

## Original Article

# miR-218 modulate hepatocellular carcinoma cell proliferation through PTEN/AKT/PI3K pathway and HoxA10

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**Abstract:** Purpose: To investigate the regulatory mechanism of miR-218 in human hepatocellular carcinoma (HCC). Methods: qPCR was used to compare the expression levels miR-218 among six hepatocellular carcinoma cell lines and normal liver tissues. After transfecting MHCC97L cells with either miR-218 mimics or miR-218 inhibitor, western blotting was used to examine the expressing patterns of cyclinD1, p21, and PTEN/AKT/PI3K signaling pathway-related proteins. MTT and colony forming assay was used to assess the capability of cell proliferation. Bioinformatic method was applied to predict the binding of miR-218 on HoxA10, and western blotting was used to examine the modulatory effect of miR-218 AND HoxA10 on PTEN/AKT/PI3K pathway in HCC. Results: The expression levels of miR-218 were frequently lower in HCC cell lines than in normal liver tissues. Over-expression of miR-218 in HCC cells significantly decreased cell proliferation whereas inhibiting miR-218 promoted cancer cell proliferation. Western blotting analysis demonstrated that tumorigenesis related protein cyclin D1 and p21, as well as PTEN/AKT/PI3K signaling pathways were actively modulated by miR-218 in HCC cells. The expression of endogenous HoxA10 was also down-regulated by miR-218 over-expression, and silencing HoxA10 directly activated PTEN in HCC cells. Conclusion: Modulation of miR-218 actively affected HCC cancer cell development. The regulatory mechanism of miR-218 in HCC cells was acting through PTEN/AKT/PI3K pathway and possibly associated with HoxA10.

**Keywords:** miR-218, HoxA10, hepatocellular carcinoma, PTEN

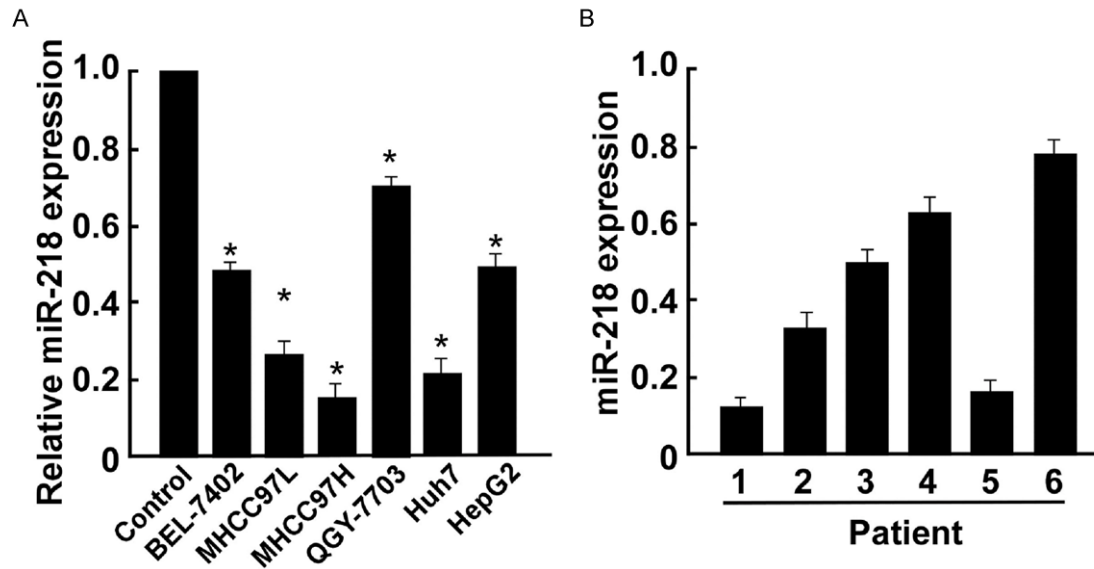
## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and it comprises more than 90% of all form of liver cancers in human [1]. The major treatment plan for patients with HCC includes hepatic resection and orthotopic liver transplantation. However, the long-term prognosis is unsatisfactory [2, 3], and the majority of the patients suffer from metastases or tumor recurrence [4, 5]. Thus, it is very important to develop new therapeutic strategies, and identify novel metastasis-associated signaling pathways or genes to suppress tumor recurrence and metastases in patients with HCC.

