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MiR-888 promotes cell migration and invasion of hepatocellular carcinoma by targeting SMAD4

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC), a type of primary liver cancer, is the second leading cause of cancer mortality worldwide. Increasing evidence suggests that dysfunction of microRNAs (miRNAs) plays an important role in human cancers. MicroRNA-888 (miR-888) has been reported to be upregulated in multiple cancers that have a high rate of metastasis. The purpose of this study was to explore the molecular mechanisms of miR-888 in HCC cell migration and invasion.

PATIENTS AND METHODS: Reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting were employed to measure the levels of miR-888 and SMAD4 in HCC tissues and cell lines. To analyse the tissues and cell lines' migratory and invasive abilities, Transwell assays were performed. To confirm that miR-888 regulates SMAD4 expression in HCC, a dual-luciferase reporter assay was applied.

RESULTS: MiR-888 was upregulated, while SMAD4 was downregulated, in HCC tissues and cell lines, and miR-888 and SMAD4 mRNA levels had a negative correlation. MiR-888 promoted cell migration and invasion *in vitro*. SMAD4 was thus confirmed as a direct and functional target of miR-888, and it could partially reverse the function of miR-888.

CONCLUSIONS: MiR-888 promoted cell migratory and invasive abilities and suppressed the expression of SMAD4 in HCC. The newly identified miR-888/SMAD4 axis provides novel insight into the progression of HCC and offers a promising target for HCC therapy.

Key Words:
MiR-888, SMAD4, Hepatocellular carcinoma, Migration, Invasion

Introduction

Hepatocellular carcinoma (HCC), the second leading cause of cancer-related deaths, accounts for 85-90% of cases of primary liver cancer[1,2]. In recent decades, although advances have made in treatment of HCC such as surgical resection, liver transplantation, and radiofrequency ablation[3], due to its high rate of recurrence and metastasis, the 5-year survival rate of HCC is only 15–40%, and the recurrence rate is 62-82% within 2 years[4]. Therefore, it is urgently necessary to further elucidate the molecular mechanisms underlying HCC invasion and metastasis and identify promising therapeutic targets to improve treatment strategies. MicroRNAs (miRNAs), a class of small noncoding RNAs that are 22–28 nucleotides in length, inhibit gene expression through binding to the 3'-untranslated region (3'-UTR) of mRNA at the posttranscriptional level[5,6]. In HCC, altered expression of miRNAs has been reported to have a relationship with the proliferation and prognosis of HCC, including miR-199, miR-196, miR-429, and miR-29a[7-10]. MiR-888, a novel microRNA, is overexpressed in multiple malignancies with a high rate of metastasis[11]. In breast cancer, miR-888 regulates cellular proliferation properties and cancer metastasis by targeting E-cadherin[12,13]. Similarly, Hovey et al[14] demonstrated that miR-888 acts as a novel cancer-testis antigen by targeting the progesterone receptor in endometrial cancer. Furthermore, Lewis et al[15] showed that miR-888 promoted prostate cell growth and migration. Similarly, miR-888 has been reported to upregulate and promote cell pro-

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liferation in HCC[16]. However, there has been no previous study showing any effect of miR-888 on migration and invasion of HCC cells, and the potential of miR-888 as a therapeutic target of HCC has not been thoroughly elucidated to date. SMAD4, a member of the SMAD protein family that is located at chromosome 18q21, could mediate multiple benign diseases and malignant carcinomas[17,18]. SMAD4 deficiency plays a significant role in initiating upper digestive tract squamous cell carcinomas and gastrointestinal tract adenocarcinomas, and the inactivation of SMAD4 predicts a poor outcome[19]. In previous studies[20-22], SMAD4 was found to act as a novel tumor suppressor in multiple tumours, including gastric cancer, pancreatic adenocarcinoma, and colorectal cancer. In osteosarcoma, Song et al[23] revealed that SMAD4 had an activity as a deubiquitinase and regulated cell proliferation, metastasis, and epithelial-mesenchymal transition. SMAD4 plays a tumor-suppressing role, and overexpression of SMAD4 can suppress cell proliferation, migration, and invasion in HCC[24,25]. Therefore, achieving a better understanding of the molecular events underlying the metastasis of HCC is important for its prevention, diagnosis, and treatment. In this study, we provided evidence that miR-888 was aberrantly upregulated in HCC tissues. The overexpression of miR-888 significantly promoted tumor invasion and migration. We discovered that SMAD4 was a functional target of miR-888. This newly identified miR-888/SMAD4 axis may provide a new potential therapeutic target for HCC treatment.

