Original Article
MicroRNA-506 suppresses invasiveness and metastasis of human hepatocellular carcinoma cells by targeting *IL8*

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Abstract: MicroRNAs (miRNAs) have been reported to be involved in tumor metastasis. In this study, we investigated the function of miR-506 in the metastasis of human hepatocellular carcinoma (HCC). We found that miR-506 is significantly downregulated in the primary tissue of metastatic HCC and in highly metastatic HCC cell lines. Over-expression of miR-506 suppressed HCC cell migration, invasion, and metastasis both *in vitro* and *in vivo*. Further-more, miR-506 was found to specifically target the 3' untranslated region (3'-UTR) of interleukin 8 (*IL8*) mRNA. Spearman's correlation analysis revealed that miR-506 expression inversely correlated with *IL8* mRNA and protein expression in HCC tissue samples. IL8 treatment reversed miR-506-induced suppression of HCC cell migration and invasiveness. Thus, miR-506 acts as a tumor suppressor that may inhibit the migration, invasiveness, and metas-tasis of HCC cells by targeting *IL8*.

Keywords: miR-506, IL8, invasion, metastasis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) ranks fifth in incidence and third in cancer mortality world-wide and brings about a heavy healthcare-related economic burden worldwide [1]. Despite promising treatment strategies, the outcomes of patients with HCC remain poor. The overall recurrence rate among patients with HCC reaches 70% [2]. Most patients with HCC die of metastasis, which has been a consistent problem in tumor prognosis and therapy. Hen-ce, there is an urgent need for determining the exact mechanisms of metastasis and for improving the current therapeutic strategies against HCC.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules (18-25 nucleotides in length) that negatively regulate expression of protein-coding genes at the post-transcription-al level through binding to the 3' untranslated region (UTR) [3]. Increasing evidence shows that miRNAs are aberrantly expressed in vari-ous human cancers and can act as tumor sup-pressors or oncogenic agents [4]. To date, sev-eral deregulated miRNAs have been shown to promote or suppress cell growth, invasiveness, and metastasis of HCC, e.g., miR-26a [5], miR-187-3p [6], and miR-331-3p [7]. Nevertheless, the relation between miRNAs and HCC metas-tasis remains elusive.

MiR-506 has been reported to be downregulat-ed and to serve as a potential tumor suppres-sor in several cancer types including pancrea-tic cancer [8], gastric cancer [9], cervical can-cer [10], and breast cancer [11]. MiR-506 is also a reliable marker of the chemotherapy response and survival in serous [12]. Regard-ing its function, miR-506 can suppress prolifer-ation and migration of gastric cancer [9], breast cancer [11], and HCC cells [13]. Of note, miR-506 levels are negatively associated with lymph node invasion and metastasis, and this miRNA may suppress the invasiveness and metastasis of colon cancer [14]. Nevertheless, the roles and mechanism of action of miR-506 in HCC metastasis remain largely unknown.

In this study, we carried out a series of *in vitro* and *in vivo* assays and found that weaker expression of miR-506 in HCCs is associated with a more aggressive tumor phenotype. Elevated miR-506 expression significantly suppressed cell migration, invasion, and epithelial–mesenchymal transition (EMT) *in vitro*. Moreover, miR-506 overexpression suppressed the growth of HCC-derived xenograft tumors and lung metastasis of HCC cells *in vivo*. Finally, our data revealed that miR-506 may inhibit HCC tumor growth and metastasis by reducing the expression of its target gene *IL8*.

Materials and methods

Cell lines and human tissue samples

Human HCC cell lines (Huh7, HepG2, HCCLM3, and MHCC97H), and normal hepatic cell line LO2 were obtained from the Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences (Shanghai, China). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum and 1% of a penicillin/streptomycin solution (Sigma, St. Louis, MO, USA) in a humidified incubator at 37°C and 5% CO_2. Thirty samples of human HCC and their matching adjacent noncancerous tissues were collected from patients that underwent a liver resection at the Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Another 10 metastatic tissue samples were also obtained from this hospital. After the resection, these tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for later RNA extraction or formalin fixing and paraffin embedding for immunohistochemistry. All the human tissue samples were obtained with informed consent, and the protocol was approved by the Ethics Committee of the Eastern Hepatobiliary Surgery Hospital.

