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Original Article

The involvement of miR-375 in the adriamycin resistance of pancreatic carcinoma cells

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Abstract: Background: Yes-associated protein 1 (YAP1) is correlated with the onset, progression, drug resistance and prognosis of pancreatic carcinoma. MicroRNA (miR)-375 is down-regulated in pancreatic cancer tissues and may target the 3'-untranslated region (3'-UTR) of the YAP1 gene. We thus investigated whether miR-375 mediates the YAP1 expression, proliferation, apoptosis, and Adriamycin (ADM) resistance of pancreatic carcinoma cells. Methods: A dual luciferase reporter assay confirmed the targeted relationship between miR-375 and YAP1. The ADM-resistant cell lines SW1990/ADM, BxPC-3/ADM, the non-resistant cell lines SW1990 and BxPC-3, and the normal pancreatic ductal cell line HPDE6-C7 were cultured and followed up by an analysis of the cell apoptosis using flow cytometry. Cultured ADM-resistant cells were transfected with an miR-375 mimic followed by measuring the YAP1 expression and the ADM-induced apoptosis by flow cytometry and the cell proliferation by EdU staining. Results: A targeted relationship exists between miR-375 and YAP1 mRNA. The SW1990/ADM and BxPC-3/ADM cells had significantly lower miR-375 than the non-resistant cells, which had a lower miR-375 expression compared to the HPDE6-C7 cells. Drug resistant cells had a remarkably higher YAP1 expression than the non-resistant cells, which had a higher YAP1 level than HPDE6-C7. Under ADM concentration (IC_{50}), the drug resistant cells showed remarkably decreased apoptosis compared to the non-resistant cells. In addition, the transfection of the miR-375 mimic significantly suppressed the YAP1 protein expression in the resistant cells, enhanced apoptosis, and inhibited proliferation. Conclusion: The down-regulation of miR-375 is correlated with the ADM resistance of pancreatic carcinoma cells. The up-regulation of miR-375 inhibits cancer cell proliferation, facilitates apoptosis, and decreases ADM resistance possibly by suppressing YAP1 expression.

Keywords: Pancreatic carcinoma, ADM, drug resistance, miR-375, YAP1

Introduction

Pancreatic cancer (PC) is a highly malignant, digestive tract tumor that is extremely difficult to diagnosis and treat [1, 2]. Chemotherapy is the major approach for treating PC. However, its efficiency is largely compromised by drug resistance [3-5].

Yes-associated protein 1 (YAP1) is the major effector and target protein in the canonical Hippo-YAP signaling pathway and can regulate target gene expression through transcriptional co-activation after nuclear translocation, thus facilitating the pathogenesis of multiple tumors [6-8]. Various studies have indicated the involvement of YAP1 in PC onset, progression, drug resistance, and prognosis [9-12], suggesting an oncogenic role of YAP1 in the occurrence and progression of PC.

MicroRNA (miR) is a type of endogenous non-coding small molecular single stranded RNA with a length of 22~25 nucleic acids in eukaryotes that can regulate target gene expression via complementary binding to the 3' untranslated region (3'-UTR) of target gene mRNA. It is estimated that microRNAs, which occupy only 1% of all human genomes, can regulate the expression of more than 30% of all human genes [13]. MiR-375 is a well-studied microRNA and has been known to exert a critical tumor

suppressor gene function in various cancers [14-17]. Various studies have shown that miR-375 is remarkably depressed in PC and exerts a tumor suppressor effect in PC [18-20]. A bioinformatics analysis showed the existence of complementary binding sites between miR-375 and YAP1 mRNA, suggesting a possible regulatory relation between them. We thus established PC cell models with Adriamycin (ADM) resistance, on which expressional profiles of miR-375 and YAP1 between drug resistant and sensitive PC cells were compared. Furthermore, the role of miR-375 in regulating YAP1 expression and proliferation, apoptosis and the ADM resistance of PC cells was also studied.

