MiR-1271 negatively regulates AKT/MTOR signaling and promotes apoptosis via targeting PDK1 in pancreatic cancer

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Abstract. – OBJECTIVE: Pancreatic cancer (PC) possesses a very poor prognosis, and its pathogenesis is not fully understood. Evidence has suggested that microRNAs play important roles in cancer development and progression, the present study was designed to study the function of miR-1271 in PC.

PATIENTS AND METHODS: PC tissues and adjacent normal tissues were collected from 17 patients. MiR-1271 and PDK1 expression were quantified by quantitative reverse-transcriptional polymerase chain reaction (RT-PCR). AKT/MTOR signaling activity and PDK1 protein expression were determined by Western blot. Cell viability and apoptosis were assessed by MTT assay and enzyme-linked immunosorbent assay (ELISA). Luciferase assay was used to verify whether miR-1271 directly targets PDK1.

RESULTS: MiR-1271 was significantly down-regulated in PC tissues compared with that in the paired normal adjacent tissue, and its expression was up-regulated dose-dependently upon cisplatin treatment in PC cells. Overexpression of miR-1271 in these cells produced a pro-apoptotic effect, similar to what caused by cisplatin treatment. Moreover, overexpression of miR-1271 inhibited AKT/MTOR signaling, which was due to the targeting relationship between miR-1271 and PDK1. Finally, knockdown of PDK1 exerted a similar effect on apoptosis to that of miR-1271 overexpression.

CONCLUSIONS: MiR-1271 is a potent tumor suppressor in PC, its pro-apoptotic function was partially mediated by reduced AKT/MTOR signaling. Targeting miR-1271 may represent an effective strategy for PC treatment.

Key Words: miR-1271, Pancreatic cancer, AKT, MTOR, PDK1, Apoptosis.

Introduction

Pancreatic cancer (PC) is a fatal malignancy in the digestive system. The onset of PC is relatively silent; thus, a large number of PC cases are discovered at advanced stages. Despite the relatively low morbidity of this malignancy, PC patients have an extremely low five-year survival rate of 8%. And given the fact that there is no targeted therapy for PC, it is critical to investigate the molecular mechanism in its pathogenesis. Currently, the risk factors for PC are not completely discovered. It is believed that PC is caused by interactions of genetic changes and environmental factors. Over the past decades, other mechanisms, such as epigenetic abnormalities, including that of non-coding RNAs, are thought to be critical in PC pathology. The small non-coding RNAs, microRNAs, are single-stranded and 21-25 nt in length. Regulation of gene expression by microRNAs is an important part in epigenetic regulation. Studies have found that microRNAs possess potentials to direct the degradation or block the translation of mRNAs when there is a complete or incomplete base pair match within 3’UTR of targeted mRNAs. Since the discovery of microRNAs in C. elegans, numerous microRNAs has been uncovered to play diverse roles in the proliferation, differentiation, and death of cells. Over the last decade, several microRNAs have been identified to have potential functions in the diagnosis, prognosis, and treatment of PC. Spatio-temporal regulation of cell behaviors by microRNAs is critical in the pathogenesis of PC. For example, two most studied microRNA families, miR-21 and miR-200, have distinct roles. MiR-21 is expressed at early stages and mainly controls PC cell proliferation.
MiR-1271 negatively regulates AKT/MTOR signaling and promotes apoptosis via targeting PDK1 in PC

while miR-200 family is related to PC progression by controlling invasion and migration of cancer cells\textsuperscript{11-13}. Apart from the two well-studied microRNAs in the pathogenesis of PC, whether the other microRNAs have multiple roles in the same context has not been fully explored.

Previous studies\textsuperscript{14-18} have identified that an important microRNA, miR-1271, exerts a tumor suppressive function in several cancers. A recent finding shows that miR-1271 affected the Epithelial-Mesenchymal Transition (EMT) process found in PC cells\textsuperscript{17}; however, whether miR-1271 has additional roles to regulate other cellular behaviors is not fully explored. In the present investigation, we show that miR-1271 is significantly down-regulated in PC tissue, and its expression is significantly up-regulated upon death stimuli by the chemotherapeutic drug cisplatin. Further studies reveal that miR-1271 positively regulates cellular apoptotic program by antagonizing the pro-survival AKT/MTOR signaling pathway. These effects are due to the direct suppression of PDK1 by miR-1271. Thus, we showed that miR-1271 mediates a novel pro-apoptotic function in PC, and it might be effective in PC treatment.

