European Review for Medical and Pharmacological Sciences 2020; 24: 6031-6038

LncRNA-HEIH suppresses hepatocellular carcinoma cell growth and metastasis by up-regulating miR-199a-3p

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Abstract. – OBJECTIVE: The aim of this study was to explore the functional changes of long non-coding ribonucleic acid (lncRNA)-HEIH on hepatocellular carcinoma (HCC) Huh7 and Hep3B cells.

PATIENTS AND METHODS: The exp changes of HEIH in 18 pairs of HCC tiss nd adjacent normal tissues were detected by titative real time-polymerase chain rea (qRT-PCR). According to its expression cha es in HCC cells silenced by sh hairpin rib nucleic acid (shRNA) transfec *ro*, these cells were divided into sh- gr nd sh-NC group. The effects of y expre d HEIH on the proliferation, mi n and HCC cells were examined h says. Western blott was d to determine the expressio anges elial-mesenchymal transi EMT) prote mentin, matrix metallo (MMP)-2 and IP-3. In addition, the of downstream effector micro RNA (mi)-199a-3 C was explored.

RESULT Compared w jacent normal tissues H was highly exp ed in HCC tissues .01). H silencing significantly inhibi he pr ation and migration, but induc ap sis of 7 cells ($p<0.05$). The expre vimen nd MMP-2 in sh-HEIH up we ark lower than those in sh-oup (urthermore, miR-199a-3p was ntified e downstream effector of HEI he expres on of miR-199a-3p increased ma and Hep3B cells with silenced H exp n ($p<0.01$). Moreover, when miR-a-3p expression was inhibited, the effects of on Huh7 and Hep3B cells were weakened, i ted as notably enhanced cell proliferation migration capabilities ($p<0.05$).

CONCLUSION LncRNA-HEIH suppresses cell growth d metastasis by up-regulating R-199a-3 ur findings suggest that HEIH may g target for HCC treatment.

Key Words:
cRNA-HEIH, Hepatocellular carcinoma (HCC), p.

Introduction

Primary liver cancer is the second leading cause of cancer-related death in the world[1]. In recent years, the incidence rate of liver cancer increases gradually by 4% in men and 3% in women[2]. Hepatocellular carcinoma (HCC) is the main type of primary liver cancer. In the latest decades, the therapeutic methods (surgical resection, liver transplantation, etc.) for HCC has dramatically improved the quality of life of patients. However, the overall 5-year survival rate is still far from satisfactory[3]. Therefore, improving the 5-year survival rate of HCC is still the direction of our efforts. The development of HCC is a complex and multi-step process implicating diverse molecules and signal transduction pathways[4]. A better knowledge of the incidence and development of HCC will be conducive to the further improvement of its treatment methods. In addition, pinpointing the molecular occurrence mechanism of HCC and discovering effective therapeutic targets can provide new approaches for HCC treatment.

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Long non-coding ribonucleic acids (lncRNAs) are a kind of RNAs unable to encode proteins. LncRNAs exert crucial effects in regulating cell cycle, growth and death[5,6]. Therefore, they are considered as vital factors that can influence the development of various diseases, including malignant tumors[7]. Multiple lncRNAs tend to be disordered in HCC, such as CCHE1[8], GIHCG[9], ZNFX1-AS1[10], LINC00052[11] and HOTAIR[12]. A fraction of lncRNAs is identified as tumor suppressor genes or oncogenes. Consistently, lncRNA HANR facilitates the occurrence and chemoresistance of HCC[13], while lncRNA uc.134 inhibits the progression of HCC[14]. Besides, lncRNAs are considered as promising therapeutic targets for HCC treatment due to their involvement in the occurrence and development of HCC[15]. Yang et al[16] reported for the first time that HEIH is a lncRNA highly expressed in HCC tissues. HEIH becomes well-known for its high expression in HBV-related HCC. Zhang et al[17] have revealed that HEIH is conducive to the invasion of HCCMHCC97L and HepG2 cells. They have also proved that HEIH exerts a tumor promoting effect on HCC development. Moreover, HEIH exhibits a high expression in many types of cancers, including colorectal cancer, melanoma, and lung cancer[18-20]. Besides, the tumor-promoting effect of HEIH in these cancers has been confirmed. Although HEIH is proved to be associated with the pathogenesis of human cancers, its function in HCC is not yet fully elaborated. Therefore, the aim of this study was to investigate the effect of HEIH on the proliferation and metastasis of human HCC cells and to reveal the potential current mechanism of HCC.

