# Interaction between miR-206 and lncRNA MALAT1 in regulating viability and invasion in hepatocellular carcinoma

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**Abstract.** MicroRNAs (miRNAs) are strongly associated to the progression of hepatocellular carcinoma (HCC), which presents a high potential for diagnosis and treatment; however, the role of miRNAs is still largely unknown. The aim of the present study was to examine the expression and the biological role of miRNA (miR)-206 in the development of HCC, and to identify the underlying molecular mechanism. Results from this study show that miR-206 was significantly downregulated in HCC tissues and cell lines. It was observed that low expression of miR-206 was linked to advanced TNM stage, tumor nodularity and venous infiltration in patients with HCC; low miR-206 expression was associated with shorter survival times. miR-206 overexpression using miR-206 mimics notably decreased the proliferative ability and increased apoptosis of MHCC97-H and HCCLM3 HCC cell lines. Overexpression of miR-206 suppressed invasiveness associated with reduced epithelial-mesenchymal transition. Moreover, the c-Met oncogene, which is upregulated in HCC tissues, was negatively associated with the expression of miR-206. Notably, it was shown that miR-206 may exert its antitumor effect through suppressing c-Met/Akt/mTOR signaling. Low expression of miR-206 was shown to be regulated by lncRNA MALAT1 in HCC. Collectively, this study presented evidence that miR-206 was controlled by lncRNA MALAT1 and partially suppressed the proliferation and invasion of HCC through the c-Met/Akt/mTOR signaling pathway. According to these results, understanding MALAT1/miR-206-dependent regulation may lead to potential approaches for diagnosis and prospective treatment of HCC.

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*Key words:* hepatocellular carcinoma, microRNA-206, c-Met/Akt/mTOR signaling pathway, lncRNA MALAT1

## Introduction

More than 500,000 patients around the world succumb to complications associated with hepatocellular carcinoma (HCC), which is one of the most prevalent cancers and the third leading cause of cancer-related death (1,2). Despite advances in medical therapy, such as regorafenib and metronomic capecitabine treatment, prognosis of HCC remains poor (3,4). Even with immunotherapy under evaluation in patients with HCC, agents such as programmed cell death-1 inhibitor have so far not brought the expected results (5-8). Thus, there is an urgent need to understand the underlying disease pathogenesis to inform and improve current therapeutic approaches.

MicroRNAs (miRNAs) are a class of small, conserved, non-coding RNA molecules with an average length of 22 nucleotides that negatively regulate protein expression by interacting with their target mRNAs (9). Previous reports have shown the significance of miRNAs function in a variety of biological processes, such as cell division, death, migration and invasion (10,11). A growing number of miRNAs have been reported to be closely related to a variety of cancer types, including HCC (12). For instance, Yin *et al* (13) demonstrated the tumor suppressor role of miR-361-5p in inhibiting viability, migration and invasion in HCC cell lines through targeting Twist1. Jiang *et al* (14) showed that miR-874, a tumor suppressor, inhibited metastasis and epithelial-mesenchymal transition in HCC by targeting sex-determining region Y-box 12. By contrast, Li *et al* (15) reported that miR-155 serves as an oncogene by increasing the invasiveness and metastasis of HCC cells. In several studies, a number of miRNAs have been linked to the clinical outcomes of patients with HCC (16,17). miR-342-3p upregulation was a factor in the poorer overall survival (OS) of patients with HCC (16). Therefore, examining the role of miRNAs in HCC may help to find potential treatment targets for this condition.

Long non-coding RNAs (lncRNAs) are a class of non-protein coding transcripts >200 nucleotides in length; they are the largest subclass of the non-coding transcriptome in humans (18). Previous studies have revealed a regulatory mechanism for lncRNAs as a competitive endogenous RNA (ceRNA) to miRNA, thus releasing the suppressive effects of miRNAs on their target mRNAs (19-21). For example, lncRNA PP7080 promoted HCC cell viability, migration and invasion through the miR-601/SIRT1 signaling axis (22).

Guan *et al* (23) showed that lncRNA TP73-AS1 promoted cell proliferation, migration and invasion of cervical cancer cell lines by targeting miR-329-3p to regulate the expression of the SMAD2 gene. Zhuang *et al* (24) demonstrated that miR-92b promoted HCC progression by targeting Smad7, and this was mediated by lncRNA XIST. Therefore, the focus of the present study was the interaction between miRNAs and lncRNAs in the development of HCC.

In the present study, miR-206, and its expression in HCC tissues and its association with the clinicopathological parameters of patients with HCC was investigated. Then, the influence of miR-206 upon HCC cell function was explored, and the connection between miR-206 and lncRNA MALAT1 was studied. Moreover, the underlying molecular mechanisms of miR-206 in HCC were also studied *in vitro*. Results suggested that miR-206 may be chosen as a new treating target and is a potential biomarker for HCC.

## Materials and methods

*Clinical specimens*. A total of 50 resected HCC and matched tumor-adjacent tissues (>5 cm away from the tumor) were obtained from patients admitted to the First Affiliated Hospital of Henan University of Science and Technology (Luoyang, China) between January 2019 and May 2020. The inclusion and exclusion criteria were strictly controlled. The details are as follows. Inclusion criteria are: i) Accurate pathological diagnosis of primary HCC; ii) Obtain complete clinicopathological and follow-up data; iii) Mainly surgical resection; iv) Willing to sign informed consent. The exclusion criteria are (1) perioperative death; (2) undergo palliative surgery; (3) have been treated with chemotherapy drugs; (4) combined with other cancers. Patient clinicopathological data is shown in Table I. Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology (Ethics approval number: 2019-006).

*miRNA expression profile data from GEO*. Data on miRNA expression profiles were downloaded from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo) using the accession number GSE10694. Based on interactive web tool GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r), differentially expressed miRNAs between HCC tumor samples and adjacent normal tissues were evaluated through the R 'limma' package (Version 4.2), which is a widely used tool that can be used to analyze data from any GEO series and significance analysis of microarray (SAM), to determine the differential expression of miRNAs among groups. The differentially expressed miRNAs were identified with general linear model, and a fold change >2 and P-value <0.05 were recognized as significantly differentially expressed miRNAs. Data were visualized as heat maps using the online tool Morpheus (a web-based tool, software.broadinstitute.org/morpheus/). Subsequently, reverse transcription-quantitative PCR (RT-qPCR) was used to confirm the miRNAs that were the most significantly differentially expressed (P<0.05).

*Reverse transcription-quantitative PCR (RT-qPCR)*. miRNA was extracted from HCC tissue or cell lines using a miRNeasy mini kit (cat no. 217004; Qiagen Sciences, Inc.) and total RNA was extracted with the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. cDNA was synthesized using a miScript II RT kit (Qiagen Sciences, Inc.). For mRNA reverse transcription, cDNA was synthesized using the PrimeScript™ First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.). On an ABI PRISM 7500 Real-time PCR equipment, miRNA and mRNA real-time qPCR were carried out according to a standard procedure from the SYBR Green PCR kit (Toyobo Life Science; Thermo Fisher Scientific, Inc.). The expression the level of miR-206 was normalized to small nuclear RNA U6, while c-Met and MALAT1 was normalized to GAPDH. The primers for qPCR analysis were as follows: miR-206 forward, 5'-GCGTGGAATGTAAGGAAGT-3'; miR-206 universal reverse, 5'-GCAGGGTCCGAGGTATTC-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; c-Met forward, 5'-CATCTCAGAACGGTTCATGCC-3' and reverse, 5'-TGCACAATCAGGCTACTGGG-3'; MALAT1 forward, 5'-ATGCGAGTTGTTCTCCGTCT-3' and reverse, 5'-TATCTGCGGTTTCCTCAAGC-3'; GAPDH forward, 5'-TCAACGACCCCTTCATTGACC-3' and reverse, 5'-CTTCCCGTTGATGACAAGCTTC-3'. qPCR amplification protocol was as follows: Initial denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec. The RT-qPCR assays were run in triplicate and the change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method (25).

*Cell lines and cultures*. The HCC cell lines MHCC97-H, Huh-7, Hep3B and HCCLM3 were acquired from the American Type Culture Collection (ATCC). 293T cells were acquired from the cell bank of Chinese Academic of Sciences, Shanghai, China. The immortalized non-tumorigenic human hepatocyte cell line, MIHA, was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. All cells were cultured in DMEM containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C under 5% $CO_2$.

