

MiR-128-3p overexpression sensitizes hepatocellular carcinoma cells to sorafenib induced apoptosis through regulating DJ-1

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Abstract. – OBJECTIVE: DJ-1 expression is elevated in a variety of tumors and is related to the survival of tumor cells under adverse stimuli. DJ-1 3'-untranslated region (3'-UTR) contains the target of miR-128-3p, and the expression of miR-128-3p is decreased in hepatoma cells. Therefore, we speculate and address in this study, that miR-128-3p can regulate DJ-1 expression in hepatocellular carcinoma (HCC) and play an important role in HCC cells survival.

MATERIALS AND METHODS: MiR-128-3p and DJ-1 expression in HCC cell lines were measured using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis. Dual luciferase reporter assay was adopted to confirm the miR-128-3p binding sequences in the 3'-UTR of DJ-1. Sorafenib-induced apoptosis was evaluated by flow cytometry, and the apoptosis-associated proteins were detected by Western blot analysis. Overexpression of miR-128-3p and DJ-1 were achieved via transfection with miR-128-3p mimic and DJ-1 plasmid, respectively.

RESULTS: We revealed that miR-128-3p expression was downregulated, while DJ-1 expression was upregulated in HCC cell lines, and DJ-1 expression can be regulated by miR-128-3p via directly binding to it. Moreover, functional assays showed that overexpression of miR-128-3p sensitized HCC cells to sorafenib-induced apoptosis, and this phenomenon was partly abolished by DJ-1. Mechanistically, PTEN/PI3K/Akt signaling pathway was found to participate in the miR-128-3p induced sensitivity to sorafenib via DJ-1.

CONCLUSIONS: We conclude that miR-128-3p overexpression sensitized HCC to sorafenib-induced apoptosis via PTEN/PI3K/Akt signaling pathway by regulating DJ-1 expression.

Key Words:

miR-128-3p, DJ-1, Hepatocellular carcinoma, Sorafenib, Apoptosis.

Introduction

Hepatocellular carcinoma (HCC), characterized by high incidence and poor prognosis, is one of the most common malignancies in the world¹⁻³. Despite the measures of prevention, diagnosis and treatment for HCC have achieved tremendous advances during the past decades, its incidence and fatality rates are still increasing yearly⁴⁻⁶. According to the data from the American Cancer Society, HCC is accounting for more than 39000 new cases and over 27000 deaths in the United State in 2016⁷. In addition, the data also show that males are more susceptible to HCC. On average, males are affected 3 times more frequently than females⁷. Various cytokines and sex hormones are considered to be involved in this gender difference, such as interleukin-6 (IL-6), estrogen and its receptors⁸⁻¹⁰. Previous investigations¹¹ have revealed that IL-6 expression was significantly overexpressed in the animal model of liver cancer induced by diethylnitrosamine, and knockdown of IL-6 could abolish this gender disparity. Traditional therapeutic measures for HCC were primarily surgical excision and chemotherapy. However, the therapeutic efficacy was disappointing^{12,13}. Therefore, it is urgent to explore the underlying mechanisms of HCC and find new promising therapeutic targets. MicroRNAs (miRNAs), a subgroup of non-coding RNAs (ncRNAs) that have no protein-coding ability, are characterized by short sequence (about 20 nucleotides) and single stranded^{14,15}. Although the exact functions of miRNAs remain unclear, accumulating evidence has suggested it may be involved in several of physiological processes, such as cell development, and DNA reparation¹⁵⁻¹⁷. Scholars^{18,19}

have revealed that miRNAs could serve as gene regulator by binding to the mRNA of target genes. MiR-128-3p, an important member of miRNAs, was demonstrated to be associated with diverse intracellular biological processes during carcinogenesis, including proliferation, apoptosis and metastasis²⁰. Aberrant expression of miR-128-3p was found in many cancers, implying it may play a critical role in the progression of tumors^{21,22}. Recently, miR-128-3p was reported to be downregulated in HCC. However, the downstream signaling pathway remains obscure²³. DJ-1, also known as Parkinson's disease-associated protein (PDAP), is a protein with multiple functions, including cysteine protease, anti-oxidative stress reaction, and tumorigenesis²⁴⁻²⁶. It was firstly identified as a tumor-related protein in mouse NH3T3 cells, and acted as an oncogene that could induce NH3T3 cells transform via the cooperation with H-RAS²⁷. Increasing evidence has suggested that DJ-1 may serve as a critical agent in the progression and development of cancers, and overexpressed DJ-1 was revealed in various of human tumors, such as breast cancer, pancreatic cancer, and gastric cancer²⁸⁻³⁰. Overexpressed DJ-1 and decreased miR-128-3p expressions were also found in HCC cell lines in our study, and miR-128-3p could regulate DJ-1 expression by directly binding to it. Further functional experiments showed that overexpressed miR-128-3p may sensitize HCC cells to sorafenib, and this effect could be abolished by DJ-1 via PTEN/PI3K/Akt signaling pathway.

