Int J Clin Exp Pathol 2016;9(5):5437-5444
www.ijcep.com /ISSN:1936-2625/IJCEP0024414

Original Article

miR-22-3p suppresses cell proliferation by regulating SP1 in hepatocellular carcinoma

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Received January 19, 2016; Accepted March 27, 2016; Epub May 1, 2016; Published May 15, 2016

Abstract: Objective: Accumulating evidence has shown that microRNAs are involved in multiple processes in cancer development and progression. miR-22-3p was a widely studied regulator which has important roles in various kinds of cancers. While, the roles and specific mechanism of miR-22-3p in HCC development are not well understood. The aim of this study was to verify miR-22-3p expression level and its roles in proliferation, cell cycle and apoptosis inHCC cell lines. To investigate the potential mechanism of how miR-22-3p works in HCC progression. Methods: RT-PCR was used to detect the expression of miR-22-3p in one normal andfour HCC cell lines. Mimics or inhibitors were used for transfecting miR-22-3p. Small interfering RNA was applied to knockdown SP1 gene expression. Cell proliferation was conducted by MTT. Cell cycle and apoptosis assays were detectedusing flowcytometry. Western blot was used to detect the proteins expression level. Results: miR-22-3p expression was much lower than normal control ($P<0.01$). Transfection of miR-22-3p by mimics could significantly inhibits the cellar proliferation, increases the G0/G1 phase cells and induces apoptosis in HCC cell lines. miR-22-3p negatively regulated SP1, CCND1 and bcl2 expressionin HCC cell lines. Knockdown SP1 by siRNA inhibited the expression of CCND1 and bcl2, which is consistent with the miR-22-3p mimics group. Conclusion: miR-22-3p can act as a tumor suppressor in HCC. It may inhibit HCC cell proliferation through up-regulating SP1 and its downstream CCDN1 and bcl2 expression. These findings will contribute to the current understanding of miR-22-3p in HCC.

Keywords: miR-22-3p, SP1, hepatocellular carcinoma

Introduction

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

miRNAs are a very large gene family, which encode small noncoding RNAs of approximately 22 nucleotides [7]. They can be classified as oncogenes or tumor suppressors at a variety of levels depending on the specific miR, the target base pair interactions and the co-factors that recognize miRs [5, 7-10]. 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 predictionof mammalian microRNA targets [11-14]. miR-22-3p is a 22-ntnoncoding RNA. Its gene is located in 17p13.3, which was originally identified in HeLa cells as a tumor-suppressing miRNA [15-17]. Subsequently, miR-22-3p was ubiquitously expressed in a variety of tissues including liver, breast, lung, skin, prostate etc. [14, 18]. Recently, several targets of miR-22-3p

were reported, including tumors uppress or PTEN, CD147, Max gene, SP1, p21, NET1 and oncogene c-myc, etc. [10, 14, 19]. Though, other researchers have studied miR-22-3p in HCC before, the specific mechanism underlying miR-22-3p in HCC development remains unclear.

In the current study, we validated the differential expression of miR-22-3p in four HCC cell lines (HepG2, Huh7, 7721 and Hep3B) and one normal liver cell line, and the function in cells proliferation, cell cycle and apoptosisof HCC. Furthermore, we investigated the potential mechanism of how miR-22-3p regulates SP1, CCND1 and bcl2 in HCC. miR-22-3p 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 specific mechanism of how miR-22-3p regulates SP1 expression in HCC cells.

Materials and methods

HCC tissues collection

The Ethics Committee of First Affiliated Hospital of Guangxi Medical University approved the study, and all patients signed a paper version of informed consent. Twenty HCC paired tissue specimens and matched adjacent tissues were obtained from General Surgery Department of the First Affiliated Hospital of Guangxi Medical University. The “normal tissue” was obtained from 10 patients with hepatic hemangioma. The tissues were obtained during surgery and immediately stored in liquid nitrogen before use.

Cell lines and culture conditions

Human HCC cell lines, including 7721, HepG2, Huh-7 and 7403 were obtained fromthe Type Culture Collection of the Chinese Academy ofSciences (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% CO_2.

