

Original Article

Up-regulation of miR-324 suppresses cell apoptosis by targeting DUSP1 in hepatocellular carcinoma

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Abstract: Backgrounds: Accumulating evidence suggested that microRNAs (miRNAs) are engaged in hepatocellular carcinoma (HCC). This study aimed to reveal the role of miR-324 in HCC and its potential mechanisms. Methods: The tissues from patients undergoing HCC resection were collected. Real time-PCR was used to test the expression of miR-324 and DUSP1 mRNA in tissues and HCC cell lines; western blot was used to detect the expression of DUSP1, AKT, MAP2K1 and p53 in the tissues and cell lines. In vitro experiment, wound healing assay was performed to test the migration ability of SK-HEP-1 cells and transwell assay was used to detect the invasion ability of SK-HEP-1 cells with the inhibitor of miR-324. Cell proliferation ability of HCC was detected by CCK-8 assay and apoptosis level was measured by flow cytometry and TUNEL assay. Luciferase assay was used to confirm whether DUSP1-3'-UTR is the target gene of miR-324. DUSP1 over expression plasmid was transfected to SK-HEP-1 cells to detect the relationship between miR-324 and DUSP1. Results: The expression of miR-324 was dramatically up-regulated in HCC tissues and cells compared with matched normal carcinoma adjacent tissue and liver cells ($P < 0.05$), meanwhile p53, MAP2K1, AKT and DUSP1 proteins showed different changes in HCC tissues. Furthermore, the migration and invasion ability of SK-HEP-1 were decreased after transfected with miR-324 inhibitor. CCK-8 assay, flow cytometry and TUNEL assay showed miR-324 significantly promoted the proliferation and inhibited apoptosis of HCC cells. Luciferase reporter assay identified the 3'-UTR of DUSP1 mRNA contained a complementary sequence for miR-324. DUSP1 could reverse anti-apoptosis role of miR-324. Conclusions: Our study provided a better understanding of miR-324 in HCC developing process. And our results may contribute to the development of miRNA-directed diagnostic and therapeutic against HCC.

Keywords: Hepatocellular carcinoma, MicroRNAs, DUSP1, tumor progression

Introduction

Primary liver cancer was the fifth most frequently diagnosed cancer globally and the second leading cause of cancer death [1, 2], with hepatocellular carcinoma (HCC) now being the third reason of cancer-related mortality worldwide [3]. And surgical resection, radiofrequency ablation and liver transplantation are the effective methods for clinical treatment of patients with liver cancer for the moment. However, the prognosis of liver cancer has not been ideal, and patients who received surgery within 2 years reappeared exceeds 50% and the 5-year survival rate for patients was less than 5% due to the late detection of the tumors and high

rate of recurrence and metastasis [4]. Hence, to further clarify the molecular mechanism of HCC invasion and metastasis is to develop new therapeutic strategies for the treatment of HCC.

MiRNAs are small endogenous, noncoding RNAs that were 21-24 nucleotides in length and directed the posttranscriptional regulation of gene expression by binding to sequences in a 3'-untranslated region (3'-UTR) of the target mRNA, resulting in upregulation or downregulation of the targeted gene [5, 6]. Mounting evidence have revealed that deregulation of miRNAs expression was related to variety of human disease such as cardiovascular disease, metabolic disorders, and particular cancer [7]. Re-

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

Table 1. Primers for qRT-PCR

Name	Sequence
miR-324	F: 5'-GCCCCAGGTGCTGGGGGT R: 5'-GATGCGGGGAGGCATAGTCAG
U6	F: 5'-CTCGCTTCGGCAGCACA R: 5'-AACGCTTCACGAATTTGCGT
DUSP1	F: 5'-GCGAAGAAGCCGAGGAGCCCCG R: 5'-CGGGCTCCTCGGCTTCTTCGC
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGTAT R: 5'-AGCCTTCTCCATGGTGGTGAAGAC

cently, some studies found that miRNAs could play important roles in liver cancer initiation and progression. For example, upregulation of miR-144 led to inhibition of cell proliferation, cell cycle progression, chemoresistance, and other malignant biological behaviors in HCC [8]. Li et al. provided an unequivocal evidence for critical oncogenic roles of the miR-675 in hepatocellular carcinoma and supported the notion that miR-675 may be an alternative bona fide promoting factor of hepatocellular carcinoma [9]. In the meantime, more and more miRNA related to liver cancer were gradually being discovered and studied.