Patients and Methods

Experimental Materials

The investigation was approved by the Ethics Committee of Jiangsu Hospital Affiliated to Nanjing University of Chinese Medicine. Signed written informed consents were obtained from all participants before the study. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). A total of 36 patients aged 45-55 years old admitted to our hospital from April 2016 to February 2017 were enrolled in this study. Paired tissue samples were obtained from each patient. U6/Neo plasmid (GenePharma, Shanghai, China), miRNeasy Mini Kit (Qiagen, Shenzhen, China), TB Green Premix Ex Taq II Kit (TaKaRa, Dalian, China), flow cytometer (Beckman, California, CA, USA), bicinchoninic acid (BCA) (Beijing Puli Lanthai, Beijing, China) and 0.22 μm filter membrane (Millipore, Billerica, MA, USA).

Research Objects

The obtained 18 HCC tissue samples were assigned into HCC group and para-cancer group. HCC Huh7 and HepG2.2.15B cells transfected with short hairpin (sh) HEIH plasmid (sh-HEIH group) and empty plasmid (sh-NC group) were used. Thereafter, HCC Huh7 cells with silenced HEIH expression were selected as research objects and transfected with inhibitor reagent (sh-HEIH+inhibitor group) and negative control (NC) reagent (sh-HEIH+NC group).

Cell Culture and Transfection

Huh7 and HepG2.2.15B cells were cultured in complete high-glucose Dulbecco Modified Eagle's medium (DMEM). The sh structure of sh-HEIH was applied in U6/Neo plasmid, and only U6/Neo plasmid sh-NC was regarded as a control. miR-199a-3p inhibitor and its scrambled NC were designed and synthesized by GenePharma (Shanghai, China). Cell transfection was performed in 6-well plates according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 6 h, the transfection reagent was discarded, and the cells were collected for subsequent assays.

Western Blotting

Transfected cells were first digested with trypsin. Cell precipitates were collected and added with cell lysate, followed by ultrasonic disruption. The supernatant was then collected, and the concentration of extracted protein was determined in accordance with the BCA kit. Next, protein samples were separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with 5% skimmed milk powder for 10 min, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody at room temperature for 2 h. Immuno-reactive bands were finally exposed by enhanced electrochemiluminescence (ECL) method.

Determination of Cell Apoptosis

Transfected cells were collected and precipitated in Eppendorf (EP; Hamburg, Germany) tubes following transient trypsinization. After washing with cold phosphate-buffered saline (PBS), the cells were incubated in cell dye solution [5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI)] and 95 μL of dye solution binding buffer for 10 min in a dark place. Finally, cell apoptosis was detected using a flow cytometer.

Detection of Cell Proliferation Via MTS

After digestion, transfected cells were uniformly inoculated into 96-well plates at a density of 5,000 cells per well, with a total volume of 200 μL. After incubation at 37°C for 48 h, 10 μL of Cell Counting Kit-8 (CCK-8) was added (Dojindo Molecular Technologies, Kumamoto, Japan) to each well, followed by incubation at 37°C for another 4 h in the dark. Absorbance at 490 nm in each cell was determined by a micro-plate reader.

Quantitative Real Time-Polymeras Chain Reaction (qRT-PCR)

Total RNAs in cells and tissues were ex ed using TRIzol reagent (Invitrogen, Carls CA, USA). To analyze the expre on of HEI PrimeScript RT Master Mix a een Premix Ex Taq II were used com mentary DNA (cDNA) synthesis qPCR, ective-ly. In addition, messen NAs Huh7 cells were isol d u easy Mini Kit to examine the pressio iR-199a-3p. Mir-X miRNA strand synt kit and Mir-X miRNA SYBR kit w utilized for reverse tr ript d qPCR. Glyceraldehyde 3-pho hate dehyd se (GAPDH) and U6 were d as internal s for mRNA and mi A, res ectively. Expr ssion level was calcu d usin e $2^{-\Delta\Delta Ct}$ method. The primer nces e sho below: HEIH: Forward: CGAC GCCATGAGACC-30; Reverse: AAC CTTGTGTGACCGA-3'; 9a-3p rd 5'-ACACTCCAGCTGG CAGTAC GCACAT-3'; Reverse: 5'-C AACTGGTGTCGTGGAGTCGGCAATTC AA CCAAT-3'; GAPDH: For-: 5'-CTCTGCTCCTCCTGTTCGAC-3'; Re-e 5'-GACTCCGACCTTCACCTTCC-3'; U6: d: 5'-CTCGCTTCGGCAGCACA-3'; Reverse: AACGCTTCACGAATTTGCGT-3'.