Major reagent and materials

Normal human pancreatic ductal epithelial cell line HPDE6-C7 and PC cell lines SW1990-31 and BxPc-3-22 were purchased from Jining Cell Culture Center (China). HEK293T cells were purchased from the Cell Bank of CAS, Shanghai (China). DMEM and Opti-MEM and fetal bovine serum (FBS) were purchased from Gibco (US). Trizol and Lipo 2000 transfection reagent were purchased from Invitrogen (US). A QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Germany). An miR-375 mimic, an miR-375 inhibitor, and miR-NC were purchased from RioBio (China). Rabbit anti-human YAP1 and anti-β-actin polyclonal antibody were purchased from Abcam (US). HRP conjugated goat anti-rabbit secondary antibody was purchased from Sangon (China). A CCK-8 cell proliferation assay kit, an Annexin V-FITC/PI apoptosis assay kit, BeyoECL Plus chromogenic regent, and a BCA protein quantification kit were purchased from Beytotime (China). An EdU Flow Cytometry Kit was purchased from Thermo (US). ADM was purchased from Sigma. A luciferase activity assay kit and a Dual-Glo Luciferase Assay System and pGL3 plasmid were purchased from Promega (US). A HERAccell 240i cell incubator was purchased from Thermo (US). A FC500MCL flow cytometry apparatus was purchased from Beckman Coulter (US).

Cell culture

SW1990, BxPC-3, and HPDE6-C7 cells were kept in a DMEM medium containing 10% FBS and cultured in a 37°C chamber with 5% CO_2. The cells were passed at a 1:4 ratio, and the cells with a log-growth phase were used for further assays.

Generation of the ADM drug resistant cell model

ADM resistant cell model preparation: 0.1 μg/mL ADM was added into the cultured medium of SW1990 or BxPC-3 cells at the log-growth phase. After 2-weeks of stable growth, the ADM concentration was elevated to 0.2 μg/mL for another round of 2-week incubation. The ADM dosage was then gradually increased to 0.4 μg/mL and 0.8 μg/mL until the stable growth and repeated passage of the SW1990 and BxPC-3 cells was reached to generate the ADM resistant PC cell lines SW1990/ADM and BxPC-3/ADM.

SW1990, BxPC-3, SW1990/ADM and BxPC-3/ADM cells were seeded into 96-well plates at 1000 cells per well density. After overnight incubation, the cells were treated with different concentrations of ADM (0, 0.01, 0.1, 1, 10, 100, 1000, and 10000 μg/mL), each of which consisted of 5 parallel replicates. After 48 h incubation, 10 μL of CCK-8 solution was added into teach well. 4 h later, the absorbance (A) values at the 450 nm wavelength of each well were measured. The inhibition rate = (1 - A450 of drug treatment group)/A450 of control group × 100%. SPSS 18.0 software was used to calculate the drug concentration for achieving 50% cell growth inhibition (IC_{50}). Resistance index (RI) = IC_{50} of drug resistant cells/IC_{50} of parental cells.

Flow cytometry analysis of cell proliferation

An EdU Flow Cytometry Kit was used to determine cell proliferation. In brief, a DMEM complete medium containing 10% FBS was used to re-suspend the cells. After incubation with 10 μM EdU for 2 h at 37°C, the cells were incubated for 48 h in culture plates. After digestion by trypsin, the cells were rinsed once in PBS and fixed with paraformaldehyde. With PBS centrifugation and washing, a 100 μL permeable buffer was added, followed by incubation in the dark for 30 min in a 500 μL reaction test buffer. The cells were rinsed by centrifugation using a 3 mL permeable buffer and re-suspended in a 500 μL wash buffer. The cell proliferation was determined by FC500MCL flow cytometry.

Dual luciferase activity assay

The full length or mutant fragment of 3'-UTR of YAP1 gene was amplified by PCR, and the products were digested by dual restriction enzymes for further ligation into a pGL3 plasmid. After transforming the competent bacteria, sequencing was performed to screen the plasmids with the correct insertion, and they were identified as pGL3-YAP1-WT, pGL3-YAP1-MUT. Lipofectamine 2000 was used to co-transfect the pGL3-YAP1-WT (or pGL3-YAP1-MUT) and the miR-372 mimic (or miR-372 inhibitor or miR-NC) into the HEK293T cells, which were incubated in a 37°C chamber with 5% CO_2 for another 48 h. A Dual-Glo Luciferase Assay System kit was used to measure dual luciferase activity.

Cell transfection and grouping

The *in vitro* cultured SW1990/ADM and BxPC-3/ADM cells were assigned into two groups: the miR-NC transfection group and the miR-372 mimic transfection group. In brief, 100 μL serum-free Opti-MEM medium was used to dilute 10 μL Lip2000, 50 nmol miR-NC or 50 nmol miR-375 mimic. After incubation for 5 min at room temperature, Lip2000 was gently mixed with miR-NC or a miR-375 mimic for 20 min of room-temperature incubation. The transfection mixture was then added into a DMEM medium containing 10% FBS for 72 h continuous incubation followed by cell collection.

