

Original Article

MiR-365 promotes hepatoma cell apoptosis through regulating DJ-1-PTEN/PI3K/AKT signaling pathway

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Abstract: PI3K/AKT signaling pathway plays an important role in tumor occurrence by reducing cancer cell apoptosis through upregulating Bcl-2 expression. PTEN can negatively regulate PTEN/AKT signaling pathway, while DJ-1 is the negative regulator of PTEN. DJ-1 upregulation is associated with various tumor occurrences. MiR-155 is significantly declined in hepatoma tissue and related to prognosis. Bioinformatics analysis showed the complementary binding site between miR-365 and DJ-1. This study aimed to investigate the role of miR-365 in regulating DJ-1-PTEN/PI3K/AKT signaling pathway and affecting hepatoma cell proliferation and apoptosis. Dual luciferase assay confirmed the targeted relationship between miR-365 and DJ-1. Hepatocellular carcinoma cell lines, HCCLM3, MHCC97-L, and HL-7702 cells were cultured in vitro. Cell proliferation was evaluated by EdU staining. Expressions of MiR-365, DJ-1, PTEN, p-AKT, and Bcl-2 expressions were measured. HCCLM3 cells were divided into five groups, including Scramble NC, miR-365 mimic, si-NC, si-DJ-1, and miR-365 mimic + si-DJ-1 groups. Cell apoptosis was determined by flow cytometry. MiR-365 targeted inhibited DJ-1 expression. MiR-365 and PTEN significantly downregulated, while p-AKT, Bcl-2 and cell proliferation obviously elevated in HCCLM3 and MHCC97-L cells compared with HL-7702 cells. MiR-365 mimic and/or si-DJ-1 transfection markedly reduced DJ-1, p-AKT, and Bcl-2 levels, upregulated PTEN expression, attenuated cell proliferation, and enhanced cell apoptosis. MiR-365 downregulated, while DJ-1 increased in hepatoma cells. MiR-365 overexpression promoted hepatoma cell apoptosis and inhibited proliferation by targeted suppressing DJ-1 expression to elevate PTEN expression and alleviate PI3K/AKT signaling pathway activity.

Keywords: Hepatoma, miR-365, DJ-1, PTEN-PI3K/AKT, apoptosis, proliferation

Introduction

Primary hepatic carcinoma is a common malignant tumor in clinic. It exhibits leading morbidity and mortality among malignancy around the world, which is only second to lung cancer and gastric cancer [1]. There are about half of hepatoma patients in China, which seriously affects quality of life and health [2, 3].

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway is involved in multiple biological processes, such as cell proliferation, survival, and apoptosis. It is closely associated with oncogenesis. B-cell lymphoma 2 (Bcl-2) is an important target gene of PI3K/AKT signaling pathway with anti-apoptotic function. PI3K/AKT signaling pathway plays a crucial role in upregulating Bcl-2 expres-

sion and facilitating tumorigenesis [4, 5]. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a negative regulator of PI3K/AKT signaling pathway, thus plays a tumor suppressor gene role in various tumors [6-8]. DJ-1 is a negative regulator of PTEN that can activate PI3K/AKT signaling pathway through inhibiting PTEN [9, 10]. It was showed that DJ-1 abnormal expression is related to the occurrence of a variety of tumors [11-13]. DJ-1 obviously elevated in hepatoma tissue, indicating that DJ-1 plays a tumor promotion role in hepatoma [14]. MiRNA is a type of endogenous single stranded noncoding RNA at the length of 22-25 nt discovered from eukaryote. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR. MiRNA expression and function in tumorigenesis receive more and more attention [15]. MiR-365 significantly reduced in

