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The role of miR-99b in mediating hepatocellular carcinoma invasion and migration

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults with a high rate of malignancy. The potent invasion and migration of HCC mainly impact the prognosis and recurrence of the disease. Our previous study found that miR-99b was highly expressed in HCC, and its expression was associated with vascular invasion. It was speculated that miR-99b may play a role in HCC invasion and migration, while the specific mechanism remains unclear.

MATERIALS AND METHODS: qRT-PCR was applied to detect expressions of miR-99b and KAI1 genes in L02, HepG2, and MHCC97H cells. HepG2 cells were transfected with miR-99b inhibitor, miR-99b mimic, and NC. Flow cytometry was used to test cell cycle and apoptosis. Dual-luciferase reporter gene assay was adopted to validate the target gene of miR-99b. Wound healing assay was used to detect cell migration. Transwell assay was performed to detect cell invasion. Western blot was performed to detect KAI1, E-cadherin, and N-cadherin expressions. Immunofluorescence assay was adopted to test Vimentin expression.

RESULTS: The expression of miR-99b was reduced in L02 while upregulated in MHCC97H. By contrast, the expression of KAI1 was increased in L02 but downregulated in MHCC97H. The transfection of miR-99b mimic inhibited HepG2 apoptosis and accelerated cell cycle. MiR-99b suppressed KAI1 gene expression through targeting its 3'-UTR. miR-99b mimic or siKAI1 transfection promoted cell invasion and migration, while their simultaneous transfection significantly enhanced cell migration and invasion. The overexpression of miR-99b or knockdown of KAI1 significantly weakened HepG2 cell adhesion, reduced E-cadherin expression, upregulated N-cadherin and Vimentin, and promoted cell epithelial-mesenchymal transition (EMT).

CONCLUSIONS: MiR-99b contributes to promote function in HCC migration and invasion through inhibiting KAI1 expression.

Key Words:
miR-99b, Cadherin, N-cadherin, Hepatocellular carcinoma, Invasion, Migration.

Introduction

Primary hepatocellular carcinoma (HCC) is common in the clinic with a high rate of malignancy, which presents a great threat to human health and life[1]. Although the comprehensive treatment based on surgery achieves a relatively good clinical effect, its postoperative recurrence seriously affects the patient's quality of life[2]. High potency of invasion and migration of HCC cells seriously restrains postoperative quality of life and prognosis[3]. Primary HCC occurrence, progression, invasion, and metastasis involve multi-step of biological processes and are associated with the regulation by various factors and signaling molecules[4]. MiRNA is a kind of endogenous non-coding small single-stranded RNA molecule in eukaryotes at the length of 21-24 nucleotides with high conservation. MiRNA regulates target gene expression by complementary binding with the 3'-UTR of target mRNA to degrade or suppress protein translation, thus mediates related biological processes[5]. Several studies indicated the involvement of miRNAs in different development stages of HCC occurrence, invasion, and metastasis[6]. Our previous investigation[7] found that miR-99b was significantly highly expressed in the tumor tissue of HCC patients, and its expression had a certain relationship with HCC vascular invasion. Therefore, this work aims to investigate the effect of miR-99b on HCC invasion and metastasis.

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Materials and Methods

Main Reagents and Materials

HepG2, MHCC97H, and HEK293 cells were got from ATCC (Manassas, VA, USA). Normal liver cell line L02 was purchased from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were bought from Gibco (Rockville, MD, USA). TRIzol and Lipofectamine 2000 were got from Invitrogen (Carlsbad, CA, USA). Reverse transcription kit and Real-time PCR kit were bought from Toyobo (Osaka, Japan). MiR-99b nucleotide fragments (mimic, inhibitor, and negative control) and PCR primers were designed and synthetized by Genepharma (Shanghai, China). Rabbit anti-human Vimentin primary antibody, rat anti-human E-cadherin primary antibody, and rabbit anti-human N-cadherin primary antibody were from Abcam (Cambridge, MA, USA). Rabbit anti-human KAI1 antibody was from CST (Danvers, MA, USA). BCA protein quantification kit and Annexin V/PI kit were provided from Beyotime (Beijing, China). [illegible] swell chamber was from Corning (Corni[illegible] USA). Matrigel was obtained from BD Bios[illegible]ce (San Jose, CA, USA). Dual-Luciferase® Re[illegible]er Assay System and pGL3-promoter were fi[illegible] Promega (Madison, WI, USA).

