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miR-139-5p inhibits epithelial–mesenchymal transition, migration and invasion of hepatocellular carcinoma cells by targeting ZEB1 and ZEB2

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ABSTRACT

Hepatocellular carcinoma (HCC) is the third most common cause of cancer deaths worldwide. miRNAs have been suggested to have important roles in HCC development. The purpose of this study was to determine the role of miR-139-5p in regulation of epithelial–mesenchymal transition (EMT) and metastasis of HCC cells. Expression levels of miR-139-5p in 49 HCC specimens with adjacent tissues and five HCC cell lines were assessed by quantitative RT-PCR. We found that miR-139-5p was down-regulated in 89.7% of the HCC tissue samples and all of the HCC cell lines. In addition, luciferase reporter assays validated direct binding of miR-139-5p to the 3′ untranslated region of zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2. Ectopic expression of miR-139-5p suppressed and miR-139-in promoted EMT, migration, and invasion in Hep3B and SMMC7721 cells. Furthermore, over-expression of ZEB1 and ZEB2 ablated the inhibitory effects of miR-139-5p on migration and invasion in HCC cells. Our study indicates that miR-139-5p functions as a suppressor of HCC EMT and metastasis by targeting ZEB1 and ZEB2, and it may be a therapeutic target for metastatic HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignant liver cancer, accounting for 83% of all cases [1]. It is estimated that the prevalence of HCC increased 3-fold between 1975 and 2007, and incidence continues to rise [2]. Previous studies have shown that aberrant expression of several microRNAs (miRNAs) is associated with HCC development and progression [3–5]. For example, up-regulation of miR-21 in HCC was found to promote its growth and spread via inhibition of phosphatase and tensin homolog (PTEN) signaling [6]. Conversely, down-regulation of miR-101 in HCC suppresses apoptosis and promotes tumorigenicity [7].

miR-139 is located within the second intron of the phosphodiesterase 2A (PDE2A) gene on chromosome 11q13.4. miR-139-5p is a common type of mature miRNA generated from a miR-139 precursor [8]. In this study, we assessed miR-139-5p expression levels in both HCC cell lines and human HCC tissue samples, and compared them to the expression of miR-139-5p in a normal liver cell line and matched non-cancerous liver tissue. Over-expression of miR-139-5p inhibited migration and invasion of Hep3B and SMMC7721 cells, and modulated epithelial–mesenchymal transition (EMT)-related gene expression. Mechanistically, we found two putative targets of miR-139-5p, ZEB1 (zinc finger E-box binding homeobox 1) and ZEB2, and the interaction of miR-139-5p with ZEB1 and ZEB2 was confirmed by luciferase reporter assays. Over-expression of ZEB1 and ZEB2 in Hep3B and SMMC7721 cells reversed the inhibitory effects of miR-139-5p. Our results indicate the tumor-suppressive role of miR-139-5p in HCC migration, so it may be a possible therapeutic target of HCC.

2. Materials and methods

2.1. Patient samples

This study was approved by the Institutional Review Broad of the Human Investigation Committee of the Second Affiliated Hospital of Harbin Medical University. HCC and matched non-cancerous liver tissue samples were surgically resected from 49 patients (35 males and 14 females) at the Department of General

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Surgery of the Second Affiliated Hospital of Harbin Medical University. All specimens were obtained from untreated patients undergoing surgery and were preserved at −80 °C. Informed consent was obtained from each patient, and samples were diagnosed by three pathologists.

2.2. Cell culture

Human HCC cell lines Bel7402, Hep3B, HepG2, Huh7, and SMMC7721, as well as the normal human liver cell line, HL-7702, were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 4 mM L-glutamine, 4.5 g/l glucose, 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, Zhejiang, China), 100 units/ml penicillin, and 100 units/ml streptomycin. All cell lines were maintained at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Transfection

Transfections were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. MiR-139-5p, miR-139-5p-inhibitor (miR-139-5p-in), and control miRNA mimics were purchased from Biomics Biotechnology (Nantong, Jiangsu, China). Full-length human ZEB1 and ZEB2 were generated from human HL-7702 cDNA by PCR and cloned into the pcDNA3.1 vector (Life Technologies).

