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Original Article

MiR-424-5p promotes cell invasion and migration by targeting CYLD in human pancreatic cancer

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Abstract: The effect and mechanism of miR-424-5p on the invasion and migration of human pancreatic cancer is unknown. Quantitative real-time PCR (qRT-PCR) was performed to detect the expression of miR-424-5p and CYLD in pancreatic cancer specimens and pancreatic cancer cells PANC-1. MiR-424-5p mimics/inhibitors and their negative controls were transfected into PANC-1 cells respectively, following by transwell, and wound healing assays, which were used for investigating the capacity of cell invasion and migration. Meanwhile, protein levels of CYLD, Smad3 and E-cadherin were examined through western blotting. Luciferase reporter assay was used to validate CYLD as a target of miR-424-5p. MiR-424-5p suppressed the expression of CYLD and was significantly upregulated in pancreatic cancer specimens and cells. Overexpression of miR-424-5p promoted cell invasion and migration, which on the contrary would be reduced by the downregulation of miR-424-5p in vitro. MiR-424-5p may increase pancreatic cancer cells invasion and migration by targeting CYLD, and thus potentially serving as a new therapeutic target for pancreatic cancer.

Keywords: CYLD, invasion, migration, MiR-424-5p, pancreatic cancer

Introduction

Pancreatic cancer (PC), which is the fourth most common cause of cancer death in the United States [1] and the sixth leading cause of cancer-related death in China [2], is one of the most lethal and aggressive malignancies. Surgical resection provides the best opportunity for cure [3]. However, only 20%-25% patients can receive comprehensive treatment based on surgery and the 5-year survival rate after complete resection is only 15-20% [4]. Patients with unresection show poorer prognosis and the 5-year survival rate is less than 1% [5]. Literatures showed that the invasion and migration of tumor cells were mainly responsible for the low 5-year survival. Additionally, because of insensitivity to chemotherapy and radiotherapy for PC, the treatment effect is not ideal. Hence, there is a dire need to explore molecular mechanism of PC and discover new therapeutic targets to improve PC treatment such as to increase the survival of PC patients.

Micro-ribonucleic acids (miRNAs) are a recently discovered class of small endogenous single non-coding RNA molecules of about 18-25nt. MiRNAs play an important role via complementary base-pairing with sequences of the messenger RNA (mRNA) 3'-untranslated region (3'-UTR), which leads to target gene mRNA degradation or translation inhibition, in various biological process, including cell survival, differentiation, apoptosis, proliferation and metastasis [6, 7]. Recent reports have indicated that aberrant upregulation or downregulation of specific miRNAs contribute to the development and progression of human cancers, which acted as either tumor suppressors or oncogenes [8, 9]. Indeed, there are multiple abnormal miRNAs expressions in the same tumor tissue and the same miRNA plays different role in specific tumor tissues. For example, miR-424-5p was downregulated and suppressed cell proliferation, invasion and migration in hepatocellular carcinoma, prostate and colon cancers [10, 11]. On the contrary, a previous study showed

that miR-424-5p was upregulated in pancreatic cancer cells and tissues, which could significantly promote tumor proliferation, migration, invasion, and apoptosis inhibition by targeting cytokine-induced signaling6 (SOCS6) [12]. However, the biological function of miR-424-5p in the pancreatic cancer has not been well studied.

We predicted that the cylindromatosis (CYLD) gene may be a potential target of miR-424-5p via the online bioinformatics TargetScan algorithm. CYLD, a multifunctional deubiquitinase, was originally identified as a gene mutated in familial.

Cylindromatosis [13] and functioned as a tumor suppressor in multiple types of cancers. The loss of CYLD promotes cellular activation, invasion, migration, proliferation, tumorigenesis and inhibits apoptosis by negatively regulating various signaling pathways, such as transforming growth factor-β (TGF-β), Wnt/β-catenin, nuclear factor kappa B (NF-κB), and c-Jun N-terminal kinase (JNK) [14-16]. Some studies have reported that CYLD was downregulated or lost in hepatocellular carcinoma [17], colorectal carcinoma [18] and breast cancer cell lines and tissues [19]. So far, CYLD has not been analyzed in human pancreatic cancer. It has been reported that miR-424-5p was upregulated in the human pancreatic cancer, which promotes the invasion and migration of several cancers. Consequently, we advance the hypothesis that miR-424-5p promotes the invasion and migration of human pancreatic cancer by targeting CYLD.