Materials and Methods

Cell Culture and Transfection

HCC cell lines (HepG2, SK-HEP-1, HuH-7, and Li-7) and normal human liver cell line, HL-7702, were all obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Both HCC cell lines and normal human liver cell lines were routinely maintained in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco BRL, Grand island, NY, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and incubated in a humidified atmosphere at 37°C with 5% CO₂. This study was approved by the Ethics Committee of Shanghai East Hospital, Tongji University. To upregulate the miR-128-3p expression in liver cancer cells, they were transfected with miR-128-3p mimic (miR-128-3p group), and cells tran-

sected with miR-negative control (miR-NC group) served as control group. To overexpress the DJ-1 in liver cancer cells, cells were transfected with DJ-1 plasmid, and cells transfected with vector served as control group. Cells were seeded in 6-well plates at a concentration of 1×10⁵ cells/well, and changed to the serum-free media at 50% confluence. After cells were incubated for 24 h, they were transfected with 50 nM miR-128-3p mimic or 150 pmol DJ-1 plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequence of specific siRNA against DJ-1 and scramble siRNA is shown in Table I.

Reverse Transcription and Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNAs of untreated or treated HCC cell lines were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and they were reverse transcribed into cDNA by the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Then, 2 µl of cDNA WERE amplified and quantified via Real-time polymerase chain reaction (RT-PCR) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; TaKaRa Bio Inc., Otsu, Shiga, Japan). The polymerase chain reaction (PCR) conditions were 94°C for 3 min, followed by 40 cycles of 94°C for 35 s and 62°C for 35 s. The primers of miR-128-3p, DJ-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 (internal loading control) were designed and obtained from Sangon Biotech Co., Ltd, (Shanghai, China). The primers of GAPDH were 5'- CCT CGT CTC ATA GAC AAG ATG GT -3' (F) and 5'-GGG TAG AGT CAT ACT GGA ACA TG-3' (R); the primers of U6 were 5'-CTC GCT TCG GCA GCA CA-3' (F) and 5'-AAC GCT TCA CGA ATT TGC GT -3' (R); the primers of miR-128-3p were 5'-GGT CAC AGT GAA CCG GTC-3' (F) and 5'-GTG CAG GGT CC G AGG T-3' (R); the primers of DJ-1 were 5'- CGC ACA GAA TTT ATC TGA GTC-3' (F), and 5'-GTC TTT AAG AAC AAG TGG AGC-3' (R); the mRNA expression levels of DJ-1 were normalized to GAPDH and the mRNA expression levels of miR-128-3p were normalized to U6.

Western Blotting

Proteins of naive or treated HCC cells were prepared by lysing cells in radio immunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% Triton X-100, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4)

Table 1. The specific siRNAs against DJ-1 and scramble siRNA.

Name	Sequence (5'-3')
siRNA1	GCGCTTGCAATTGTTGAAGCCCTGA
siRNA2	AAGTGAAGGCTCCACTTGTCTTAA
siRNA3	AGGCTCCACTTGTCTTAAAGACTA
NC siRNA	AAGGAATCGACCTTCTGTCTGTAA

followed by ultrasonication on ice. After centrifuged at 12000 rpm/min for 30 min, the supernatant was collected and the protein concentration was measured by a protein assay reagent (Bio-Rad Laboratory, Hercules, CA, USA). After that, proteins from each group were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transferred onto a nitrocellulose membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were immersed into 5% low fat dried milk for 2 hours at room temperature to block non-specific sites, followed by incubated overnight with corresponding primary antibodies that against DJ-1 (1:1000, ab18257, Abcam, Cambridge, MA, USA), caspase-3 (1:2000, ab13585, Abcam, Cambridge, MA, USA), PARP (1:5000, ab32064, Abcam, Cambridge, MA, USA), PTEN (1: 2000, ab32199, Abcam, Cambridge, MA, USA), pPTEN (1:2000, ab109454, Abcam, Cambridge, MA, USA), AKT (1:1000, ab8805, Abcam, Cambridge, MA, USA), pAKT (1:1000, ab81283, Abcam, Cambridge, MA, USA). Next, horseradish peroxidase (HRP)-conjugated secondary antibodies were applied to the membranes for 2 h at room temperature. GAPDH was used as an internal control, and signals were detected by enhanced chemiluminescent (ECL) reagents.

Plasmid Construction and Dual Luciferase Activity Assay

The fragment of DJ-1 that binds to miR-128-3p was produced by PCR and then it was inserted into a luciferase vector psi-CHECK (Promega, Madison, WI, USA) and named wild-type (Wt) plasmid. The mutant DJ-1 fragment that complementary to miR-128-3p was also established as described above and named mutant-type (Mut) plasmid. HEK-293T cells were seeded into 24-well plates and cultured for 24 h, and then they were co-transfected with Wt or Mut plasmids and miR-128-3p mimic or miR-NC via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured using the dual lucifera-

se reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocols. Renilla luciferase activity was normalized to firefly luciferase activity.

Cell Apoptosis Analysis

Cell apoptosis rate of treated liver cancer cells was measured by flow cytometry analysis with Annexin-V/PI double staining. Briefly, treated liver cancer cells were collected after cultured in DMEM medium overnight, and re-suspended with 500 ml binding buffer which supplemented with 1% annexin V-FITC and PI. After liver cancer cells were incubated at room temperature (protected from light) for 15 min, they were analyzed using flow cytometry.

