

Original Article

miR-103 promotes hepatocellular carcinoma cell proliferation and migration in the simulation transition zone of RFA through PI3K/Akt signaling pathway by targeting PTEN

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Abstract: Radiofrequency ablation (RFA) is a potentially curative therapy for nontransplantable hepatocellular carcinoma (HCC). However, as tumor size increases, incomplete RFA can increase rates of local recurrence and tumor progression. As such, there remains a need to identify potential biologic mechanisms mediating HCC response to thermal ablation. Our results revealed that miR-103 was markedly upregulated in recurrent HCC tissues treated with RFA as first-line treatment and in HCC lines after heat stress in vitro, simulating the marginal zone of RFA treatment. Gain-of-function and loss-of-function studies showed that miR-103 ectopic overexpression promoted, but miR-103 silencing reduced, heat-exposed HCC proliferation, and migration in vitro. Western blotting displayed that proteins related with proliferation and migration were significantly changed in different groups. Furthermore, PTEN may be a potential target of miR-103 and miR-103 could activate the PI3K/Akt pathway by suppressing PTEN expression. Taken together, these studies provide experimental evidence supporting a role for miR-103 in HCC response to heat stress.

Keywords: Hepatocellular carcinoma, radiofrequency ablation, miR-103, proliferation, migration, PI3K/AKT signaling

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the third most common cause of cancer death worldwide [1]. Radio frequency ablation (RFA) has greatly expanded treatment options for non-transplantable HCC patients while achieving short-term outcomes similar to surgical resection with less morbidity [2-4]. However, as tumor size increases, suboptimal RFA treatment for HCC has increased rates of local recurrence and tumor progression, particularly for tumors beyond 3 cm in size, and overall survival remains poor for these patients [5]. As such, there remains a need to identify potential biologic mechanisms mediating HCC response to thermal ablation.

Accumulating evidence suggests that abnormal expression of miRNAs is involved in human

tumorigenesis and progression [6]. In particular, several miRNAs are suppressor genes in some tumors, but are oncogenes in other tumors [7, 8]. So far, an increasing number of miRNAs which can serve as potential diagnostic or therapeutic targets of HCC have been studied. For example, miR-1269 [9], miR-494 [10], and miR-492 [11] play key roles in HCC metastasis by downregulating mRNA targets involved in metastasis. Recently, it was reported that miR-103 acts as a potential oncogene by targeting AKAP12 in HCC [12]. However, the role of miR-103 in tumorigenic processes of HCC subjected to thermal ablation is not fully understood.

In the present study, we found that miR-103 was markedly upregulated in recurrent HCC tissues after RFA therapy and in HCC cell lines after heat stress in vitro. The upregulation of miR-103 could enhance the proliferation and

migration of heat-exposed HCC cells. Our data also suggested that the role of miR-103 in heat-exposed HCC cells might involve the regulation of PI3K/Akt signaling pathway by targeting PTEN.

Materials and methods

Tissues and cells

Fresh recurrent HCC tissue samples from HCC patients, and their matched adjacent non-tumor tissues (> 5 cm laterally from the edge of tumor region) were obtained from the Institute of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University, China. The samples had been clinically and histopathologically diagnosed according to the World Health Organization criteria. Tumor and non-cancerous tissues were confirmed histologically by hematoxylin and eosin staining. All samples were collected from consenting individuals according to the protocols approved by the Ethics Review Board at Third Military Medical University. HCC cell lines Hep3B, MHCC97-L, HepG2, HC-CLM3, HuH-6 and Bel-7402 were routinely maintained in DMEM medium supplemented with 10% fetal bovine serum.

In vitro heat stress treatment

Heat stress treatment was performed as described previously. Briefly, cells collected in 1.5 mL microcentrifuge tubes and heat stressed at temperatures (45°C) or control (37°C) for 10 min in an isothermic water bath, and then plated in 96-well tissue culture plates and incubated in a 37°C, 5% CO₂ incubator.