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

Total RNAs containing miRNAs were isolated with the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), and RNA concentrations were determined on a NanoDrop 2000. MiRNAs or mRNAs were reverse-transcribed to generate cDNA using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China) or oligo-dT primers, respectively. qRT-PCR analyses were performed by means of the Mir-X™ miRNA qRT-PCR SYBR® Kit (Takara) for miR-506 and the TB Green™ Fast qPCR Mix (Takara) for *IL8*. U6 was analyzed as an internal control for miRNA, whereas *GAPDH* served as an internal control for mRNA. Relative quantification analysis was conducted by the $2^{-\Delta\Delta CT}$ method.

Cell transfection

miR-506 mimic and its negative control (miR-ctrl), and miR-506 inhibitor (anti-miR-506) and its negative control (NC) were purchased from RiboBio (Guangdong, China). Cells (5 × 10^5/ well) were seeded at 60% confluence in a 6-well plate. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the cells with the miR-506 mimic or miR-ctrl. After 48 h of transfection, the cells were collected for the following experiments.

Wound-healing assay

Cells transfected with the miR-506 mimics or anti-miR-506 were seeded in 6-well plates and allowed to grow to ~95% confluence. A linear wound was created with a 200 μL sterile pipette tip, and the cells were washed with the serum-free medium to remove floating ones. The wounded monolayers were photographed at 0 and 48 h by means of an inverted microscope to assess the rate of gap closure [15].

Transwell and three-dimensional (3D) spheroid invasion assays

For the Transwell invasion assay, 10^5 cells were seeded in the upper chambers of 24-well Transwell plates (Corning Costar, Lowell, MA, USA) precoated with Matrigel (BD Biosciences, Franklin, NJ, USA). The medium containing 20% of fetal bovine serum was added to the lower compartment as a chemoattractant. After incubation for 48 h, the cells on the upper surface of the filters were removed with a cotton swab. Invading cells on the bottom were fixed and stained with 0.5% crystal violet and photographed in five random visual fields under a microscope. A 3D spheroid invasion assay was performed as previously described [16]. In brief, 2 × 10^3 transfected cells were mixed with 20% Matrigel and seeded in 24-well plates coated with 100% Matrigel (BD Biosciences). The medium was changed every other day. Ten
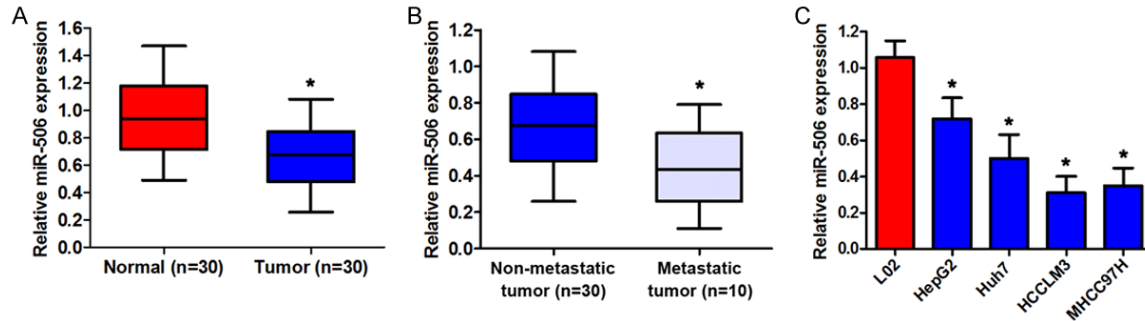
Figure 1. The expression of miR-506 in HCC tissue samples and cell lines. A. Expression levels of miR-506 were measured by qRT-PCR in 30 pairs of samples of HCC and adjacent nontumorous tissue. B. Expression levels of miR-506 in nonmetastatic (n = 30) and metastatic (n = 10) tumors. C. qRT-PCR analysis of miR-506 expression in four human HCC cell lines, with the human hepatocyte LO2 cell line as a control. The average miR-506 expression was normalized to U6 expression. *P < 0.05.

days later, tumor cell outgrowth was visualized by microscopy.

Detection of target proteins of miR-506

The concentration of IL8 in the culture medium was detected by an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, USA). Total protein was isolated from HCC cells and subjected to western blot analysis as described elsewhere [17]. The primary antibodies were anti-E-cadherin, anti-vimentin, and anti-β-actin antibodies (Proteintech, Wuhan, China).

