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MicroRNA-20b promotes proliferation of H22 hepatocellular carcinoma cells by targeting PTEN

JING HE[1*], MIMI MU[1*], YULAN LUO[1], HELONG WANG[1], HUA MA[1], SHUJUN GUO[1], QIANG FANG[2], ZHONGQING QIAN[1], HEZUO LU[1,3] and CHUANWANG SONG[1]

Departments of [1]Immunology and [2]Microbiology and Parasitology, Bengbu Medical College, and Anhui Key Laboratory of Infection and Immunity at Bengbu Medical College, Bengbu, Anhui 233030; [3]Clinical Laboratory, The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui 233004, P.R. China

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Abstract. MicroRNAs (miRNAs/miRs) are small, noncoding RNA molecules that are closely associated with the occurrence and development of tumors. miR-20b is overexpressed in hepatocellular carcinoma cell lines and tissues. However, it is not clear whether miR-20b can promote the proliferation of hepatocellular carcinoma cells. In the present study, the proliferation of H22 mouse hepatocellular carcinoma cells was detected using the Cell Counting Kit-8 assay. MiRanda software was used to predict the binding sites of miR-20b to the 3'-untranslated region (3'-UTR) of phosphatase and tensin homolog (*PTEN*). The 3'-UTR sequence of the *PTEN* gene was amplified using the polymerase chain reaction in H22 cells. The recombinant plasmid or empty plasmid was co-transfected with miR-20b mimics or miR-20b scramble into HeLa cells, and luciferase activity was assessed by Dual-Luciferase® Reporter Assay System 24 h post-transfection. In the present study, miR-20b knockdown significantly inhibited the proliferation of H22 mouse hepatocellular carcinoma cells. In addition, miR-20b inhibition upregulated the expression of *PTEN*, and it was revealed that miR-20b may directly target the 3'-untranslated region of the *PTEN* gene. Downregulation of *PTEN* partially reversed the anti-proliferative effect of miR-20b on H22 cells. In conclusion, miR-20b may promote H22 cell proliferation by targeting *PTEN*, providing a rationale for further study investigating novel therapeutic strategies for liver cancer.

Correspondence to: Dr Chuanwang Song, Department of Immunology, Bengbu Medical College, and Anhui Key Laboratory of Infection and Immunity at Bengbu Medical College, 2600 Donghai Avenue, Bengbu, Anhui 233030, P.R. China
E-mail: chuanwangsong@163.com

Dr Hezuo Lu, Clinical Laboratory, The First Affiliated Hospital of Bengbu Medical College, 287 Chang Huai Road, Bengbu, Anhui 233004, P.R. China
E-mail: lhz233003@163.com

[*]Contributed equally

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Introduction

Annually, ~780,000 patients are diagnosed worldwide with liver cancer; this type of cancer accounted for ~740,000 cases of mortality in 2012. Between 70 and 90% of primary liver cancer cases are hepatocellular carcinoma (1-3). Viral infection with hepatitis B or C, and alcohol consumption, are among the most common factors that promote the occurrence and development of liver cancer. However, despite investigation of the underlying causes and pathogenesis of hepatocellular carcinoma, survival remains poor (4,5).

MicroRNAs (miRNAs/miRs) are a type of noncoding RNA that contain 19-22 nucleotide pairs. miRNAs inhibit post-transcriptional gene expression by binding to the 3'-untranslated region (3'-UTR) of the target gene (6). miRNAs are associated with tumor cell proliferation, differentiation, apoptosis, migration and invasion (7), and have been implicated in hepatocellular carcinoma growth (8). miR-20b belongs to the miR-106a-363 gene cluster, and together with the miR-17-92 and miR-106b-25 clusters, it forms the miR-17 gene (9), which is active in the oncogenesis of certain types of human tumor (10). Increased miR-20b expression has previously been associated with decreased survival rate in gastric cancer (11), promotion of proliferation and migration of prostate cancer cells (12), and proliferation and DNA synthesis of breast cancer cells (13). miR-20b expression is altered in hepatocellular carcinoma, although its activity has not yet been described. In the present study, it was suggested that miR-20b promoted the proliferation of the mouse hepatocellular carcinoma H22 cell line by directly targeting the phosphatase and tensin homolog (*PTEN*) gene and negatively regulating its expression. *PTEN* was at least partially involved in the promotion of H22 cell proliferation by miR-20b.