Patients and Methods

Patients and Clinical Samples

A collection of 53 patients who sought treatment at Qingdao No. 6 People's Hospital from 2015 to 2017 were included in this study, and we obtained 53 pairs of HCC and corresponding paracancerous tissues. Surgically excised tissues, which had not been treated with radiotherapy or chemotherapy before surgery, were immediately snap-frozen in liquid nitrogen and stored at −80°C. Informed consent was obtained from each patient, and the study was approved by the Ethical Committee of Qingdao No. 6 People's Hospital.

Cell Lines and Culture Conditions

The normal human hepatocyte cell line L-02 and two human HCC cell lines HuH-2 and HepG2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2.

RNA Isolation and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

We verified the total mRNAs or miRNAs expression using qRT-PCR assays *via* the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) or the MIR Cut and Separation of miRNAs Kit (Tiangen, Beijing, China) from HCC tissues and cells. First, reverse transcription was performed using PrimeScript™ II 1st Strand Complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit (TaKaRa, Dalian, China) to synthesize the first cDNA chain. Second, a SYBR Prime Script miRNA RT-PCR Kit and SYBR Premix Kit (both purchased from TaKaRa, Dalian, China) were employed to quantify miR-888 and SMAD4, which were normalized against U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Relative expression of mRNA or miRNA was calculated in accordance with the $2^{-\Delta\Delta Ct}$ method. Primers were as follows: miR-888 forward, 5'-ATGTGGCAGATCCCACAGGAGTTT-3', reverse, 5'-ACTGGGTTTGACTTCGTAGCCCTT-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; SMAD4 forward, 5'-CGGACATTACTGGCCTGTTC-3', reverse, 5'-TAGGGCAGCTTGAAGGAAACC-3', GAPDH forward, 5'-CTGGGCTACACTGAGCACC-3', reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'.

Transfection

MiR-888 mimic and inhibitor were utilized to overexpress or knockdown miR-888 and to research the influence of miR-888 on cell migration and invasion. Similarly, a SMAD4 expressing plasmid (pcDNA3.1-SMAD4) was used to overexpress SMAD4, and then the ability of migration and invasion were detected in miR-888 overexpressing HuH-2 cells. The specified cells were plated into 6-well plates and cultured overnight in phenol-red free medium, which can reduce the presence of hormones. The used medium was replaced before transfection, and the specific vectors were transfected into HuH-2 cells using Lipofectamine 3000 Reagent (Invitrogen, Carls-

bad, CA, USA) according to the manufacturer's instructions.

Protein Extraction and Western Blotting

The HCC cells were lysed to extract their total protein on ice, which was performed using radioimmunoprecipitation assay (RIPA) Lysis Buffer containing phenylmethylsulfonyl fluoride (PMSF) (both from Beyotime, Shanghai, China). After half an hour of hydrolysis on ice, they were centrifuged, and we collected the supernatant. The concentration of protein was detected by using a bicinchoninic acid (BCA) Reagent Kit (Solarbio, Beijing, China), and we determined the absorbance using a microplate reader. After separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), we transferred the proteins onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated it with rabbit anti-SMAD4 monoclonal antibody (1:1,000; Abcam, Cambridge, MA, USA) at 4°C overnight. After that, the blots were incubated with anti-rabbit antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h after washing with TBST buffer (Tris-buffered saline and Tween-20, pH 8.0) three times. Finally, the Western blot signal was detected by chemiluminescence performed on the Bio-Rad Gel Doc XR instrument (Bio-Rad, Hercules, CA, USA).

Transwell Assay

Cell invasion and migration were performed using Transwell chambers with or without Matrigel (Clontech, Mountain View, CA, USA), the membrane pore size of which was 8 μm (Costar, Beijing, China). A total of 5×10^5 cells were resuspended in FBS-free medium, and 200 μL of the suspension was seeded into the top chamber, which was then placed in a 24-well plate followed by adding 500 μL of medium (containing 10% FBS) into the lower chamber, which acted as an inducer. After incubation at 37°C for 48 h, the cells still on the topside of the chamber were removed by a cotton swab. After fixing and staining the cells with methanol and 0.1% crystal violet, the membranes were separated and photographed under a microscope.

