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Increased miR-142 and decreased DJ-1 enhance the sensitivity of pancreatic cancer cell to adriamycin

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Abstract. – OBJECTIVE: Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway is related to tumorigenesis by up-regulating survivin. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) can suppress PI3K/AKT signaling pathway, while DJ-1 is the negative regulator of PTEN. DJ-1 up-regulation is closely correlated with t[illegible] currence, progression, and drug resista[illegible] pancreatic cancer. MicroRNA-142 (MiR-1[illegible] is significantly declined in pancreatic cance[illegible] sue. Bioinformatics analysis demonstrated [illegible] complementary binding site exist[illegible]tween mi[illegible] 142 and DJ-1. This investigatio[illegible]e, aimed to study the role of miR-142 [illegible]ne r[illegible]ation of DJ-1-PTEN/PI3K/AKT/Sury[illegible] signali[illegible]athway as well as in pancreatic [illegible]r cell [illegible] apoptosis, and adriam[illegible]in [illegible]

MATERIALS AN[illegible]METH[illegible]ual luciferase assay was pe[illegible]med to as[illegible]the target**ed relationship [illegible]n miR-142 a[illegible]-1. MiR-142, DJ-1, an[illegible]T[illegible]pressions [illegible]SW1990 cells and drug esistan[illegible]1990/ADM cells were compared[illegible]V1990/ADM [illegible]were divided into five gro[illegible] including mimi[illegible] miR-142 mimic, si[illegible]interfe[illegible] normal co[illegible]trol (si-NC), si-DJ-1[illegible]d miR-[illegible]mimic + si-DJ-1 groups. DJ-1, PTE[illegible]hos[illegible]ylated [illegible]KT (p-AKT), and Survivin [illegible]ons w[illegible]tested. Cell apoptosis [illegible]dete[illegible]d by[illegible] cytometry. Cell proliferation was e[illegible]y EdU staining.

R[illegible]ULTS: M[illegible]2 targeted inhibited DJ-1 expre[illegible]on. MiR-14[illegible], PTEN, and cell apoptosis sig**ni[illegible]n-regulated, while DJ-1, p-AKT, [illegible]vivin, [illegible] cell proliferation significantly el[illegible]ated in SW1990/ADM cells compared with [illegible]990 cells. MiR-142 mimics and/or si-DJ-1 t[illegible]ction markedly reduced DJ-1, p-AKT, and Sur[illegible]vin expressions enhanced PTEN level, attenuated cell proliferation, enhanced cell apoptosis, and weakened ADM resistance.

[illegible]SIONS: MiR[illegible]2 over-expression w[illegible]ened ADM resistance in pancreatic cancer [illegible]s by targetin[illegible]J-1 to enhance PTEN expres[illegible] and attenua[illegible]PI3K/AKT signaling pathway a[illegible]ty.

Key Wo[illegible]
MiR-142, DJ-1, PTEN, PI3K/AKT, Adriamycin, Pancre[illegible]ncer, Drug resistance.

Introduction

Pancreatic carcinoma (PC) is a type of malignancy with poor curative effect and prognosis[1,2]. Chemotherapy is an important method in the treatment of PC. However, drug resistance is an adverse factor that affects the curative effect[3]. Therefore, investigating the mechanism of drug resistance in the PC is of clinical significance to increase the effect of chemotherapy as well as improve the prognosis.

Survivin is a critical anti-apoptotic factor that participates in regulating cell proliferation[4,5]. It was found that Survivin is one of the target genes of phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway. PI3K/AKT signaling pathway activation promotes Survivin transcription and expression, suppressed cell apoptosis, and facilitated cell proliferation. It is closely correlated with various tumor occurrence, progression, and drug resistance[6-8]. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor gene that negatively regulates PI3K/AKT signaling pathway and tumorigenesis[9-11]. DJ-1 is a negative regulator of PTEN

