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miR-199b exerts tumor suppressive functions in hepatocellular carcinoma by directly targeting JAG1

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Abstract. – OBJECTIVE: To investigate the regulating roles of miR-199b in hepatocellular carcinoma (HCC).

PATIENTS AND METHODS: The expression of miR-199b of 45 human HCC tissues and the corresponding para-cancerous tissue samples were detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). Western blot was employed to investigate the level of JAG1. Transwell assay was used to monitor the ability of cell migration and invasion. Cell proliferation was tested by CCK-8 assay and luciferase reporter assay was done to clarify whether JAG1 was a target of mir-199b.

RESULTS: miR-199b expression level was decreased in 45 paired tumor tissues in contrast with the corresponding para-carcinoma tissues. The expression level of miR-199b was closely associated with TNM stage, tumor size, and 5-year overall survival. Transwell assay result showed that miR-199b inhibited HCC cell migration and invasion. Cell counting kit-8 (CCK-8) results demonstrated that miR-199b could suppress HCC cell proliferation. Luciferase reporter assay suggested that Jagged1 (JAG1) was a direct target of miR-199b in HCC cells. miR-199b could negatively regulate JAG1 expression by targeting JAG1.

CONCLUSIONS: miR-199b exerted tumor suppressive functions in HCC by targeting JAG1, and it may be a potential target treatment for hepatocellular carcinoma.

Key Words:
miR-199b, Hepatocellular carcinoma, Migration, Invasion, Proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common prevalent malignancies in the world[1]. Since it's hard to diagnose the disease in the earlier stage, patients are reached the metastatic stage once diagnosed. Moreover, metastasis is the prominent reason of HCC patient death. Surgery and radiotherapy play crucial roles in the treatments of HCC[1, 2]. However, the 5-year survival rate of HCC remains poor. Hence, it is necessary to find the novel biomarkers for the diagnosis and therapy of HCC. MicroRNAs (miRNAs) can bind to the 3'-UTRs of target mRNA directly, negatively regulating the expression level of gene[3,4]. miRNAs function as oncogene or suppressor through targeting different genes. Increasing evidence indicated that miRNAs play critical roles in development and progression of HCC. For example, miR-370[5], miR-200a[6], miR-144[7] was down-regulated in HCC and miR-487a[8], miR-765[9], miR-761[10] was up-regulated in HCC. Previous studies suggested that miR-199b was aberrantly expressed in multiple cancers, including acute myeloid leukemia[11] and non-small cell lung cancer[12]. Additionally, miR-199b was involved in multiple biological processes, including proliferation, migration and growth. However, the underlying mechanism of miR-199b in HCC still needs to be further elucidated. Therefore, the current study aimed to demonstrate the potential role of miR-199b in HCC proliferation, invasion and migration and investigate the underlying mechanisms. Jagged1 (JAG1) is one of the ligand for Notch receptors that interacts with other Notch receptors[13]. It has been reported that JAG1 was associated with various tumors, such as breast carcinoma[14], non-small cell lung carcinoma (NSCLC)[15] and prostate carcinoma[16]. Additionally, a number of miRNAs were reported to regulate JAG1 to inhibit cell proliferation, growth and invasion, such as miR-186, miR-489, miR-34b[17-19]. However, the specific effects of JAG1 in HCC remain unknown.

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In this study, we detected the miR-199b expression levels in HCC tissues cells. Results indicated that miR-199b expression was decreased in HCC tissues as well as cells. miR-199b re-expression could suppress the proliferation, migration and invasion abilities of HCC cells. In addition, the results indicated that miR-199b could directly bind to the 3'-UTRs of JAG1, negatively regulating JAG1 expression in HCC. These results firstly demonstrated that miR-199b exerted suppressive functions and could be used as a new therapeutic marker of HCC.

Patients and Methods

Cell Culture

Human HCC cell line (Huh7) and immortal normal liver cell line HL7702 were purchased from Cell Resource Center, Chinese Academy of Science (Shanghai, China) and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) with 5% CO_2 at 37°C.

Tissues Samples

A total of 45 human HCC tissues and the corresponding para-cancerous tissue samples were collected from 2013 to 2015 at the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All samples were stored at –80°C. The clinicopathological characteristics were shown in Table I. Written informed consents were offered by all patients. This work was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China).