HepG2 Cell Culture a[illegible] miR-99b Transfection

HepG2 cells were [illegible]ured [illegible]EM supplement by 10% FBS[illegible] U/mL pe[illegible]in, and 100 μg/mL streptom[illegible]d maintaine[illegible]7°C and 5% CO_2. The [illegible] we[illegible]d for transfection when the density [illegible]ched 50-[illegible]MiR-99b nucleotide fragment[illegible]iR-99b mi[illegible]'-CACCCGUAGAAC[illegible]CCUUGCG-3', mi[illegible]c negative control[illegible]5'-UC[illegible]CGAACGUGUCACGUTT-3', mi[illegible]itor, [illegible]'-CGCAAGGUCGGUUCU[illegible]G-3', i[illegible]bitor negative control, [illegible]CAGU[illegible]UUU[illegible]GUAGUACAA-3') and [illegible]ctami[illegible]were diluted by Opti-MEM and [illegible]ubated [illegible]5 min at room temperature, res[illegible]ly. Then, they were gently mixed and a[illegible]ns for 30 min at room temperature. [illegible]xt, the cells were cultured in an incubator for [illegible]h and the medium was changed. After 48 h, t[illegible]s were collected for the following experiment[illegible]. MHCC97H and L02 cells were treated by the same method.

Target Prediction

The online miRNA target gene prediction database, microRNA.org, was used to predic[illegible] sible target genes of miR-99b. It wa[illegible] miR-99b presented a good compl[illegible]tary relationship with the 3'-UTR of KA[illegible]e luciferase reporter gene assay was conducte[illegible]t the target regulation of miR-99b o[illegible]AI1.

Luciferase Reporter[illegible]ne Carrier Construction

HEK293 cell geno[illegible]as [illegible]d as a [illegible]plate to amplify the [illegible]TR [illegible]AI1 ge[illegible]primer sequence, [illegible]CCGTCC[illegible]AT[illegible]GGGTAGAGGGAC[illegible]nd 5'-GG[illegible]GCTATCCCCATC[illegible]C[illegible]'). After PC[illegible] product was collected, [illegible] was d[illegible] digested by XbaI/NotI, insert[illegible]to luciferase[illegible]ter carrier pGL-3M, an[illegible]rmed to DH5[illegible]mpetent cells. After t[illegible]positive clone was selected by PCR, it was [illegible]uenced by [illegible]on (Shanghai, China). The [illegible]mid with cor[illegible] sequence was named pGL3-K[illegible]'UTR ar[illegible]ansfected to the cells.

Lucifer[illegible]eporter Gene Assay

HEK293 cells were cultured in DMEM sup[illegible] with 10% FBS and 1% penicillin-strep[illegible]my[illegible] and seeded in 24-well plate at 5×10^4/cm^2. After the cell density reached 60-70%, 200 ng pGL3-KAI1-3'UTR, 50 nmol miR-99b mimic (or mimic negative control), and 50 ng internal reference pRL-TK mixture were transfected to cells together with Lipofectamine 2000. After incubation for 6 h, the medium was changed and cells were further cultured for 48 h. After being washed by phosphate-buffered saline (PBS) for two times, the cells were added with 100 μL lysis for 15 min and centrifuged at 1000 rpm for 5 min. A total of 50 μL supernatant was added with 50 μL luciferase substrate and detected in chemiluminescence apparatus to determine luciferase activity. Next, the liquid was added with 50 μL termination reaction liquid to test gastro-vascular luciferase activity. The ratio of luciferase activity and gastro-vascular luciferase activity was used as the relative gene expression level.

siRNA Transfection

KAI1 siRNA (CAGCAAGGTCCCCAAGTAC) or negative control siRNA (TTCTCCGAACGTGTCACGT) was transfected to the cells using the same method as miRNA transfection. The cells were divided into five groups, including mimic NC group, miR-99b mimic group, si-NC group,

si-KAI1 group, and miR-99b + si-KAI1 group. The cells were used for the following experiments at 48 h after transfection.

