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miR-222 enhances hepatocellular carcinoma cell proliferation, migration, and anti-apoptosis via targeting PTEN

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ABSTRACT: Increasing evidence indicates that miRNAs affect the progression of hepatocellular carcinoma (HCC). The target genes and binding sites of miR-222 were predicted by TargetScan website. RT-PCR was used to detect the expression levels of miR-222 and Phosphatase and tensin homolog (PTEN) in HCC tissues and HCC cell lines (HCC-LM3, SMCC7721, and HepG2). CCK-8 assay, flow cytometry, scratch test, and Transwell test were used to detect the proliferation, apoptosis, migration, and invasion capabilities of HCC cells following miR-222 modulation, respectively. Western blotting was performed to analyze the effect of miR-222 inhibitor on the expression of PTEN, Bcl-2, and Bax. The results showed that miR-222 expression was significantly up-regulated, while PTEN expression was significantly down-regulated in HCC tissues and cell lines. miR-222 inhibitor significantly inhibited the proliferation, migration, and invasion of HepG2 cells and promoted the apoptosis of HepG2 cells and the expression of PTEN. In addition, miR-222 expression was negatively correlated with PTEN, and HCC patients with high miR-222 expression and low PTEN expression were significantly associated with larger tumor size, well/moderate histological differentiation, and metastasis. These results suggest that miR-222 inhibitor exerts an anticancer effect on HCC cells, possibly by targeting the target gene PTEN, and provide a theoretical basis for potential new therapeutic targets for HCC.

KEYWORDS: miR-222, phosphatase and tensin homolog, hepatocellular carcinoma, proliferation, migration

INTRODUCTION

Hepatocellular carcinoma (HCC) has the sixth highest incidence among malignant tumors and the fourth highest mortality among cancer-related deaths worldwide [1]. The incidence of liver cancer is very high in China [2], and the number of cases of primary liver cancer accounts for 50% of the total number of cases in the world [3]. Studies have shown that the occurrence of liver cancer is a multi-step process, and the changes of multiple genes and signaling pathways are involved together to drive the transformation of normal cells into highly malignant neoplasms [4]. There are intermediate steps in the occurrence of liver cancer, which are successively accompanied by the inactivation of a variety of genes and abnormal protein expression [5]. Existing studies have only revealed some possible key events in the course of liver cancer, so the research on the regulatory mechanism of liver cancer-related genes and proteins is particularly important for the prevention, early diagnosis, and treatment of liver cancer.

As a kind of small non-coding RNA, miRNAs are widely present in eukaryotes with high conservation, timing, and tissue specificity [6]. miRNAs are often arranged in clusters, and miRNAs in the same gene cluster are often co-expressed [7]. miRNAs play an important role in tumors. Previous studies have confirmed that the abnormal expression of protein-coding genes will lead to the occurrence and development of tumors [8]. The abnormal expression of miRNAs in tumors and their ability to inhibit the synthesis of target proteins provide new ideas for a deeper understanding of tumors [9]. Abnormal expression of miR-222 has been reported in colon cancer [10], pancreatic cancer [3], gastric cancer [11], bladder cancer [12], and glioblastoma [13], and its downstream proteins mainly include p27, PTEN, and TIMP3.

Phosphatase and tensin homolog (PTEN), a member of the protein tyrosine phosphatases (PTP) gene family, is the first tumor suppressor with double phosphatase activity discovered and named by 3 research groups in 1997 [14]. PTEN is localized at 10q23.3 and is approximately 200 kb in length, consisting of 9 exons and 8 introns [15]. PTEN expression can be regulated at multiple levels such as gene level (mutation or

deletion, etc.) [16], transcriptional level (transcription factors) [17], post-transcriptional level (miRNAs or endogenous competition, etc.), and post-translational level (drugs, E3 ligases or deubiquitinizing enzymes, etc.) [18]. PTEN protein limits tumor formation by preventing uncontrolled cell growth [19]. It also helps control cell migration and adhesion and maintains the stability of genetic information [20]. Studies suggest that a slight decrease in PTEN level in human body may lead to tumor susceptibility and may also cooperate with other carcinogenic factors to promote tumor development or affect drug response [21, 22]. Gong et al [23] established that miR-222 promoted ovarian cancer invasion and migration by targeting PTEN, but whether miR-222 could affect behavior by targeting PTEN in HCC has not been reported.