Previous study showed that to identify plasma levels of miR-324 as a potential diagnostic biomarkers for early stage lung cancer, and detection of plasma miR-324 levels may also serve as a prognostic marker for lung cancer patients [10]. MiR-324 could also be used as one of the biomarker in the diagnosis of HCC [11], but the mechanism remains unclear. Dual-specificity phosphatase-1 (DUSP1, also called MKP-1, ERP) was initially identified in cultured murine cells [12]. DUSP1 is one member of dual-specificity phosphatases which were recognized as key players for inactivating different mitogen-activated protein kinase (MAPK) isoforms [13, 14]. DUSP1 played a role in cell proliferation, differentiation and transformation, cycle arrest, and apoptosis mainly by regulation of MAPK signaling [15, 16]. Previous study showed that the miRNA could target to DUSP1, which regulated the development of tumor cells through DUSP1 and its pathway [17]. Hence, we hypothesized that miR-324 suppressed HCC cell apoptosis and induced the cell proliferation and migration through directly regulating DUSP1 in this study.

Materials and methods

Patients and samples

Hepatocellular carcinoma and their corresponding non-tumor tissues were collected at the time of surgical resection from 100 patients with liver cancer from 2014 to 2015 at Guangdong General Hospital. All specimens were confirmed pathologically. Human tissues were immediately frozen in liquid nitrogen and stored at -80°C refrigerator. Informed consent was signed by all patients and the study was approved by the Ethics Committee of Guangdong General Hospital. All of the included patients met the following criteria: pathologically and histologically confirmed HCC, no history of any other malignant tumors, and no neoadjuvant therapy prior to the surgery.

Cell culture and transfection

The following human HCC cell lines were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA): HepG2, SK-HEP-1, Huh-7, and Hep3B. The normal human liver LO2 cell line was also employed as normal control. All the cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco, CA, USA), 1% of 100 U/ml penicillin and 1% of 100 mg/ml streptomycin sulfates. The cells were incubated in humidified incubators with 5% CO₂ at 37°C.

MiR-324 inhibitor and the inhibitor control (Negative control, NC) were synthesized chemically in Suzhou GenePharma Co., Ltd. (Suzhou, China). Human DUSP1 gene was constructed into pcDNA3.1+ vector by Life Technologies (Invitrogen, CA, USA), and the empty vector was served as the negative control. MiR-324 inhibitor and pcDNA3.1+HA-DUSP1 or pcDNA3.1+HA empty vector were transfected after the cells were cultured to 70-80% confluence by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from the cell lines and frozen tissue specimens with TRIzol reagent (Thermo Fisher Scientific). Complementary DNA was generated using a miScript Reverse Transcription Kit (Qiagen NV, Venlo, Netherlands). Primers for miR-324, U6 small nuclear

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

RNA (snRNA) (internal control), DUSP1 and GAPDH were purchased from Suzhou GenePharma. The expression level of miRNA was defined based on the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, using the expression level of the U6 snRNA as a reference gene. Each polymerase chain reaction (PCR) was performed in triplicate. The primers for the examined genes were presented in **Table 1**.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was used for cell proliferation analysis following the manufacturer's instruction. SK-HEP-1 and HepG2 cells with established stable expression after transfected with NC, miR-324 inhibitor, miR-324 inhibitor+pcDNA3.1 empty vector or miR-324 inhibitor+pcDNA3.1-DUSP1 were seeded at a density of 5×10^3 cells per well in 96-well plates and incubated for various periods of time (0 h, 24 h, 48 h, 72 h). The absorbance at 450 nm was measured using a electroluminescence immunosorbent assay reader (Thermo Fisher Scientific, Waltham, MA).

Flow cytometry and TUNEL analysis of cell apoptosis

Cells were collected and washed twice with cold phosphate-buffered saline solution (PBS) to remove floating cells before analysis by the Annexin V-APC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China). Apoptosis was evaluated with a flow cytometry analyzer (BD Biosciences, CA). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL) detection kit (Roche, China) was used to demonstrate cell apoptosis followed the manufacturer's instructions. After DAPI counterstain, the tissue section was examined and photographed with a fluorescence microscope.

