

Original Article

miR-24-3p suppresses cellular proliferation in hepatocellular carcinoma

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Abstract: Deregulation of microRNAs is a frequent event in the tumorigenesis and tumor progression. The aim of this study was to investigate the potential role of miR-24-3p expression in hepatocellular Carcinoma (HCC). The expression level of miR-24-3p was detected in HCC tissues and HCC cell lines compared with their corresponding non-cancerous tissues, normal tissues and normal liver cell line by quantitative real-time PCR. In vitro function assays including cell cycle arrest, apoptosis, migration and invasion were further explored. We found that miR-24-3p was reduced in HCC tissues and HCC cell lines compared with their corresponding non-cancerous tissues, normal tissues and normal liver cell line ($P < 0.001$). In addition, functional assays showed that over-expression of miR-24-3p induced HCC cell cycle arrest and apoptosis. Mechanistically, we found that miR-24-3p could regulate CCND1 and Caspase3 expression in hepatocellular carcinoma cell lines, while has no effects on BCL2. In addition, we found that knockdown Sp1 by siRNA has no effects on miR-24-3p expression. Our findings suggested that miR-24-3p acted as a tumor suppressor in hepatocellular carcinoma. It inhibited hepatocellular carcinoma cells proliferation through regulate CCND1 and Caspase3 expression. These findings of this study contribute to the current understanding of miR-24-3p in hepatocellular carcinoma and miR-24-3p may have a potential role in prognosis and therapy.

Keywords: miR-24-3p, hepatocellular carcinoma, tumor suppressor

Introduction

Liver cancer is the fifth most prevalent form of cancer and the third leading cause of cancer-related death worldwide, immediately following lung and colon cancer [1]. Among them, hepatocellular carcinoma (HCC) representing over 90% of all cases of primary liver cancer, is the most common form of adult liver cancer [2]. Despite recent progress in detection and treatment for early HCC, the 5 years survival rate of HCC patients is still very poor [3, 4]. And even many oncogenes and tumor suppressors have been reported, the molecular mechanisms underlying HCC progression are still known little [2, 5, 6].

Recently, important advances have been achieved with the introduction of microRNAs (miRNAs). miRNAs are a very large gene family, which encode small noncoding RNAs of approximately 22 nucleotides [7]. They can be classified as oncogenes or as tumor suppressors

at a variety of levels depending on the specific miR, the target base pair interactions and the co-factors that recognize miRs [6-10]. Growing evidence have suggested that miRNAs have important roles in the regulation of diverse biological processes, and their deregulation or dysfunction participates in various processes of cancer development, including proliferation, apoptosis, metabolism, cellular differentiation and prediction of mammalian microRNA targets [11-14]. MiR-24-3p, a master regulator from the gene cluster of miR-23a-27a-24-2, is found to be deregulated in a variety of cancers and acts as a tumor oncogene or suppressor in specific cell type [15-20]. Study has shown that miR-24 could stimulate myeloid cell proliferation as an oncogene by the suppression of mitogen-activated protein kinase (MAPK) phosphatase-7 [21]. miR-24 has also been implicated to regulate apoptosis by targeting fas-associated factor 1 (FAF1) in prostate cancer cells or through negative effect on Caspase-9 and apoptotic peptidase activating factor 1 [22, 23]. It is also

noteworthy that miR-24 level was associated with the presence of intrahepatic metastasis and liver cirrhosis in human hepatocellular carcinoma [24]. Recently, Mishra demonstrated that miR-24 could function as a tumor suppressor independent of p53 by targeting and repressing dihydrofolate reductase (DHFR) in CRC cell lines [20]. However, little knowledge is known about the potential role and mechanism of miR-24-3P in HCC.

In the current study, we validated the differential expression of miR-24-3p in HCC tissues and hepatocellular carcinoma cell lines compared with their corresponding non-cancerous tissues, normal tissues and normal liver cell line, and the potential roles of miR-24-3p in cell cycle arrest and apoptosis of HCC were detected. Furthermore, we investigated the potential mechanism. miR-24-3P might be a tumor suppressor and preserves as a potential therapeutic target in HCC. To the best of our knowledge, this is the first study to investigate the expression, potential roles and mechanism of miR-24-3P in HCC.