In this study, we found that miR-222 was significantly up-regulated in HCC clinical specimens and PTEN, as a functional target gene of miR-222, was down-regulated. Further experiments were carried out *in vitro* using HCC cell line HepG2. The effects of miR-222 on the expression of PTEN, proliferation, and invasion of HCC cells were analyzed by RT-PCR, Western blotting, CCK-8, flow cytometry, cell scratch test, and Transwell assay.

MATERIALS AND METHODS

Clinical sample collection

Collection of 40 pairs of frozen tissue samples from liver cancer patients and non-tumor tissue samples at Gongli Hospital of Shanghai Pudong New Area. This study obtained the informed consent of the patients themselves and was in line with local ethics. This plan was approved by the Ethics Committee of Gongli Hospital of Shanghai Pudong New Area before implementation.

Cell culture

The human normal liver cell line (L-02) and liver cancer cell lines (HCC-LM3, SMCC7721, and HepG2) were purchased by ATCC (Manassas, VA, USA). The L02, HCC-LM3, and HepG2 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) in a humidified incubator containing 5% CO_2 at 37 °C. The SMCC7721 cell was cultured in RPMI-1640 (Gibco) supplemented with 10% FBS.

Cell transfection

miR-222 inhibitor and miR-222 NC were obtained from RiboBio (Guangzhou, China). The HepG2 cells at logarithmic growth stage were inoculated into 6-well plates for culture overnight. miR-222 inhibitor and miR-222 NC were added to lipofectamine 2000 transfection reagents according to the instructions. The mixture was then added to 6-well plates and cultured for 6–8 h. After refreshing the medium, cells were cultured for an additional 48 h.

Dual-luciferase reporter assay

The wild type (WT) or mutant (Mut) PTEN promoters were constructed into PGL3-basic plasmid (YouBia, Changsha, Hunan, China). HepG2 cells (1×10^5 cells/well) were plated in 48-well plate and then recombinant plasmid of pGL3-PTEN-wt, pGL3-PTEN-Mut, miR-222 NC, and miR-222 mimic was co-transfected using Lipofectamine™ 3000 (Thermo Fisher Scientific, Waltham, MA, USA). HepG2 cells were collected 48 h after transfection, and the luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Thermo Fisher Scientific).

RT-PCR assay

Total RNA from tissues or HepG2 cells was extracted with Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). The reverse transcription (RT) of mRNA was carried out using Oligo (dT) and random primers. The RT system of 10 µl were carried out by SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) on an Applied 7300 Real-Time PCR System (Thermo Fisher Scientific). β-actin was used as an internal reference for mRNA, and U6 were used as an internal reference for miRNAs. The relative expression was calculated through the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

HepG2 cells transfected with miR-222 inhibitor, miR-222 NC, or non-transfected controls for 48 h were washed with precooled culture medium. Then cells were incubated with Cell Cycle and Apoptosis Analysis Kit (MedChemExpress, Monmouth Junction, NJ, USA USA) and analyzed with a flow cytometer (Attune NxT; Thermo Fisher Scientific).

CCK-8 assay

Cell Counting Kit-8 (CCK-8, MedChemExpress) was applied to detect cell proliferation ability. The HepG2 cells transfected with miR-222 inhibitor, miR-222 NC, and non-transfected controls were plated into 96-well plates (1×10^3 cells/well) for 6, 12, 24, and 48 h. Then 10 µl of CCK-8 solution was added to each well and incubated for 2 h. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad Laboratories).