Wound Healing Assay

To determine the changes in relati gration, the healing changes after eratch were measured to reflect the ges of cel migration. In short, the cells venly inoculated into 6-well plates. When nfluence reached 100%, the effect o ll proli was eliminated by replacing t iginal medi serum-free medium. T straig lines in well were drawn usi 10 μ ar head. The beginning of draw r ed as and images were co ou red af ncubation for 24 h.

Statisti Anal

Statistical Product rvice Solutions (SPSS) 19.0 Corp., Armon , USA) was used for a istical analysis. The *t*-test was used to com- the differen between two groups. $p<0.05$ considered s tically significant.

Results

HEIH Was Highly Expressed In ues

The xpression of HEIH in 18 pairs of HCC tissues and adjacent normal tissues was quantitatively analyzed by qRT-PCR. The results showed that HEIH expression in HCC tissues was markedly higher than that in para-cancerous tissues ($p<0.01$) (Figure 1). This indicated that HEIH might be associated with the occurrence and development of HCC.

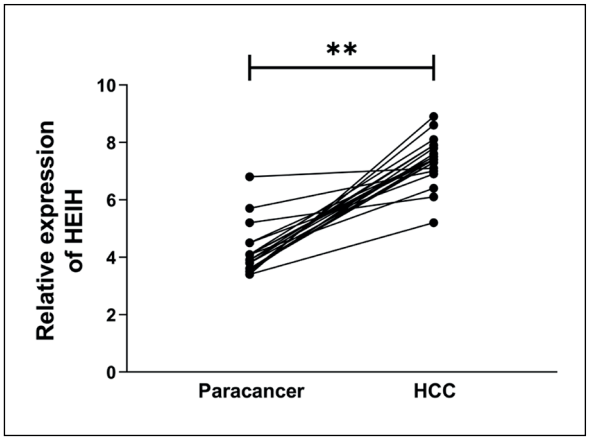


Figure 1. HEIH expression in HCC tissues and para-cancer tissues. HEIH is highly expressed in HCC tissues compared with para-cancer tissues ($p<0.01$) (**$p<0.01$).

HEIH Silencing Inhibited the Proliferation and Migration of Huh7 Cells

To testify the authenticity of the above hypothesis, HEIH was silenced in Huh7 cells by shRNA transfection. QRT-PCR data revealed that sh-HEIH transfection significantly reduced HEIH expression in HCC cells in comparison with NC transfection ($p<0.01$). Next, the effects of HEIH silencing on the growth and metastasis of Huh7 cells were assessed by functional assays. According to MTS results, cell proliferation in sh-HEIH group was significantly inhibited ($p<0.05$). In addition, in contrast to sh-NC group, sh-HEIH group exhibited remarkably shortened cell migration distance and lower wound healing rate ($p<0.01$) (Figure 2).

Silencing of HEIH Suppressed Invasion but Triggered Apoptosis of Huh7 Cells

Western blotting manifested that the expressions of matrix metalloproteinase (MMP)-2, MMP-3 and vimentin were overtly lower in sh-HEIH group than those in sh-NC group ($p<$ indicated that suppressing HEIH e sion p moted the invasion of HCC cells. cytometry was then adopted to detect the p ge of apoptotic Huh7 cells with silenced HE ssion. It was found that the percenta of apop lls in sh-HEIH group was rema y higher th sh-NC group ($p<0.01$) ure 3) hese fin suggested that the in ion on H expression induced the apoptos CC s.