Materials and methods

HCC tissues collection

Twenty paired tissue specimens of HCC and matched adjacent tissues were obtained from Department of General Surgery in First affiliated hospital of Guangxi Medical University. The “normal tissue” was obtained from 10 patients with hepatic hemangioma. The tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use. All patients wrote the informed consent, and the study was approved by the Ethics Committee of First affiliated hospital of Guangxi Medical University.

Cell lines and culture conditions

Human hepatocellular carcinoma cell lines, including 7721, HepG2, Huh-7 and 7403 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The normal cell 7702 was preserved in our central laboratory. All cell lines were routinely cultured in the Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO₂.

microRNA and siRNA

All of the miRNA mimics and inhibitors were purchased from Thermo Scientific Dharmacon. The HepG2 cells were transfected at a density of 2×10⁴ cells per well in a 24-well culture plates with either 50 nM of hsa-miR-24-3p or negative-control mimics, or with either 100 nM of hsa-miR-24-3p or negative-control inhibitors by using the Dharma FECT 4 transfection reagent (Thermo Scientific Dharmacon) according to the manufacturer's instructions. Cells were incubated for 48 h with the microRNA mimics or inhibitors prior to RNA purification for gene expression analysis, and were incubated for 72 h for protein expression analysis. To knockdown human SP1, small interfering RNA targeting was performed using Lipofectamine 2000 (Invitrogen) transfection reagent. After 48 h, the protein expression level was measured.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using the PrimeScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Shiga, Japan). Real-time PCR was performed using SYBR Premix Ex Taq™ II kit (TaKaRa) according to the manufacturer's protocol on an ABI 7500 QPCR system (Stratagene, La Jolla, CA, USA). All of the reactions were run in triplicate. The delta-Ct method for relative quantification of gene expression was used to determine miRNAs expression levels. Forward primers for miR-24-3p and u6 sn-RNA were 5'-AAGCTGCCAGT-TGAAGAACTGTA-3' and 5'-CTCGCTTCGGCAGC-ACA-3', and Universal reverse Primer 5'-AAC-GCTTCACGA ATTTGCGT-3', respectively. All primers above were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Western blot

Western blot was performed according to standard protocols using Immobilon-P PVDF membranes (Millipore). For immunoblotting, membranes were incubated with the primary antibody (0.5 g/mL) for 2 h, followed by 1 h incubation with HRP-conjugated secondary antibody (0.2 lg/mL). The primary antibodies and appropriate secondary antibodies were from Abcam (Abcam, CA, USA). Finally, the blots

