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MiR-451a suppresses cell proliferation, metastasis and EMT via targeting YWHAZ in hepatocellular carcinoma

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been identified to participate in the progression of hepatocellular carcinoma (HCC). However, the function of miR-451a in HCC remains unknown. The aim of this study was to determine the function of miR-451a by construction of several experiments in HCC tissues and cells.

PATIENTS AND METHODS: The expression level of miR-451a in 69 paired of HCC and adjacent normal tissue samples was detected using quantitative Real-time polymerase chain reaction (qRT-PCR). MiR-451a expression in HCC derived cell lines was detected as well. By transfecting with miR-451a mimics or inhibitor, the expression level of miR-451a in HepG2 or Huh-7 cells was up- or down-regulated. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and colony formation analysis were employed to evaluate the changes of cell proliferation. Cell migration and invasion abilities were measured via transwell assay. Meanwhile, the underlying mechanism of miR-451a in HCC was demonstrated and verified by dual-luciferase assay and Western blotting, respectively. Rescue experiments were used to identify the downstream molecule of miR-451a in HCC.

RESULTS: MiR-451a expression in HCC tissue samples was significantly lower than that of adjacent normal samples. Meanwhile, the level of miR-451a in HCC cell lines was significantly down-regulated when compared with normal human hepatic cell line LO2. MTT assay and colony formation analysis showed that over-expressed miR-451a remarkably inhibited proliferation of HepG2 cells, whereas down-regulated miR-451a promoted growth of Huh-7 cells. Transwell results indicated that up-regulation of miR-451a significantly decreased HCC cell invasion and migration, while down-regulation remarkably increased cell metastasis. Furthermore, YWHAZ was identified as a direct target for miR-451a in HCC cells.

CONCLUSIONS: The expression level of miR-451a was decreased in HCC tissues and cell lines. Moreover, miR-451a inhibited the proliferation, invasion and migration of HCC cells via targeting YWHAZ. Our findings indicated that miR-451a could serve as a novel target for HCC diagnosis and biological therapy.

Key Words:
MiR-451a, Hepatocellular carcinoma (HCC), Proliferation, Metastasis, YWHAZ.

Introduction

Hepatocellular carcinoma (HCC) accounts for 85-90% of primary liver cancer. HCC is the fifth most common morbidity and third most common mortality cancer worldwide[1]. In recent years, comprehensive treatment based on surgical resection has made great progress. However, the overall 5-year survival rate of HCC is far from satisfactory[2]. Metastasis and recurrence of HCC have become a bottleneck restricting long-term survival of patients[3-5]. Therefore, it is of great significance to explore the molecular mechanism of HCC development, invasion and metastasis, and to find novel therapeutic targets. In recent years, researches[6] have found that microRNAs (miRNAs) play an important role in the development, diagnosis and treatment of various tumors, including HCC. MiRNAs are a class of endogenous, non-coding, and single-stranded RNAs with 21 to 23 nucleotides in length. It can bind completely or partially to the 3' non-coding region (3'-UTR) of target mRNA molecule. This may result in its cleavage or translational blockade, specific inhibition of gene expression, and involvement in the regulation of life processes[7,8]. For instance, down-regulation of miR-194-5p inhibits breast cancer cell proliferation, invasion and migration *via* repressing Wnt/β-catenin signaling axis[9]. In

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human gastric cancer, miR-17-92 acts as an oncogene and regulates nuclear factor-kappa B (NF-κB) signaling pathway by targeting TRAF3[10]. Meanwhile, miR-106b-5p promotes cell growth and inhibits cell apoptosis in non-small-cell lung cancer (NSCLC) through regulating BTG3[11]. Furthermore, miR-101 decreases the proliferation and metastasis of pancreatic cancer by down-regulating STMN1[12]. Previous works[13] have demonstrated that miR-451a regulates the growth and metastasis of multiple tumors through different targets. However, its exact role and function in HCC has not been elucidated. In our study, we first detect the relative expression of miR-451a in 69 pairs of HCC tumor tissues and matched normal tissues, as well as in HCC cell lines. Subsequently, HepG2 and Huh-7 cells were transfected with miR-451a mimics or inhibitor to over-express or knockdown miR-451a. Several functional experiments, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, colony formation assay and transwell assay were employed to demonstrate the influence of miR-451a on cell growth, invasion and migration. Western blotting and dual-luciferase analysis were used to explore the downstream molecules of miR-451a in HCC. Our study demonstrated that miR-451a acted as a tumor suppressor in HCC, and significantly inhibited tumor progression *via* YWHAZ. Our findings indicated that miR-451a could be used as a potential target for HCC biotherapy.

Patients and Methods

HCC Tissues and Cell Lines

A total of 69 paired HCC tissues and adjacent normal tissues were taken from the specimen library of Henan Traditional Chinese Medicine Hospital. Patients received no radiotherapy, chemotherapy or immunotherapy before or during surgery. The collection of HCC tissue specimens was approved by the Ethics Committee of Henan Traditional Chinese Medicine Hospital. Informed consent was obtained from each patient before the study. All specimens were washed with sterile and non-enzymatic water within 30 min after excision, and then placed in liquid nitrogen for subsequent use.