Cell migration assay

HepG2 cells transfected with miR-222 inhibitor, miR-222 NC, and non-transfected controls were inoculated into 12-well plates with 1×10^5 cells per well. After cell growth reached 80%, lines were drawn with the gun head perpendicular to the culture plate, and PBS was added to flush the deleted cells. The plates were placed under the microscope (Leica Microsystems, Wetzlar, Germany) and photographed to record the migration of 0 h cells. Serum-free medium was added into the culture plate and transferred to the incubator for

further culture for 24 h. After the culture plate was removed, it was placed under the microscope to take pictures and record the cell migration in 24 h.

Transwell invasion assay

Transwell invasion assays were performed using 24-well plates (Corning Life Science, Corning, NY, USA). Each Transwell chamber was added with 100 µl of Matrigel and placed in the incubator for 30 min. After the Matrigel was solidified, the excess Matrigel was removed. The HepG2 cells transfected with miR-222 inhibitor, miR-222 NC, and non-transfected controls were resuspended in 200 µl of serum-free medium and added to the upper chamber of the Transwell. Then, 600 µl of complete medium containing 10% FBS was added to the lower chamber. After 24 h incubation in a humidified 37 °C/5% CO_2 incubator, cells in the Transwell chamber were fixed and stained with 0.1% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Then, PBS was added for washing, and the chambers were placed under the microscope for observation.

Western blot analysis

The protein was extracted from HepG2 cells with RIPA Lysis (Thermo Fisher Scientific) and detected using the BCA Protein Assay Kit (Thermo Fisher Scientific). Then the equal protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Billerica, MA, USA). After blocking in 5% BSA at room temperature for 1 h, the membranes were incubated with primary antibodies against PTEN (1:1000, ab137337, Abcam, Cambridge, UK), Bcl2 (1:1000, ab94583, Abcam), Bax (1:1000, ab32503, Abcam), and GAPDH (1:1000, ab9485, Abcam) overnight at 4 °C, followed by incubation with secondary antibody (Goat Anti-Rabbit IgG H&L (HRP); 1:1000; ab205718; Abcam) for 1 h. Lastly, immunoreactivities were visualized by chemiluminescence using the ECL kit (MedChemExpress) and analyzed with Image J software.

Statistical analysis

Data are represented as the mean ± SEM of at least 3 independent experiments. SPSS 20.0 statistical software and GraphPad Prism statistical software were applied for statistical analyses. The difference between 2 groups was compared using two-tailed student's t-test, or one-way analysis of variance (ANOVA). p-values less than 0.05 were considered statistically significant.

RESULTS

The expression of miR-222 and PTEN in HCC patient specimens and HCC cell lines

The expression levels of miR-222 and PTEN in 40 pairs of HCC tissues and adjacent tissues were detected by RT-PCR. The results showed that miR-222 expression was significantly up-regulated (Fig. 1A), while PTEN was significantly down-regulated (Fig. 1B) in HCC tissues compared with adjacent tissues. Furthermore, we explored the correlation between miR-222 and PTEN in HCC tissues. Linear regression analysis showed that the expression of miR-222 was negatively correlated with PTEN in HCC tissues ($r = -0.4935$, $p = 0.0001$, Fig. 1C). The expressions of miR-222 and PTEN in human normal hepatocyte cell line (L-02) and HCC cell lines (HCC-LM3, SMCC7721, and HepG2) were further detected. The results showed that the expression of miR-222 was higher in HCC cells than in L-02 cells (Fig. 1D), and the expression of PTEN was lower in HCC cells than in L-02 cells (Fig. 1E).

To clarify the clinicopathological role of miR-222 and PTEN in HCC, 40 patients were divided into miR-222 high expression group ($n = 23$) and miR-222 low expression group ($n = 17$) or PTEN high expression group ($n = 21$) and PTEN low expression group ($n = 19$) according to the cut-off value. As shown in Table 1, patients with high miR-222 expression and low PTEN expression were significantly associated with larger tumor size ($p = 0.041$), well/moderate histological differentiation ($p = 0.027$), and metastasis ($p = 0.026$). Taken together, these results suggest that miR-222 expression is up-regulated and PTEN expression is down-regulated in HCC, which is closely associated with poor HCC.