Plasmid Construction and Luciferase Reporter Assay

TargetScan (http://www.targetscan.org/vert_71/) was applied to predict the binding sequences, and SMAD4 was determined to be a direct target of miR-888. The 3'-UTR sequences of SMAD4 were cloned and inserted into the pmirGlo luciferase vector (WT). To verify the miR-888 directly targeted the 3'-UTR of SMAD4, a Quick Change Multi Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) was performed to mutate the SMAD4 3'-UTR (MUT). Additionally, an miR-888 mimic or a mimic NC were inserted into the pmirGlo vector as controls.

Huh-7 cells with 80% confluence in 6-well plates were cotransfected with luciferase reporter vectors containing the wild type or the mutant SMAD4 mRNA and miR-888 mimic or NC using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocols.

Statistical Analysis

Each experiment was repeated at least three times, and all quantitative data are presented as the mean ± standard deviation (SD). All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons between two groups were conducted with *t*-tests, and one-way analysis of variance (ANOVA) was used to analyze three or more groups. $p<0.05$ was considered to be statistically significant.

Results

Expression of MiR-888 and SMAD4 in HCC Tissues and Cells

The mRNA levels of miR-888 were detected in 53 paired HCC and corresponding paracancerous tissue specimens using qRT-PCR. We found that miR-888 was upregulated in all 53 HCC tissues compared with the corresponding paracancerous tissues ($p<0.001$) (Figure 1A). We further analyzed and assessed the expression of miR-888 in a normal human hepatocyte cell L-02 and two HCC cell lines. As the results show, the levels of miR-888 in HCC cell lines Huh-7 ($p=0.0003$) and HepG2 ($p=0.0013$) were higher than that in the normal human hepatocyte cell L-02 (Figure 1B). Furthermore, the expression levels of SMAD4 in HCC cells were also assessed by qRT-PCR. The relative expression level of SMAD4 was downregulated in HCC cells Huh-7 ($p=0.0019$) and HepG2 ($p=0.0027$) *vs.* L-02 (Figure 1C), which is contrary to the results of miR-888. Then, we found that

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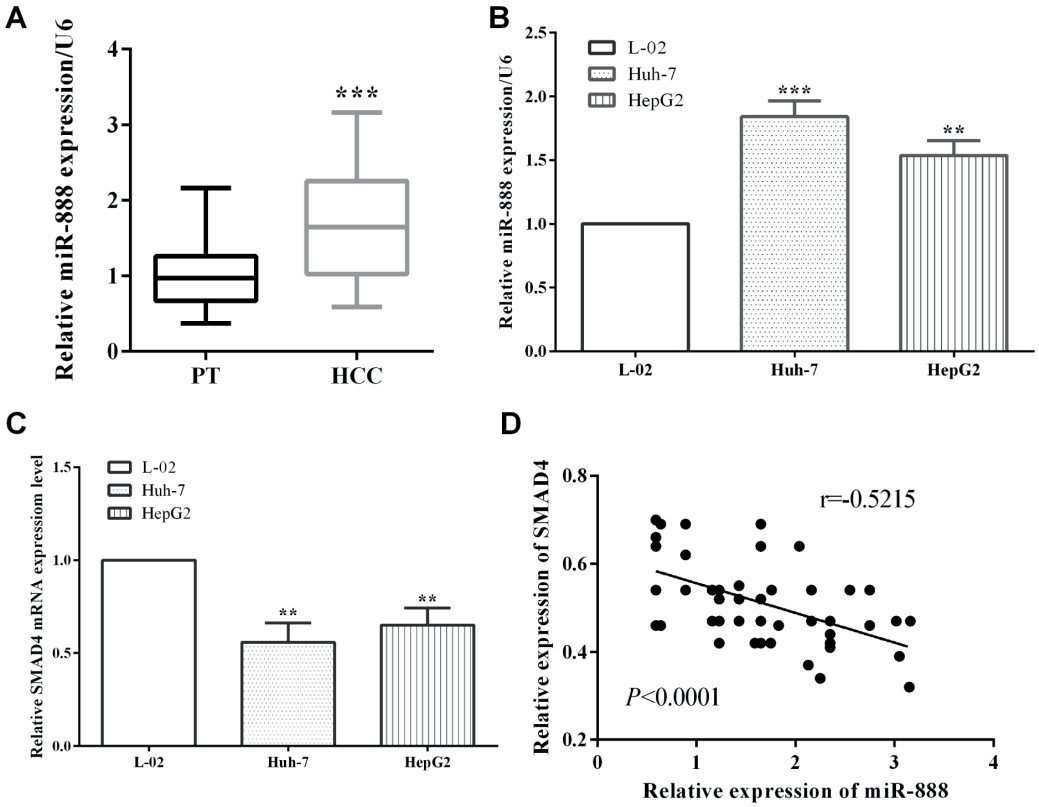

