Table S1. Relative gene expression was calculated from the qRT-PCR data using the 2^{−ΔΔCT} method.

2.7. Western blotting

Whole protein was extracted from tissues and cells using RIPA buffer (Roche, Basel, Switzerland). A BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine protein concentration. An equal amount of protein was separated by 10% SDS-PAGE, and then transferred into PVDF membranes (Millipore, Billerica, MA, USA). The membrane was probed with a HRP-conjugated secondary antibody. The protein bands were visualized using an electrochemiluminescence reagent (ECL; Pierce Chemical Co., Rockford, IL). GAPDH was used as a loading control, and quantitative analysis was performed using Image J software.

2.8. MTT assay

A total of $2 \times 10^3$ cells per well were seeded in 96-well plates and incubated overnight before cell proliferation was examined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay every 24 h for 4 days. The optical density was measured at 450nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. Each assay was repeated independently 3 times in triplicate.

2.9. Wound healing assay

Cells were grown to 90% confluence in a 6-well plate. A wound was made in the middle of the well using a 20-μl pipette tip, and then wash with PBS to remove detached cells. The cells were incubated in medium containing 2% serum for 24 h, and migration rates at the wound site were observed using a microscope (Nikon, Tochigi, Japan) at 0 and 24 h. All experiments were performed in triplicate.

2.10 Transwell assay

Migration (without Matrigel) and invasion (with Matrigel) assays were conducted using transwell 24-well plates (8-μm pores; Millipore). A total of $5 \times 10^4$ cells were suspended in the upper chamber in 150 μL serum-free medium, and 600 μL normal growth medium was placed in the lower chamber. Cells which migrated/invaded through to the under-side of the membrane were fixed, stained, and counted in 5 random fields of view.
2.11 Luciferase reporter assay

Target genes of miR-1252-5p were screened for with the Starbase 3.0 program (http://starbase.sysu.edu.cn/). Then, a luciferase reporter assay was conducted to evaluate whether NEDD9 was a direct target gene of miR-1252-5p. Luciferase reporter plasmids predicted to interact with miR-1252-5p (pmiR-GLO-WT-NEDD9–3'UTR), with a corresponding mutated sequence (pmiR-GLO-MUT-NEDD9–3'UTR), were synthesized and purified by GenePharma. Panc-1 and BxPC-3 cells were plated into a 24-well plate and transfected using Lipofectamine 2000 (Invitrogen, USA). After 48 h, cells were collected, and luciferase activities were examined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), according to the manufacturer’s protocol. Renilla luciferase activity was used to normalize the corresponding firefly luciferase activity.

2.12 Chromatin immunoprecipitation (CHIP) assay

We performed the CHIP assay according to the manufacturer’s instructions by a ChIP assay kit (Millipore). Briefly, the cells were fixed with 1% formaldehyde to covalently crosslink proteins to DNA, sheared to 300-500 bp in length. Then the PCR assay was performed to measure enrichment of DNA fragments in the putative Myb-binding sites in the miR-1252 promoter.

2.13 Animal experiments

The animal experiments were approved by the Institutional Animal Care and Use Committee of Affiliated hospital of Jiangnan University. The female BALB/c nude mice (5-week-old) were fed under standard conditions. The BxPC 3 cells (2 × 10^6) were injected subcutaneously into the posterior flank of the mice. Tumor size was measured every 5 days, and was calculated by using the formula (volume = length × width^2 / 2). When tumor volumes reached 50–100 mm³, treatment was initiated as follows: agomir NC, agomiR 1252-5p.

2.14 Statistical analyses

All statistical analyses were performed using the PASW Statistics software program (version 19; SPSS, Chicago, IL, USA) and GraphPad Prism (version 5; GraphPad Inc., La Jolla, CA, USA). A two-tailed p-value < 0.05 was considered to be significant. Data are presented as the mean ± standard deviation. Significant differences were analyzed by using paired student’s t test or one-way analysis of variance (one-way ANOVA) with Tukey’s post hoc test. Spearman correlation test was conducted to analyze the association between miR-1252-5p and NEDD9 expression of PAC tissues. The Kaplan–Meier method and the log rank test were performed to analyze the OS. Cox’s hazards regression model was conducted to evaluate independent prognostic factors.