Patients and Methods

PC Samples

The tissue samples for PC were collected during the period from April 2014 to March 2016 in Anhui Provincial Hospital. All patients were informed, and they agreed to donate their surgically resected tissues for scientific research. The study design was approved by the Ethics Committee of Anhui Provincial Hospital. All the patients did not receive chemotherapy before surgery. The tissues were histologically examined by 3 experienced independent pathologists. Tissues were snap frozen in liquid nitrogen and rapidly transferred to -80°C for long-term storage.

Cell Culture

The pancreatic cancer cell line PANC1 was obtained from American Type Culture Collection (ATCC). Cells were maintained in DMEM medium supplied with 10% FBS. Cells were cultured at 37°C in a humidified atmosphere supplied with 5% CO\textsubscript{2}. Cells were propagated every 36 h.100 U/mL penicillin and 100 U/mL streptomycin were added to the culture medium to avoid contamination. For the transfection of miR-1271, miR-1271 inhibitor and small-interfering RNA (siRNA) for PDK1, X-treme GENE siRNA Transfection Reagent (Roche, Basel, Switzerland) was applied according to the manufacturer’s protocol. SiRNA transfection concentration was 150 nM, and the concentration for miR-1271 and its inhibitor was 100 nM unless otherwise stated.

Quantitative PCR

MicroRNAs were isolated from tissues or cells using the miRcute isolation Kit (TIANGEN, Beijing, China). Synthesis of the first strand cDNA was carried out using PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan), the amplification step of qRT-PCR was carried out using SYBR GREEN Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). All the primer sets were purchased from Genepharma (Shanghai, China). The expression of miR-1271 was normalized to U6, and the expression of PDK1 was normalized to β-actin. There are three biological repeats for each treatment and three technical repeats for each sample.

Cell Viability Assay

Cell viability was performed using the 3-(4,5-di-methyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) reagent. In brief, cells were grown in 96 well plates, seeded at the density of 5000 cells per well. After culturing for 36 h, cells were treated with cisplatin at concentrations of 5 μM, 10 μM, 20 μM, and 40 μM for 72 h. 20 μl MTT solution (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium of each well and normally incubated with cells for 4 h. The supernatant in each well was then discarded and 200 μl DMSO was added to each well. The plate was then read on a spectrophotometer (Tecan, Salzburg, Austria) for the value of OD490nm. Five biological repeats were performed for each concentration.

Cell Death Enzyme-linked Immunosorbent Assay (ELISA)

We analyzed the apoptosis product DNA fragmentation and histone release using the Cell Death Detection ELISA Plus Kit (Roche, Basel, Switzerland). After cells were transfected and incubated for 72 h, the experimental procedure was processed according to the manufacturer’s protocol to facilitate the immunoreaction directed by anti-DNA-POD and anti-histone-biotin antibodies. The color was then visualized by ABTS solution in the kit, and absorbance value of OD405 nm was measured under a spectrophotometer (Tecan, Salzburg, Austria). Five biological repeats were performed for each treatment.
Western Blot
The whole cell lysate was prepared using RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration of cell lysates was then quantified using bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Western blot was performed as previously described\(^\text{19, 50}\). 50 μg protein was loaded in each well. Proteins were resolved on 10% or 15% SDS-PAGE gels and electro-blotted on a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Danvers, MA, USA). The membranes were blocked with 5% skimmed milk followed by primary antibody incubation at 4°C overnight. The horseradish peroxidase (HRP)-conjugated secondary antibodies (ZSGB, Beijing, China) were used to detect the primary antibodies. All the primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), the host of all the antibodies is a rabbit.

Luciferase Assay
The PDK1 3'-untranslated region (3'UTR) luciferase construct was constructed by inserting the 3'UTR of PDK1 into the downstream of firefly luciferase gene in pMiRGLO vector (Promega, Madison, WI, USA). Mutation of the seed sequence binding site was introduced by Fast Site-directed Mutagenesis Kit (TIANGEN, Beijing, China). The constructs were cotransfected with miR-1271 into PANC-1 cells for 24h. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used for final detection. The experiments were repeated for three times.