*Cell transfection*. miR-206 mimics, mimics negative control (NC), miR-206 inhibitor and inhibitor NC were purchased from Chang Jing Bio-Tech, Ltd. For MALAT1 upregulation, lncRNA MALAT1 (NCBI reference sequence, NR_002819.4) was inserted into pcDNA3.1 vector (cat no. V87020; Thermo Fisher Scientific, Inc.) with the *Xba*I and *Eco*RI restriction sites. Similarly, the open reading frame (ORF) of c-Met (NCBI GenBank accession number, J02958.1) was cloned into pcDNA3.1 vector with the *Hind*III and *Bam*HI restriction sites. In addition, MALAT1-targeted small interfering (si)RNA (si-MALAT1) and NC Scramble siRNA (si-Scramble) were also purchased from Chang Jing Bio-Tech, Ltd. Transfections of the miRNA mimics (10 nM), miRNA inhibitors (10 nM), pcDNA vectors (2 $\mu$g) and 25 nM of siRNAs, including the respective NCs, were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37˚C for 24 h, according to manufacturer's instructions. Non-transfected cells are used as control group (Blank). Sequences of miR-206 mimics, inhibitor, MALAT1 siRNA and corresponding controls are

as follows: miR-206 mimics, 5'-UGGAAUGUAAGGAAG UGUGUGG-3'; mimics NC, 5'-UAAGGGGAUGAGUGG AUAUGUG-3'; miR-206 inhibitor, 5'-CCACACACUUCC UUACAUUCCA-3'; inhibitor NC, 5'-CUCUAACACUCU ACCUCACCUA-3'; si-MALAT1, 5'-CAGAAGGUCUGA AGCUCAUACCUAA-3'; si-Scramble, 5'-AGGCAUCAUAAG UGAACUCGUACCA-3'. All subsequent experiments were performed 24 h after transfection.

*Cell viability assay*. Cell viability was assessed by MTT assay. HCCLM3 and MHCC97-H cells ($2x10^4$) were seeded in 96-well plates at 24-h post-transfection. At 0, 24, 48, 72 and 96 h, 20 $\mu$l MTT reagent (5 mg/ml) was added to each well and further incubated at 37˚C for 4 h. Then the culture medium was removed from each well, and 150 $\mu$l DMSO was added to each well to dissolve the purple MTT-formazan crystals for 10 min at 37˚C. The absorbance of each well at 490 nm was determined using a microplate reader.

*Apoptosis assay*. HCCLM3 and MHCC97-H cells ($5x10^5$ cells/well) were seeded in 6-well plates overnight at 37˚C, transfected for 24 h with miR-206 mimics, miR-206 mimics + pcDNA-c-Met, or mimics NC. Apoptotic rates were determined using the Annexin V-FITC Apoptosis Detection Kit (Abcam). Cells were digested with 0.25% trypsin (MilliporeSigma; Merck KGaA), centrifuged at 300 g for 5 min, resuspended in 20 $\mu$l binding buffer and incubated with 5 $\mu$l Annexin V-FITC and 1 $\mu$l PI in a dark room at room temperature for 20 min. The stained cells were then analyzed using BD FACSCalibur Flow Cytometer System (BD BioSciences). Data analysis was performed using BD FACSuite™ software (Version 6.0, BD Biosciences).

*Caspase-3 activity*. Caspase-3 activity assay was performed using Caspase-3 colorimetric assay kit (BD Biosciences) according to the manufacturer's protocol. The results were evaluated using a microplate reader (Bio-Rad, California, USA) at 405 nm.

*Wound-healing assay*. HCCLM3 and MHCC97-H cells were seeded into six-well plates at a density of $3x10^5$ cells/well overnight at 37˚C, the cells were transfected for 24 h with miR-206 mimics, miR-206 mimics + pcDNA-c-Met, or NC oligonucleotides. A new 1 ml pipette tip was used to scratch the center of the cell layer once it had reached 70-80% confluence. Afterward, serum-free medium was added and cultivated for 24 h at 37˚C and 5% $CO_2$. The distances between the wound sides were captured in the same fields at the baseline and 48 h later using an inverted microscope (magnification, x200). The percentage of the wound healing was quantified using the ImageJ Software (version 1.49; National Institutes of Health).

*Matrigel invasion assays*. Cell invasive ability was examined using 24-well Transwell chambers with an 8 $\mu$m pore size (Corning, Inc.) at 37˚C. Briefly, The HCCLM3 and MHCC97-H cells at $1x10^5$ density were put in the top chamber with 200 $\mu$l serum-free DMEM. Furthermore, 500 $\mu$l complete DMEM and 10% FBS were added to the bottom chamber. The cells was washed with PBS twice 48 h after transfection, and stained with 0.1% crystal violet (Baomanbio Biomart Co, Shanghai, China) for 15 min at 37˚C. The cells that invaded the bottom chamber were counted under a light microscope (magnification, x200; Olympus Corporation).

*Luciferase reporter assay*. The biological prediction website microRNA.org was employed to analyze and predict the target genes of miR-206. To create the wild-type (wt) c-Met-3'-UTR vector and the mutant c-Met-3'-UTR vector, respectively, the 3'-UTR of c-Met and the fragment of Met 3'-UTR mutant were separately inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA). The 293T cells ($1x10^5$) were cultured in 24-well plates and transfected for 48 h at 37˚C using Lipofectamine 2000 with 0.5 $\mu$g wild type c-Met 3' UTR and mutant c-Met 3'UTR reporter plasmid, together with 50 nM miR-206 mimics, 100 nM miR-206 inhibitor or the respective NC. The relative firefly luciferase activity normalized with *Renilla* luciferase was measured 48 h after transfection by using the Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's instructions. Three duplicates of each experiment were carried out.

*Western blot*. Total protein was extracted from cells using the RIPA lysis buffer 48 h after transfection (Beyotime Institute of Biotechnology). A BCA protein assay reagent kit was used to measure the protein contents in the supernatant after cell lysates were centrifuged at 12,000 x g for 20 min at 4˚C (Beyotime Institute of Biotechnology). Proteins (40 $\mu$g/lane) were separated by SDS-PAGE using 8% gels, and then electroblotted on PVDF membranes. Primary antibodies against c-Met (1:1,000; BF8218, Affinity Biosciences, Ltd.), E-cadherin (1:1,000, sc-8426, Santa Cruz Biotechnology, Inc.), N-cadherin (1:1,000, sc-8424, Santa Cruz Biotechnology, Inc.), fibronectin (1:1,000, sc-8422, Santa Cruz Biotechnology, Inc.), vimentin (1:2,000, sc-6260, Santa Cruz Biotechnology, Inc.), phosphorylated (p)-c-Met (1:1,000, AF8121, Affinity Biosciences, Ltd.), p-mTOR [1:1,000; cat no. 5536, Cell Signaling Technology, Inc. (CST)], mTOR (1:1,000; cat no. 4517; CST), p-AKT (1:1,000; cat no. 5106, CST), AKT (1:1,000; cat no. 2920, CST) and β-actin (1:2,000; sc-47778, Santa Cruz Biotechnology, Inc.) were added to membranes and incubated at 4˚C overnight. Subsequently, the membranes were incubated with secondary antibody (Horseradish Peroxidase-conjugated IgG, 1:5,000, sc-516102, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 37˚C. The protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific Inc., Waltham, USA), and the relative gray value was analyzed were quantified using the ImageJ Software (version 1.49; National Institutes of Health).

*Statistical analysis*. Statistical analysis was performed using SPSS (version 18.0; SPSS, Inc.). Data are presented as mean ± SD. Paired or unpaired Student's t-test was used to compare differences between two groups; one-way ANOVA followed by Tukey's post hoc test was used to compare differences between multiple groups. $\chi^2$ test and Fisher's exact test were used to investigate the relationships between miR-206 levels and clinicopathological characteristics. Pearson's correlation coefficient was used to examine the correlation between the expression levels of miR-206 and lncRNA MALAT1 or c-Met mRNA. Kaplan-Meier survival curves were analyzed