Cells from two treatment groups were inoculated into a 6-well plate. When they reached 50% confluence, the cells were treated with 1.6 μg/mL ADM for 48 h continuous incubation. Cell apoptosis was measured by flow cytometry as described in the following section.

Trypsin was used to digest the cells, which were collected and incubated with 10 μM EdU for 2 h, followed by 48 h incubation. An EdU Flow Cytometry Kit was used to measure the EdU positive rate of cells to reflect their proliferation potency.

qRT-PCR for measuring gene expression

Trizol was used to extract total RNA. A QuantiTech SYBR Green RT-PCR Kit was used to measure gene expression using one-step qRT-PCR and using RNA as the template. The qRT-PCR reaction was performed in a 20 μL system, including 10.0 μL 2 × QuantiTech SYBR Green RT-PCR Master Mix, 1.0 μL forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTech RT Mix, and ddH_2O. The qRT-PCR conditions were: 45°C for 5 min, and 94°C for 30 s, followed by 40 cycles, each at 95°C for 5 s and 60°C for 30 s. Gene expression was measured on a Bio-Rad CFX96 real-time fluorescent quantitative PCR cycler.

Western blot

Cells from all treatment groups were digested by trypsin and collected. Total proteins were extracted by adding a 100 μL RIPA lysis buffer. Protein concentration was measured using the BCA approach. 40 μg protein samples were loaded on to SDS-PAGE (10% separating gel and 4% condensing gel) for electrophoresis (45 V, 150 min). The proteins were transferred to a PVDF membrane (250 mA, 100 min). The membrane was blocked by 5% defatted milk powder at room temperature. A primary antibody (1:2000 diluted YAP1, and 1:10000 diluted β-actin) was added for a 4°C overnight incubation. The membrane was washed in PBST three times and incubated with an HRP conjugated secondary antibody (1:1000 dilution) for a 60 min room temperature incubation. The membrane was washed in PBST three times. BeyoECL Plus working solution A and solution B were mixed in equal volumes and were added onto the protein blotting membrane. After incubation in the dark for 2-3 min, the membrane was exposed, and the film was developed for scanning to store the data.

Cell apoptosis assay

Cells were digested by trypsin and collected, followed by PBS rinsing. Cells were re-suspended in a 100 μL Annexin V Binding Buffer and sequentially stained with 10 μL Annexin V-FITC and 5 μL PI. After 15 min room temperature incubation, 400 μL Annexin V Binding Buffer was added, and cell apoptosis was measured using FC500 MCL flow cytometry (Beckman Coulter).

Statistical analysis

SPSS 18.0 was used for the statistical analysis of the data. Measurement data were presented as the mean ± standard deviation (SD) and

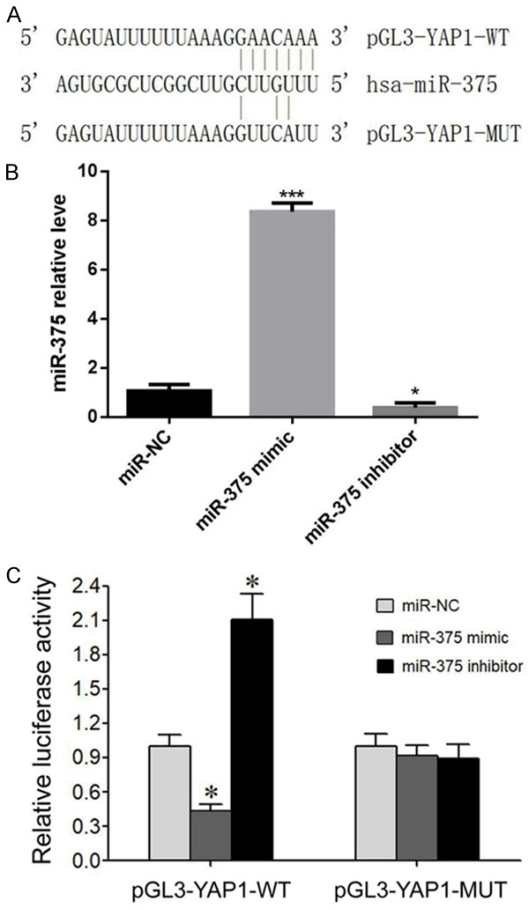

Figure 1. Targeted regulation between miR-375 and YAP1 mRNA. A. Illustration for functioning sites between miR-375 and 3'-UTR of YAP1 mRNA. B. Dual luciferase gene reporter assay. *, $P<0.05$ compared to the miR-NC group. C. Relative luciferase activity quantification.

compared using Student's *t*-test for comparison between two groups. A one-way analysis of variance (ANOVA) was used for comparing measurement data among multiple groups, followed by a Bonferroni comparison between two groups. Statistical significance was defined as $P<0.05$.