Materials and methods

Cell culture and transfection. Mouse H22 hepatocellular carcinoma cells were purchased from Nanjing Keygen

Biotech Co., Ltd. (Nanjing, China). Cells were cultured in RPMI-1640 complete medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a humidified incubator containing 5% CO_2. Cells were passaged once every 3-4 days. miR-20b inhibitor and a scrambled RNA control were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells (10^6 cells/ml) were transfected with the miR-20b inhibitor and control (20 nmol/l) at 37˚C for 24 h, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) reagents according to the manufacturer's protocols. The sequences were as follows: miR-20b inhibitor sequence, 5'-CUACCU GCACUAUGAGCACUUUG-3'; miR-20b inhibitor control sequence, 5'-CAGUACUUUUGUGUAGUACAA-3'; miR-20b mimics sequence forward, 5'-CAAAGUGCUCAUAGUGCA GGUAG-3' and reverse, 5'-ACCUGCACUAUGAGCCAC UUUGUU-3'; miR-20b mimics control sequence forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3'.

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit (CCK)-8 assay (Beyotime Institute of Biotechnology, Haimen, China). Briefly, H22 cells were seeded in 96-well plates at $3x10^3$ cells/well, and were incubated with 10 μl CCK-8 reagent for 2 h at 37˚C. Absorbance was measured at 24, 48, 72 and 96 h at 450 nm using a microplate reader.

Construction of dual luciferase recombinant plasmids. Total RNA was isolated from H22 cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed to first strand cDNA using TransScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. The miR-20b binding site in the *PTEN* 3'-UTR region sequence was predicted using the miRanda algorithm. The 3'-UTR sequence of the *PTEN* gene was then amplified by polymerase chain reaction (PCR) using TransStart FastPfu DNA Polymerase (TransGen Biotech Co., Ltd., Beijing, China). The reaction conditions were: 95˚C predenaturation, 1 min; 95˚C denaturation, 20 sec; 57˚C annealing, 20 sec; 72˚C extension, 30 sec; 40 cycles. The length of the amplified product was ~318 bp. The primer PCR amplification sequences were as follows: Forward 5'-CCGCTCGAGCCCTCCTTGCTATCCT-3' and reverse 5'-GAATGCGGCCGCTCCCGATGAAACCTC-3'. The miRNA reporter vector plasmid (pmiR-RB-Report system; Applied Biosystems; Thermo Fisher Scientific, Inc.) and the amplified 3'-UTR *PTEN* gene sequence were double digested with *Xho*I and *Not*I. The digested products were separated using electrophoresis on a 1.5% agarose gel, and the gels were cut to recover the purified product. The ligation reaction of the purified product was performed at 16˚C using the TaKaRa DNA Ligation kit Ver. 2.0 (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol, and the ligation product was transformed into competent DH5α cell lines (Beyotime Institute of Biotechnology, Haimen, China) for expansion culture for 16 h at 37˚C. The pmiR-RB-Reporter-PTEN 3'-UTR dual luciferase recombinant plasmid was extracted by concentration from bacterial culture supernatant using DNA extraction kit (Axygen Biosciences, Union City, CA, USA). Recombinant plasmids were digested by *Xho*I and *Not*I restriction enzymes and sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing.

Luciferase activity assay. The recombinant plasmid or empty plasmid was co-transfected with miR-20b mimics or miR-20b scramble into HeLa cells. The detection of luciferase activity was divided into four groups: (i) pmiR-RB-Report empty plasmid+miR-20b scramble; (ii) pmiR-RB-Report empty plasmid+miR-20b mimics; (iii) pmiR-RB-Report-PTEN 3'-UTR recombinant plasmid+miR-20b scramble; and (iv) pmiR-RB-Report-PTEN 3'-UTR recombinant plasmid+miR-20b mimics. Luciferase activity was assessed by the luciferase activity assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. HeLa cells were washed with PBS and lysed with freshly prepared lysis buffer (Promega Corporation, Madison, WI, USA). The luciferase activity was evaluated in 96-well black plates and the results were expressed as the ratio of *Renilla* and firefly luciferase fluorescence.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from H22 cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was then reverse transcribed to cDNA (Easy Script First-Strand cDNA Synthesis SuperMix kit; TransGen Biotech Co., Ltd.) and amplified by PCR (TransStart Tip Green qPCR SuperMix kit; TransGen Biotech Co., Ltd.), according to the manufacturer's protocol (Beijing TransGen Biotech Co., Ltd., Beijing, China). The PCR conditions were 94˚C for 30 sec; 94˚C for 5 sec, 60˚C for 30 sec, for a total of 40 cycles. The $2^{-\Delta\Delta Cq}$ method (14) was used to determine the relative expression of each gene; miR-20b expression was normalized against U6 small nuclear RNA as an internal reference. Primer sequences were as follows: miR-20b RT primer, GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCTACCT; forward, 5'-ATG CCAAAGTGCTCATAGTG-3'; reverse, 5'-GTGCAGGGT CCGAGGT-3'; U6 RT primer, 5'-AACGCTTCACGAATT TGCGT-3'; forward, 5'-CTCGCTTCGGCAGCACA-3'; and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