2.4. Quantitative RT-PCR analysis

Total RNA, including miRNA, was extracted from the cells or tumor tissues, using Trizol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was generated from total RNA (1 μg) from each sample, using a reverse transcription kit (Life Technologies). The following primers were used in this study:

E-cadherin: 5′-GATAGAGAA CGCATTGCCACATAC-3′ (sense) and 5′-CTGATGACT CCTGTGTTCCTCTTA-3′ (anti-sense);
N-cadherin: 5′-GAGATCCTACTGGACGGTTCG-3′ (sense) and 5′-TCTTGGCGAATGATCTTAGGA-3′ (anti-sense);
Vimentin: 5′-CAGAGAGAGGAAGCCGAAAA-3′ (sense) and 5′-TCCTCT TCGTGGAGTTTCTT 3′ (anti-sense);
β-actin: 5′-AGCGAGCATCCCCCAAAGTT-3′ (sense) and 5′-GGGCACGAAGGCTCATCATT-3′ (anti-sense).

For miRNA, reverse transcription was performed using a miRNA reverse transcription kit (Qiagen, Valencia, CA, USA). Quantification of miRNA was performed using the TaqMan MiRNA assay (Life Technologies), and the U6 transcript was used as an internal control to normalize RNA input. Mature miR-139-5p expression was measured using a miRNA primer assay kit (Qiagen). The $^{\Delta\Delta}$Ct method was used for relative quantification of RNA expression.

2.5. Wound healing assay

After transfection of the miRNA mimics, Hep3B and SMMC7721 cells (1×10^6/well) were seeded onto 6-well plates. Wounds were created by dragging a 1000-μl pipette tip through the cell monolayer. Cells were allowed to migrate for 36 h. The gap area was then photographed, and migration distances were measured.

2.6. Cell invasion assay

Invasion activities of the cells were determined with a cell invasion assay used according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, Hep3B and SMMC7721 cells (5×10^4/well) were seeded in the upper chamber of insert with 1% FBS medium after transfection, while the lower chamber insert contained complete culture medium with 10% FBS medium to trap invading cells. After incubation overnight, the cells that penetrated the Matrigel-coated membranes and migrated into the lower chamber were stained with crystal violet (0.1%) and photographed. In each sample, invasion ability was quantified by counting crystal violet-stained cells.

2.7. Protein extraction and Western blot analysis

Cellular protein was extracted using lysis buffer with a proteinase inhibitor, and protein concentrations were measured with the Protein BCA Assay Kit (Beyotime, Nantong, Jiangsu, China). Protein was separated by electrophoresis on 10% SDS-PAGE, then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight at 4 °C with 1% BSA containing antibodies against ZEB1, ZEB2, E-cadherin, N-cadherin, Vimentin, or β-actin (all from Santa Cruz Biotechnology, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody. The membranes were developed using a SuperSignal West Pico Chemiluminescent Substrate (Thermal Scientific, Waltham, MA, USA).

2.8. Luciferase assay

The 3′ untranslated region (UTR) of ZEB1 and ZEB2 (containing their potential binding sites) and mutated controls were cloned and inserted into the pMIR-Report vector (Life Technologies). MiRNA mimics were then transfected into Hep3B and SMMC7721 cells containing wild-type or mutant 3′ UTR pMIR-Report plasmids. After 24 h, the activities of firefly luciferase and Renilla luciferase in the cell lysates were measured with the Dual-Luciferase Assay System (Promega, Madison, WI, USA), used according to the manufacturer's instructions.

2.9. Statistical analysis

All the experiments were performed at least three times independently, and typical results are shown with values expressed as means ± SEM. An analysis of variance (ANOVA) and Student's t-test were used to estimate the differences among groups. A p value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS software (version 19.0).