HEIH Silenc Increas MiR-199a- xpression

Cancer iR-199a-3p h been widely recogni as a suppressor miRNA in HCC[21,22]. It shows st bility to inhibit multiple processes, su growth, migration, i ion and angiogenesis[22,23]. In this study, the elation betwe HEIH and miR-199a-3p was

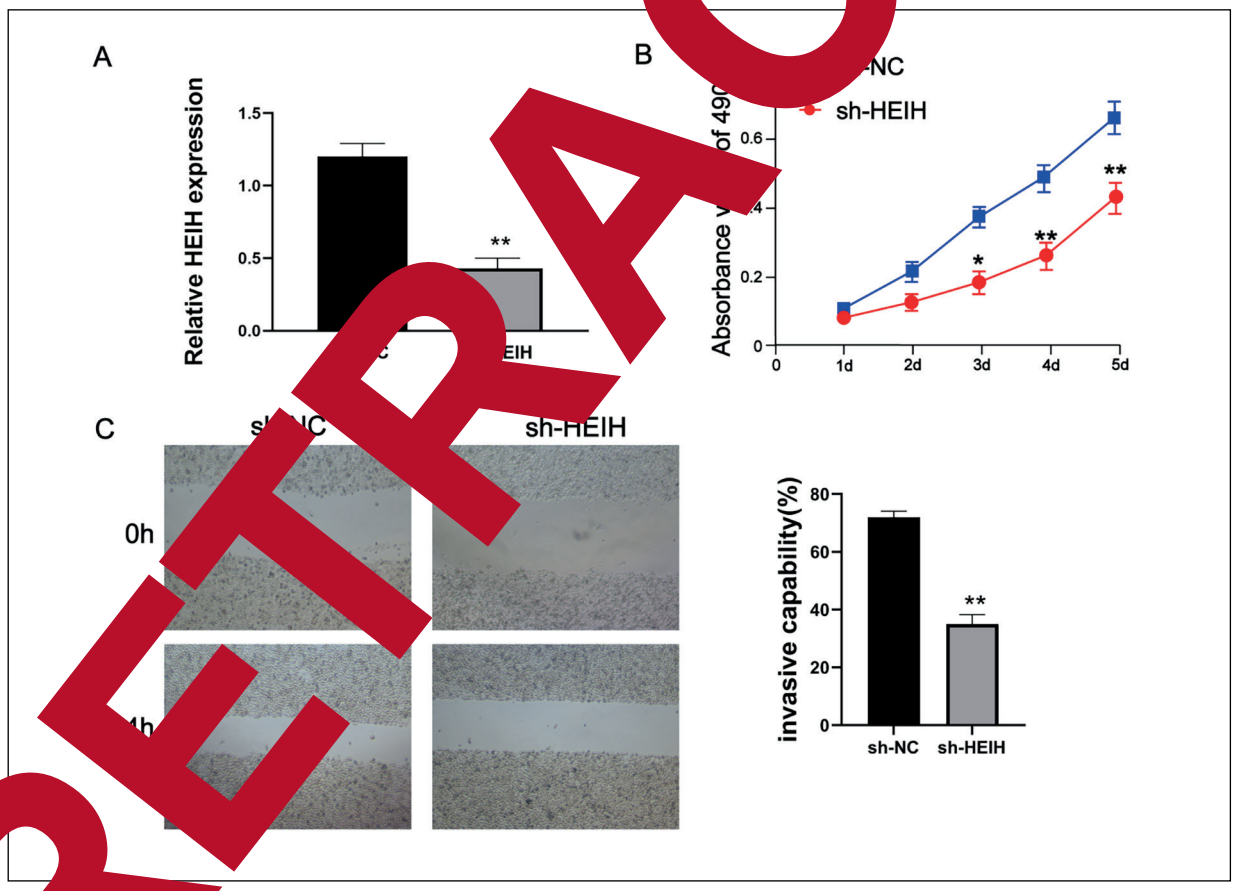


Figure 2. Influences of HEIH expression on HCC cell proliferation and migration. **A,** HEIH expression in Huh7 cells transfected with shRNA. HEIH is lowly expressed in sh-HEIH group compared with that in sh-NC group ($p<0.01$). **B,** Effects of silenced HEIH expression on Huh7 cell proliferation. Absorbance at 490 nm of cells in sh-HEIH group is notably lower than that in sh-NC group ($p<0.05$). **C,** Effects of silenced HEIH expression on the migration of Huh7 cells. Sh-HEIH group exhibits a prominently lower wound healing rate than sh-NC group (magnification: 40×) ($p<0.01$) (*$p<0.05$, **$p<0.01$).