Statistical Analysis

All statistical analyses were performed using GraphPad (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA). All data were presented as the mean $\pm$ SEM. Data from this study were subjected to one-way analysis of variance, followed by post-hoc test Least Significant Difference (LSD) if the overall ANOVA was significant. Differences between means were considered significant if $p < 0.05$.

Results

Decreased miR-128-3p Expression and Increased DJ-1 Expression Was Found in the HCC Cell Lines

Previous studies have revealed that miR-128-3p expression was downregulated in both HCC tissues and cell lines. In this study, we confirmed this result by qRT-PCR in HCC cell lines. It indicated that in four HCC cell lines (HepG2, SK-HEP-1, HuH-7, and Li-7), miR-128-3p expression was indeed significantly downregulated, as compared to a normal human liver cells (HL-7702) (Figure 1A). Meanwhile, we also found that DJ-1 mRNA expression was predominantly higher in the four HCC cell lines than that in HL-7702 cells (Figure 1B). Results from Western blot analysis showed that DJ-1 protein expression was also higher in the four HCC cell lines than that in HL-7702 cells (Figure 1C and 1D).

MiR-128-3p Directly Regulated DJ-1 Expression in HCC Cells

In order to figure out whether there was a correlation between the expression of miR-128-

3p and DJ-1, relative DJ-1 mRNA expression was measured by qRT-PCR in miR-128-3p overexpressed liver cancer cells. We firstly verified the miR-128-3p expression in liver cancer cells transfected with 5 nmol/l or 10 nmol/l miR-128-3p by qRT-PCR, and results showed that miR-128-3p expression was significantly increased in both 5 nmol/l and 10 nmol/l treated cells (Figure 2A). The relative DJ-1 mRNA expression (Figure 2B) and protein expression (Figure 2C) were both significantly decreased in the miR-128-3p overexpressed liver cancer cells. These results suggested that miR-128-3p may negatively regulate the DJ-1 expression. To further confirm this result, dual-luciferase activity assay was performed. We

co-transfected plasmid containing 3'UTR of DJ-1 downstream of firefly luciferase reporter along with miR-128-3p mimic or its negative control RNA (miR-NC). A significant decrease in luciferase activity was detected in DJ-1 Wt plasmid and miR-128-3p mimic treated cells, which was abolished when the miR-128-3p site was break from DJ-1 (Figure 2D).

Sorafenib Induced Apoptosis of HCC Cells was Promoted by miR-128-3p Overexpression

To evaluate the possible association between miR-128-3p expression and sensitivity of HCC cells to sorafenib, cell apoptosis at 5 μ M sora-