Results
3.1. MiR-1252-5p expression is downregulated in PAC specimens and cell lines

Using qRT-PCR, we first assessed miR-1252-5p expression levels in cell lines (ASPC-1, CAPAN-2, SW1990, Panc-1, BxPC 3, and HPNE cells). MiR-1252-5p expression was significantly reduced in the 5 PAC cell lines compared with HPNE cells (Fig. 1A). BxPC 3 and Panc-1 cells exhibited the lowest and highest levels of miR-1252-5p expression, respectively, and were therefore selected for subsequent gain- and loss-of-function experiments, respectively. The expression of miR-1252-5p in TT from 18 patients was significantly lower than that in matched NAT (P < 0.01; Fig. 1B and 1C). Expression of miR-1252-5p was also assessed in a TMA containing 102 human TT and matched NAT pancreatic specimens using ISH. According to the calculated staining score, the expression levels of miR-1252-5p were significantly lower in TT compared to NAT (P = 0.01; Fig. 1D-E). These results suggest that miR-1252-5p may be correlated with tumor progression in PAC.

3.2. Clinical significance of miR-1252-5p expression in patients with PAC

To determine the role of miR-1252-5p in PAC progression, we evaluated the relationship between miR-1252-5p expression and clinicopathological characteristics in 102 patients with PAC. We observed that low expression of miR-1252-5p was significantly correlated with node invasion (P = 0.013; Fig. 2A) and high histologic grade (P = 0.010; Fig. 2B), but not with tumor diameter (P = 0.465; Fig. 2C), neural invasion (P = 0.189; Fig. 2D) or T stage (P = 0.277; Fig. 2E). Additionally, Kaplan-Meier curves indicated that PAC patients with low miR-1252-5p expression had a notably decreased OS (P = 0.034; Fig. 2F).

As shown in Table 1, Cox regression survival analysis indicated that downregulated miR-1252-5p expression was an indicator of poor OS (hazard ratio [HR] = 1.79; 95% confidence interval [CI]: 1.00-3.23; P = 0.051), as were large tumor diameter (P = 0.061), advanced T stage (P = 0.032), and positive neural invasion (P = 0.006). Multivariate analyses indicated that low expression of miR-1252-5p was an independent prognostic indicator of poor OS (HR = 1.59; 95% CI: 1.01-2.50; P = 0.046), as were advanced T stage (P = 0.038), and positive neural invasion (P = 0.029).

3.3. MiR-1252-5p inhibits proliferation, migration and invasion in PAC

To explore the biological role of miR-1252-5p in PAC, gain- and loss-of-function studies were conducted in BxPC3 and Panc-1 cells, respectively. It was found that miR-1252-5p was effectively upregulated in BxPC3 cells and downregulated in Panc-1 cells (P < 0.01; Fig. 3A and 3B). MTT assays indicated significantly inhibited cell proliferation in the miRNA mimic group compared with the NC group (Fig. 3C), while significantly enhanced cell proliferation was observed in the inhibitor group (Fig. 3D). Western blotting of markers of cell proliferation and apoptosis showed that miR-1252-5p overexpression significantly inhibited expression of PCNA and Bcl-2, but increased expression of Bax. MiR-1252-5p knockdown led to the opposite change of these markers (Fig. 3E). We next examined whether miR-1252-5p influences cell motility and invasion. After 24 h of transfection, it was found that the wound closure was significantly reduced in the miR-1252-5p-transfected BxPC3 cells (Fig. 3F) and increased in anti-miR-1252-5p transfected Panc-1 cells (Fig. 3G). Transwell analysis also demonstrated that anti-miR-1252-5p
significantly promoted, whereas miR-1252-5p significantly reduced, the migration and invasion of Panc-1 and BxPC3 cells, respectively \( (P < 0.05; \text{Fig. 3H and I}) \). Further animal experiments showed that agomiR 1252-5p treatment significantly suppressed both tumor volume (Fig. 3J) and weight (Fig. 3K). qRT-PCR assay verified enhanced levels of miR-1252-5p expression in tumors treated with miR-1252-5p agomiR-treated BxPC3 cells compared with control tumors (Fig. 3L). The expression levels of Ki-67 were decreased in tumors from miR-338-3p agomiR treated mice compared to that in tumors from control mice (Fig. 3M). These results indicate that miR-1252-5p inhibits PAC proliferation, migration and invasion.