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that can enhance the activity of the PI3K/AKT signaling pathway[12,13]. DJ-1 is found abnormally increased in several tumor tissues[14-16]. It was showed that DJ-1 significantly elevated in the tumor tissue[17] and peripheral blood of PC patients, suggesting the potential tumor suppressor gene role of DJ-1 in PC tumorigenesis[16,18]. MiRNA is a type of endogenous single-strand non-coding RNA with a length of 22-25 nt which has been discovered from an eukaryote. It plays a degrading or inhibiting role in the transcription of more than 1/3 mRNAs by binding with the 3'-UTR. Its relationship with tumorigenesis attracts more and more attention[19]. MiR-142 significantly down-regulated in PC tissue and cells, revealing that miR-142 might be a tumor suppressor gene in PC[20,21]. Bioinformatics analysis revealed a complementary binding site between miR-142 and DJ-1. This study aimed to evaluate the role of miR-142 in the regulation of DJ-1 as well as in PC cell proliferation, apoptosis, and adriamycin (ADM) resistance by establishing ADM resistant PC cell line.

Materials and Methods

Main Reagents and Materials

Human normal pancreatic cell line HPC was purchased from Aolu Biotechnology (Shanghai, China). Human PC cell line SW1990 was purchased from Baili Bio (Shanghai, China). Roswell park memorial institute 1640 (RPMI-1640) medium, option, essential (Opti-MEM), penicillin and streptomycin were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Science Co. Ltd. (Carlsbad, CA, USA). Total extraction kit SPLIT RNA Extraction Kit was provided by Lexogen (Vienna, Austria). TransScript Green One-Step qRT-PCR SuperMix was purchased from TransGen Biotech (Beijing, China). miR-NC, miR-142 mimic, miR-142 inhibitor and EdU flow cytometry detection reagent were purchased from Ribobio (Guangzhou, China). Rabbit anti-human DJ-1, p-AKT and AKT antibodies were got from Abcam Biotechnology (Cambridge, MA, USA). Mouse anti-human PTEN and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was derived from Bio-Rad Laboratories (Hercules, CA, USA). Annexin-V/propidium iodide (PI) apoptosis detection kit and cell counting kit 8 (CCK-8) cell viability detection kit were purchased from Dojindo Laboratories (Kumamoto, Japan). TransFast™ Transfection Reagent was purchased from Promega (Madison, WI, USA). Luciferase reporter plasmid pLUC was purchased from Novect (Beijing, China). Luciferase activity analysis kit LightSwitch was obtained from Active Motif (Saranac Lake, NY, USA). Adriamycin was purchased from Meiji Seika (Chuo-Kyo, Japan).

Cell Culture

HPC-Y5 and SW1990 cells were cultured in RPMI-1640 medium with 10% FBS and 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO_2. The cells were passaged at 1:4. This investigation has been approved by the Ethics Committee of Hospital Affiliated to Mudanjiang Medical University, Mudanjiang, China.

SW1990/ADM Model Establishment

SW1990 cells in logarithmic phase were treated by 0.1 μg/ml ADM for 24 h. Then, the medium was changed until the cells can grow and pass on stably. Next, the concentration of ADM was gradually increased to 0.2 μg/ml, 0.4 μg/ml, 0.8 μg/ml, 1.6 μg/ml, and 3.2 μg/ml. The cell can grow stably in 3.2 μg/ml ADM to establish a cisplatin-resistant SW1990/ADM cell line. SW1990 and SW1990/ADM cells were treated by ADM at 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml. After 48 h, 10 μl CCK-8 cells were added into the cells for 4 h and tested at 450 nm to obtain the absorbance value (A450). Inhibitory rate = (1- ADM group A450)/control A450 ×100%. IC_{50} was calculated by SPSS software (SPSS, Inc., Chicago, IL, USA). Resistance index (RI) = IC_{50} of SW1990/ADM/IC_{50} of SW1990.

Dual-Luciferase Assay

The PCR products containing the full length of DJ-1 gene 3'-UTR or mutant segment were cloned into pLUC followed by being transformed into DH5α competent cells and subsequent selection of the plasmid with the correct sequence. Then pLUC-DJ-1-wt (or pLUC-DJ-1-mut) was co-transfected into HEK293T cells using TransFast™ Transfection Reagent together with miR-142 mimic (or miR-142 inhibitor, or miR-NC). The luciferase activity was measured according to the Light Switch manual after cultured for 48 h.