miR-222 directly regulates PTEN

The TargetScan website was used for bioinformatics prediction of miR-222 target genes and the binding sites in the non-coding regions. The prediction results showed that miR-222-5p has binding site at nucleotides 1269-1275 of the PTEN 3′UTR (Fig. 2A). The positions of the introduced mutations in the 3′UTR of PTEN mRNA were shown in Fig. 2B. Dual-luciferase reporter gene assay (Fig. 2C) showed that miR-222 mimic expression remarkably decreased the luciferase activity of PTEN mutation. These results confirmed that the 3′UTR of PTEN mRNA was a direct target gene of miR-222.

miR-222 inhibitor promotes apoptosis and inhibits proliferation, migration, and invasion of HepG2 cells

Down-regulation of miR-222 significantly inhibits the proliferation, migration, and invasion of HepG2 cells and induces apoptosis. Transfection efficiency was measured using qRT-PCR, and the results demonstrated that the relative expression of miR-222 was decreased by the miR-222 inhibitor. Flow cytometry was performed to assess the effects of miR-222 on HepG2 cell apoptosis. The statistical results of the apoptosis rate showed that compared with the control and miR-NC groups, the apoptosis rate of the miR-222 inhibitor group significantly increased (Fig. 3A,B). The

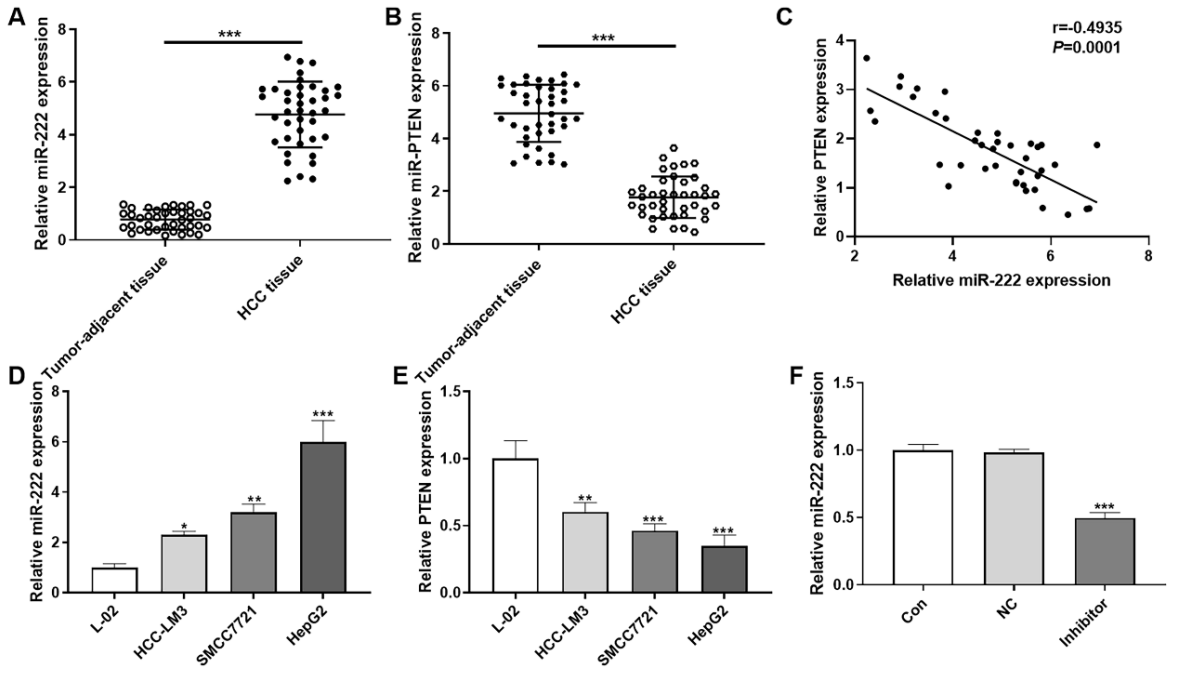


Fig. 1 Up-regulation of miR-222 and down-regulation of PTEN in HCC tissues and HCC cell lines. (A and B) The RT-PCR analysis of the mRNA expression level of miR-222 (A) and PTEN (B) in HCC tissue and Tumor-adjacent tissue ($n = 40$). (C) Negative relationship between the miR-222 level and PTEN expression in 40 HCC tissues. (D and E) The RT-PCR analysis of the mRNA expression level of miR-222 (D) and PTEN (E) in human normal hepatocyte cell line (L-02) and HCC cell lines (HCC-LM3, SMCC7721, and HepG2). (F) The RNA expression of miR-222 in HepG2 cells was detected by RT-PCR analysis after miR-222 inhibitor transfection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Tumor-adjacent tissue, L-02, or Control group. miR, microRNA; NC, negative control; HCC, hepatocellular carcinoma.

