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Downregulated miR-328 suppressed cell invasion and growth in hepatocellular carcinoma via targeting PTEN

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Abstract. – OBJECTIVE: To investigate the regulatory effect of miR-328 on biological behaviors of hepatocellular carcinoma (HCC) cells, such as invasion and proliferation.

PATIENTS AND METHODS: The expressions of miR-328 were detected in 48 pairs of HCC tissue samples and matched adjacent tissues, as well as in 3 kinds of HCC cell lines via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Further, we analyzed the effects of miR-328 inhibition on cell invasion, proliferation, cell apoptosis, and cell cycle. Dual-luciferase activity assay was performed to examine the potential target gene PTEN which was predicted by an online database. Protein levels were detected using Western blot assay.

RESULTS: The expression of miR-328 was significantly increased in HCC tissue samples. Decreased miR-328 in HCC cells significantly attenuated cell invasion and proliferation capacities, promoted cell apoptosis and induced cell cycle arrest at G0/G1 phase. Moreover, PTEN was verified as a target gene of miR-328 by dual-luciferase activity assay, qRT-PCR and Western blot. Furthermore, the silence of PTEN neutralized the suppressive effect of decreased miR-328 on cell growth and metastasis.

CONCLUSIONS: MiR-328 is involved in the development of HCC via regulating PTEN, which might provide a new target for HCC diagnosis and therapy.

Key Words:
miR-328, Hepatocellular carcinoma (HCC), Proliferation, Invasion.

Introduction

Primary hepatocellular carcinoma (HCC) is a malignant tumor that originates in hepatocytes or intrahepatic cholangiocarcinoma. Due to the high degree of malignancy and the prone to hematogenous metastasis, early diagnosis of HCC is very difficult[1]. Therefore, most patients are already in the middle and late clinical stages of HCC when receiving treatment and often have a poor prognosis[2]. At present, the treatment of HCC is mainly surgical excision combined with radiotherapy and chemotherapy is still the mainstream therapy of HCC. The 5-year survival rate of HCC patients after surgery is 20% to 30%. Local and distant metastases are a major threat to the treatment effect of HCC and also a major cause of poor prognosis[3]. Therefore, exploring novel therapeutic target molecules and tumor markers for HCC are of great importance to the treatment and prognosis of HCC.

MicroRNAs (miRNAs) are single-stranded non-coding small RNAs of about 22 nucleotides in length. Mature miRNAs selectively integrate into RNA-induced silencing complexes (RISC) to form miRISC complexes[4]. MiRNAs play important roles in gene expression regulation by targeting the mRNA 3'-UTR and inducing the cleavage or degradation of the target mRNA[5]. Previous reports have revealed that abnormal expression of miRNAs is closely related to the occurrence, development and prognosis of HCC. For example, miR-302b could repress tumor cells growth of HCC *in vivo* and *vitro* by targeting AKT2[6]. MiR-145 also suppressed HCC cell growth *via* regulating Akt signaling pathway[7]. MiR-202 was reported as a potential tumor suppressor and participated in carcinogenesis of HCC by suppressing LRP6[8].

Researches have confirmed that miR-328 is closely related to the occurrence and development of tumors. Pan et al[9] showed that the expression of miR-328 and BRCP in human breast cancer cells was inversely proportional, and miR-328 reduced the expression of BCRP by targeting 3'-

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UTR of BCRP mRNA. The interaction between miR-328 and ATP binding cassette transporter (ABC)/ATP-binding cassette subfamily G member 2 (ABCG2) inhibited the drug resistance of colorectal cancer cells[10]. Arora et al[11] showed that miR-328 not only, but also increase the invasion and metastasis capacity of lung cancer cells by up-regulating protein kinase C (PRKCA). Delic et al[12] found that miR-328 promoted the infiltration of astroglioma cells into peripheral tissues through activating the SFRP1-dependent Wnt signaling pathway. However, the specific mechanism of miR-328 in the development of HCC has not been explored yet.

This study aimed to investigate the role of miR-328 in metastasis and proliferation of HCC *in vitro*, confirming that miR-328 might be used as a new potential target for the treatment of HCC.

Patients and Methods

Clinical Samples and Cell Lines

Primary hepatocellular carcinoma specimens and corresponding para-cancer tissue specimens from 48 patients were collected in People's Hospital of Rizhao. Patients were not treated with radiotherapy and chemotherapy before surgery. All patients included in the study signed informed consent. This study was approved by the Hospital Ethics Committee.