Cell Transfection and Grouping

SW1990/ADM cells were divided into five groups, including mimic-NC, miR-142 mimic,

Table I. Primers for the PCR.

Genes		Primers
miR-142	Forward	5'-GCCACAAGGAGGGCTGGG
	Reverse	5'-GAGCGCCGAGGAAGATG
DJ-1	Forward	5'-CGGGGTGCAGGCTT
	Reverse	5'TCCGGTTTTCCTGC
PTEN	Forward	5'-CACACGACGGGAAGAC
	Reverse	5'-CCTCTGGTCCTGG
GAPDH	Forward	5'-ACACCCACTC
	Reverse	5'-TCCACCAC

si-NC, si-DJ-1, and miR-142 mimic + si-DJ-1 groups. Nucleotide fragments and TransFast™ Transfection Reagent were added to Opti-MEM for incubation at room temperature for 20 min, respectively. After that, they were added to the cells and cultured in Opti-MEM. After incubation for 6 h, the medium was changed to RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin and further cultured for 48 h followed by detection.

Quantitative Real Time-PCR (qRT-PCR)

Total RNA was extracted using SPLIT RNA Extraction Kit for PCR analysis using TransScript Green One-Step qRT-PCR SuperMix. The reaction system consisted of 1 μg RNA template, 0.3 μM primers, 10 μl 2×TransStart Tip Green qPCR SuperMix, 0.4 μl RT Enzyme Mix, 0.4 μl Passive Dye II, and ddH_2O. The reverse transcription condition was 37°C for 15 min and 98°C for 5 min. The PCR reaction was performed on ABI 7500 (Applied Biosystems, Foster City, USA) with the condition as follows: 45°C for reverse transcription for 5 min, 94°C for pre-denaturation for 30 s, followed by 40 cycles of 94°C for 5 s, and 60°C for 30 s. The primers were listed in Table I.

Western Blot

Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer. A total of 50 μg protein was separated by 12% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for 3 h and transferred to a membrane. After that, the membrane was blocked and incubated with primary antibody at 4°C overnight (DJ-1, PTEN, p-AKT, Survivin, and β-actin at 1:500, 1:300, 1:200, 1:300, and 1:800, respectively) followed by incubation with secondary antibody (1:10000) for 60 min after washed by Phosphate Buffered Saline Tween-20 (PBST) for three times. At last, the protein expression was detected using chemiluminescence (ECL) (Amersham Biosciences, Little Chalfont, Buckinghamshire, England).

Flow Cytometry

Cells were re-suspended in 100 μl binding buffer, incubated with 5 μl Annexin V-FITC and 5 μl PI for 15 min under dark. Then, 400 μl Annexin V Binding Buffer was added into the cells followed by analysis of cell apoptosis by FC 500 MCL/MPL flow cytometer (Beckman Coulter Inc., CA, USA).

EdU Staining

100 μl of 15 μM EdU solution was added into cells and incubated at 37°C for 60 min. After incubated for 48 h, cells were digested by trypsin and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation in 1% saponin and suspension in phosphate buffered saline (PBS). At last, cells were stained with 500 μl 6-FAM Azide at room temperature under dark for 30 min and tested on FC 500 MCL/MPL flow cytometer r (Beckman Coulter Inc., Brea, CA, USA).

Statistical Analysis

All data analyses were performed by SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between the two groups. Tukey's post hoc test was used to validate the ANOVA for comparing measurement data among groups. $p<0.05$ was considered as statistical significance.