Table 1 Correlation between miR-222 or PTEN and the characteristics of liver cancer patients.

Factor	Case (n)	miR-222		p value	PTEN		p value
		Low	High		Low	High	
Sex							
Male	29	13	16	0.629	14	15	0.873
Female	11	4	7		5	6	
Age (years)							
⩽60	27	13	14	0.298	13	14	0.906
>60	13	4	9		6	7	
Maximum tumor size (cm)							
⩽5.0	15	3	12	0.026	4	11	0.041
>5.0	25	14	11		15	10	
Histological grade							
Well/moderate differentiation	20	12	8	0.025	13	7	0.027
Poor differentiation	20	5	15		6	14	
Metastasis							
No	33	11	22	0.011	13	20	0.026
Yes	7	6	1		6	1	

Values are expressed as the mean ± standard error of the mean.

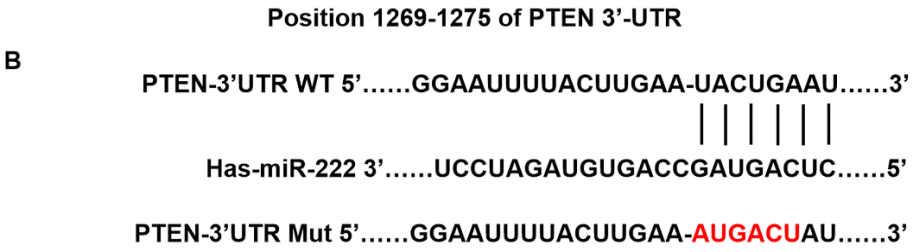
proliferation of HepG2 cells was detected by CCK-8 assay. The results showed that the proliferation rate in the miR-222 inhibitor group was significantly reduced compared with that in the miRNA-NC control group (Fig. 3C).

The migration and invasion ability of HepG2 cells was assessed using a wound healing assay and Transwell chambers, respectively. The results showed that miR-222 inhibitor significantly inhibited the migration (Fig. 4A,B) and cell invasion ability (Fig. 4C,D) of HepG2 cells. These results confirmed that miR-222 inhibitor can promote apoptosis and inhibit proliferation,

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}	Predicted relative K_D
Position 1269-1275 of PTEN 3' UTR hsa-miR-222-5p	5' ...GGAAUUUUACUUGAA-UACUGAAU... 3' UCCUAGAUGUGACCGAUGACUC	7mer-A1	-0.15	83	-0.14	0	N/A	N/A

B



C

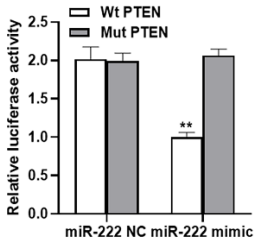


Fig. 2 miR-222 targets the 3′UTR of PTEN. (A) miR-222 was predicted to target 3′UTR of PTEN. (B) The mutation introduced in the 3′UTR of PTEN mRNA. (C) The luciferase reporter assay was detected in HepG2 cells cotransfected with the recombinant reporter plasmid and miR-222 NC/mimic, firefly luciferase activity was normalized to the Renilla luciferase control. ** $p < 0.01$ vs. miR-222 NC-Wt PTEN group. miRNA, microRNA; UTR, untranslated region; mut, mutant; Wt, wild-type; NC, negative control; PTEN, phosphatase and tensin homolog.