HCC cell lines (HepG2, HuH7, SMMC772) and normal human liver-derived cell lines (L02) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences (Shanghai, China). The above cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% cyanine-streptomycin in a constant temperature incubator at 37°C and 5% CO_2 (Gibco, Rockville, MD, USA). Then, cells were digested with 0.25% trypsin for subsequent experiments when cell fusion reached 80% to 90%.

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and complementary Deoxyribose Nucleic Acid (cDNA) was synthesized using SYBR Prime Script miRNA RT-PCR Kit and PrimeSeript RT Master Mix (TaKaRa, Otsu, Shiga, Japan) according to the instruction. SYBR Green II was used for qRT-PCR to detect miR-328 and mRNA levels. U6 was selected as the internal reference for miR-328, and GAPDH was taken as the internal reference for PTEN. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of miRNA and mRNA. Primer sequences used in this study were as follows: miR-328, F: 5'-GCAGGAGGGGCTCAGGG-3', R: 5'-CGACGAGCAAAAAGCTTGT-3'; PTEN, F: 5'-CAATGACAGCCATCATCAAAGAG -3', R: 5'-CAATGACAGCC ATCATCAAAGAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cells Transfection

MiR-328 inhibitor and NC-inhibitor were transfected into HepG2 cells using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA). About 48 h after transfection, cells were collected for follow-up experiments.

SiRNA interference assays were also performed using Lipofectamine™ 2000 liposome as mentioned above. Briefly, 6 μL of Lipofectamine™ 2000 were added to 500 μL Opti-MEM and incubated for 5 min at room temperature; next, 5 μL PTEN siRNA were added to the mixture at room temperature for 20 min before dropped into a petri dish cultured with HepG2 cells. After 48 h of transfection, qRT-PCR was used to examine the interference efficiency of siRNA targeting PTEN.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was detected using the CCK-8 kit (Dojindo, Kumamoto, Japan). After 48 h of cell culture, 8 μL of CCK-8 solution and 100 μL of serum-free medium were added to each group of cells. After incubation for 90 min, the absorbance at 450 nm wavelength was measured using a microplate reader (OD450). The experiments were independently repeated 3 times.

Cell Apoptosis Analysis

Cells were seeded into 6-well plates and transfected with miR-328 inhibitor or NC-inhibitor. Apoptotic cells were detected 72 h later. The cells were digested with trypsin without EDTA (Ethylene Diamine Tetraacetic Acid) and then harvested. Cells were resuspended by adding 250

μL of 1 × Binding Buffer per tube and adjusted the cell concentration of 1 x 10^6/ml. After that, 100 μL cell suspension were drawn and mixed with 5 μL of Annexin V/FITC and 10 μL of 20 μg/mL Propidium Iodide (PI) solution (Dojindo, Kumamoto, Japan), and incubated at room temperature for 15 min in the dark. A total of 300 μL Binding Buffer were finally added to the reaction tube and the cell apoptosis was examined by a flow cytometer.

Cell Cycle Analysis

Cells of each group were harvested 48 h after transfection and washed with pre-chilled phosphate-buffered saline (PBS). After fixed with 75% ethanol for 12 h, the cells were stained with 0.5 μL of 100 μg/mL propidium iodide staining solution at room temperature for 10 min. Flow cytometry was used to analyze changes in DNA content of HepG2 cells and the results were calculated by randomized software ModFit LT.

Transwell Assay

Transwell chambers coated with Matrigel were used to detect cell invasion. A total of 1×10^5 cells were seeded into the upper chamber with 100 μL serum-free medium, and 600 μL of a 10% FBS-containing medium was added to the lower chamber. Transwell chambers were placed at 37°C for 24 h. After that, cells in the lower chamber were fixed and stained with crystal violet. The number of cells was counted under a light microscope and the fold change in the number of cells was then calculated.

Dual-Luciferase Assay

Dual-luciferase reporter plasmids containing wild-type and mutant PTEN-3'-UTR were constructed. The HEK293T cells were seeded into 96-well plates, and the constructed pmirGLO/PTEN-3' UTR and pmirGLO/PTEN-3' UTR mut fluorescent reporter vectors were co-transfected with miR-328, miR-328 inhibitor or NC-inhibitor, respectively. Luciferase activity was measured by the Promega (Madison, WI, USA) Dual-Luciferase reporter system 48 h after transfection.