Cell Transfection, miRNAs

Human miRNA-199b mimics, miRNA-199b inhibitor and JAG1 siRNA as well as the corresponding control were obtained from RiBoBio (Guangzhou, China). Huh7 cells were transfected with miRNA-199b mimics or inhibitor by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Table I. miR-199b expression and clinicopathological features in 45 paired hepatocellular carcinoma tissues.

Clinicopathological features	miR-199b expression[a]		*p*-value
	Up-regulated (n = 35)	Down-regulated (n = 10)	
Age (years)			0.4764
≤ 60	20	4	
> 60	15	6	
Gender			1.0000
Male	16	5	
Female	19	5	
Tumor (cm)			0.1513
< 5	21	3	
≥ 5	14	7	
Local invasion			0.0079
T1+T2	10	8	
T3+T4	25	2	
Distant metastasis			0.0115
M0	15	8	
M1	30	2	
TNM stage			0.0211
I+II	9	7	
III+IV	26	3	
BCLC stage			0.0041
BCLC A	22	1	
BCLC B	13	9	
Cirrhosis			0.0653
Yes	17	6	
No	18	4	

TNM: tumor-node-metastasis; BCLC stage: Barcelona clinic liver cancer. [a]The mean expression level of miR-199b was used as the cutoff.

HCC cells were transfected with plasmids using X-treme GENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). The sequences for miR-199b mimics and inhibitor are as follows: miR-199b mimics: 5'-CCCA GUGUUUAGAC-UAUCUGUUC-3'; miR-199b inhibitor: 5'-AA-CAGGTAGTCTGAACACT-3'; negative control: 5'-TAACACGTCTATACGCCCA-3'; the follow-ing siRNA sequences were used: JAG1 siRNA: 5'-GGCCAAGCCUUGUGUAAAUTT-3'; con-trol siRNA sequence: 5'-CAGUACUUUUGU-GUAGUACAA-3'

RNA Extraction, Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated from HCC cells and tissues by TRIzol regent (Invitrogen, Carls-bad, CA, USA) in accordance with the manufac-turer's recommendation. Complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase regent kit (Thermo Fisher, Waltham, MA, USA). qRT-PCR was conducted by SYBR *Premix Ex* Taq™ (TaKaRa, Otsu, Shiga, Japan) on the system of CFX96 Real-time PCR Detec-tion (Bio-Rad, Hercules, CA, USA). The primers of miR-199b, U6 were synthesized from RiBoBio (Guangzhou, China). The primers of JAG1, glyc-eraldehyde 3-phosphate dehydrogenase (GAP-DH) were synthesized from Biosune (Shanghai, China). The expressions of miR-199b and JAG1 were respectively normalized to U6 and GAP-DH expression. The $2^{-\Delta\Delta Ct}$ was used to evaluate the relative expression of Gene. The above reac-tions were performed in three times. The prim-ers sequences of GENE were as follows: GAPDH forward sequence was 5'-AAGGTGAAGGTCG-GAGTCAAC-3' and GAPDH reverse sequence was 5'-GGGGTCATTGATGGCAACAATA-3'; JAG1 forward sequence was 5'-CTCATCAGC-CGTGTCTCAAC-3' and JAG1 reverse sequence was 5'-GGCACACACACTTAAATCCG-3'.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was used to extract the total proteins from Huh7 cells in line with the manufacturer's protocols. The protein concentration was analyzed with bicin-choninic acid (BCA) protein Assay Kit (Beyo-time, Shanghai, China). After being separated with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) which was blocked with 5% skim milk in Tris-buffered saline and Tween 20 (TBST) at room temperature for 2 h and then probed with specific anti JAG1 antibody (Abcam, Cambridge, MA, USA). Subsequently, the PVDF membrane was incubated by the proper secondary antibody (Abcam, Cambridge, MA, USA) and the chemi-luminescence (ECL, Millipore, Billerica, MA, USA) was used to visualize the proteins. GAPDH (Abcam, Cambridge, MA, USA) was used as the internal control.