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 2×10^4 cells per well in a 24-well culture plates witheither 50 nM of hsa-miR-22-3p or negative-control mimics, or with either 100 nM of hsa-miR-22-3p or negative-control inhibitors by 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 by 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 PmiRcute miRNAcDNA kit (TIANGEN BIOTECH, BEIJING, CO., LTD). Real-time PCR was performed using miRcute miRNAQpcr Detection kit (TIANGEN BIOTECH, BEIJING, CO., LTD) according to the manufacturer’s protocol on an ABI7500qPCR system (Life Technologies, USA). All of the reactions were runin triplicate. The delta-Ct method for relative quantification of gene expression was used to determine miRNAs expression levels. Forward andreverse primers for miR-22-3p and u6 sn-RNA were 50-AAGCTGCCAGTTGAAGAACTGTA-30 and Universal Primer (TIANGEN BIOTECH, BEIJING, CO., LTD), and 50-CTCGCTTCGGCAGCACA-30 and 50-AACGCTTCACGAATTTGCGT-30, respectively. All primers above were synthesized by TIANGEN BIOTECH, BEIJING, CO., LTD.

Western blot

Western blot was performed according to standard protocols using Immobilon-P 0.22 μm PVDF membranes (Millipore). For immunoblotting, membranes were incubated with theprimary antibody (0.5 g/mL) for 16 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 were washed, and the signals were visualized using the ECL plus Kit (Amersham, Buckinghamshire, UK).

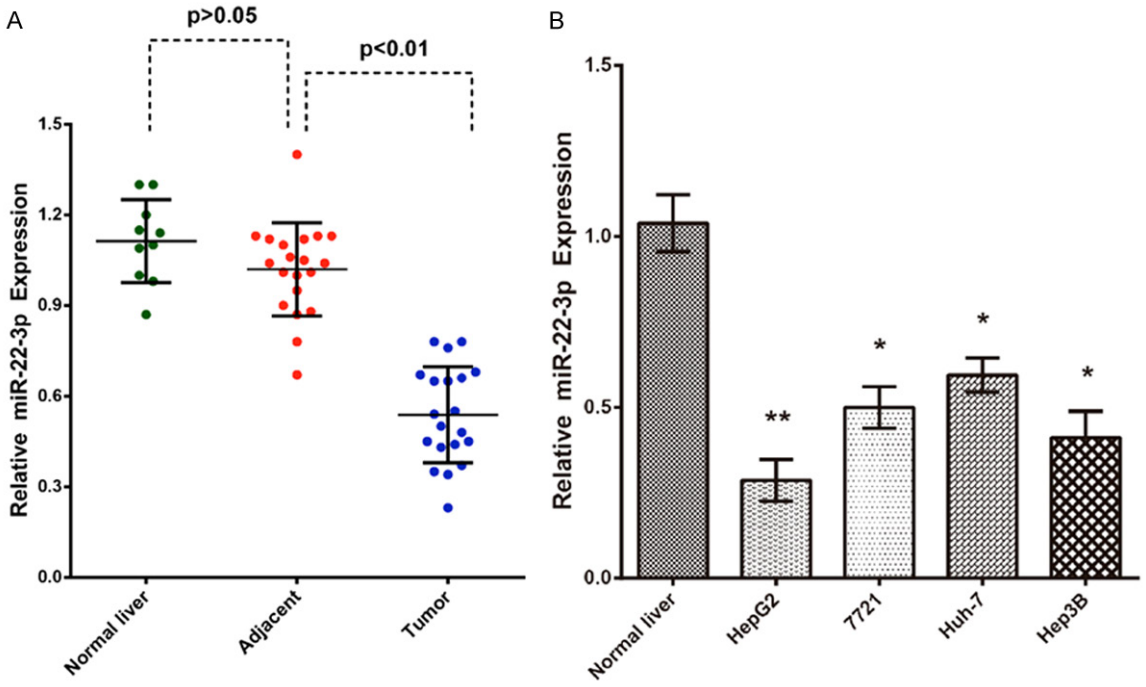

Figure 1. miR-22-3p expression was decreased in HCC. A: miRNA-22-3p expression in 10 miRNomes of human normal liver, 20 HCC and matched 19 adjacent tissues was analyzed by real-time qRT-PCR. B: miR-22-3p expression in HCC cells. *P<0.01, **P<0.001.

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 FACSC alibur 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 AnnexinV-FITC and PI (BioVision, USA). The cells were gently vortexed and resuspended in binding buffer at a concentration of 3×10^6/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 μg/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.

Statistical analysis

All experiments were carried out at least 3 times with triplicate samples. Statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS, Chicago, IL, USA). The significance of the data was determined using Student's t test, with error bars representing the mean ± SD. Statistical difference was considered significant if *P* values were less than 0.05.