Cell Culture and Transfection

HEK 293T cell line, human normal hepatic cell line (LO2), and human hepatoma cell lines (HepG2, Huh-7, Hep3B, and SSMC-7721) were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle' Medium (DMEM) medium (Gbico, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gbico, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO_2 incubator. For transfection, miR-451a mimics, miR-451a inhibitor, and pcDNA-YWHAZ plasmid over-expressing YWHAZ were supplied by Genephrama Co. (Shanghai, China). Briefly, a total of 1×10^5 HepG2 or Huh-7 cells were seeded into 6-well plates. MiR-451a mimics or inhibitor was transfected into HepG2 or Huh-7 cells according to the instructions of Lipofectamine 3000 kit (Invitrogen, Carlsbad, CA, USA). NC and INC were used as negative control group and inhibitor negative control group, respectively. Hep G2 cells in logarithmic growth phase were transfected with pcDNA-YWHAZ and Lipofectamine 3000. 48 h after transfection, cells were collected for subsequent experiments.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in HCC tissues and experimental cells were extracted from using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA). By using cDNA as a template, the relative expression of miR-451a was detected by the SYBR Green (TaKaRa, Dalian, China) method according to the instructions. U6 was used as an internal reference. Relative expression level of miR-451a was calculated by the $2^{-\Delta\Delta Ct}$ method.

MTT Assay

Cells transfected with miR-451a mimics or inhibitor were first seeded into 96-well plates, followed by culture in a 37°C, 5% CO_2 incubator for 1, 2, 3, and 4 day, respectively. 20 μL of MTT solution (5 mg/mL) (Beyotime, Shanghai, China) were added to each well, and cultured for 4 h. After discarding the old culture solution, 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were added to each well and shaken at room temperature for 10 min. Optical density (OD) value at the wavelength of 490 nm was measured by a microplate reader.

Colony Formation Analysis

Cells transfected with miR-451a mimics or inhibitor treatment was seeded into 6-well plates at a concentration of 300 cells per well. After 15 days of culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), formed colonies were fixed with methanol and stained with crystal violet. Finally, the number of colonies with over 50 cells was counted and recorded.

Transwell Assay

For invasion and migration assays, 8 μm pore size inserts (Millipore, Billerica, MA, USA) were used. For cell invasion, the chamber was pre-coated with matrigel (BD, Franklin Lakes, NJ, USA). HepG2 or Huh-7 cells transfected with miR-451a mimics or inhibitor were seeded into the upper chamber at a density of 3×10^4 cells per well, and maintained in serum-free DMEM medium. Meanwhile, 600 μL DMEM containing 10% FBS were added in the lower chamber. After incubation for 48 h, cells on the upper chamber were wiped using cotton swabs. Cells on the lower surface of the chamber were fixed with methanol and stained with crystal violet. Invasive cells were observed under a microscope (ZEISS, Heidenheim, Germany) at 400 × magnification, and the number of invasive cells was recorded. Five fields were randomly selected for each sample. For cell migration, the insert was not pre-coated with matrigel, and the remaining steps were the same as invasion assay.

Luciferase Reporter Gene Assay

Luciferase reporter gene assay (Biovision, Milpitas, CA, USA) was used to verify targeted regulation of YWHAZ by miR-451a. HepG2 cells were seeded into 96-well plates at a density of 3 x 10^4 cells/well. Next, the cells were co-transfected with miR-451a mimics or NC and a plasmid containing miR-451a binding to wild type YWHAZ 3'-UTR sequence or mutant sequence. 48 h later, relative luciferase activities were determined by a dual luciferase reporter gene detection system.

Western Blotting

Transfected HepG2 or Huh-7 cells were fully lysed with lysis buffer (Beyotime, Shanghai, China). After reaction on ice for 5 min, the concentration of extracted protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Extracted protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h, and transferred onto 0.45 μm nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies of YWHAZ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA, USA) overnight. On the next day, the membranes were washed with phosphate-buffered saline and Tween-20 (PBST) solution (Beyotime, Shanghai, China), followed by incubation with corresponding secondary antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. After the membrane was completely washed, immunoreactive bands were exposed using enhanced chemiluminescence (ECL) luminescent solution (Millipore, Billerica, MA, USA).

Statistics Analysis

Statistical Product and Service Solutions (SPSS) 24.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Graphpad Prism 6.0 software (La Jolla, CA, USA) was applied for image editing. Experimental data were displayed as mean ± SD. *t*-test was used to compare the difference between two groups. $p<0.05$ was considered statistically significant.