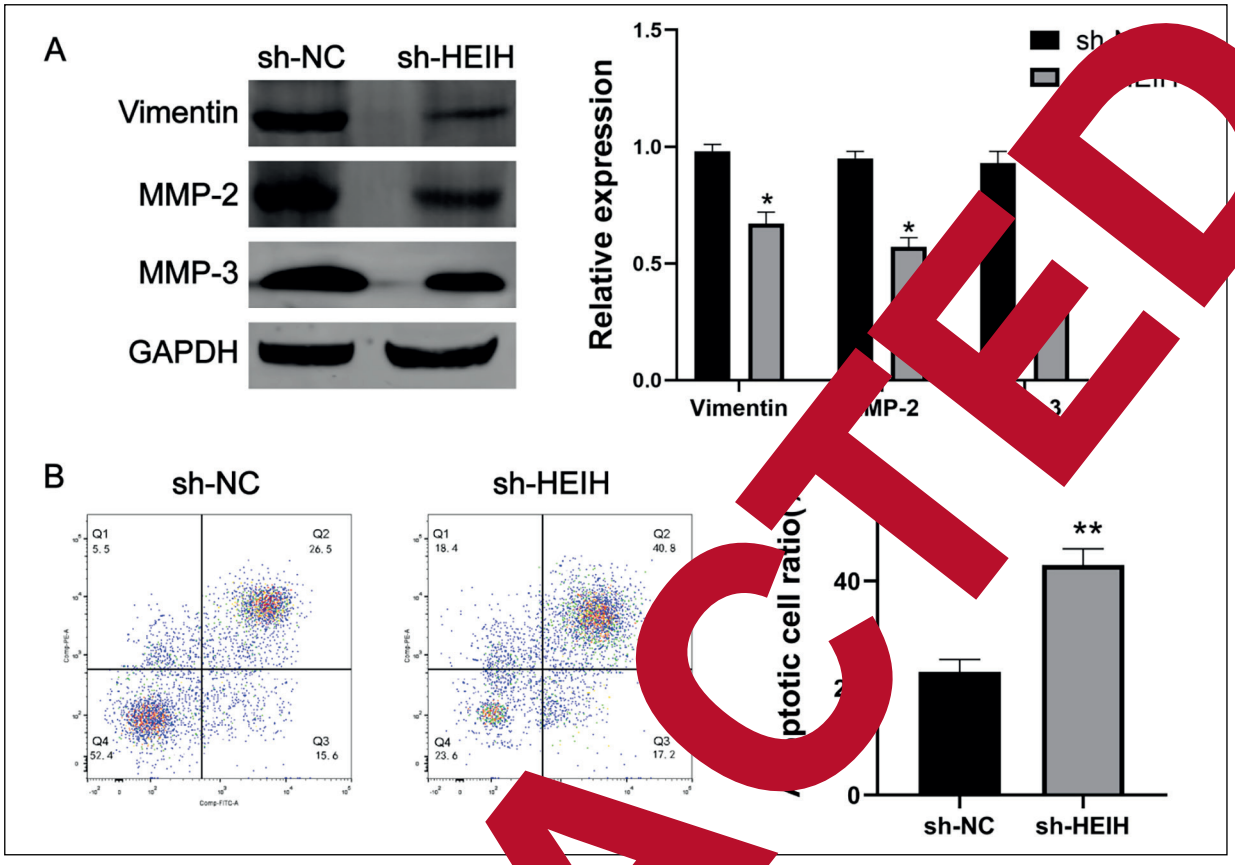


Figure 3. Impacts of suppressing HEIH expression on HCC cell invasion and apoptosis. **A,** Effect of HEIH on HCC cell invasion. In comparison with sh-NC group, sh-HEIH group significantly reduced expressions of epithelial-mesenchymal transition (EMT) proteins ($p<0.05$). **B,** Effect of HEIH expression on HCC cell apoptosis. The percentage of apoptotic cells in sh-HEIH group is markedly higher than sh-NC group ($p<0.01$) (*$p<0.05$, **$p<0.01$).

explored to explain the inhibitory effect of HEIH silencing on tumors. RT-PCR illustrated that sh-HEIH significantly up-regulated the expression of miR-199a-3p through silencing HEIH expression in both Huh7 and Hep3B cells compared with sh-NC ($p<0.01$) (Figure 4).