Results

Targeted relationship between miR-375 and YAP1 mRNA

The bioinformatics analysis results showed the existence of complementary biding sites between miR-375 and 3'-UTR of YAP1 mRNA (**Figure 1A**). Compared with the negative control (miR-NC), the transfection of the miR-375 mimic significantly unregulated the miR-375 expression, but the transfection of the miR-375 inhibitor significantly reduced the miR-375 expression (**Figure 1B**). The dual luciferase gene reporter assay showed that the transfection of miR-375 mimic significantly decreased the relative luciferase activity in the HEK293T cells which were transfected with pGL3-YAP1-WT. The transfection of the miR-375 inhibitor, on the other hand, significantly elevated the relative luciferase activity of the HEK293T cells which were transfected with pGL3-YAP1-WT. The transfection of the miR-375 mimic or the miR-375 inhibitor, however, had no effect on the relative luciferase activity of the HEK293T cells transfected with pGL3-YAP1-MUT (**Figure 1C**). These results suggest that miR-375 can target the 3'-UTR of YAP1 mRNA to inhibit its expression.

High levels of drug resistance in SW1990/ADM and BxPC-3/ADM cells

The SW1990 cells had IC_{50} values of 3.17 ± 0.11 μg/mL, and the drug resistant cell line SW1990/ADM had IC_{50} of 36.75 ± 0.63 μg/mL, making the RI of the SW1990/ADM cells 11.59 (**Figure 2A** and **Table 1**). The BxPC-3 cells had IC_{50} values of 1.86 ± 0.08 μg/mL, and the BxPC-3/ADM cells had IC_{50} value of 25.63 ± 1.92 μg/mL, making its RI 13.77 (**Figure 2C**, **Table 1**).

Under 3.17 μg/mL ADM treatment, the SW1990 cells had a relatively higher apoptotic rate, but the SW1990/ADM cells had a relatively lower apoptosis rate (**Figure 2B**). Using 1.86 μg/mL ADM treatment, the BxPC-3 cells had relatively higher apoptotic rate, but the BxPC-3/ADM cells had a lower apoptosis rate (**Figure 2D**).

Down-regulation of miR-375 and up-regulation of YAP1 in drug resistant PC cells

The qRT-PCR results showed that, compared to the normal pancreatic ductal epithelial cell line HPDE6-C7, the PC cell lines SW1990 or BxPC-3 had a significantly decreased miR-375 level, which was further down-regulated in respective ADM resistant cell lines (**Figure 3A**). In addition, the qRT-PCR results showed that compared to the HPDE6-C7 cells, the PC cell lines SW1990 and BxPC-3 had significantly elevated YAP1 mRNA expressions, which were much higher in the ADM resistant cell lines