Vector construction and luciferase reporter assay

The wild-type (wt) 3'-UTR of the *IL8* sequence containing a putative binding site for miR-506 was inserted into the pmirGLO vector (Promega, Madison, WI, USA). The mutant (mt) 3'-UTR of *IL8* was created by mutating the seed region of the miR-506-binding site using the QuikChange Lightning Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). For the luciferase reporter assay, HEK293 cells were cotransfected with the luciferase reporters with either miR-506 mimics or miR-ctrl. After 48 h, the cells were harvested, and luciferase activity was measured by means of the Dual-Luciferase Reporter Assay System (Promega). *Renilla* luciferase served for normalization.

In vivo tumor growth and metastasis assay

Six-week-old BALB/c nude mice were acquired from Shanghai Slac Laboratory Animal Co., Ltd.

(Shanghai, China) and were housed and maintained under specific pathogen-free conditions according to the guidelines. All the animal experiments were approved by the Scientific Investigation Board of the Second Military Medical University (Shanghai, China). HCCLM3 cells (2×10^6) infected with lentiviral vectors expressing the miR-506 (LV-miR-506) or negative control (LV-NC) (Hanbio, Shanghai, China) were inoculated subcutaneously into the flanks of nude mice (five mice per group). The mice were closely monitored for tumor growth, and tumor size was measured every 5 days. Tumor volume (V) was calculated via the formula $V = 0.5 \times length \times width^2$. After 30 days, the mice were euthanized, and tumors were collected and weighed. As a metastasis model, 