qRT-PCR

Total RNA was extracted using the TRIzol method and quantified on Eppendorf protein nucleic acid detector. Then, the RNA was reverse-transcripted to cDNA using ReverTra Ace RT Kit. The reverse transcription system in 20 μl contained 2 μl total RNA, 1 μl dNTP (10 mmol/L), 4 μl RT Buffer (5×), 2 μl RT primer (1 μmol/L), 1.5 μl reverse transcriptase, 0.5 μl RNase inhibitor, and ddH_2O. Reverse transcription was performed at 16°C for 30 min, 42°C for 15 min, and 85°C for 5 min. The cDNA was stored at -20°C. Then, the cDNA was used for PCR reaction, and the primers used were as follows. miR-99bP_{RT}, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATFCGCACTGGATACGACCGCAAG-3', miR-99bP_F, 5'-GTGCAGGGTCCGAGGT-3', miR-99bP_R, 5'-TCACCCGTAGAACCGACCTT-3'; U6P_F, 5'-ATTGGAACGATACAGAGAAGATT-3', U6P_R, 5'-GGAACGCTTCACGAATTTG-3'; KAI1P_F, 5'-TGTCCTGCAAACCTCCTC[illegible]3', KAI1P_R, 5'-CCATGAGCATAGTGACTG[illegible], β-actinP_F, 5'-GAACCCTAAGGCCAAC-3', β-actinP_R, 5'-TGTCACGCACGATTTCC-3'. PCR reaction system in 10 μl contained 4.5 μl 2×SYBR Green Mixture, 0.5 μl primer (2.5 μm/L), 1 μL cDNA, and 3.5 μl ddH_2O. PCR reaction was performed on ABI 7[illegible] amplifier at 40 cycles of 95°C for 15 s, 60°C for 30 s, [illegible]°C for 30 s. U6 and β-actin were used as references for miRNA and mRNA. Each sample was repeated for three times. Comparative Ct method ($2^{-\Delta\Delta CT}$) was applied for quantitative analysis.

Western Blot

Total protein was extracted and quantified by bicinchoninic acid (BCA) method. A total of 30 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane after blocking it in 5% skim milk at room temperature for 60 min, the membrane was incubated in primary anti-body (KAI1 1:300, E-cadherin 1:400, N-cadherin 1:300, β-actin 1:1000) at 4°C overnight. After washed by TBST for three times, the membrane was further incubated in horseradish peroxidase (HRP)-tagged secondary anti-body (1:5000) at room temperature for 60 min. At last, the membrane was treated by enhanced chemiluminescence (ECL) and scanned to collect data. Relative protein level = target band gray value/β-actin band gray value.

Immunofluorescence Analysis

The cells were collected and washed by PBS for 5 min. After fixed in 4% paraformaldehyde at room temperature for 40 min, cells were washed with PBS and treated by 0.5% Triton X-100 for 30 min. Then, the cells were blocked in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 at room temperature for 60 min and incubated in primary antibody (1:200) diluted in PBS containing 1% BSA and 0.1% Triton X-100 at 4°C overnight, washed by PBS, then were further incubated in Alexa Fluor tagged Goat Anti-Rabbit IgG H&L (1:400) containing BSA at room temperature for 60 min. At last, the cells were stained with 0.1% 4',6-diamidino-2-phenylindole (DAPI) for 1 min and observed under a microscope.

Cell Cycle Detection

The cells were digested with 0.25% enzyme and fixed in 70% ethanol at 4°C overnight. After washed by PBS for twice, the cells were resuspended in 500 μL PBS and treated with 50 μg/mL RNase A at 37°C for 30 min. Next, the cells were incubated on ice for 5 min to stop the reaction. Then, the cells were treated with 0.1% Triton X-100 for 30 min and added with 100 μg/ml PI staining at 4°C for 30 min. At last, the cells were detected by flow cytometry to calculate G0/G1, S, and G2/M cell ratio.

Cell Apoptosis

The cells were collected after digestion and centrifuged at 1000 g for 5 min. The cells were resuspended in 195 μl Annexin V-FITC buffer and 5 μl Annexin V-FITC. After mixed, the cells were further added with 10 μl PI and incubated at room temperature avoid of light for 10-20 min. At last, the cells were tested on flow cytometry.

Wound Healing Assay

When the cell density reached 80%, a 10 μl tip was applied to scratch a line on the cell surface. Then, the cells were washed with PBS and cultured for 48 h. The relative distance of cell migration was observed under microscope and photograph was taken.