Statistical Analysis
Data were presented as means ± SEM, for tumor samples, paired t-test was used to compare the miR-1271 and PDK1 expression. One way ANOVA or unpaired t-test were performed to measure the significance of the difference. The Student-Newman-Keuls method was used for ANOVA post-hoc test. A \( p \)-value less than 0.05 was an indication of statistical significance.

Results

**The Expression of miR-1271 is Down-regulated in PC Tissues and Up-regulated in PC Cells Upon Cisplatin Treatment**
To assess the potential function of miR-1271, we collected 17 paired samples for PC tissue and the adjacent normal tissue. QRT-PCR revealed that miR-1271 was significantly down-regulated in PC tissues (Figure 1A), which is consistent with the previous report\(^\text{17}\). To fully understand the role of miR-1271 in the apoptosis of PC cells, we treated pancreatic cancer cell line PANC-1 with the chemotherapeutic drug, cisplatin. This drug dose-dependently inhibits cell survival to a great extent (Figure 1B). Of note, we found that, during the treatment course, miR-1271 expression was dose-dependently up-regulated (Figure 1C). This finding suggests that miR-1271 might have a promoting effect on cell death and mediate the anti-cancer effect of cisplatin.

**Ectopic Expression of miR-1271 in PC Cells Induces Apoptosis**
To further explore the possible mechanism of miR-1271 on cell death, we utilized apoptosis ELISA, which specifically detects the DNA fragmentation and histone release, two specific products of apoptosis. We transfected PANC-1 cells with a low dose (50 nM) and a high dose (100 nM) of miR-1271 for 72 h, cisplatin treatment at 20 μM was used as a positive control. Treatment of miR-1271 resulted in increased release of apoptosis products in a dose-dependent manner (Figure 2A). We found that treatment of miR-1271 induced significantly up-regulated expression of cleaved-caspase-3, a hallmark of apoptosis, which was comparable to the degree induced by cisplatin (Figure 2B and 2C). These findings strongly suggest that miR-1271 is a prominent apoptosis inducer in PC.

**Ectopic Expression of miR-1271 in PC Cells Inhibits the AKT/MTOR Signaling**
We further explored the mechanism by which miR-1271 regulates cell survival. The PI3K/AKT/MTOR signaling has an essential role in regulating this cell survival in PC. We found that, compared with negative control (NC) transfected cells, miR-1271 transfection decreased the phosphorylation of AKT and MTOR, whereas miR-1271 inhibitor transfection could exert an opposite effect. (Figure 3A and 3B). This finding suggests that decreased AKT/MTOR signaling might be partially responsible for miR-1271 induced apoptosis.

**PDK1 is Targeted by miR-1271**
In silico analysis found that miR-1271 can target multiple targets. One intriguing potential target, PDK1, has a critical function in activating
MiR-1271 negatively regulates AKT/MTOR signaling and promotes apoptosis via targeting PDK1 in PC

Figure 1. The expression of miR-1271 is downregulated in PC tissues and upregulated in PC cells upon cisplatin treatment. 

A. The relative expression of miR-1271 in PC tissues was significantly downregulated compared with normal tissues (N) (B) MTT assay confirmed cell death induced by increasing dose of cisplatin in PANC-1 cells. C. The relative expression of miR-1271 in PC tissues was dose-dependently upregulated in PANC-1 cells treated with cisplatin. *p < 0.05 vs. control.