In this study, we first evaluated the expression of miR-424-5p in human pancreatic cancer tissues and cells. And then we investigated the effect of miR-424-5p on the invasion and migration of PANC-1 cells by wound healing and transwell assays. Finally, we identified that the CYLD was the target gene of miR-424-5p using dual luciferase report assays, in which miR-424-5p downregulated the CYLD by both overexpression and expression inhibition methods *in vitro*.

Methods

Clinical tissue specimens

Specimens of PC and adjacent matched noncancerous tissues from 12 patients were obtained postoperatively at General Hospital of the Second Artillery, Beijing, China. All tissue specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C. All samples were diagnosed correctly based on clinical and pathological evidence. Informed consent was provided before admission into the trial and the Investigational Ethics Committee in our hospital reviewed and approved the trial.

Cell culture

Human pancreatic cancer cell line PANC-1 was selected and obtained from Central Experiment Laboratory of Cancer Hospital Chinese Academy of Medical Sciences. Human normal pancreatic ductal epithelial cell line HPDE6-C7 was purchased from Jennio Biotech Corp., Guangzhou, China. Cells were maintained in Dulbecco's modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), in a humidified incubator with 5% CO_2 atmosphere at 37°C.

Cell transfection

The miR-424-5p mimics/inhibitors and the negative controls (NC) respectively were purchased from GenePharma (GenePharma, China). PANC-1 cells were seeded into 6-well culture plates and transfected at 70-80% confluence with Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. The transfection efficiency was detected after 36 h via qRT-PCR assay.

RNA extraction and quantitative real-time PCR

Total RNA was obtained from the frozen tissues and cells using the TRIZOL Reagent (GenePharma, China) according to the manufacturer's instructions. CDNA was synthesized from total RNA with oligo (dT) primers by using the Omniscript cDNA Kit (GenePharma, China). Then MiR-424-5P level was measured by quantitative PCR using Taqman assay kits (GenePharma, China), and U6 small nuclear RNA was used as an normalization. For CYLD mRNA detection, β-actin mRNA was examined as a housekeeping gene to normalize reference in expression level. The primers used in this study were as follows:

MiR-424-5p, forward 5'-CAGCAGCAATTCATGT-3'reverse 5'-TGGTGTCGTGGAGTCG-3'; U6, for-

ward 5'-GCACCCGTCCAAGAGAGTC-3'; reverse 5'-GGTTCCATCCGTACAGCCT-3'; CYLD, forward 5'-GCACCCGTCCAAGAGAGTC-3'; Reverse 5'-GGTTCCATCCGTACAGCCT-3'; β-actin, forward 5'-CGTGGACATCCGCAAAGA-3'; reverse 5'-GAAGGTGGACAGCGAGGC-3'; Each PCR reaction was run in triplicate and gene relative expression was calculated as $2^{-\Delta\Delta Ct}$.

Western blotting

Total protein was extracted from PANC-1 cells or PC tissues which were lysed in RIPA buffer supplemented with protease inhibitors. The BCA protein assay kit (TIANGEN, China) was used to determine the protein concentration. Samples were electrophoresed in 10% SDS-PAGE gel at 120 V. After transferring to polyvinylidene fluoride (PVDF) membranes at 18 V for 2 h and blocking for 1 h with 5% non-fat milk (Bio-rad, USA), the membranes were incubated with primary antibodies and corresponding secondary antibodies, and then exposed to X-ray films. The primary antibodies used were against CYLD (Sigma-Aldrich, USA), Smad3 (Cell Signaling Technology, USA), E-cadherin (Bioworld, China) and α-Tubulin (Santa Cruz, USA). α-Tubulin was used as control.

Luciferase reporter assay

PANC-1 cells were cultured in 24-well plates for 24 h until 70-80% confluence. The CYLD 3'-UTR was cloned into the luciferase reporter vector pGL3 (WT/Mut iGenebio, China), and it was cotransfected with miR-424-5p mimics or mimics NC into PANC-1 cells using the Lipofectamine 3000 according to the manufacturer's instruction. 40 hours later, the Dual Luciferase Reporter Assay Kit (Promega, USA) was applied to measure luciferase activities. Three independent experiments were executed.