Transwell Assay

Matrigel was slowly unfrozen at 4°C and diluted by three times volume of serum-free medium.

A total of 100 µl matrigel was put onto the upper surface of the Transwell chamber and incubated at 37°C for 60 min. The cells were resuspended in serum-free medium at 1×10^6/mL and added to the upper chamber at 200 µl. Another 600 µl complete medium was added to the lower chamber. After 48 h incubation, the cells on the filter membrane and matrigel were removed by swab. Then, the membrane was fixed by 4% paraformaldehyde for 20 min and stained by 0.1% crystal violet for 30 min. The membrane was observed under a microscope, and 5 visual fields were randomly selected for calculation.

Statistical Analysis

All data analysis was performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurement data was presented as mean ± standard deviation. One-way ANOVA followed by Dunnett post hoc test were applied for group comparison. $p < 0.05$ was considered as statistical significance.

Results

MiR-99b Highly Expressed in HCC

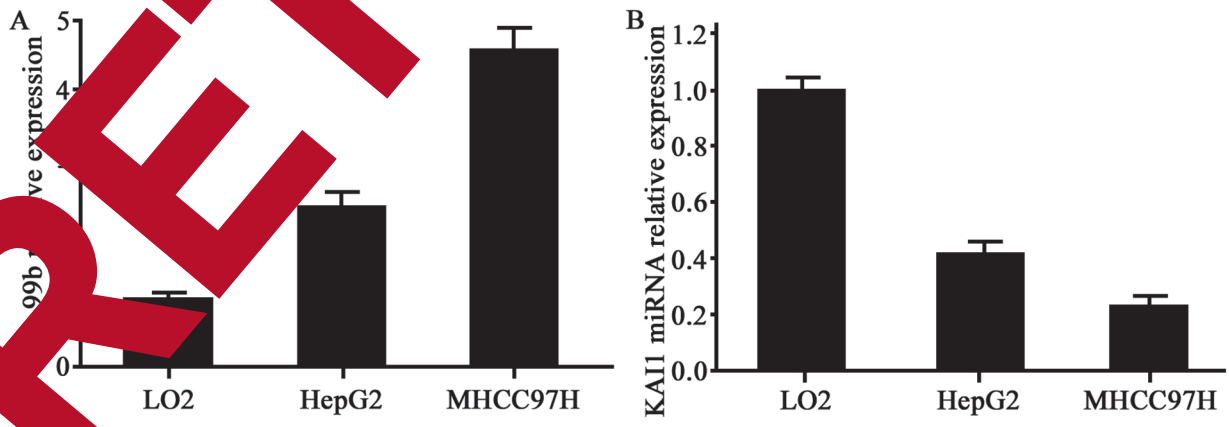
qRT-PCR revealed that miR-99b exp sion was significantly higher in human H compared with normal liver line LO ($p<0.05$). Its level in MHCC9 ith high metastasis potential was nifica higher than that in HepG2 cell h wea tion and invasive ability (Fig 1A ggs that the growing le f m may be related to HCC in n and m is. KAI1 showed opposite expression trend, the level of which was reduced in cells with a strong invasive ability (Figure 1B), indicating th might be a targeting inhibitory rela p tween miR-99b and KAI1.

MiR-99b Overexpression Inh l Cell Apoptosis and Promote Cell C

Compared with NC p, in the mimic group the apopt rate w statist decreased (p = 0.01 while significantly elevated in miR-99b to oup (p 001) (Figure 2A), sug ing R-99b yed an inhibitory eff on HCC si ne overexpression R-99b signi promoted HepG2 c y increasing ll ratio in S phase and G2/M s The cells were blocked in G0/G1 hase after pression of miR-99b wa regulated (Fig 2B).

-99b Targ g Inhibited Express

s transfected with pGL3-KAI1-3'UTR, 9b mimic transfection significantly upregulated miR-99b level, while the relative activity was significantly reduced (p 0.0 , revealing that miR-99b may target on the 3'-UTR of KAI1 gene (Figure 3A). Moreover, after the transfection of miR-99b mimic induced the miR-99b expression (Figure 3B), KAI1 level in HepG2 cells were markedly decreased (Figure 3C and D). The opposite result was shown with transfection of miR-99b inhibitor, indicating that miR-99b can inhibit KAI1 expression in HepG2 cells.