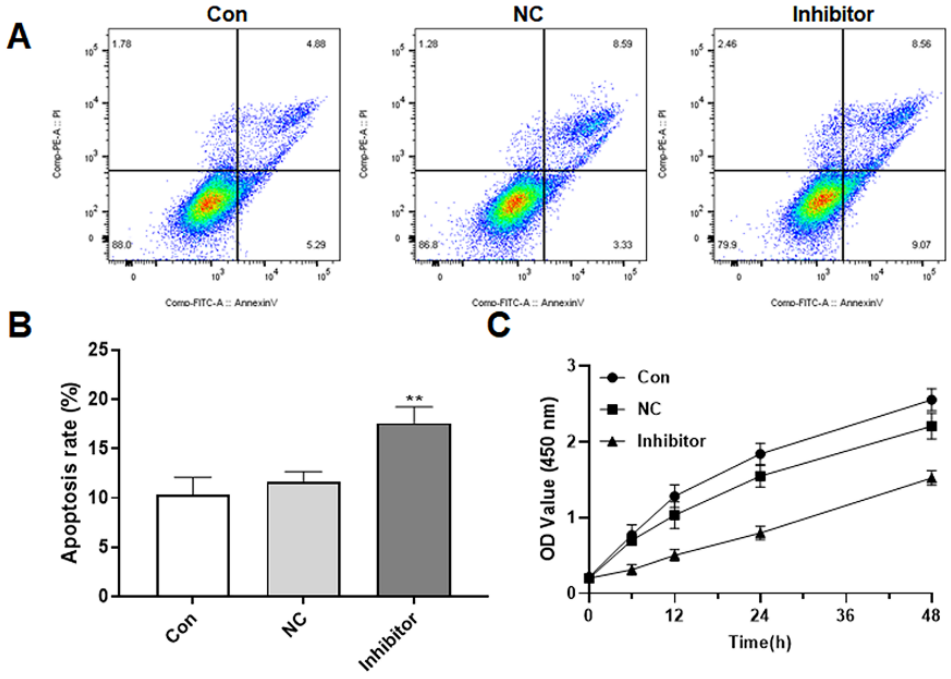


Fig. 3 miR-222 inhibitor promotes the apoptosis rate and reduces the proliferation of HepG2 cells. (A) Flow cytometry assay of HepG2 cell apoptosis with miR-222 inhibitor transfection. (B) Statistical HepG2 cell apoptosis rate of (A). (C) CCK-8 assay of HepG2 cell proliferation with miR-222 inhibitor transfection. ** $p < 0.01$ vs. Control group. miR, microRNA; NC, negative control; Con, control; OD, optical density.