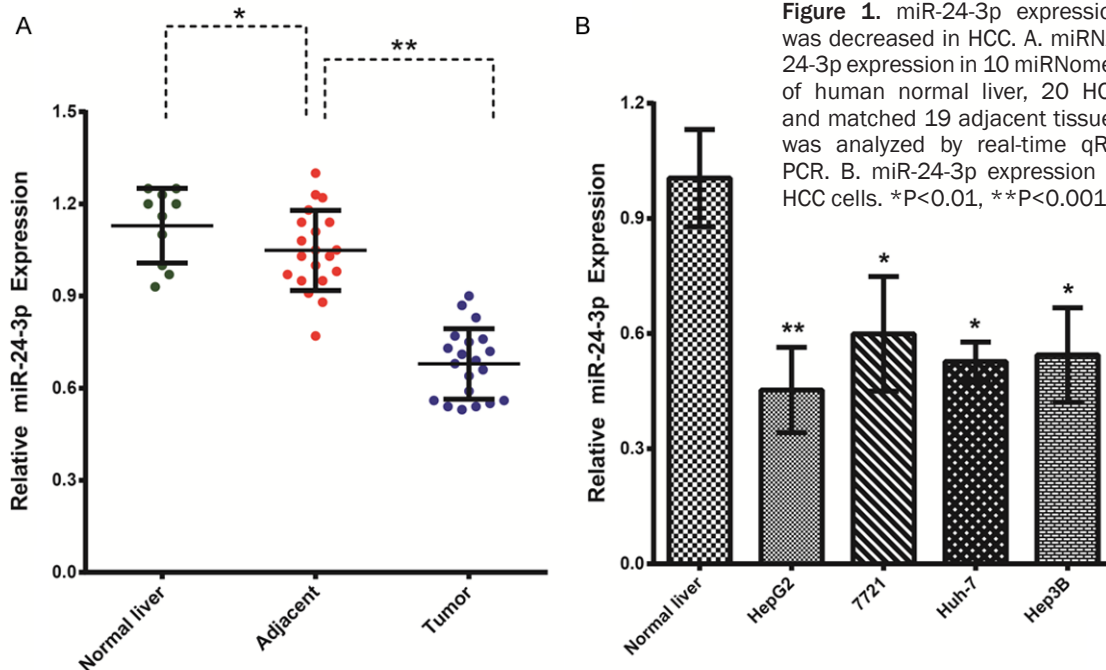


Figure 1. miR-24-3p expression was decreased in HCC. A. miRNA-24-3p expression in 10 miRNomes of human normal liver, 20 HCC and matched 19 adjacent tissues was analyzed by real-time qRT-PCR. B. miR-24-3p expression in HCC cells. *P<0.01, **P<0.001.

were washed, and the signals were visualized using the ECL plus Kit (Amersham, Buckinghamshire, UK).

Measurement of cell cycle

Cells (1×10^6) were fixed in 70% ethanol at -20°C overnight. After being washed, the cells were incubated with 0.25 mg/mL RNase A at 37°C for 30 min. Then, 5 μL of propidium iodide (PI, KeyGen, Nanjing, China) was added to the cell suspension and further incubated at room temperature for 30 min in the dark. The mixture was analyzed for cell cycle by using the FACS Calibur Flow Cytometer (BD, USA).

Measurement of apoptosis

HepG2 cells were plated in 6-well plates at a density of 2×10^6 cells/well and grown for 24 h. For the first sample, the enumeration of apoptotic cells was performed using Annexin V-FITC and PI (BioVision, USA). The cells were gently vortexed and resuspended in binding buffer at a concentration of $3 \times 10^6/\text{mL}$, and 5 μL of Annexin V-FITC and 10 μL of PI was added to 100 μL of the cell suspension. The samples were mixed for 15 min in the dark at room temperature, and 400 μL of PBS was then added to the solution. A FACScan (Becton Dickinson, San Jose, CA, USA) was used to

count cells (1×10^3) at an excitation wave length of 490 nm. Cell Quest software was used for data collection and processing. With the second sample, cells were resuspended in a buffer containing 2 $\mu\text{g}/\text{mL}$ Hoechst 33342 and PI at 28°C for 30 min and washed three times (5 min each) with PBS (pH 7.2) before detecting apoptosis by fluorescence microscopy.

In vitro cell wound healing assay and invasion assay

Confluent cells were wounded using a 200- μL pipette tip in six well culture plates and incubated in DMEM with 10% of fetal bovine serum. The wound width was photographed at 0 h and 24 h under a phase-contrast microscope. The migration distances were measured and quantified. The computer-assisted image software was used to quantify cell migration relative to the grid line border. Invasion assays were performed with the invasion assay kit (BD Bioscience, CA, USA), according to the manufacturer's instructions. Briefly, 1×10^5 cells were resuspended in serum-free medium and plated in the upper chamber. Medium with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation at 37°C , cells on the lower surface of the membrane were stained, photographed, and counted under a microscope.