Wound-healing assay

Wound-healing assay was performed using SK-HEP-1 cells. Cells were trypsinized and seeded in equal numbers into 6-well culture plates, and allowed to grow until confluent. When serum starvation for 24 hours, an artificial homogenous wound (scratch) was created onto the cell monolayer with a sterile 100 μ L

tip. After scratching, the cells were washed with serum-free medium, complete media was added, and microscopic images (20 \times magnification) of the cells were collected at 0 and 48 hours.

Cell invasion assay

Transwell chamber was used to examine cell invasion capability. SK-HEP-1 cells were transfected with miR-324 inhibitor and pcDNA3.1-DUSP1 or pcDNA3.1 empty vector following to the manufacture's information. Transfected cells were trypsinized and resuspended, 2.0×10^4 cells in 200 μ L RPMI 1640 medium were placed into the upper chambers (8-mm pore size; Corning, MA) after 6 h transfection. The lower chambers were filled with 500 μ L complete medium with 10% FBS. The cells on the upper side of the inserts were softly scraped off after incubation for 12 h at 37°C. Cells that migrated to the lower side of the inserts were fixed with 4% paraformaldehyde and stained with crystal violet (1 μ g/ml), and then the cells from five independent, randomly chosen visual fields were counted under an immunofluorescence microscope ($\times 100$ magnification) for quantification of cells.

Luciferase reporter assay

Luciferase reporter assay was performed according to the manufacturer's instructions. Briefly, cells (3.0×10^4) were seeded in triplicate in 24-well plates overnight. Next, 100 ng of pcDNA3.1-DUSP1-3'-UTR (wild type/mutant) or control-luciferase plasmid plus 1 ng of pcDNA3.1 empty vector renilla plasmid (#E2810; Promega, USA) were transfected into the cells using Lipofectamine® 2000 (Thermo Fisher Scientific). Three independent experiments were performed and the data was presented as the mean \pm standard deviation (SD).

Statistical analyses

Data were presented as the mean value \pm SD and analyzed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by an analysis of one-way ANOVA analysis or two-tailed Student t-test. *P*-value of less than 0.05 was considered to be statistically significant. All experiments were performed at least three independent times.

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

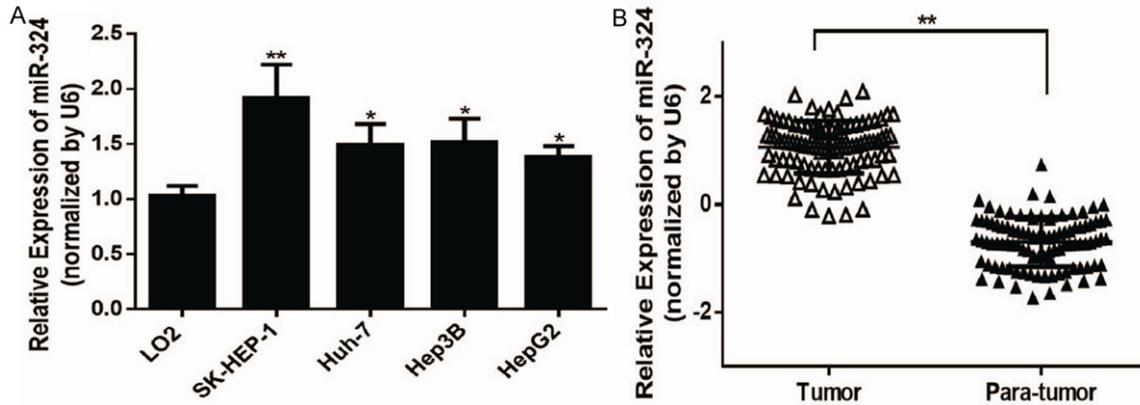


Figure 1. Upregulation of miR-324 in HCC tissues and cell lines. A. The relative miR-324 expression in HCC lines was much higher than that in the normal liver cell LO2 detected by qRT-PCR. B. The expression of miR-324 was increased in 100 HCC tissues than their matched adjacent non-tumor liver tissues. The data is shown as the means \pm SD. * $P < 0.05$; ** $P < 0.001$.

Table 2. Relationship between miR-324 and clinicopathological features of HCC patients

Clinicopathological parameters	Number of cases (%)	Expression of miR-324		P-value
		High (15/100)	Low (85/100)	
Sex				1.000
Male	53	45	8	
Female	47	40	7	
Age (years)				0.2843
≤ 60	48	37	11	
> 60	52	47	5	
Diameter(cm)				1.0000
≤ 5.0	59	50	9	
> 5.0	41	35	6	
Histological differentiation				0.1726
Well	33	28	5	
Moderate	48	41	7	
Poor	19	13	6	
Liver cirrhosis				0.7832
Yes	37	30	7	
No	63	54	9	
Recurrence				0.0062*
Yes	31	21	10	
No	69	64	5	

Note: *Statistically significant ($P < 0.05$).