10^6 lentivirus-infected HCCLM3 cells in 0.1 mL of PBS were injected into nude mice via the tail vain (six mice per group). After 6 weeks, the mice were euthanized, and the lungs were excised, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The metastatic nodules were counted microscopically to assess the development of pulmonary metastases.

Immunohistochemical (IHC) analysis

This analysis was carried out as previously described [18]. Briefly, the paraffin-embedded specimens were deparaffinized and rehydrated, then processed for antigen recovery and endogenous-peroxidase blocking. After blocking with goat serum, the slides were incubated with primary antibodies against Ki67, vimentin, E-cadherin, and IL8 (Proteintech) at 4°C over-
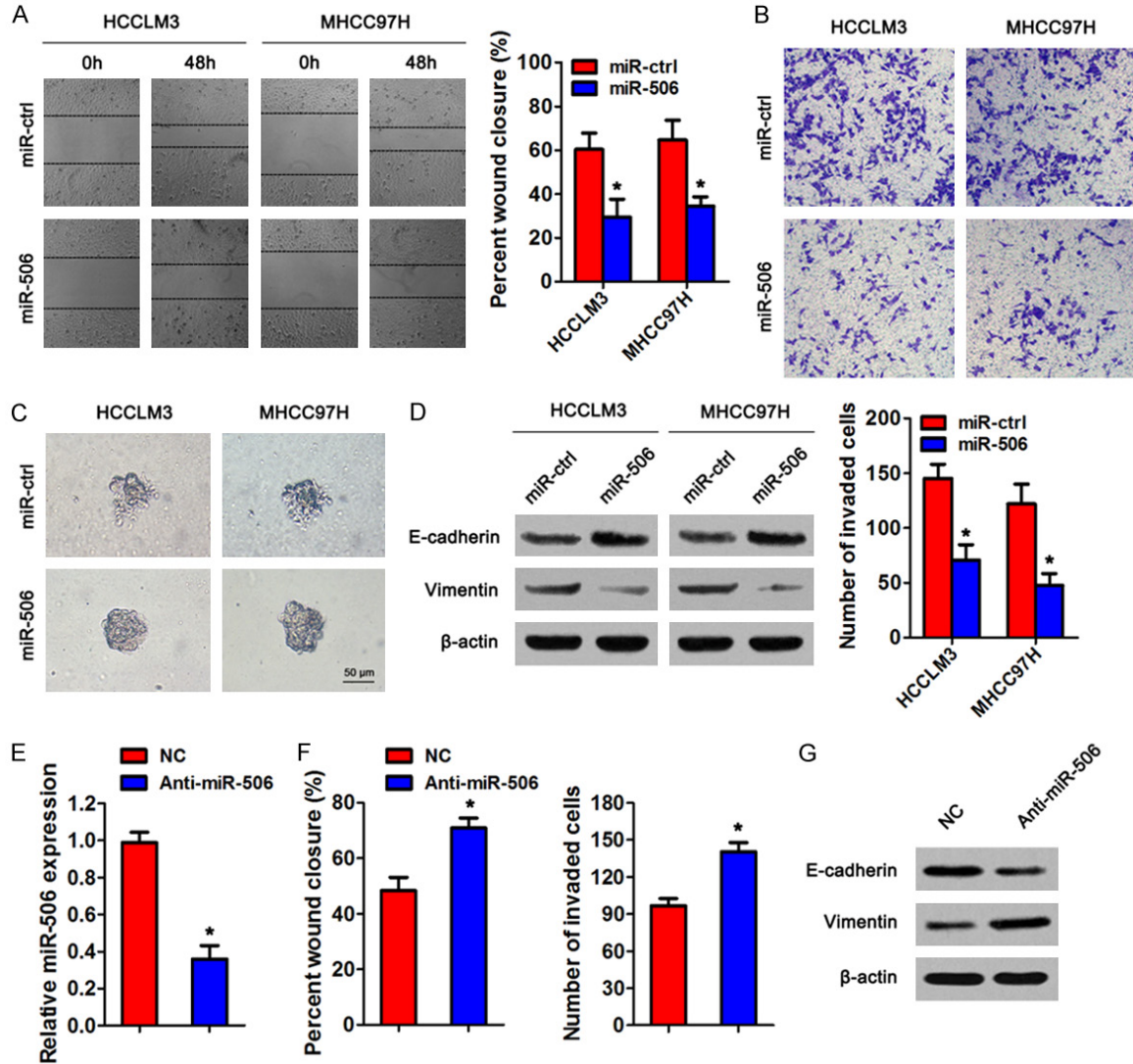
Figure 2. MiR-506 suppresses cell migration, invasion and EMT in vitro. HCCLM3 and MHCC97H cells were transiently transfected with miR-506 mimics or miR-ctrl, respectively. A. A wound-healing assay was performed to detect the cell migration ability. B. Cell invasion was determined by a Transwell assay. C. Representative micrographs of the indicated cultured cells in a Matrigel-based 3D spheroid invasion assay. D. Western blot analysis of EMT markers E-cadherin and vimentin in HCCLM3 and MHCC97H cells transfected with miR-506 mimics or miR-ctrl. β-Actin served as an internal control. E. qRT-PCR analysis of miR-506 expression in HepG2 cells transfected with anti-miR-506 or negative control. F. Wound-healing assay and Transwell invasion assay. G. Western blot analysis of EMT markers in the transfected HepG2 cells. *P < 0.05.