Statistical analysis

All experiments were carried out at least 3 times with triplicate samples. The differences between groups were analyzed by one-way ANOVA when there were more than two groups. The significance of the data was determined using Student's t test, with error bars representing the mean $\pm$ SD. In all cases, differences were considered to be statistically significant at $P < 0.05$. All analyses were performed with SPSS 17.0 (Chicago, IL, USA).

Results

The expression of miR-24-3p is down-regulated in hepatocellular carcinoma tissues and cell lines

We first verified the expression level of miR-24-3p in twenty paired of HCC and adjacent tissues, and 10 normal tissues by RT-PCR. As shown in **Figure 1A**, we found that tumor tissues showed aberrant down regulation of miR-24-3P compared with adjacent non-tumor tissues and normal tissues ($P < 0.01$, $P < 0.001$). We also detected miR-24-3p expression in four hepatocellular carcinoma cell lines and one normal liver cell line 7702 by real-time PCR. As shown in **Figure 1B**, we found that miR-24-3p has a much lower expression in four hepatocellular carcinoma cell lines than that of immortalized normal liver cell line 7702 ($P < 0.01$). This specific expression mode indicates that miR-24-3p might play important roles in hepatocellular carcinoma progression and also highlighted the expression profile of miR-24-3p in hepatocellular tumor types.

Overexpression of miR-24-3p inhibits cell proliferation in hepatocellular carcinoma

Upon the above results, we detected whether miR-24-3p could change the capacity of hepatocellular carcinoma cells in cell cycle arrest, apoptosis, migration and invasion. We transfected HepG2 cells with miR-24-3p mimics, inhibitor and control, respectively. As expected, transfection of miR-24-3p mimics in HepG2 cells resulted an increase in miR-24-3p expression compared with mimic-NC cells ($P < 0.001$) (**Figure 2A**). And miR-24-3p inhibitor transfection group down-regulate its expression comparing the control group ($P < 0.01$) (**Figure 2B**).

Then, we analyzed cell cycle distribution in miR-24-3p mimics transfected HepG2 cells by flow cytometry. In comparison with mimic-NC, miR-24-3p overexpression group showed cell cycle arrest in G2/M phase 48 h after transfection (**Figure 2C**). Overexpression of miR-24-3p could induce the apoptosis of hepatocellular carcinoma cells compared with the NC and sh-NC group (**Figure 2C**). Furthermore, we found that overexpression of miR-24-3p could inhibit the migration and invasion of HepG2 cells. While, miR-24-3p inhibitor got the reverse results (**Figure 2D** and **2E**). Taken together, our results indicated that miR-24-3p could control cell proliferation by inducing cell cycle arrest, apoptosis, and inhibiting cell migration and invasion in HCC.

miR-24-3P regulates CCND1 and Caspase3 expression while has no influence on BCL2 expression

Next, we sought to investigate the molecular mechanism responsible for the anti-tumor effects of miR-24-3P in HCC. As miRNAs are known to suppress hundreds of miRNA targets, resulting in global changes in the cellular phenotype of cells [20, 21]. CCND1 is a member of the highly conserved cyclin family which is characterized by abundant expression throughout the cell cycle. CCND1 functions as regulator of CDK kinases and forms a complex with CDK4 or CDK6 promoting G1/S cell cycle transition. Here, we detected a down-regulated expression of CCND1 in miR-24-3P mimics group and up-regulated expression in inhibitor group. Both Caspase3 and bcl2 were apoptosis related protein, and we found a higher expression of Caspase3 in miR-24-3P mimics group and a lower expression in inhibitor group compared to the nature control. While, has no influence on bcl2 expression by western blot (**Figure 3A**). SP1 is an important gene participates in regulating cell cycle, apoptosis, proliferation and invasion [22-24]. Here, we also detected the influence of SP1 on miR-24-3P expression by RT-PCR. And as shown in **Figure 3B**, we found that knockdown SP1 by siRNA has no influence on miR-24-3P. Taken together, our results suggest that miR-24-3P could regulates CCND1 expression, while has no influence on bcl2 expression. And SP1 has no influence on miR-24-3P expression.