Results

Expression of miR-324 was frequently upregulated in HCC tissues and cell lines

To demonstrate whether miR-324 was correlated with the progression in HCC cell lines, tissues and matched with adjacent non-tumor

liver tissues collected from 100 patients by qRT-PCR. The results confirmed that the expression of miR-324 was significantly increased in cell lines SK-HEP-1, Huh-7, Hep3B and HepG2 compared with that in normal HCC cell line LO2 (Figure 1A). As shown in Figure 1B, the expression of miR-324 was found increased in HCC tissues with matched adjacent non-tumor liver tissues ($P < 0.05$). However, no statistically significant relationships was found between miR-324 and any of the clinicopathological parameters except for recurrence ($P = 0.0062$) (Table 2).

Effect of miR-324 on the proliferation of HCC cells

First of all, the miR-324 inhibitor in SK-HEP-1 and HepG2 cells which were all proved to have good inhibitory effect showed in Figure 2A ($P < 0.05$).

To investigate the effects of miR-324 on the proliferation of HCC cell lines, CCK-8 assay was performed. The OD=450 nm value in the CCK-8 assay revealed that miR-324 inhibited SK-HEP-1 and HepG2 cell proliferation compared with the miR-324 negative control group (Figure 2B and 2C). Annexin V staining showed that the per-