RNA interference. Transfection of interfering RNA fragments was performed with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells (10^6 cells/ml) were transfected 48 h at 37˚C with 30 nM *PTEN* small interfering (si)RNA or scramble siRNA, which was synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). PTEN siRNA sense, 5'-GGUGAA ACUAUACUUUACATT-3' and antisense, 5'-UGUAAAGUA UAGUUUCACCTT-3'; scramble siRNA sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'.

Western blotting. Western blot analyses were performed as previously described (15). Briefly, cells were harvested and lysed using NP-40 lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration of the cell lysates was quantified using the bicinchoninic acid assay. Equal

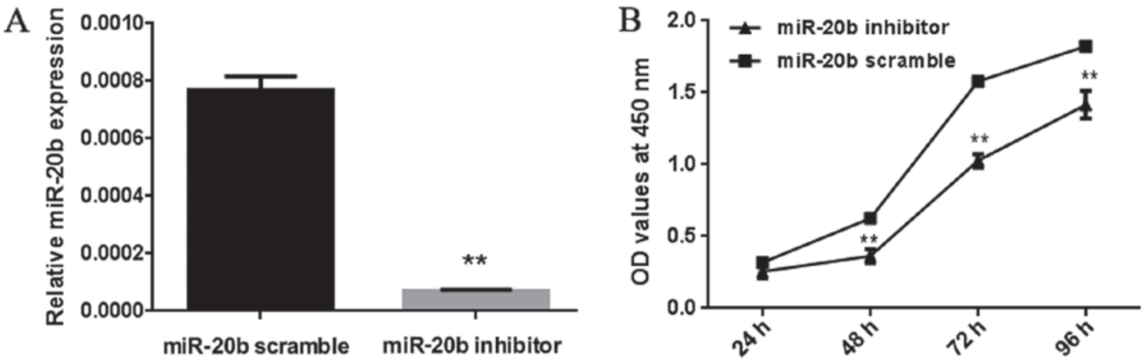

Figure 1. miR-20b promotes H22 cell proliferation *in vitro*. H22 cells were transfected with miR-20b inhibitor or scrambled miR-20b. (A) Effects of the miR-20b inhibitor on miR-20b expression were confirmed by reverse transcription-quantitative polymerase chain reaction. (B) Cell proliferation was assessed in both groups using the Cell Counting kit-8 method. **P<0.01. miR-20b, microRNA-20b; OD, optical density.

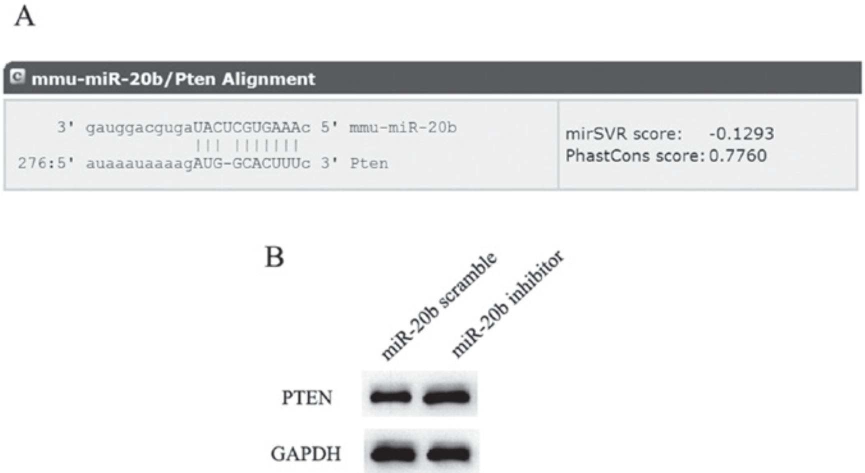

Figure 2. miR-20b negatively regulates PTEN expression in H22 cells. (A) The miRanda algorithm predicted that *PTEN* was a target gene for miR-20b binding. (B) Western blotting demonstrated that PTEN expression was increased in H22 cells transfected with miR-20b inhibitor compared with the controls. miR-20b, microRNA-20b; PTEN, phosphatase and tensin homolog.