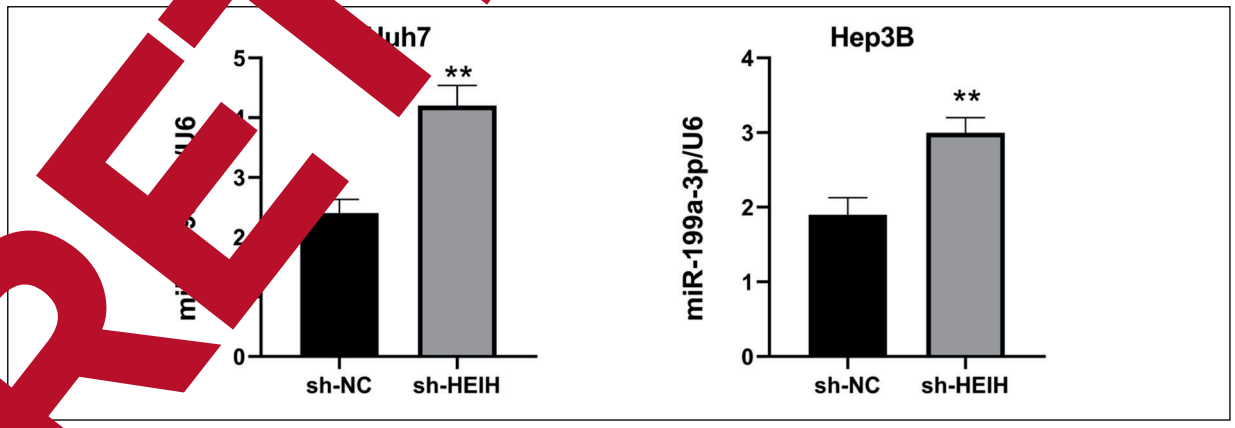


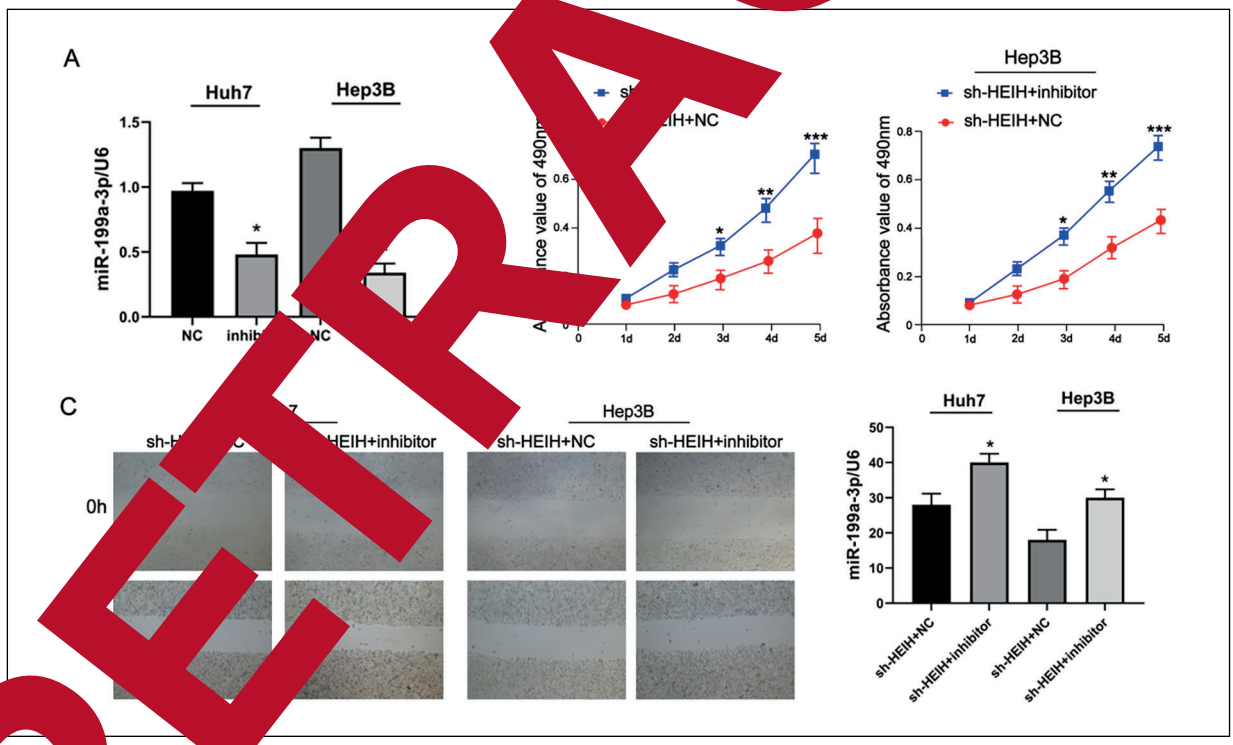
Figure 4. Effect of HEIH silencing on miR-199a-3p expression. In contrast to cells in sh-NC group, those in sh-HEIH group have a markedly up-regulated expression of miR-199a-3p ($p<0.01$) (**$p<0.01$).

