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Original Article

miR-150 represses metastasis of hepatocellular carcinoma by targeting HMGA2

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Abstract: MicroRNAs (miRNAs) play critical roles in carcinogenesis and tumor progression. However, the mechanism of miR-150 funcions as a tumor suppressor or oncogene remains controversial. In this study, we found that miR-150 was significantly increased in hepatocellular carcinoma (HCC) tissues and cell lines. Overexpression of miR-150 suppressed HCC cell metastatic ability. miR-150 was a negative regulator of HMGA2 by directly combining with the 3'-UTR, and overexpression of miR-150 significantly inhibited the mRNA and protein expression levels of HMGA2. In addition, si-HMGA2 could partially attenuate the pro-oncogenic effects of anti-miR-150 on HCC cells. HMGA2 was inversely correlated with miR-150 in HCC tissues. Taken together, these results suggested that miR-150 inhibited HCC metastasis by targeting HMGA2 and might be used as a therapeutic target for the development of novel HCC treatment.

Keywords: miR-150, HMGA2, hepatocellular carcinoma, migration, invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and ranks the fifth most common cause of cancer-related mortality worldwide [1]. Although new HCC therapeutic approaches have been improved, the 5-year overall survival is less than 30% due to therapy resistance and metastasis [2]. Thus, a deep analysis of its molecular mechanisms represents a critical step in the development of new therapeutic strategies to treat and prevent HCC metastatic progression.

microRNAs (miRNAs), a class of small non-coding RNAs 21-23 nucleotides in length that post-transcriptionally regulate target genes through binding the 3'-untranslated region (UTR) to induce mRNA degradation and inhibit mRNA translation [3]. It has been reported that miRNAs are involved in many cellular processes, such as proliferation, development, differentiation and tumorigenesis [4, 5]. Dysregulation of several miRNAs have been found in HCC [5, 6]. For example, miR-150 functions as a tumor suppressor or oncogene in different type of cancers, including colorectal cancer, pancreatic cancer, non-small cell lung cancer [7-9], but its detailed role in HCC is unclear.

In this study, we investigated the expression of miR-150 and HMGA2 in HCC tissues and their matched adjacent normal tissues. miR-150 overexpression substantially suppressed HCC cells proliferation and motility. The high-mobility group AT-hook 2 (HMGA2) was identified as a novel target of miR-150, and HMGA2 silencing could attenuate the effects of anti-miR-150.

Materials and methods

HCC tissues and cell culture

Primary HCC specimens and their matched normal specimens were obtained from 18 patients undergoing hepatic resection in The First Hospital of Yulin during January, 2012 to December, 2014, and were snap-frozen in liquid nitrogen and stored at -80°C until use. All patients between the ages of 46 and 71 (mean age, 57.8 years) have not received any preoperative chemo- or radiotherapy. Both tumor and

normal tissues were histologically confirmed by HE (hematoxylin and eosin) staining. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committee of the hospital.

The two human HCC cell lines (HepG2 and SMMC-7721) and HEK293 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), while human liver cell line LO2 were obtained from ATCC. All cell lines were cultured in DMEM medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum. All cell lines were incubated at 37°C with 5 % CO_2.

RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA, following the manufacturer's instructions, was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To evaluate miR-150 expression, total RNA was reverse-transcribed using miRNA-specific primers (5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACTGGT-3'). To evaluate HMGA2 mRNA expression, total RNA was reverse-transcribed using the PrimeScript RT reagent Kit following the manufacturer's instructions (Takara, Dalian, China). qRT-PCR was performed using SYBR Premix Ex Taq II on an ABI7500 PCR machine. Relative mRNA and miRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. U6 and β-actin were used as controls for miRNA and mRNA level, respectively. Each experiment was measured in triplicate. The following primers were used to perform qRT-PCR: Forward: 5'-ATCCAGTGCGTGTCGTG-3' and reverse: 5'-TGCTTCTCCCAACCCTTGT-3' for miR-150; Forward: 5'-CTCAAAAGAAAGCAGAAGCCACTG-3' and reverse: 5'-TGAGCAGGCTTCTTCTGAACAACT-3' for HMGA2.