3. Results

3.1. Expression levels of miR-139-5p in HCC tissues and cell lines

Expression levels of miR-139-5p in HCC tissues were significantly lower in 89.7% (44 of 49) of the HCC patient samples compared to matched - noncancerous tissue samples (Fig. 1A). In addition, we examined the expression of miR-139-5p in cultured cell lines, including the normal liver cell line, HL-7702, and five HCC cell lines (Bel7402, Hep3B, HepG2, Huh7 and SMMC7721). We found that miR-139-5p expression was down-regulated in all five HCC cell lines when compared to the normal liver cell line (HL-7702) (Fig. 1B). Hence, miR-139-5p is down-regulated in HCC, which may contribute to HCC pathogenesis.

3.2. miR-139-5p directly targets ZEB1 and ZEB2

Since miRNAs mainly function through inhibiting their target mRNAs by binding to the 3′ UTR, we searched the putative target genes of miR-139-5p in online miRNA target prediction databases

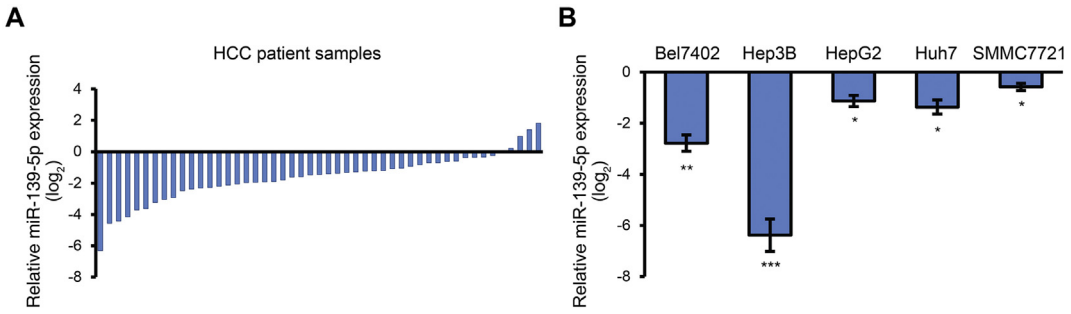


Fig. 1. Expression levels of miR-139-5p in HCC tissues and cell lines. (A) Quantitative RT-PCR analysis of the expression levels of miR-139-5p in 49 HCC tissue samples compared to pair-matched adjacent non-cancerous liver tissues. (B) Quantitative RT-PCR analysis of miR-139-5p expression in five HCC cell lines (Bel7402, Hep3B, HepG2, Huh7 and SMMC7721) normalized to that in normal liver cell line HL-7702. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal liver cells.

(Targetscan and microRNA.org) [9,10], and found that ZEB1 and ZEB2 are potential targets of miR-139-5p. A dual-luciferase reporter system was used with co-transfection of miR-139-5p and a luciferase reporter plasmid containing a wild-type or mutant 3′ UTR of human ZEB1 or ZEB2 (Fig. 2A). Luciferase activity was significantly inhibited by miR-139-5p, but increased by miR-139-5p-in co-transfected with wild-type ZEB1 or ZEB2 3′ UTR, and miR-139-5p and miR-139-5p-in failed to inhibit the expression of luciferase constructs with mutated target sites, suggesting that miR-139-5p directly targets the 3′ UTR of ZEB1 or ZEB2 (Fig. 2B–C). Moreover, a change in mRNA and protein expression levels of ZEB1 and ZEB2 in response to miR-139-5p over-expression or inhibition was verified by RT-PCR and Western blotting in Hep3B and SMMC7721 cells (Fig. 2D–F), confirming that miR-139-5p negatively regulates ZEB1 and ZEB2 expression by directly targeting their 3′UTR regions.