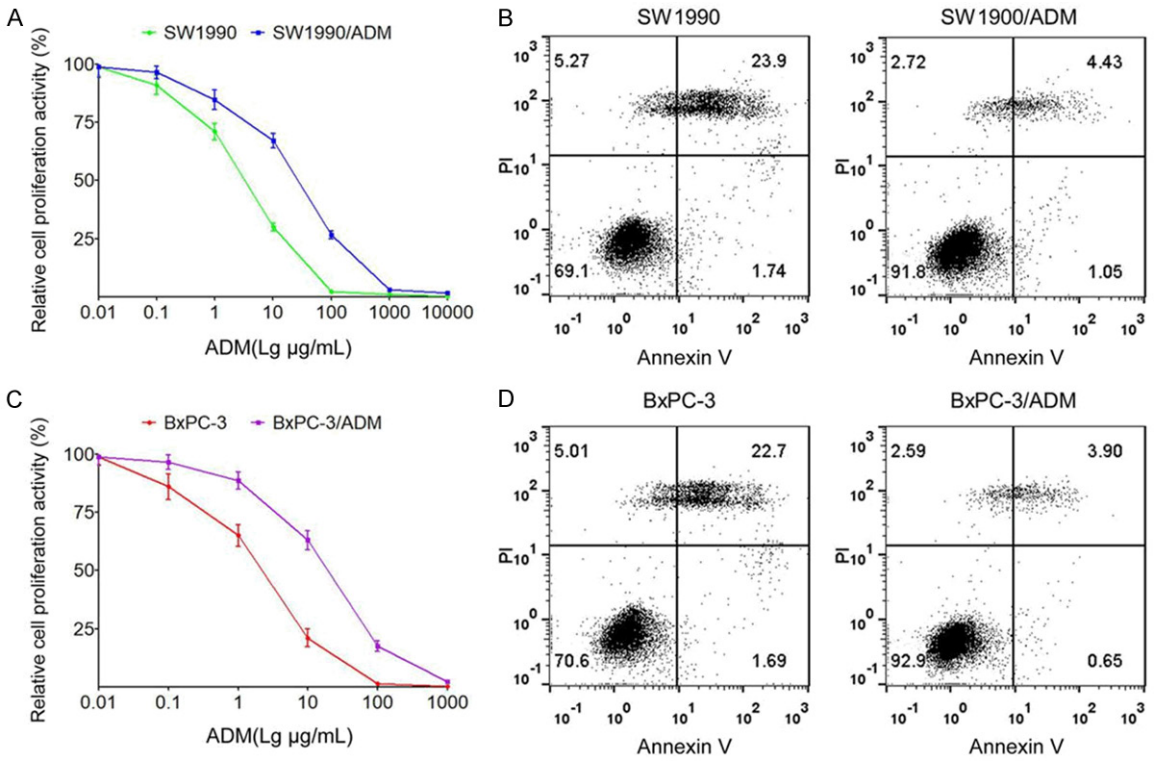

Figure 2. High levels of drug resistance of SW1990/ADM and BxPC-3/ADM cells. A. CCK-8 assay of the proliferation activity of SW1990 and SW1990/ADM cells. B. miR-375 expression after the transfection of an miR-375 mimic or inhibitor. C. CCK-8 assay for the proliferation activity of the BxPC-3 and BxPC-3/ADM cells. C. Flow cytometry for the apoptotic rate of the SW1990 and SW1990/ADM cells. D. Flow cytometry for the apoptotic rate of the BxPC-3 and BxPC-3/ADM cells. Compared with miR-NC, **P*<0.05 and ****P*<0.001.

Table 1. Drug resistance index of SW1990/ADM and BxPC-3/ADM cells

Cell	Drug resistance index
SW1990/ADM	11.59
BxPC-3/ADM	13.77

(**Figure 3A**). The Western blot results showed that, compared to the HPDE6-C7 cells, the PC cell lines SW1990 and BxPC-3 had a significantly increased YAP1 protein expression, and the respective ADM resistant cell lines showed much higher YAP1 protein expression (**Figure 3B**).

Over-expression of miR-372 suppressed YAP1 expression and decreased the ADM resistance of PC cells

The qRT-PCR results showed that, compared to the miR-NC group, the miR-375 mimic transfection into the SW1990/ADM (transfection efficiency: 65%) and BxPC-3/ADm (transfection efficiency: 71%) cells showed a significantly decreased YAP1 mRNA level (**Figure 4A**). The Western blot results showed that, compared to the miR-NC group, the miR-375 mimic transfection significantly decreased YAP1 protein expression in the SW1990/ADM and BxPC-3/ADM cells (**Figure 4B**). Flow cytometry found that the transfection of miR-375 mimic significantly increased apoptosis in the SW1990/ADM and BxPC-3/ADM cells under ADM treatment (**Figure 4C**), but the cell proliferation potency was significantly decreased (**Figure 4D**).