### 3.4. MiR-1252-5p inhibits EMT in PAC

We then explored whether miR-1252-5p modulated PAC progression via regulating EMT by examining the relationship between miR-1252-5p expression and EMT markers (E-cad and Vim) in 102 human PAC tissues. PAC tissues exhibiting high miR-1252-5p expression also had higher E-cad scores than those with low miR-1252-5p expression \( (P < 0.01; \text{Fig. 4A and 4B}) \). On the contrary, Vim expression score of PAC tissues exhibiting high miR-1252-5p expression was significantly lower than those with low miR-1252-5p expression \( (P < 0.01; \text{Fig. 4C and 4D}) \). Western blotting assays revealed that miR-1252-5p enhances E-cad expression and decreases N-cad, Vim, ZEB1, Twist and Snail expression in BxPC 3 cells, while anti-miR-1252-5p induced the opposite in Panc-1 cells (Fig. 4E and 4F). Moreover, we determined EMT marker expression by qRT-PCR and observed similar results \( (P < 0.01; \text{Fig. S1A-B}) \). Collectively, these results implicate that miR-1252-5p suppressed EMT process in PAC.

### 3.5. NEDD9 is a direct target of miR-1252-5p in PAC

Using a miRNA target algorithm (Starbase), we then predicted potential targets and observed conserved putative miR-1252-5p binding sites at the 3′-UTR of NEDD9 (Fig. 5A). We selected NEDD9 as a potential target for further experiments because our previous study \(^{13}\) found that NEDD9 was overexpressed in PAC tumor tissues compared with the NAT pancreas tissues, and high NEDD9 expression was significantly correlated with clinical staging, lymph node metastasis and poor prognosis in patients with PAC. By qRT-PCR and western blotting, we found that overexpression or knockdown of miR-1252-5p markedly decreased or enhanced the mRNA and protein expression levels of NEDD9 in PAC cells, respectively (Fig. 5B-5E). Moreover, we found that the protein expression of NEDD9 in human PAC tissues expressing high levels of miR-1252-5p was significantly lower than those expressing low levels (Fig. 5F). An inverse correlation between miR-1252-5p and NEDD9 mRNA expression was validated in human PAC tissues by Spearman's correlation analysis \( (P = 0.0285; \text{Fig. 5G}) \). Next, a luciferase reporter assay confirmed that overexpression or knockdown of miR-1252-5p significantly decreased or enhanced the luciferase activity of wild-type (WT) NEDD9 3′-UTR, respectively \( (P < 0.05; \text{Fig. 5H-5I}) \). Altering miR-1252-5p expression did not significantly change the luciferase activity of mutant (MUT) NEDD9 3′-UTR cells (Fig. 5H-5I). Taken together, these data indicate that NEDD9 is a downstream target of miR-1252-5p in PAC.

### 3.6. NEDD9 mediates the biological functions of miR-1252-5p in PAC cells
To confirm the biological role of NEDD9 in the effects of miR-1252-5p on PAC, NEDD9 expression was successfully upregulated by transfecting pcDNA3.1-NEDD9 (Fig. 6A-B) or knocked down by transfecting siRNA targeting NEDD9 (Fig. 6C 6D). NEDD9 overexpression promoted cell proliferation of BxPC3 cells (Fig. S2A), while silencing NEDD9 in Panc-1 cells resulted in a significantly reduced cell proliferation (Fig. S2B). To validate NEDD9 as a functional mediator of miR-1252-5p, MTT, wound healing and transwell assays were performed. Rescue experiments showed that up-regulation of NEDD9 restored the miR-1252-5p overexpression-inhibited proliferation (P < 0.01, Fig. 6E), migration (P < 0.01, Fig. 6G), invasion (P < 0.01, Fig. 6I), and EMT process (P<0.01, Fig. 6K) in BxPC3 cells, while knockdown of NEDD9 blocked the miR-1252-5p inhibitor-enhanced biological effects on Panc-1 cells (P < 0.01, respectively, Fig. 6F, 6H, 6JandFig.6L). These results support the hypothesis that NEDD9 acts as a functional mediator of miR-1252-5p in PAC.