Transwell Assays

Transwell chambers (Corning, NY, USA) with or without Matrigel (Clontech, Mountain View, CA, USA) were used to detect the HCC cell mi-gration and invasion abilities. Huh7 cell transfect-ed with miRNA or JAG1 siRNA were suspended in 150 μL RPMI-1640 and then were seeded into the top chamber. RPMI-1640 medium including 20% fetal bovine serum (FBS) was added into the bottom chamber. Being incubated at 37°C for 24 hours, the cells on the upper chamber were removed by cotton swabs while 100% methanol and 0.2% crystal violet were respectively used to fix and stain the cells stayed on the bottom cham-ber. The inverted microscope (Olympus, Tokyo, Japan) was used to count the number of migration or invasion cells.

Cell Proliferation

Cell counting kit 8 (CCK-8, Beyotime, Shang-hai, China) assay was performed to detect the proliferation ability of HCC cell. Huh7 cells transfected with miRNA or JAG1 siRNA were suspended and seeded into 96-well plate. After being cultured for 24 hours, CCK-8 solution was added into each well and the Huh7 cells were cul-tured at 37°C for 2 hours. Then, OD450 was de-tected by microplate reader (BioTeke, Winooski, VT, USA). The proliferation was measured at 0 h, 24 h, 48 h, 72 h respectively, after transfections.

Luciferase Assay

PCR was conducted to amplify the JAG1 3'-UTR. The amplified 3'-URRs were cloned into the pGL3-basic vector (Promega, Madison, WI, USA). The complementary sequences of miR-199b in JAG1 3'-UTR were mutated by Site-Di-rected Mutagenesis Kit (TaKaRa, Otsu, Shiga, Japan). HCC cell lines were co-transfected with the miR-199b mimics and JAG1-3'UTR-WT or JAG1-3'UTR-Mut plasmids using Lipofectamine

2000. 48 hours after transfection, the dual-luciferase reporter assay was conducted to analyze the luciferase activities. The results were normalized to the luciferase of renilla.

Statistical Analysis

All the above assays were performed at least three times. Statistical Product and Service Solutions (SPSS) Version 17.0 (SPSS Inc., Chicago, IL, USA) and the Student's *t*-test or one-way ANOVA and Scheffe's post-hoc test were jointly used for statistical analysis. Kaplan-Meier method and log-rank test were applied to estimate the survival rates and compare the survival curves, respectively. The data was indicated as means ± SD and was considered statistically significant when $p<0.05$.

Results

Expression of miR-199b was Decreased in HCC Cells and Tissue Samples

The miR-199b expression was measured in 45-paired HCC tissue samples as well as the matched adjacent normal tissues using qRT-PCR. It was indicated that the miR-199b expression was decreased significantly in HCC tissue samples compared to noncancerous tissues (Figure 1A). The miR-199b expression in HCC cells was also measured using qRT-PCR. The qRT-PCR result demonstrated that miR-199b expression level in HCC cells was significantly reduced in contrast with the normal liver cells HL-7702 (Figure 1B). The correlation between miR-199b expression and clinicopathological features of HCC patients was also analyzed. The HCC patients were divided into miR-199b up-regulated group and down-regulated group with the mean miR-199b expression level being regarded as the cutoff. The results showed that miR-199b expression level was relevant to TNM stage and tumor size while other clinicopathological features such as cirrhosis were not influenced significantly by miR-199b (Table I). In addition, the miR-199b expression was found to have a relevance to the patient's survival. The overall survival rate of patients with HCC expressed in low miR-199b was lower than those with higher miR-199b expression (Figure 1C). Furthermore, spearman correlation analysis revealed that JAG1 mRNA levels were negatively correlated with miR-199b levels in HCC tissues (Figure 1D).