Quantitative real-time PCR for genes and miRNAs

Total RNA from cultured cells and fresh surgical pancreatic tissues was extracted using Trizol Reagent according to the manufacturer's instructions. The primers used were as follows: PTEN forward 5'-CTTTGTGCTGAAAGACATTATGAC-3', reverse 5'-GGCTTTGTCTTTATTTGCTTTGTC-3'; BIM forward 5'-GCATCATCGCGGTATTCGGT-3', reverse 5'-CGCAGGCTGCAATTGTCTAC-3'; CyclinD1 forward 5'-TTGTTGAAGTTGCAAAGTCCTGG-3', reverse 5'-ATGGTTTCCACTTCGCAGCA-3'; FASL forward 5'-CTTGGTAGGATTGGGCC-TGG-3', reverse 5'-CTGGCTGGTAGACTCTCGGA-3'; P21 forward 5'-TGCCGAAGTCAGTTCCTTGT-3', reverse 5'-CATTAGCGCATCACAGTCGC-3'; GA-

PDH forward 5'-TGGGTGTGAACCATGAGAAGT-3', reverse 5'-TGAGTCCTTCCACGATACCAA-3'.

Wound healing assays

Cells were seeded on six-well plates with DMEM containing 10% fetal bovine serum (FBS) and grown to confluence. The cells were scratched with a sterile 200-AL pipette tip to create artificial wounds. At 0 and 21 h or 24 h after wounding, phase-contrast images of the wound healing process were photographed digitally using an inverted Olympus IX50 microscope with a 10 X objective lens. Eight images per treatment were analyzed to determine averaging position of the migrating cells at the wound edges by digitally drawing lines using the Image-Pro Plus software (Media Cybernetics).

MTT assays

Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay as described previously. Absorbance was determined by a spectrophotometer at 540/620 nm (GDV, Roma, Italy).

Luciferase reporter assays

Heat-exposed HEPG2 cells were co-transfected with pmirGLO-PTEN-3'-UTR-wt or pmirGLO-PTEN-3'-UTR-mut along with miR-103 mimic or negative control miRNA using Lipofectamine 2000. A renilla luciferase-expressing plasmid pRL-TK (Promega) was co-transfected as an internal control. At 24 h posttransfection, cells were harvested and luciferase activity was determined using the Dual Luciferase Reporter Assay Kit.

For MMP2 promoter reporter system, the MMP2 proximal promoter (-1256/+299) was amplified from human genomic DNA by PCR. Both PCR products and pGL3-cyclin D1-luciferase reporter plasmid were digested with KpnI and HindIII and ligated together to form the (-1256/+299) MMP2-Luc reporter plasmid.

Western blotting

Western blotting analysis was performed as described previously. The antibodies used were as follows: anti-GAPDH, anti-PTEN and anti-FASL antibody (Cell Signaling, USA), anti-Akt antibody (BioLegend, San Diego, CA), anti-p-Akt