MicroRNA (miRNAs) are a group of 19- to 25-nucleotide-long RNAs that bind to the 3'-untranslated regions (3'-UTR) of targeted mRNAs to induce gene or protein degradation

[6]. MicroRNAs have been found to play important roles in embryogenesis, organ maturation, cell differentiation and death [7-9]. Particularly in hepatocellular carcinoma, many of the miRNAs are associated with human cancer, acting as either oncogenes or tumor-suppressing genes depending on the downstream targets [10, 11].

In the present study, we investigated whether microRNA 218 (miR-218), one of the tumor suppressor miRNAs, was involved in the regulation of HCC. We first compare the expression level of miR-218 between HCC and normal live cell lines, as well as between carcinoma tissues and adjacent tissues in HCC patients. We then use functional essays to stimulate and suppress the expression of miR-218 in HCC cell lines to examine its effect on cancer cell proliferation. Finally, we used bioinformatics and biochemistry to investigate the possible signaling



**Figure 1.** Expression of miR-218 in HCC and normal liver tissues. A. The expression levels of miR-218 in six HCC cell lines, including BEL-7402, MHC97L, MHC97H, QGY-7703, Huh7 and HepG2 were assessed with quantitative RT-PCR, and normalized to the expression level in THLE-2 cells. B. The expression levels of miR-218 in carcinoma tissues were examined by quantitative RT-PCR in six HCC patients and compared with the expression level in adjacent tissues. (\*,  $P < 0.05$ ).

pathways that might associate with the regulation of miR-218 on HCC cells.

## Materials and methods

### Materials

We purchased HCC cell lines, BEL-7402, MHC97L, MHCC97H, QGY-7703, Huh7, HepG2, and normal liver cell line THLE-2 from ATCC (ATCC, USA). The cell lines were cultured in Dulbecco's modified Eagle medium (Gibco, USA) supplemented with 10% fetal calf serum and transfected them with Lipofectamine 2000 (Invitrogen, USA). The miR-218 mimics (miR-218-mimics) and inhibitor (miR-218-In), and their non-specific control microRNAs (miR-218-NC, miR-218-NC-In), HoxA10 siRNA and non-specific control siRNA were all purchased from RiboBio (RiboBio, Guangzhou, China). Human carcinoma or normal tissues were collected from patient in the department of Hepatic Surgery at the First Affiliated Hospital of Harbin Medical University. All patients signed consent forms. All experimental procedures were reviewed and approved by the Ethic Committee at the First Affiliated Hospital of Harbin Medical University.