Figure 1. Expression of miR-888 and SMAD4 in HCC tissues and cells. ***A,*** In hepatocellular carcinoma, the average miR-888 expression level was higher than in paracancerous tissues. ***B,*** miR-888 is expressed at higher levels in HCC cells Huh-7 and HepG2 *vs.* the normal human hepatocyte cell L-02. ***C,*** opposite to miR-888, SMAD4 is downregulated in Huh-7 and HepG2 *vs.* the normal human hepatocyte cell L-02. ***D,*** miR-888 has a negative association with the SMAD4 mRNA level. **, $p<0.01$; ***, $p<0.001$; PT, paracancerous tissues; HCC, hepatocellular carcinoma.

miR-888 expression was inversely correlated with the SMAD4 level in 53 HCC clinical specimens (Figure 1D). These results indicated that miR-888 is upregulated in HCC tissues and that decreased SMAD44 levels may play an essential role in the development and progression of HCC.

MiR-888 Promotes the Migration and Invasion of HCC Cells

To determine its effect on migratory and invasive abilities, miR-888 mimic or miR-888 inhibitor were transfected into Huh-7 cells, and we then evaluated the cells' migratory and invasion abilities. After transfection with the miR-888 mimic, the intracellular level of miR-506 was increased in Huh-7 cells compared with the level in cells transfected with miR-NC ($p=0.0159$), and in contrast, the expression level of miR-888 was statistically reduced ($p=0.0038$) when we transfected the miR-888 inhibitor into Huh-7 cells (Figure 2A). Furthermore, we validated that transfection of the miR-888 mimic clearly promoted the migratory ($p=0.0080$) and invasive ($p=0.0104$) abilities of Huh-7 cells. In contrast, their migratory ($p=0.0365$) and invasive ($p=0.0030$) capacities were suppressed by transfection of the miR-888 inhibitor, as shown in Figure 2B. These results indicate that miR-888 could improve the migration and invasion of HCC cells.

MiR-888 Targets to SMAD4 and Inhibits its Expression

To study the mechanism of miR-888 affecting HCC cell migration and invasion, TargetScan was applied to predict the candidate target genes, and we found SMAD4 to be a target gene of miR-888. According to the prediction, the binding site of miR-888 on SMAD4 was located at 1362–1368 in the mRNA 3'-UTR, which was 5'-UUUUUCUUUUGCACUUUUGAGUC-3'. Thus, the binding sites were mutated from 5'-…UUUGAGU…-3' to 5'-…AAACUCA…-3', and both of them were inserted into pmirGlo luciferase vectors, which were named WT and MUT,

respectively (Figure 3A). To verify miR-888 direct binding to SMAD4, luciferase reporter assays were carried out. As expected, the luciferase activity in the reporter vector with the wild type SMAD4 3'-UTR was reduced (p=0.0012) by the miR-888 mimic, but nevertheless, the activity of the mutated SMAD4 3'-UTR showed no variations (p=0.4400) in Huh-7 cells (Figure 3B). In addition, the SMAD4 mRNA level was repressed (p=0.0012) by the miR-888 mimic, whereas it was increased (p=0.070) after transfection with the miR-888 inhibitor (Figure 3C). Taken together, these data indicate that SMAD4 is a direct target of miR-888.

SMAD4 Could Partially Reverse the Function of miR-888

To demonstrate that miR-888 regulates HCC cell migration and invasion through SMAD4, the SMAD4 overexpressing plasmid and miR-888 mimic were coexpressed in Huh-7 cells. As shown in Figure 4A, mRNA and protein levels of SMAD4 were increased (p=0.0133) when cotransfected with SMAD4 and the miR-888 mimic *vs.* only transfection of the miR-888 mimic into Huh-7 cells. The number of migrating cells was increased (p=0.0010) when they were transfected with the miR-888 mimic, and this partial function was reversed when they were co-transfected with SMAD4 (p=0.0226). Similarly, the cells' invasive activity was reduced (p=0.0009) when they were cotransfected with the miR-888 mimic and SMAD4 overexpressing plasmids compared with only being transfected with the miR-888 mimic, which increased invasion (p=0.0079) compared with the negative control (Figure 4B).