night and treated with a horseradish peroxidase (HRP)-conjugated secondary antibody, then incubated with 3,3'-diaminobenzidine (DAB) for color development. The array was counterstained with hematoxylin and was examined under a microscope. Staining intensity was scored visually by two experienced pathologists independently as follows: 0 = no staining, 1 = weak staining, 2 = moderate straining, and 3 = strong staining. Five visual fields with tumor cells were randomly selected and scored based on the percentage of positively stained cells (0-100%). The final IHC score was calculated by multiplying the intensity score with the percentage of positive cells [19].

Statistical analysis

Numerical data were presented as mean ± SD. All the statistical analyses were conducted in the SPSS software, version 18.0. The significance of the differences in variables was
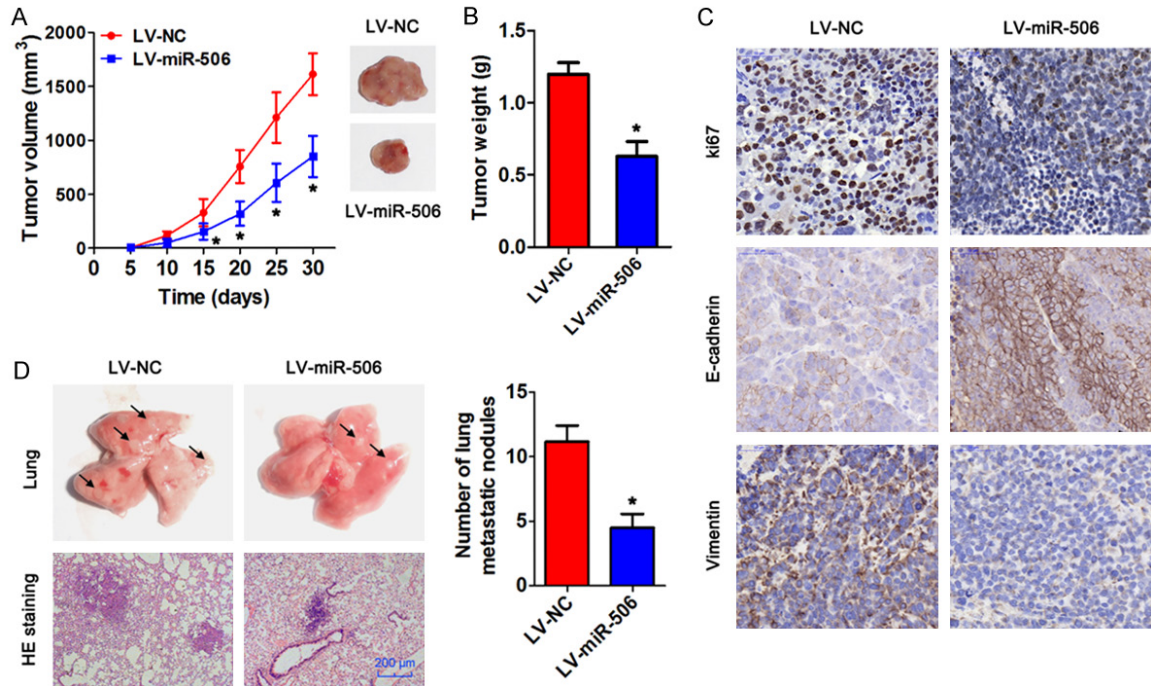
Figure 3. MiR-506 inhibits tumor growth and metastasis in vivo. HCCLM3 cells infected with lentivirus LV-miR-506 or LV-miR-NC were intravenously injected into nude mice (n = 5), and the tumors were collected after 30 days. A. The volume of the xenograft tumors. B. The weight of the xenograft tumors. C. IHC staining of Ki67, E-cadherin, and vimentin in subcutaneous tumors. Bar = 50 μm. D. Representative H&E-stained sections of the lung tissues collected from the LV-NC and LV-miR-506 groups are shown in the left panel. Quantitative analysis was carried out by counting the metastatic nodules in five randomly selected visual fields. Bar = 200 μm. *$P < 0.05$.

assessed by Student's *t* test. The association between miR-506 and IL8 expression was evaluated by Spearman's correlation analysis. Data with *P* values < 0.05 were considered significant.

Results

MiR-506 is frequently downregulated in HCC

In this study, we first determined the expression level of miR-506 in 30 pairs of HCC samples and matching adjacent nontumorous tissues by qRT-PCR. As shown in **Figure 1A**, the expression level of miR-506 was significantly lower in HCC tissues compared to the corresponding adjacent normal tissues. In addition, miR-506 expression was significantly lower in metastatic-HCC tissue samples than in non-metastatic-HCC tissue samples (**Figure 1B**). We also measured miR-506 levels in four HCC cell lines with different metastatic potentials: HepG2, Huh7, HCCLM3, and MHCC97H. The results showed that miR-506 expression levels were lower in all four HCC cell lines than in the normal hepatic cell line LO2. This was espe-

cially true for the highly metastatic HCC cell lines HCCLM3 and MHCC97H, which were used in the subsequent experiments (**Figure 1C**). The results above suggest that miR-506 may participate in HCC metastasis.