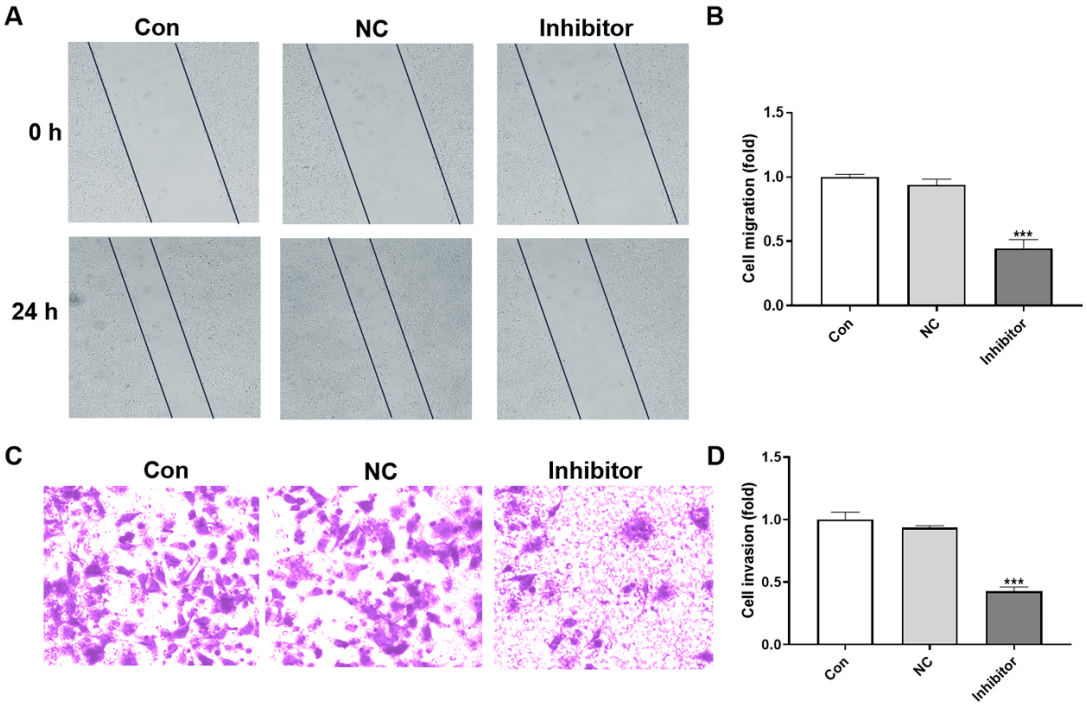


Fig. 4 miR-222 inhibitor inhibits HepG2 cell migration and invasion. (A and B) The cell migration ability was detected by a wound healing assay. (C and D) The cell invasion ability was assessed using Transwell chambers. *** $p < 0.001$ vs. Control group. miR, microRNA; NC, negative control; Con, control.

migration, and invasion of HepG2 cells.

miR-222 inhibitor inhibits the expression of PTEN in HePG2 cells

We examined the protein expression of PTEN in HepG2 cells after miR-222 inhibitor transfection. The results of Western blotting showed that the protein levels of PTEN were significantly up-regulated in miR-222 inhibitor transfected HepG2 cells (Fig. 5A,C). In addition, the protein expression of the apoptotic proteins (Bax and Bcl-2) was examined. Consistent with the flow cytometry results, miR-222 inhibitor significantly inhibited the expression of Bcl-2 and increased the protein expression level of Bax (Fig. 5B,D,E).

DISCUSSION

In this study, we predicted the target genes of miR-222 and its effect on the biological behavior of HCC cells. The current data demonstrate that (1) PTEN is the target gene of miR-222, with a predicted binding site in its 3′UTR; (2) High expression and low expression of PTEN in HCC tissues and HCC cell lines are negatively correlated with the poor prognosis of HCC patients; (3) miR-222 inhibitor promoted HepG2 cell apoptosis and PTEN expression; (4) miR-222 inhibitor inhibited the proliferation, migration, and invasion of HepG2 cells. These data reveal that miR-222 inhibitor prevents the progression of HCC cells by targeting the 3′UTR of PTEN mRNA, providing a possible therapeutic target for the treatment of HCC.

Increasing evidence suggests that miRNAs play an important role in the molecular mechanism of HCC development. The expression of miR-222 is increased in most tumors, acting as an oncogene [24]. In lung adenocarcinoma [25], colorectal cancer [26], and breast cancer [27], the high expression of miR-222 is associated with poor prognosis. miR-222 may be involved in tumor proliferation, cell cycle regulation, apoptosis, and metastasis [28]. It promoted the proliferation of epithelial ovarian cancer [29] and breast cancer [30] by down-regulating p27(kip1). At present, researchers have explored the role of miR-222 in HCC. Wong et al [31] found that there were 40 significantly differentially expressed miRNAs in HCC tissues compared with non-HCC normal tissues, and miR-222 was the most significantly up-regulated miRNA and exerted migration advantage by enhancing AKT signaling pathway, but inhibition of miR-222 activity seemed to have no effect on cell viability of Hep3B and HKCI-9 cells. Liu et al [32] found that miR-222-3p was highly expressed in HCC cell lines and was involved in the proliferation, migration, and invasion of HCC cells. In our study, we found that miR-222-5p was significantly up-regulated in HCC patient tissues and cell lines. Clinically, its overexpression correlated with shorter overall survival. Inhibition of miR-222-