Results

MiR-451a Was Lowly Expressed in HCC tissues and Cell Lines

We first detected miR-451a expression in 69 pairs of HCC tissues and adjacent normal tissues by qRT-PCR. As shown in Figure 1A, the expression level of miR-451a in HCC tissues was significantly lower than that of adjacent normal tissues. Subsequently, we detected miR-451a level in 4 HCC-derived cell lines (Huh-7, HepG2, SMMC7721, Hep3B) and 1 human normal hepatic cell line (LO2). QRT-PCR found that miR-451a was lowly expressed in HCC cells (Figure 1B). These results suggested that miR-451a acted as a tumor suppressor in HCC. To further detect the effect of miR-451a on HCC *in vitro*, we overexpressed and down-regulated miR-451a in HepG2 and Huh-7 cells, respectively. Results showed that the expression of miR-451a in HepG2 cells transfected with miR-451a mimics was significantly increased compared with NC group. However, miR-451a expression in Huh-7 cells treated with

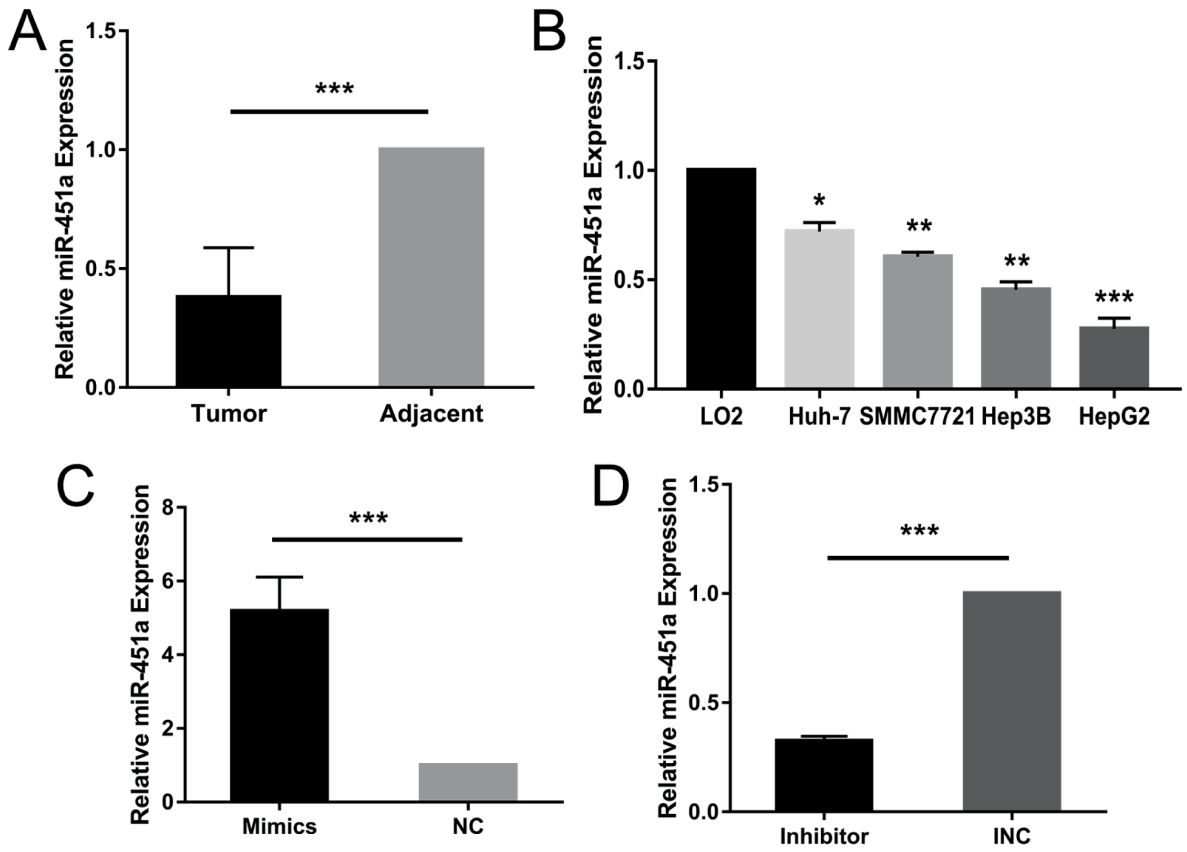

Figure 1. MiR-451a was decreased in HCC tissues and cell lines. ***A,*** Analysis of miR-451a expression in paired 69 HCC tissues and adjacent normal tissues. ***B,*** Analysis of miR-451a expression in 4 HCC cell lines (HepG2, SMMC7721, Hep3B, Huh-7) and 1 human normal hepatic cell line (LO2). ***C,*** Expression of miR-451a in HepG2 cells transfected with miR-451a mimics. ***D,*** Expression of miR-451a in Huh-7 cells transfected with miR-451a inhibitor. *p<0.05, **p<0.01, ***p<0.001.

miR-451a inhibitor was significantly decreased than that of INC group (Figure 1C, 1D).