3.7 The SRC/STAT3 signaling pathway is involved in the biological roles of miR-1252-5p in PAC

Recently, it has been shown that NEDD9 acts through SRC and STAT3 to promote invasion in melanoma, cervical cancer and ovarian cancer. To investigate whether NEDD9 is involved in the activation of oncogenic signaling pathways, including SRC and STAT3, in PAC development, we examined the phosphorylation states of these proteins. NEDD9 upregulation at least partially rescued the level of phosphorylated SRC and STAT3 inhibited by miR-1252-5p overexpression in BxPC-3 cells, while modulating NEDD9 expression did not alter the levels of both total proteins (Fig. 7A). Furthermore, inhibition of miR-1252-5p induced activation of SRC/STAT3 signaling, and NEDD9 knockdown at least partially inhibited these effects (Fig. 7B). In addition, MTT assays showed that NEDD9 stimulated proliferation of BxPC-3 (Fig. 7C) and Panc-1 cells (Fig. 7D), which was significantly impeded in both cell lines incubated with PP1, an inhibitor of SRC. Wound healing and western blotting assays showed that NEDD9-induced migration and EMT rely on SRC activity in BxPC-3 (Fig. 7E and 7G) and Panc-1 cells (Fig. 7F and 7H). Collectively, these data indicate that the SRC/STAT3 pathway is involved in the biological function of miR-1252-5p/NEDD9 in PAC.

3.8 Myb inhibits miR-1252-5p expression through binding its promoter

We used the JASPER bioinformatics software program to search a 2 kb region upstream of the transcription start site (TSS) of miR-1252. Two Myb-binding motifs from 1963 to −1972 and −352 to −361 were identified, named A and B (Fig. 8A), and ChIP assays confirmed that Myb protein was indeed recruited to these two binding sites in both cells (Fig. 8B and 8C). Subsequently, reduced luciferase activity in the wt miR-1252 promoter was observed after overexpression of Myb in both cells (Fig. 8D and 8E). These effects were not observed when the A and/or B sites were mutated. We found that ectopic expression of Myb inhibited, while silencing of Myb increased, miR-1252-5p expression in Panc-1 and BxPC-3 (Fig. 8F and 8G) cells. On the contrary, expression of NEDD9 protein in PAC cells was decreased or increased after expression of Myb was silenced or upregulated, respectively (Fig. 8H and 8I). We further revealed that Myb expression was negatively correlated with miR-1252-5p expression, but positively with NEDD9 expression in human PAC tissues (Fig. 8J and 8K).
3.9 MiR-1252-5p inhibits Myb-induced cell biological behaviors in PAC cells

We then explored if miR-1252-5p played a role in the Myb-induced phenotypes in PAC cells. As shown in Fig.9A-9D, Myb overexpression significantly increased BxPC 3 and Panc-1 cell growth (Fig.9A and 9B), migration (Fig.9C and 9D), while miR-1252-5p overexpression significantly reversed these effects. Further western blotting assays observed that Myb overexpression significantly enhanced NEDD9 expression, the activation of SRC/STAT3 pathway and the EMT process, while miR-1252-5p overexpression significantly reversed these effects (Fig.9E). Taken together, our findings indicate that miR-1252-5p counteract Myb-induced PAC growth, EMT process and the subsequent activation of SRC/STAT3 via targeting NEDD9.

Discussion

Although significant progress has been made in cancer therapy, PAC still develops resistance to current standard therapies, resulting in a poor prognosis. Evidence suggests that miRNAs serve significant roles in the regulation of genes during the development of various cancer types, including PAC. In this study, we observed significantly downregulated expression of miR-1252-5p in PAC tissues and cell lines for the first time. We demonstrated that miR-1252-5p plays a suppressive oncogene role in PAC by directly targeting NEDD9 to inhibit activation of SRC/STAT3 signaling, consequently impeding PAC progression. Specifically, miR-1252-5p expression was inhibited by Myb at the transcriptional level. These data indicate that miR-1252-5p may be useful as a biomarker for prediction of prognosis, and may be a potential therapeutic target for PAC.