Western Blotting

Tissue and cell proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated by polyacrylamide gel electrophoresis (10% resolving gel and 5% laminated gel) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membranes were then immersed in Tris-buffered saline and Tween (TBST) (Beyotime, Shanghai, China) containing 5% nonfat dry milk for 1 h incubation at room temperature. The hybridized membrane was cut and incubated with PTEN antibody (1:1000) and β-actin antibody (1:3000) at 4°C overnight. After that, the hybridized membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000). Protein bands were detected with electrochemiluminescence (ECL) reagents (Thermo Fisher Scientific, Waltham, MA, USA) and photographed using the Alpha gel imaging and chemiluminescence system. The results were analyzed with Image J analysis software.

Statistical Analysis

All quantitative data were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Differences between two groups were compared using two-sided unpaired *t*-test. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). If there were significant differences, Dunnett's test was used to compare each group. $p < 0.05$ was considered statistically significant.

Results

MiR-328 Expression Was Elevated in HCC Tissues

In order to clarify the role of miR-328 in the development of HCC, we first used qRT-PCR to detect the expression of miR-328 in tumor specimens from patients with HCC. The results showed that miR-328 expression was significantly up-regulated in HCC tissues compared with normal liver tissues (Figure 1A). Thus, we hypothesized that miR-328 was involved in the malignant process of HCC.

Downregulation of miR-328 Inhibited HCC Cell Metastasis In Vitro

To investigate the effect of miR-328 on cell metastasis, we first detected miR-328 expression in selected hepatoma cell lines. As results, it was found that the expression of miR-328 was relatively higher in HepG2 cells compared with the normal human liver-derived cell line (L02) (Figure 1B). Therefore, HepG2 cells