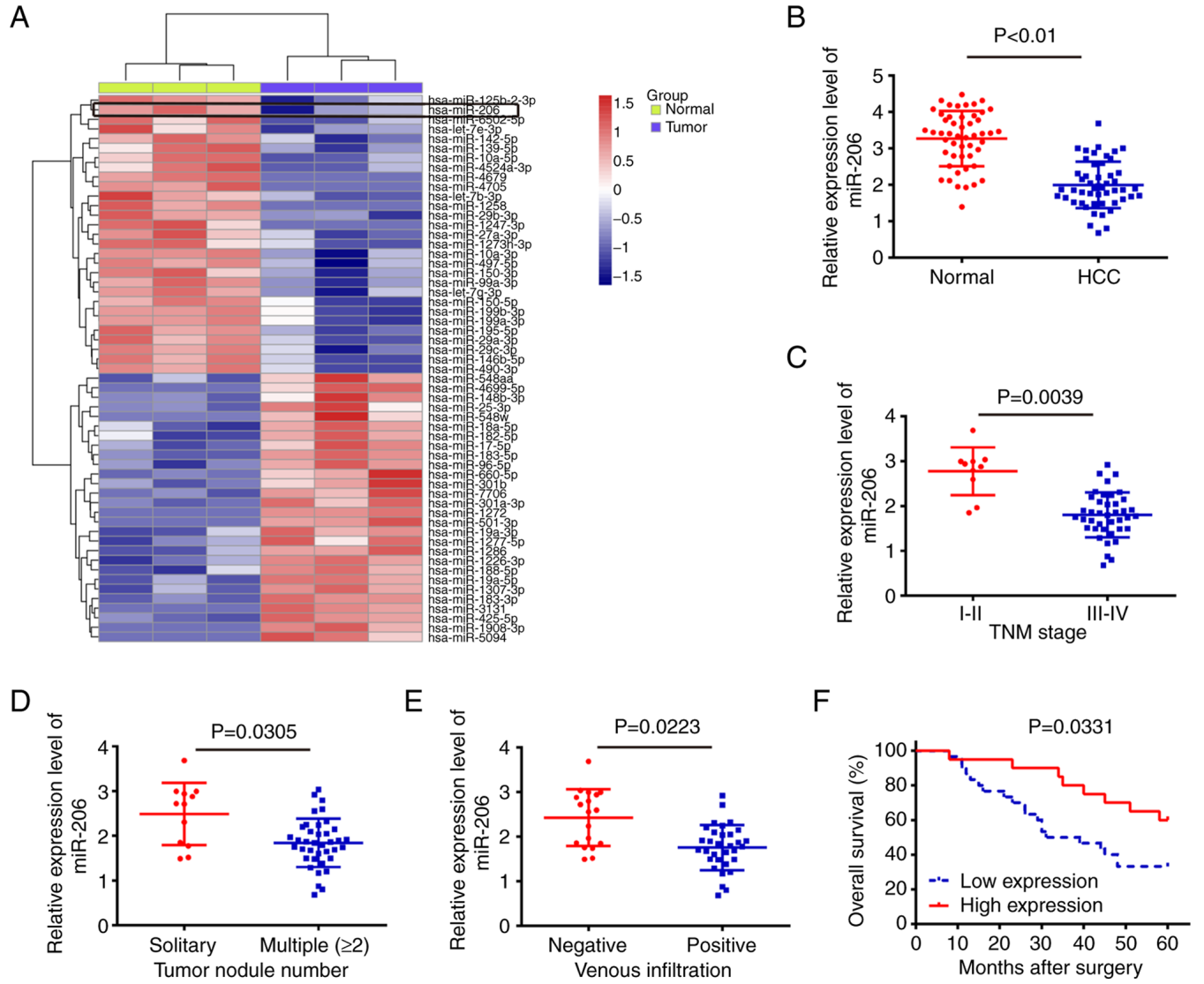


Figure 1. miR-206 is downregulated in HCC tissues and is associated with clinicopathologic features. (A) Heatmap of normalized expression levels of microRNAs in HCC tissues and matched tumor-adjacent tissues, based on the GSE10694 array dataset. Blue indicates low expression levels; red indicates high expression levels. (B) miR-206 expression was detected by reverse transcription-quantitative PCR in HCC and matched tumor-adjacent tissues (n=50). P<0.01 vs. Normal. The relationship between miR-206 and clinicopathological features of patients with HCC, including (C) TNM stage, (D) tumor nodule number and (E) venous infiltration. (F) Overall survival of patients with high or low miR-425-5p expression. HCC, hepatocellular carcinoma; miR, microRNA.

by log-rank. P≤0.05 was considered to indicate a statistically significant difference.

## Results

*miR-206 expression is downregulated in HCC tissues and is associated with clinicopathological features*. To explore the role of miRNAs in HCC, differentially expressed miRNAs from the GEO dataset GSE10694 were analyzed. Cluster analysis based on miRNA expression demonstrated a significant difference between HCC and adjacent normal tissue; 29 miRNAs were upregulated and 27 miRNAs were downregulated (Fig. 1A). Among aberrantly expressed miRNAs, miR-206 had the lowest expression level in the HCC group. Notably, miR-206 has previously been reported to function as a tumor suppressor in various types of cancers, including lung adenocarcinoma, breast cancer and colorectal cancer (26-28). Although miR-206 has been reported to function as tumor suppressor in various tumors including HCC, we conducted this study to further determine the roles of miR-206 in HCC, especially in clinical pathological characteristics. To validate the expression of miR-206 identified from the miRNA microarray assay, RT-qPCR was conducted to investigate the expression of miR-206 in 50 pairs of HCC tissue. The results showed that the expression of miR-206 in HCC tissues was significantly lower compared with that of their matched adjacent normal tissues (Fig. 1B).

Subsequently, the 50 patients with HCC were divided into two groups, the miR-206 high-expression group and the miR-206 low-expression group, using the mean expression level of miR-206 as a cut-off value. The association between miR-206 expression and clinicopathological characteristics of the patients is summarized on Table I. Low miR-206 expression was shown to be associated with advanced tumor nodularity, TNM stage and venous infiltration (Fig. 1C-E). The Kaplan-Meier survival curve revealed that patients in the

Table I. Association between miR-206 and clinicopathological features of patients with hepatocellular carcinoma.

| Clinicopathological feature | Total (n=50) | miR-206 expression | | P-value |
|---|---|---|---|---|
| | | High | Low | |
| Sex | | | | 0.8045 |
| Male | 34 | 14 | 20 | |
| Female | 16 | 6 | 10 | |
| Age, years | | | | 0.7256 |
| ≥50 | 29 | 11 | 18 | |
| <50 | 21 | 9 | 12 | |
| Tumor size, cm | | | | 0.1220 |
| ≥5 | 19 | 5 | 14 | |
| <5 | 31 | 15 | 16 | |
| Tumor nodule number | | | | 0.0445[a] |
| Multiple (≥2) | 38 | 12 | 26 | |
| Solitary | 12 | 8 | 4 | |
| TNM stage | | | | 0.0088[a] |
| I-II | 10 | 8 | 2 | |
| III-IV | 40 | 12 | 28 | |
| Venous infiltration | | | | 0.0223[a] |
| Negative | 18 | 11 | 7 | |
| Positive | 32 | 9 | 23 | |
| Differentiation | | | | 0.0771 |
| Well and moderate | 30 | 9 | 21 | |
| Poorly | 20 | 11 | 9 | |
| Serum AFP level, $\mu$g/l | | | | 0.2971 |
| ≤400 | 27 | 9 | 18 | |
| >400 | 23 | 11 | 12 | |

[a]P<0.05. AFP, α-fetoprotein.

low miR-206 expression group had a worse 5-year OS rate compared with those in the high miR-206 expression group (Fig. 1F). These findings suggested that miR-206 may be useful as a biomarker for predicting the clinical prognosis of patients with HCC.

*Overexpression of miR-206 suppresses cell viability and induces cell apoptosis*. To determine the effects of miR-206 in HCC, the expression of miR-206 was investigated by RT-qPCR in the HCC cell lines MHCC97-H, Huh-7, Hep3B and HCCLM3; the immortalized non-tumorigenic human hepatocyte cell line, MIHA, was used as a NC. RT-qPCR demonstrated that all HCC cell lines had significantly lower levels of miR-206 compared with MIHA cells, which is similar to the findings in HCC tissues (Fig. 2A). miR-206 mimics were transfected into MHCC97-H and HCCLM3 cells because they exhibited the lowest expression levels of miR-206 compared with the other cell lines; the results showed a significant increase in miR-206 expression (Fig. 2B). miR-206 overexpression resulted in a significant reduction in cell viability of MHCC97-H and HCCLM3 cells compared with cells transfected with mimics NC (Fig. 2C). Furthermore, the effects of overexpression of miR-206 on cell apoptosis were determined by caspase-3 activity and flow cytometry. Overexpression of miR-206 significantly promoted caspase-3 activity and induced apoptosis in both MHCC97-H and HCCLM3 cells compared with the respective mimics NC groups (Fig. 2D-F). These results demonstrated that miR-206 inhibited HCC cell viability and induced apoptosis.

