MicroRNA-200b inhibits the proliferation of hepatocellular carcinoma by targeting DNA methyltransferase 3a

XING-YU LI, XING-ZI FENG, JIAN-ZHONG TANG, KUN DONG, JUN-FENG WANG, CHUN-CHENG MENG, JUN WANG, YI-WO MO and ZHI-WEI SUN

Department of Hepatobiliary Surgery, The First People's Hospital of Yunnan Province, Kunming, Yunnan 650032, P.R. China

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Abstract. Aberrant microRNA (miRNA or miR) expression has been reported to contribute to the pathogenesis of hepatocellular carcinoma (HCC). However, the involvement of specific miRNAs in HCC remains to be elucidated. The present study aimed to investigate the potential role of miR-200b and the mechanism underlying its function in hepatocarcinogenesis. The results of the present study demonstrated that the expression levels of miR-200b were significantly reduced in HCC tissue samples, as compared with normal liver (NL) and para-tumorous (PT) tissue samples. The results also revealed that miR-200b expression levels in HepG2 cells were significantly decreased compared with those in L02 cells. In addition, western blotting and reverse transcription-quantitative polymerase chain reaction demonstrated that the expression levels of DNA methyltransferase 3a (DNMT3a), a possible target gene for miR-200b, were significantly higher in HCC tissue samples, as compared with those in NL and PT tissue samples. Furthermore, the data suggested that DNMT3a was a direct target gene of miR-200b. Upregulated miR-200b expression in HepG2 cells led to a decrease in DNMT3a expression levels, and an inhibition of cell proliferation. These results suggested that miR-200b has an important role in hepatocarcinogenesis and acts by downregulating DNMT3a expression. Thus, miR-200b may be a promising target for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-associated mortality, and is one of the most common malignant tumors with poor prognosis worldwide (1,2). In clinical settings only a limited number of patients with HCC are eligible for potentially curative treatment options such as surgical resection followed by orthotopic liver transplantation (3,4). Therefore, the development of effective therapeutic strategies for the treatment of greater numbers of patients with HCC is imperative. However, the pathogenesis of HCC remains unclear. HCC has long been considered the result of various genetic alterations that ultimately led to malignant transformation (5,6). Cancer development is no longer thought to be induced by genetic and genomic alterations alone, but is also considered to be the result of epigenetic alterations (7,8).

MicroRNAs (miRNAs or miRs) are a type of highly conserved non-coding small RNA that post-transcriptionally regulate the expression of their target genes (9). miRNAs have been demonstrated to regulate numerous aspects of cell activity, including metabolism, differentiation and development, proliferation, apoptosis and viral infection (10-15). Previous findings demonstrated that miRNAs have important roles in numerous types of malignant tumors, including HCC (16). In addition, numerous studies have demonstrated the existence of prognostic miRNAs in clinical tissue specimens of primary HCC, and miRNAs have been shown to have important regulatory roles in hepatocarcinogenesis (17-20).

The present study aimed to investigate the expression levels of miRNA-200b as well as those of its potential target DNA methyltransferase 3a (DNMT3a) in HCC.

Materials and methods

Patients and cell lines. Histologically normal liver (NL) tissue samples were obtained from 10 patients with gallbladder stones during a biopsy procedure. HCC and adjacent non-malignant para-tumorous (PT) tissue specimens were obtained from 44 hepatitis B-positive patients by radical hepatectomy. The patients, 33 males and 11 females, had undergone treatment at The First People's Hospital of Yunnan Province (mean age, 53.7±11.6 years). HCC was confirmed in the tissue samples by pathological examination and were obtained with written informed consent from the patients. The present study was approved by the Institutional Review Board of the Fourth Military Medical University (Xian, China). The HepG2 and L02 cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured at 37˚C with 5% CO_2 in RMPI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA)

Correspondence to: Mr. Zhi-Wei Sun, Department of Hepatobiliary Surgery, The First People's Hospital of Yunnan Province, 157 Jinbi Road, Kunming, Yunnan 650032, P.R. China
E-mail: sunzhiwei616@163.com

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supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) until cell density reached ~70-80%.