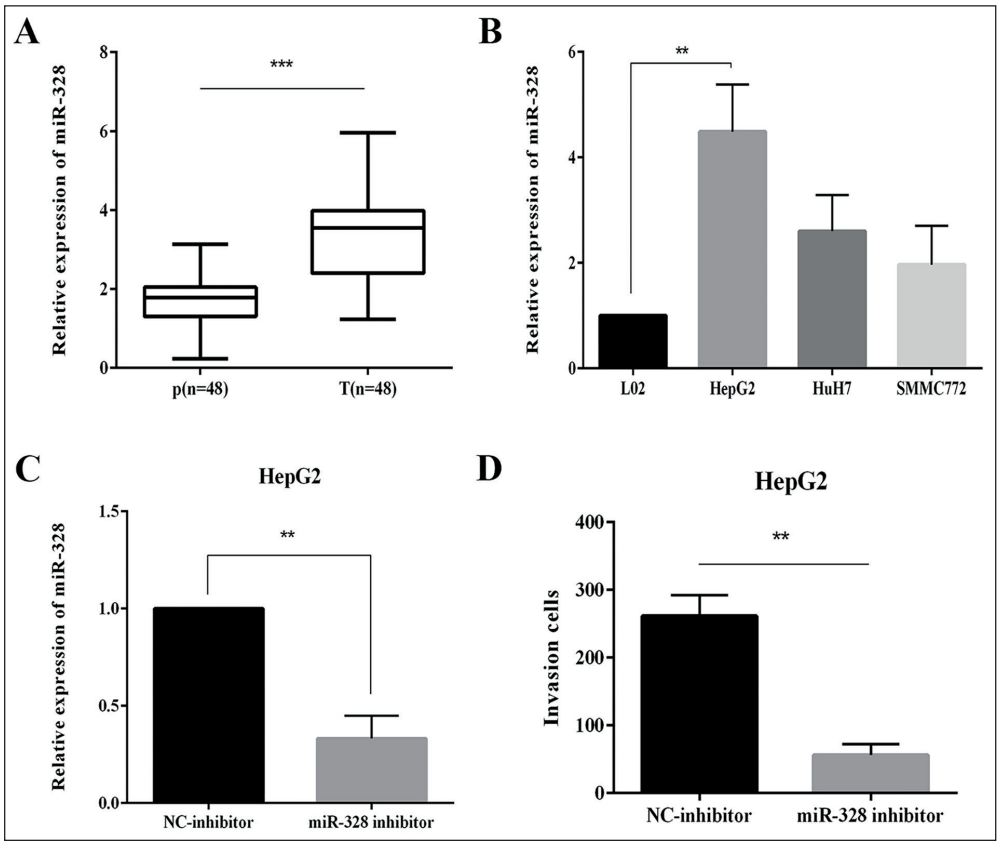

Figure 1. Downregulation of miR-328 inhibited HCC cell metastasis *in vitro*. ***A,*** Analysis of miR-328 expression in HCC tissue samples and adjacent tissues. ***B,*** Analysis of miR-328 expression in HCC cell lines. ***C,*** Analysis of transfection efficiency of miR-328 inhibitor and NC-inhibitor in HCC cells. ***D,*** Transwell assay was performed to determine the metastasis capacity of transfected HCC cells. RNA level was detected by qRT-PCR and GAPDH was used as an internal control. Data of three independent experiments were presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

were selected to further analyze the effect of miR-328 on cell invasion. MiR-328 inhibitor and NC-inhibitor were transfected into HepG2 cells, respectively (Figure 1C), and transwell assay was performed to detect the changes of cell invasion. The results showed that cell metastasis capacity was significantly inhibited in cells transfected with miR-328 inhibitor after 48 h (Figure 1D).

Downregulation of miR-328 Inhibited HCC Cell Proliferation In Vitro

In order to identify the effect of miR-328 on cell proliferation, we transfected miR-328 inhibitor and NC-inhibitor into HepG2 cells, and cell proliferation ability was measured using the CCK-8 assay. The results showed that cell proliferation capacity was significantly inhibited in cells transfected with miR-328 inhibitor after 48 h (Figure 2A).

Downregulation of miR-328 Promoted Cell Apoptosis and Induced Cell Cycle Arrest at G0/G1 Phase

To investigate the role of miR-328 in cell proliferation, we selected HepG2 cells for analyzing the effect of miR-328 on apoptosis and cell cycle. First, miR-328 inhibitor was transfected into HepG2 cells and the effect of miR-328 on cell apoptosis was analyzed by flow cytometry. The results showed that compared with the cells transfected with NC-inhibitor, the ratio of apoptotic cells was significantly decreased in cells transfected with miR-328 inhibitor for 48 h (Figure 2B). These results demonstrated that down-regulation of miR-328 could significantly increase the ability of cell apoptosis.

Similarly, the effect of miR-328 on cell cycle distribution was also measured by flow cytometry. The results indicated that compared with the cells transfected with NC-inhibitor, the number of cells in the G0/G1 phase increased signifi-

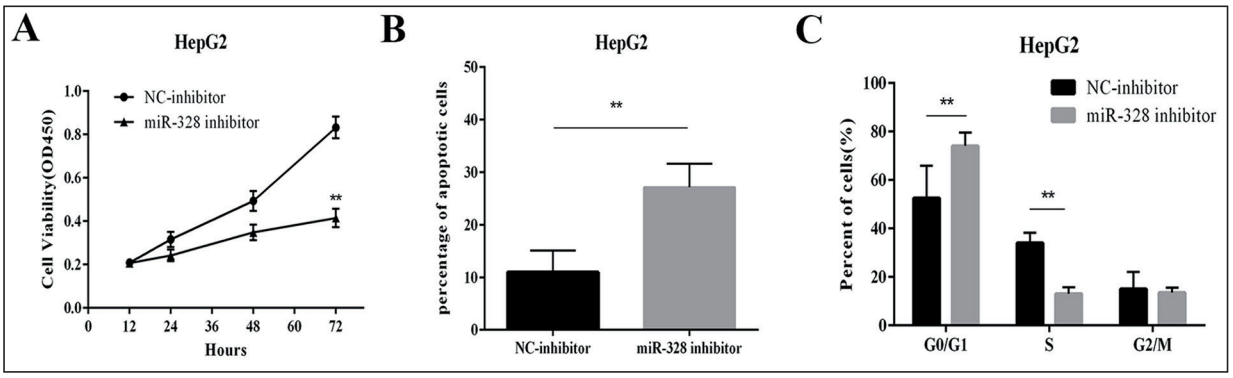

Figure 2. Downregulation of miR-328 suppressed HCC cell proliferation *in vitro*. ***A,*** CCK-8 assay was performed to determine the proliferation capacity of transfected HCC cells. B: Flow cytometric analysis was performed to detect the apoptotic rates of transfected HCC cells. ***B,*** Flow cytometry analysis was performed to detect cell cycle of transfected HCC cells. $^{**}p < 0.01$.

cantly after miR-328 inhibitor was transfected for 48 h, while the cells of S phase significantly decreased (Figure 2C). Our data further revealed that down-regulation of miR-328 expression could significantly induce the cell cycle distribution in the G0/G1 phase.