MiR-451a Affected Proliferation of HCC Cells

To evaluate the influence of miR-451a on cell proliferation, we employed MTT assay and colony formation assay in established cells. MTT assay revealed that over-expression of miR-451a markedly inhibited proliferation of HepG2 cells. However, knockdown of miR-451a significantly promoted Huh-7 cell growth compared with negative control group (Figure 2A, 2B). Meanwhile, the number of colonies in HepG2 cells treated with miR-451a mimics was significantly less than those treated with NC. However, the number of colonies in Huh-7 cells transfected with miR-451a inhibitor was more than those transfected with INC (Figure 2C, 2D). These data indicated that miR-451a inhibited proliferation of HCC cells.

MiR-451a Influenced Invasion and Migration of HCC Cells

To test the influence of miR-451a on HCC cell metastasis, we detected invasion and migration abilities cells *via* transwell assay. Results indicated that the invasion of HepG2 cells over-expressing miR-451a was remarkably deceased. However, knocking down miR-451a could significantly increase the invasion of Huh-7 cells (Figure 3A). On the other hand, migration ability of HepG2 cells was significantly suppressed by miR-451a mimics transfection. However, miR-451a inhibitor remarkably promoted the migration of Huh-7 cells (Figure 3B). These results suggested that miR-451a inhibited invasion and migration of HCC cells.

YWHAZ Functioned as a Target for miR-451a in HCC

To further explore the underlying mechanism of miR-451a in HCC, we searched and cross-contrast-

ed several databases, including TargetScan, PiTar, miRBase. Results predicted that YWHAZ was a potential target of miR-451a in HCC, and the binding region was shown in Figure 4A. To verify our assumption, we conducted dual-luciferase reporters containing mutant or wild type binding region of YWHAZ 3'-UTR. They were co-transfected with miR-451a mimics in HepG2 cells. Luciferase activity was significantly reduced in wild type group, while no difference was found in mutant group (Figure 4B). Meanwhile, we detected the protein level of YWHAZ in cells. Western blotting demonstrated that the protein level of YWHAZ in HepG2 cells treated with miR-451a mimics was significantly lower than those transfected with NC. However, Huh-7 cells treated with miR-451a inhibitor expressed remarkably higher YWHAZ protein level compared with INC group (Figure 4C). These