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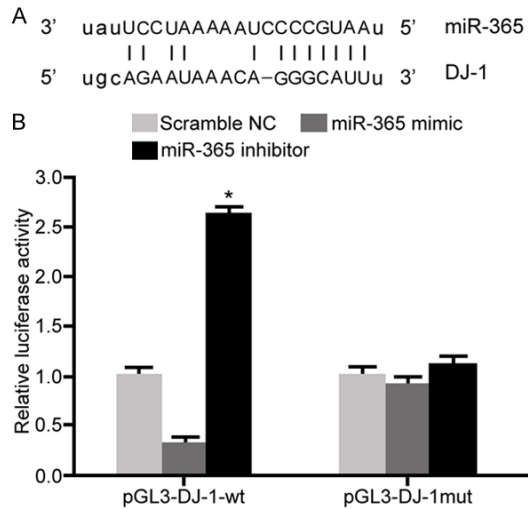


Figure 1. MiR-365 targeted regulated DJ-1 expression. A. The binding site between miR-365 the 3'-UTR of DJ-1 mRNA; B. Dual luciferase assay. * $P < 0.05$, compared with miR-NC.

hepatoma tissue and cells, revealing the potential cancer suppressor gene role of miR-365 in hepatoma [16, 17]. Bioinformatics analysis showed the complementary binding site between miR-365 and DJ-1. This study aimed to investigate the role of miR-365 in affecting hepatoma cell proliferation and apoptosis by regulating DJ-1.

Materials and methods

Main reagents and materials

Human high malignant degree hepatoma cell line HCCLM3, low malignant degree hepatoma cell line MHCC97-L, and normal hepatocyte HL-7702 were purchased from Baili Biotechnology (Shanghai, China). High-glucose DMEM, Opti-MEM, FBS, penicillin, and streptomycin were got from Gibco (USA). EasyPure RNA Kit and real-time PCR reagent TransScript Green One-Step qRT-PCR SuperMix were obtained from TransGen Biotech (Beijing, China). miR-NC, miR-365 mimic, and miR-365 inhibitor were bought from Ribobio (Guangzhou, China). Mouse anti human DJ-1, PTEN, and p-AKT primary antibodies were got from Abcam (USA). Rabbit anti human Bcl-2 and β -actin primary antibodies were obtained from GeneTex (USA). EdU flow cytometry reagent was bought from Sigma (USA). HRP conjugated secondary antibody was derived from Genetimes (Shanghai, China). Annexin-V/PI apoptosis detection kit

was purchased from Keygentec (Jiangsu, China). FuGENE[®] 6 Transfection Reagent, luciferase reporter plasmid pGL3, and Dual-Luciferase[®] Reporter Assay System were obtained from Promega (USA).

Cell culture

HCCLM3, MHCC97-L, and HL-7702 cells were cultured in high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were passaged at 1:4.

Dual-luciferase reporter gene assay

The PCR products containing the full length of DJ-1 gene 3'-UTR or mutant segment were cloned to pGL3. Next, it was transformed to DH5 α competent cells and sequenced to select the plasmid with correct sequence. Then pGL3-DJ-1-wt (or pGL3-DJ-1-mut) was co-transfected to HEK293T cells using FuGENE[®] 6 Transfection Reagent together with miR-365 mimic (or miR-365 inhibitor, or miR-NC). The luciferase activity was detected after cultured for 48 h.

Cell transfection and grouping

HCCLM3 cells were cultured in vitro and divided into five groups, including Scramble NC, miR-365 mimic, si-NC, si-DJ-1, and miR-365 mimic + si-DJ-1 groups, which were all synthesized by Shanghai GenePharma company. Nucleotide fragments and FuGENE[®] 6 Transfection Reagent were added to Opti-MEM and incubated at room temperature for 5 min, respectively. Then they were mixed at room temperature for 20 min and added to the cells cultured in Opti-MEM. After 6 h incubation, the medium was changed back to high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were used for detection after 48 h.