PTEN Was Directly Targeted by miR-328

TargetScan (www.targetscan.org) was used for miR-328 target gene prediction, and PTEN was selected as a potential target gene based on the prediction (Figure 3A). Luciferase activities of HCC cells transfected with the wild-type or the

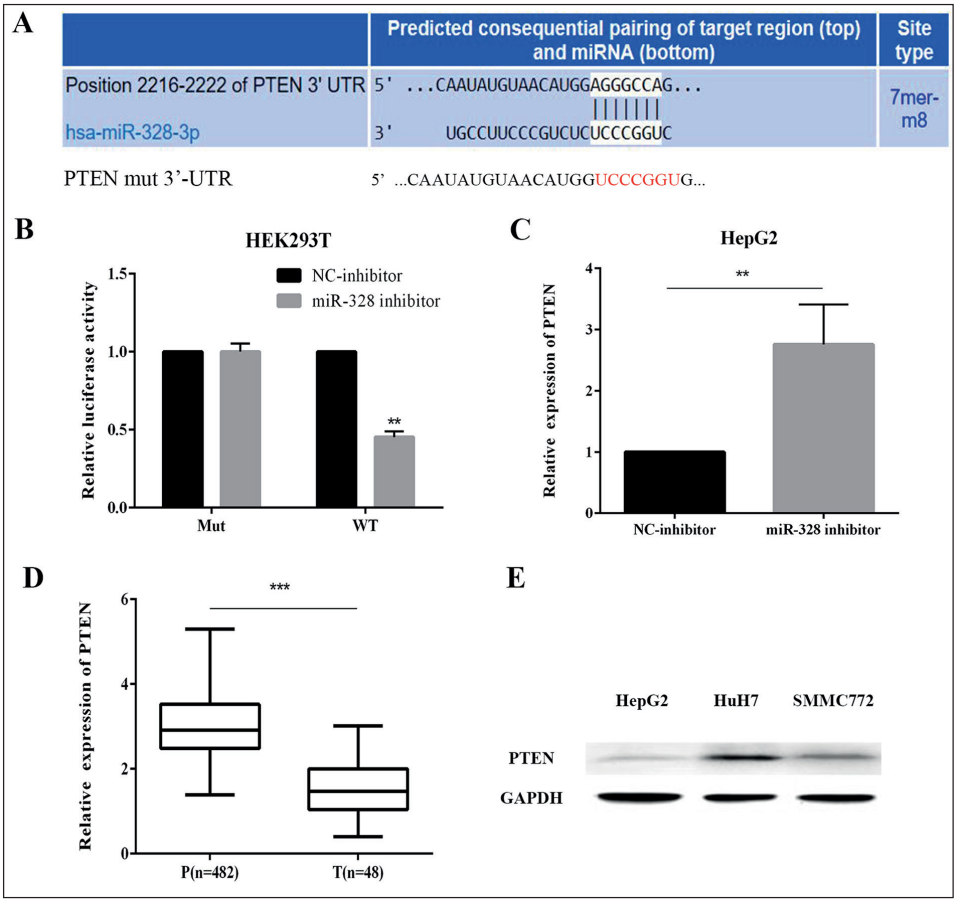

Figure 3. PTEN was directly targeted by miR-328. ***A,*** PTEN was selected as the potential downstream of miR-328 *via* bioinformatics analysis. ***B,*** Luciferase activities in HCC cells transfected with the wild-type or the mutated PTEN 3'-UTR together with miR-328 inhibitor or NC-inhibitor. ***C,*** Analysis of PTEN mRNA expression level of HCC cells transfected with miR-328 inhibitor or NC-inhibitor. ***D,*** Analysis of PTEN expression in HCC tissue samples and adjacent tissues. ***E,*** Analysis of PTEN protein expression level of HCC cell lines. Data of three independent experiments are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

mutated PTEN 3'-UTR together with miR-328 inhibitor or NC-inhibitor also confirmed this result (Figure 3B). At the cellular level, we observed that miR-328 inhibitor significantly increased the expression of PTEN in HepG2 cells (Figure 3C). Moreover, we detected the expression of PTEN in the tumor tissues of HCC patients using qRT-RCR and found that PTEN expression was down-regulated in HCC tissues compared with normal liver tissues (Figure 3D). Consistent with above results, the expression of PTEN was significantly lower in hepatoma-derived cell lines than in L02 cells (Figure 3E). All these identified that PTEN is a direct target gene of miR-328.