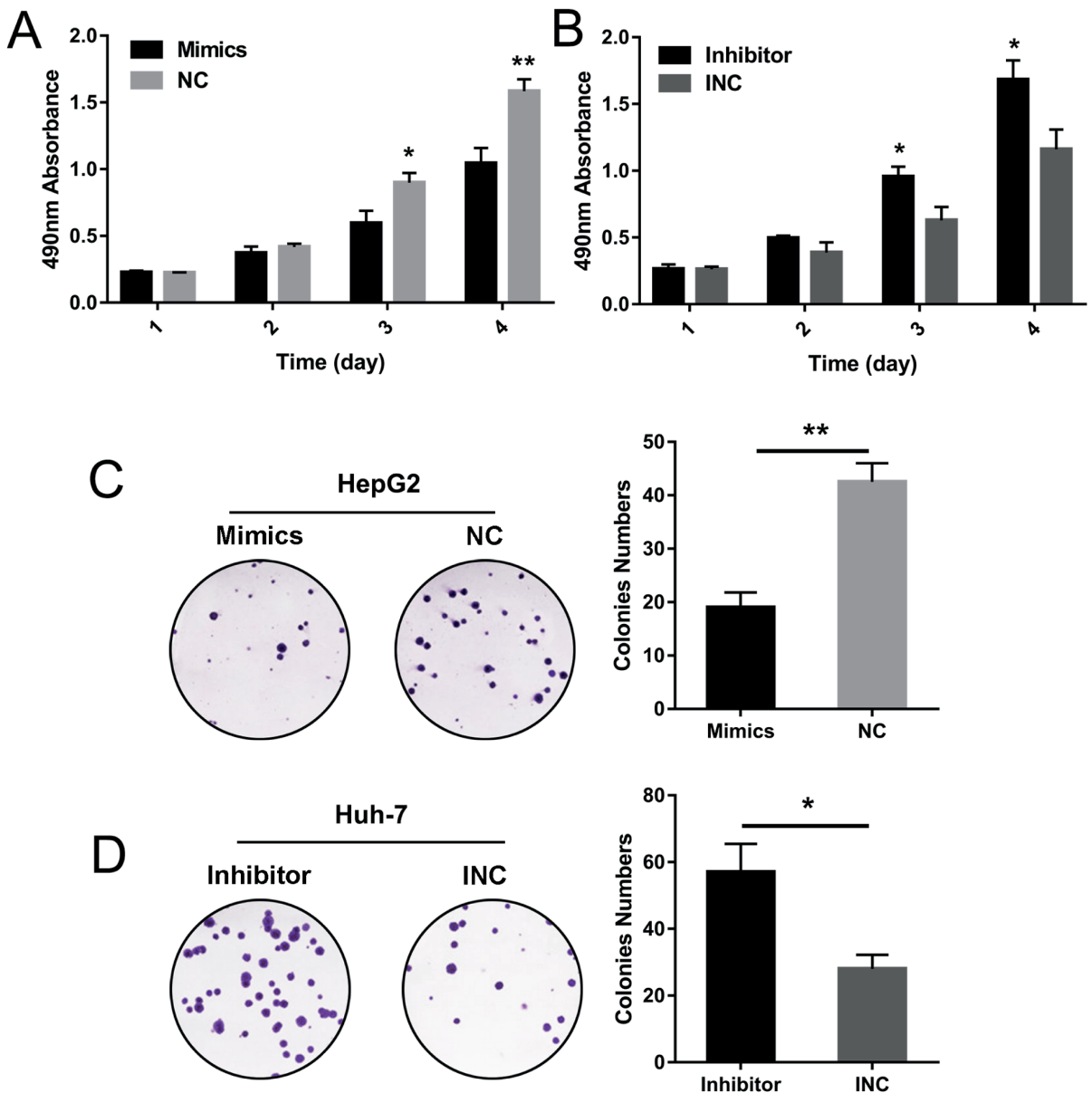

Figure 2. MiR-451a affected proliferation of HCC cells. ***A-B,*** MTT assay was performed to determine the proliferation of HepG2 (A) or Huh-7 ***(B)*** cells transfected with miR-451a mimics or inhibitors compared to each negative control. ***C-D***, Colony formation assay was performed to determine the growth of HepG2 (C) or Huh-7 (D) cells transfected with mimics or inhibitor, respectively. *p<0.05, **p<0.01.