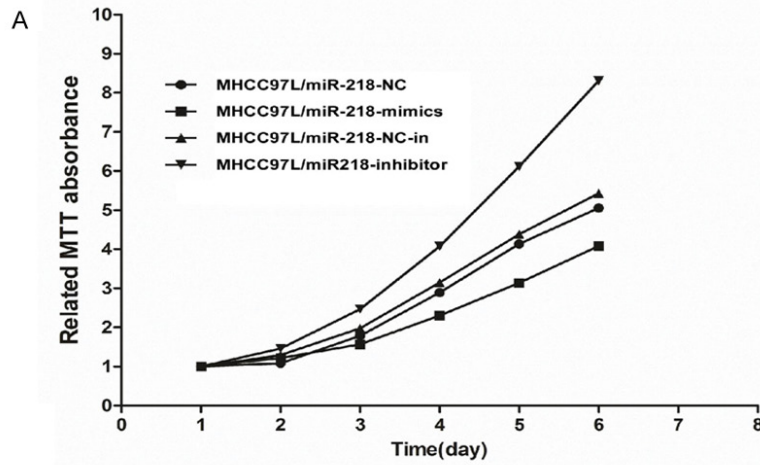
### Real-time Q-PCR

Total RNA were extracted from HCC cells or human tissues with an RNeasy Mini Kit (Qiagen, USA). The cDNA was reverse-transcribed by Superscript III (Invitrogen, USA). We quantified miR-218 expression by real-time quantitative polymerase chain reaction (PCR) using the Sybergreen PCR Master Mix kit (Applied Biosystems, USA) and the ABI-Prism 7300 System. The primers for miR-218 are: 5'-CGGG-ATCCGACCAGTCGCTGCGGGGCTTTCCTTTGTGCTTGATCTAACCATGTGGTGAACGATGGAAA-3' and 5'-CCCAAGCTTTGCAGGAGAGCACGGTGCTTCCGCGGTGCTTGACAGAACCATGTTCCGTTTCATCGTTC-3. We also used glyceraldehyde 3-phosphate dehydrogenase as internal control. For quantification, we calculated the copy ratios of miR-218 messenger RNA (mRNA) and then normalized to normal liver tissues.

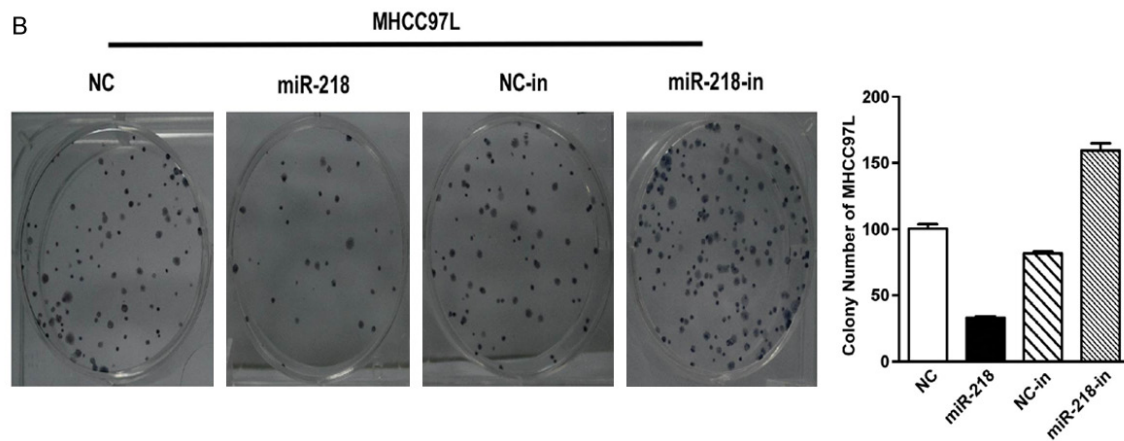
### MTT assay and colony forming assay

For MTT assay, HCC Cells were aliquoted into a 96-well plate (10,000 per 100  $\mu$ L/well), incubated for 24 h, and treated with miR-218 mimics, miR-218 inhibitors and non-specific controls for 1 to 6 days. After replacing the medium

## miR-218 suppresses hepatic cancer cell



**Figure 2.** miR-218 inhibited HCC cell proliferation. (A) MHCC97L cells were transfected with miR-218-mimics and miR-218-inhibitor, along with their corresponding control siRNAs. Cell proliferations were examined with MTT assay for 6 consecutive days. (B) MHCC97L cells were transfected with miR-218-mimics and miR-218-inhibitor, along with their corresponding control siRNAs. Representative images were shown for the cell colonies at 2 DIV. (C) The average numbers of formed cell colonies per dish for (B) examined.



with 100  $\mu$ L DMEM containing 10% fetal bovine serum, 20  $\mu$ L MTT solution (Sigma Aldrich, USA) was added and plates were incubated for another 2 h. The absorbance at 490 nm was measured to determine the number of viable cells in each well. For colony forming assay, 10,000 cells were seeded onto a 10 cm culture dish and incubated in an incubator at 37C with 5% CO<sub>2</sub>. After 2 days culture with miR-218 mimics, miR-218 inhibitors or non-specific controls, cells were fixed by ice-cold methanol for 30 min and stained by Crystal violet for 10 min. Colonies (more than 50 cells) were then counted. Statistical significance was calculated from triplicates. All experiments were done in triplicates.