3.3. Inhibitory effects of miR-139-5p on cell migration, invasion and EMT in HCC cells

Down-regulation of ZEB1 and ZEB2 by miR-139-5p in HCC prompted us to investigate whether miR-139-5p can suppress HCC migration, invasion and EMT. We selected the Hep3B and SMMC7721 cell lines because they have the lowest and highest levels of miR-139-5p expression among the five cell lines that we tested, to conduct further functional studies. We first transfected miR-139-5p, miR-139-5p-in, or control miRNA into HCC cells (Fig. 3A), then assessed the role of miR-139-5p in regulating HCC cell migration with wound healing assays (Fig. 3B). The relative distance of cell migration was reduced approximately 40% by miR-139-5p and increased by 50% after miR-139-5p inhibition, indicating that miR-139-5p suppresses cell migration in Hep3B and SMMC7721 cells (Fig. 3C). In cell invasion assays, over-expression of miR-139-5p significantly inhibited but miR-139-5p-in significantly promoted the numbers of invading cells in both the Hep3B and SMMC7721 cell lines (Fig. 3D–E). Since EMT is closely related to cancer cell metastasis ability, we next examined EMT markers in control-, miR-139-5p-, or miR-139-5p-in-transfected HCC cells. Over-expression of miR-139-5p resulted in increased expression of E-cadherin and decreased expression of N-cadherin and Vimentin (Fig. 3F–G), while miR-139-5p had the opposite effects. Taken together, these results indicate that miR-139-5p suppresses HCC cell migration, invasion and EMT.

3.4. Over-expression of ZEB1 and ZEB2 ablates the inhibitory effects of miR-139-5p on migration and invasion in HCC cells

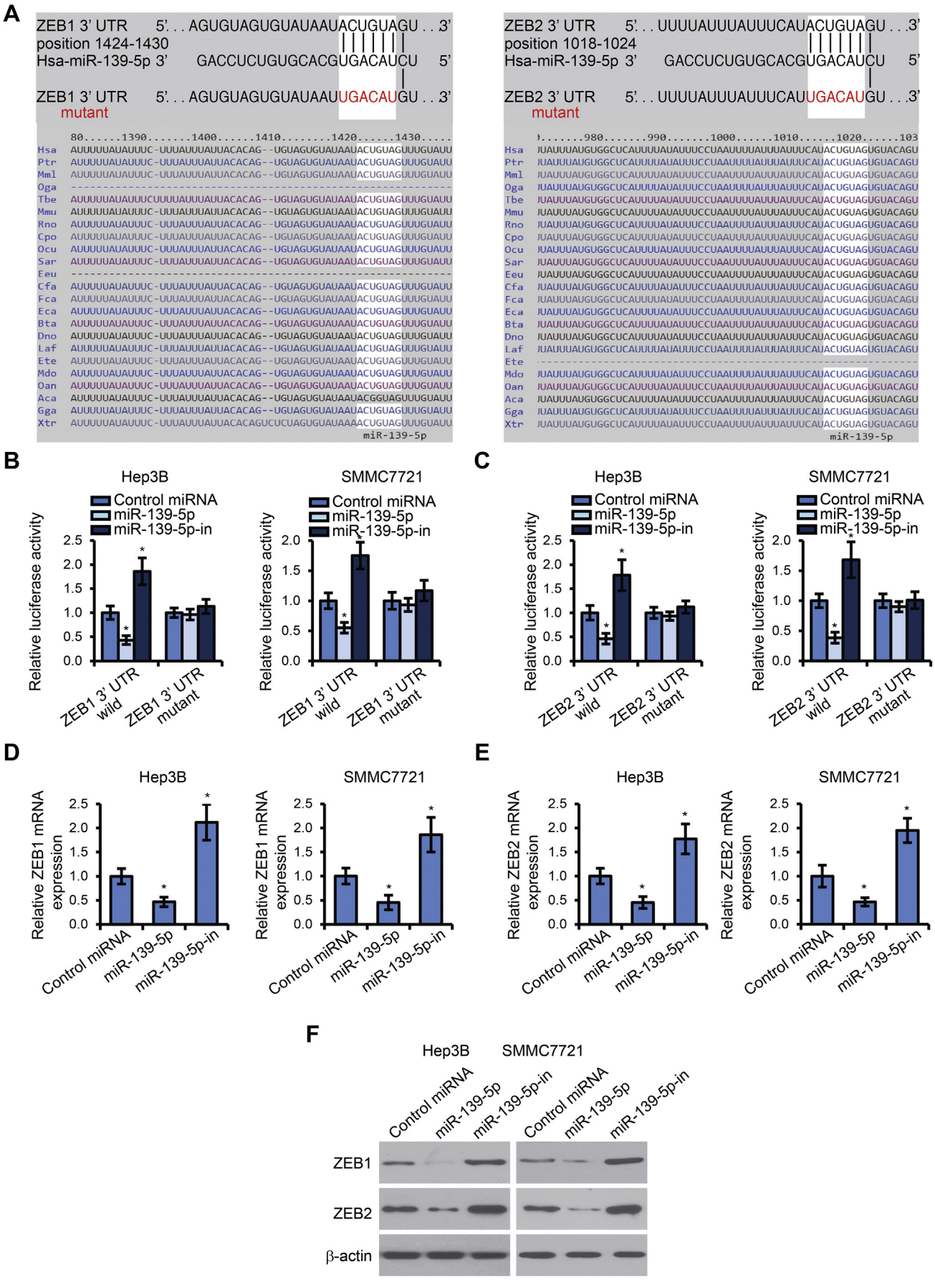
To determine whether the role of miR-139-5p in HCC is mediated by ZEB1 and ZEB2, we over-expressed both ZEB1 and ZEB2 with miR-139-5p in Hep3B and SMMC7721 cells (Fig. 4A). Wound healing migration assays showed that over-expression of ZEB1 and ZEB2 fully abolished suppression of cell migration induced by miR-139-5p in both Hep3B and SMMC7721 cells (Fig. 4B http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0116170 - pone-0116170-g003). Invasion assays further confirmed that over-expression of ZEB1 and ZEB2 reversed the inhibitory effects of miR-139-5p on HCC cell invasion (Fig. 4C http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0116170 - pone-0116170-g003). These results confirm that ZEB1 and ZEB2 are regulated by miR-139-5p and miR-139-5p down-regulation, and may participate in HCC carcinogenesis and progression through potentiation of ZEB1 and ZEB2 expression.