Results

The expression of miR-22-3p is down-regulated in HCC tissues and cell lines

We first verified the expression level of miR-22-3p in twenty paired of HCC, adjacent tissues

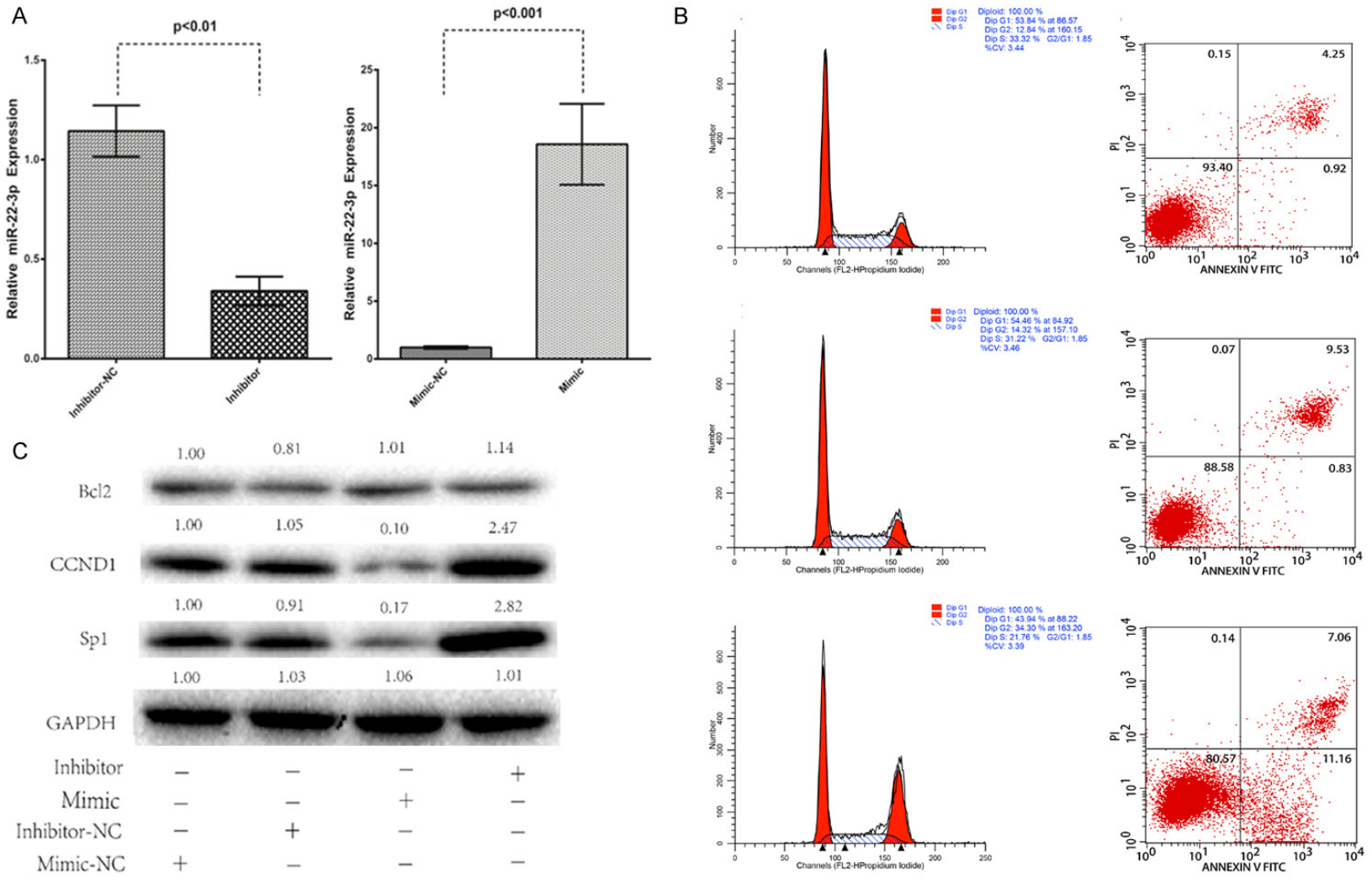

Figure 2. Overexpression of miR-22-3p in hepatocellular carcinoma induces cell cycle arrest and apoptosis, and miR-22-3p negatively regulates SP1 and results in altered expression of CCND1 and bcl2. A: Transfection of miR-22-3P mimics to HepG2 cells increase the expression of miR-22-3P and inhibitor down regulate the expression of miR-22-3P by real-time qRT-PCR. B: HepG2 cells transfected with miR-22-3P mimics compared with the NC and sh-NC for 48 h were harvested and analyzed by FACS, and the cell cycle distributions and apoptosis were calculated. C: miR-22-3P mimics and inhibitor regulate Sp1, CCND1 and Bcl2 expression, which were tested western blot.