Cell migration and invasion assay

The wound-healing assay and transwell assay were adopted for assessing the migration of PANC-1 cells. For the wound-healing assay, PANC-1 cells transfected with the miR-424-5p mimics/inhibitors and the negative controls were cultured in 6-well plates until confluence and then an artificial wound approximately every 1.5 mm was created with a 200-μl pipette tip. After wounding, the cells were washed with serum-free medium. Serial images were obtained at 0, 24 and 48 h. For transwell migration assay, PANC-1 cells (1×10^5) were suspended in transwell chambers (8 μm; Corning, USA) with serum-free medium. In the bottom chamber (12-well plates), medium containing 10% FBS served as the chemoattractant. After incubating for 24 h, the migrated cells adhering to the lower surface were fixed with methanol, stained with a dye solution containing 0.5% crystal violet, and then counted with a microscope (Olympus Corp., Tokyo, Japan). Cell invasion assay was similar to that of the migration assay except that the upper chambers were first covered with 50 mg/ml matrigel.

Statistical analysis

The SPSS 19.0 software was used for the statistical analysis. Data from at least three independent experiments was presented as the mean ± SD. The two-tailed Student's t-test was used to assess the differences between two groups of data. A paired Student's t-test was used to evaluate the statistical significance in the comparing of paired tissues. All tests performed were two sided. *P* value < 0.05 was considered statistically significant.

Results

MiR-424-5p is upregulated and CYLD is downregulated in pancreatic cancer tissues and PANC-1 cells

To investigate the function of miR-424-5p in the development of human pancreatic cancer and its underlying biological mechanism, we first detected the expression of miR-424-5p and CYLD in the tumor tissues and paired adjacent non-cancer tissues of 12 patients by qRT-PCR. We found that miR-424-5p expression was significantly upregulated with a median of 5.78-fold (range 1.09- to 14.13-fold) in PC tissues compared with the matched non-tumor tissues (**Figure 1A**; $P < 0.05$); on the contrary, compared with non-cancer tissues, expression of CYLD showed a 58.4% (range 21.72% to 88.31%) reduction in PC tissues (**Figure 1B**; $P < 0.05$). Subsequently, qRT-PCR demonstrated that miR-424-5p was also upregulated in PANC-1 cells compared with the pancreatic ductal epithelial cells HPDE6-C7 (**Figure 1C**; $P < 0.05$).

Next, we utilized Pearson correlation analysis to further explore the biological relationship