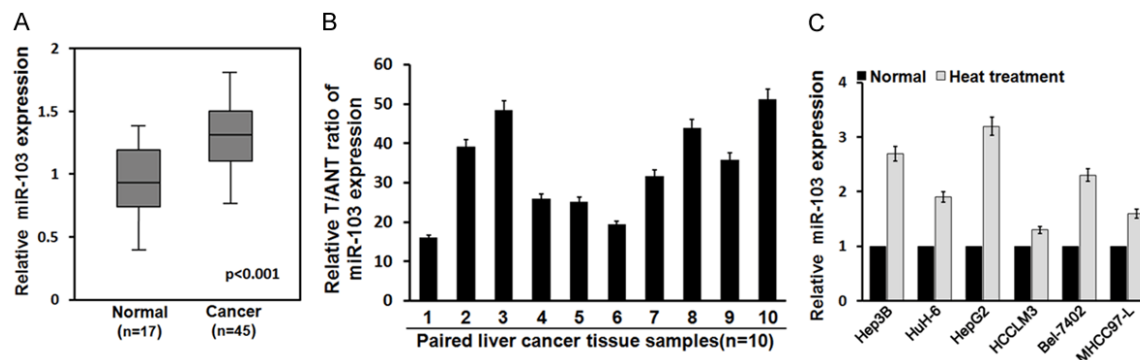


Figure 1. A. miR-103 is upregulated in recurrent HCC tissues and cell lines after heat stress. miR-103 levels remained low in normal liver tissues but became dramatically elevated in HCC tissues according to analyzing The Cancer Genome Atlas (TCGA) HCC miRNA sequencing data sets (Normal, n = 17; hepatocellular carcinoma, n = 45). $P < 0.001$, 2-tailed Student's t-test. B and C. Real-time PCR analysis of miR-103 expression in 10 pairs of recurrent HCC samples after RFA therapy (T) and adjacent normal tissues (ANT), and in 6 cultured hepatocellular carcinoma cell lines after heat stress. Transcript levels were normalized by U6 expression. Error bars represent the mean $\pm$ s.d. of three independent experiments. * $P < 0.05$.

antibody (EnoGeneBiotech, New York, USA), anti-p21 antibody (Santa Cruz Biotechnologies, Heidelberg, Germany), anti-Bim and anti-MM-P9 antibody (Abcam, Cambridge, UK), anti-pRb and anti-p-pRb antibodies (Cell Signaling, USA). The membranes were stripped and re-probed with an anti-GAPDH antibody as the loading control.

Statistical analyses

All statistical analyses were carried out using SPSS statistical software (SPSS Inc., Chicago, IL, USA). The 2-tailed Student's t-test was used to evaluate the significance of the differences between two groups of data in all pertinent experiments; a P value < 0.05 was considered significant.

Results

miR-103 is upregulated in recurrent HCC tissues and cell lines after heat stress

By analysis of the The Cancer Genome Atlas (TCGA) hepatocellular carcinoma miRNA sequencing data sets, we found that miR-103 levels were significantly upregulated in human HCC tissues (n = 45) compared with that in normal tissues (n = 17) ($P < 0.001$) (Figure 1A). To investigate the role of miR-103 in HCC response to RFA therapy, we further verified the miR-103 levels in paired tissues and HCC cell lines after heat stress by real-time PCR analysis. As shown in Figure 1B and 1C, miR-103 levels were differentially increased in 10 hepatocellular carcinoma tissues (T) compared to that in the adja-

cent normal tissues (ANT) (Figure 1B), and more increased in six heat-exposed hepatocellular carcinoma cell lines than that in normal HCC cells (Figure 1C). Collectively, these results suggest that miR-103 is upregulated in HCC and might be involved in progression.

miR-103 promotes heat-exposed HEPG2 cell proliferation and migration

To investigate the role of miR-103 in heat-exposed HCC cells, MTT assay was used to compare the cell proliferation viability in each group. HCC cell line HEPG2 after heat treatment was engineered to overexpress or silence miR-103 by transfection of miR-103 mimic or miR-103 inhibitor (Figure 2A). We found that cell proliferation viability increased in the miR-103 overexpression group, and decreased in the miR-103 inhibitor group, compared with the control group (Figure 2B).

Consistent with abovementioned results, wound healing assays indicated that miR-103 overexpression dramatically enhanced the migratory capacities of HCC cells (Figure 2C, 2D).

miR-103 activates PI3K/AKT signaling pathway

Since PI3K/AKT signaling is one of the most important pathways in maintaining survival and proliferation and is frequently activated in HCC, we then examined the role of miR-103 in PI3K/AKT signaling pathway. As shown in Figure 3A, 3B, overexpressing miR-103 significantly increased, but silencing miR-103 decreased, the