MiR-1252-5p was found to be downregulated in human PAC cell lines and tissues compared with normal pancreatic cells and NAT. Furthermore, low miR-1252-5p expression was closely associated with node invasion and high histologic grade, but not with T stage, neural invasion or tumor diameter. Low expression of miR-1252-5p was identified by multivariate Cox regression analysis as an independent predictor of poor OS for PAC patients who underwent tumor resection. Intriguingly, gain- and loss-of-function experiments revealed that overexpression of miR-1252-5p inhibited the proliferation, migration, invasion, and EMT of PAC cells, while knockdown of miR-1252-5p enhanced these aggressive behaviors. EMT, characterized by acquiring a migratory and invasive mesenchymal phenotype, is a major contributor to cancer-cell aggressiveness and metastasis. EMT has also been demonstrated to be regulated by miRNAs such as miR-202 and miR-200c. In the present study, expression of epithelial (E-cad) and mesenchymal markers (Vim, N-cad, ZEB1) were increased or decreased, respectively, in PAC cells when miR-1252-5p expression was upregulated, and the opposite was achieved when miR-1252-5p expression inhibited.

The major finding of the present study is that miR-1252-5p functions as a tumor-suppressive miRNA in human PAC through inhibiting the cell mobility, invasiveness and proliferation that underlies metastasis, via directly inhibiting its downstream target, NEDD9. Firstly, miR-1252-5p negatively regulated NEDD9 expression at the mRNA and protein levels in PAC cells. Secondly, miR-1252-5p expression was inversely correlated with the expression of NEDD9 in PAC tissues. Finally, we identified the complementary
sequence of miR-1252-5p in the 3'UTR of NEDD9 mRNA, and overexpression or knockdown of miR-1252-5p accordingly altered the luciferase activity with the WT 3'UTR but not the mutant 3'UTR of NEDD9.

NEDD9 is a member of the non-catalytic Crk-associated substrate (CAS) family of scaffolding proteins, which functions as a mediator of oncogenic proteins and regulates a number of metastatic signaling molecules. Elevated NEDD9 expression has been reported in many cancer types, and is typically associated with tumor growth and invasion. Data from Gabbasov et al. showed that cancer cell-intrinsic NEDD9 expression promoted ovarian carcinoma development and invasion via induction of genes associated with oncogenic signaling and cancer stem cell properties (ALDH1a1 and ALDH1a2). Another study revealed that reduced expression of miR-451 increased chemoresistance in patients with metastatic castration-resistant prostate cancer by targeting NEDD9. Our work and other previous studies have determined the role of NEDD9 in PAC. We show that high expression of NEDD9 is significantly correlated with clinical staging, lymph node metastasis, histologic stage and significantly shorter survival time. Contrastingly, other studies have reported that NEDD9 acts as a tumor suppressor in breast cancer and chronic myelogenous leukemia (CML). An early study by Minn et al. found that downregulation of NEDD9 was part of a gene expression signature predicting breast cancer cell metastasis to the lung. In a p210 Bcr/Ab1 mouse model of CML, knockdown of NEDD9 promoted the development, infiltration of myeloid cells in several tissues in CML. In the current research, silencing of NEDD9 decreased miR-1252-5p loss-of-function-induced proliferation and migration of PAC cells, while ectopic expression of NEDD9 rescued miR-1252-5p overexpression-inhibited aggressive behavior of PAC cells, suggesting that miR-1252-5p inhibits NEDD9-mediated PAC progression.

NEDD9 has been shown to exert its oncogenic function by enhancing activation of the SRC and STAT3 signaling pathways. Employing genetically engineered mouse models (targeted disruption of Nedd9, Nedd9−/− genotype) in ovarian carcinoma, Gabbasov et al. showed that mice with the Nedd9−/− genotype exhibited decreased tumor growth and incidence of ascites via reduced expression and activation of signaling proteins, including SRC/STAT3. SRC, a 60-kDa member of the non-receptor tyrosine kinase family, is upregulated in 70% of pancreatic tumors. Inhibition of SRC signaling may retard pancreatic tumor growth and enhance gemcitabine cytotoxicity in xenografts of human pancreatic cancer in mouse models. In the current study, we examined whether SRC/STAT3 signaling was essential for the effect of miR-1252-5p/NEDD9 on PAC progression. We found that ectopic expression of NEDD9 rescued the inhibited SRC/STAT3 phosphorylation caused by miR-1252-5p overexpression, while the opposite results were observed in NEDD9- and miR-1252-5p-silenced cells. Further functional assays (MTT, wound healing and western blotting) showed that NEDD9-stimulated PAC progression was substantially inhibited by PP1. These data demonstrate that miR-1252-5p inhibits NEDD9-mediated biological behavior of PAC via the SRC/STAT3 pathway.