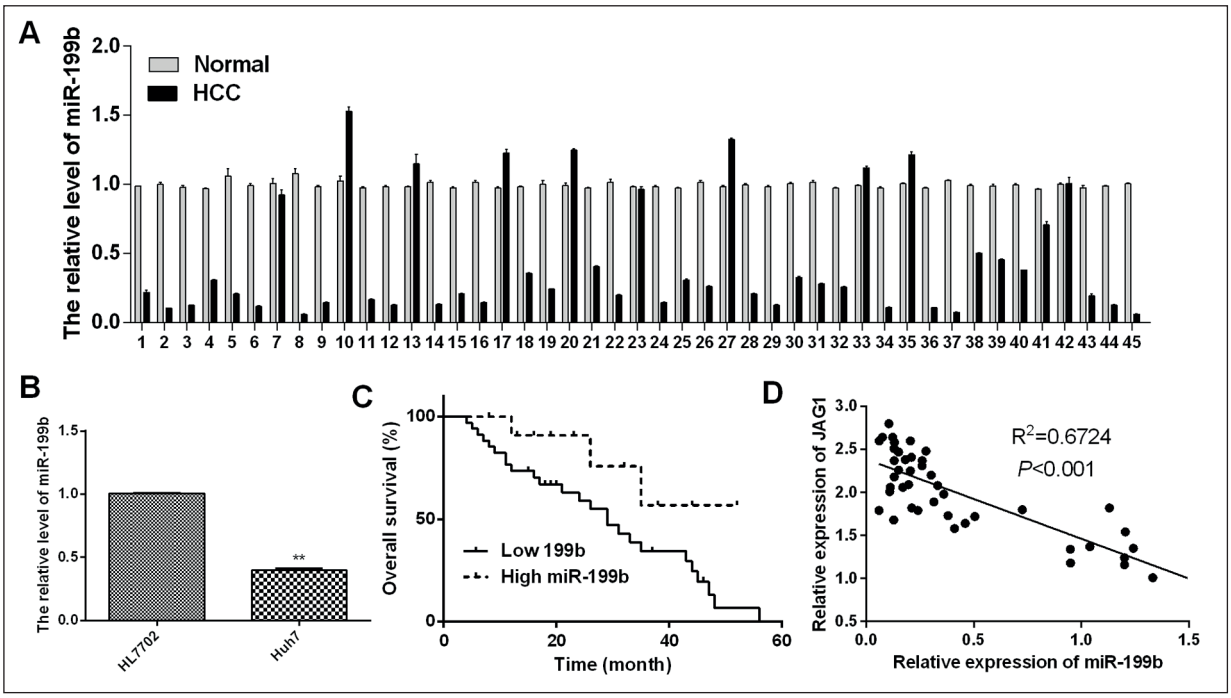

Figure 1. miR-199b expression was reduced in HCC tissues. ***A,*** miR-199b expression level was detected by qRT-PCR in HCC tissues ***B,*** miR-199b expression levels in HCC cells and normal liver cell HL7702 were measured by qRT-PCR. ***C,*** miR-199b expressions were related to overall survival in HCC. ***D,*** An inverse correlation between the levels of JAG1 mRNA and miR-199b expression was observed in HCC tissues.

miR-199b Inhibits Cell Migration and Invasion, Proliferation in HCC

From the clinicopathological analysis, we knew that the expression level of miR-199b was relevant to TNM stage. We suggested that miR-199b played critical roles in HCC progression. To better understand the function of miR-199b in HCC, Huh7 cells were transfected with miR-199b mimics or inhibitor (Figure 2A). CCK-8 assays indicated that miR-199b mimics could reduce Huh7cell proliferation significantly compared with the control group (Figure 2B). However, miR-199b inhibitor increased Huh7 cell proliferation (Figure 2B). Next, we detected the roles of miR-199b in regulating HCC cell invasion and migration abilities. Transwell assay results showed that miR-199b overexpression decreased migration and invasion abilities of Huh7 cells. However, miR-199b inhibitor increased migration and invasion abilities of Huh7 cells (Figure 2C and 2D).

miR-199b Negatively Regulates JAG1 by Targeting the 3'-UTR of JAG1

To further understand the mechanism of miR-199b in inhibiting HCC more clearly, TargetScan and Mirnda were used to predict the targets of miR-199b. The results indicated JAG1 was a candidate gene of miR-199b and one binding site from 135 to 141 of JAG1 3'-UTR was found. The complementary binding sites were shown in Figure 3A. To determine the predicted results, the luciferase reporter vector contains the JAG1 3'-UTR was constructed. Then, the luciferase reporter assay was conducted in HCC cells. The results showed that miR-199b remarkably reduced the JAG1 3'-UTR-WT luciferase activity. However, miR-199b had no significant influence on the JAG1 3'-UTR-Mut luciferase activity (Figure 3B). Furthermore, we also detected the mRNA expression as well as the protein expression levels of JAG1 in HCC cell lines transfected with miR-