qRT-PCR

Total RNA was extracted using EasyPure RNA Kit and adopted for PCR reaction by TransScript Green One-Step qRT-PCR SuperMix. The reaction system contained 1 μ g RNA template, 0.3 μ M primers, 10 μ L 2 \times TransStart Tip Green qPCR SuperMix, 0.4 μ L RT Enzyme Mix, 0.4 μ L Dye II, and ddH₂O. The reverse transcription condition was 37 $^{\circ}$ C for 15 min and 98 $^{\circ}$ C for 5 min. The PCR reaction was composed of 45 $^{\circ}$ C reverse transcription for 5 min, 94 $^{\circ}$ C pre-denaturation for 30 s, followed by 40 cycles of 94 $^{\circ}$ C

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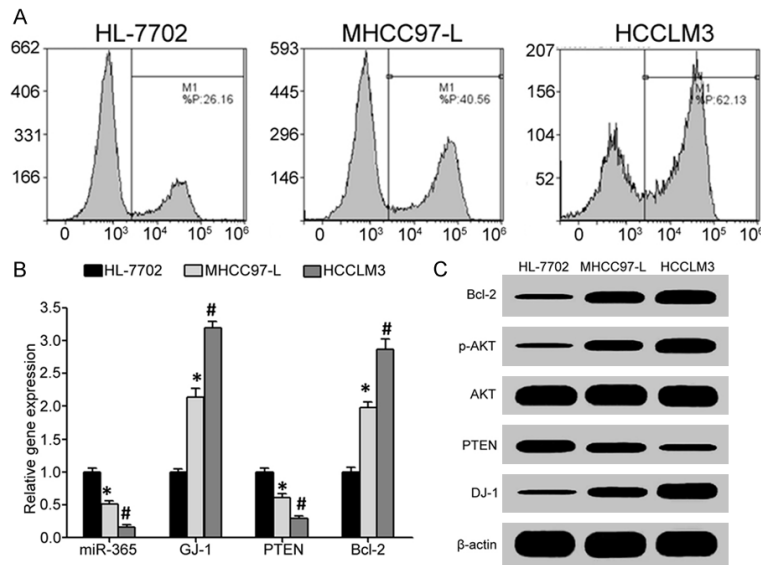


Figure 2. MiR-365 downregulation and DJ-1 overexpression were associated with malignancy enhancement in hepatoma cells. A. Flow cytometry detection of cell proliferation; B. qRT-PCR detection of gene expression; C. Western blot detection of protein expression. * $P < 0.05$, compared with HL-7702 cells; # $P < 0.05$, compared with MHCC97-L cells.

for 5 s and 60°C for 30 s. Real-time PCR was performed on Bio-Rad CFX96/CFX connect to test the relative expression.

Western blot

Total protein was extracted by RIPA from cells. A total of 50 µg protein was separated by 12% SDS-PAGE for 3 h and transferred to membrane. Next, the membrane was blocked and incubated in primary antibody at 4°C overnight (DJ-1, PTEN, p-AKT, Bcl-2, and β-actin at 1:200, 1:200, 1:100, 1:200, and 1:600, respectively). Then the membrane was incubated in secondary antibody (1:5000) for 60 min after washed by PBST for three times. At last, the protein expression was detected by ECL chemiluminescence.

Flow cytometry

The cells were resuspended in 500 µl binding buffer and incubated in 5 µl Annexin V-FITC and 5 µl PI avoid of light for 15 min. Next, the cells were added with 5 µl PI and tested on CytoFLEX flow cytometry to evaluate cell apoptosis.

EdU staining

The cells were added with 15 µM EdU solution at 37°C for 60 min. After incubated for 48 h,

the cells were digested by trypsin and collected. After fixed in 4% paraformaldehyde, the cells were incubated in 1% saponin and resuspended in PBS. At last, the cells were stained by 500 µl 6-FAM Azide at room temperature avoid of light for 30 min and tested on CytoFLEX flow cytometry (Beckman).

Statistical analysis

All data analyses were performed on SPSS 18.0 software. The measurement data were depicted as mean ± standard deviation and compared by one-way ANOVA. $P < 0.05$ was considered as statistical significance.

