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LncRNA HAGLR exacerbates hepatocellular carcinoma through negatively regulating miR-6785-5p

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Abstract. – OBJECTIVE: The purpose of this study was to explore the role of long non-coding RNA (lncRNA) HAGLR in exacerbating the development of hepatocellular carcinoma (HCC) by targeting microRNA-6785-5p (miR-6785-5p).

PATIENTS AND METHODS: HAGLR levels in 46 HCC tissues and paracancerous tissues were detected. The relationship between HAGLR level and clinical features of HCC patients was analyzed. After knockdown of HAGLR, proliferative, and metastatic potential changes in Bel-7402 and Hub7 cells were assessed. Thereafter, the interaction between HAGLR and miR-6785-5p, as well as the involvement of miR-6785-5p in HAGLR-regulated HCC phenotypes were finally determined.

RESULTS: It was found that HAGLR level was higher in HCC tissues than paracancerous ones and correlated with rates of lymphatic metastasis and distant metastasis but not with age, gender, and tumor staging in HCC patients. Survival analysis uncovered that HAGLR level was negatively linked to overall survival in HCC. After knockdown of HAGLR, proliferative, and metastatic potentials in Bel-7402 and Hub7 cells were attenuated. MiR-6785-5p was proven as the target gene binding to HAGLR. It was lowly expressed in HCC species, and negatively correlated with HAGLR level. Moreover, rescue experiments demonstrated that miR-6785-5p was responsible for HAGLR-regulated HCC phenotypes.

CONCLUSIONS: LncRNA HAGLR stimulates proliferative and metastatic potentials in HCC via negatively regulating miR-6785-5p level, thus exacerbating the development of HCC.

Key Words:
LncRNA HAGLR, MiR-6785-5p, HCC, Malignant development.

Introduction

Hepatocellular carcinoma (HCC) is featured by insidious onset, difficulties in early stage detection and high mortality[1,2]. About 600,000 people die of HCC every year globally, and the morbidity and mortality of HCC rank fifth and second, respectively[2,3]. In China, the number of HCC patients accounts for 55% of global HCC cases. The incidence of HCC is much higher in Southeast coastal areas of China[4]. More males are affected by HCC than females[5]. Multiple factors are responsible for the development of HCC, including Hepatitis B virus, alcohol-induced cirrhosis, chemical carcinogens (i.e., flavonoids), and environmental factors[6,7]. Since evident clinical symptoms and signs in the early stage of HCC are lacked, most of HCC patients are initially diagnosed in the advanced stage and thus miss the optimal surgical treatment window[7,8]. Therefore, only palliative treatments, such as TACE, radiotherapy, chemotherapy, and traditional Chinese medicine, are available to these HCC patients. However, these treatments are not effective and can even cause a series of adverse effects[9,10]. It is extremely important to find new therapeutic targets of HCC[10,11].

Genomics studies have shown that only about 1% of genes can be transcribed into protein-coding RNAs. Most of genes are transcribed into non-coding RNAs[11,12]. Among them, long non-coding RNA (lncRNA) is a member of non-coding RNAs with longer than 200 nucleotides. They used to be considered as byproducts of transcription without biological functions[13,14]. Later, their vital functions in life activities have been identified[15]. It is currently suggested that lncRNAs are promising candidates in disease diagnosis and treatment[13,16]. Through literature review, lncRNAs are extensively involved in almost every aspect of tumor development[17,18].

This paper aims to illustrate the role of lncRNA HAGLR in the malignant progression

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of HCC and the underlying mechanism. The findings in this paper may provide a new direction in effectively prevention and treatment of HCC.

Patients and Methods

HCC Species

A total of 46 paired HCC tissues, including tumor tissues and paracancerous ones (3 cm away from the tumor edger) were surgically resected from HCC patients. Their clinical data were recorded. Tumor staging was conducted based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by Ethics Committee of China-Japan Union Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study.

Cell Culture

HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and normal hepatocytes (LO2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted using 1×tyrpsin + ethylenediaminetetraacetic acid (EDTA).

Transfection

Cells were cultured to 30-40% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the cells were collected for the following use (Promega, Madison, WI, USA).

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration and Invasion Assay

A total of 200 μL of suspension (5×10^5 cells/mL) was inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, the bottom cells were reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. Next, the migratory cells were counted in 10 random fields per sample (magnification 200×). Invasion assay was similarly conducted in transwell chamber pre-coated with Matrigel.

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

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences used are shown below: HAGLR forward: 5'-GGGCTGGTACAGACTAGGGA-3' and reverse: 5'-TAAGCAGGTCAGAAGGGCG-3'. GAPDH forward: 5'-AACAGCCTCAAGATCATCAGCAA-3' and reverse: 5'-GACTGTGGTCATGAGTCCTTCCA-3'. MiR-6785-5p forward: 5'-ACACTCCAGCTGGGTGGGAGGGCGTGGATG-3' and reverse: 5'-TGGTGTCGTGGAGTCGTGGGAGGGCGTGGATG-3'. U6 forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Luciferase Assay

Cells inoculated in 24-well plates were co-transfected with HAGLR-WT/HAGLR-MUT and miR-6785-5p mimics/miR-6785-5p inhibitor, followed by lysis for determining relative Luciferase activity 48 h later.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The differences between the groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between HAGLR level and clinical features of HCC patients. Pearson correlation test was applied for evaluating the relationship between expression levels of HAGLR and miR-

6785-5p in HCC species. Kaplan-Meier method with the log-rank test was used for the survival analysis. $p<0.05$ was considered as statistically significant.