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

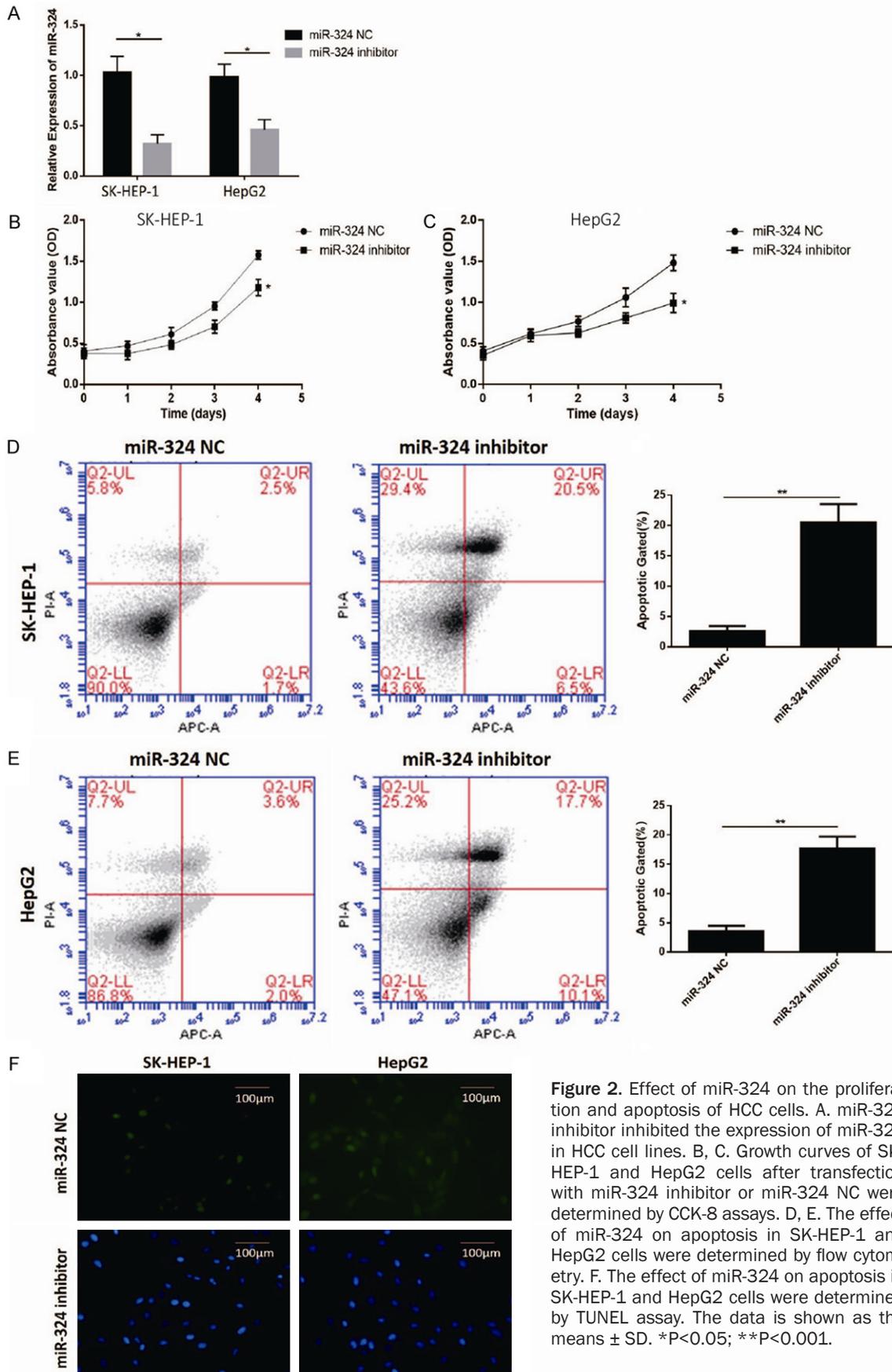


Figure 2. Effect of miR-324 on the proliferation and apoptosis of HCC cells. **A.** miR-324 inhibitor inhibited the expression of miR-324 in HCC cell lines. **B, C.** Growth curves of SK-HEP-1 and HepG2 cells after transfection with miR-324 inhibitor or miR-324 NC were determined by CCK-8 assays. **D, E.** The effect of miR-324 on apoptosis in SK-HEP-1 and HepG2 cells were determined by flow cytometry. **F.** The effect of miR-324 on apoptosis in SK-HEP-1 and HepG2 cells were determined by TUNEL assay. The data is shown as the means \pm SD. * $P < 0.05$; ** $P < 0.001$.