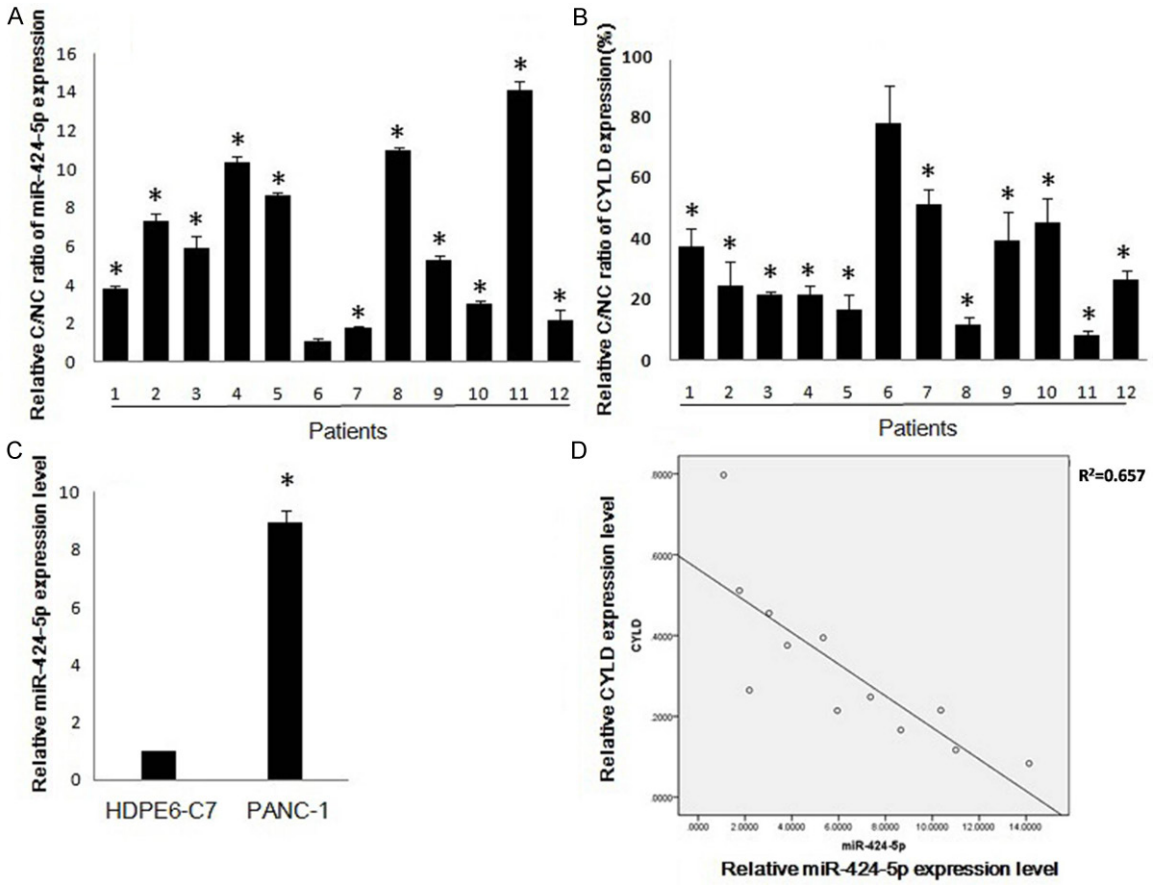

Figure 1. Comparison of miR-424-5p and CYLD expression levels in pancreatic cancer (PC). (A and B) The expressions of miR-424-5p and CYLD in 12 paired PC tissues (C) and non-cancer tissues (NC) were measured by qRT-PCR. (C) Relative miR-424-5p expression levels in HDPE6-C7 and PANC-1 cells by qRT-PCR. (D) MiR-424-5p expression levels were statistically significant inversely correlated with CYLD in a total of 12 tumors by Pearson correlation analysis. *$P < 0.05$.

between CYLD and miR-424-5p in PC specimens. As shown in **Figure 1D**, the expression of miR-424-5p is statistically significant inversely correlated with CYLD (R^2=0.657, P=0.001) in a total of 12 tumors. The data imply that the function of miRNA-424-5p in the development of PC is implemented via directly targeting CYLD.

Overexpression of miR-424-5p promotes PANC-1 cells invasion and migration

To assess whether miR-424-5p plays a role in the development and progression of PC, we selected PANC-1 cells for experiments. PANC-1 cells were first transfected with miR-424-5p mimics or negative control. After 48 h, expression of miR-424-5p increased significantly compared with NC by qRT-PCR (**Figure 2A**; P < 0.05). And then, wound healing assay and transwell assay were performed to examine the biological significance of miR-424-5p in PC metastasis. As shown in **Figure 2B**, wound-healing assay, which was used for examining the cell migration, revealed that overexpression of miR-424-5p significantly accelerated cell migration (after 48 h, 26.32±3.51 vs. 53.28±4.47, P < 0.05). Transwell assay also demonstrated that overexpression of miR-424-5p markedly quickened the migrative capacity of PANC-1 cells compared with negative control cells (**Figure 2C** migration, 119.33±11.31 vs. 48.46±7.73/HP; P < 0.05). Finally, Transwell assay with matrigel was conducted to analyze the effects of miR-424-5p on the invasion of PANC-1 cells and showed that overexpression of miR-424-5p significantly accelerated the invasion of PANC-1 cells (**Figure 2C** invasion, 123.21±9.62 vs. 50.33±7.02/HP; P < 0.05). Collectively, the above results suggest that miR-424-5p promotes cell invasion and migration *in vitro*.