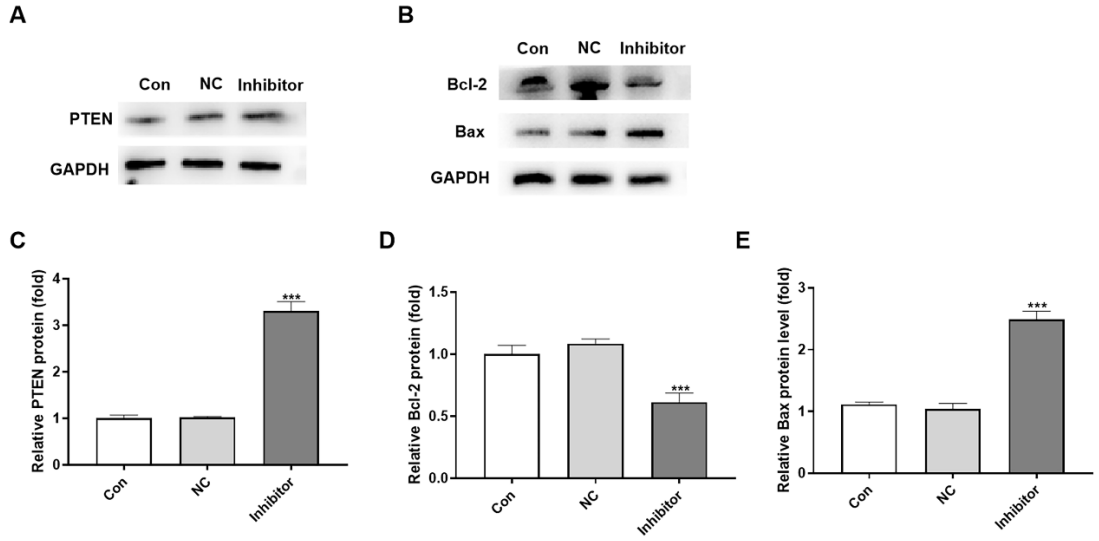


Fig. 5 miR-222 inhibitor promotes the expression of PTEN in HepG2 cells. (A and B) The expression of PTEN (A), Bcl-2, and Bax (B) protein in HepG2 cells with miR-222 inhibitor transfection was assessed by Western blotting. (C–E) The corresponding gray value statistics of PTEN, Bcl-2, and Bax protein. *** $p < 0.001$ vs. Control group. miR, microRNA; NC, negative control; Con, control.

5p further inhibited the proliferation, migration, and invasion and promoted apoptosis of HepG2 cells.

PTEN can regulate the phosphatase activity of various protein molecules in cells, resulting in the deletion and mutation of this gene, which plays an important role in the occurrence and development of tumors [33]. PTEN gene is the only tumor suppressor gene with phosphatase activity discovered so far in human. It dephosphorylates PIP3, the key second messenger in the PI3K/AKT pathway, thereby suppressing oncogenic signaling and tumor progression [34]. Previous studies have demonstrated an inverse correlation between PTEN expression levels and prognosis in HCC patients, with PTEN loss associated with significantly poorer survival outcomes [35]. Our results showed that PTEN was down-regulated in HCC tissues and cell lines and was closely related to the grade, differentiation, and metastasis of HCC. In addition, the PTEN 3′UTR region was predicted to have a binding site with miR-222-5p by TargetScan, and dual-luciferase assay results showed that miR-222 mimic significantly reduced the luciferase activity of PTEN mutant.

The limitation of this paper is that we did not further validate the effect of miR-222 on HepG2 biological behavior after PTEN overexpression by rescue experiments, which were further confirmed at the animal level.

CONCLUSION

In summary, our study demonstrated that miR-222 was up-regulated and PTEN was down-regulated in HCC patients and HCC cell lines. This inverse correlation was clinically significant, as the miR-222 high/PTEN low expression pattern strongly correlated with poor patient prognosis. miR-222 inhibitor promoted apoptosis and inhibited the expression level of PTEN, cell proliferation, migration, and invasion of HePG2 cells.

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