Western blot analysis

The cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China). Proteins were separated by 10% SDS-PAGE gel and transferred to membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with a primary anti-HMGA2 antibody (Proteintech Group Inc, China), followed by incubation with HRP-conjugated secondary antibody. Signals were visualized using ECL Substrates (Pierce, Rockford, IL, USA). β-actin was used as an endogenous control.

miRNA and siRNA transfection

The oligonucleotides of pre-miR-150 was synthesized (Beijing AuGCT DNA-SYN Biotechnology Co.Ltd) and cloned into the pcDNA6.2-GW vector. The sense was 5'-AATTCCTCCCCATGGCCCTGTCTCCCAACCCTTGTACCAGTGCTGGGCTCAGACCCTGGTACAGGCCTGGGGGACAGGGACCTGGGGACA-3'. The anti-miR-150 was purchased from GenePharma (Shanghai, China). The oligonucleotides of si-HMGA2 (5'-GAAATGGCCACAACAAGTTGT-3') and scramble (5'-CCTAAGGTTAAGTCGCCCTCG-3') were synthesized (Beijing AuGCT DNA-SYN Biotechnology Co.Ltd).

Transfection was performed using Lipofectamine® 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Luciferase reporter assays

The wild-type HMGA2 3'UTR sequence (HMGA2-Wt) for miR-150 was synthesized (Beijing AuGCT DNA-SYN Biotechnology Co.Ltd) and cloned into pmirGLO vector. The sequence was the following: Sense: 5'-CTATAGTTTATTTTTGTGGGAGATC-3'. The mutated HMGA2 3'UTR sequences was also cloned and named HMGA2-Mut. The primer sequence was the following: Sense: 5'-CTATAGTTTATTTTTGTCCCAGATC-3'.

For the luciferase assay, HEK293 cells were seeded in 96-well plates and cotransfected using 0.1 μg miR-150 along with 0.1 μg HMGA2-Wt or HMGA2-Mut reporter plasmid. Twenty-four hours after transfection, the luciferase assay was measured using the Dual-Luciferase Assay System (Promega), luciferase activitiy was normalized using Renilla.

Cell proliferation assay

Cells were seeded in 96-well plates and incubated at 37°C overnight and then transfected. The treated cells were cultured for 24 h, 48 h and 72 h respectively. Twenty ul MTT solution (5 mg/ml, 0.5% MTT) was added to each well and the cells were cultured for 4 h. Next the supernatant was discarded, and 150 ul dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at a wavelength of 492 nm in a microplate spectrophotometer.