MicroRNA arrays. MicroRNA arrays were conducted as previously described (21) on miR-200b from ten NL tissue samples, and 44 HCC and corresponding non-malignant PT tissue samples. Briefly, 100 ng RNA was extracted from each tissue sample using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and an RNeasy Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The tissue samples were subsequently hybridized by labeling with the miRCURY Hy3/Hy5 Power labeling kit (Exiqon A/S, Vedbæk, Denmark) and hybridizing on the miRCURY LNA™ Array (v.11.0). They were then scanned using an Axon GenePix 4000B microarray scanner (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the manually homogenized tissue samples as well as the cell lines using TRIzol® reagent, according to the manufacturer's protocol. Reverse transcription of 5 ng RNA to cDNA was performed using a QuantiMir RT kit (System Biosciences, Mountain View, CA, USA). The following primers (Shanghai GenePharma Co., Ltd., Shanghai, China) were used: miR-200b forward, 5'-TCATCATTACCAGGCAGTATTA-3', and reverse, 5'-TCC ATCATTACCCGGCAGTATTA-3'; U6 forward, 5'-CTCGCT TCGGCAGCACA-3', and reverse, 5'-AACGCTTCACGA ATTTGCGT-3'; DNMT3a forward, 5'-CAATGACCTCTC CATCGTCAAC-3', and reverse, 5'-CATGCAGGAGGCGGT AGAA-3'; and β-actin forward, 5'-GAACGGTGAAGGTGA CAG-3', and reverse, 5'-TAGAGAGAAGTGGGGTGG-3'. miR-200b was amplified in a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: Denaturation at 95˚C for 10 min, and then 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec, and 72˚C for 10 sec. DNMT3a was amplified as follows: Denaturation at 95˚C for 10 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. U6 RNA was used as an miRNA internal control, and β-actin was used to normalize the expression levels of total:mRNA in each sample. Values were calculated as ratios normalized to U6 or β-actin. The expression level of miRNA was defined based on the quantification cycle (Cq), and relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Transfection of miR-200b mimics. Synthesized Dharmacon miR-200b mimics were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). HepG2 cells ($2x10^6$) were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in 5% CO_2. When the HepG2 cells reached 30-50% confluence, they were transfected with miR-200b mimics (60 nM) or miRNA mimic control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were then harvested and protein expression levels were measured by western blotting 72 h post-transfection.

Western blot analysis. Western blotting was performed as previously described (23). Briefly, total protein was extracted using radioimmunoprecipitation buffer (Sigma-Aldrich) and 10 μg of each sample was separated by SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% (v/v)Tween 20 (TBST; EMD Millipore) and then incubated with primary antibodies with gentle agitation for 12 h at 4˚C. The membranes were then washed three times with TBST and incubated with goat anti-rabbit and goat anti-mouse peroxidase-conjugated secondary antibody (dilution, 1:3,000; Beyotime Institute of Biotechnology, Haimen, China; cat nos. A0208 and A0216). Rabbit anti-human monoclonal anti-DNMT3a (dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 3598) and mouse anti-human anti-GAPDH antibodies (dilution, 1:1,000; ProMab Biotechnologies, Inc., Richmond, CA, USA; cat. no. 20035) were used. Protein bands were visualized by chemiluminescence detection (EMD Millipore) and the quantification of band density was conducted using Image J (version 1.5; National Institutes of Health, Bethesda, MD, USA). GAPDH was used as an internal control, and all values were calculated as ratios normalized to GAPDH.

Identification of targets of miR-200b. TargetScan (www.targetscan.org) was used to identify potential targets of miR-200b. An important enzyme in DNA methylation, DNMT3a, was identified as one of the potential targets.

Luciferase activity assay. The 3'-untranslated region (UTR) of DNMT3a was amplified by PCR and inserted into a pGL3 vector (Promega Corporation, Madison, WI, USA). The PCR was conducted on a MyCycler thermal cycler under the following conditions: 50˚C for 30 min; 95˚c for 95 min; and 40 cycles of 95˚C for 30 sec and 55˚C for 30 sec. A pGL3 construct containing DNMT3a 3'-UTR with point mutations in the seed sequence was synthesized using 30 reactions of the Quik-Change Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The primers used for DNMT3a were as follows: Forward, 5'-GCTCTAGACGAAAAGGGT TGGACATCAT-3', and reverse, 5'-GCTCTAGAGCCGAGG GAGTCTCCTTTTA-3'.