MiR-506 suppresses HCC cell migration, invasion, and EMT in vitro

Next, we explored the potential involvement of miR-506 in the regulation of HCC metastasis. We transfected miR-506 mimics or the control (miR-ctrl) into HCCLM3 and MHCC97H cells. The wound-healing assay revealed that miR-506 overexpression suppressed cell migration of HCC cell lines (**Figure 2A**). Similarly, the invasion ability of HCC cells decreased after miR-506 overexpression in the Transwell and Matrigel-based 3D invasion assays (**Figure 2B, 2C**). In addition, we evaluated the expression of EMT markers in HCC cells by western blotting. The level of epithelial marker E-cadherin increased and that of mesenchymal marker vimentin decreased after overexpression of miR-506 (**Figure 2D**), suggesting that miR-506 inhibits the EMT of HCC cells. In contrast, miR-
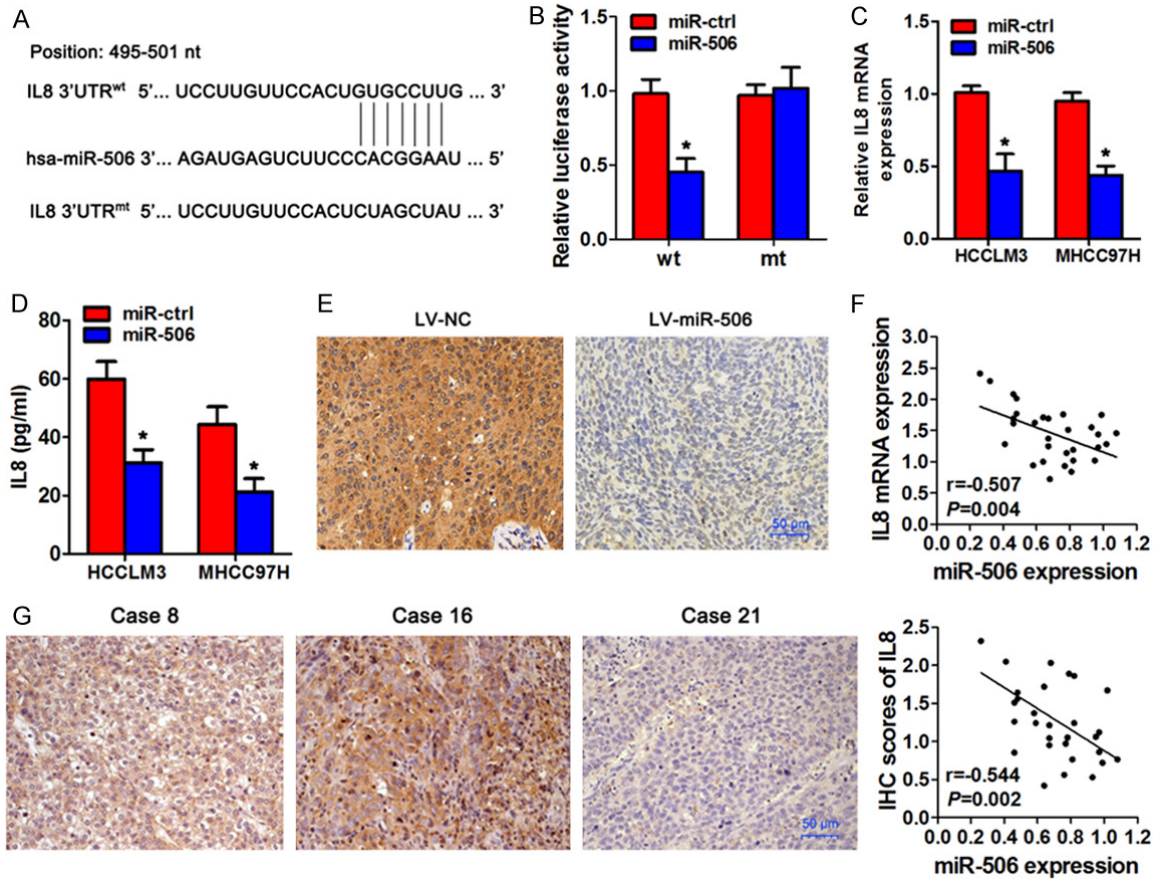
Figure 4. IL8 is a direct target gene of miR-506 in HCC cells. A. A schematic diagram of miR-506-binding sites in the IL8 3'-UTR. The sequence of miR-506 was compared with wild-type (wt) or mutant (mt) putative target sites in the 3'-UTR of IL8. B. Relative luciferase activity in HEK293T cells cotransfected with miR-506 or miR-ctrl and the wt or mt IL8 3'-UTR luciferase reporter construct. C. qRT-PCR analyses of IL8 expression in miR-506-overexpressing HCCLM3 and MHCC97H cells. D. IL8 protein levels were quantified by an ELISA in HCCLM3 and MHCC97H cells and in the cell culture supernatant after transient transfection with a miR-506 mimic. E. IHC staining of IL8 in subcutaneous tumors. F. The correlation between miR-506 levels and IL8 mRNA levels in 30 HCC tissue samples. G. The correlation between miR-506 levels and IL8 protein levels in 30 HCC tissue samples. $*P < 0.05$.

506 knockdown using anti-miR-506 in HepG2 cells significantly increased cell migration and invasion, and promoted EMT compared to the negative control (**Figure 2E-G**). These observations suggest that miR-506 suppresses HCC cell migration, invasion, and EMT *in vitro*.