aliquots of sample protein were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature with 5% non-fat dry milk for 2 h, and were subsequently incubated with anti-GAPDH antibody (cat. no. AF0006; Beyotime Institute of Biotechnology) and anti-PTEN antibody (cat. no. AF1426; Beyotime Institute of Biotechnology) at a 1:1,000 dilution, overnight at 4˚C. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology) for 2 h at room temperature and bands were visualized by enhanced chemiluminescence using Immobilon™ Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Data are expressed as the mean ± standard error of the mean from at least three experiments. Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance followed by the least significant difference test was used for comparison between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-20b promotes the proliferation of H22 cells in vitro. The effects of miR-20b on the proliferation of H22 cells were investigated using the CCK-8 assay, by comparing proliferation between cells that were transfected with miR-20b inhibitor or miR-20b scramble control. Compared with the transfected control group, transfection with the miR-20b inhibitor significantly reduced miR-20b expression levels in H22 cells (Fig. 1A). CCK-8 assay results at 48, 72 and 96 h revealed a significant inhibition in the proliferation of miR-20b-transfected H22 cells compared with that in the control cells (Fig. 1B).

miR-20b negatively regulates PTEN expression in H22 cells. Investigation of the mechanism of action of miR-20b was guided by the miRanda prediction of *PTEN* as a potential target gene of miR-20b (microRNA.org) (Fig. 2A). *PTEN* is a tumor suppressor gene which inhibits the proliferation of various types of tumor, including bladder cancer, breast cancer and colon carcinoma (16). In the present study, downregulation of miR-20b in H22 cells significantly upregulated *PTEN*