HEIH Inhibited the Growth and Metastasis of Huh7 and Hep3B Cells by Modulating MiR-199a-3p

HCC Huh7 cells were selected and transfected with miR-199a-3p inhibitor to inhibit miR-199a-3p expression, so as to further determine whether HEIH silencing up-regulated the expression of miR-199a-3p and was related to the function of HEIH. QRT-PCR results demonstrated that the expression of miR-199a-3p in Huh7 cells was indeed significantly inhibited after transfection of miR-199a-3p inhibitor reagent ($p<0.01$). Subsequently, sh-NEIN and miR-199a-3p inhibitor were co-transfected into Huh7 cells, and the changes in cell growth and metastasis were reevaluated. Experimental results showed that co-transfection of sh-HEIH and miR-199a-3p inhibitor weakened the inhibition on cell proliferation by sh-HEIH, manifested as evidently higher absorbance at 450 nm in sh-HEIH+inhibitor group than sh-HEIH+NC group ($p<0.05$). In addition, co-transfection with sh-HEIH and miR-199a-3p inhibitors weakened the inhibitory effect of sh-HEIH on cell migration as well. Wound healing rate in sh-HEI group was overtly higher than th sh-H H+NC group ($p<0.05$). To sum the growth and metastasis of Huh7 cells w pressed by mediating the expression of mi 3p after silencing HEIH expression r anoth C cel line Hep3B, a similar tre was observe silencing prominently s essed e prolife and migration of H cells <0.05). When the expression of m a-3 s inhi , the anti-growth and i-m effect HEIN silencing on H B cells y All our findings den rated that HE cing inhibited the g metastasis o ep3B cells by regulati iR-19 (Figure 5).

Discussion

Currently, the rall survival rate of HCC pa- is relatively . Meanwhile, high metastasis



Figu Impacts of HEIH silencing and miR-199a-3p inhibition on the growth and metastasis of Huh7 cells. **A,** Expression of uh7 and Hep3B cells transfected with miR-199a-3p inhibitor or NC. It is discovered that compared with sh- +NC group, the expression of miR-199a-3p in sh-HEIH+inhibitor group evidently declines ($p<0.05$). **B,** Changes in the feration of the two cell lines co-transfected with shRNA and miR-199a-3p inhibitor. In contrast to cells in sh-HEIH+NC those in sh-HEIH+inhibitor group display a significantly increased absorbance at 450 nm ($p<0.01$). **C,** Changes in cell mi after co-transfection with shRNA and miR-199a-3p inhibitor. Wound healing rate of cells in sh-HEIH+inhibitor group ignificantly higher than that in sh-HEIH+NC group (magnification: 40×) ($p<005$) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