Results

MiR-142 Regulates DJ-1 Expression

MicroRNA.org online prediction revealed a targeted binding site between miR-142 and 3'-

Table II. IC50 of SW1990/ADM cells

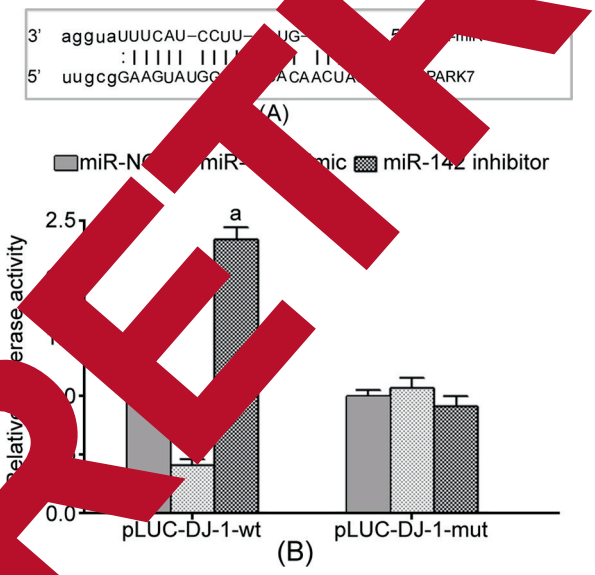
Group	IC50 (μg/ml)
miR-NC	22.71±1.13
miR-142 mimic	9.14±0.76a
si-NC	24.28±1.09
si-DJ-1	8.65±0.62b
miR-142 mimic+ si-DJ-1	5.75±0.45cd

[a]$p<0.05$, compared with miR-NC, [b]$p<0.05$, compared with si-NC, [c]$p<0.05$, compared with miR-142 mimic, [d]$p<0.05$, compared with si-DJ-1 group.

UTR of DJ-1 mRNA (Figure 1A). Dual luciferase assay showed that miR-142 mimics or inhibitor transfection significantly decreased or increased the relative luciferase activity of HEK293 cells transfected by pLUC-DJ-1-wt, while it showed no statistical effect on the luciferase activity in HEK293 cells which were transfected by pLUC-DJ-1-mut (Figure 1B), suggesting a regulatory relationship between miR-142 and DJ-1 mRNA.

MiR-142 Down-Regulated, While DJ-1 Over-Expressed in SW1990/ADM Ce

The IC_{50} of SW1990 cells was 0.86 μg/m
le it was 23.75 μg/ml in SW1990/ADM cell
RI was 27.61. The qRT-PCR showed that mi
and PTEN mRNA levels were significantly lo
while DJ-1 mRNA level was
in SW1990 cells compared w
HPC-Y5



Fi 1. MiR-142 targeted regulated DJ-1 expression. ***A,*** binding site between miR-142 the 3'-UTR of DJ-1 mRNA. ***B,*** Dual luciferase assay. *$p<0.05$, compared with miR-NC.

cells. Similar results were observed in SW1990/ADM cells compared with SW1990 (Figure 2A). Western blot showed that DJ-1 protein expression was markedly higher, while PTEN prote
sion was significantly lower in SW19 DM c
ls compared with SW1990 cells an C-Y5 cells (Figure 2B). Flow cytometry de rated that the cell apoptotic rate was sig ifican er (Figure 2C), while cell prolife n (Figu was significantly stronger in 990/ADM ce that in SW1990 cells t d by 0 g/ml A

MiR-142 Over- re tenu ADM Resista in SW /AD ells

3.2 μg/ml was used e the impact of m regulation or DJ-1 reduction o e AD sitivity of SW1990/ADM cells. MiR-142 mimic or si-DJ-1 transfection sig y enhanced F level, reduced p-AK nd Survivin expressions (Figure 3A and B), nuated cell iferation (Figure 3C), enhan- cell apopto (Figure 3D), and weakened A resistance ble II).