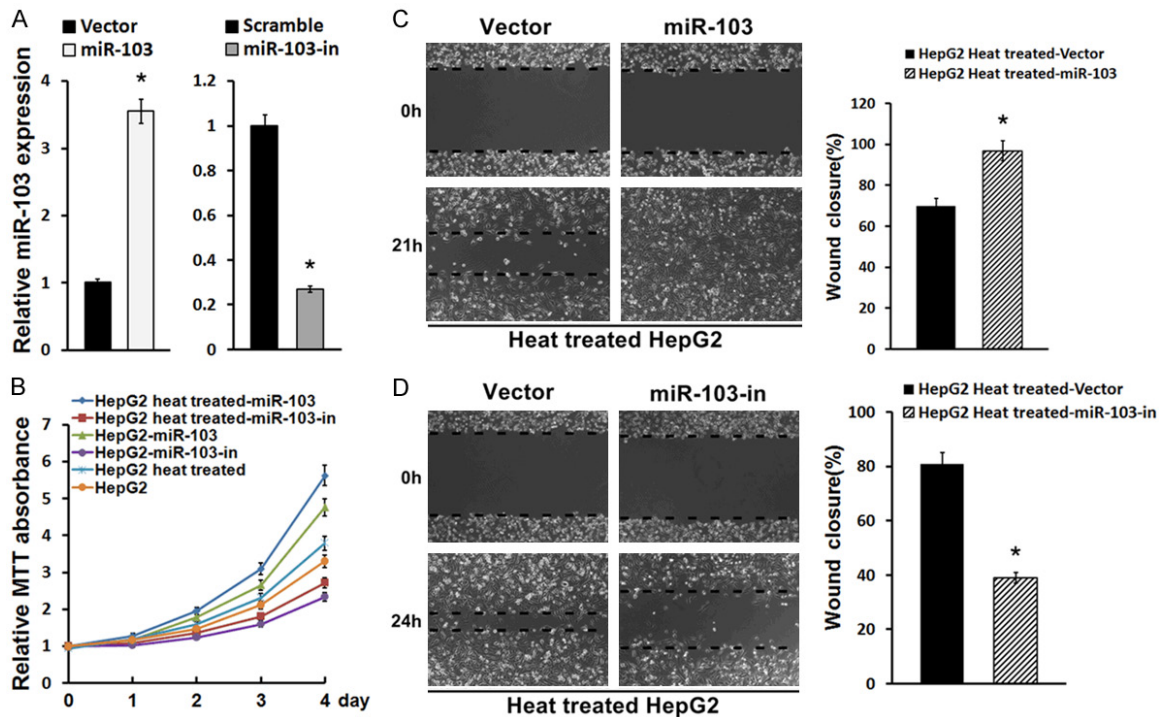


Figure 2. A. miR-103 promotes heat-exposed HEPG2 cell proliferation and migration. Real-time PCR analysis of miR-103 in Vector-transduced, miR-103-overexpressing and miR-103-silenced heat-exposed HEPG2 cell lines. Transcript levels were normalized to U6 expression. B. The cell proliferation viability was determined by MTT assay. A 490 absorption was assayed after culture from 1 to 4 days. C. Wound healing assays indicated that miR-103 overexpression dramatically enhanced the migratory capacities of HCC cells (* $P < 0.05$). D. Wound healing assays indicated that miR-103 inhibition dramatically reduce the migratory capacities of HCC cells (* $P < 0.05$). All of the data are mean $\pm$ SEM of three independent experiments.

mRNA and protein levels of CyclinD1, p21, Bim, and FasI, four downstream effectors of PI3K/Akt signaling. In addition, the expression of phosphorylated Akt and pRb in HEPG2 cells were also significantly altered in the miR-103-deregulated HEPG2 cells (Figure 3C, $P < 0.05$). Moreover, the expression of matrix metalloproteinase-9 (MMP-9) protein, a key factor of HCC invasiveness regulated by the PI3K/Akt signaling pathway, was also upregulated in the miR-103 overexpression group and downregulated in the miR-103 inhibitor group ($P < 0.05$), respectively. The increase in MMP-2 expression by miR-103 was further supported by a luciferase reporter assay (Figure 3D), suggesting that miR-103 enhances MMP-2 transcriptional activity.