Discussion

Hepatocellular carcinoma is one of the most prevalent malignant tumours, and it is ranked second in terms of cancer deaths[1,2]. Therefore, in order to improve the prognosis of HCC pa-

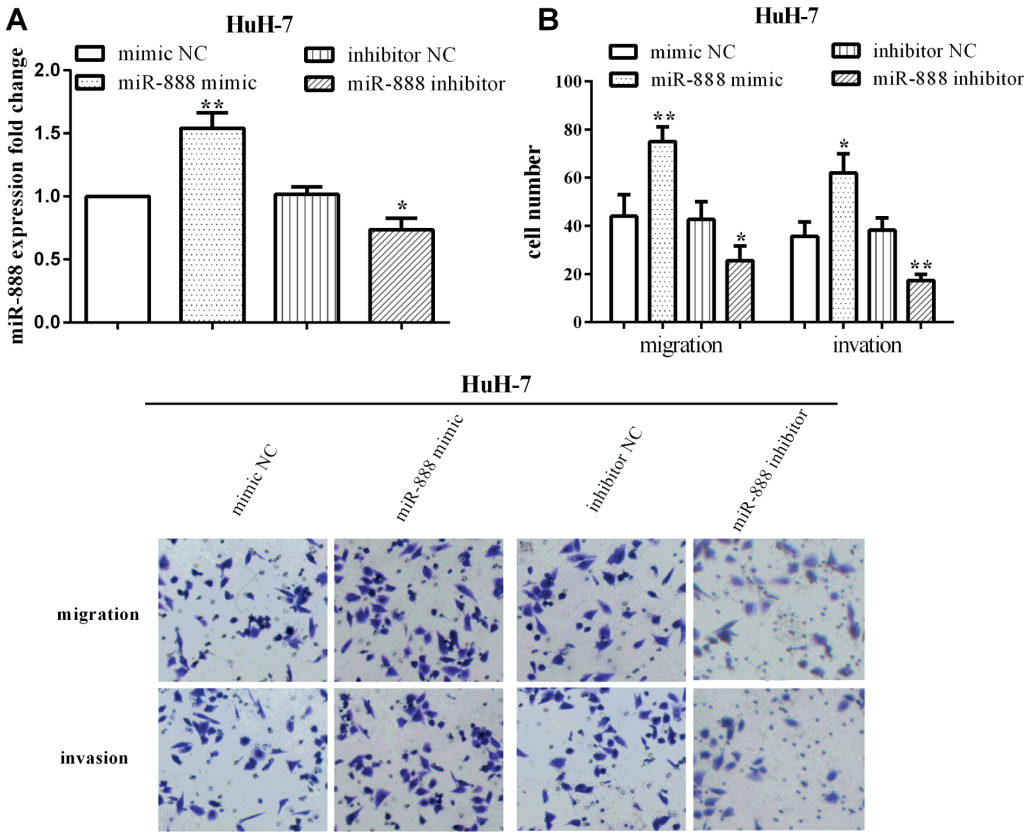

Figure 2. MiR-888 inhibits the migration and invasion of HCC cells. ***A,*** the miR-888 level was upregulated when transfected with the miR-888 mimic, while it was downregulated when transfected with the miR-888 inhibitor. ***B,*** The number of migrating and invasive cells was increased when we transfected the miR-888 mimic into Huh-7 cells. However, the migratory and invasive ability was reduced when they were transfected with the miR-888 inhibitor. *, $p<0.05$; **, $p<0.01$