miR-24-3p in hepatocellular carcinoma

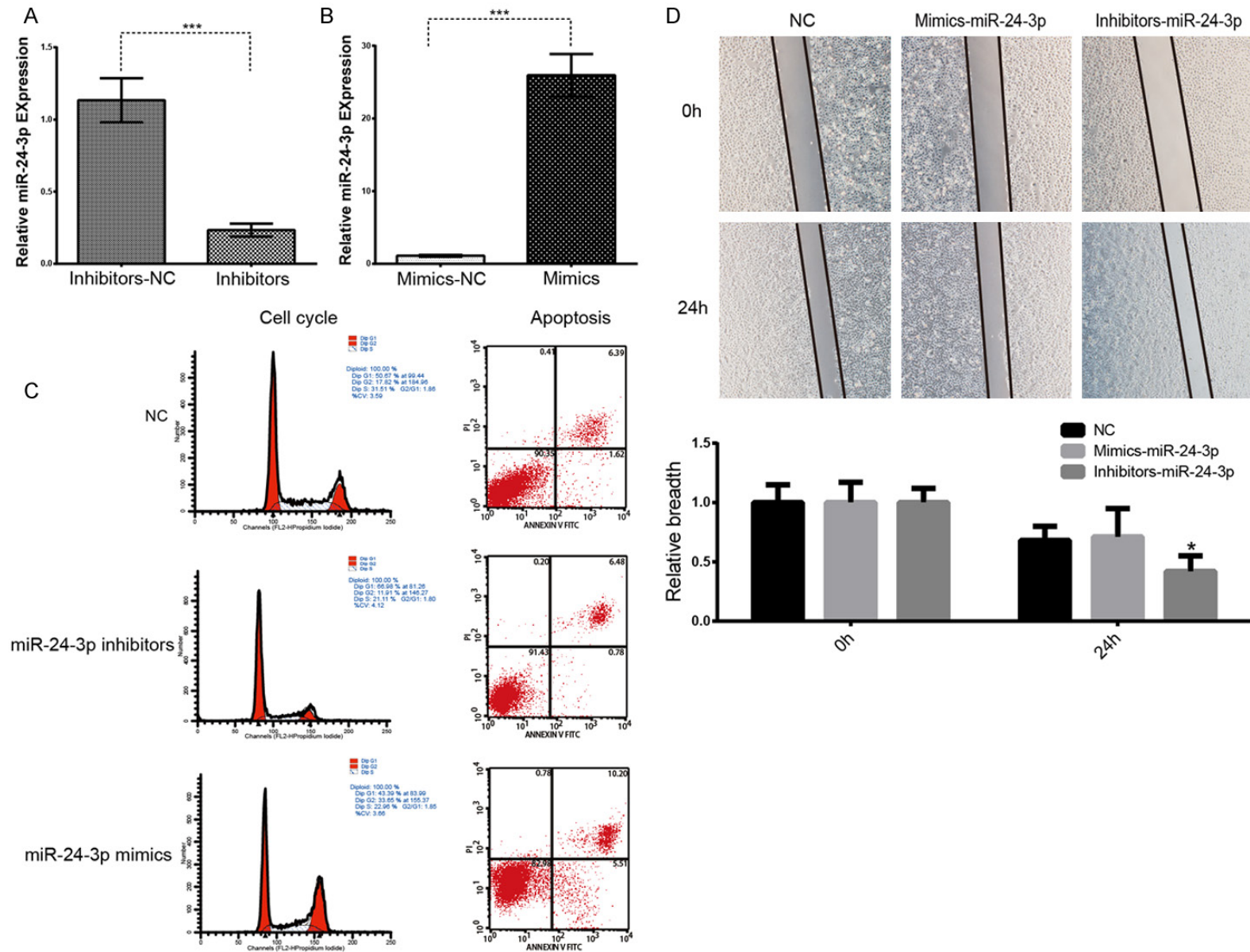
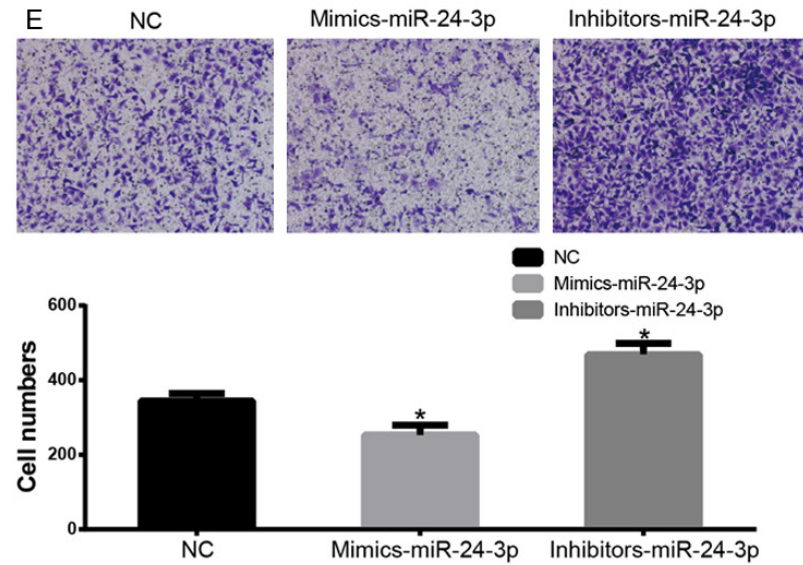