Figu 1. MiR-99b and KAI1 expression in different liver cell lines. ***A,*** qRT-PCR detection of miR-99b expression. ***B,*** qRT-PCR detection of KAI1 mRNA expression.

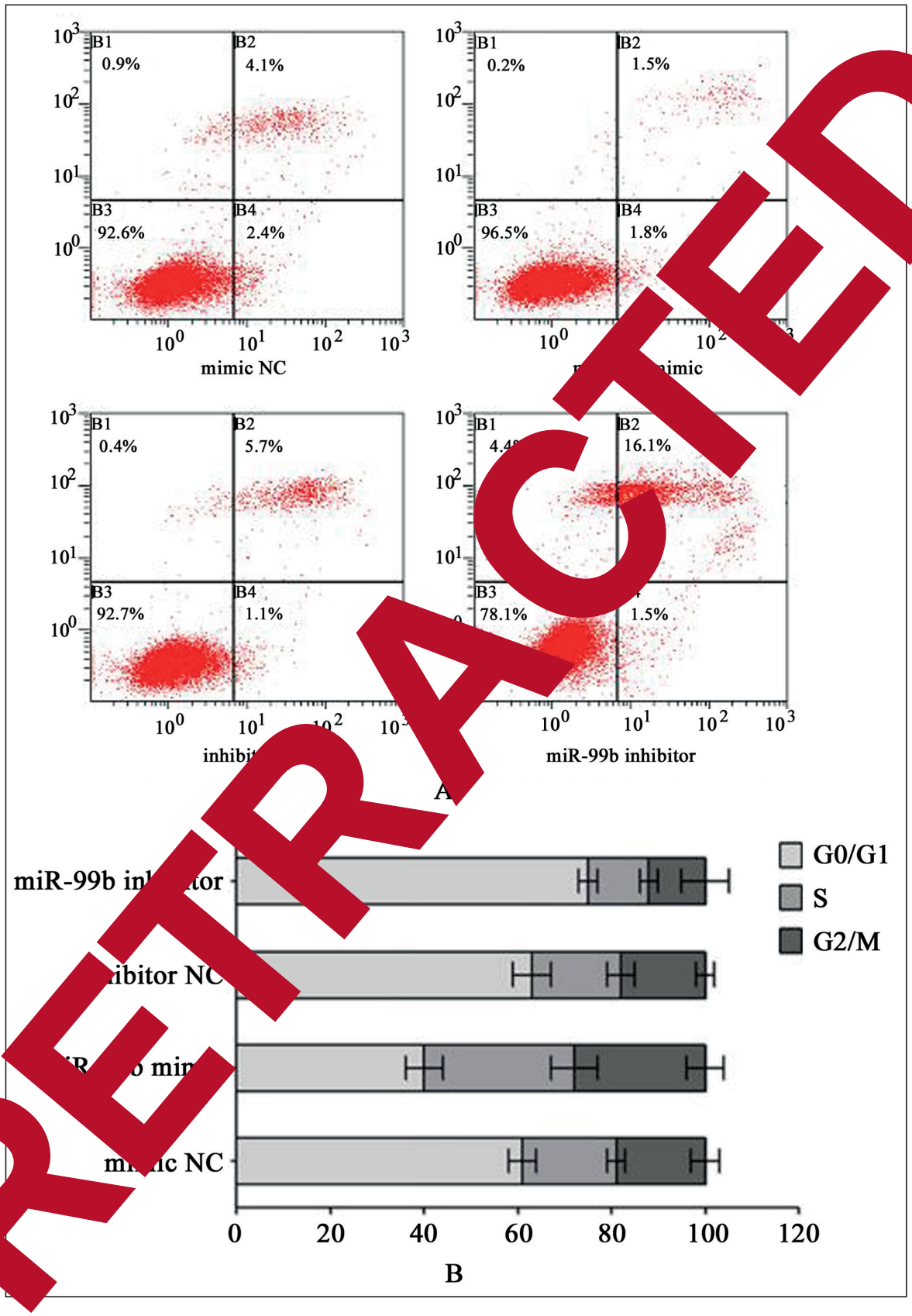


Figure 2. Flow cytometry detection of cell apoptosis *(A)* and cell cycle *(B)*.