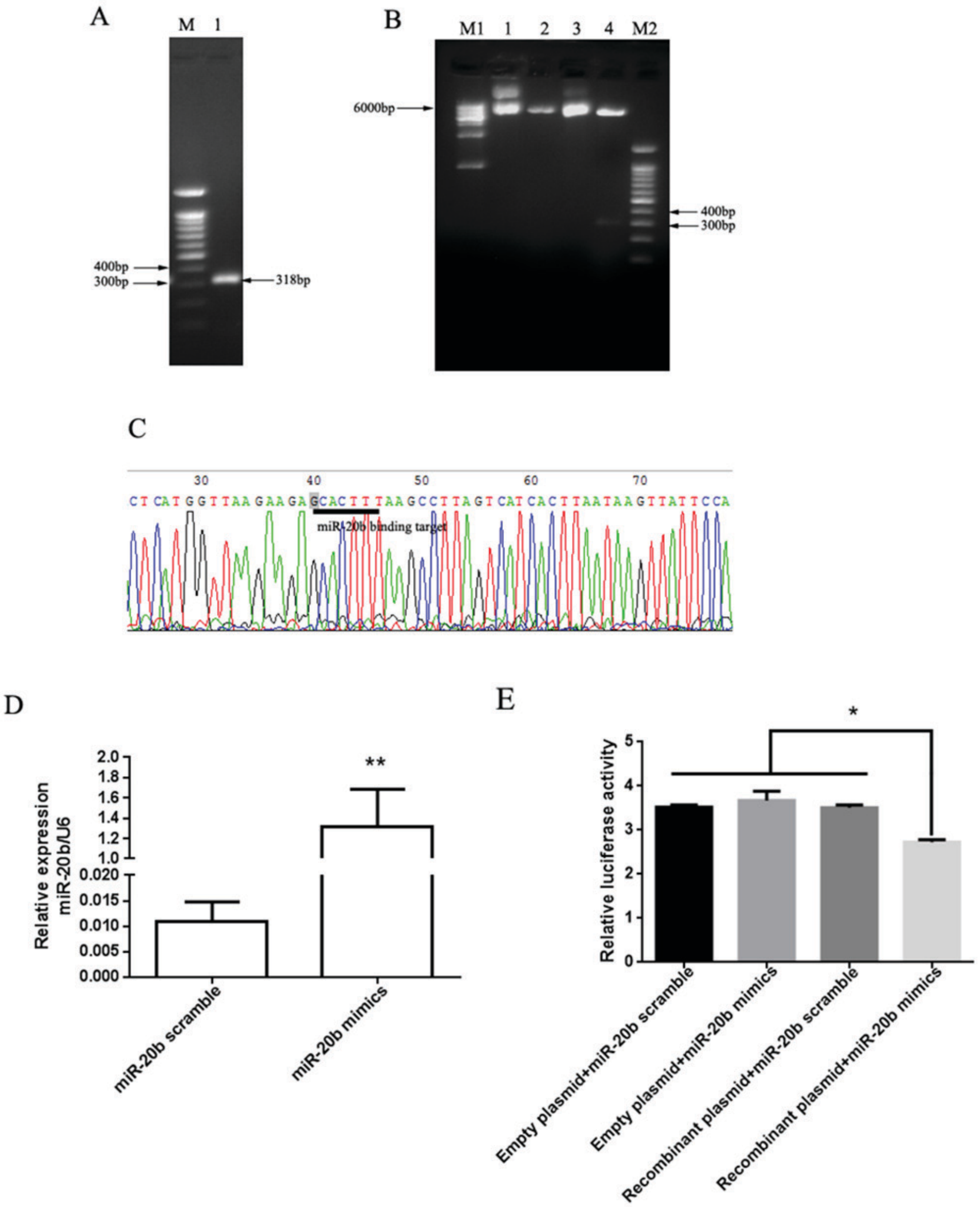

Figure 3. *PTEN* is a direct target gene of miR-20b. (A) *PTEN* 3'-UTR including the miR-20b binding sequence was amplified by polymerase chain reaction and amplification products were separated by 1% agarose gel electrophoresis. (B) pmiR-RB-Reporter-PTEN 3'-UTR recombinant plasmids and blank plasmids were digested with *Xho*I and *Not*I, and the digested products were separated by 1% agarose gel electrophoresis. (C) Partial sequences of recombinant plasmids including the miR-20b binding site. (D) Successful miR-20b mimic transfection was confirmed by reverse transcription-quantitative polymerase chain reaction. **P<0.01, compared with miR-20b scramble (E) Following recombinant or blank plasmid co-transfection with miR-20b mimics or miR-20b scramble into H22 cells, a dual luciferase reporter assay was used to measure luciferase activity. *P<0.05, compared with recombinant plasmids+miR-20b mimics. 3'-UTR, 3'-untranslated region; miR-20b, microRNA-20b; PTEN, phosphatase and tensin homolog.

expression (Fig. 2B). These findings suggested that the expression of *PTEN* was negatively regulated by miR-20b.

PTEN is a direct target gene of miR-20b. The direct targeting of *PTEN* by miR-20b was confirmed with a dual luciferase reporter vector assay. The *PTEN* 3'-UTR fragment containing the miR-20b binding site (Fig. 3A) was cloned and inserted into the pmiR-RB-Reporter plasmid vector system. The pmiR-RB-Reporter-PTEN 3'-UTR dual luciferase recombinant plasmid vectors were identified by double enzyme digestion electrophoresis (Fig. 3B) and DNA sequencing (Fig. 3C). The miR-20b mimics or control, and recombinant or empty plasmids were co-transfected into H22 cells. Post-transfection of H22 cells with miR-20b mimics, the expression levels