*miR-206 overexpression suppresses cell migration and invasion*. Whether miR-206 overexpression could modify the metastatic potential of HCC was investigated, as the ability of HCC to spread is a significant determinant in the poor prognosis of patients (29). Overexpression of miR-206 significantly reduced the migratory and invasive capacity of MHCC97-H and HCCLM3 cells (Fig. 3A-C). Epithelial-mesenchymal transition (EMT) is an important contributor in the early stage of the metastatic cascade (30). To determine if miR-206 affects EMT in HCC cells, Western blot was performed to examine the expression of EMT related proteins. The findings showed that overexpression of miR-206 in MHCC97-H and HCCLM3 cells increased the expression of E-cadherin, a known epithelial marker, and decreased the levels of the mesenchymal-markers

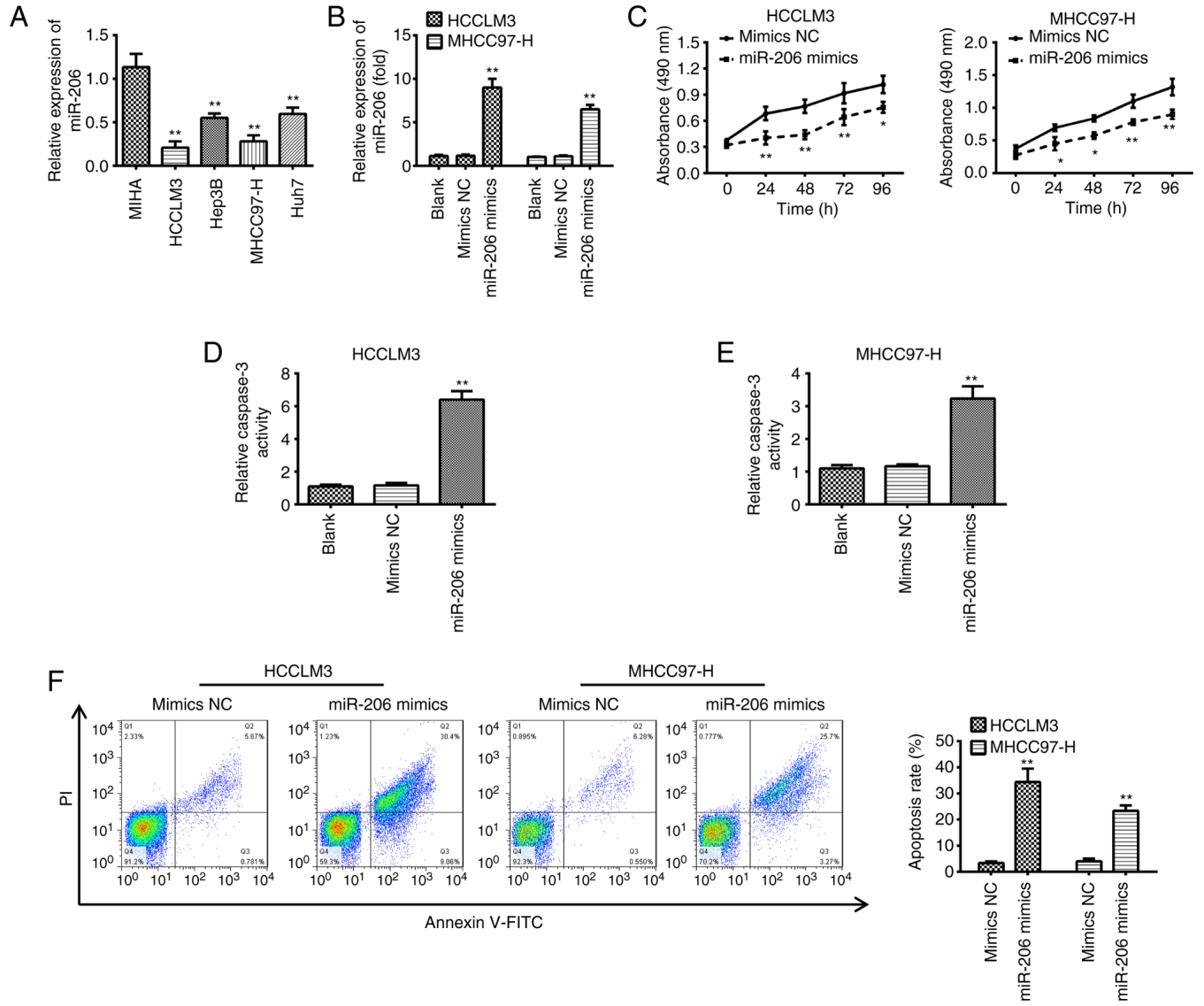


Figure 2. Overexpression of miR-206 suppresses cell viability and induces apoptosis. (A) miR-206 expression of in the immortalized non-tumorigenic human hepatocyte cell line MIHA, which was used as the NC, and the HCC cell lines MHCC97-H, HCCLM3, Huh-7, and Hep3B. Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01 vs. MIHA. (B) Reverse transcription-quantitative PCR was used to assess the miR-206 transfection efficiency. (C) The MTT assay was used to examine cell viability. A commercial kit was used to measure the caspase-3 activity in (D) HCCLM3 and (E) MHCC97-H cells. (F) Flow cytometry was used to investigate apoptotic rates. Data are presented as the mean ± SD of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 vs. Blank or mimics NC. HCC, hepatocellular carcinoma; miR, microRNA; NC, negative control.

N-cadherin, Vimentin and Fibronectin (Fig. 3D). These results suggested that miR-206 overexpression inhibited cell migration and invasion by regulating the process of EMT.

*c-Met is a direct target of miR-206*. To identify specific gene targets of miR-206 by which it might anti-oncogenic behavior *in vitro*, the public algorithm microRNA.org (microrna.org) was used. Among hundreds of predicted targets, c-Met was chosen for the reason that it was not only identified as an oncogene, but also as a direct target of miR-206 in various types of cancers (31-33). The target sites for miR-206 in the c-Met sequence are shown in Fig. 4A. To validate whether c-Met was a direct target of miR-206, a dual-luciferase reporter assay was conducted in 293T cells, which showed that overexpression of miR-206 significantly reduced luciferase activity of wt c-Met-3'-UTR when compared with negative control, whereas inhibition of miR-206 promoted the luciferase activity of wt c-Met-3 3'-UTR (Fig. 4B); the activity of the mutant-type c-Met-3 3'-UTR displayed no obvious change. In addition, the protein expression level of c-Met in transfected HCC cells was investigated by western blotting. The results showed that c-Met was downregulated in MHCC97-H and HCCLM3 cells transfected with miR-206 mimics and upregulated in cells transfected with the miR-206 inhibitor (Fig. 4C). Moreover, RT-qPCR was used to assess the levels of c-Met in the 50 pairs of HCC tumor and matched tumor-adjacent tissues. When compared with matched tumor-adjacent tissues, the data revealed that c-Met was considerably elevated in HCC tissues (Fig. 4D). Pearson's correlation analysis revealed an inverse relationship between the levels of miR-206 and c-Met in 50 HCC tissues (Fig. 4E). These findings indicated that miR-206 suppressed the expression of the oncogene c-Met and may serve as a tumor suppressor.