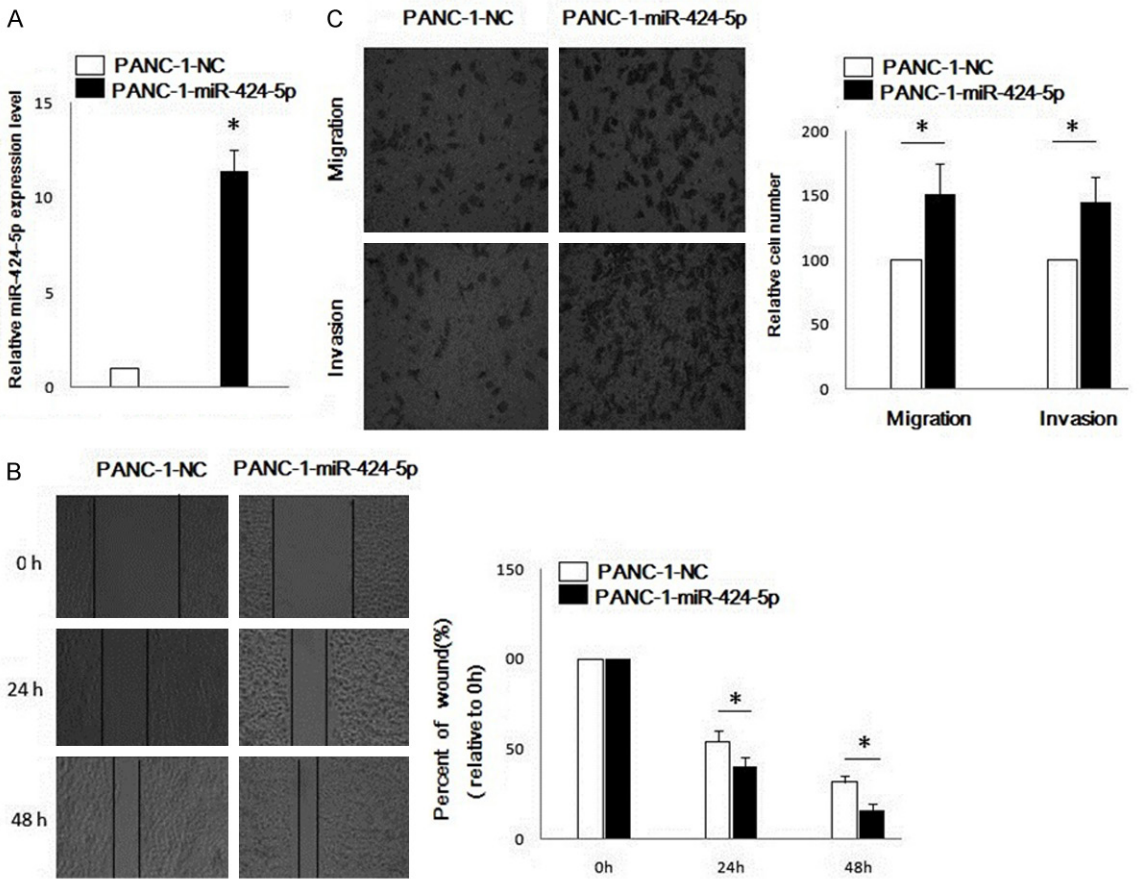

Figure 2. MiR-424-5p promotes invasion and migration of PANC-1 cells *in vitro*. A. Expression of miR-424-5p in PANC-1 cells transfected with negative control (NC) or miR-424-5p mimics by qRT-PCR. B. Representative images and the migration rates from a wound healing assay at 0, 24, 48 hours in PANC-1 cells transfected with NC or miR-424-5p mimics. C. Transwell migration and invasion assays were conducted using PANC-1 cells transfected with miR-424-5p mimics or NC. Representative images are shown on the left, and the quantification of 10 randomly selected fields is shown on the right. *$P < 0.05$.

Downregulation of miR-424-5p inhibits PANC-1 cells invasion and migration

As shown in **Figure 3A**, PANC-1 cells were successfully transfected with the miR-424-5p inhibitors. Compared with negative control cells, the migrative capacity of PANC-1 cells transfected with miR-424-5p inhibitors was significantly retarded in wound-healing assay (**Figure 3B**, after 48 h, 71.52±9.82 vs. 53.28±4.47; P < 0.05) and transwell assay (**Figure 3C** migration, 26.67±5.68 vs. 54.0± 7.54/HP; P < 0.05). Moreover, transwell assay with matrigel showed that miR-424-5p inhibition markedly decreased the invasive capacity of PANC-1 cells compared with negative control cells (**Figure 3C** invasion, 22.67±3.52 vs. 49.83±6.28/HP; P < 0.05). These results suggest that miR-424-5p inhibition reduced the invasion and migration of PANC-1 cells *in vitro*.