HepG2 cells ($2x10^5$) were transfected using Lipofectamine® 2000 with the appropriate plasmids in 24-well plates. The cells were then harvested and lysed with reporter gene cell lysis buffer (Beyotime Institute of Biotechnology) in order to conduct a luciferase activity assay 48 h post-transfection using a dual luciferase reporter assay system (Promega Corporation). Relative luciferase activity levels were normalized to Renilla luciferase activity, which served as an internal control.

MTT assay. An MTT assay was used to determine the effects of ectopic miR-200b mimics on HepG2 cell proliferation. Briefly, HepG2 cells were transfected with miR-200b mimics using Lipofectamine® 2000 and then seeded into 96-well plates at $5x10^3$ cells/well in 200 μl RPMI-1640 medium for 72 h. MTT solution [0.5 mg/ml in 20 μl phosphate-buffered saline (PBS); Sigma-Aldrich] was added to each well and incubated for 4 h at 37˚C. An enzyme-labeled instrument (Thermo Fisher Scientific, Inc.) was used to measure the absorbance of each

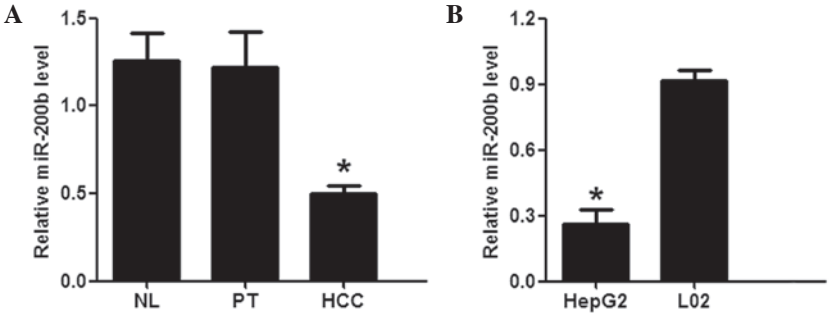

Figure 1. miR-200b expression levels are downregulated in HCC tissue samples and HepG2 hepatoma cell lines. (A) The expression levels of miR-200b in the NL, PT, and HCC tissue samples from 44 patients with hepatitis B-positive HCC, as determined by RT-qPCR. *P<0.05, vs. the NL/PT groups. (B) The expression levels of miR-200b in the HepG2 and L02 cell lines, as determined by RT-qPCR. *P<0.05, vs. the L02 cells. The values are presented as means ± standard error of the mean. All experiments were performed in triplicate, and similar results were obtained from each experiment. miR, microRNA; NL, normal liver; PT, para-tumorous; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