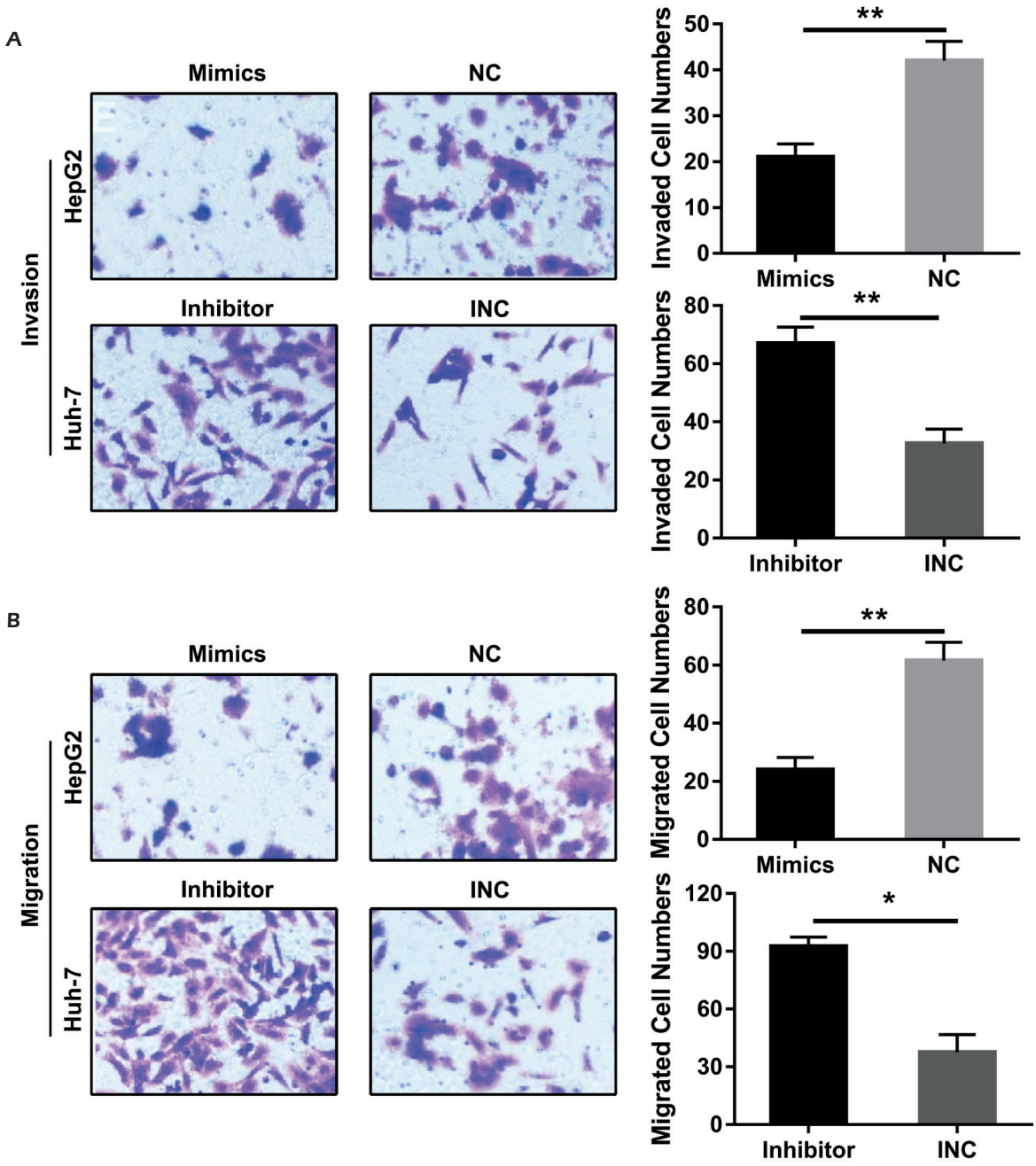

Figure 3. MiR-451a affected invasion and migration of HCC cells. A, Transwell invasion assay was used to detect the invasion ability of HepG2 cells transfected with miR-451a mimics or Huh-7 cells transfected with miR-451a inhibitor. B, Transwell migration assay was used to detect the migration ability of HepG2 cells transfected with miR-451a mimics or Huh-7 cells transfected with miR-451a inhibitor. Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01.

data indicated that YWHAZ was a direct target for miR-451a in HCC cells.

MiR-451a Suppressed Proliferation and Metastasis of HCC via YWHAZ

The above results identified that YWHAZ was a direct target of miR-451a. Subsequently, we designed rescue experiments by over-expressing YWHAZ in HepG2 cells using pcDNA-YWHAZ. As shown in Figure 5A, after co-transfection with miR-451a mimics and pcDNA-YWHAZ, the protein level of YWHAZ reduced by restore of miR-451a expression. Next, we detected the proliferation and invasion capacities of miR-451a

mimics group, control group and miR-451a+pcDNA-YWHAZ group. MTT assay found that cell growth inhibited by miR-451a mimics was rescued by YWHAZ recovery (Figure 5B). Similarly, cell invasion ability was also restored by pcDNA-YWHAZ (Figure 5C). These results confirmed that miR-451a suppressed cell proliferation and metastasis *via* repressing YWHAZ expression.

Discussion

HCC remains one of the most common malignant tumors due to its high recurrence rate. Meanwhile, high recurrence rate is one of the main factors affecting the survival rate and prognosis of patients[14,15]. Therefore, related biomarkers have been explored to guide early diagnosis, treatment and prognosis[16]. MiRNAs are a class of endogenous non-coding RNAs with 21-23 nt in length, which can regulate gene-coding genes[17]. MiRNAs are widely found in eukaryotes. Previous studies have indicated that miRNAs participate in various processes of tumorigenesis, such as development, cell differentiation, cell proliferation and apoptosis[18]. Meanwhile, studies on the expression profiles of miRNAs have identified multiple molecular markers associated