Overexpression of miR-506 inhibits tumor growth and metastasis in vivo

We then determined the influence of miR-506 on the tumorigenic potential and metastasis *in vivo*. HCCLM3 cells infected with a lentiviral vector expressing miR-506 or negative control were subcutaneously injected into the flanks of nude mice. The mice were euthanized after 30 days, and the tumors were collected. The results revealed that the mice injected with

miR-506-overexpressing tumor cells had significantly smaller tumor volumes and tumor weight than did the mice injected with the control tumor cells (**Figure 3A, 3B**). We next detected the expression levels of Ki67, E-cadherin, and vimentin in the tumors processed by IHC staining. As **Figure 3C** indicates, the expression of Ki67 in the groups with miR-506 overexpression was significantly lower relative to the negative control. In addition, epithelial marker E-cadherin expression increased, whereas mesenchymal maker vimentin expression decreased after miR-506 overexpression. To further elucidate the effect of miR-506 on tumor metastasis *in vivo*, lentivirus-infected HCCLM3 cells were injected into nude mice through the tail vein. At 6 weeks after the injection, we found that miR-506 overexpression
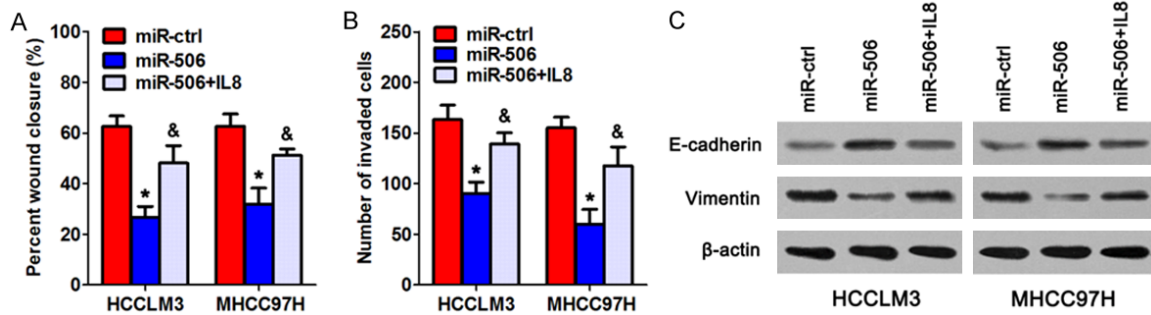
Figure 5. IL8 attenuates the effects of miR-506 on migration, invasiveness, and EMT of HCC cells. HCCLM3 and MHCC97H cells transfected with miR-506 mimics or miR-ctrl were treated with 100 pg/mL recombinant IL8 protein. A. A wound-healing assay was performed to assess the cell migration ability. B. A Transwell invasion assay was conducted to evaluate cell invasion. C. The expression levels of E-cadherin and vimentin were assessed by western blotting. β-actin served as an internal control. *$P < 0.05$ as compared with the miR-ctrl group; &$P < 0.05$ as compared with the miR-506 group.

significantly decreased the number of metastatic nodules in lungs (**Figure 3D**). These findings suggest that miR-506 suppresses the growth of xenograft tumors and lung metastasis derived from HCC cells.

IL8 expression was directly targeted by miR-506

To further investigate the mechanism of miR-506 action in the regulation of HCC metastasis, the miRanda software was employed to predict the potential targets of miR-506. *IL8* was identified as a potential effector of miR-506 according to the putative binding site in the 3'-UTR of *IL8* mRNA (**Figure 4A**). To test whether miR-506 directly targets *IL8* 3'-UTR, we inserted the target region sequence of the *IL8* 3'-UTR (wt) or the mutated sequence (mt) into a luciferase reporter vector and coexpressed one of these vectors with either miR-506 mimics or miR-ctrl in HEK293 cells. As shown in **Figure 4B**, overexpression of miR-506 resulted in a significant decrease in the luciferase activity of the construct containing the wt 3'-UTR of *IL8* but had no effect on the mt 3'-UTR reporter. In addition, miR-506 overexpression significantly suppressed the mRNA expression of *IL8* in HCCLM3 and MHCC97H cells and reduced IL8 protein levels in the culture supernatant as compared to their respective controls (**Figure 4C**, **4D**). Accordingly, subcutaneous tumors formed by miR-506-overexpressing HCCLM3 cells showed less IL8 staining (**Figure 4E**). We next investigated the relation between miR-506 expression and IL8 mRNA and protein expression in HCC tissues by Spearman's cor-

relation analysis. The results revealed that miR-506 expression inversely correlated with IL8 expression in HCC tissues (**Figure 4F**, **4G**). Taken together, these data indicated that miR-506 represses *IL8* expression via its specific seed region in the 3'-UTR of *IL8* mRNA.