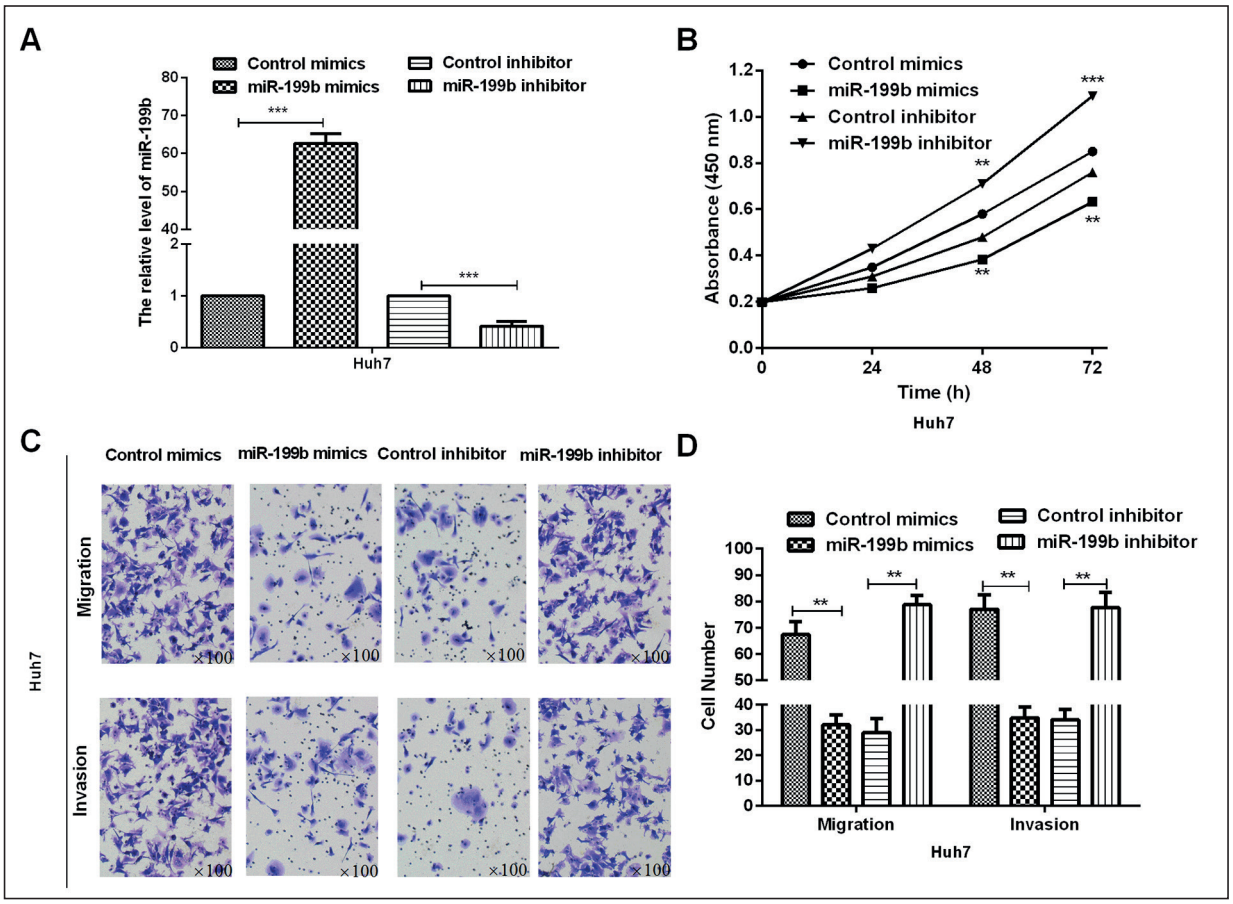

Figure 2. miR-199b prevents cell migration, invasion and proliferation in HCC. ***A,*** Transfection efficiency of the transfected HCC cell lines. ***B,*** CCK-8 assay in the transfected HCC cell lines. ***C,*** Transwell assay in the transfected Huh7 cell lines. ***D,*** The number of invasive and migratory Huh7 cells with different transfections.