Figure 2. Ectopic expression of miR-1271 in PC cells induces apoptosis. A. Apoptosis ELISA show increased apoptosis in low dose (miR-1271-Lo) and high dose (miR-1271-Hi) miR-1271 transfected cells. Cisplatin was used as positive control. B. Western blot image for apoptosis marker cleaved-caspase-3. C. Quantification of the Western blot images, miR-1271 significantly upregulated cleaved-caspase-3 expression to a level comparable to cisplatin treatment. *p < 0.05 vs. negative control (NC).
Figure 3. MiR-1271 regulates AKT/MTOR pathway by targeting PDK1. **A**, Western blot images for AKT/MTOR signaling. **B**, Quantification of the Western blot images in **A** showed the phosphorylation of AKT and MTOR were significantly inhibited by the miR-1271 and promoted by miR-1271 inhibitor. **C**, The putative binding site and mutagenesis of the seed sequence binding site in PDK1 3’UTR. **D**, Luciferase assay showed decreased luciferase activity upon miR-1271 transfection in PDK1-WT 3’UTR group, which was abrogated by PDK1-Mut 3’UTR. **E**, Western blot images for PDK1 expression. **C**, Quantification of the Western blot images in **E** showed decreased PDK1 expression upon miR-1271 transfection, which was abrogated by the miR-1271 inhibitor. **G**, The relative expression of PDK1 in PC tissues was significantly upregulated compared with normal tissues. *p < 0.05 vs. NC.
AKT pathway in its upstream (Figure 3C). To confirm whether the putative binding between miR-1271 and PDK1 is authentic in PC cells, we performed luciferase assay. MiR-1271 could significantly reduce the luciferase activity yielded by wild-type PDK1 3’UTR construct, but could not achieve a same effect in the mutant PDK 3’UTR construct (Figure 3D). Moreover, transfection of miR-1271 inhibited PDK1 expression, which was reversed by its inhibitor (Figure 3E and 3F). Notably, the negative relationship of PDK1 and miR-1271 was also consistent in tissue samples, PDK1 expression was significantly up-regulated in PC tissues (Figure 3G). These findings indicate that miR-1271 regulates AKT/MTOR signaling via directly targeting the upstream regulator PDK1 in PC.

Knockdown of PDK1 in PC Cells Induces Apoptosis

To assess whether the decreased level of PDK1 contributes to the apoptosis of PC cells. We employed the approach of siRNA mediated gene silencing to knockdown the endogenous expression of PDK1. As shown in Figure 4A-4C, knockdown of PDK1 induced caspase-3 cleavage. Measurement by ELISA also revealed a 4-fold increase of apoptosis products in PDK1 siRNA transfected cells (Figure 4D). These data confirm that decreased level of PDK1 caused by miR-1271 can contribute to the apoptosis of PC cells. Based on our data, we propose the following model for the regulatory role of miR-1271 on cell survival in PC (Figure 4E). AKT/MTOR signaling is critical for PC cell survival under various conditions, and its signaling activity is greatly dependent upon PDK1. MiR-1271 directly targets PDK1, and inhibits the downstream AKT/MTOR signaling, which leads to compromised cell survival. Apart from PDK1, miR-1271 may also function to promote apoptosis through other possible anti-apoptotic targets (dashed lines), which was previously identified in other cancer types14,20.

Discussion

In the present investigation, we demonstrated that miR-1271 has a prominent cytotoxic effect on PC cells through induction of apoptosis. Our results showed that miR-1271 could be induced by cell death stimuli such as the chemotherapeutic drug cisplatin and that ectopic expression of miR-1271 in PC cells induced apoptotic cell death, which was similar to that of cisplatin treatment. Of note, we discovered that the upstream kinase PDK1, which acts upstream of AKT/MTOR signaling, was a direct target of miR-1271 in PC cells. Thus, our study establishes a molecular axis composed of miR-1271, PDK1 and AKT/MTOR pathway, which is critical for the apoptosis regulation in PC cells.

Despite the emerging studies showing the regulatory functions of microRNAs in the pathogenesis of PC, our knowledge in microRNA mediated tumorigenesis is far from clear due to the base sequence diversity of numerous microRNAs. Currently, there are few microRNAs that have well-characterized functions in PC. MiR-21 is extensively studied in PC, yet its tumor-promoting role has been revealed in various cancers21-23. Apart from the classical miR-21/PTEN axis, many of the tumor suppressive genes have been identified as direct targets of miR-21. These molecular interactions function in a context-dependent manner to contribute to many aspects of tumorigenesis. For example, targeting of PTEN and PDCD4 by miR-21 resulted in drug resistance24; miR-21-SLUG signaling also affects the motility of PC by altering the EMT process25. Overexpression of miR-200a and miR-200c both had an alleviation on invasive cell capacity in PC stem cells26. Importantly, the tumor suppressive function of miR-200 family is effective in suppressing gemcitabine-resistant pancreatic cancer cells27, which further supported the notion that replacement therapy using microRNAs is a promising approach for treating this challenging disease.