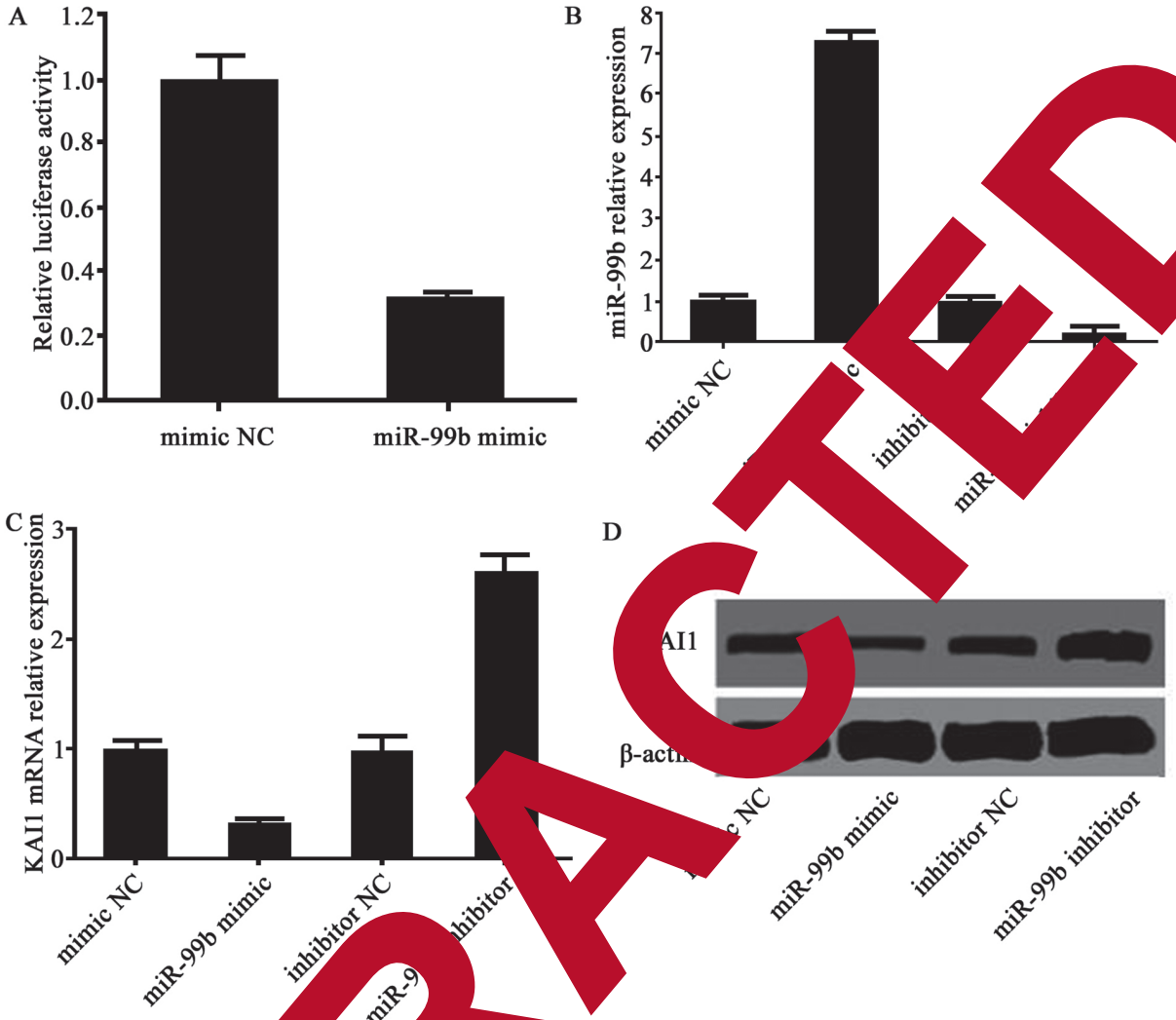


Figure 3. MiR-99b targe[ting] in[duces] KAI1 expression. ***A,*** Dual-luciferase reporter gene assay. ***B,*** qRT-PCR detection of miR-99b expression i[n] HepG2 ce[lls]. qRT-PCR detection of KAI1 mRNA expression in HepG2 cells. ***D,*** Western blot detection of KAI1 pro[tein] expression in [HepG2] cells.