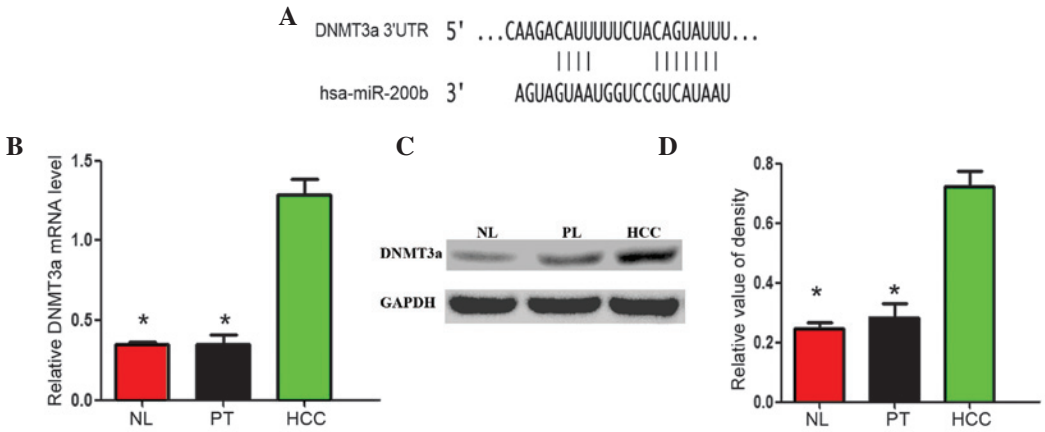

Figure 2. Expression levels of the miR-200b target gene DNMT3a are increased in HCC tissues samples compared with NL tissue samples. (A) Bioinformatics analysis suggested that DNMT3a was an important enzyme in DNA methylation, and a potential target of miR-200b. (B) The mRNA expression levels of DNMT3a in NL, PT, and HCC tissue samples were determined using reverse transcription-quantitative polymerase chain reaction. (C and D) Protein expression levels of DNMT3a in NL, PT and HCC tissue samples. The data are presented as means ± standard error of the mean. All experiments were performed in triplicate and yielded similar results. *P<0.05, vs. the HCC group. NL, normal liver; PT, para-tumorous; HCC, hepatocellular carcinoma; miR, microRNA; 3'-UTR; 3'-untranslated region; DNMT3a, DNA methyltransferase 3a.

well at 570 nm. Data were obtained from three independent experiments.

TUNEL staining. DNA fragmentation of apoptotic cells was detected using a TUNEL kit (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, $2x10^5$ cells were cultured on cover slips for 48 h at 37˚C with 5% CO_2. Following miR-200b mimic transfection for 72 h, the cells were fixed in 4% paraformaldehyde solution (EMD Millipore) in PBS for 30 min at room temperature. The cells were incubated with methanol solution containing 0.3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity, and then incubated in the TUNEL reaction mixture for 60 min at 37˚C. Nuclei were visualized with DAPI staining (0.5-10 μg/ml; Beyotime Institute of Biotechnology) for 10 mins. The cells were then visualized by fluorescence microscopy (DM4000B; Leica Microsystems GmbH, Wetzlar, Germany). Apoptotic cells were counted from four randomly selected fields in each sample.

Flow cytometry. Apoptotic cells were detected using double-staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, the cells were harvested 48 h post-transfection and stained with anti-Annexin V conjugated

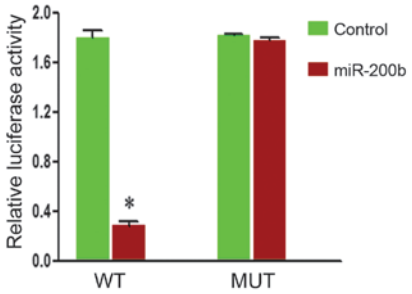

Figure 3. Ectopic miR-200b expression inhibits WT but not MUT DNMT3a 3'-UTR reporter activity in HepG2 cells. Cells were co-transfected with miR-200b and either a WT or MUT DNMT3a 3'-UTR reporter construct. A luciferase activity assay was performed 48 h following co-transfection. The data are presented as means ± standard error of the mean. All experiments were performed in triplicate, and similar results were obtained from each experiment. *P<0.05, vs. the WT control group. miR, microRNA; WT, wild-type; MUT, mutant; 3'-UTR, 3'-untranslated region; DNMT3a, DNA methyltransferase 3a.