IL8 attenuates the effects of miR-506 on cell migration, invasion, and EMT

To determine whether miR-506 functionally targets *IL8* 3'-UTR, we added 100 pg/mL recombinant IL8 protein to the culture medium of HCC cells to simulate overexpression of IL8. As presented in **Figure 5A**, **5B**, treatment with IL8 significantly attenuated the miR-506-induced suppression of HCC cell invasion and migration. Furthermore, IL8 treatment reversed the upregulation of E-cadherin as well as downregulation of vimentin caused by miR-506 overexpression (**Figure 5C**). Therefore, *IL8* was a direct target gene through which miR-506 suppressed the EMT process and invasiveness of HCC cells.

Discussion

In this study, we analyzed the biological role of miR-506 in human HCC metastasis. We found that miR-506 is significantly downregulated in metastatic HCC tissues and cell lines compared with nonmetastatic HCCs and HCC cells with a low metastatic potential. Overexpression of miR-506 remarkably inhibited the migration and invasiveness of HCC cells *in vitro* and metastasis *in vivo*. In terms of the mechanism, we identified *IL8* as a direct and functional target gene of miR-506. This finding deepens our

understanding of the mechanisms underlying HCC metastasis.

MiR-506 is a member of the family of X chromosome-linked miRNAs, which was first identified by Bentwich and colleagues [20]. MiR-506 usually acts as a tumor suppressor by regulating tumor cell characteristics, including proliferation, cell cycle distribution, and apoptosis. Moreover, several lines of evidence indicate that miR-506 may perform novel functions in tumor invasion and metastasis. For example, Zhang *et al.* have demonstrated that downregulation of miR-506 is associated with colon cancer metastasis, whereas miR-506 suppresses tumor cell invasion and metastasis [14]. Li *et al.* have reported that miR-506 overexpression inhibits gastric cancer cell migration, invasion, and EMT [9]. In our study, we found that miR-506 expression levels were low in HCC cell lines and HCC tissue samples. Weak expression of miR-506 is highly associated with distant metastasis and has been shown to be a strong and an independent predictor of shorter disease-free survival among patients with HCC. We confirmed that restoration of miR-506 expression suppresses HCC cell migration and invasion *in vitro* and inhibits the growth of xenograft tumors and lung metastasis *in vivo*.

EMT is a critical process in tumor invasion and metastasis [21]. Epithelial cells lose their epithelial adherence, polarity, and cell-cell contacts, and these changes facilitate cell motility and invasion [22]. In our study, we demonstrated that ectopic expression of miR-506 significantly increases E-cadherin expression and concomitantly decreases vimentin expression in HCC cells, suggesting that miR-506 inhibits HCC metastasis by suppressing EMT.

Previous studies have revealed that miR-506 can regulate the expression of sphingosine kinase 1 (SPHK1) [23], snail family zinc finger 2 (SNAI2) [24], and zeste homolog 2 (EZH2) [14]. Here, we identified *IL8* as a direct and functional target gene of miR-506. IL8 is well known as an inflammatory chemoattractant and contributes to the formation of a tumor microenvironment and to the progression of cancer. Thus, IL8 is involved in mobilization of immature myeloid cells and acceleration of tumorigenesis [25]. Studies indicate that high levels of IL8 contribute to poor prognosis of HCC, and IL8 promotes inflammation and the invasive and metastatic abilities of HCC cell lines [26]. In our study, administration IL8 reversed the miR-506-mediated suppression of migration, invasion, and EMT of HCC cells. Moreover, miR-506 expression negatively correlated with IL8 expression in human HCC specimens. These data indicate that *IL8* is a direct target gene and that the IL8 protein attenuates the effects of miR-506 on the migration, invasiveness, and EMT of HCC cells.

In conclusion, our experiments indicate that miR-506 suppresses the migration, invasiveness, and metastasis of HCC cells and specifically targets the 3'-UTR region of *IL8* mRNA. These findings suggest that miR-506 may function as a tumor suppressor and could be a therapeutic target in HCC.

Acknowledgements

Disclosure of conflict of interest

None.

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