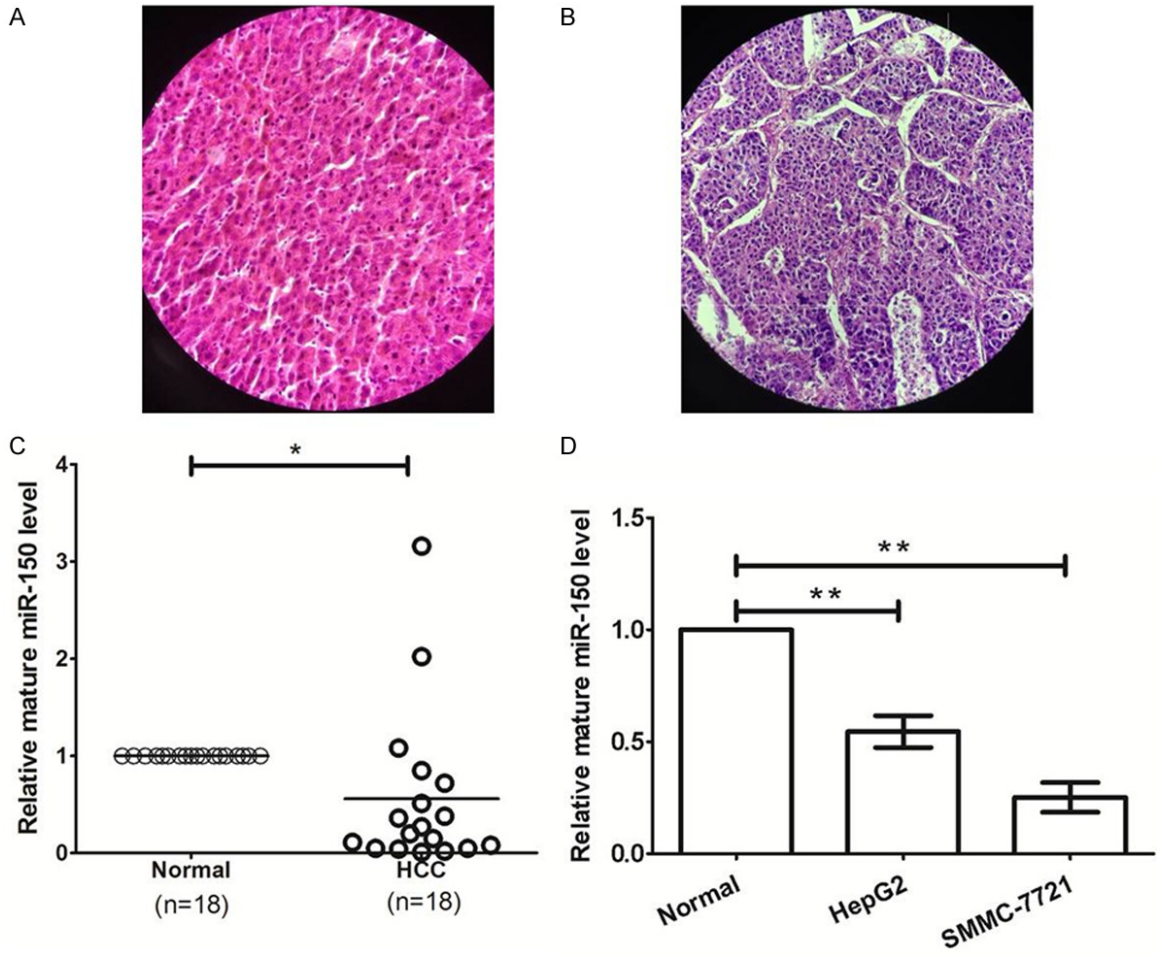

Figure 1. The expression of miR-150 was reduced in HCC tissue specimens and cell lines. Representative images of HE staining for normal (A) and HCC (B) tissues (40×). (C) Expression level of miR-150 between HCC and normal tissue samples using qRT-PCR. (D) The expression of miR-150 in 2 HCC cell lines (HepG2 and SMMC-7721) and a control cell line (LO2) using qRT-PCR. Data were expressed as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group.

Migration and invasion assays

The treated cells were plated equally in the upper compartments of the 12-well Boyden chamber. Transwell assay was then performed with uncoated or coated Matrigel for migration or invasion, respectively. After 24 hours, the migrated and invaded cells located on the lower side of the chamber were fixed with methanol, stained with crystal violet, air dried and photographed.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software package (Chicago, IL, USA). Differences between groups were determined by Student's *t* test. Data were expressed as the mean ± standard deviation (SD) from at least three independent experiments. $P<0.05$ was considered statistically significant.

Results

Decreased expression of miR-150 in HCC

As shown in **Figure 1A**, **1B**, both tumor and normal tissues were histologically confirmed by HE staining. Then, the miR-150 expression in 18 paired HCC tissues was detected by qRT-PCR. The results showed that miR-150 expression was significantly decreased in HCC tissues compared with matched controls ($P=0.037$, **Figure 1C**). Furthermore, we also found that miR-150 was downregulated in 2 HCC cell lines compared with the normal hepatic cells LO2 ($P<0.001$, **Figure 1D**).