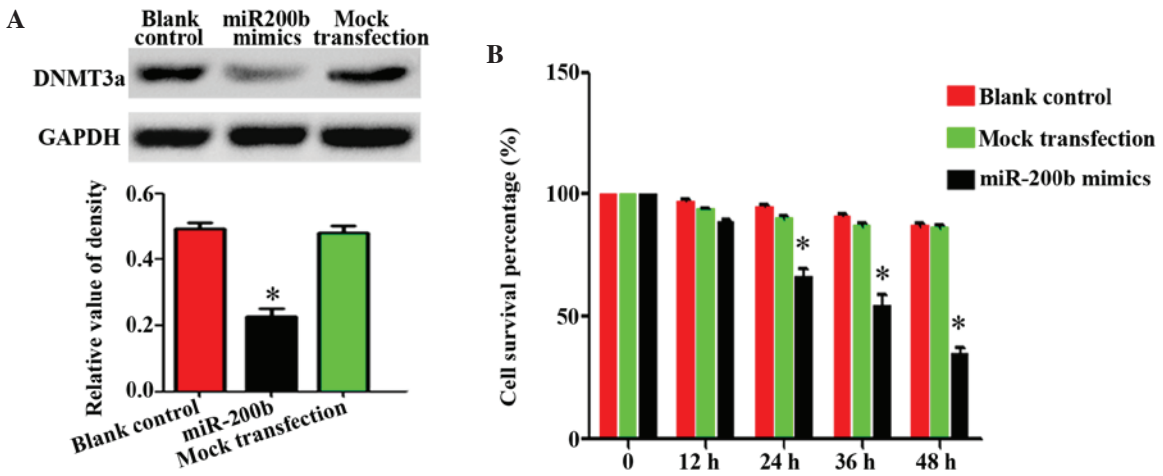

Figure 4. Ectopic miR-200b decreases DNMT3a expression levels and HepG2 cell proliferation. (A) miR-200b mimics were transfected into HepG2 cells, and the protein expression levels of DNMT3a were detected by western blotting. (B) At 0, 12, 24, 36, and 48 h post-miR-200b transfection, cell proliferation was measured by MTT assay. The data are presented as means ± standard error of the mean. All experiments were performed in triplicate, and similar results were obtained for each experiment. *P<0.05, vs. the blank control and mock transfection groups. miR, microRNA; DNMT3a, DNA methyltransferase 3a.

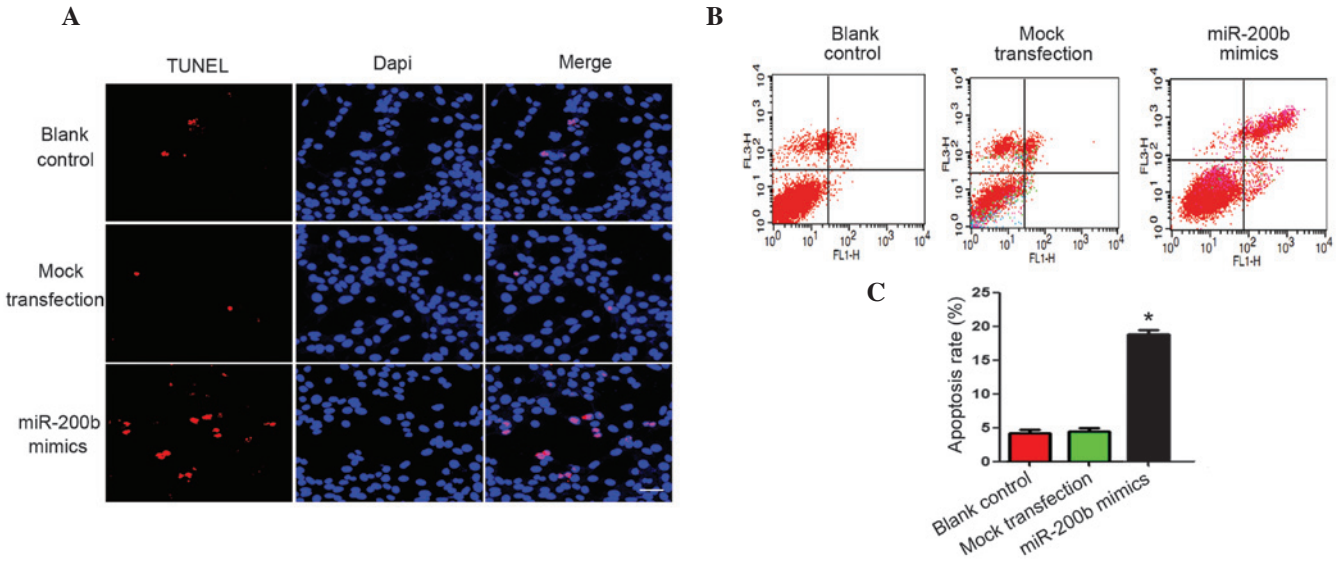

Figure 5. Ectopic miR-200b markedly increased apoptosis of HepG2 cells compared with the blank and negative controls. (A) miR-200b mimics were transfected into HepG2 cells, and apoptosis levels were determind 48 h post-transfection by TUNEL staining. Red, apoptotic cells; blue; DAPI. Magnification, x800. (B and C) The apoptosis levels were also determined by flow cytometry. The lower left quadrants represent live cells, the upper left represent early apoptotic cells, the lower right represent late apoptotic cells and the upper right quadrant represents dead cells. The data are presented as means ± standard error of the mean. All experiments were performed in triplicate, and similar results were obtained for each experiment. *P<0.05, vs. the blank control/mock transfection groups. miR, microRNA; DAPI, 4',6-diamidino-2-phenylindole.