Subsequently, we explored the underlying mechanism that contributed to the downregulation of miR-1252-5p in PAC. It has been shown that the interactions between transcription factors and miRNAs are critical in many pathologic conditions. The MYB proto-oncogene, located on the 6q22-24 chromosomal
region, has been widely accepted to act as a critical oncogenic driver of many types of cancer, including PAC. Our results uncovered two putative binding sites of Myb in the region upstream of miR-1252 locus. The subsequent ChIP assay and luciferase assay demonstrated that Myb could negatively regulate miR-1252-5p expression by directly binding its promoter. We further confirmed that there was a negative correlation between Myb and miR-1252-5p in the PAC cells and tissues.

**Conclusion**

To conclude, we demonstrate for the first time that miR-1252-5p expression is downregulated in human PAC tissues and cell lines, and that the decreased expression is associated with malignant clinicopathological features and poor prognosis of PAC patients. Mechanistically, miR-1252-5p inhibits the proliferation, migration, invasion and EMT of PAC cells by directly targeting NEDD9-mediation of SRC/STAT3 signaling. Our study also demonstrated that miR-1252-5p is inhibited by Myb at the transcription level. There is a MYB/miR-1252-5p/NEDD9 axis that results in PAC progression. Our findings have enriched the body of knowledge about the molecular mechanisms underlying PAC and provide potential targets for future therapeutic invention.

**Abbreviations**

miRNAs, MicroRNAs; PAC, pancreatic cancer; OS, overall survival; NEDD9, neural precursor cell expressed, developmentally downregulated 9; EMT, epithelial-mesenchymal transition; UTR, untranslated region; NSCLC, non-small cell lung cancer; FOX2, Fork head box 2; E-cad, E-cadherin; Vim, Vimentin; N-cad, N-cadherin; TT, tumor tissues; NAT, non-cancerous adjacent tissues; AJCC, American Joint Committee on Cancer; LNA, locked nucleic acid; CHIP, Chromatin immunoprecipitation; CAS, Crk-associated substrate; CML, chronic myelogenous leukemia

**Declarations**

**Acknowledgements**

Not applicable.

**Authors' contributions**

Conception and design: XYZ and WTL.

Acquisition of data: XYZ, SYY and WTL.

Analysis and interpretation of data: XYZ, WTL, SYY, ZY, HBS.

Writing, review, and/or revision of the manuscript: XYZ, BCQ.
All authors read and approved the final manuscript.

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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.

**Ethics approval and consent to participate**
All procedures performed in the present study involving human specimens were approved by the Ethics Committee of the affiliated hospital of Jiangnan University, Wuxi, Jiangsu Province. Written informed consent was provided by all patients prior to surgery.

**Consent to participate**
All patients provided written informed consent prior to their inclusion within the study.

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

**References**


**Tables**

Table 1. Prognostic factors in Cox’s proportional hazards model
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Median OS (95%CI) (months)</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>HR (95%CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR (95%CI)</td>
</tr>
<tr>
<td><strong>Tumor size, cm</strong></td>
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<tr>
<td>&lt; 3.5</td>
<td>15.7 (11.7-18.9)</td>
<td>1.0</td>
<td>0.061</td>
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<td>≥3.5</td>
<td>12.2 (10.1-13.4)</td>
<td>1.62 (0.98-2.69)</td>
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<td><strong>T stage</strong></td>
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<td>T1-2</td>
<td>18.8 (12.9-24.3)</td>
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<td>T3</td>
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<tr>
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<td>Poor</td>
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<tr>
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<td><strong>Neural invasion</strong></td>
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<tr>
<td>Negative</td>
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<tr>
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<td>2.06 (1.23-3.46)</td>
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<td><strong>Vascular invasion</strong></td>
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<tr>
<td><strong>MiR-1252-5p score</strong></td>
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<td>High</td>
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<tr>
<td>Low</td>
<td>12.2 (10.9-13.5)</td>
<td>1.79 (1.00-3.23)</td>
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</table>

NOTE: Tumor classification and stage were referred to the 7th edition of UICC (2009) on cancer staging system.