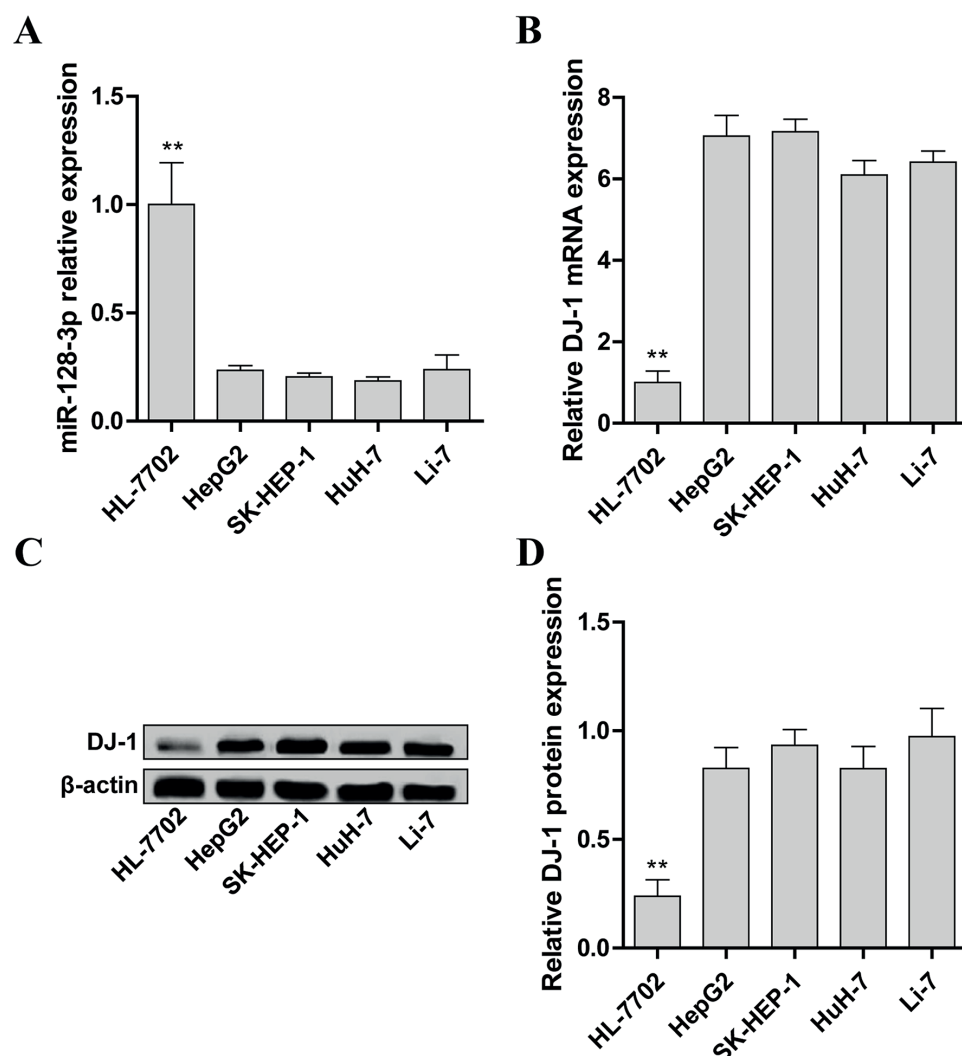


Figure 1. Aberrant expression of miR-128-3p and DJ-1 in hepatocellular carcinoma (HCC) cell lines. (A) The relative expression of miR-128-3p was reduced in the HCC cell lines (HepG2, SK-HEP-1, HuH-7, and LI-7) compared to HL-7702. (B) Expression of DJ-1 mRNA in HCC cell lines was detected by qRT-PCR. (C and D) Western blot analysis was performed to examine the expression of DJ-1 protein (** $p < 0.01$).

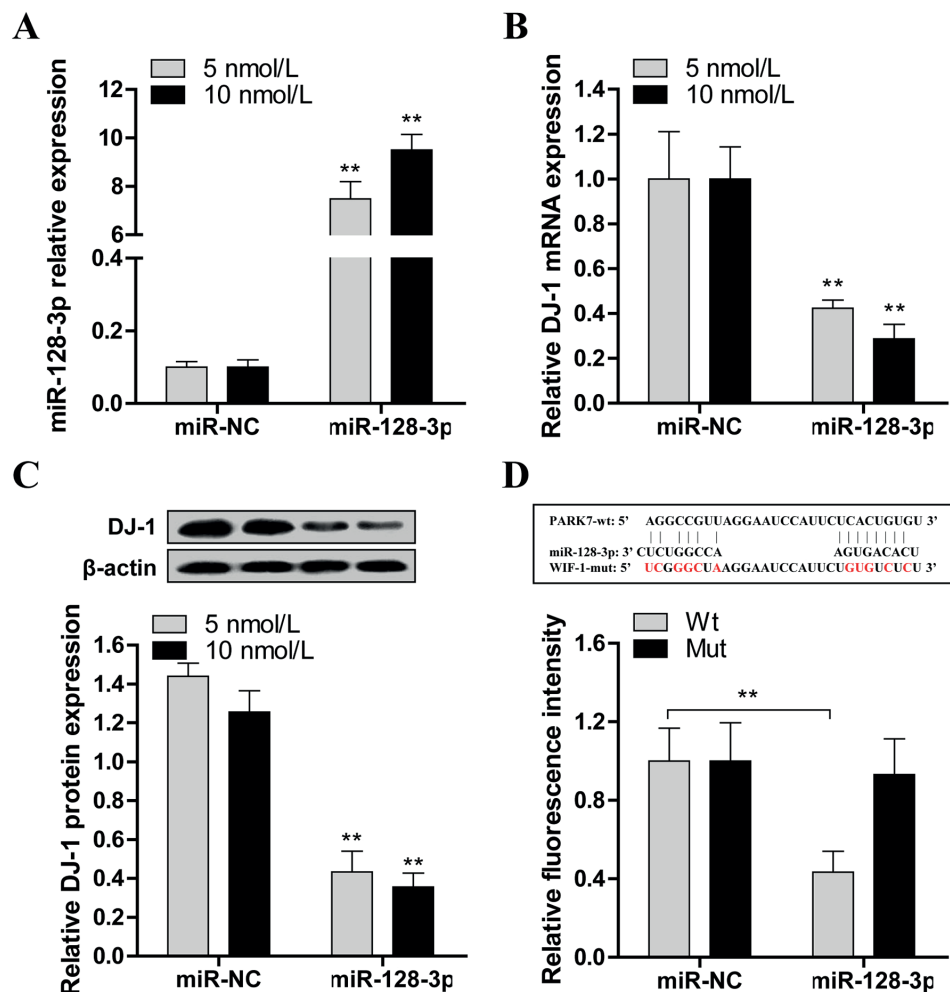


Figure 2. MiR-128-3p directly regulates DJ-1 expression in hepatocellular carcinoma (HCC) cell. (A and B) The relative mRNA expression of miR-128-3p and DJ-1 in liver cancer cells treated with negative control RNA (miR-NC) or miR-128-3p mimics (5 nmol/l or 10 nmol/l) was measured by qRT-PCR. (C) Western blot analysis was used to detect the protein expression of DJ-1 in liver cancer cells treated with miR-NC or miR-128-3p (5 nmol/l or 10 nmol/l). (D) Upper panel: the sequence of complementary site of miR-128-3p, DJ-1 (PARK7) and its mutant. Lower panel: luciferase activity driven by DJ-1 3'-UTR was attenuated by adding miR-128-3p mimics (** $p < 0.01$).

fenib or dimethyl sulfoxide (DMSO) was examined in both miR-128 mimic and miR-NC transfected liver cancer cells by flow cytometry. Results showed a significant increase in cell apoptosis rate of liver cancer cells pre-treated with miR-128 mimic, under 5 μ M sorafenib than those cells pre-treated with miR-NC then sorafenib (Figure 3A and 3B). In addition, Western blot analysis was used to determine the caspase-3 and PARP protein expression in miR-128-3p overexpressed liver cancer cells treated by DMSO or sorafenib. Results indicated that in both miR-NC and miR-128-3p mimic transfected liver cancer cells, sorafenib induced ca-

spase-3 and PARP protein expression was significantly higher than DMSO (Figure 3C and 3D). And under the treatment of sorafenib, caspase-3 and PARP protein expression showed a significant increase in the miR-128-3p treated liver cancer cells when compared with those treated with miR-NC (Figure 3C and 3D).