to FITC and PI for 15 min at room temperature. The cells were then detected using fluorescence-activated cell sorting FACS using a FACS Calibur obtained from BD Biosciences (Franklin Lakes, NJ, USA). The data were analyzed using CellQuest software (version 5.1; BD Biosciences).

Statistical analysis. All the experiments were repeated in triplicate, and the data were expressed as the mean ± standard error of the mean. The results were analyzed using the Student's t-test and one-way analysis of variance. The statistical analyses were conducted using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) and P<0.05 was considered to indicate a statistically significant result.

Results

miR-200b expression levels are downregulated in HCC tissue samples and HepG2 cell lines. To investigate miR-200b expression in HCC tissue samples, RT-qPCR was used to quantify miR-200b expression levels. miR-200b expression levels were significantly decreased in the HCC tissue samples, as compared with those in the NL and non-malignant PT tissue samples (P<0.05; Fig. 1A). The expression levels of miR-200b were then evaluated in the HepG2 and L02 cells by RT-qPCR. The expression levels of miR-200b were significantly lower in the HepG2 cells, as compared to those in the L02 cells (P<0.05; Fig. 1B). These results indicated that miR-200b

expression levels are significantly decreased in HCC tissue samples and cell lines, suggesting that miR-200b is associated with hepatocellular carcinogenesis.

In silico prediction of DNMT3a as an miR-200b target. TargetScan was used to identify the potential targets of miR-200b. An important enzyme in DNA methylation, DNMT3a, was identified as one of the potential targets of miR-200b. The predicted binding site of miR-200b with the DNMT3a 3'-UTR is shown in Fig. 2A.

The expression levels of the potential miR-200b target gene DNMT3a are significantly increased in HCC tissue samples compared with NL tissue samples. The mRNA and protein expression levels of the miR-200b potential target gene DNMT3a were evaluated by RT-qPCR and western blot analysis. Compared with mRNA expression levels of DNMT3a in the NL (0.318±0.047) or PT (0.326±0.082) tissue samples, the mRNA expression levels of DNMT3a in the HCC (1.295±0.093) tissue samples were significantly higher ($P<0.05$; Fig. 2B). In addition, compared with the protein expression levels of DNMT3a in the NL (0.214±0.037) and PT (0.280±0.068) tissue samples, the protein expression levels of DNMT3a in the HCC (0.722±0.014) tissue samples were also significantly higher ($P<0.05$; Fig. 2C and D).

DNMT3a is the direct target of miR-200b. To examine miR200b-DNMT3a interactions, DNMT3a complementary sites, with or without mutations, were cloned into the 3'-UTR of the firefly luciferase gene and co-transfected with miR-200b mimics or a negative control in HepG2 cells. The presence of miR-200b led to a significant reduction in the relative luciferase activity levels in the wild-type construct of the DNMT3a 3'-UTR in HepG2 cells ($P<0.05$). However, the mutant construct of the DNMT3a 3'-UTR reversed the suppressive effect of miR-200b in HepG2 cells (Fig. 3). These results suggest that DNMT3a is a direct target of miR-200b.

Ectopic miR-200b decreases the expression levels of DNMT3a and suppresses HepG2 cell proliferation. Western blotting was conducted to detect the protein expression levels of DNMT3a following miRNA-200b mimic transfection in HepG2 cells. DNMT3a protein expression levels were significantly decreased in the mimic group (0.214±0.021), as compared with those in the blank control (0.527±0.035) or mock transfection control group (0.513±0.013; $P<0.05$; Fig. 4A and B).