4. Discussion

HCC is an aggressive cancer with poor prognosis. Despite decades of progress in treatment of HCC, the overall 5-year survival rate remains less than 12%, largely because of intra-hepatic metastases or post-surgical recurrence. In this study, we demonstrated that miR-139-5p is down-regulated in HCC, and miR-139-5p inhibits cell migration, invasion, and EMT by targeting ZEB1 and ZEB2.

miR-139-5p is down-regulated in multiple human cancers. It has been reported that miR-139-5p is the most down-regulated miRNA in colorectal cancer (CRC) tissue [11], and it is frequently down-regulated in invasive breast carcinoma [8]. Consistent with previous studies, we found that miR-139-5p was under-expressed in all five HCC cell lines and in 89.7% of the HCC tissue samples. However, the mechanism by which miR-139-5p is down-regulated in cancer tissues is still controversial. Au et al. found that EZH2 represses miR-139-5p expression in human HCC through H3K27 methylation [12]. However, miR-139 may be expressed differently from its host gene, PDE2A [8]. Furthermore, although miR-139-5p was under-expressed in colorectal cancer tissue [11,13–16], the expression levels of pre-miR-139 were unchanged in comparison to non-cancerous samples [17], indicating a post-transcriptionally regulatory mechanism occurring during miR-139 maturation.

miR-139-5p functions as a metastastic suppressor in multiple human cancers. Ectopic expression of miR-139 was found to suppress metastasis and progression of HCC [18], colorectal cancer [17], and breast cancer [8]. Several targets of miR-139-5p have been identified, such as Rho-Kinase 2 and c-Fos in HCC [18,19] and type 1 insulin-like growth factor receptor (IGF-1R) in CRC [14]. miR-139-5p may be involved in the TGF-β, Wnt, Rho, and APK/PI3K signaling cascades in the breast cancer cell line, MCF7 [8]. Notably, in all the studies reported, the miR-139-5p target-binding sites were found to be located in the 3′ UTR of targeted mRNA.