Results

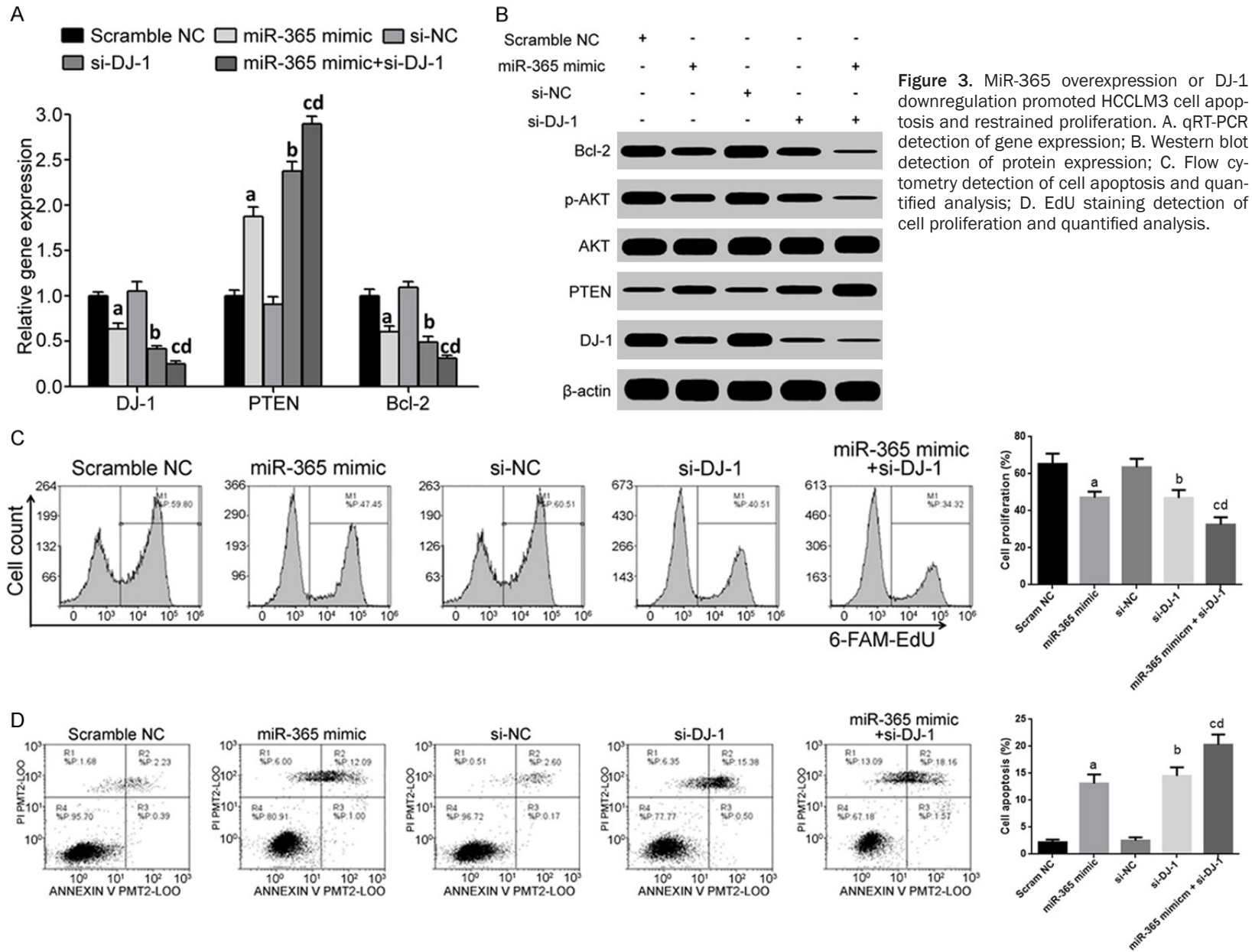
MiR-365 targeted regulated DJ-1 expression

MicroRNA.org online prediction showed the targeted binding site between miR-365 and 3'-UTR of DJ-1 mRNA (Figure 1A). Dual luciferase assay revealed that miR-365 up- or downregulation significantly declined or increased the relative luciferase activity of HEK293 cells transfected by pGL3-DJ-1-wt, while it exhibited no statistical impact on the luciferase activity in HEK293 cells transfected by pGL-DJ-1-mut (Figure 1B), indicating the regulatory relationship between miR-365 and DJ-1 mRNA.