Discussion

PC has a relatively high incidence and has become the fifth most common cancer. It is estimated that the incidence of PC is as high as 8.3 per 100,000, with the mortality rate reaching 7.8% [21]. Chemotherapy is an important treatment approach for PC, but drug resistance adversely affects treatment efficiency [3-5]. Therefore, an investigation of the chemotherapy drug resistance of PC is of critical impor-

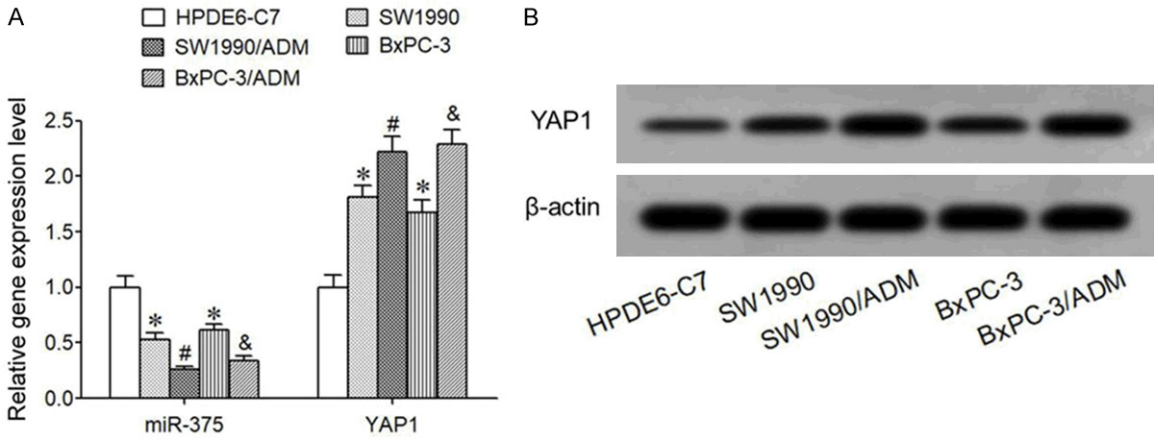

Figure 3. Down-regulation of miR-375 and up-regulation of YAP1 in PC resistant cells. A. qRT-PCR for miR-375 and YAP1 mRNA expression. B. Western blot for measuring YAP1 protein expression. *, *P*<0.05 compared to the HPDE6-C7 cells; #, *P*<0.05 compared to the SW1990 cells; &, *P*<0.05 compared to the BxPC-3 cells.