and recurrence rates become huge obstacles to the treatment of HCC. Specific lncRNAs exert regulatory roles in the growth, apoptosis, migration and invasion of HCC cells[15]. In this study, we mainly aimed to explore the effect of HEIH on HCC. The difference in HEIH expression in 18 pairs of HCC tissues and para-cancer tissues was detected by qRT-PCR. The results showed that HEIH was highly expressed in HCC tissues compared with para-cancer tissues, which is consistent with the findings of previous studies[17,19]. It is suggested that HEIH level increases in tumor microenvironment. To further determine whether elevated HEIH participates in regulating the growth and metastasis of HCC cells, human HCC cell lines (Huh7 and Hep3B) were transfected with shRNA to silence HEIH expression. Huh7 cell line with silenced HEIH expression was selected for subsequent functional assays. According to the results, HEIH silencing notably reduced the proliferation and migration of Huh7 cells but induced their apoptosis. Of note, miR-199a-3p was highly expressed in HCC cells with silenced HEIH expression. Therefore, it was regarded as a downstream target molecule of HEIH in HCC. In the case of in[illegible] miR-199a-3p expression, the functions of [illegible] Huh7 and Hep3B cells were weakened.

Up to date, there are few studies on th[illegible] mor-promoting effect of HEIH on human canc[illegible] Overexpression of HEIH has bee[illegible] confirmed [illegible] promote the migration and inva[illegible] nor cells, which may also increase the [illegible] ity [illegible] n-small cell lung cancer A549 and [illegible] -1 cells [illegible] ilar results have been observed [illegible] rect[illegible] and melanoma cells[18]. H[illegible] ntroversies exist in the function[illegible] EIH in [illegible] Yang et al[16] have initially prov[illegible] at HEIH is a[illegible] odulate the cell cycle ar[illegible] C cells, indica[illegible] the involvement of [illegible] H in [illegible] ting the proliferation of HCC cel[illegible] Highly exp[illegible] HEIH facilitates the invas[illegible] of HCC MHC[illegible] ells but does not infl[illegible] e its proliferation abi[illegible]ty[17]. In addition, epith[illegible] mese[illegible]nal transition (EMT) is an important [illegible] roce[illegible] the pr[illegible]ression and metastasis of can[illegible] n this s[illegible], the results of western [illegible] show[illegible] t the [illegible] ressions of matrix metalloproteinase [illegible] MMP-3 and vimentin were signi[illegible]ntly low[illegible] sh-HEIH group than those in s[illegible] group. All these results suggested that af[illegible] e expression of HEIH in Huh7 Hep3B cells, cell proliferation and migration significantly inhibited. However, cell apoptosis induced. The findings of this study are partially consistent with those in previous researches, illustrating that HEIH is conducive to the proliferation and metastasis of HCC cells. A[illegible] data imply that HEIH silencing may [illegible] poten[illegible] therapeutic target for HCC treatm[illegible]

LncRNAs have a very c[illegible] functional mechanism. Physically, lncRNA[illegible] combine with DNAs, proteins, mR[illegible]s and [illegible]As to modulate the expression. [illegible]ization and of binding partners[21]. A[illegible]g these different [illegible] anisms of action, ther[illegible] increa[illegible]ly more interactions between lnc[illegible] a[illegible] iRNA[illegible] Meanwhile, lncRNAs [illegible] K[illegible]NAs [illegible] from mRNAs like [illegible] olecular [illegible] [illegible] present study, the c[illegible]nking betwe[illegible] and miR-199a-3p w[illegible]d. MiR-199a[illegible] ranks third among [illegible]NAs [illegible] highest expression in the normal liver, which i[illegible]kably down-regulated in [illegible] Besides, miR[illegible]3p exerts a crucial e[illegible] in driving the occurrence and development [illegible]CC. In recent years, it becomes one of the [illegible]st extensively [illegible]earched miRNAs in HCC[23-[illegible] this study, [illegible] expression changes of miR-[illegible] in HCC[illegible]s with lowly expressed HEIH were [illegible] QRT-PCR results demonstrated that miR-199a-3p was highly expressed in HCC [illegible]lls with low expression of HEIH, suggesting that [illegible]3p expression is negatively regulated by [illegible] ubsequent functional analysis manifested that when miR-199a-3p expression was inhibited, the tumor-promoting effect of HEIH silencing on Huh7 and Hep3B cells was weakened. The above results prove that HEIH functions in the growth and metastasis of HCC cells, which may be partially realized by negative regulation on miR-199a-3p.

Conclusions

The novelty of this study was that HEIH silencing may be a promising target for inhibiting HCC cell growth and metastasis. In addition, HEIH silencing exerts its anti-tumor impact mainly by up-regulating miR-199a-3p. However, the results of this study still need to be verified *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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