Discussion

AKT signaling pathway is involved in multiple biological behaviors, such as cell proliferation, apoptosis, cell cycle, etc. It is closely associated with embryonic development, angiogenesis, and tumorigenesis. The kinase activity of PI3K phosphorylates PIP2 to PIP3, which phosphorylates AKT at Ser473 and Thr308 with the help of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and 3-phosphoinositide-dependent protein kinase 2 (PDK2). Phosphorylated AKT further participates in gene transcription and translation that regulates cell proliferation, cell cycle, apoptosis, and invasion[22]. As the strongest factor in inhibitor of apoptosis protein (IAPs) family, Survivin antagonizes cell apoptosis by suppressing Caspase-3 and Caspase-7 activities[4]. In addition, Survivin up-regulation is related to the enhancement of cell proliferation[5]. Survivin is one of the important target genes of PI3K/AKT signaling pathway. PI3K/AKT pathway promotes Survivin gene transcription and expression, and inhibits cell apoptosis, and accelerates cell proliferation. It is related to multiple cancers occurrence, progression, and drug-resistance, including gastric cancer[6], endometrial cancer[7], and colorectal cancer[8]. PTEN is the only discovered tumor suppressor gene with the dual activity of

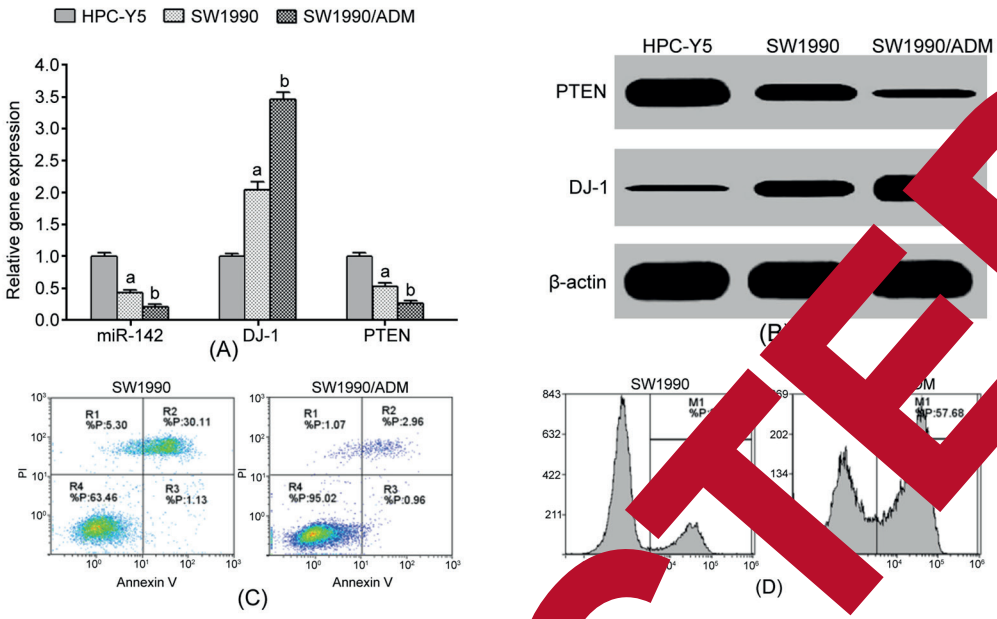


Figure 2. MiR-142 down-regulated, while DJ-1 over-expressed in 1990/ADM cell qRT-PCR detection of gene expression. ***B,*** Western blot detection of protein expression. ***C,*** Flow cyto y detection of c poptosis. ***D,*** EdU staining detection of cell proliferation. [a]$p<0.05$, compared with HPC-Y5 cells,[b]$p<0.05$ pared with S 0 cells.

protease and phosphatase. It plays an in role in tumorigenesis through negatively r a- ting PI3K/AKT signaling pathway, such as lbladder carcinoma[9], breast cancer[10], and prost cancer[11]. DJ-1, also known as P on gene (PARK7), is related to Huma l reces- sive inheritance early-ons arkins disea- se (PD)[23,24]. DJ-1 involv multip processes, such as ati- chaperone, cell pro ion, a sis, transfor- mation, and and receptor ling tran- sduction[25,26]. D ces the ac of the PI3K/AKT si ing p y by attenuating the inhibitory ct of PTE I3K/AKT[12,13]. DJ-1 up-regu is found in m tumor tissues, such a east c cer[14] and lun cancer[15]. It was sho that D significantly elevated in the tum d peri al blood[16,18] of PC pa- tients, s ng th tential oncogene role of in PC ris. MiR-142 significantly do egulat C tissue and cells, revealing that R-142 mig t be a tumor suppressor gene in P matics analysis revealed a com- mentary inding site between miR-142 and 1. Establishment of drug-resistant cancer cell *vitro* is of great significance in exploring the chanism of cancer resistance and screening chemotherapy drugs. This study investigated the role of miR-142 in the regulation of DJ-1, PC cell proliferation, apoptosis, as well as ADM resistan- tablishing ADM resistant PC cell line.