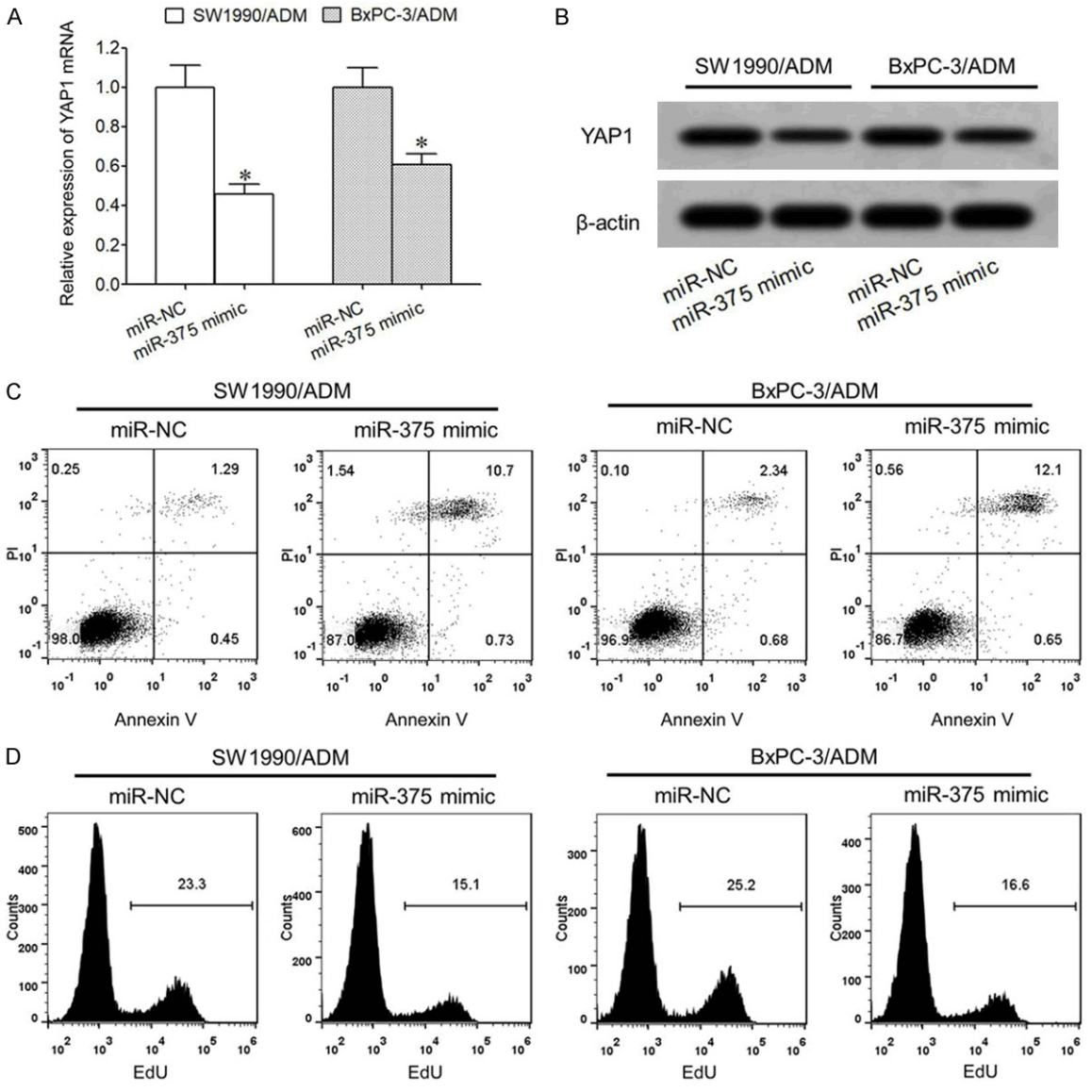

Figure 4. The up-regulation of miR-372 to inhibit YAP1 expression and suppress the ADM resistance of PC cells. A. qRT-PCR for measuring YAP1 mRNA expression; B. Western blot for the protein expression assay; C. Flow cytometry for cell apoptosis; D. Flow cytometry for cell proliferation. *, $P<0.05$ comparing to miR-NC group.

tance for improving treatment efficiency, guiding individualized treatment, and improving patient prognosis.

YAP1 is a pluripotent intracellular connecting protein and transcriptional co-activator, and it is the major effector downstream of Hippo-YAP signal transduction. Its expressional level and functional activity are under phosphorylated regulation by the upstream kinase cascade [22]. Located in human chromosome 11q13, YAP1 codes for a protein with a molecular size of 65KD [23]. YAP1 can specifically recognize and bind to intracellular transcriptional factors containing the PPXY sequence through its WW structural domain, to modulate the transcription and expression of various genes, thus facilitating cell proliferation, inhibiting apoptosis, leading to the loss of contact inhibition and the potential malignant transformation of tumor cells [23, 24]. Various studies have shown that the enhanced expression and function of YAP1 are involved in the onset, progression, drug resistance, and prognosis of PC [9-12], indicating its oncogenic role in PC. MiR-375 is a well-studied microRNA. The down-regulation of miR-375 is related with the onset, progression and drug resistance of multiple tumors, including gastric cancer [16], breast cancer [15, 17], and colon cancer [14], thus playing critical tumor suppressor roles. Multiple studies have demonstrated the down-regulation of miR-375 in PC, indicating its necessary anti-tumor function in PC [18-20]. A bioinformatics analysis also revealed the complementary binding sites between miR-375 and YAP1 mRNA, indicating a possible regulatory role between these two factors. Although miR-375 has been found to be significantly downregulated in multiple types of cancer, it suppresses the core hallmarks of cancer by targeting several important oncogenes like AEG-1, YAP1, IGF1R, and PDK1 [25]. However, the exact role of miR-375 in the drug resistance of PC and its mechanism remain poorly understood. Our present study focused on investigating the role of miR-375 in the drug resistance of PC and showed the involvement of miR-375 in PC drug resistance possibly through regulating YAP1 expression.