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

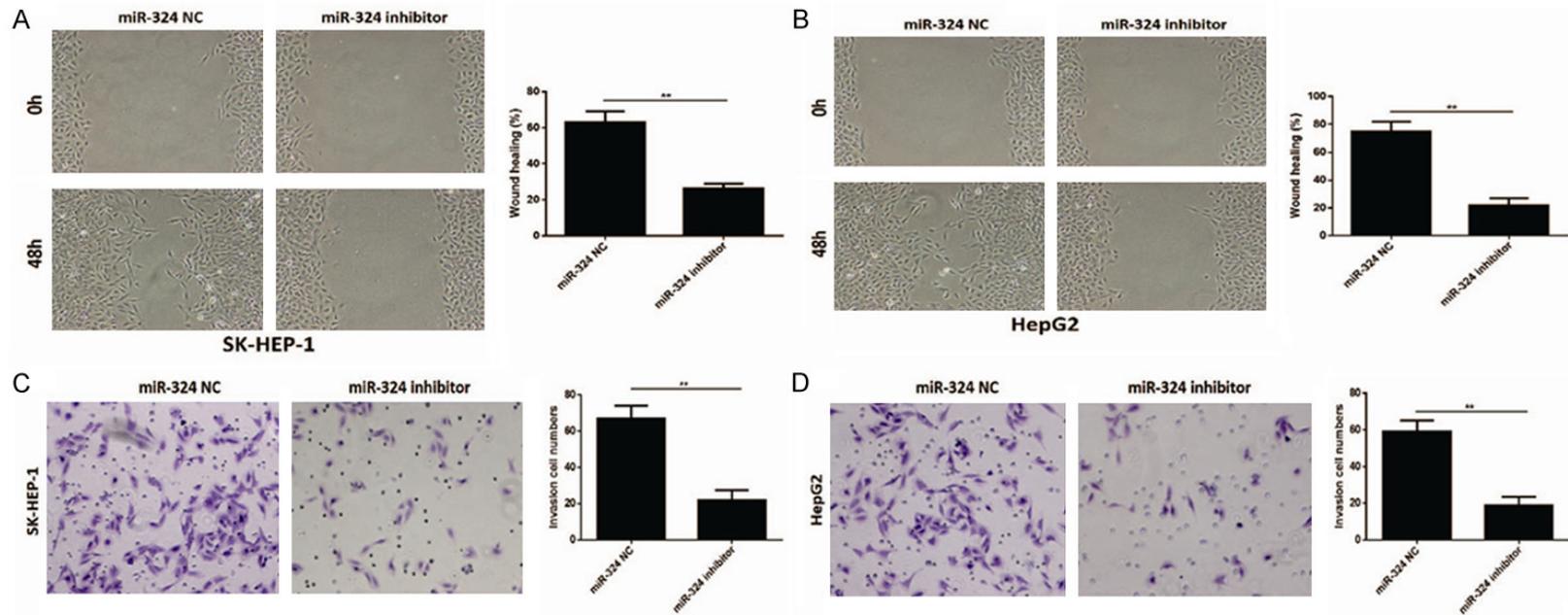


Figure 3. Effect of miR-324 on migration and invasion of HCC. A, B. The wound healing rate in SK-HEP-1 and HepG2 cells transfected with miR-324 inhibitor were significantly decreased compared with negative control. C, D. The number of invaded cells in the SK-HEP-1 and HepG2 cells transfected with miR-324 were significantly decreased compared with the negative control. The data is shown as the means \pm SD. *P<0.05; **P<0.001.

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

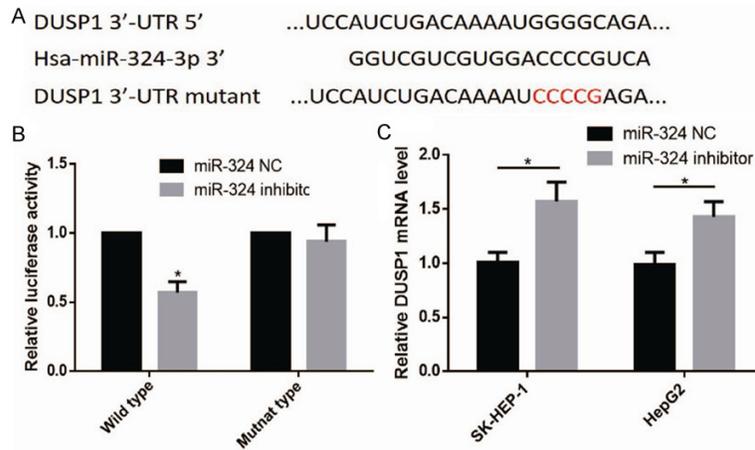


Figure 4. Function of miR-324 in HCC cells partially attributed to DUSP1. A. Prediction consequential pairing of target region between miR-324 and DUSP1 by bioinformatic method and construction of reporter gene plasmids and point mutation plasmids. B. miR-324 significantly suppressed the luciferase activity that carried wild-type DUSP1 but not the mutant DUSP1. C. Downregulated of miR-324 significantly increased the mRNA of DUSP1 in SK-HEP-1 and HepG2 cells compared with negative control group. The data is shown as the means \pm SD. * $P < 0.05$; ** $P < 0.001$.

centage of apoptotic cells following miR-324 inhibitor was drastically increased relative to that in control groups (Figure 2D and 2E). TUNEL staining revealed that cell apoptosis of SK-HEP-1 and HepG2 cells transfected with miR-324 inhibitor were significantly increased compared with miR-324 NC group (Figure 2F).

Effect of miR-324 on migration and invasion of HCC

To further investigate the biological significance of miR-324 in HCC, wound healing assay and transwell assay were performed to detect the effect of miR-324 on migration and invasion of HCC cells. Wound-healing assay showed that the mobility of SK-HEP-1 and HepG2 cells transfected with miR-324 inhibitor evidently decelerated in rate in within 48 hours compared with negative controls showed in Figure 3A and 3B ($P < 0.05$). Transwell assay showed that SK-HEP-1 and HepG2 cells transfected with miR-324 inhibitor resulted in a significant decrease in invasive potential ($P < 0.05$) (Figure 3C and 3D). Taken together, the expression of miR-324 suppressed the migration and invasion of HCC cells.

Function of miR-324 in HCC cells partially attributed to DUSP1

To investigate the underlying mechanism by which miR-324 regulates progression of HCC,

bioinformatic algorithms, including TargetScan and Star base, to predict the potential target of miR-324. Bioinformatics analysis of miRNA recognition sequences on miR-324 revealed the presence of DUSP1 binding sites. Furthermore, we constructed the luciferase reports carrying the wild type and mutant type of DUSP1 3'-UTR (Figure 4A). As shown in Figure 4B, luciferase assays indicated that the wild type of 3'-UTR caused a significant reduction in luciferase activity, but mutant of 3'-UTR of DUSP1 showed no change in the luciferase activity compared with the control group. However, real-time PCR results showed that cells transfected with miR-324 inhibitor promoted the expression of DUSP1 mRNA (Figure 4C). Taken together, the results strongly suggested that miR-324 could significantly suppress the expression of DUSP1 through targeting the 3'-UTR.