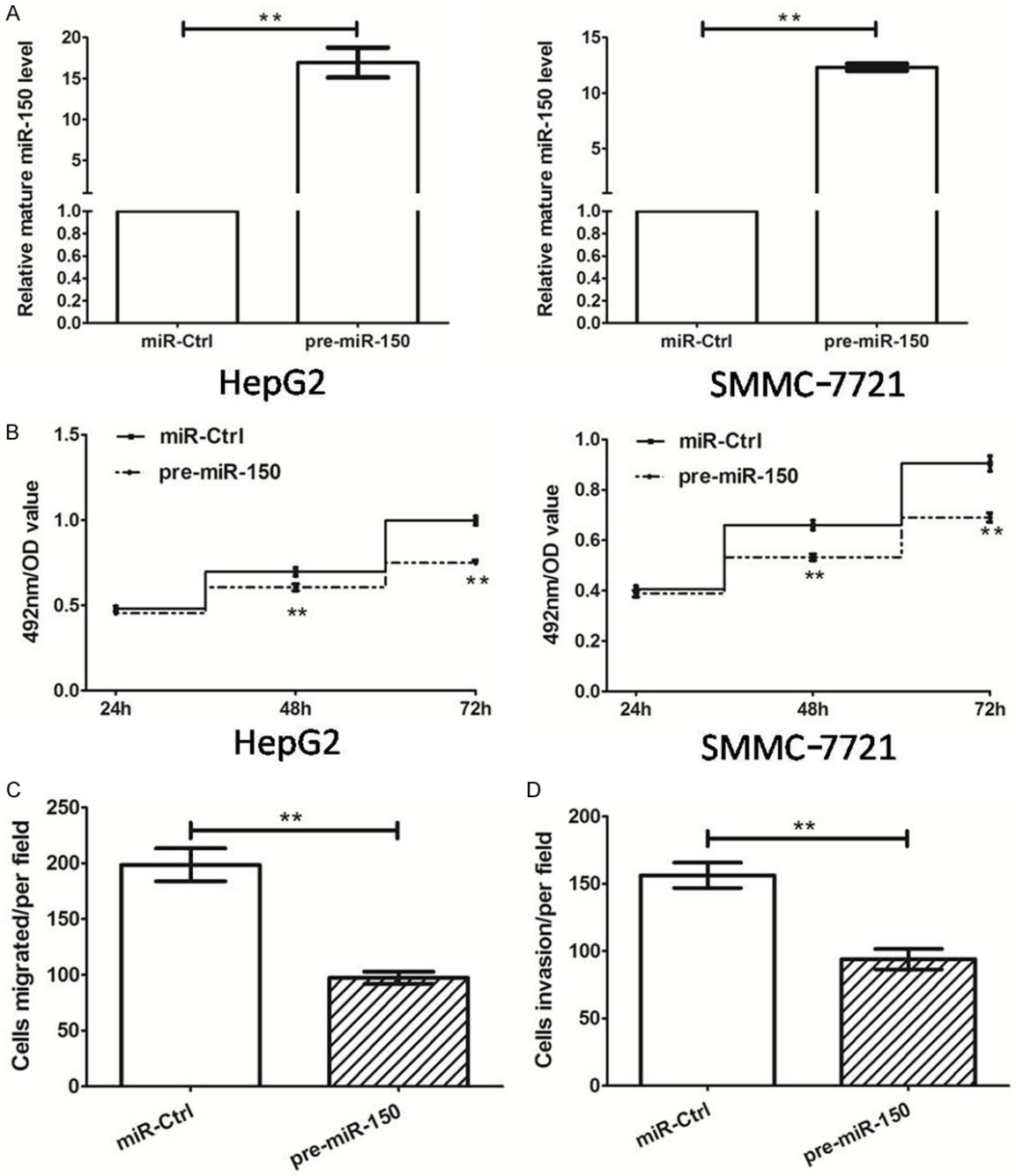

Figure 2. miR-150 inhibited HCC cell lines migration and invasion. A. Expression of miR-150 in HepG2 and SMMC-7721 cells transfected with miR-150 or miR-Ctrl control. B. The proliferation of HepG2 and SMMC-7721 cells transfected with miR-150 or miR-Ctrl control using MTT assay. C. HepG2 migration assay after transfection with miR-150 or miR-Ctrl control. D. HepG2 invasion assay after transfection with miR-150 or miR-Ctrl control. Data were expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01.