A dual luciferase gene reporter assay showed that the transfection of the miR375 mimic significantly depressed the relative luciferase activity in HEK293T cells transfected with pGL3-YAP1-T, and the miR-375 inhibitor transfection significantly elevated the relative luciferase activity of HEK293T cells transfected with pGL3-YAP1-WT, indicating targeted regulation between miR-375 and the YAP1 gene. Using the CCK-8 assay, the IC_{50} values of both parental and drug resistant cells against ADM were calculated and showed a significantly higher IC_{50} of the resistant PC cells compared to their parental PC cell lines. Under the treatment of ADM equivalent to the IC_{50} of parental cells, the drug resistant cells showed an apoptotic rate compared to the parental cells, indicating drug resistance and the successful generation of ADM resistant PC cell lines. Both the gene and protein assays showed that compared to normal pancreatic ductal epithelial cells, the PC cells had significantly decreased miR-375 expression, which was further down-regulated in the drug resistant cells. YAP1 expression was significantly increased in the PC cells and was much higher in the drug resistant cells. These results showed the role of miR-375 down-regulation in YAP1 expressional potentiation, suggesting a correlation between YAP1 up-regulation and the ADM resistance of PC in addition to PC pathogenesis. In a correlation study between miR-375 and PC, Bhatti et al. found significantly decreased miR-375 expression in PC tissues and cell lines, including MIA-Pa-Ca-2, HUP-T3, PSN-1, and tumor adjacent tissues [26]. Calatayud et al. found that compared to the normal control group, the PC tumor tissues showed decreased miR-375 expression [27]. Zhou et al. also showed decreased miR-375 in PC tumor tissues compared to the adjacent tissues [28]. Yonemori et al. found that compared to the tumor adjacent tissues, PC patients had abnormally decreased miR-375 expression in tumor tissues and the PANC-1 or SW1990 cell line, along with an abnormally increased expression of its target gene ZFP36L2 [29]. Zhou et al. further showed that compared to tissues adjacent to the tumors, the PC tissues presented abnormally decreased miR-375 expression, which was correlated with tumor clinical phase and lymph node metastasis [20]. This study found the correlation between miR-375 down-regulation and the occurrence or drug resistance of

PC. Consistent with those studies, Niu et al. performed a correlation study between YAP1 and PC, and found significantly elevated YAP1 expression in PC tissues compared to the adjacent tissues [30]. Diep et al. also showed significantly higher YAP1 expression in PC tissues compared to the tissues adjacent to the tumor, and abnormally elevated YAP1 expression in PC cell lines AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1 compared to the normal pancreatic ductal epithelial cell line HPDE6 [31]. We found remarkably increased YAP1 expression in PC cells, indicating an oncogenic role of YAP1 in PC.

This study showed that the transfection of the miR-375 mimic significantly decreased YAP1 expression in SW1990/ADM and BxPC-3/ADM drug resistant cells, enhanced the apoptosis of those cells that can maintain stable growth in ADM, inhibited cell proliferation, and decreased drug resistance. In a study of the biological effect of miR-375 on PC cells, Zhou et al. showed that the over-expression of miR-375 in the PC cell lines PANC-1 and BxPC03 significantly inhibited cell proliferation and facilitated apoptosis, but the down-regulation of miR-375 facilitated PC cell proliferation and inhibited apoptosis by the targeted regulation of the PDK1 gene [28]. Yonemori et al. found that the over-expression of miR-375 inhibited the proliferation, migration, and invasion potency of PC cells by targeting the inhibition of ZFP36L2 expression [29]. Song et al. showed the tumor suppressor role of miR-375 in PC, and its down-regulation was related to PC onset. In particular, the over-expression of miR-375 can target and inhibit PDK1 expression to suppress PC cell PANC-1 proliferation, to induce cell cycle arrest, to facilitate cell apoptosis, and to inhibit the *in vivo* growth or tumorigenesis of PANC-1 cells [19]. All these studies showed that up-regulation of miR-375 may exert tumor suppressor functions to inhibit malignant biological properties of PC cells, as supported by our observations. Currently, few studies have been performed regarding the role of miR-375 in the drug resistance of PC. In a correlation study about miR-375 and PC cell drug resistance, Basu et al. found that the over-expression of miR-375 could decrease the proliferation activity of PC cells and elevated their sensitivity against benzyl isothiocyanate (BITC) via targeting IGFBP5 [32]. In contrast to this study [32], we investigated the targeted regulation between miR-375 and YAP1 and revealed the role of miR-375 in suppressing the ADM resistance of PC cells by inhibiting YAP1 expression, which has not been previously described and is thus the novelty of this study. However, the exact mechanism by which the downstream target gene of YAP1 exerts the modulation of PC cell drug resistance has not been fully illustrated and requires further investigation. Moreover, only an *in vitro* assay was performed here without any confirmation on animal models, making it another limitation of the current study. In addition, as this study only focuses on investigating the role of miR-375 in the drug resistance of PC cells, no clinical data were included. In the future, we plan to collect clinical data to verify this finding.

Conclusion

The down-regulation of miR-375 is correlated with the ADM resistance of PC cells. The up-regulation of miR-375 inhibits PC cell proliferation, facilitates cell apoptosis, and decreases ADM drug resistance possibly by suppressing YAP1 expression.

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Disclosure of conflict of interest

None.

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