MiR-324 regulates apoptosis by affecting the expression of DUSP1

As showed in Figure 5A, we detected parts of protein level which were related to apoptotic pathway HCC cells. When the expression of miR-324 was downregulated by inhibitor, the protein level of DUSP1 and p53 were increased. Otherwise, the protein level of MAP2K1 and AKT were decreased. Furthermore, MAP2K1 and p53 showed different level changes compared with β -actin consistent with apoptosis change in SK-HEP-1 cells transfected with miR-324 NC, miR-324 inhibitor, pcDNA3.1-NC or pcDNA3.1-DUSP1 (Figure 5B), and the cells also showed different apoptosis after transfection in SK-HEP-1 and HepG2 cell lines (Figure 5C).

Discussion

In the present study, we showed that the expression of miR-324 was significantly increased in tissues of HCC patients compared with its carcinoma adjacent tissues. Over expression of miR-324 promoted migration, proliferation and

MiR-324 suppresses hepatocellular carcinoma cell apoptosis

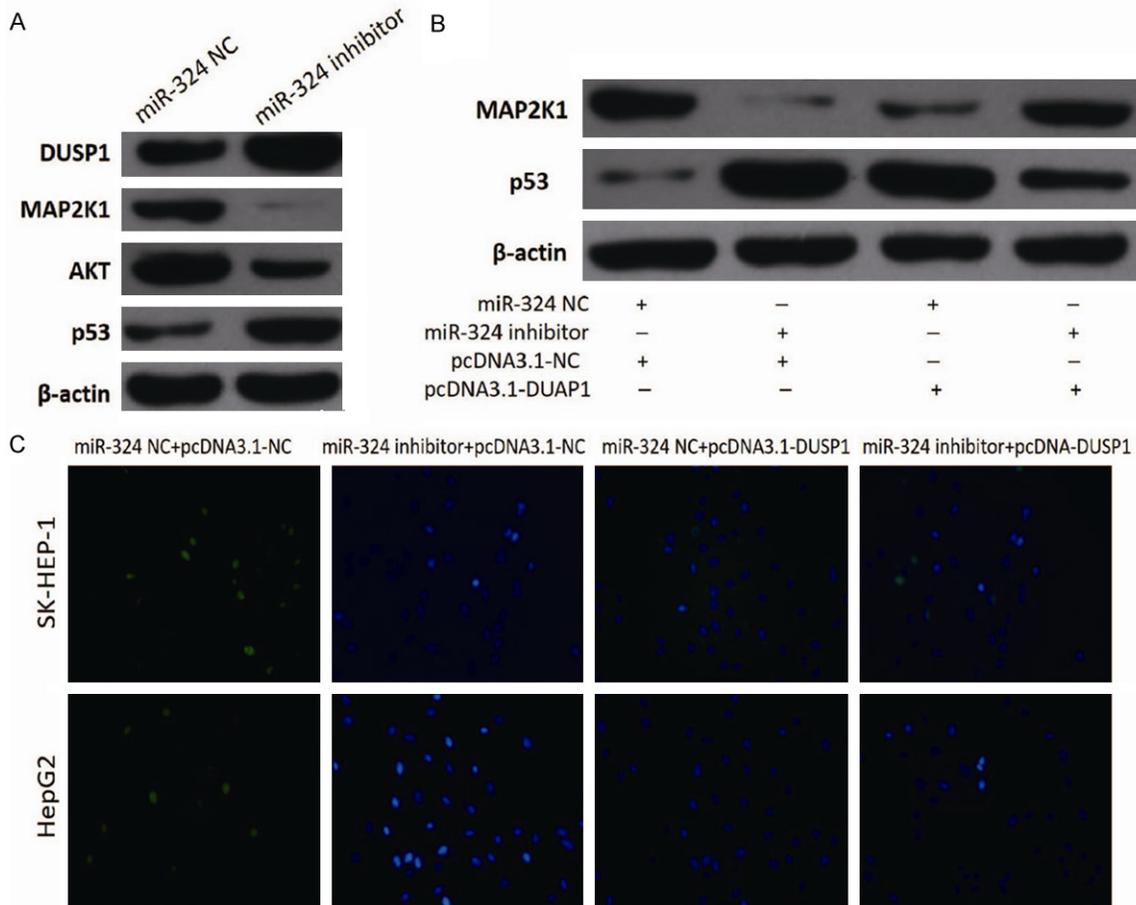


Figure 5. MiR-324 regulates apoptosis by affecting the expression of DUSP1. A. DUSP1, MAP2K1, AKT and p53 in HCC cells affected by miR-324 inhibitor. B. The protein level of MAP2K1 and p53 protein in HCC cells transfected with miR-324 inhibitor and/or pcDNA3.1-DUSP1. C. Different apoptosis after transfection in SK-HEP-1 and HepG2 cell lines detected by TUNEL assay. The data is shown as the means \pm SD. * $P < 0.05$; ** $P < 0.001$.

inhibited apoptosis of HCC cells. Furthermore, we demonstrated DUSP1 as a target gene of miR-324 in SK-HEP-1 cells. Restoration of DUSP1 partly reverse miR-324 suppressed cell apoptosis in HCC cells.

Lots of evidence indicated that miRNAs are important regulators in various cellular processes and are recently extensively investigated relating to cancer initiation, progression, diagnosis and treatment [6, 8, 18]. Dysregulation of miRNAs were often found in HCC, and it was confirmed that most of them played an important role in the progression and development of HCC [5]. For example, miR-384 was significantly downregulated in HCC cells and tissues, and this resulted in HCC cell proliferation was suppressed [19]. Previous study also showed that miR-1299 inhibited cell prolifera-

tion and might be a target for HCC therapy [20]. MiR-324 was detected dysregulation expression and as a potential prognostic markers in breast cancer [21]. Next, researchers showed that that miR-324 could be highly promising as diagnostic biomarkers for early stage LSCC with miR-1285 [10]. In the present study, we found that the expression of miR-324 was increased in different degrees both in tissues and cells of HCC. The proliferation, apoptosis and metastasis of HCC cells were changed when we inhibited or over-expressed miR-324. So we think that the up-regulation of miR-324 was one of the reasons for the development of liver cancer.