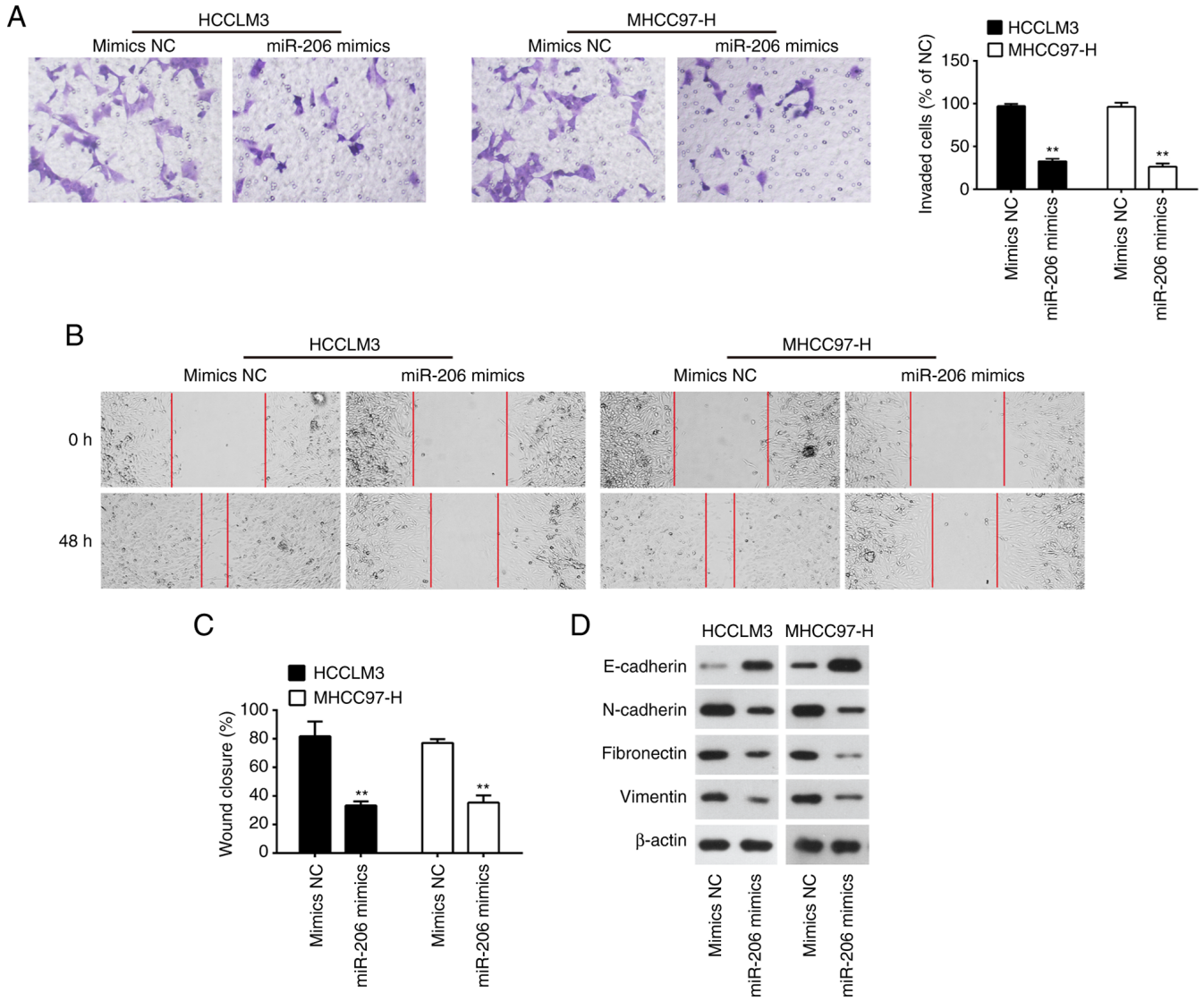


Figure 3. Overexpression of miR-206 suppresses cell migration and invasion. (A) Transwell Matrigel and (B and C) wound healing assays were performed to analyze the effects of miR-206 on MHCC97-H and HCCLM3 cell migration and invasion, respectively (magnification, x200). Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01 vs. mimics NC group. (D) The protein expression levels of epithelial marker (E-cadherin and N-cadherin) and mesenchymal markers (Vimentin and Fibronectin) were assessed by western blotting; β-actin was used as a loading control. miR, microRNA; NC, negative control.

*c-Met reverses the inhibitory effects of miR-206 overexpression on HCC cells.* The aforementioned results led to the investigation of whether miR-206 exerts its antitumor effect through c-Met in HCC. To achieve this, pcDNA-c-Met overexpression vector was co-transfected in miR-206-overexpressing MHCC97-H and HCCLM3 cells (Fig. 5A). pcDNA-c-Met transfection reversed the inhibitory effects of miR-206 mimics on cell viability (Fig. 5B and C). In MHCC97-H and HCCLM3 cells co-transfected with miR-206 mimics and pcDNA-c-Met, the increased apoptotic rate caused by miR-206 was considerably reduced by c-Met overexpression (Fig. 5D). c-Met overexpression also reversed the inhibitory effects of miR-206 mimics on cell migration and invasion in MHCC97-H and HCCLM3 cells (Fig. 5E and F). These findings suggested a role of the target gene c-Met in the tumor suppressor function miR-206.

*miR-206 suppresses c-Met/AKT/mTOR signaling in HCC cells.* Given that the AKT/mTOR signaling pathway is one of the most important downstream pathways of c-Met (34), and that c-Met has been shown to be a target of miR-206, it was speculated that the c-Met/AKT/mTOR signaling pathway may be involved in the anticancer effect of miR-206 in HCC cells. To investigate this, western blotting was performed to determine the changes in the expressions of the downstream molecules of AKT/mTOR signaling, including p-Met, total c-Met, p-AKT, total AKT, p-mTOR and total mTOR. The results showed that miR-206 mimics transfection lowered the protein expression levels p-c-Met, p-AKT and p-mTOR, but did not significantly alter the levels of total c-Met, AKT and mTOR (Fig. 6). These data suggested the role of miR-206 in suppressing the activity of c-Met/AKT/mTOR signaling in HCC cells.

*lncRNA MALAT1 serves as an endogenous sponge of miR-206 in HCC.* Previous studies confirmed that lncRNAs may function as ceRNAs by interacting with miRNAs and functionally freeing the target genes of bound miRNAs (35,36). Previous

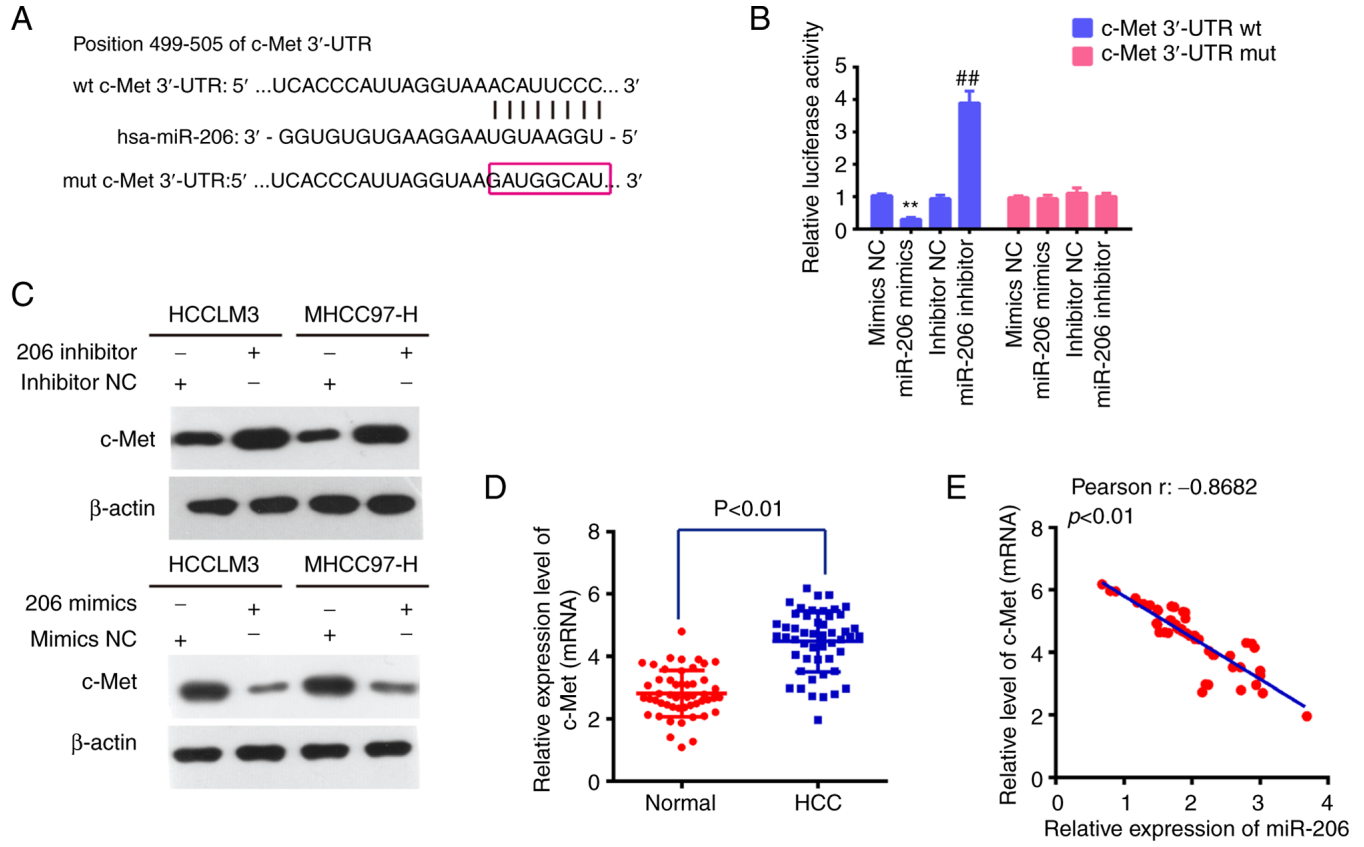


Figure 4. c-Met is a direct target of miR-206. (A) The putative target site of miR-206 and c-Met. The red box shows the mutated target site of miR-206 and c-Met. (B) Relative luciferase activity of c-Met wt or mut 3'-UTR in 293T cells following transfection with the miR-206 mimics, inhibitor or corresponding NC, as indicated). Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01 vs. mimics NC; $^{\#\#}$P<0.01 vs. inhibitor NC. (C) c-Met protein expression after transfection with miR-206 mimic or miR-206 inhibitor was measured by western blotting; β-actin was used as a loading control. (D) Reverse transcription-quantitative PCR was used to determine the mRNA expression levels of c-Met in HCC and matched tumor-adjacent tissues (n=50). Data are presented as the mean ± SD of three independent experiments. (E) Correlation between miR-206 and c-Met in HCC tissues was determined by Pearson's correlation coefficient. HCC, hepatocellular carcinoma; miR, microRNA; mut, mutant; NC, negative control; UTR, untranslated region; wt, wild-type.