miR-150 inhibits metastatic potential of HCC cells

To assess the effects of miR-150 on cell metastasis. Firstly, the expression of pre-miR-150 was evaluated by qRT-PCR, the result demonstrated that the expression of miR-150 in the HepG2 and SMMC 7721 cells transfected with pre-miR-150 was significantly increased compared with the miR-Ctrl group (P<0.001, **Figure 2A**). Overexpression of miR-150 significantly inhibited cell proliferation activity of HepG2 and

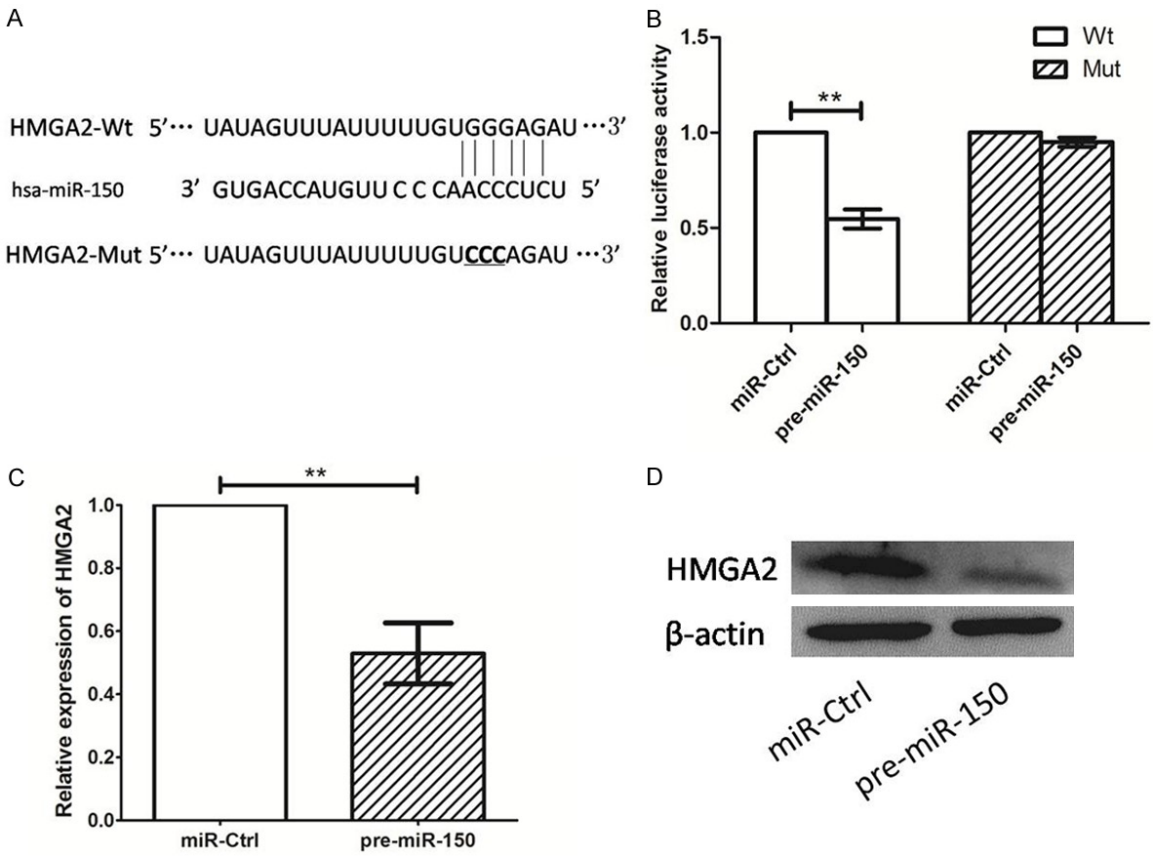

Figure 3. HMGA2 was a direct target of miR-150. A. The potential matching between miR-150 and HMGA2 3′-UTR (HMGA2-Wt)/(HMGA2-Mut) sequence were shown. B. HEK-293 cells were co-transfected with miR-150 and HMGA2-Wt or HMGA2-Mut. Luciferase activity was performed. C. The expression of miR-150 in HepG2 cells transfected with miR-150 or miR-Ctrl control using qRT-PCR. D. HMGA2 protein level was detected by western blot in HepG2 cells transfected with miR-150 or miR-Ctrl control. Data were expressed as mean ± SD. *P<0.05, **P<0.01 compared with the control group.

SMMC-7721 cells compared with the miR-Ctrl group (P<0.05, **Figure 2B**). Furthermore, the migration and invasion assays showed that miR-150 significantly suppressed the migration and invasion abilities of HepG2 cells (P<0.001, P=0.001, **Figure 2C**, **2D**).

HMGA2 is a direct target gene of miR-150

TargetScan 6.2 was used to identify the target of miR-150 in HCC and HMGA2 was predicted to be a potential target of miR-150 (**Figure 3A**). To further confirm this result, luciferase reporter assay showed that miR-150 significantly inhibited luciferase activities in HMGA2-Wt but not HMGA2-Mut in HEK293 cells (P<0.001, **Figure 3B**). In addition, miR-150 overexpression significantly suppressed both the mRNA and protein levels of HMGA2 (**Figure 3C**, **3D**).

HMGA2 attenuates the tumor suppressive effects of miR-150

Further to study whether HMGA2 could attenuate the tumor suppressive effects of miR-150 on HCC cells, the expression levels of HMGA2 and miR-150 were identified in HCC cells transfected with si-HMGA2 and anti-miR-150 respectively (**Figure 4A-C**). MTT assay, migration and invasion showed that si-HMGA2 could partially attenuate pro-oncogenic effects of anti-miR-150 (**Figure 4D-F**).

miR-150 is inversely correlated with HMGA2 expression in HCC tissues

Expression of HMGA2 in 18 paired HCC tissues was examined by qRT-PCR, and we found that expression of HMGA2 was obviously increased in HCC tissue samples compared with the adja-