D luciferase assay showed that miR-142 mimics or inhibitor transfection significantly re- duced or increased the relative luciferase activity of HEK293 cells transfected by pLUC-DJ-1-wt, while it did not exhibit any statistical effect on the luciferase activity in HEK293 cells tran- sfected by pLUC-DJ-1-mut, suggesting a regu- latory relationship between miR-142 and DJ-1 mRNA. MiR-24 and PTEN mRNA levels were significantly lower, while DJ-1 mRNA expres- sion was significantly higher in SW1990 cells compared with HPC-Y5 cells. Similar results were observed in SW1990/ADM cells compa- red with SW1990. It indicated that miR-142 and PTEN reduction, and DJ-1 elevation are related not only to PC tumorigenesis, but also ADM resi- stance enhancement. Lu et al[20] reported that miR- 142 significantly declined in PC cell line PANC- 1, SW1990, Hup, and CFPAC-1 compared with normal pancreatic cell HPC-Y5. Moreover, miR- 142 significantly declined in PC tumor tissue compared with adjacent normal control. In this study, the miR-142 level was markedly lower in PC cells than normal pancreatic cells, suggesting that miR-142 down-regulation was a tumor-pro- moting factor in PC, which was similar with Lu et al[20] findings. Ohuchida et al[21] established