The expression of DUSP1 has been found to correlate with cancer development and progression [22]. Up-regulation of DUSP1 in the

early phase of cancer helped the tumor to evade JNK1-induced apoptosis, whereas down-regulation of DUSP1 allowed for proliferation and increased tumor mass in the more advanced stages of tumorigenesis [23]. DUSP1 was one member of dual-specificity phosphatases which was recognized as key players for inactivating different mitogen-activated protein kinase (MAPK) isoforms [24]. Increased expression of DUSP1 was also observed in other tumors, including colon, bladder, gastric, breast, and lung cancer, which consequently inhibited tumor cell apoptosis [16, 25, 26]. But on the other hand, previous studies found that the expression of DUSP1 is different in different tumors. For example, the expression of DUSP1 in hepatocellular carcinoma (HCC) decreased slightly compared with normal liver tissues as an ERK inhibitor, played a role in inhibiting the hepatocarcinogenesis [27]. The changes in the proliferation, apoptosis and metastasis of hepatocellular carcinoma cells in the present study may be due to the involvement of DUSP1 proteins.

The findings of the present study were in line with those of previous evidence, which showed the increased expression of miR-324 in cancer cell lines and human tissues. The expression of miR-324 enhanced HCC cell proliferation and migration suggesting that increased expression of miR-324 corrected with the malignant potential of HCC. Furthermore, we identified DUSP1 as a target of miR-324 in HCC cells. And DUSP1 was involved in p53 activation via the p38 MAPK/HSP27 pathway. Downregulating the DUSP1 may interrupt the positive regulatory loop between DUSP1 and p53, and then promote HCC development and progression. Therefore, miR-324 was directly regulated the expression of DUSP1 protein, which was involved in the MAPK pathway, and ultimately affect the growth and metastasis of liver cancer cells through affecting the expression of protein included in the pathway.

In conclusion, our study indicated that miR-324 was up-regulated in HCC patients and cells. In addition, miR-324 promoted proliferation and inhibited cell apoptosis of HCC by targeting DUSP1. Our study provided a better understanding of miR-324 function in liver cancer development, which may also be benefit for the development of miRNA-directed diagnostic and therapeutic against HCC.

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Disclosure of conflict of interest

None.

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