DJ-1 Partly Abolished the Effect of miR-128-3p Overexpression on Sorafenib Induced Apoptosis

To determine whether DJ-1 involves in the effects of miR-128-3p overexpression on sorafenib induced apoptosis, DJ-1 overexpressed liver

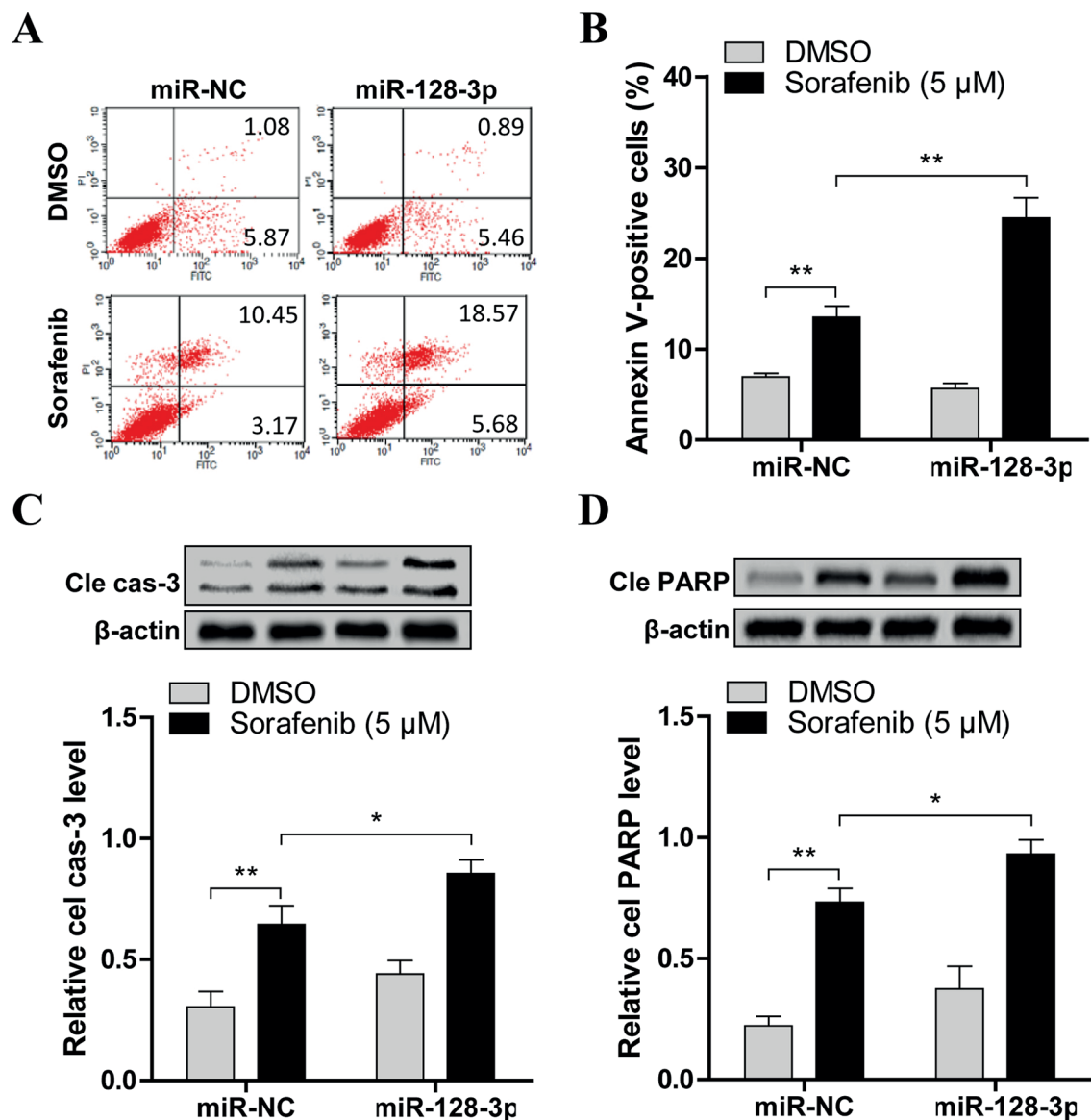


Figure 3. MiR-128-3p overexpression sensitizes hepatocellular carcinoma (HCC) cells to sorafenib induced apoptosis. After transfected with miR-128-3p mimic or miR-NC, HCC cells were treated with sorafenib (5 μ M) or DMSO for 24 h, then (A and B) cell apoptosis rate was measured by flow cytometry, and (C and D) the protein expression of caspase-3 and PARP was detected by Western blot analysis (* $p < 0.05$, ** $p < 0.01$).