A
ZEB1 3' UTR 5'. . . AGUGUAGUGUAUAAUACUGUAGU . . 3'
position 1424-1430
Hsa-miR-139-5p 3' GACCUCUGUGCACGUGACAUCU 5'
ZEB1 3' UTR 5'. . . AGUGUAGUGUAUAAUUGACAUGU . . 3'
mutant
ZEB2 3' UTR 5'. . . UUUUAUUUAUUUCAUACUGUAGU . . 3'
position 1018-1024
Hsa-miR-139-5p 3' GACCUCUGUGCACGUGACAUCU 5'
ZEB2 3' UTR 5'. . . UUUUAUUUAUUUCAUUGACAUGU . . 3'
mutant
miR-139-5p
B
Hep3B
SMMC7721
Control miRNA
miR-139-5p
miR-139-5p-in
Relative luciferase activity
ZEB1 3' UTR wild
ZEB1 3' UTR mutant
C
ZEB2 3' UTR wild
ZEB2 3' UTR mutant
D
Relative ZEB1 mRNA expression
E
Relative ZEB2 mRNA expression
F
ZEB1
ZEB2
β-actin

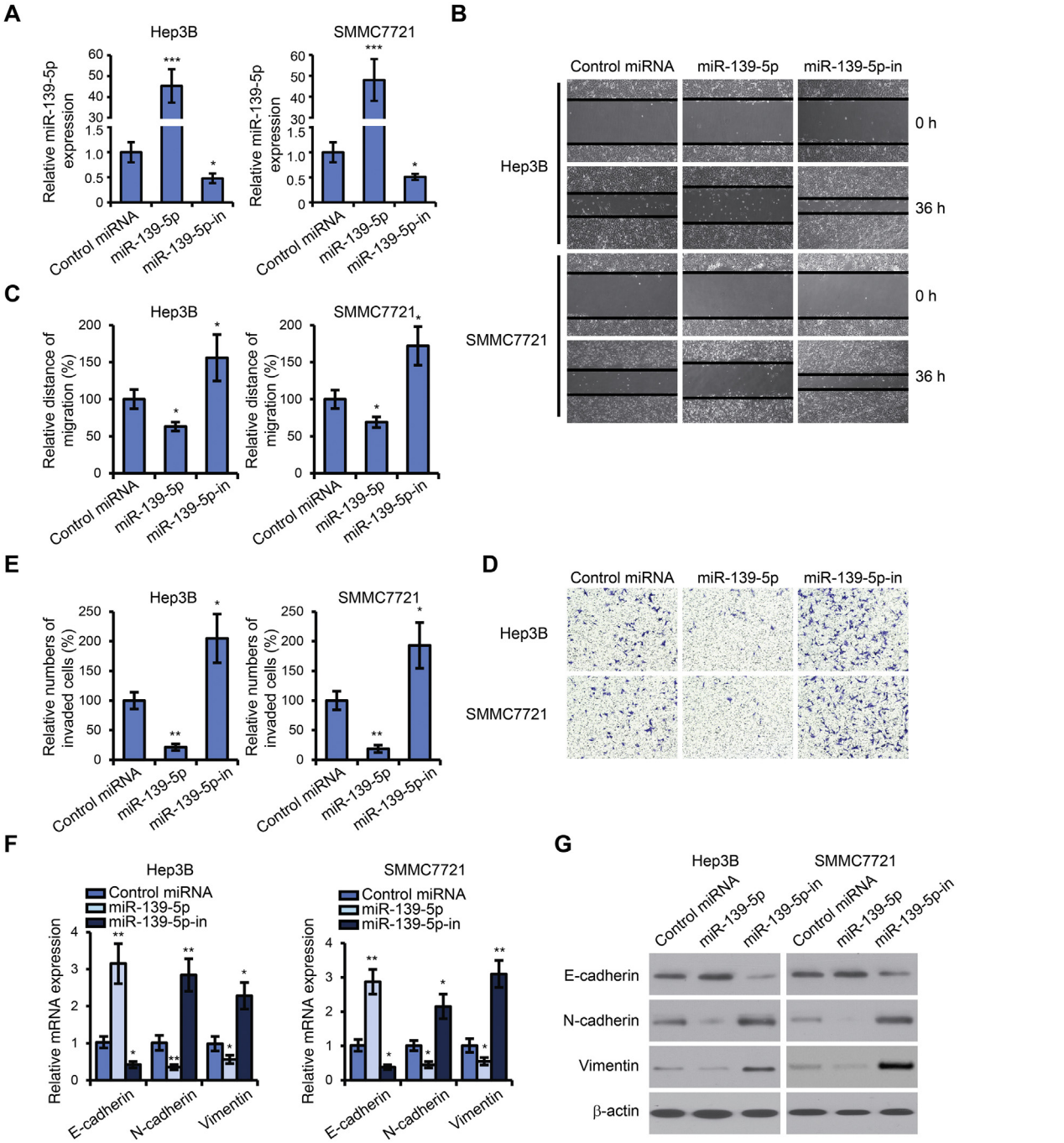


Fig. 3. miR-139-5p regulates cell metastasis ability and EMT marker expression. (A) miR-139-5p levels in Hep3B and SMMC7721 cells transfected with control miRNA, miR-139-5p, or miR-139-5p-in. (B) Wound healing assay to assess migration of Hep3B and SMMC7721 cells transfected with miR-139-5p, miR-139-5p-in, or control miRNA. (C) Quantification of relative migration distance (%). (D) Cell invasion assay to determine the invasion abilities of Hep3B and SMMC7721 cells transfected with miR-139-5p, miR-139-5p-in, or control miRNA. (E) Quantification of relative numbers of invaded cells (%). (F) RT-PCR analysis of *E-cadherin*, *N-cadherin*, and *Vimentin* in Hep3B and SMMC7721 cells, in response to miR-139-5p over-expression and inhibition. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to control miRNA group. (G) Western blot analysis of E-cadherin, N-cadherin and Vimentin in Hep3B and SMMC7721 cells, in response to miR-139-5p over-expression and inhibition. β-actin was used as loading control.

Fig. 2. ZEB1 and ZEB2 are direct targets of miR-139-5p. (A) Putative miR-139-5p binding sites in the 3′UTR region of ZEB1 and ZEB2. Six nucleotides (red) were mutated in the luciferase reporter plasmids carrying ZEB1 or ZEB2 3′UTR. (B) Luciferase activity of Hep3B or SMMC7721 cells transfected with plasmids carrying a wild-type or mutant 3′UTR of ZEB1, in response to miR-139-5p over-expression or inhibition. (C) Luciferase activity of Hep3B or SMMC7721 cells transfected with plasmids carrying a wild-type or mutant 3′ UTR of ZEB2, in response to miR-139-5p over-expression or inhibition. (D–E) mRNA levels of *ZEB1* (D) and *ZEB2* (E) examined by RT-PCR in Hep3B and SMMC7721 cells transfected with miR-139-5p, miR-139-5p-in, or control miRNA. (F) ZEB1 and ZEB2 protein levels were analyzed by Western blot in Hep3B and SMMC7721 cells transfected with miR-139-5p, miR-139-5p-in, or control miRNA. β-actin was used as loading control. $^{*}p < 0.05$ compared with the control miRNA group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