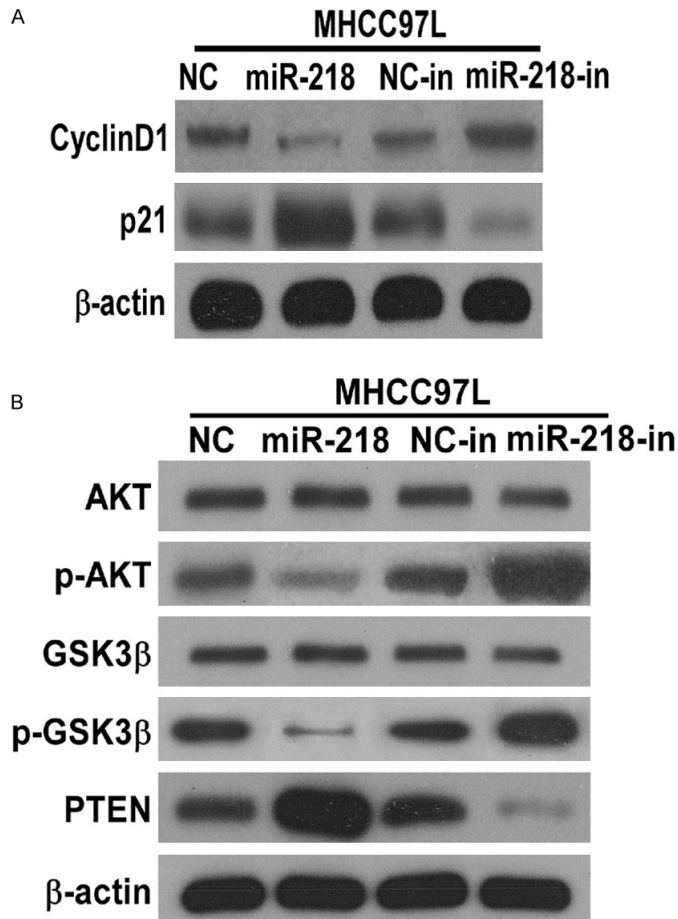
### Western blot analysis

Proteins were extracted in Lysis Buffer (Cell Signaling Technology, USA). The extracts were separated by 12% SDS-PAGE and transferred

to PDMF membrane (Millipore, USA). After 1 hour blocking of 5% nonfat milk, primary antibodies were added on the membrane at 4°C over night. The membrane was then washed 10mins for 3 times with TBS/T and incubated with secondary antibody for 2 hour at room temperature. The signal was detected by ECL Plus system (GE Healthcare, UK). Antibodies against cyclin D1, p21, AKT, phosphorylated-AKT (p-AKT), GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$  (p-GSK-3 $\beta$ ), PTEN, HoxA10 and  $\beta$ -actin (control) were all purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

### Statistical analysis

Student's *t* test was used for comparison between groups. Statistical analyses were conducted with SPSS software for windows (version 15.0). Data were presented as mean  $\pm$  standard errors. *P* < 0.05 was considered statistically significant.



**Figure 3.** miR-218 regulated PTEN/AKT/PI3K pathway in HCC. (A) MHC97L cells were transfected with miR-218-mimics and miR-218-inhibitor, along with their corresponding control siRNAs. Western blotting analysis was used to examine the cell cycle proteins Cyclin D1 and p21, and (B) PTEN/AKT/PI3K signaling pathway related proteins, including AKT/phosphorylated-AKT, GSK-3β phosphorylated-GSK-3β PTEN.

## Results

### *Expression of miR-218 was down-regulated in hepatocellular carcinoma*

We first applied quantitative RT-PCR to examine the expression levels of miR-218 in hepatocellular carcinoma tissues, and compared with the expression level in normal liver tissues. As compared to the expression level in normal liver cell line THLE-2, miR-218 was significantly down-regulated in all examined HCC cell lines, including BEL-7402, MHCC97L, MHCC97H, QGY-7703, Huh7 and HepG2 (**Figure 1A**,  $p < 0.05$ ). We then assessed human carcinoma and adjacent tissues from 6 patients with HCC.