MiR-99b Inhibiting [KAI1] Promoted Cell Mig[ration] and Inv[asion]

We f[ound] that miR-99b expr[ession] was significantly higher in [HCC] cell line with high metastatic p[otential] than that in HCC cell line with lower metastatic potential and normal liver cell line, suggesting involvement of miR-99b in the progression of HCC invasion and migration. The overexpression of miR-99b in HepG2 cells significantly enhanced wound healing ability and matrigel [invasion] activity (Figure 4B and 4C). Dual-luciferase reporter gene and miR-99b overexpression assay validated that KAI1 was the target gene of miR-99b. Furthermore, miR-99b mimic transfection or si-KAI1 significantly down-regulated KAI1 protein expression in HepG2 cells (Figure 4A). The migration and invasion of HepG2 cells were significantly enhanced after KAI1 level was down-regulated. The overexpression of miR-99b together with KAI1 reduction showed the apparent cell migration and invasion, indicating that the increase of miR-99b and inhibition of KAI1 show a synthetic effect on HCC cell migration and invasion (Figure 4B and 4C). Our data found that E-cadherin level was markedly reduced while N-cadherin expression was enhanced in HepG2 cells. Immunofluorescence assay demonstrated that miR-99b overexpression or si-KAI reduced the adhesion among cells, and mesenchymal morphology emerged from epithelial status. Vimentin protein expression was also elevated in miR-99b mimic + si-KAI1 group (Figure 4D).

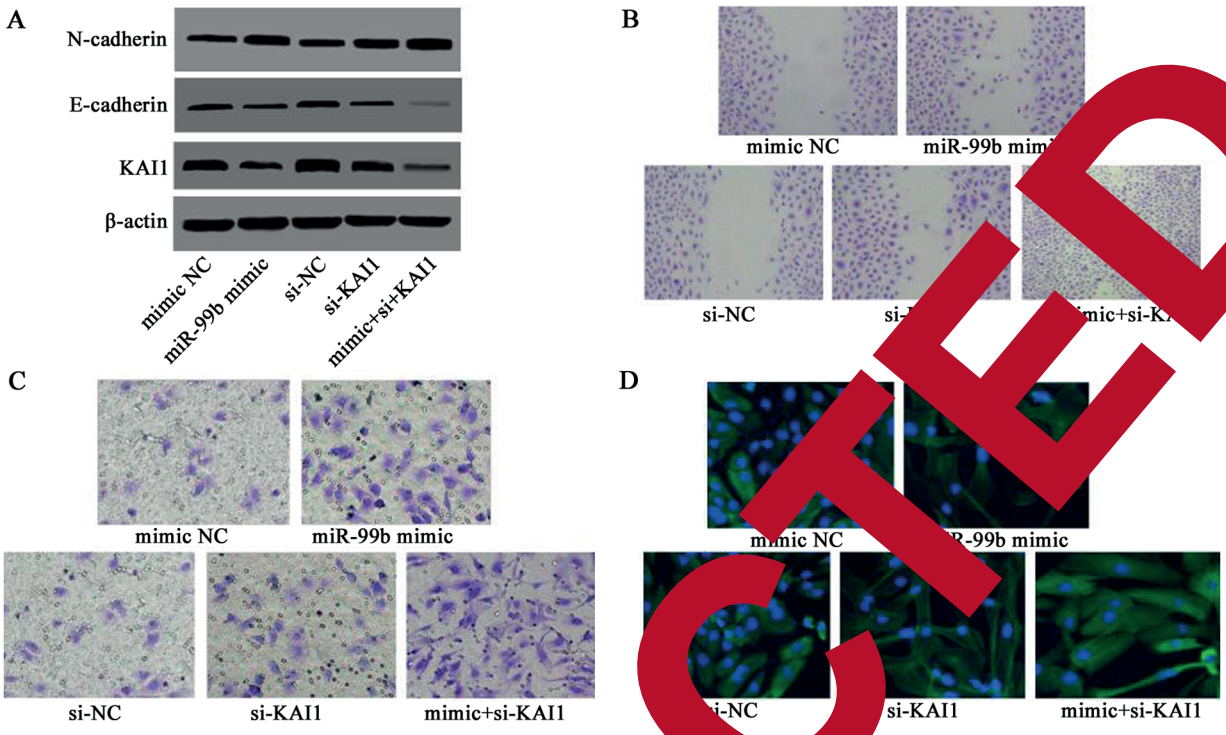


Figure 4. MiR-99b targeting inhibited KAI1 to promote cell migrati ***A,*** Western blot detection of protein expression in HepG2 cells. ***B,*** Wound healing assay ion of cell migrat Transwell assay detection of cell invasion. ***D,*** Immunofluorescence detection of Vimentin pr