Figure 2. Overexpression of miR-24-3p in hepatocellular carcinoma induces cell cycle arrest and apoptosis. A, B. Transfection of miR-24-3p mimics to HepG2 cells increase the expression of miR-24-3p and inhibitor down regulate the expression of miR-22-3P by real-time qRT-PCR. C. HepG2 cells transfected with miR-24-3P mimics for 48 h were harvested and analyzed by FACS, and the cell cycle distributions and apoptosis were calculated compared with the NC and sh-NC. D, E. HepG2 cells transfected with miR-24-3p mimics and inhibitor for 48 h were harvested, and the migration and invasion assay were conducted.



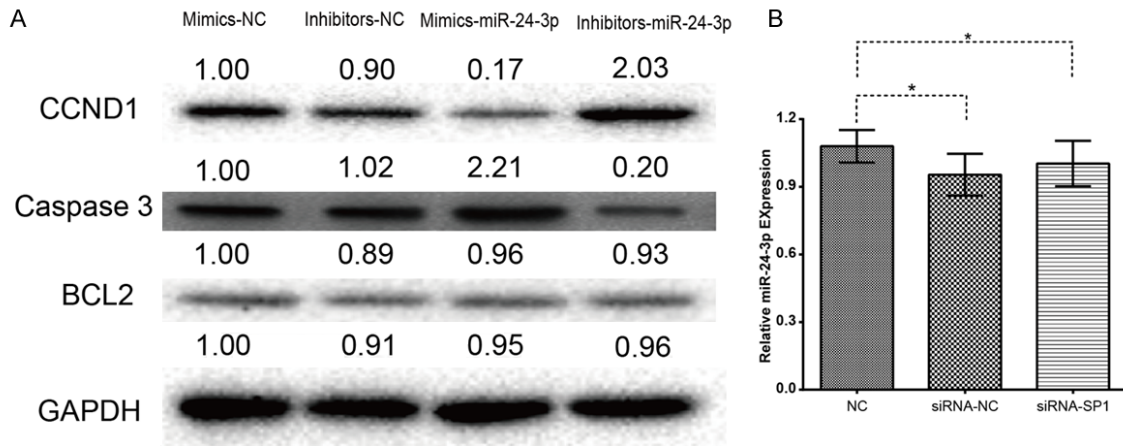


Figure 3. miR-24-3p regulates CCND1 and Caspase3 expression, while has no influence on bcl2 expression; SP1 has no influence on miR-24-3p expression. A. Transfection of miR-24-3P mimics and inhibitor to HepG2 cells, miR-24-3P mimics and inhibitor regulate CCND1 and Caspase3 expression, while has no influence on Bcl2 by western blot. B. Knockdown SP1 expression by siRNA has no influence on miR-24-3P expression (* $P < 0.01$, ** $P < 0.001$).