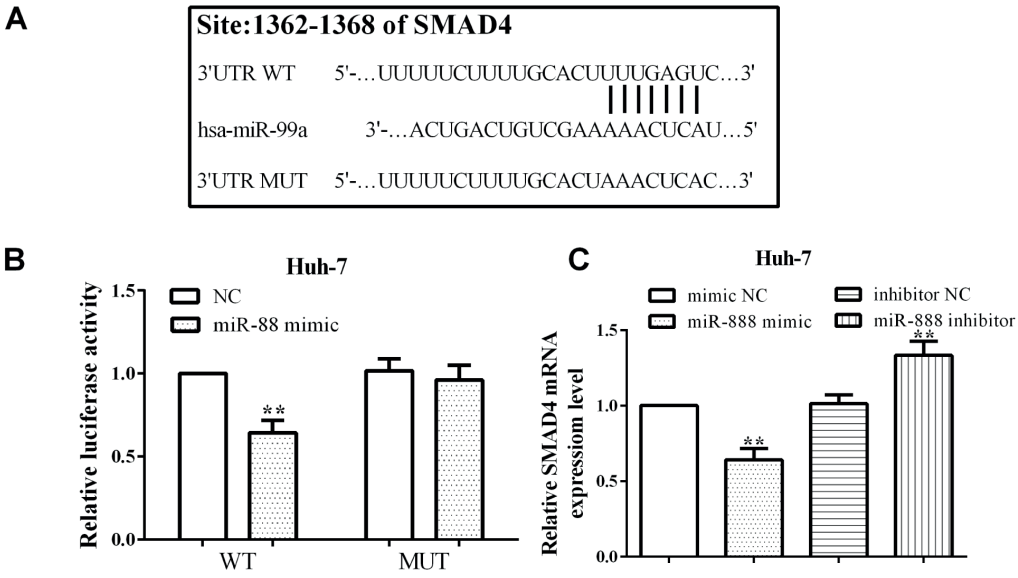

Figure 3. MiR-888 targets to SMAD4 and inhibits its expression. ***A,*** The complementary sequences of miR-888 and SMAD4 and mutant sequences of SMAD4 3'-UTR. ***B,*** The luciferase reporter assay demonstrated reduced expression when cotransfected with miR-888 and WT, whereas there were no variations in MUT. ***C,*** The SMAD4 mRNA level was decreased when we overexpressed miR-888. In contrast, miR-888 was upregulated when we inhibited miR-888. **, $p<0.01$; WT, wild type of SMAD4 3'UTR; MUT, mutant of SMAD4 3'UTR.

tients, great efforts to better understand the molecular mechanisms underlying HCC metastasis are warranted. Increasing evidence has indicated that miRNAs play vital roles as diagnostics biomarkers and therapeutic targets for human cancers because miRNAs can regulate gene expression through binding to 3'-UTRs[5,6]. However, the molecular mechanism by which miR-888 regulates the progression and development of HCC has not been thoroughly investigated. MiR-888 has been reported to be upregulated in several cancers with high metastasis rates[11]. Similar findings reported by Huang et al[13] indicated that miR-888 regulated tumor metastasis in breast cancer. Although miR-888 has been reported to upregulate and promote cellular proliferation[16], there has been no previous study on the effect of miR-888 on cell migration and invasion in HCC. Our results were consistent with previous findings that miR-888 was upregulated in the HCC cell lines Huh-7 and HepG-2. We also found that miR-888 could suppress human HCC cell migration and invasion using Transwell assays, indicating that miR-888 might act as a tumor suppressor and play an important role in HCC metastasis. Furthermore, we found that SMAD4 was a target of miR-888 and its expression is regulated by miR-888. This research revealed the relative expression level of SMAD4 was lower in HCC tissues and cell lines *vs.* normal tissues. SMAD4 is widely expressed in tumors and could function as an important factor in regulating cell invasiveness, survival and tumour-stroma interactions to expedite cancer progression. SMAD4 is a member of the SMAD family, which regulates cell growth and differentiation[17,26]. In previous studies[20-22], SMAD4 was found to be downregulated and act as a cancer suppressor factor in multiple tumors. Song et al[23] discovered that SMAD4 could regulate cell proliferation, metastasis and epithelial-mesenchymal transition in osteosarcoma. Additionally, SMAD4 is frequently downregulated and exerts a tumour-suppressing role in hepatocellular carcinoma[24,25]. Our results are consistent with these previous findings. SMAD4 is downregulated in HCC, and its expression is correlated with miR-888. We also showed that SMAD4 may be involved in the biological function of miR-888. Ssimilar findings were reported by Lewis et al[15] that miR-888 promotes prostate cell growth and migration through repressing SMAD4 expression. In our study, overexpression of SMAD4 restored miR-888's promoting effect on the migration and invasion of HCC cells, and these results demonstrated that SMAD4 is a direct functional