cancer cell line was established by treating cells with DJ-1. Results from qRT-PCR showed that transfection of DJ-1, but not vector control, into liver cancer cells significantly increased the DJ-1 mRNA expression at the presence of miR-128-3p negative control (miR-NC) (Figure 4A). As shown in Figure 2B, miR-128-3p significantly decreased the expression of DJ-1, and this effect could be partly reversed by the application of DJ-1 plasmid (Figure 4A). Flow cytometry assay was performed to further evaluate the effects of DJ-1 on the

miR-128-3p induced sensitivity to sorafenib. Liver cancer cells treated with miR-128-3p showed a significant increase in sorafenib induced cell apoptosis compared to miR-NC treated group (Figure 3A, 3B and 4B). At the presence of miR-128-3p, liver cancer cells treated with DJ-1 showed a remarkable decrease in sorafenib induced cell apoptosis compared with vector treated cells (Figure 4B). Results from Western blot analysis indicated that caspase-3 and PARP protein expression were significantly higher in miR-128-3p treated cells

than those in miR-NC group (Figure 4C and 4D). Moreover, in the miR-128-3p overexpressed cells, DJ-1 treated cells showed a significant downregulation of caspase-3 and PARP protein expression when compared to vector treated cells (Figure 4 and 4D).

PTEN/PI3K/Akt Signaling Pathway is Involved in the Protective Function of DJ-1 on HCC Cells Against Sorafenib

To explore the potential signaling pathways that involved in the protective effects of DJ-1

on liver cancer cells against sorafenib, Western blot analysis was used to examine the protein expressions of PTEN/pPTEN and AKT/pAKT. In the vector treated groups, the pPTEN/PTEN rate and pAKT/AKT rate of liver cancer cells treated with miR-128-3p showed a remarkable downregulation compared to miR-NC treated cells (Figure 5A and 5B). In the miR-128-3p overexpressed liver cancer cells, the pPTEN/PTEN rate and pAKT/AKT rate were significantly higher in the DJ-1 treated group than those in vector group (Figure 5A and 5B). In addition, li-