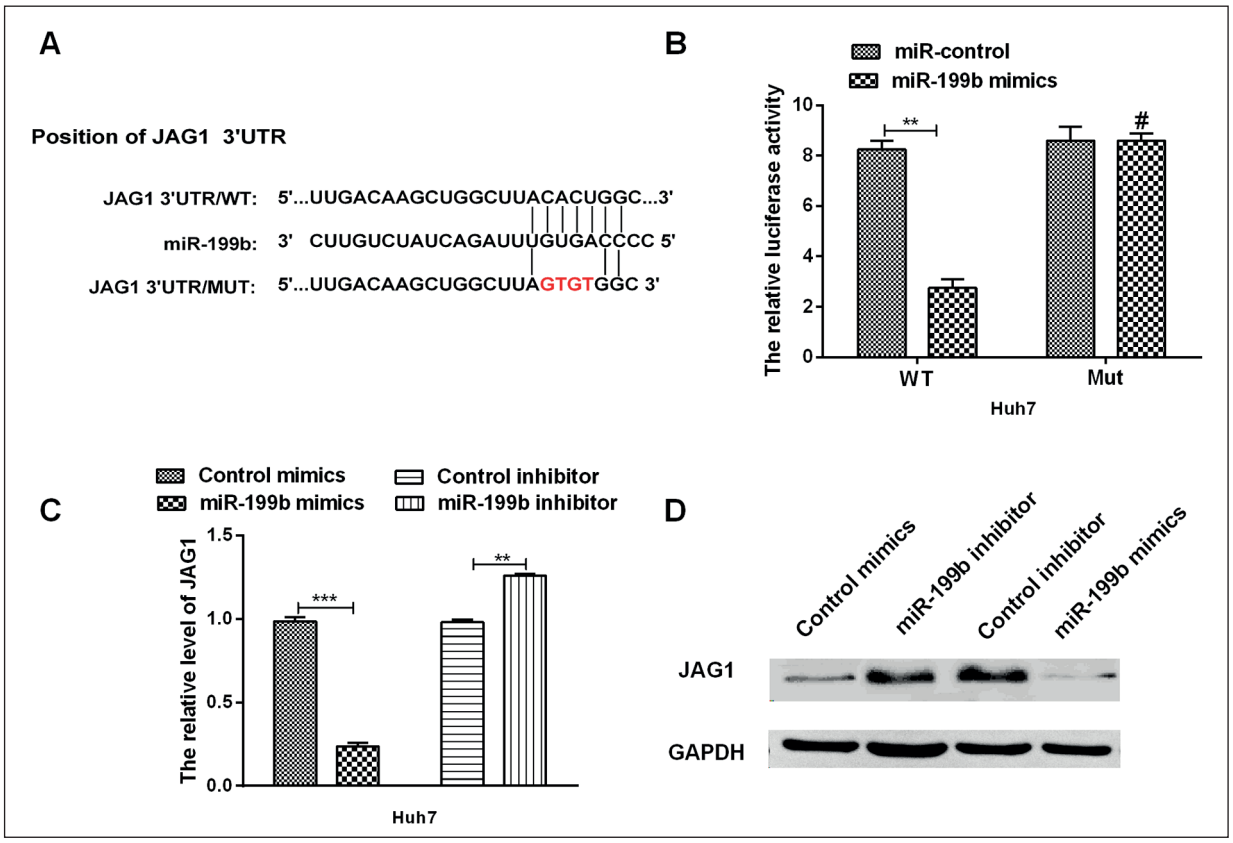

Figure 3. miR-199b negatively regulated JAG1 by targeting its 3'-UTR. ***A,*** The binding sequence of miR-199b in the JAG1 3'-UTR. WT: wild type; Mut: Mutant. ***B,*** Luciferase abilities of Huh7 cells, which were co-transfected with WT or Mut luciferase reporter and miR-199b mimics. ***C,*** The expression of JAG1 on mRNA level in transfected Huh7 cell lines was measured by qRT-PCR. ***D,*** The expression of JAG1 on protein level was measured by Western blot in the transfected HCC cell lines.

199b mimics or inhibitor. These results indicated that re-expression of miR-199b in HCC cell lines reduced JAG1 expressions both on mRNA and protein levels (Figure 3C and 3D). Down-expression of miR-199b by specific miR-199b inhibitor increased JAG1 mRNA and protein expression levels in HCC cell lines (Figure 3C and 3D). These results suggested that miR-199b could bind to the JAG1 3'-UTR directly and negatively regulate JAG1 expression in HCC cells.