PTEN is a direct target of miR-103

Previous studies have shown that PTEN functions as a tumor suppressor gene by specifically negatively regulating the PI3K/Akt signaling pathway. Through searching the Target Scan and miRbase, we found that 3'-UTR of human

PTEN contained a putative region that could match to the sequence of miR-103 (Figure 4A).

Western blotting analysis was used to verify whether PTEN was indeed the target of miR-103 (Figure 4B). Moreover, luciferase assay showed that miR-103 overexpression attenuated, while inhibition of miR-103 elevated the reporter activities driven by the 3'UTRs of PTEN (Figure 4C). However, ectopic expression of the miR-103 did not exhibit repressive effects on the reporter activities driven by the mutant 3'UTRs of PTEN (Figure 4C). To further validate that PTEN was a direct downstream target of miR-103, we investigated the relationship between miR-103 and PTEN expression in recurrent HCC tissue after RFA therapy and found that PTEN levels are inversely correlated with miR-103 expression levels (Figure 4D).

Discussion

Evidence generated over the past few years suggested residual HCC cells can progress rapidly after RFA [3, 4]. Several reports demon-

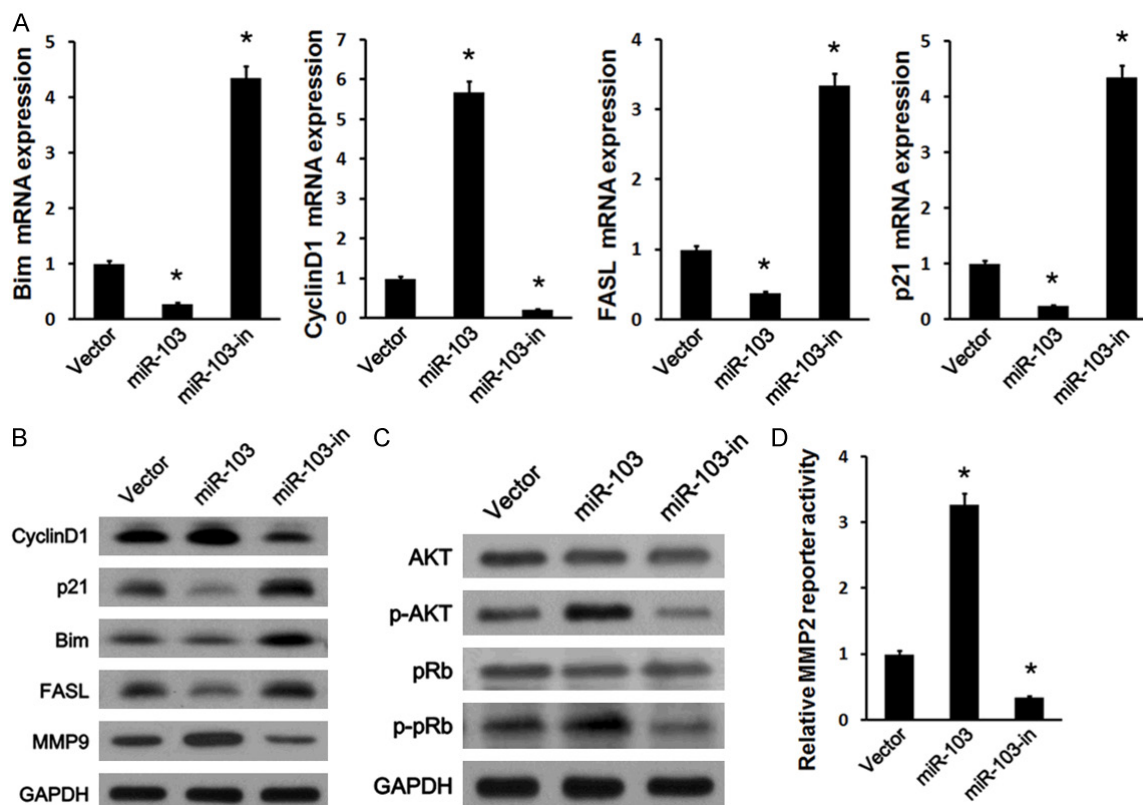


Figure 3. A. miR-103 activates PI3K/Akt signaling pathway. Real-time PCR analysis revealed that miR-103 regulates the expression levels of multiple PI3K/Akt downstream genes of CyclinD1, p21, Bim and Fasl. B. Western blotting analysis of CyclinD1, MMP9, p21, Bim and Fasl protein levels. C. Western blotting analysis revealed that the expressions of p-Akt, p-pRb in HEPG2 cells were also significantly altered in the miR-103-deregulated HEPG2 cells. GAPDH was used as a loading control. D. miR-103 regulates the promoter activity of MMP2. Error bars represent the mean $\pm$ s.d. of three independent experiments. *P < 0.05.