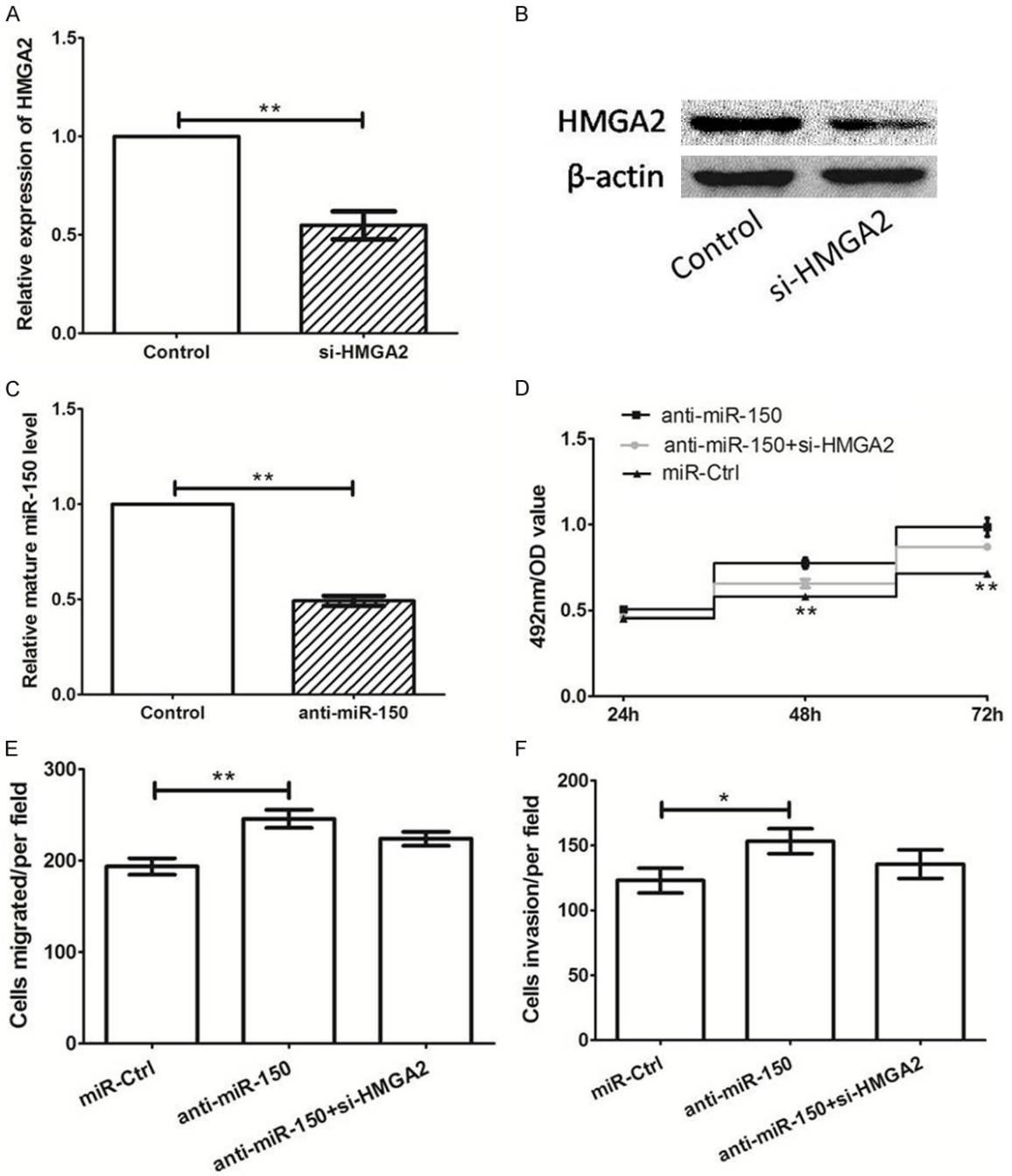

Figure 4. si-HMGA2 attenuated pro-oncogenic effects of anti-miR-150 on HepG2 cells. A. HMGA2 protein level of miR-150 in HepG2 cells transfected with si-HMGA2 using qRT-PCR. B. HMGA2 mRNA level in HepG2 cells transfected with si-HMGA2 using western blot. C. Expression of miR-150 in HepG2 cells transfected with anti-miR-150 using qRT-PCR. D. MTT assay was performed in HepG2 cells transfected with anti-miR-150 with/without si-HMGA2. E. HepG2 migration after transfection with anti-miR-150 with/without si-HMGA2. F. HepG2 Invasion after transfection with anti-miR-150 with/without si-HMGA2. Data were expressed as mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

cent normal tissues (P<0.001, **Figure 5A**). Furthermore, HMGA2 mRNA level was inversely correlated with miR-150 level in HCC tissues (r=-0.692, P=0.001, **Figure 5B**).

Discussion

In the present study, we have characterized miR-150 role in HCC tumor development and