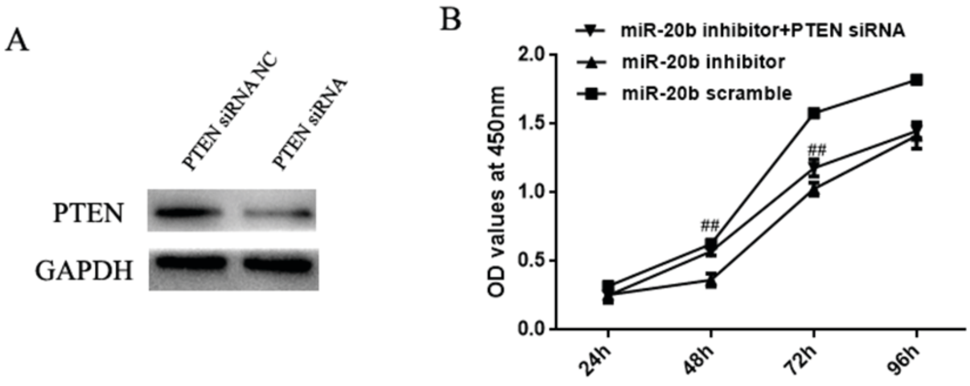

Figure 4. Downregulation of *PTEN* partially reverses the anti-proliferative effects of miR-20b inhibitor on H22 cells. (A) *PTEN*-specific siRNA was used to transfect H22 cells, and PTEN protein expression was assessed by western blotting 48 h post-transfection. (B) H22 cells were transfected with miR-20b inhibitor or miR-20b scramble, and with *PTEN* siRNA. Cell proliferation was assessed using the Cell Counting kit-8 assay. $^{\#\#}$P<0.01 compared with miR-20b inhibitor. miR-20b, microRNA-20b; OD, optical density; NC, negative control; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA.

of miR-20b were increased by ~120-fold compared with the control group (Fig. 3D). The results of the dual luciferase reporter assay revealed that miR-20b significantly reduced recombinant plasmid luciferase activity (Fig. 3E). These results indicated that miR-20b may directly target *PTEN* in H22 cells.

Downregulation of PTEN partially reverses the anti-proliferative effects of miR-20b inhibitor in H22 cells. To confirm that the effect of miR-20b on H22 cell proliferation may be mediated directly by targeting *PTEN*, H22 cells were transfected with *PTEN* siRNA and miR-20b inhibitor. Initially, total protein was extracted from the siRNA-transfected H22 cells and *PTEN* protein expression was assessed by western blotting. As illustrated in Fig. 4A, *PTEN* expression was downregulated in response to *PTEN* siRNA. The CCK-8 results (Fig. 4B) revealed that the miR-20b inhibitor significantly reduced H22 cell growth, whereas the *PTEN* siRNA partially reversed this effect.

Discussion

In the present study, downregulation of miR-20b inhibited H22 cell growth. It is well known that miRNAs participate in oncogenesis by regulating the expression of their target genes (17). miR-20b is a member of the miR-106a-363 and miR-7 gene families (9), which are active in various types of human tumor (10). For example, miR-20b activity is increased in liver, gastric and breast tumors (18-20), and it is also a plasmatic marker for non-small cell lung cancer (21). In addition, anti-angiomiR-miR-20b has been described as a potential therapeutic target for refractory large B-cell lymphoma (22). In this study, miR-20b was revealed to promote H22 cell growth by targeting *PTEN*.

PTEN is a tumor suppressor gene located on chromosome 10 at 10q23.31 (23). It codes for a lipid phosphatase that dephosphorylates the second messenger inositol triphosphate and negatively regulates the phosphoinositide 3-kinase pathway (24,25). *PTEN* regulates tumor cell migration, cell cycle progression and apoptosis (26). The loss of *PTEN* expression has been demonstrated to promote transforming growth factor-β-induced cell invasiveness, and *PTEN* deletion is associated with progression and liver metastasis in colon cancer (27). In addition, *PTEN* is regulated by miRNAs in ovarian, colon and breast cancer, glioma and other malignant tumors (28-31). Notably, miR-20b regulates proliferation and migration of prostate cancer cells by directly targeting *PTEN* (12). Chu *et al* (32) demonstrated that the expression of PTEN is inhibited by miR-205, which is why miR-205 promotes proliferation and invasion of ovarian cancer cells. miR-106a promotes proliferation of prostate cancer cells by regulating the expression of PTEN (33). PTEN expression is significantly lower in primary hepatocellular carcinoma than in normal liver tissue, and its expression is correlated with tumor stage, invasion and metastasis (34). In the present study, miR-20b negatively regulated *PTEN* expression, whereas *PTEN* siRNA partially reversed the anti-proliferative effect of the miR-20b inhibitor on H22 cells. Therefore, it was suggested that miR-20b promoted H22 cell growth by directly targeting and downregulating *PTEN* expression, and *PTEN* expression may be involved in the effects of miR-20b on H22 cell growth. The importance of the present study is that it may further reveal the growth mechanism of hepatocellular carcinoma, and also provide a theoretical basis for clinical tumor therapy with miR-20b as the target.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CWS and HZL conceived and designed the experiments. JH, MMM, YLL, and HLW performed the experiments. CWS, HM, SJG, QF, ZQQ and HZL analyzed and interpreted the data, and wrote the manuscript. HZL revised the paper. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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