MiR-365 downregulation and DJ-1 overexpression were associated with malignancy enhancement in hepatoma cells

Flow cytometry demonstrated that HCCLM3 cells exhibited the strongest proliferative ability, followed by MHCC97-L and HL-7702 cells (Figure 2A). qRT-PCR showed that miR-365 and PTEN mRNA levels were significantly lower, while DJ-1 and Bcl-2 mRNA expression were obviously higher in hepatoma cells compared with HL-7702 cells (Figure 2B). The difference was more significant in HCCLM3 cells with higher malignancy than that in MHCC97-L cells (Figure 2B). Western blot revealed that DJ-1, p-AKT, and Bcl-2 protein levels were markedly

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higher, while PTEN expression was apparently lower in hepatoma cells compared with normal hepatocytes (**Figure 2C**). It indicated that miR-365 downregulation may play a role in elevating DJ-1, suppressing PTEN, enhancing PI3K/AKT signaling pathway, and upregulating Bcl-2, and strengthened malignant phenotype.

MiR-365 overexpression or DJ-1 downregulation promoted HCCLM3 cell apoptosis and restrained proliferation

After transfection of miR-365 mimic, the expression of miR-365 was increased significantly (more than 50% increase). Meanwhile, miR-365 mimic and/or si-DJ-1 transfection markedly reduced DJ-1, p-AKT, and Bcl-2 levels (**Figure 3A** and **3B**), upregulated PTEN expression (**Figure 3A** and **3B**), attenuated cell proliferation (**Figure 3C**), and enhanced cell apoptosis (**Figure 3D**).

Discussion

PI3K/AKT signaling pathway is involved in multiple biological behaviors, such as embryonic development, organ formation, angiogenesis, cell proliferation, apoptosis, and cell cycle, etc. It plays a promoting role in tumorigenesis, progression, and metastasis of cancer cells [18, 19]. 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 [20]. B-cell lymphoma 2 (Bcl-2) is an important anti-apoptotic factor in mitochondrial dependent apoptosis pathway with the function of antagonizing apoptosis, promoting proliferation, and facilitating survival. It was found that Bcl-2 is one of important target genes of PI3K/AKT signaling pathway. PI3K/AKT pathway activation accelerates various tumorigenesis by upregulating Bcl-2 expression, such as bladder cancer [4] and prostate cancer [5]. PTEN blocks the phosphorylation of PIP2 into PIP3 and the phosphorylation activation of AKT by PIP3, so as to negatively regulate PI3K/AKT signaling pathway [21]. It plays a tumor suppressor gene role a variety of cancers, including gallbladder carcinoma [6], breast cancer [7], and prostate can-

cer [8]. DJ-1 is a negative regulator of PTEN, which can activate PI3K/AKT signaling pathway to suppress cell apoptosis and promote cell survival [9, 10]. Several studies revealed that DJ-1 was significantly upregulated in lung cancer [12], pancreatic cancer [13], and breast cancer [11], and closely related to tumor occurrence, development, metastasis, survival, and prognosis. DJ-1 was found obviously elevated in the tumor tissue of liver cancer patients, suggesting its tumor promotion role in liver cancer [14]. MiR-365 significantly reduced in hepatoma tissue and cells, revealing the potential cancer suppressor gene role of miR-365 in hepatoma [16, 17]. Bioinformatics analysis showed the complementary binding site between miR-365 and DJ-1. This study aimed to investigate the role of miR-365 in affecting hepatoma cell proliferation and apoptosis by regulating DJ-1.