To determine the role of miR-200b deregulation in hepatocarcinogenesis, an MTT assay, a TUNEL assay, and flow cytometry were used to determine the proliferation and apoptosis rates of HepG2 cells following miR-200b mimic exposure. The MTT assay demonstrated that at 24, 36 and 48 h following miR-200b mimic exposure, the proliferation rate of HepG2 cells was reduced to 68, 42 and 36.3% of the control, respectively (Fig. 4C). The differences between the miR-200b mimic-transfected and control groups at the above-mentioned time points were statistically significant ($P<0.05$), whereas the difference between the blank and negative control groups were not ($P>0.05$; Fig. 4C). The apoptosis of HepG2 cells post-transfection with miR-200b mimics was evaluated by TUNEL assay and flow cytometry. Apoptosis levels were markedly increased in the miR-200b mimic group, as compared with the blank and negative control groups (Fig. 5).

Discussion

The present study aimed to investigate the possible role of miR-200b in hepatocarcinogenesis and to identify its target gene. The results demonstrated that, compared with NL and PT tissue samples, miR-200b expression levels were significantly reduced in HCC tissue samples. In addition, miR-200b expression levels in HepG2 cells were significantly decreased, as compared with those in L02 cells. Western blotting and RT-qPCR demonstrated that the expression levels of DNMT3a, a possible target gene for miR-200b, were significantly higher in HCC tissue samples, as compared with NL and PT tissue samples. Furthermore, the results of the present study demonstrated that DNMT3a was the direct target gene of miR-200b. Upregulated miR-200b expression in HepG2 cells led to a decrease in DNMT3a expression levels, and had an inhibitory effect on cell proliferation. These data suggested that miR-200b is an important factor in hepatocarcinogenesis, and acts by downregulating DNMT3a expression. miR-200b may therefore be a promising target for HCC treatment.

miRNAs are non-coding RNAs 19-25 nucleotides in length that have been demonstrated to regulate gene expression by inducing translational inhibition or cleavage of their target mRNAs through base pairing at partially or fully complementary sites (24-26). Numerous miRNAs have been demonstrated to function as tumor suppressor or oncogenes by regulating their target genes (27-29). Previous studies have demonstrated that various miRNAs are abnormally expressed in malignant HCC cells or tissue samples, as compared with normal hepatocytes or tissue samples (18,30-32). In the present study, the results revealed that miR-200b expression levels were significantly decreased in HCC tissue samples and in HepG2 cells, as compared with those in non-malignant liver tissue samples and L02 cells.

Genomic stability is regulated by both genetic and epigenetic mechanisms (33). Promoter hypermethylation mediated by DNMTs is widely accepted as the predominant mechanism underlying the epigenetic inactivation of tumor suppressor genes (TSGs) (34,35). Previous studies have demonstrated that viral genes have important roles in regulating DNA methylation (36-38). However, the epigenetic mechanisms underlying virus-associated cancers are poorly understood. Numerous studies have suggested that hypermethylation is responsible for the silencing of TSGs in hepatocarcinogenesis (39-42). In addition, data from previous studies support a role for miRNAs as targets and effectors in aberrant mechanisms underlying DNA hypermethylation (43-45). The present study demonstrated that there exists an interaction between miR-200b and DNMT3a. Firstly, *in silico* analysis suggests that DNMT3a, an important enzyme in DNA methylation, may be one of the possible targets of miR-200b. Secondly, the results demonstrate that the mRNA and protein expression levels of DNMT3a are inversely correlated with miR-200b expression in HCC. Ectopic miR-200b expression led to a reduction in the expression levels of DNMT3a. Furthermore, DNMT3a was shown to be a direct target of miR-200b, as demonstrated by a luciferase activity assay. The results of the present study also demonstrate

that ectopic miR-200b expression significantly suppressed the proliferation of HepG2 cells and induced apoptosis. These data suggest that miR-200b regulates DNMT3a expression and has a tumor suppressive role in HCC development.

Since the number of samples used in the present study was relatively small, further investigation with a larger number of samples is required. Furthermore, the regulatory mechanism underlying miR-200b expression downregulation in HCC requires investigation. In conclusion, the results of the present study suggest that miR-200b is an important factor in hepatocarcinogenesis, and acts by downregulating DNMT3a expression. Thus, miR-200b is a promising target for HCC treatment.

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