CYLD is a direct downstream target of miR-424-5p

To further unravel the mechanism underlying the function of miR-424-5p in PANC-1 cells, the online bioinformatics TargetScan algorithm was performed to search for potential mRNA targets of miR-424-5p. CYLD was selected from multitudinous candidates (**Figure 4A**). To determine whether CYLD was the direct target of miR-424-5p, a dual-luciferase reporter was performed. We cloned a fragment of the CYLD 3'-UTR mRNA containing a miR-424-5p binding site into the luciferase reporter vector pGL3. PANC-1 cells were transfected with either miR-424-5p mimics or negative control, along with pGL3-CYLD 3'-UTR or pGL3-CYLD 3' UTR-mut. As shown in **Figure 4B**, overexpression of miR-424-5p induced a 43.9% decrease in luciferase activity of pGL3-CYLD 3'-UTR compared

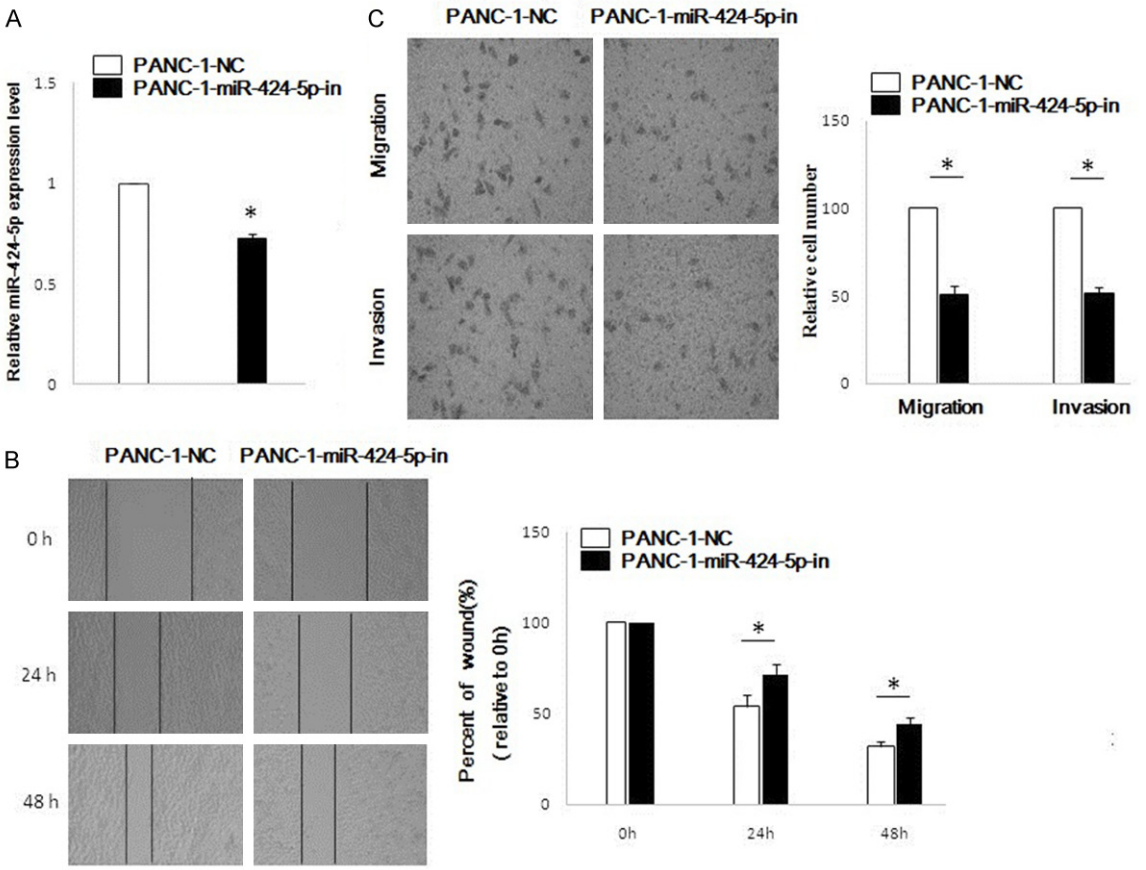

Figure 3. MiR-424-5p inhibition reduces invasion and migration of PANC-1 cells *in vitro*. A. Expression of miR-424-5p in PANC-1 cells transfected with negative control (NC) or miR-424-5p inhibitors by qRT-PCR. B. Representative images and the migration rates from a wound healing assay at 0, 24, 48 hours in PANC-1 cells transfected with NC or miR-424-5p inhibitors. C. Transwell invasion and migration assays were conducted using PANC-1 cells transfected with miR-424-5p inhibitors or NC. Representative images are shown on the left, and the quantification of 10 randomly selected fields is shown on the right. *$P < 0.05$.