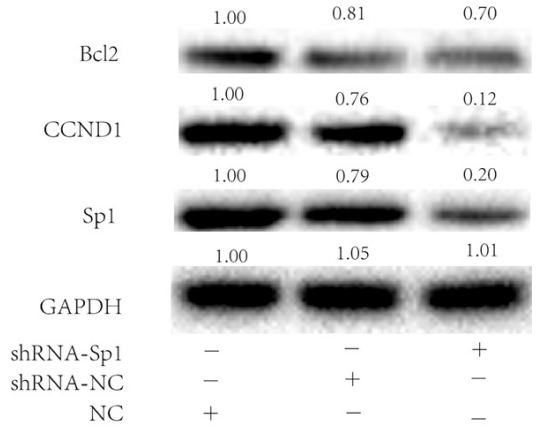

Figure 3. Knockdown of Sp1 reduces CCND1 and bcl2 expression. Sp1, CCND1 and Bcl2 protein expression in HepG2 cells was analyzed by western blot after transfection with Sp1 siRNA or NC duplex for 48 h.

and normal tissue by RT-PCR. As shown in **Figure 1A**, we found that tumor tissues showed aberrant down regulation of miR-22-3p compared with adjacent non-tumor tissues and normal tissues (P<0.01). We also detected miR-22-3p expression four HCC cell lines and one normalliver cell line 7702 by real-time PCR. Asshown in **Figure 1B**, we found that miR-22-3p has a much lower expressionin four HCC cell lines than that of immortalized normal liver cell line 7702 (P<0.01). This specific expression mode indicates that miR-22-3p might play important roles in HCC progression and also highlighted the expression profile of miR-22-3p in hepatocellular tumor types.

Overexpression of miR-22-3p in HCC cells induces cell cycle arrest and apoptosis

Upon the above results, we detected whether miR-22-3p could change the capacity of hepatocellular carcinoma cells in cell proliferation, cell cycle and apoptosis. We transfected HepG2 cells with miR-22-3p mimics, inhibitorand control, respectively. As expected, transfection of miR-22-3p mimics in HepG2 cells resulted an increase in miR-22-3p expression compared with negative control (NC)-transfected cells (P<0.001) (**Figure 2A**). And miR-22-3p inhibitor transfection group down-regulate its expression comparing the control group (P<0.01) (**Figure 2A**). Then, we analyzed cell cycle distribution in miR-22-3p treated HepG2 cells by using flow cytometry. In comparison with NC and sh-NC group, miR-22-3p overexpression group showed cell cycle arrestin G2/M phase 48 h after transfection, characterized by the presence of nearly of 43.94% cells in the G1 phase of the cell cycle, the presence of about 21.76% of cells in the S phase, and the presence of nearly 34.30% of cells in the G2/M phase (**Figure 2B**). Furthermore, we found that overexpression of miR-22-3p could induce the apoptosis of hepatocellular carcinoma cells compared with the NC and sh-NC group. (**Figure 2B**). Taken together, our results indicate that miR-22-3p works as a tumor suppressor and contributes to inhibit proliferation and induce cell cycle arrest and apoptosis.

miR-22-3p negatively regulates SP1 and results in altered expression of CCND1 and bcl2

Next, we sought to investigate the molecular mechanism responsible for the anti-tumor effects of miR-22-3p in HCC. As miRNAs are known to suppress hundreds of miRNA targets, resulting in global changes in the cellular phenotype of cells [20, 21]. SP1 was an important gene participates in regulating cell cycle, apoptosis, proliferation and invasion [22-24]. Though, the previous studies have shown that SP1 was a target gene of miR-22-3p in gastric cancer [14]. To further investigate whether miR-22-3p could regulate SP1 expression in hepatocellular carcinoma cells, we conducted western blot assay in different groups. As shown in **Figure 2C**, SP1 was significantly down-regulated in the miR-22-3p overexpression group compared with negative control, and also showed an up-regulated expression in the inhibitor group compared with control. Furthermore, we also detected a down-regulated expression of cell cycle related protein CCND1 and apoptosis related protein bcl2 in miR-22-3p mimics group and up-regulated expression in inhibitor group (**Figure 2C**). Taken together, our results suggest that miR-22-3p negatively regulates SP1, CCND1 and bcl2 expression. miR-22-3p might through up-regulating SP1 and cell cycle related protein CCND1 and bcl2 to inhibit cell proliferation and induce apoptosis.