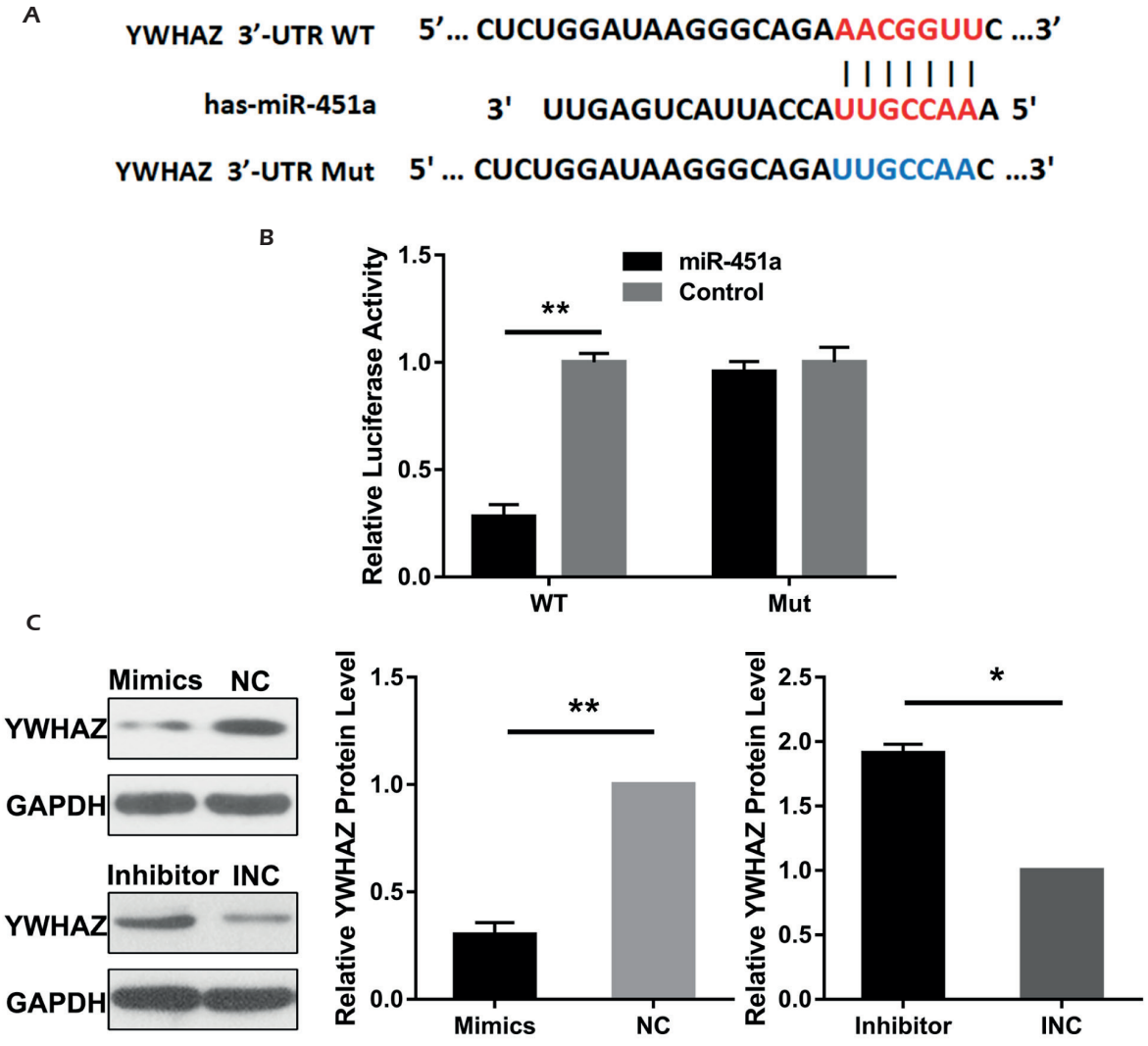

Figure 4. YWHAZ was a direct target of miR-451a. ***A,*** The predicted binding sites of miR-451a in the 3'-UTR of YWHAZ. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C,*** Protein levels of YWHAZ and GAPDH were measured by Western blotting in miR-451a over-expression HepG2 cells and miR-451a knockdown Huh-7 cells. The relative protein level of YWHAZ was normalized to GAPHD. Data were presented as mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01.