studies have shown that MALAT1 regulates miR-206 expression in a number of different cancers. For example, MALAT1 regulate osteosarcoma progress by modulating CDK9 expression via sponging miR-206 (37). In addition, MALAT1 was also reported to promote gallbladder cancer development by acting as a molecular sponge to miR-206 (38). Thus, it was investigated whether expression of miR-206 is also regulated by lncRNAs MALAT1 in HCC. To determine whether miR-206 interacted with MALAT1 in HCC, MALAT1 expression levels were first measured in HCC tissues. The results of RT-qPCR indicated that the expression of MALAT1 was significantly higher in HCC tissues compared with that in tumor-adjacent tissues (Fig. 7A), which is consistent with previous studies (39-41). Furthermore, a negative correlation was observed between MALAT1 expression and miR-206 levels in HCC tissues (Fig. 7B). Taken a step further, we examined the expression levels of MALAT1 in HCC cells. The data showed that MALAT1 was remarkably increased in MHCC97-H, Huh-7, Hep3B and HCCLM3 compared with that in the immortalized non-tumorigenic human hepatocyte cell line, MIHA, especially in MHCC97-H and HCCLM3 cells (Fig. 7C). Subsequently, we found that lncRNA MALAT1 had a complementary sequence of miR-206 using Starbase v.2.0 (Fig. 7D). Then, dual-luciferase reporter assay was performed to further investigate the interaction of miR-206 with MALAT1. Luciferase reporter gene assay showed that overexpression of miR-206 could significantly inhibit the reporter activity of wt-MALAT1, whereas inhibition of miR-206 promoted the reporter activity. Similarly, we observed that the luciferase activity did not change significantly when the targeted sequence of MALAT1 was mutated in the miR-206-binding site (Fig. 7E). To further confirm the relationship between miR-206 and MALAT1, we also detected the expression of MALAT1 in miR-206 mimics transfected MHCC97-H cells and miR-206 inhibitor transfected Hun7 cells. As shown in Fig. 7F, overexpression of miR-206 markedly decreased the expression of MALAT1, whilst knockdown of miR-206 promoted the expression of MALAT1. Furthermore, MHCC97-H cells which had high original MALAT1 expression levels were selected to silence MALAT1 expression by siRNA, while Huh7 cells which had low original MALAT1 expression levels were selected to enhance the expression of MALAT1 by pcDNA-MALAT1. The interfering and overexpressing efficiencies were confirmed by RT-PCR (Fig. 7G). We subsequently tested whether MALAT1 influenced the miR-206 expression. miR-206 was significantly increased after knockdown of MALAT1, whereas the miR-206 expression level could also be repressed by MALAT1 overexpression (Fig. 7H). These findings may explain why miR-206 was expressed at low levels in HCC.

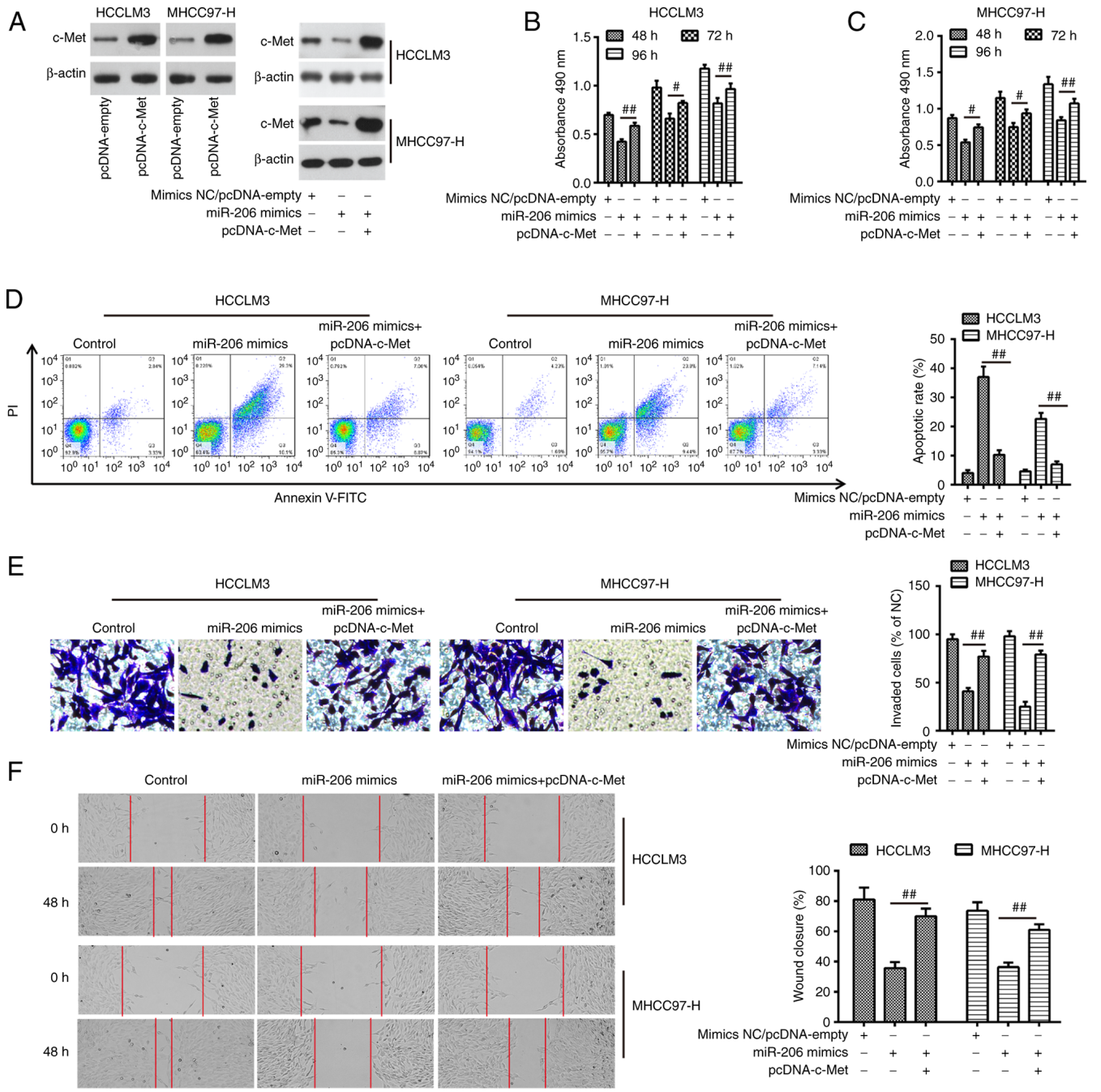


Figure 5. c-Met overexpression reverses the inhibitory effects of miR-206 mimics in HCC cells. (A) c-Met protein expression was determined by western blotting; β-actin was used as a loading control. Cell viability was measured by MTT assay in transfected (B) HCCLM3 and (C) MHCC97-H cells. (D) Apoptotic rates were determined by flow cytometry. (E) Transwell Matrigel invasion and (F) wound healing migration assays were used to examine how miR-206 affected HCC cell migration and invasion, respectively (200x magnification). Data are presented as the mean ± SD of three independent experiments. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. miR-206 mimics. HCC, hepatocellular carcinoma; miR, microRNA.

## Discussion

In the present study, it was shown that miR-206 expression is downregulated in human HCC tissues and cell lines, and significantly associated with poor prognosis. Furthermore, miR-206 overexpression reduced cell viability, induced apoptosis and suppressed migration and invasion through AKT/mTOR pathway by downregulating c-Met. Data from this study also revealed that the miR-206 expression was regulated by lncRNA MALAT1 in HCC. These results suggested that miR-206 may function as a tumor suppressor in HCC and may serve as a novel and promising therapeutic target for non-small cell lung cancer (NSCLC).