Silencing of PTEN Recovered the Carcinogenesis Effect of miR-328

To manifest the relationship of miR-328 and PTEN, we measured the expression correlation between miR-328 and PTEN in HCC tissues. The results indicated that the expression of PTEN was negatively correlated with the expression of miR-328 in HCC tissues (Figure 4A).

Subsequently, we investigated whether PTEN was contributed to the functional effects of miR-328 in HCC tumorigenesis. We silenced the expression of PTEN by transfected with siRNA-PTEN in miR-328-decreased HepG2 cells (Figure 4B). PTEN inhibition not only increased the metastasis and proliferation capacities, but also attenuated cell apoptosis and arrested cell cycle distribution at G0/G1 phase (Figure 4C-F). These results indicated that miR-328 promoted HCC tumorigenesis partially by regulating PTEN.

Discussion

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules with a length of 18-22 nt and play an important role in the regulation of cell differentiation, proliferation, apoptosis and metabolism[13]. The miRNAs bind to the 3'-untranslated region (3'-UTR) of the target mRNA and inhibit the expression of the target gene to regulate a variety of biological processes. It is reported that miR-328 exert tumor promoting and anti-tumor effects in different types of tumors. However, there has been no report on the role of miR-328 in HCC yet. This study focuses on the relationship between miRNA and the development of HCC. The present study demonstrated that miR-328 was significantly upregulated in HCC tissues compared with adjacent tissues, implying that miR-328 might play a potential role in the development of HCC. Besides, downregulated miR-328 could inhibit HCC cell invasion and proliferation, promoting cell apoptosis and arrest cell cycle at G0/G1 phase. All these findings suggested that downregulated miR-328 exerts suppressive effect on cell metastasis and proliferation of HCC.

The mode of action of miRNAs is generally achieved by completely or incompletely combining with the 3'-UTR of the target gene, thereby directly degrading mRNA or inhibiting protein translation[14]. Therefore, finding and determining the functional targets of miRNAs are of great significance in clarifying the mechanism of biological function of corresponding miRNAs in tumors[15]. In this study, we used TargetScan to predict PTEN as the potential target of miR-328. Dual-luciferase reporter system, Western blot and qRT-PCR were conducted to confirm the direct binding relationship between PTEN and miR-328.

Phosphate and tension homologous deleted on chromasome ten (PTEN), also known as MMAC1 (mutated in multiple advanced cancer 1) and TEP1 (TGF-regulated and epithelial cell-enriched phosphatase), is located on chromosome 10q23.3 and consists of 9 exons encoding a protein consisting of 403 amino acids with phosphatase activity[16]. PTEN protein can inhibit the occurrence and development of tumors by antagonizing the activity of phosphorylase such as tyrosine kinase[17]. Previous reports showed that after PTEN was overexpressed in glioblastoma, the growth and invasion of the tumor cells were significantly inhibited, and the tyrosine kinase FAK (focal adhesion kinase) activity was also repressed[18,19]. Besides, abnormal expression of PTEN may also exist in prostate cancer[20], endometrial cancer[21], ovarian cancer[22], breast cancer[23], lung cancer[24], bladder cancer[25], thyroid cancer[26], head and neck squamous cell carcinoma[27], melanoma[28], lymphoma[29] and other tumors. PTEN is considered to be a tumor suppressor downstream of the p53 gene that is extensively and closely related to tumorigenesis. However, the underlying upstream mechanism of PTEN in HCC has not been well identified yet. In the present study, we initially revealed that PTEN was directly targeted by miR-328, and PTEN expression was negatively correlated with miR-328 in HCC tissue. Moreover, silencing of PTEN could abolish tumor suppressive