with the negative control. Moreover, no significant variation in luciferase activity was observed by introducing point mutations in the miR-424-5p-binding seed region.

We had discovered that CYLD mRNA was downregulated in PC tissues and PANC-1 cells. To test the effect of miR-424-5p on CYLD and how downstream suppressor of CYLD is negatively regulated by miR-424-5p in PC, PANC-1 cells were transfected with the miR-424-5p mimics/inhibitors and cultured for 48 h, and then, western blotting assay was performed to detect the expression of CYLD, Smad3 and E-cadherin protein. As shown in **Figure 4C**, CYLD and E-cadherin protein level was decreased in PANC-1 cells transfected with the miR-424-5p mimics and upregulated when miR-424-5p was knocked down with miR-424-5p inhibitors. Smad3 expression was contrary to that of CYLD and E-cadherin.

Collectively, these results suggested that miR-424-5p could directly bind to the CYLD 3'-UTR region to regulate CYLD in PANC-1 cells.

Discussion

MicroRNAs (miRNAs) have been identified and demonstrated to play important roles in the development of various solid tumors and hematologic malignancies [20, 21]. MiRNAs may function as tumor suppressors or oncogenes because of their ability to affect the expression of genes and proteins that regulate cell proliferation, migration and invasion. However, the molecular mechanisms of miRNAs modulation in the process of tumorigenesis are not fully