Increasing evidence indicates that miRNAs may be valuable as a target for diagnostic and therapeutic purposes in various malignancies, including HCC (42,43). In particular, previous studies have revealed that a number of miRNAs are dysregulated and exert essential roles in HCC progression; these miRNAs include miR-342-3p, miR-125b and miR-9-5p (16,44,45). Li *et al* (46) found that miR-20b-5p promoted HCC cell viability, migration and invasion by downregulating cytoplasmic polyadenylation element-binding protein 3. Cao *et al* (47) reported

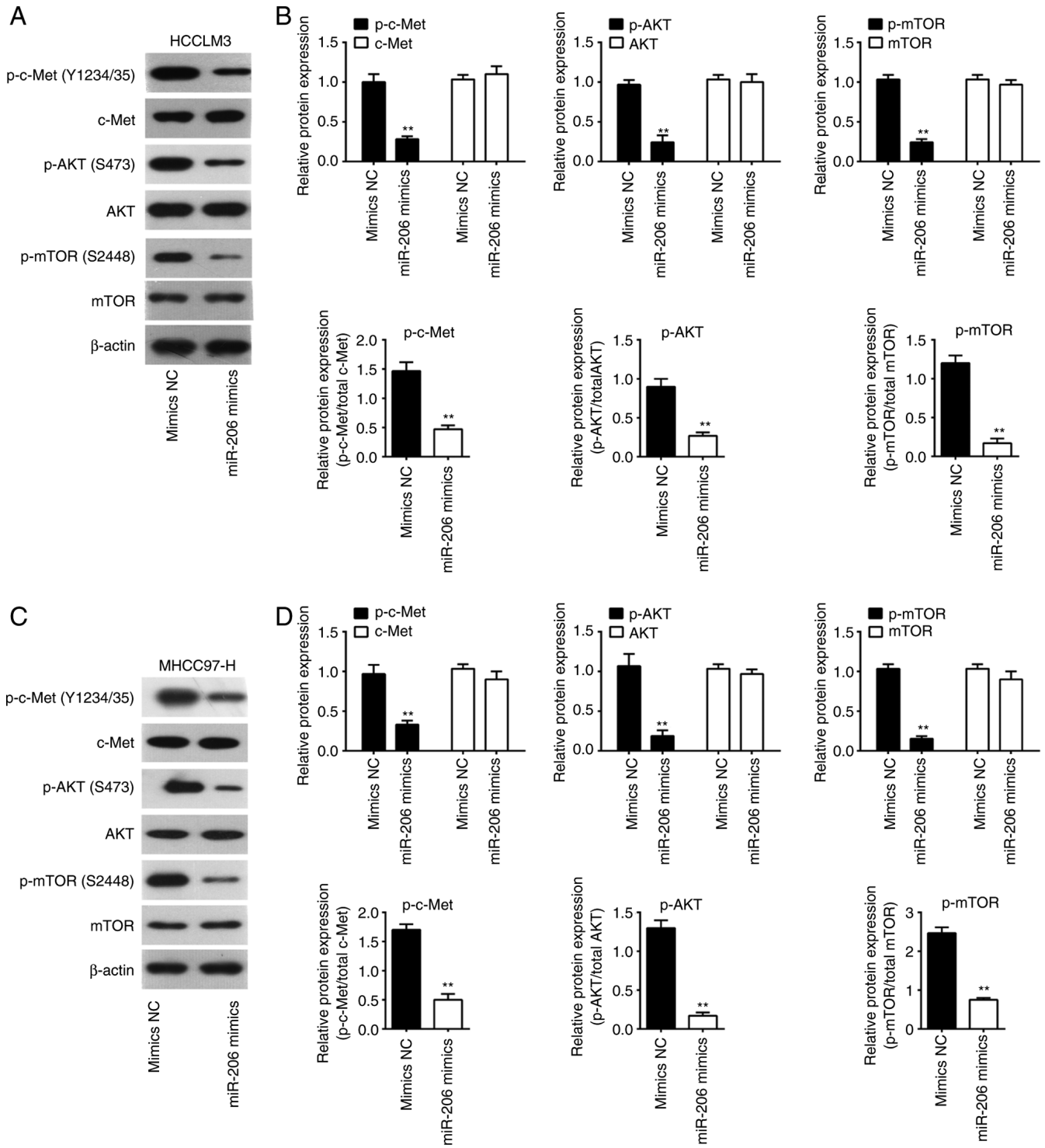


Figure 6. Overexpression of miR-206 blocks activation of AKT/mTOR pathway. (A and B) western blot was used to identify the protein expression levels of c-Met, p-c-Met, AKT, p-AKT, p-mTOR and mTOR in HCCLM3 cells after miR-206 mimics transfection. (C and D) western blot was used to identify the protein expression levels of c-Met, p-c-Met, AKT, p-AKT, p-mTOR and mTOR in MHCC97-H cells after miR-206 mimics transfection. Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01 vs. mimics NC. HCC, hepatocellular carcinoma; miR, microRNA; NC, negative control; p-, phosphorylated.

that miR-23b was a tumor suppressor which may regulate HCC migration and invasion by targeting Pyk2 regulation of EMT. More significantly, in non-human primates with chronic HCV infection, the administration of locked nucleic acids specific for miR-122 inhibited long-term viral viability, supporting its use as a therapeutic agent for HCC (48). Additionally, a multi-center phase IIA trial involving patients with HCC and the miR-122 antagonist miravirsen showed a decreased viral burden in patients with HCC following miravirsen treatment (49). Morpholino-anti-miR-487a oligomers were also used to effectively silence miR-487a in mouse models, which prevented the progression of HCC tumors without causing any harm to the mice in terms of weight loss, obvious abnormalities or animal mortality (50). The present study validated that miR-206 was