Similar to the results of cell lines, the expression level of miR-218 was down-regulated in human hepatocellular carcinoma (**Figure 1B**).

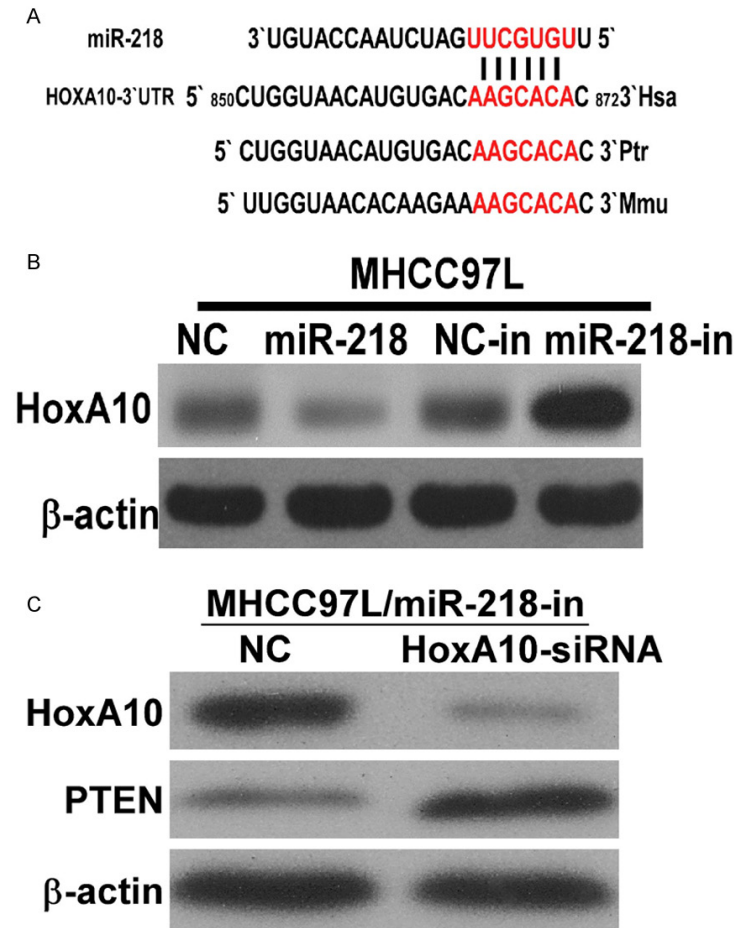
### *miR-218 modulated cell proliferation in HCC cancer cells*

We then investigated whether miR-218 had a functional role in regulating HCC. We transfected MHC-97 L cells with either miR-218 mimics (miR-218-mimics) or miR-218 inhibitor (miR-218-in) to either up-regulate or down-regulate the expression level of miR-218 in HCC cells. MHC97L cells were also transfected with corresponding non-specific controls (miR-218-NC and miR-218-NC-in). Through a MTT assay, we found that cell proliferation was significantly reduced while miR-218 was up-regulated, whereas proliferation was increased by miR-218 inhibition in MHC97L cells 4 days after transfection (**Figure 2A**). The regulatory effects of miR-218 were also confirmed with the colony formation assay, in which upregulation of miR-218 inhibited whereas down-regulation of miR-218 promoted the growth of MHC97L cells (**Figure 2B, 2C**).

### *miR-218 regulated PTEN/AKT/PI3K in HCC cancer cells*

We then asked what signaling pathways were involved during the regulation of miR-218 on HCC cells. Firstly, we speculated that cell cycling machinery was modulated in HCC cells by miR-218. The MHC97L cells were transfected with either miR-218 mimics or miR-218 inhibitor and western blotting analysis was used. We found that the core cell-cycle regulator Cyclin D1 and its related protein p21 were actively modulated by miR-218, as up-regulation of miR-218 reduced Cyclin D1 and increased p21 whereas down-regulation of miR-218 increased Cyclin D1 and reduced p21 (**Figure 3A**).