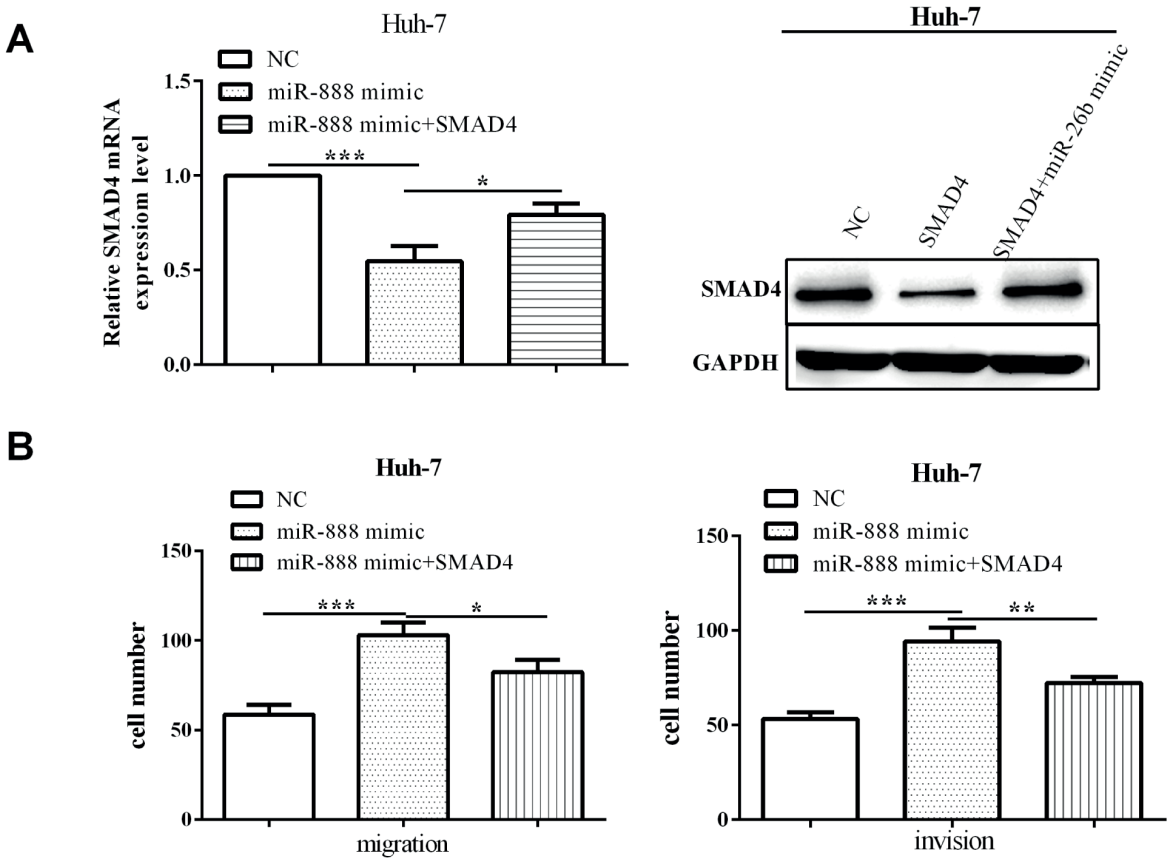

Figure 4. SMAD4 can partially reverse the function of miR-888. ***A,*** SMAD4 mRNA and protein levels were increased when we over-expressed SMAD4 in Huh-7 cells. ***B,*** The number of migrating and invasive cells was increased after transfection with the miR-888 mimic, which was partially reversed when the cells were co-transfected with SMAD4. *$p<0.05$, **, $p<0.01$, ***, $p<0.001$

target gene of miR-888 and that miR-888 functions as a tumor promoter through regulation of SMAD4.

Conclusions

We have demonstrated that miR-888 acts as a tumor promoter in HCC by promoting cancer migration and invasion. Furthermore, we showed that miR-888 expression has an inverse correlation with SMAD4 and directly targets it by binding to its 3'-UTR and that overexpression of SMAD4 partially reversed the function of miR-888. This newly identified miR-888 may provide further insight into the progression of HCC and offers a promising therapeutic target for the treatment of HCC. Additional studies to investigate the function of the miR-888/SMAD4 axis in the tumorigenesis and progression of HCC are needed.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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