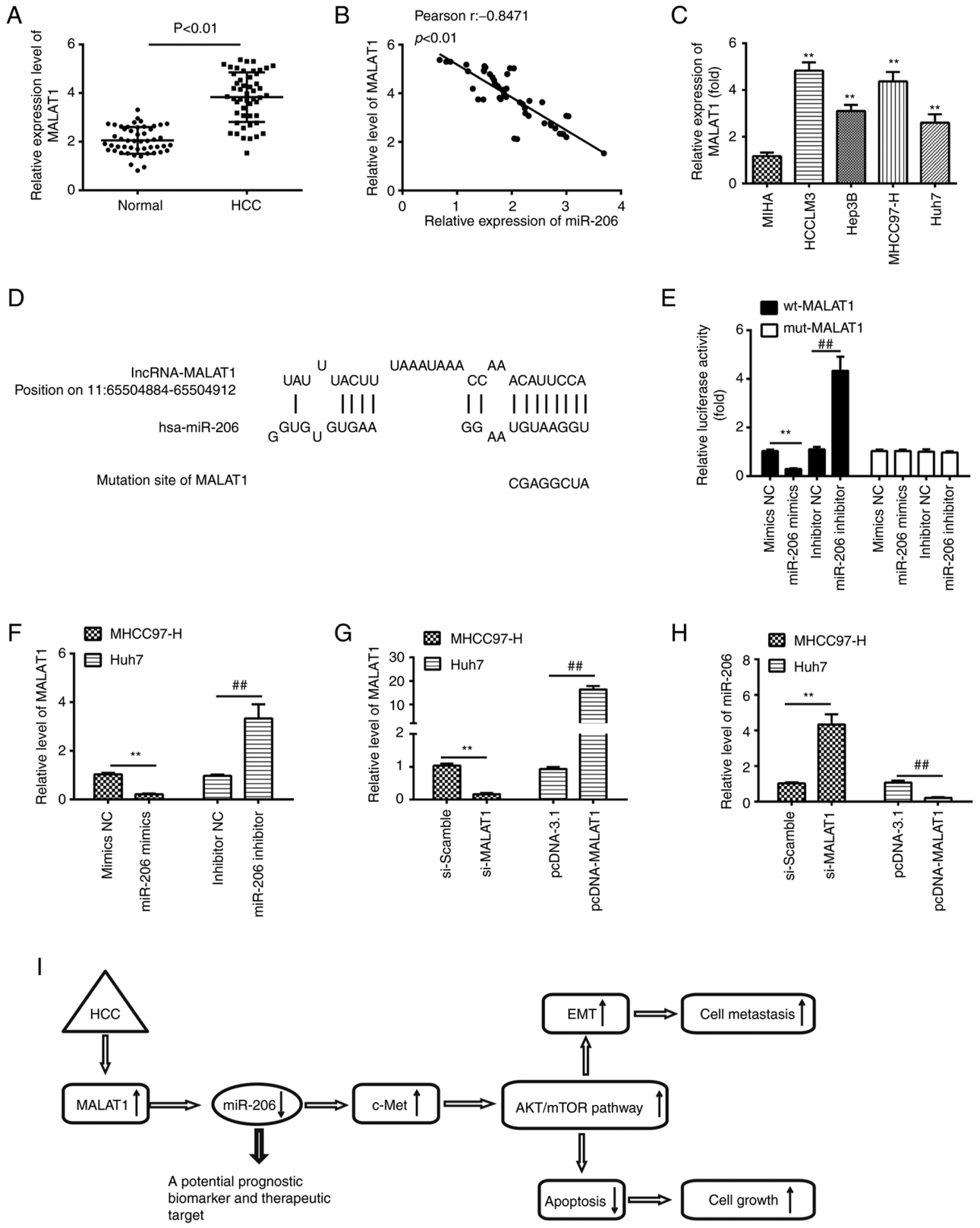


Figure 7. LncRNA MALAT1 negatively regulates miR-206 expression in HCC. (A) Expression of MALAT1 in HCC and matched tumor-adjacent tissues (n=50). (B) A negative correlation was identified between MALAT1 and miR-206 in a cohort with 50 patients with HCC. (C) The expression of MALAT1 in HCC cell lines MHCC97-H, HCCLM3, Huh-7 and Hep3B; MIHA used as NC. Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01 vs. MIHA. (D) Schematic representation of the predicted binding site for miR-206 and lncRNA MALAT1. (E) Relative luciferase activity of MALAT1 wt or mut in 293T cells following transfection with the miR-206 mimics, inhibitor or corresponding NC. Data are presented as the mean ± SD of three independent experiments. $^{**}$P<0.01; $^{\#\#}$P<0.01. (F) lncRNA MALAT1 expression levels following transfection with miR-206 mimic or miR-206 inhibitor were measured by reverse transcription-quantitative PCR. $^{**}$P<0.01; $^{\#\#}$P<0.01. (G) Verification of successful downregulation of MALAT1 by siRNA and overexpression of MALAT1 by pcDNA-MALAT1 vector transfections in MHCC97-H and Huh-7 cells. $^{**}$P<0.01; $^{\#\#}$P<0.01. (H) MALAT1 knockdown increased, whereas overexpression of MALAT1 decreased miR-206 expression in HCCLM3 and Huh-7 cells. $^{**}$P<0.01; $^{\#\#}$P<0.01. (I) lncRNA MALAT1 increases c-Met expression via sequestering miR-206 at post-transcription level, leading to the activation of AKT/mTOR signaling pathway, thus promotes HCC cell growth, migration and invasion. HCC, hepatocellular carcinoma; hsa, *Homo sapiens*; lncRNA, long non-coding RNA; miR, microRNA; NC, negative control.

lowly expressed in HCC tissues and cell lines, and its expression was significantly associated with advanced tumor nodularity, TNM stage and venous infiltration. Notably, our data provided evidence that miR-206 may have great potential as a prognostic biomarker for HCC.

A number of previous studies have reported that miR-206 acts as a tumor suppressor in various types of human tumors. For instance, Liu *et al* (51) demonstrated that miR-206 suppressed the viability of head and neck squamous cell carcinoma cells by targeting HDAC6. Similarly, Shao *et al* (19) discovered that miR-206 had an inhibitory effect on gastric cancer cell proliferation. Xiao *et al* (52) demonstrated that miR-206 inhibited clear-cell renal cell carcinoma proliferation through inducing cell cycle arrest by targeting the cell cycle related genes CDK4, CDK9 and CCND1. In addition, the function of miR-206 was explored in HCC. For example, Liu *et al* (53) reported miR-206 could suppress HCC cell dedifferentiation and liver cancer stem cells expansion by targeting EGFR signaling. Another study performed by Liu *et al* showed that miR-206 prevented HCC by attenuating TGFβ1 overproduction, disrupted the communication of malignant hepatocytes with CTLs (cytotoxic T lymphocytes) and Tregs (regulatory T cells) (54). In the present study, overexpression of miR-206 suppressed cell viability and invasion, and induced apoptosis in HCC cells. Previous studies have highlighted the notable effects of miR-206 during the process of EMT and metastasis (32,55). The current study results demonstrated that overexpression of miR-206 resulted in a significant upregulation of E-cadherin and a significant downregulation of N-cadherin, Vimentin and Fibronectin in MHCC97-H and HCCLM3 cells. These data suggested that miR-206 may suppress the proliferative and invasive abilities of HCC cells and may serve as a tumor suppressor in HCC.

c-Met is a well-known oncogene linked to development of numerous malignancies (56,57). For example, upregulation of c-Met promotes the progression and development of multiple myeloma (58). Notably, it has also been shown in several types of cancers including NSCLC and colorectal cancer that miR-206 exerted its tumor-suppressive effects by targeting c-Met (32,59). For example, miR-206 targets c-Met to inhibit tumor cell proliferation, migration and colony formation in NSCLC (60). Given the relationship between miR-206 and c-Met (31-33), it was hypothesized that, in HCC, the anti-oncogenic function of miR-206 was achieved through suppressing c-Met expression. In the present study, c-Met was identified to be a target of miR-206 using online informatics tools. It was also shown that there was an inverse correlation between miR-206 and c-Met in tumor tissues. Notably, c-Met overexpression reversed the suppressive effects induced by miR-206 mimics on HCC cell invasion and viability. Interestingly, a previous study reported the similar results that miR-206 inhibited HCC cell proliferation and migration by modulating c-MET expression (61). In this study, we further determined that this targeting relationship between miR-206 and c-Met affected the AKT/mTOR pathway signaling pathway, thus affecting cell proliferation, invasion and migration. The protein expression levels of AKT/mTOR pathway, one of downstream signaling pathways regulated by c-Met (62,63) were assessed, and it was revealed that miR-206 suppresses the activity of c-Met/AKT/mTOR signaling pathway. These results suggested that miR-206 suppressed c-Met to inhibit AKT/mTOR signaling pathway and therefore inhibit the malignancy of HCC cells.

Although the present study data showed that miR-206 was expressed at a low level in HCC tissues and cell lines, it was still unclear how miR-206 is altered. lncRNAs regulate the expression and activity of miRNAs by acting as miRNA sponges (64). Thus, whether there are lncRNAs that regulate the expression of miR-206 in HCC is a critical issue. In the present study, lncRNA MALAT1 was speculated to be a potential regulator of miR-206 according to previous studies. For example, Sun *et al* (65) showed that MALAT1 promoted bone marrow-derived mesenchymal stem cell differentiation into endothelial cells by sponging miR-206. In addition, MALAT1 regulates osteosarcoma progress by modulating CDK9 expression by sponging miR-206 (37). MALAT1 silencing suppressed cell viability, migration, invasion and vasoformation of hemangioma endothelial cells through modulating miR-206 (66). However, it is not known whether MALAT1 and miR-206 interact with each other directly or indirectly in HCC cells. Expression of MALAT1 was upregulated in HCC tissues, and there was an inverse correlation between miR-206 and MALAT1 expression in HCC tissues. Furthermore, silencing MALAT1 by siRNA increased, whereas overexpression of MALAT1 by pcDNA-MALAT1 repressed the expression of miR-206. All data suggest that downregulation of miR-206 is regulated by MALAT1 in HCC.

However, this study has limitations. For example, the sample size was small, thus the relationship between miR-206 and clinicopathological features of HCC should be further explored in a large number of samples. Previous studies have reported that miR-206 serves an important role in various tumor cells by targeting c-Met, such as in NSCLC (32) and gastric cancer (67). Thus, the relationship between miR-206 and c-Met was investigated, attempting to explain how the lowly expressed miR-206 may serve a role in the development of HCC. Results from the present study demonstrated that miR-206 may function as a tumor suppressor by targeting c-Met. However, whether there are additional functional targets of miR-206 in HCC needs to be further investigated.

In conclusion, miR-206 is downregulated in HCC tissues and cell lines. We also find that lncRNA MALAT1 increases c-Met expression via sequestering miR-206 at post-transcription level, leading to the activation of AKT/mTOR signaling pathway, thus promotes HCC cell growth, migration and invasion. miR-206 is likely controlled by the lncRNA MALAT1 and may function as a tumor suppressor by blocking the c-Met/AKT/mTOR signaling pathway to induce apoptosis and inhibit HCC cell viability and invasion (Fig. 7I). Our findings takes a further step into the mechanism of miR-206 in HCC, and it is suggested that developing targets to miR-206 may lead to new approaches to treat HCC.

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## Availability of data and materials

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

## Authors' contributions

JW, GY and BZ performed the experiments, contributed to data analysis and wrote the paper. JW, GY, BZ and ZZ analyzed the data. YF conceptualized the study design, contributed to data analysis and experimental materials. JW and YF confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

This research was approved by the ethics committee of the First Affiliated Hospital of Henan University of Science and Technology (Luoyang, China) (Ethics approval number: 2019-006). All patients provided written informed consent.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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