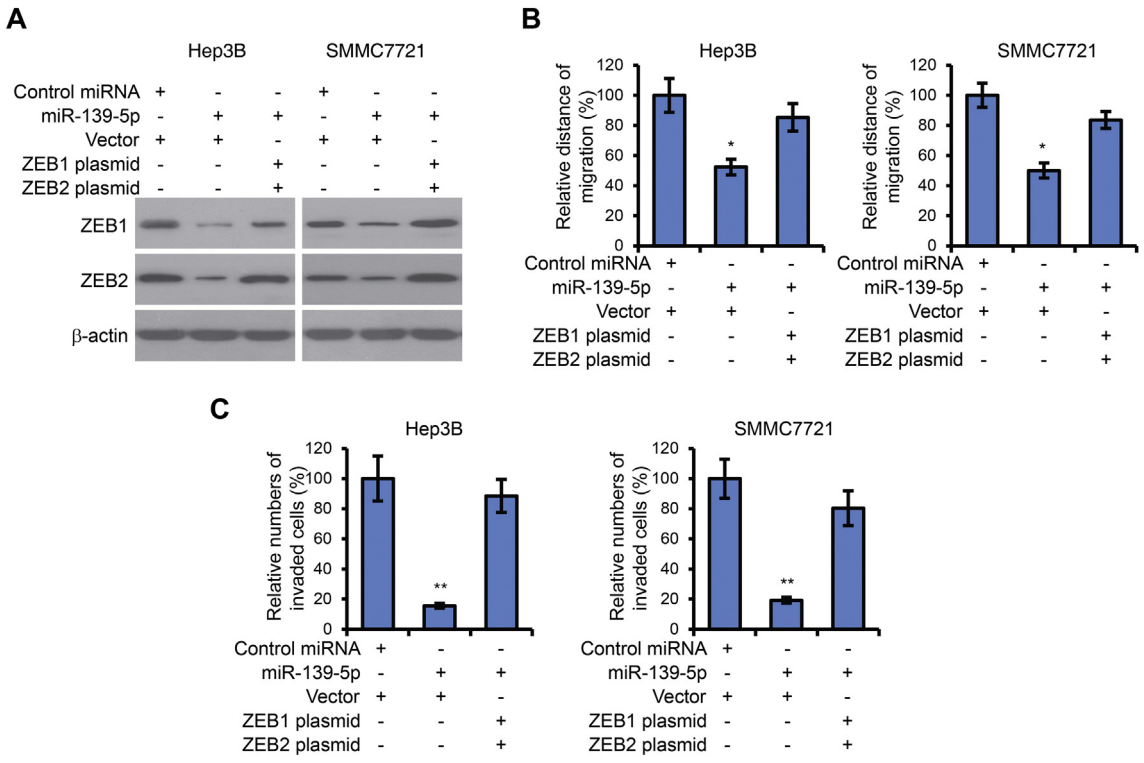


Fig. 4. The role of ZEB1 and ZEB2 in miR-139-5p-regulated cell migration and invasion. (A) Western blotting analysis of ZEB1 and ZEB2 expression in Hep3B and SMMC7721 cells transfected with indicated molecules. (B) Wound healing assay to determine the migration of Hep3B and SMMC7721 cells transfected with indicated molecules. (C) Cell invasion assay to assess the invasion abilities of Hep3B and SMMC7721 cells transfected with indicated molecules.*$p < 0.05$, **$p < 0.01$ compared with control miRNA and vector transfected cells.

Our results demonstrate that miR-139-5p is associated with EMT in HCC. Loss of E-cadherin is considered to be a fundamental event in EMT, and EMT is an essential process in tumor progression and metastasis [20–22]. In our study, ectopic expression of miR-139-5p significantly increased E-cadherin expression. Accordingly, expression of N-cadherin and Vimentin was reduced when miR-139-5p was over-expressed in Hep3B and SMMC7721 cells.

We identified ZEB1 and ZEB2 as targets of miR-139-5p. They both contain two C2H2-type zinc fingers that mediate their binding to paired CAGGTA/GE-box-like promoter elements, and function as transcriptional repressors [23–25]. It has been reported that ZEB1/2 inhibits EMT by targeting E-cadherin in HCC cells [25]. In our study, luciferase reporter assays detected direct binding of miR-139-5p to the 3′ UTR of ZEB1/2 transcripts. Furthermore, over-expression of ZEB1/2 rescued, at least in part, the migration activity of HCC cell lines induced by ectopic expression of miR-139-5p. Taken together with previous studies, it seems that miR-139-5p serves as a pivotal mediator in regulation of HCC metastasis.

In summary, we have found that down-regulation of miR-139-5p in HCC cells is highly associated with their migration activity. ZEB1/2 mediates miR-139-5p-regulated EMT and migration of HCC cells. Thus, miR-139-5p may be a diagnostic marker and a potential target of anti-cancer therapy of HCC.

Conflicts of interest

There are no conflicts of interest in this work.

Acknowledgments

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.062.

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