The tumor suppressive function of miR-1271 in cancers has been reported. In endometrial cancer cells, overexpression of miR-1271 inhibited CDK1 and cancer cell growth14. While in prostate cancer, it targets ERG to suppress cell proliferation15. In HepG2 cells, miR-1271 interferes with the insulin growth pathway28, suggesting its broad anti-growth function in multiple cancer cells. Using a miRNA array, Liu et al17 recently identified that miR-1271 is significantly down-regulated in PC tissues; they found that miR-1271 is involved in the EMT process and that two signature transcriptional factors ZEB1 and TWIST1 are the direct targets. However, whether miR-1271 is involved in other cell biological events was not investigated. Consistent with their finding, we also captured a decreased expression of miR-1271 in PC tis-
sues. Our results showed that cisplatin dose-dependently increased the miR-1271 expression, suggesting that miR-1271 may be the mediator of cisplatin-induced cell death. We further confirmed that forced expression of miR-1271 alone could induce prominent apoptosis comparable to that induced by cisplatin. Given the highly cytotoxic effect of cisplatin on other normal cells such as the neurocytes, we assume the possibility that synergistic treatment with miR-1271 and a lower dose of chemotherapeutic drugs could reduce the adverse effects without significantly affecting its efficacy. However, this should be further tested by in vivo studies.

Figure 4. Knockdown of PDK1 in PC cells induces apoptosis. A, Western blot images for cleaved-caspase-3 and PDK1. B, Quantification of the Western blot images in (A) showed decreased PDK1 expression upon PDK1 siRNA (Si-PDK1) transfection. C, Quantification of the Western blot images in (A) showed increased cleaved-caspase-3 expression upon PDK1 siRNA (Si-PDK1) transfection. D, Apoptosis ELISA showed increased apoptosis upon PDK1 siRNA (Si-PDK1) transfection. *p < 0.05 vs. NC. E, Proposed model for the mechanism of miR-1271 action in PC cell survival. MiR-1271 directly targets PDK1, inhibits the downstream AKT/MTOR signaling, which leads to reduced cell survival. Apart from PDK1, miR-1271 may also function to promote apoptosis through other possible anti-apoptotic targets (dashed lines).
The AKT/MTOR pathway functions downstream of the oncogenic KRAS signaling. It is central in building a complex network that is closely related to multiple cellular events such as cell differentiation, cell survival, apoptosis, and autophagy. Strong AKT/MTOR activity confers cancer cells to survive through stresses such as nutrition depletion and chemical death stimuli. Previous researches have shown that miR-21, miR-375, miR-221, and miR-181a are critical regulators in AKT/MTOR signaling. Our work identifies that AKT/MTOR pathway is also inhibited by miR-1271, which partially explains the high proliferation potential of PC cells. Importantly, cisplatin induces apoptosis possibly by activating the miR-1271/AKT/MTOR axis; further researches are needed to examine whether the miR-1271/AKT/MTOR pathway is involved in the development of drug resistance in PC cells.

The present study identifies PDK1, which functions upstream of AKT, is a direct target of miR-1271. Considering that microRNAs may have multiple targets in the same context, we cannot exclude the possibility that other regulatory mechanisms by various targets may mediate the pro-apoptotic effect. Intriguingly, several anti-apoptotic factors such as IGFR1, MTOR, BCL2, and CDK1 have been identified to be direct targets of miR-1271 in gastric cancer and endometrial cancer. We speculate that these factors may also have functional relevance in miR-1271 mediated apoptosis of PC cells.

**Conclusions**

We identify that the down-regulation of miR-1271 may partially contribute to the development of PC. Overexpression of miR-1271 attenuates AKT/MTOR signaling and induces apoptosis via targeting PDK1. Our results reveal that AKT/MTOR signaling and induces apoptosis via targeting PDK1. Our results identify PDK1, which is a critical regulator of AKT/MTOR signaling. The AKT/MTOR pathway is involved in the development of drug resistance in PC cells.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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