strated that hepatoma cells that were exposed to sublethal heat for 10 minutes adopted molecular and functional characteristics of hepatic progenitors, coupled with increased proliferation, up-regulation of genes that are involved in EMT, and an enhanced malignant potential in vivo [13]. However, it is not known whether microRNAs may play a role in HCC response to heat stress from RFA therapies.

Previous evidence has demonstrated that miR-103 is overexpressed in human colorectal and endometrial cancer and upregulation of miR-103 contributes to cancer cell proliferation, migration, and drug resistance by different mechanisms [14]. In our study, we found that miR-103 was markedly upregulated in recurrent HCC tissues treated with RFA as first-line treatment and in HCC cell lines after heat stress in vitro, simulating the marginal zone of RFA treatment. Moreover, it also promotes the

proliferation, and migration of heat-exposed HEPG2 cells.

Numerous studies showed that activation of the PI3K/Akt signaling pathway was essential to the development and progression of HCC and could modulate the malignant behavior of HCC, such as cell proliferation, invasiveness, and metastasis. In our experiment, upregulation of the miR-103 specifically increased, but silencing miR-103 decreased, the expression of phosphorylated Akt and p-pRb. Consistently, the mRNA and protein levels of, CyclinD1, p21, Bim, and Fasl, four downstream effectors of PI3K/Akt signaling, were also significantly altered. Moreover, the expression of MMP-9 protein, a key factor of HCC invasiveness regulated by the PI3K/Akt signaling pathway, was upregulated in the miR-103 overexpression group and downregulated in the miR-103 inhibitor group. The increase in MMP-2 expression by miR-103