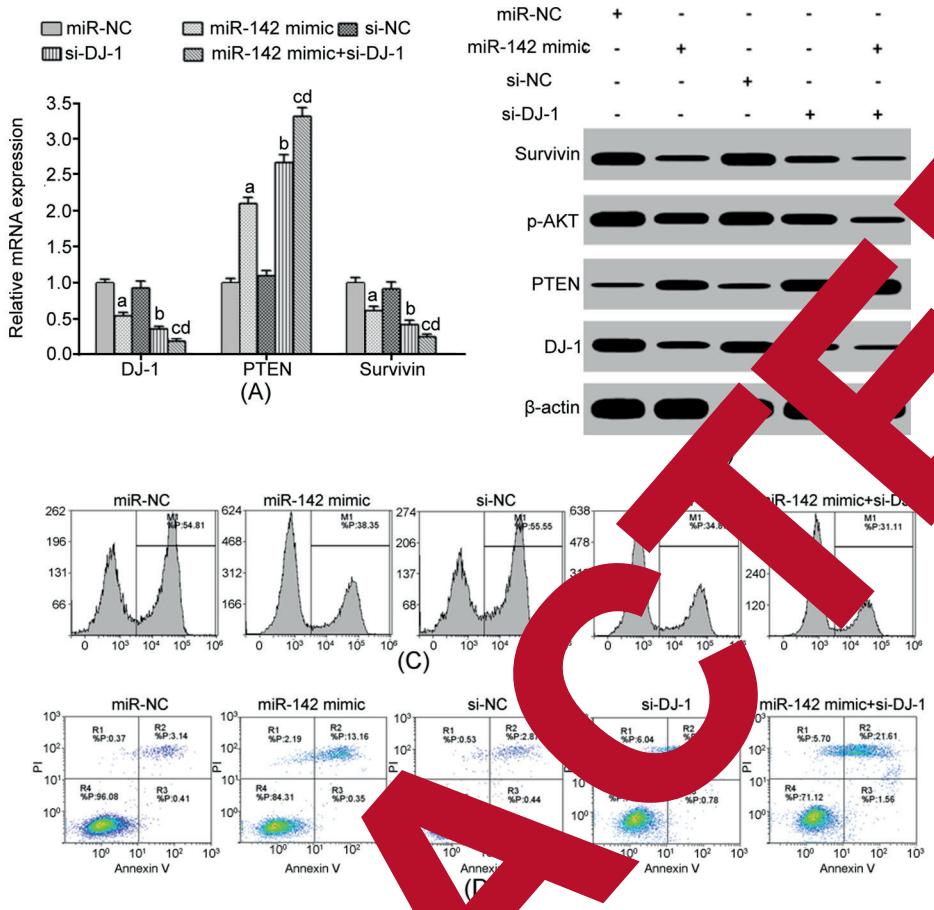


Figure 3. MiR-142 over-expression att[enuates] ADM resi[stance] in SW1990/ADM cells. ***A,*** qRT-PCR detection of gene expression. ***B,*** Western blot detection of pr[otein]. ***C,*** Flo[w cyto]metry detection of cell apoptosis. ***D,*** EdU staining detection of cell proliferation. [a]$p<0.05$, com[pared] with [miR-]NC, [b]$p<0.0$[5, co]mpared with si-NC, [c]$p<0.05$, compared with miR-142 mimic, [d]$p<0.05$, compared with si-DJ-1[+si-NC].

Gemcitabine resi[stant] cell line b[y] Gemcitabine to treat [human] SUIT-2 an[d] PAN-1. It was found [that] mi[R-142] significantly decreased in Ge[m]itabine resis[tant] cell line compared with pa[rental] drug-sensitive [cells]. In this work, miR-1[42] expres[sion] in SW199[0] cells was markedly lower t[han] HPC-Y5, revealing that miR-142 [down]regulation m[ay] be associated with PC drug re[sistance], whi[ch] was in accordance with [Ohu]chida [et al's] findings. Chen et al[18] discovered that DJ-1 [was] apparently up-regulated in the peripheral blood of PC patients compared with h[ealthy] [people]. Tsiaousidou et al[17] demonstrated that DJ-1 abnormally elevated in PC tumor tissue and was negatively correlated with chemotherapy sensitivity, indicating that DJ-1 over-expression was associated with PC tumorigenesis and drug resistance. In this study, DJ-1 increased in PC cells, while its increasing amplitude was larger in drug-resistant cells, suggesting that DJ-1 was a promoting factor in PC occurrence and drug resistance, which was confirmed by Chen et al[18] and Tsiaousidou et al[17]. Further investigation showed that miR-142 mimics and/or si-DJ-1 transfection markedly enhanced PTEN level, reduced p-AKT and Survivin expressions, attenuated cell proliferation, enhanced cell apoptosis, and weakened ADM resistance. Lu et al[20] showed that miR-142 declined, while PC cell proliferation and invasion enhanced under hypoxia condition. MiR-142 over-expression attenuated PC cells SW1990 and PANC-1 proliferation, EMT, and invasion by restraining hypoxia-inducible factor 1 (HIF-1α). MacKenzie et al[27] exhibited that miR-142 up-regulation weakened PC cells MIA PaCa-2 and Capan-1 proliferation through targeting HSP-70 expression. Ohuchida et al[21] also showed that miR-142 level was significantly higher in patients

with better survival and prognosis after Gemcitabine treatment, suggesting that miR-142 may be related to drug resistance. This research found that miR-142 elevation reduced PC malignancy and attenuated drug resistance, which was supported by MacKenzie et al[27] and Ohuchida et al[21]. Chen et al [18] reported that DJ-1 level was higher in Gemcitabine resistant MIA PaCa-2 cells compared with parental cells, while down-regulation of DJ-1 enhanced Gemcitabine sensitivity and apoptosis in MIA PaCa-2 cells, which was similar with our results. We revealed that miR-142 reduction plays a role in down-regulating PTEN, enhancing PI3K/AKT signaling pathway, and promoting PC drug resistance.

Conclusions

We found that miR-142 down-regulation and DJ-1 over-expression are associated with DM resistance in PC cells. MiR-142 over-expression weakened ADM resistance in pancreatic cancer cells by targeting DJ-1 to enhance PTEN expression and attenuate PI3K/AKT signaling pa... activity.

Acknowledgments

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Conflict of Int...

The Authors de... that t... ...e no conflict of interest.

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