Dual luciferase assay revealed that miR-365 up- or downregulation significantly declined or increased the relative luciferase activity of HEK293 cells transfected by pGL3-DJ-1-wt, while it exhibited no statistical impact on the luciferase activity in HEK293 cells transfected by pGL3-DJ-1-mut, indicating the regulatory relationship between miR-365 and DJ-1 mRNA. It was showed that miR-365 and PTEN mRNA levels were significantly lower, while DJ-1 and Bcl-2 mRNA expression were obviously higher in hepatoma cells compared with HL-7702 cells (**Figure 2B**). The difference was more significant in HCCLM3 cells with higher malignancy than that in MHCC97-L cells. It indicated that miR-365 downregulation and DJ-1 elevation were related to hepatoma tumorigenesis and malignancy. Tao reported that miR-365 expression obviously declined in mouse hepatoma model induced by c-Myc and AKT/Ras gene overexpression [22]. Liu demonstrated that miR-365 level markedly declined in liver cancer tissue compared with adjacent normal control, while its expression was associated with TNM stage and lymphatic metastasis [17]. Liu also found that miR-365 also reduced in liver cancer cell line compared with normal hepatocytes [17]. Chen revealed that miR-365 apparently attenuated in liver cancer tissue compared with normal liver tissue, which was correlated with high TNM stage, low differentiation, and poor prognosis [16]. In this study, miR-365 expression significantly reduced in hepatoma cells, indicating its tumor suppressor role in liver cancer, which was similar with Tao [22], Liu

[17], and Chen [16]. Liu showed that DJ-1 obviously elevated, while PTEN level declined in liver cancer tissue compared with normal control. They exhibited markedly negative correlation. Moreover, DJ-1 expression was closely related to vascular invasion, cell differentiation, and Edmondson pathological grading [14]. Qiu discovered that DJ-1 abnormal increased in liver cancer tissue and correlated to poor overall survival [23]. They were similar with our results. MiR-365 mimic and/or si-DJ-1 transfection markedly reduced DJ-1, p-AKT, and Bcl-2 levels, upregulated PTEN expression, attenuated cell proliferation, and enhanced cell apoptosis. Tao exhibited that miR-365 overexpression significantly restrained liver cancer tumorigenesis and reduced tumor size in mouse hepatoma model induced by c-Myc and AKT/Ras gene overexpression, suggesting its tumor suppressor role in liver cancer [22]. Liu found that miR-365 upregulation inhibited liver cancer cell proliferation, clone formation, migration, invasion, and tumorigenicity through targeted suppressing ADAM10 [17]. Chen demonstrated that miR-365 overexpression markedly inhibited liver cancer cell HepG2 migration and attenuated clone formation [16]. In this study, miR-365 upregulation obviously weakened liver cancer cell malignancy, which was in accordance with Liu [17] and Chen [16]. Liu exhibited that DJ-1 shRNA transfection into HepG2 cells significantly increased PTEN expression, downregulated p-AKT level, declined cell proliferation, adhesion, and invasion, and suppressed tumor growth [24]. Qiu showed that the possibility of liver cancer in DJ-1 knockout mice apparently reduced, whereas in vitro investigation found that DJ-1 overexpression obviously enhanced MHCC97-L cell proliferation [23]. In this study, DJ-1 siRNA alleviated the malignant biological characteristics of liver cancer cells, which was similar with Liu [24] and Qiu [23]. This study revealed the role of miR-365 reduction in DJ-1 upregulation and hepatoma cell malignancy enhancement. MiR-365 overexpression attenuated the inhibitory role of DJ-1 on PTEN to facilitate liver cancer cell apoptosis and suppress proliferation.

Conclusion

MiR-365 downregulated, while DJ-1 increased in hepatoma cells. MiR-365 overexpression promoted hepatoma cell apoptosis and inhibited proliferation by targeted suppressing DJ-1

expression to elevate PTEN expression and alleviate PI3K/AKT signaling pathway activity.

Acknowledgements

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Disclosure of conflict of interest

None.

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