Discussion

Primary HCC represents t malig-nant tumor in the clinic wi gh m dity up to 50/100,000, along with rate o and metastasis[8]. In mos ca patients died of rec ce a stasis even after surgery[9]. The e, the me m of HCC invasion and re and effecti lecular markers are of t ance for ear diagno-sis, metastasis, recurren diction, prevention, and progn [9-12]. The effec iRNAs on HCC invasio d metastasis have be concerned, and beco import rgets for HCC diagnosis and tre [13-16] NAs rticipate in several im-portan al pro s of HCC, such as cell proliferat apop , invasion[19], and metas-. Our g iously found[7] that miR-99b was erexpres n primary HCC tumor tissue, and l had a certain relationship with vas- suggesting that miR-99b may be ociated with HCC invasion and metastasis.

We showed that, compared with normal liver c iR-99b level was abnormally elevated in H C cells. It was highly expressed in MHCC97H cells, which have a strong invasive ability, indicating that miR-99b might be related to HCC cell invasive enhancement. Flow cytometry revealed that miR-99b overexpression inhibited HepG2 cell apoptosis, and changes cell cycle by up-regulation of S phase and G2/M phase. On the contrary, miR-99b inhibition induced higher HepG2 cell apoptosis and blocked cell cycle in G0/G1 phase. The results demonstrated that miR-99b has a promoting effect on HCC cell survival and proliferation. HepG2 cells wound healing ability and invasive ability markedly improved after miR-99b mimic transfection, indicating that miR-99b has promoting effect on HCC cell migration and invasion. It also explained the phenomenon that high level of miR-99b was found in MHCC97H cells. Sato et al[21] compared miRNA expression between tumor tissue and normal tissue in HCC patients and found that miR-99b elevation was closely associated with HCC metastasis and recurrence, which is consistent with our findings. This study used online prediction software microRNA.org to predict the potential target genes of miR-99b, and found that the 3'-UTR of KAI1 has a good complementary relationship with miR-99b. KAI1 locates on human chromosome 11p11.2,

is a member of transmembrane 4 superfamily (TM4SF)[22]. KAI1 is downregulated in a variety of tumors, such as pancreatic cancer[23], esophageal cancer[24], lung cancer[25], and colorectal cancer[26]. Some researches[27] confirmed that KAI1 presented the inhibitory role on tumor invasion and metastasis, while its downregulation was involved in promoting multiple tumors' progress, invasion, and metastasis. In the process of HCC occurrence and development, KAI1 was also validated in the inhibition of cancer invasion and metastasis[28,29]. It was demonstrated that KAI1 can reduce cell deformation by promoting the interaction between cells and extracellular matrix[30]. Dual-luciferase report assay revealed that miR-99b targeted on the 3'-UTR region of KAI1. MiR-99b mimic transfection largely decreased the level of KAI1 in HepG2 cells, confirming that KAI1 is the target gene of miR-99b. Moreover, KAI1 siRNA enhanced HepG2 cell migration and invasion, which was similar with miR-99b mimic effect. MiR-99b elevation and KAI1 knockdown simultaneously further promoted HepG2 cell migration and invasion. E-cadherin is one of the key me rs of cadherin family. It locates in the ju between cells and regulates cell adhesi by forming zipper structure[31]. E-cadherin re tion or dysfunction will promote tumor migration and invasion[31]. E-cad is consi ered to be an important mar epitheli- al feature, whereas N-cad n and mentin are two important make mesen cell characteristics. N-cadhe an up-regulation are r d to cell EMT, motility enhancer , and inva bility improvement. Co with the pre study[34] that miRNA as mor modulator in hepatocellular ca cinoma, esults showed that miR-99b inhibit KAI ssion and function, re e cell adhesion, an romote EMT to acce e HCC gration and invasion.

Co usions

M 99b pla n important role in HCC migra d invasion through suppressing KAI1 e function, along with the reduction cell adhesion and promotion of EMT.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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