Secondly, we examined the effect of miR-218 on PTEN/AKT/PI3K signaling pathway (**Figure**



**Figure 4.** miR-218 regulated PTEN/AKT/PI3K pathway through HoxA10. A. The predicted binding site of miR-218 on 3'-UTR of HoxA10. B. MHC97L cells were transfected with miR-218-mimics and miR-218-inhibitor, along with their corresponding control siRNAs. Western blotting analysis was used to examine the expression of HoxA10 in MHC97L cells. C. MHC97L cells were first transfected with miR-218-inhibitor. Then siRNA was used to knock down HoxA10 (HoxA-siRNA). Non-specific scrambled siRNA (NC) was used as control. Western blotting was used to examine the expression level of PTEN protein.

**3B).** We found that, while miR-218 was up-regulated, phosphorylated AKT and phosphorylated BSK3β were reduced and PTEN protein was increases in MHC97L cells. On the other hand, when miR-218 was down-regulated, phosphorylated AKT and phosphorylated BSK3β were increased and PTEN protein was decrease in MHC97L cells.

*HoxA10 was involved in the regulation of miR-218 on PTEN/AKT/PI3K*

Finally, through bioinformatic methods including TargetScan, Pictar, miRANDA, we found that HoxA10 was very likely to be bound with miR-

218 (**Figure 4A**). The western blotting assay confirmed our hypothesis. The results showed that up-regulation of miR-218 reduced, whereas down-regulation of miR-218 increased the expression level of HoxA10 in MHC97L cells (**Figure 4B**). Furthermore, after miR-218 was down-regulated in MHC97L, the application of HoxA10 targeted siRNA significantly up-regulated PTEN protein (**Figure 4C**), suggesting that HoxA10 was directly associated with miR-218 in regulating PTEN/AKT/PI3K pathway in HCC cells.

### Discussions

In the present study, we reported that miR-218 was frequently down-regulated in in both hepatoma cell lines and human HCC tissues. This result is in line with previous study showing that miR-218 was lowly expressed in patients with Hepatitis C [12]. However, while it had been reported that downregulation/upregulation of miRNAs played important role in tumorigenesis including hepatocellular carcinoma [13-16], little is known about the exact role of miR-218 in regulating HCC in human or animals. In the present study, for the first time ever, we demonstrated that up-regulation of miR-218 inhibited whereas its down-

regulation promoted cancer cell proliferation, thus providing the direct evidence of an active role of miR-218 in regulating hepatocellular carcinoma.

It was shown before that, in oral squamous cell carcinoma, ectopic miR-218 suppressed Rictor and Akt S473 phosphorylation to inhibit PTEN/mTOR pathway to induce cancer cell apoptosis [17]. In the present study, we found that that PTEN/AKT/PI3K pathway was modulated by miR-218 in HCC cells. The up-regulation of miR-218 inhibited the activity of PTEN/AKT pathway whereas the down-regulation of miR-218 induced the phosphorylation of AKT and

GSK3 $\beta$  to activate the tumor suppressing PTEN/mTOR pathway. Thus, It seems like the similar regulatory mechanism of miR-218 was involved in multiple forms of cancers while PTEN/AKT pathway was involved as common down streaming target. Interestingly, further functional analysis of our study revealed that HoxA10 was very likely the gene associated with miR-218 in regulating PTEN/AKT/PI3K pathway in hepatocellular carcinoma, unlike the modulator of Rictor as the direct target of miR-218 in oral cancer [17]. Those findings suggest that independent intermediate pathways are very likely to be involved in the regulations of miR-218 on pathogenesis in various types of cancer cells.

In conclusion, we presented that miR-218 was lowly expressed in HCC cells. The overexpression of miR-218 reduced, whereas inhibition of miR-218 increased cancer cell proliferation. Also, we demonstrated the regulatory mechanism of miR-218 in HCC cells was through PTEN/AKT pathway with possible association of HoxA10. The results of this study showed that targeting miR-218 might provide a novel therapeutic strategy for the treatment of patients with hepatocellular carcinoma.

#### Disclosure of conflict of interest

The authors declare no conflict of interest.

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