Knockdown of JAG1 Markedly Reversed miR-199b-Medicated Inhibition of HCC Cell Activities

Following the siRNA-mediated knockdown of JAG1, qRT-PCR analysis confirmed that JAG1 siRNA was sufficient to inhibit JAG1 expression in Huh7 cells (Figure 4A). Subsequently, CCK-8 assays were used to determine the capacities of Huh7 cells cotransfected with miR-199b inhibitor and JAG1 siRNA or control siRNA. Results showed that cell proliferation was significantly decreased in Huh7 cells transfected with miR-199b inhibitor and JAG1 siRNA compared with the cells treated with miR-199b inhibitor and control siRNA (Figure 4B). Subsequently, transwell assays were performed to detect the invasion and migration abilities of Huh7 cells with different transfections. Results indicated that the deletion of JAG1 markedly reversed miR-199b inhibitor-medicated promotion of HCC cell invasion and migration (Figure 4C and 4D).

Discussion

Recently, the dysregulation of miRNAs has been revealed in several cancers, including HCC[20]. Increasing evidence indicated that miRNAs was considered as biomarkers and therapeutic target in various cancers[21-23]. miR-199b[11] is located on chromosome 9q34.11. Previous studies showed

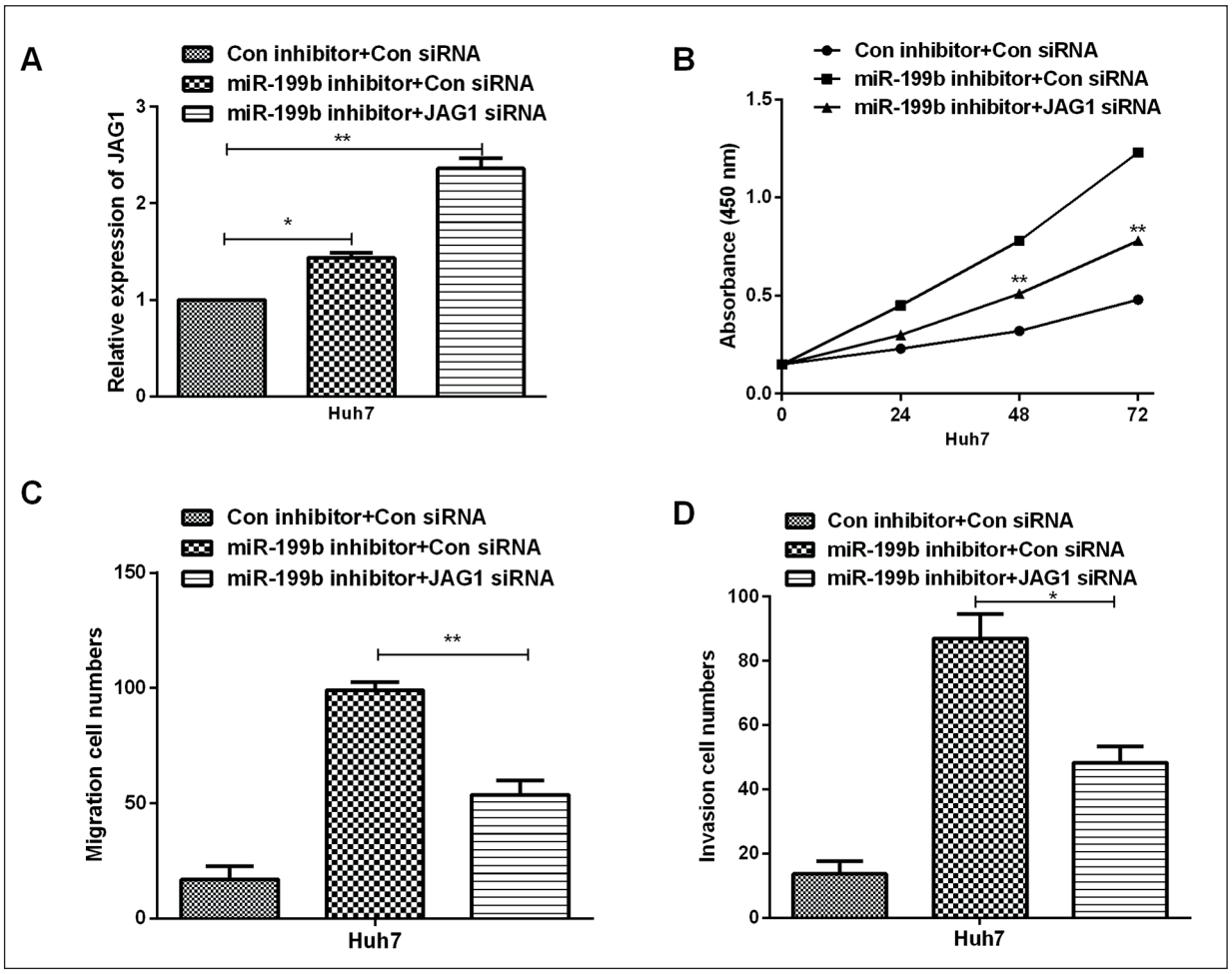

Figure 4. Knockdown of JAG1 markedly reversed miR-199b-medicated inhibition of HCC cell activities. ***A,*** The JAG1 mRNA expression level was detected by qRT-PCR in HCC cells co-transfected with JAG1 siRNA and miR-199b inhibitor. ***B,*** CCK-8 assay in HCC cells co-transfected with JAG1 siRNA and miR-199b inhibitor. ***C, D,*** Results of transwell assays in HCC cells co-transfected with JAG1 siRNA and miR-199b inhibitor. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $^{\#}p > 0.05$.

that miR-199b was down-expressed in different kinds of cancers, including prostate carcinoma[24], colorectal carcinoma[25], breast carcinoma[26]. Additionally, miR-199b was related to the tumor size and TNM stage[27] and involved in multiple biological processes, including proliferation, migration and growth[28-30]. However, the biological effects of miR-199b were not elusive in HCC. Here, we measured the miR-199b expression levels in HCC tissues and cells. The results of qRT-PCR indicated that miR-199b was reduced in HCC tissues and cells in contrast with the corresponding controls. Moreover, miR-199b was related to the TNM stage. This