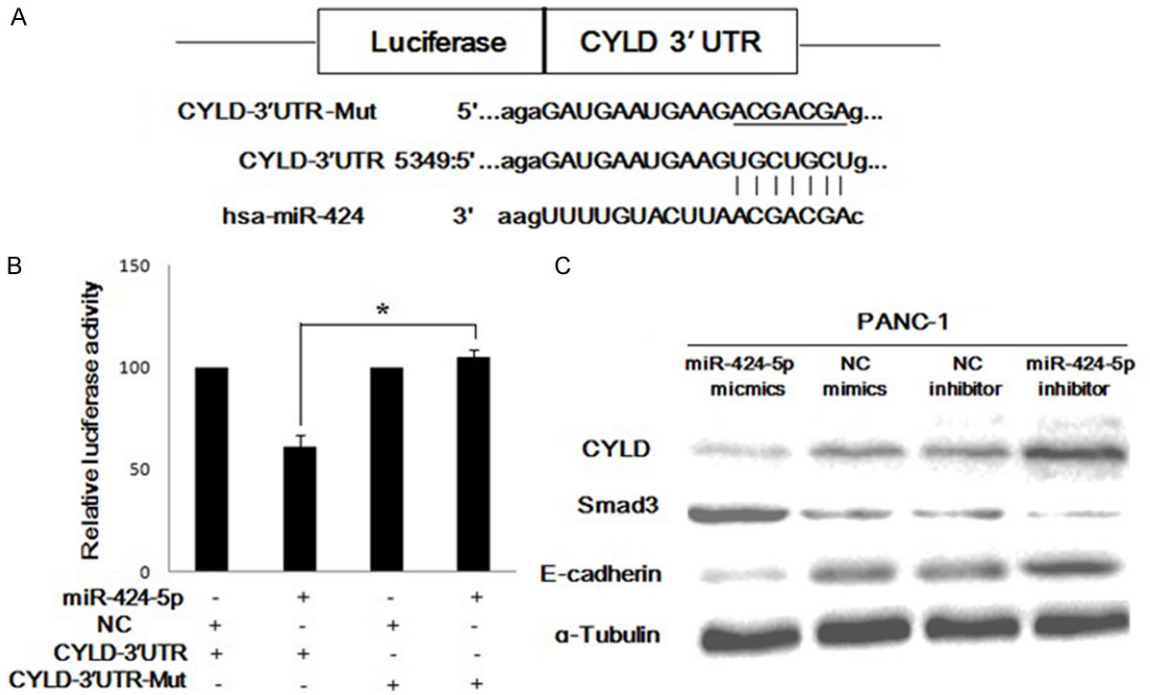

Figure 4. CYLD is a direct target of miR-424-5P in PC. A. Diagram of the mature miR-424-5p sequence and miR-424-5p target site in the 3'-UTR of CYLD mRNA. B. Luciferase activity assay of PANC-1 cells transfected with pGL3-CYLD-3'-UTR or pGL3-CYLD-3'-UTR-mut and miR-424-5p mimics or negative control. C. Western blotting analysis of protein expression of CYLD, Smad3 and E-cadherin in PANC-1 cells transfected with miR-424-5p mimics/inhibitor and their negative controls. α-Tubulin was used as control. *$P < 0.05$.

understood. In the present study, we found that miR-424-5p was significantly upregulated in PC tissues and PANC-1 cells compared with the matched adjacent non-cancerous tissues and HPDE6-C7 cells. Additionally, high expression of miR-424-5p significantly promoted PANC-1 cells invasion and migration, while inhibition of miR-424-5p had the opposite effect. These results indicate that miR-424-5p may be a novel oncogene that plays critical roles in the regulation of PC tumor metastasis.

CYLD, which has a putative binding site for miR-424-5p, has been reported to be a negative regulator in several types of cancers. However, this effect was not detected in PC. As described before, CYLD, a multifunctional deubiquitinase, regulates diverse biological processes including proliferation, differentiation, apoptosis, invasion and migration [22-24]. Down regulation or deletion of CYLD has been observed in a variety of human cancers including colorectal cancer, breast cancer and hepatocellular carcinoma, which suggests CYLD may function as a tumor suppressor. In this study, we found that CYLD was significantly down-regulated in PC tissues and PANC-1 cells. Additionally, overexpression of miR-424-5p decreased the expression of CYLD and inhibition of CYLD gene expression could elevate the ability of PANC-1 cells invasion and migration, while downregulation of miR-424-5p had the opposite effects. Furthermore, we identified CYLD as a potential target gene of miR-424-5p via a bioinformatics method. Luciferase reporter assay showed that downregulation of CYLD was modulated by miR-424-5p specifically targeting the CYLD 3'-UTR. These results demonstrated that CYLD is a bona fide target of miR-424-5p.

To further explore the underlying mechanisms of PANC-1 cell invasion and migration induced by miR-424-5p, we examined the expression of Smad3 and E-cadherin using western blotting assay. Jae Hyang Lim *et al.* [15] showed that CYLD negatively regulates TGF-β signaling via deubiquitinating K63-ubiquitinated Akt to inhibit Smad3. TGF-β signaling, including either Smad-dependent or independent signaling, is generally activated and involved in promoting invasion, angiogenesis, and metastasis in a wide array of human cancers [25-27]. It has

been reported that epithelial-mesenchymal transition (EMT) was a critical step for the invasion and metastasis of many cancers [28] and Beuran M *et al.* [29] also showed a strong correlation between the EMT and the local progression and metastasis of pancreatic cancer. Smad3 is a potent inducer of EMT and it can initiate and maintain EMT to promote invasion and migration [30]. In addition, E-cadherin, a cell-cell adhesion molecule, is often lost or down regulated, which highly correlated with the tumor invasion and metastasis [31]. In our study, we observed that overexpression of miR-424-5p suppressed the expression of CYLD, TGF-β/Smad3 signaling was activated and the expression of E-cadherin was remarkably down-regulated; while expression inhibition of miR-424-5p had the opposite effects. Therefore, miR-424-5p could mediate promotion of invasion be through the down-regulation of CYLD and subsequent activation of TGF-β/Smad3 signaling in PC.

In conclusion, miR-424-5p was significantly upregulated in PC tissues and PANC-1 cells, and high expression of miR-424-5p could strengthen the invasion and migration ability of PANC-1 cells, due to the downregulated expression of CYLD, which thus activates TGF-β/Smad3 signaling pathway. Therefore, miR-424-5p may be useful as a potential therapeutic target for PC. However, further studies are required to address the relationships between miR-424-5p or CYLD expression and its clinical relevance in PC patients.

Disclosure of conflict of interest

None.

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