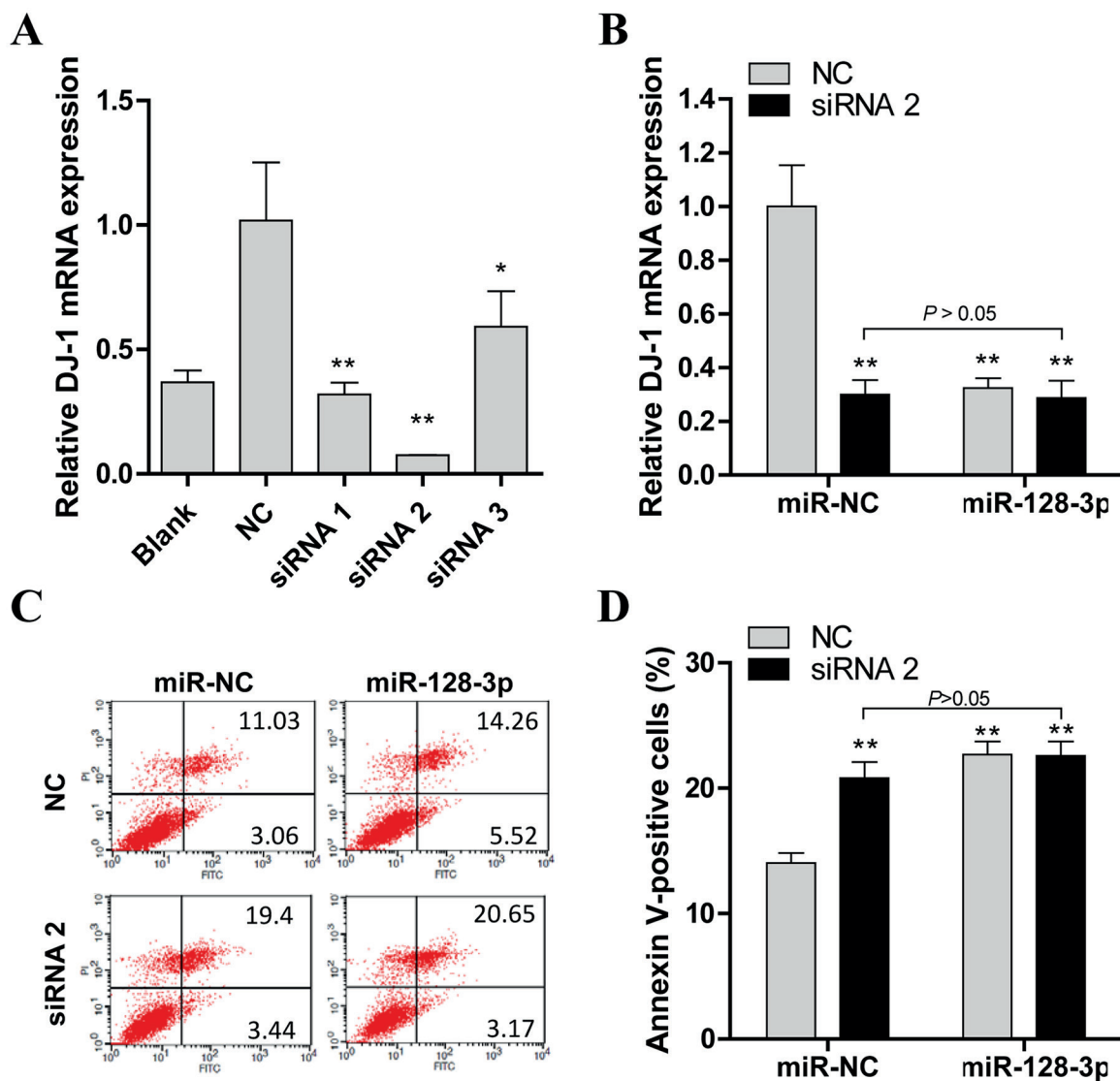


Figure 4. DJ-1 partly abolishes the effect of miR-128-3p overexpression on sorafenib induced apoptosis. (A) The relative mRNA expression level of DJ-1 was examined in the miR-NC or miR-128-3p and DJ-1 or vector treated liver cancer cells by qRT-PCR. (B) Cell apoptosis of liver cancer cells treated with miR-NC or miR-128-3p and DJ-1 or vector was measured by flow cytometry analysis. (C and D) Caspase-3 and PARP protein expressions were detected by Western blot analysis in liver cancer cells treated with miR-NC or miR-128-3p and DJ-1 or vector (* $p < 0.05$, ** $p < 0.01$).

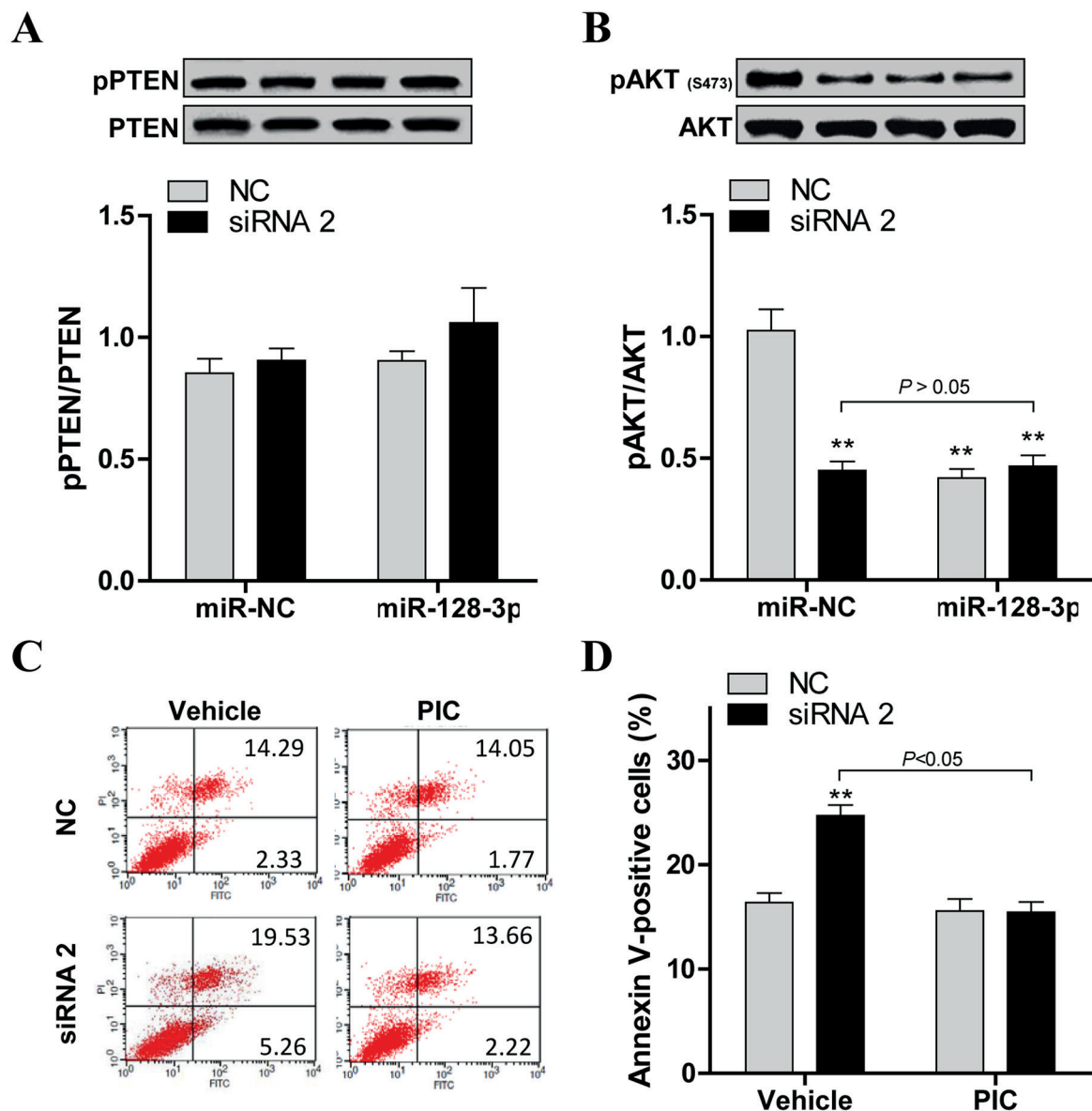


Figure 5. PTEN/PI3K/Akt signaling pathway was involved in the protective function of DJ-1 on liver cancer cells against sorafenib. (**A** and **B**) Western blot analysis was used to examine the rate of pPTEN/PTEN and pAKT/AKT in liver cancer cells co-transfected with miR-128-3p mimic or miR-NC and DJ-1 siRNA2 or NC. (**C** and **D**) After treated with PTEN inhibitor, PIC (10 nmol/l), DJ-1 treated liver cancer cells were subjected to examine cell apoptosis induced by sorafenib using flow cytometry (* $p < 0.05$, ** $p < 0.01$).