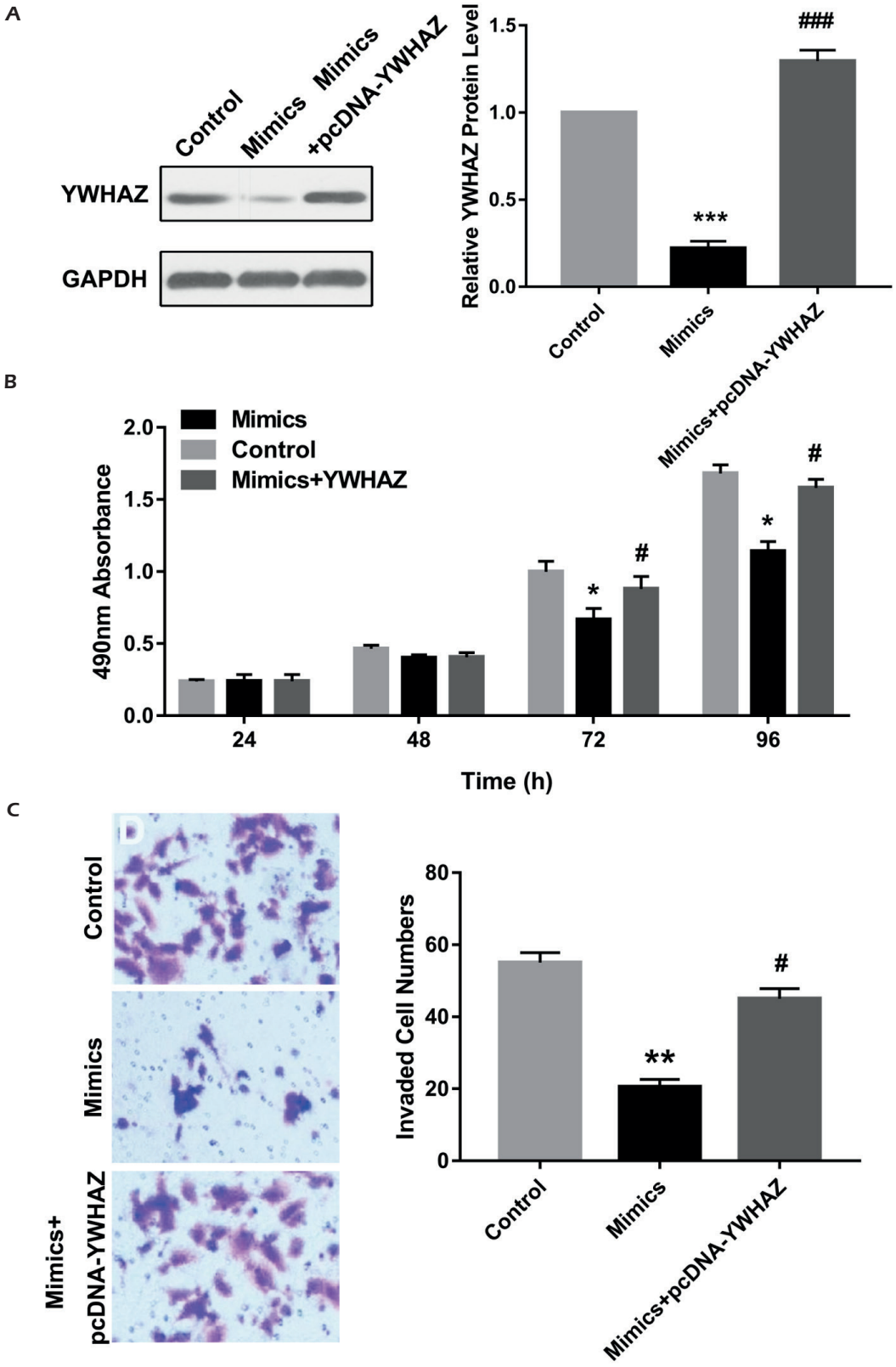

Figure 5. YWHAZ rescued the effects of miR-451a overexpression in HepG2 cells. ***A,*** Western blotting analyses of YWHAZ. GAPDH was used as an internal reference. ***B,*** Analysis of cell proliferation ability by MTT assay in control, mimics, or mimics + pcDNA-YWHAZ treated HepG2 cells. ***C,*** Cell invasion ability was measured by transwell assay. Data were represented as mean ± SD of three replicates. **p*<0.05, ***p*<0.01, ****p*<0.001, #*p*<0.05, ### ****p*<0.001.

with human tumor diagnosis, staging, progression, prognosis and therapeutic responsiveness[7,19]. MiR-451a has been proved as a tumor suppressor in several types of cancers. For example, in non-small cell lung cancer, it suppresses cell invasion and migration *via* regulating ATF2. In renal cell carcinoma, miR-451a affects oncogenic pathways and acts as an anti-tumor miRNA. In lung adenocarcinoma (LAC), together with miR-126-3p, it is related to clinic-pathological features and serves as a protective factor from LAC[13,20,21]. Moreover, miR-451a can enhance sensitive of breast cancer to Tamoxifen and inhibit cell growth. MiR-451a is lowly expressed in papillary thyroid carcinoma (PTC), targeting AKT/mTOR pathway and inhibiting the pathway activity[22,23]. In our study, we first detected the level of miR-451a in 69-paired HCC tissues and matched normal tissues. Results found that the expression of miR-451a in HCC tumor tissues was significantly decreased. Meanwhile, miR-451a was lowly expressed in HCC-derived cell lines than human normal hepatic cell line. These data indicated that miR-451a acted as a tumor suppressor in several malignant tumors. Subsequently, we found that over-expression of miR-451a obviously inhibited HepG2 cell proliferation, invasion and migration. However, knockdown of miR-451a promoted Huh-7 cell growth and metastasis. These data showed that miR-451a inhibited HCC development and progression. MiRNAs do not encode proteins, but are widely involved in the regulation of gene expression by interacting with the 3' non-coding regions of target mRNAs[17]. Here, we searched several databases and found that YWHAZ was a potential target for miR-451a. Dual-luciferase reporter gene assay verified that miR-451a could directly bind to the 3'-UTR of YWHAZ. Western blotting analysis indicated miR-451a up-regulation significantly reduced the protein level of YWHAZ in HepG2 cells, while miR-451a down-regulation increased YWHAZ protein expression in Huh-7 cells. YWHAZ, as an important regulator of cell growth and apoptosis, is involved in the regulation of various tumor pathways[24,25]. In HCC, YWHAZ was identified as a biomarker for HCC prognosis. In ovarian cancer, inhibition of YWHAZ could suppress tumorigenicity and drug resistance[24]. Moreover, YWHAZ is over-expressed in adenocarcinoma of esophago-gastric junction, serving as an independent factor for prognosis[25]. YWHAZ can also be regulated by several miRNAs including miR-375 in ccRCC and miR-22 in HCC[26]. Furthermore, we restored YWHAZ expression in miR-451a over-expressed HepG2 cells. Subsequent experiments found that cell proliferation and invasion abilities decreased by miR-451a mimics was rescued by YWHAZ recovery. All these findings indicated that miR-451a inhibited HCC cell growth, invasion and migration *via* repressing YWHAZ expression.

Conclusions

We showed that miR-451a was down-regulated in HCC tissues and cells. Moreover, it inhibited HCC cell proliferation, migration and invasion through repressing YWHAZ. We provided a novel insight for HCC diagnosis and biological therapy. Further *in vivo* studies are still needed for deeper research in the future.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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