revealed that miR-199b may be related to the development process of HCC. Next, we verified the biological function of miR-199b in HCC cells. As illustrated in Figure 2, ectopic miR-199b expression inhibited the proliferation, migration as well as invasion abilities of HCC cells, suggesting that miR-199b functioned as an anti-tumor miRNA in HCC. The finding of our research was consistent to the work of Zhan et al[31] who demonstrated that miR-199a/b-5p inhibited hepatocellular carcinoma progression by post-transcriptionally suppressing ROCK1. In their study, ROCK1 was identified as a direct target of miR-199a/b-5p and implicated in the suppressive effects mediated by miR-199a/b-5p in HCC progression while in our research, we demonstrated that JAG1 was a novel target gene of miR-199b and regulated the functions of miR-199b in HCC development. Moreover, previous invetigations showed that JAG1 played crucial roles in the development and differentiation of various cancers. According to Reedijk et al[14] high-level expression of JAG1 is associated with poor overall survival of breast

cancer patients. Chang et al[15] found that JAG1 is associated with poor survival through inducing metastasis in lung cancer. Qiu et al[32] reported that high Jagged1 expression is associated with poor outcome in primary glioblastoma. In current research, JAG1 was identified as a target of miR-199b in HCC cells. Luciferase assay results suggested that miR-199b bound to JAG1 directly through targeting the JAG1 3'-UTR. Additionally, qRT-PCR and Western blot results indicated that miR-199b negatively regulated JAG1 expressions not only on mRNA level, but also on protein level in HCC cell lines. These results indicated that miR-199b exerts tumor suppressive functions in hepatocellular cancer by targeting JAG1 directly. There were several studies supported our findings. For example, Qu et al[33] indicated that miR-199b prevented ligamentum flavum cells from osteogenetic differentiation by targeting JAG1.

Conclusions

We found that miR-199b was down-regulated not only in HCC tissues but also in HCC cells and its reduced expression were related to the poor clinicopathological features of HCC patients and it can be used as a target molecule for the treatment of hepatocellular carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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