ver cancer cells pre-treated with DJ-1 and PIC (PTEN inhibitor, 10 nmol/l) were incubated with sorafenib for 24 h, and flow cytometry was then performed to examine the cell apoptosis. As the results showed, cell apoptosis was remarkably lower in the DJ-1 treated cells than that in vector group, and the DJ-1 induced downregulation of cell apoptosis was reversed by the application of PIC (Figure 5C and 5D).

Discussion

Non-coding RNAs (ncRNAs), including lncRNAs, miRNAs, and circular RNAs (circRNAs), have long been considered to be insignificant since they were uncovered in human cells³¹⁻³³. However, with developing of corresponding researches, ncRNAs were demonstrated to be involved in diverse physiological progresses and

diseases³⁴⁻³⁶. Accumulating researches^{31,37} have suggested that ncRNAs may participate in the pathogenesis of many human tumors by regulating the oncogenic or suppressive gene expression. MiRNAs, as one of the most important member of ncRNAs, have been well documented in the tumorigenesis of many human tumors, including HCC³⁸⁻⁴¹. Yu et al⁴² have reported overexpressed miR-196b in HCC tissues and cells, which may promote cell migration and invasion by directly interacting with FOXP2 in HCC. Wu et al⁴³ have found that there was a remarkably inverse association between miR-124 expression level and TNM stages, and miR-124 could inhibit tumor progression by directly targeting KLF4 in HCC. MiR-128-3p is a novel miRNA that was firstly identified in T-cell acute lymphoblastic leukemia cell lines by an unbiased Plant Homeodomain Finger 6 (PHF6) 3'UTR-microRNA library screen⁴⁴. Recently, miR-128-3p was also reported to participate in the initiation and development of HCC. Huang et al²³ have reported that miR-128-3p expression was downregulated in HCC, and it may inhibit HCC cell proliferation by modulating PIK3R1. In the present study, miR-128-3p expression was also found to be decreased in HCC cells, which was consistent with previous study. DJ-1 was frequently considered as an oncogene, and overexpressed DJ-1 was found in various human cancers, and evidence indicated that DJ-1 may be directly produced and released by the tumor cells. It implied that DJ-1 expression may be a potential biomarker for the diagnosis and prognosis of cancers⁴⁵. Recently, Chen et al⁴⁶ found that DJ-1 expression could be regulated by miR-4639 in Parkinson's Disease (PD); however, whether DJ-1 interacts with miRNAs in tumors remains undetermined. DJ-1 overexpression was also found in HCC in our study, and it can be regulated by miR-128-3p. In the following functional assays, we revealed that miR-128-3p overexpression in liver cancer cells significantly increased sorafenib induced cell apoptosis, and the effect could be abolished by the application of DJ-1, suggesting that miR-128-3p may act as an oncogenic agent in HCC via DJ-1. Phosphatase and tensin homologue deleted on chromosome ten (PTEN), a tumor suppressor, was frequently found to be damaged in human tumors. Evidence showed that knock-down of PTEN could result in Akt activation, suggesting that PTEN may exhibit its tumor suppressive effect via inhibition of Akt signaling pathway^{47,48}. Recently, DJ-1 was reported to

play as a PTEN regulator by directly interacting with it⁴⁹⁻⁵¹. Therefore, we examined whether the PTEN/PI3K/Akt signaling pathway participates in the sensitivity of HCC to sorafenib induced via miR-128-3p/DJ-1 pathway. We showed that the PTEN/Akt signaling pathway was significantly inhibited in the miR-128-3p overexpressed liver cancer cells, and it could be reversed by the application of DJ-1. In addition, we found that the downregulation of sensitivity of liver cancer cells to sorafenib induced by DJ-1 overexpression was reversed by the application of PIC, which further supported that DJ-1 could affect the phosphatase activity of PTEN.

Conclusions

We firstly showed the downregulation of miR-128-3p and upregulation of DJ-1 in HCC cell lines by qRT-PCR; meanwhile, we found that DJ-1 could be targeted by miR-128-3p and there was a negative correlation between them. Furthermore, overexpressed miR-128-3p could sensitize HCC cells to sorafenib-induced apoptosis, and this effect could be abolished by DJ-1 via PTEN/PI3K/Akt signaling pathway.

Acknowledgements

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Authors' declaration of personal interests

(1) Xinlai Guo and Hongbin Wang performed the experiment, they were responsible for data acquisition and analysis. (2) Qinghua Li and Jie Zhong conceived and designed the study. (3) Junekong Yong and Jie Zhong were responsible for statistical analysis. (4) Xinlai Guo and Hongbin Wang drafted the manuscript. (5) Qinghua Li and Jie Zhong revised it accordingly. All authors have read and approved the final manuscript.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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