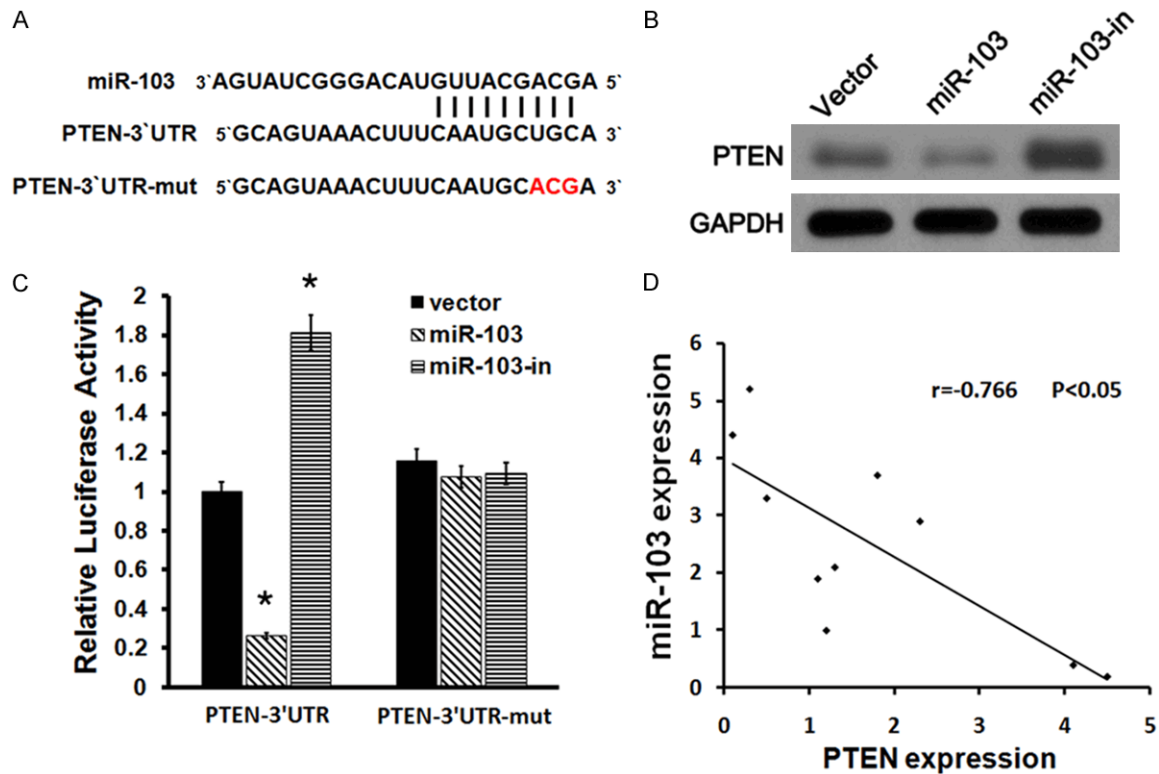


Figure 4. A. PTEN is a direct target of miR-103. Predicted miR-103 target sequence in 3'UTRs of PTEN. The mutated 3'UTRs of PTEN containing the altered nucleotides were indicated. B. The PTEN protein expression was measured in heat-exposed HEPG2 cells transfected with miR-103 mimic and inhibitor, by western blot. C. Luciferase assay of pGL3-PTEN-3'UTR and pGL3-PTEN-3'UTR-mut reporter in the miR-103-overexpressing or miR-103-silenced and control cells. D. Pearson's correlation scatter plot of the fold change of the levels of miR-103 and PTEN mRNA in 10 recurrent HCC tissue samples. ($R = -0.766$, $P = 0.009$). Error bars represent the mean $\pm$ s.d. of three independent experiments. * $P < 0.01$.

was further supported by a luciferase reporter assay, suggesting that miR-103 could enhance MMP-2 transcriptional activity. These results implied that miR-103 promoted proliferation and migration of HCC cells at least partly through the PI3K/Akt pathway.

The identification of target genes is a key step in assessing the role of aberrantly expressed miRNA in cancer. It may be valuable for further development of miRNA-based gene diagnosis and therapy. With the help of two bioinformatics software, Targetscan and miRBase, we predicted the potential targets of miR-103 and found that PTEN has binding sites for miR-103 in its 3'UTR.

PTEN was the first tumor suppressor gene which has a bispecific phosphatase activity and was closely associated with carcinoma [15]. Furthermore, previous studies have shown that PTEN played an important role in miRNA medi-

ated effects on cell growth, apoptosis, adhesion and migration by suppressing the PI3k/Akt pathway [16, 17]. In our experiment, although bioinformatics analysis predicted that PTEN was a target gene of miR-103, it was necessary to verify the results experimentally in HCC cells because the miR-mediated effects on gene expression and cellular functions are cell-specific. Therefore, we used western blotting and luciferase assay to confirm our conjecture. The results showed that PTEN was downregulated in the miR-103-up group and upregulated in miR-103-down group. To further validate that PTEN was a direct downstream target of miR-103, we investigated the relationship between miR-103 and PTEN expression in recurrent HCC tissue after RFA therapy and found that PTEN levels are inversely correlated with miR-103 expression levels. These results indicated that miR-103 might act as an oncogene by repressing PTEN expression in HCC.

In summary, our study has revealed that upregulation of miR-103 exerts a promoting effect on proliferation and migration of HCC cells with a potential involvement of PI3K/Akt signaling pathway via regulation of PTEN expression, which may help improve our understanding of the pathogenesis and therapies of HCC recurrence after RFA therapy.

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Disclosure of conflict of interest

None.

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