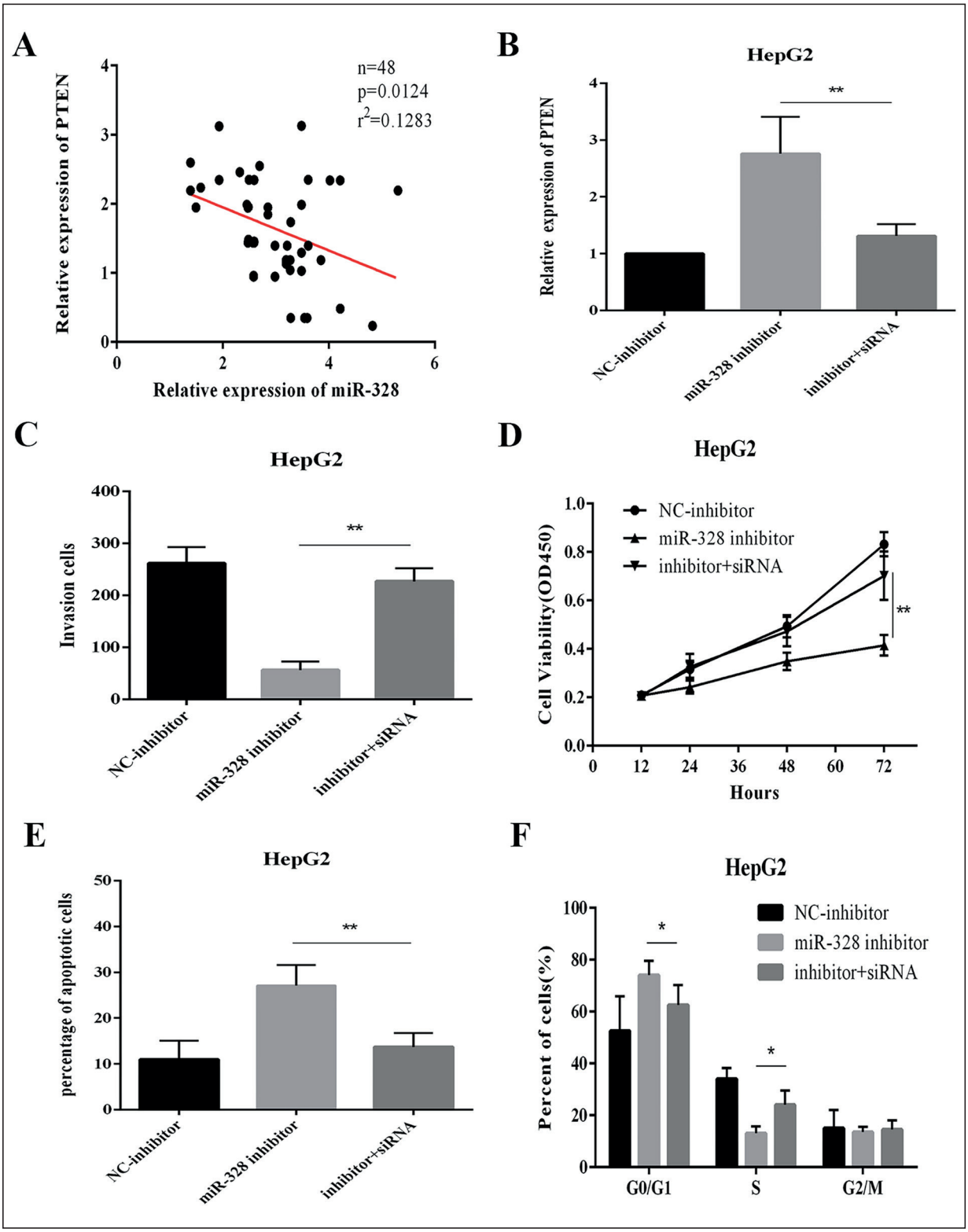

Figure 4. Silencing of PTEN recovered the carcinogenesis role of miR-328. ***A,*** Analysis of the relationship between miR-328 and PTEN expression. ***B,*** Analysis of transfection efficiency of si-PTEN in HCC cells. ***C,*** Reduced PTEN rescued the suppressed cell invasion by miR-328. ***D,*** Reduced PTEN rescued the suppressed cell proliferation by miR-328. ***E,*** Reduced PTEN attenuated the cell apoptosis. ***F,*** Reduced PTEN attenuated the cell cycle distribution at G0/G1 phase. Data of three independent experiments are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$.

effect of miR-328 on HCC cell metastasis and proliferation. This evidence indicated that miR-328 might be the upstream regulator of PTEN involved in HCC tumorigenesis.

Conclusions

We demonstrated that downregulated miR-328 had tumor-suppressive effect on HCC metastasis and proliferation *via* targeting PTEN *in vitro*. Our findings may help to elucidate the molecular mechanisms underlying HCC progression and suggest miR-328/PTEN axis as an important mechanism in the development of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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