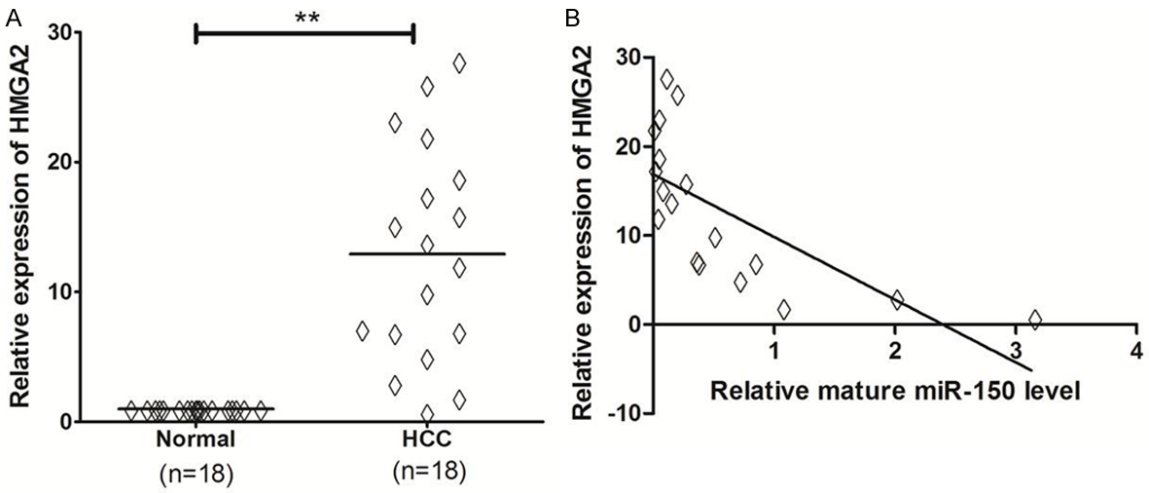

Figure 5. miR-150 was negatively correlated with HMGA2 in HCC tissues. A. Expression level of HMGA2 between HCC and normal tissue samples using qRT-PCR. B. HMGA2 mRNA level was inversely correlated with miR-150 level in HCC tissues. $^{**}P<0.01$ compared with the normal tissues.

metastasis. In addition, we identified HMGA2 as a direct target of miR-150 in HCC cells. HMGA2 silencing attenuated the pro-oncogenic effects of anti-miR-150 on HCC cells. Moreover, HMGA2 was inversely correlated with miR-150 in HCC tissues.

The miR-150 gene is located on chromosome 19q13. Aberrant miR-150 expression could contribute to the malignant phenotype of several tumors. Yin et al. reported that miR-150 was significantly higher in NSCLC tissues compared with normal tissues, and the level of miR-150 in NSCLC was strongly correlated with lymph node metastasis, distant metastasis and clinical TNM stage. Overall survival rate of patients with high miR-150 expression was significantly poorer compared with patients with low miR-150 [9]. In contrast, miR-150 overexpression significantly suppressed tumour cell proliferation migration, invasion and promoted cell apoptosis by targeting c-Myb in colorectal cancer [7]. Li et al. [10] found that miR-150 expression was significantly decreased in HCC tissues compared with non-tumor tissues. miR-150-5p overexpression suppressed cancer cell migration and invasion in vitro by targeting MMP14. In our study, we found that miR-150 was also significantly down-regulated in HCC tissues and cell lines. Overexpression of miR-150 significantly suppressed migration and invasion of HCC cells. These results suggest that miR-150 could function as a tumor suppressor gene inhibiting HCC metastasis, although the downstream regulatory mechanism of miR-150 differed from that of Li et al.

HMGA2 is a small nonhistone chromosomal protein that can modulate transcription by altering chromatin architecture [11]. It is also known to play important roles in human malignancies and carcinogenesis. Accumulating evidence suggests that HMGA2 was increased in a variety of tumors, including bladder cancer [12], thyroid cancer [13] and ovarian cancer [14]. The aberrant expression of HMGA2 was usually regulated by different miRNAs, such as miR-485, miR-204, miR-145, and then inhibited cell proliferation, promoted metastasis and epithelial-mesenchymal transition [12-15]. In the present study, we identified that HMGA2 was a target of miR-150 in HCC cells, and si-HMGA2 could partially attenuate pro-oncogenic effects of anti-miR-150 on HCC cells. Furthermore, HMGA2 was inversely correlated with miR-150 in HCC samples.

In conclusion, our present study has identified a novel metastasis suppressor miRNA, miR-150 is significantly decreased in HCC and miR-150 overexpression represses migration, and invasion of HCC cells by negatively regulating HMGA2 expression. Therefore, miR-150 might be considered as a biomarker for HCC, thus it could be used in an anti-metastatic therapy in HCC treatment.

Disclosure of conflict of interest

None.

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