Knockdown of SP1 reduces CCND1 and bcl2 expression

In order to confirm whether the effects of miR-22-3p in HCC cell lines were through regulate

cell cycle related protein CCND1 and apoptosis related protein bcl2, we knockdowned the expression of SP1 by siRNA. The expression was inhibited at approximately 80% of protein expression level after knockdown of the SP1 gene in HepG2 cells (**Figure 3**). Consistently, knockdown of SP1 markedly reduced cell cycle related proteins CCND1 and apoptosis related protein bcl2 expression (**Figure 3**). These results suggested that SP1 might though regulating CCND1 and bcl2 expression to regulate cell proliferation, cell cycle arrest and apoptosis in HCC cells and both of them could be regulated by miR-22-3p and SP1 axis.

Discussion

In this study, we confirmed that miR-22-3p has adecreased expression in human HCC cell lines compared with normal liver cell line, which is consistent with the previous reports. The expression of miR-22-3P correlated with HCC cells proliferation, cell cycle and apoptosis ability. Furthermore, we discovered that miR-22-3p could negatively regulate SP1 expression in HCC cells, and resulted in a typical downregulation of CCND1 and bcl2 expression.

Previous reports suggested that miR-22-3P has controversial expression in cancer development, such as the up-regulated expression in prostate cancer, and the down-regulated expression in gastric cancer, breast cancer, multiple myeloma and cholangiocarcinoma, exhibiting various abilities in tumor cells proliferation, cellcycle and apoptosis [9, 10, 13, 14, 19]. As the existed systemic therapy for HCC has been quite ineffective, discovering more about the potential therapy for HCC patients is urgent [4, 25]. Recently, re-expression of miRNAs is considered to have substantial clinical potential in cancer therapy [20, 21]. So it is obviously suggested that re-expression of miR-22-3p may have considerable potential for clinical treatment of HCC patients, especially for those with poor miR-22-3p expression, such as gastric cancer, breast cancer, multiple myeloma and HCC [14, 19, 26]. Human HDAC4, an important epigenetic modifier, is identified to be a direct target of miR-22-3p in HCC [26]. A current report also revealed that miR-22-3p can repress Max expression and Myc-Max complex regulate cellcycle progression [27]. These results, taken together with SP1 we studied here, are consistent with current opinions that a single miRNA can target multiple mRNAs, to post-transcriptionally regulate gene expression [28, 29]. Till now, we know much less about the specific mechanism of how miR-22-3p influence tumor progression in HCC. And according to the known knowledge, interesting future work may be carried out to investigate more about the mechanism. HCC is the fifth most frequently diagnosed cancerand the third leading cause of cancer related death worldwide [1]. Despite the improvement of operation technique, radio-chemotherapy regimens, and perioperative attendance, its five-year survival rate remains less than 20% [1, 30, 31]. Discovering more about the effective molecules will probably be a key to monitoring cancer progression or development of HCC, and give information on the need for therapy. miRNAs have been demonstrated to have close relationship with HCC [21, 32, 33]. In this study, we demonstrated that miR-22-3p expression is decreased in human HCC cell lines compared with normal liver cellline, which is consistent with the previous reports. Transfection of miR-22-3p mimics in HepG2 cells significantly inhibited cells proliferation and apoptosis, and down regulated SP1 and its downstream CCND1 and bcl2 expression. Taken together, our results suggested that miR-22-3p acted asa tumor suppressor played an essential role in cells proliferation and apoptosis. The anti-tumor effects of miR-22-3p may through negatively regulate SP1 and downstream CCND1 and bcl2 expression.

In summary, the present study provides evidence to support that miR-22-3P has a low expression in HCC cell lines. Overexpression of miR-22-3P in HCC cell lines could inhibit cells proliferation and induce apoptosis. Furthermore, we demonstrated that miR-22-3P negatively regulates SP1 and downstream cell cycle related protein CCDN1 and apoptosis related protein bcl2 expression. These findings imply that miR-22-3P might be a suitable candidate for anticancer therapy in HCC.

Acknowledgements

This study was supported by Major Projects Of National Science and Technology (no. 2012ZX10002010001009), Guangxi Scientific Research and Technological Development Projects (no. 14124003-4) and Key Laboratory of Early

Prevention and Treatment for Regional High Frequency Tumor, Ministry of Education (GKE-2015-ZZ06).

Disclosure of conflict of interest

None.

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