Discussion

Mounting studies have indicated that miRNAs can play regulatory roles in gene expression associated with cancer development and could act as an effective biomarker for the tumor diagnosis, prognosis or even therapy [25, 26]. The aberrant expression of miR-24 is a frequent event in various kinds of cancers [15-20], suggesting that miR-24 play an important role in the tumorigenesis and tumor progression. The miR-24-3p is encoded in two distinct clusters. One is the miR-24-1 cluster which encompasses miR-24-3p, miR-23b and miR-27b on chromosome 9, and the other encompasses miR-23a, miR-27a and miR-24-2 on chromosome 19. It is noticeable that the functional disparities of miR-24 in different types of cancer may result from its diverse target genes or distinction among tissue types and cellular circumstances. miR-24-3p is thought to regulate cell cycle progression by suppression of E2F2 and c-Myc [27]. Studies showed that miR-24 could directly target p27(Kip1) and p16(Ink4a) in primary keratinocyte and different cancer-derived cell lines to promoting cell proliferation [28]. However, Mishra et al. and others demonstrated that miR-24 expression has a down regulated expression in human CRC which was associated with lymph node metastasis and advanced diseases, suggesting that miR-24 could function as a tumor suppressor [29]. Till now, we have little knowledge about the roles and underling mechanism of miR-24-3p in HCC. And

here, we analyzed the expression of miR-24-3p in HCC tissues and cell lines. We found that miR-24-3p was downregulated in HCC tissues and cell lines compared with corresponding controls. To explore the potential roles and mechanism of miR-24-3p in HCC, we performed in vitro functional studies. And we found that over-expression of miR-24-3P could induce cell cycle arrest and apoptosis, and also inhibit cell migration and invasion. And miR-24-3P could regulate the expression of CCND1, Caspase3 expression. while has no influence on bcl2 expression in HCC cells. And SP1 has no influence on miR-24-3P expression. While, it's a pity that we didn't further detected the potential target gene of miR-24-3P in HCC.

As the existed systemic therapy for HCC has been quite ineffective, so discovering more about the potential therapy for HCC patients is urgent [3, 30]. Recently, re-expression of miRNAs is considered to have substantial clinical potential in cancer therapy [31, 32]. It is obviously suggested that re-expression of miR-24-3p might have considerable potential for clinical treatment of HCC patients, especially for those with poor miR-24-3p expression, such as gastric cancer, glioma and HCC [13, 33, 34]. And according to the known knowledge, interesting future work may be carried out to investigate more about the mechanism.

In this study, we confirmed that miR-24-3P has a decreased expression in human HCC tissues

and cell lines compared with adjacent tumor tissues, normal liver tissues and normal liver cell line, respectively. In vitro studies, overexpression of miR-24-3P could induce cells cell cycle arrest and apoptosis, and also inhibit cell migration and invasion. Furthermore, we discovered that miR-24-3P could regulate CCND1 and Caspase3 expression, while has no influence on bcl2 expression in HCC cells. And we also found that SP1 has no influence on miR-24-3P expression.

Conclusions

In summary, the present study provides evidence to support that miR-24-3P has a low expression in HCC cell lines and HCC tissues. Overexpression of miR-24-3P in HCC cell lines could induce cell cycle arrest and apoptosis, and also inhibit cell migration and invasion. Furthermore, we demonstrated that miR-24-3P could regulates CCND1 and Caspase3 expression, while has no influence on bcl2 expression in HCC cells. And knockdown the expression of SP1 also could not influence miR-24-3P expression. These findings imply that miR-24-3P might be a tumor suppressor and suitable candidate for anticancer therapy in HCC.

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Disclosure of conflict of interest

None.

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