Results

HAGLR Was Upregulated In HCC

The most differentially expressed lncRNA HAGLR between HCC tissues and normal tissues through bioinformatics prediction was selected to be analyzed in this paper. The results showed that HAGLR was highly expressed in collected HCC tissues, suggesting the potential oncogenic role in HCC (Figure 1A). Compared with HCC patients without metastases, HAGLR level was higher in those suffering from lymphatic metastasis or distant metastasis (Figure 1B). Moreover, clinical features of included HCC patients were analyzed. The data showed that HAGLR level was positively linked to rates of lymphatic metastasis ($p=0.032$) and distant metastasis ($p=0.021$), while it was unrelated to age, gender and tumor staging in HCC patients ($p>0.05$) (Table I). Besides, survival analysis illustrated that high level of HAGLR was unfavorable to the prognosis in HCC (Figure 1C). As expected, HAGLR was identically upregulated in HCC cell lines (Figure 1D).

HAGLR Stimulated Proliferative and Metastatic Potentials In HCC

sh-HAGLR was constructed, and its transfection efficacy in Bel-7402 and Hub7 cells was tested (Figure 2A). CCK-8 assay results showed decreased viability in HCC cells transfected with sh-HAGLR (Figure 2B). In addition, migratory and invasive potentials in HCC cells were suppressed after transfection of sh-HAGLR (Figure 2C).

Interaction Between HAGLR and MiR-6785-5p

In Bel-7402 and Hub7 cells transfected with sh-HAGLR, miR-6785-5p was markedly upregulated (Figure 3A). Contrary to HAGLR, miR-6785-5p was lowly expressed in HCC tissues (Figure 3B), and its level was negatively linked to rates of lymphatic metastasis and distant metastasis in HCC (Table I). Similarly, HAGLR level was negatively regulated by miR-6785-5p in HCC cells

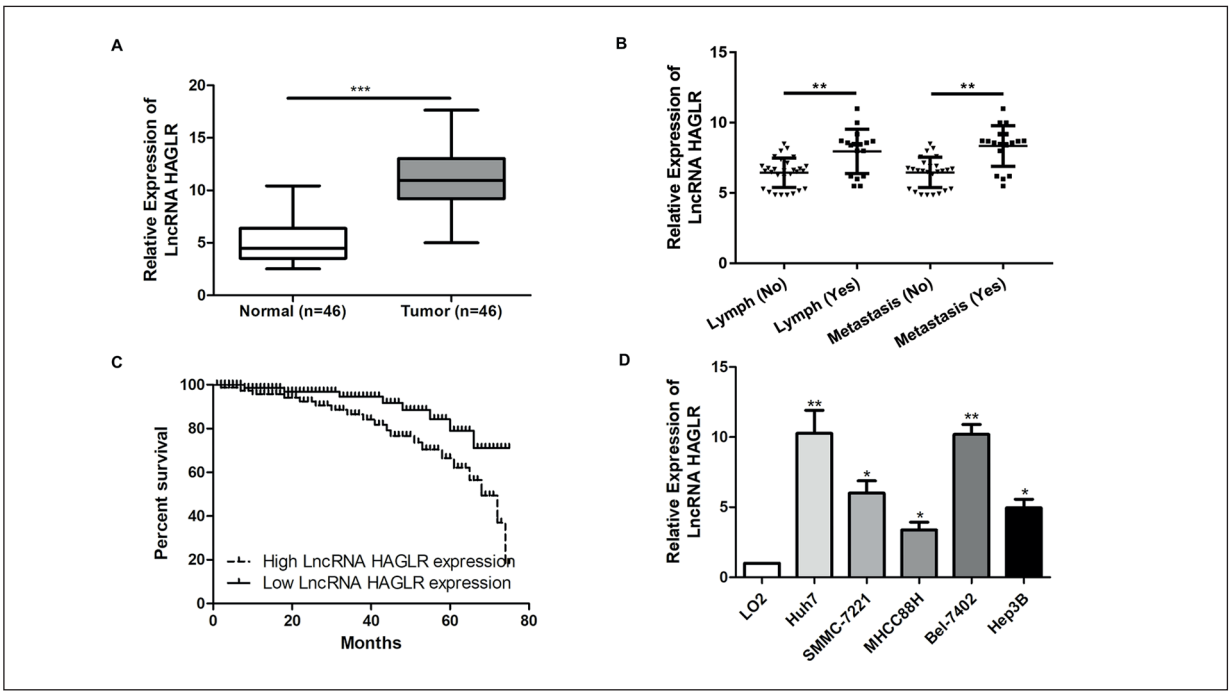

Figure 1. HAGLR is upregulated in HCC. **A**, HAGLR levels in HCC tissues (n=46) and paracancerous tissues (n=46). **B**, HAGLR levels in HCC patients either with lymphatic, distant metastasis or not. **C**, Kaplan-Meier curves in HCC patients with a high or low level of HAGLR. **D**, HAGLR levels in LO2 cell line and HCC cell lines. Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of LncRNA HAGLR and miR-6785-5p expression with clinicopathologic characteristics of hepatocelluar carcinoma.

Parameters	No. of cases	HAGLR expression		*p*-value	MiR-6785-5p expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.369			0.446
< 60	19	11	8		7	12	
≥ 60	27	12	15		13	14	
Gender				0.376			0.552
Male	23	13	10		9	14	
Female	23	10	13		11	12	
T stage				0.134			0.293
T1-T2	27	16	11		10	17	
T3-T4	19	7	12		10	9	
Lymph node metastasis				0.032			0.026
No	29	18	11		9	20	
Yes	17	5	12		11	6	
Distance metastasis				0.021			0.024
No	27	19	10		8	19	
Yes	19	6	13		12	7	

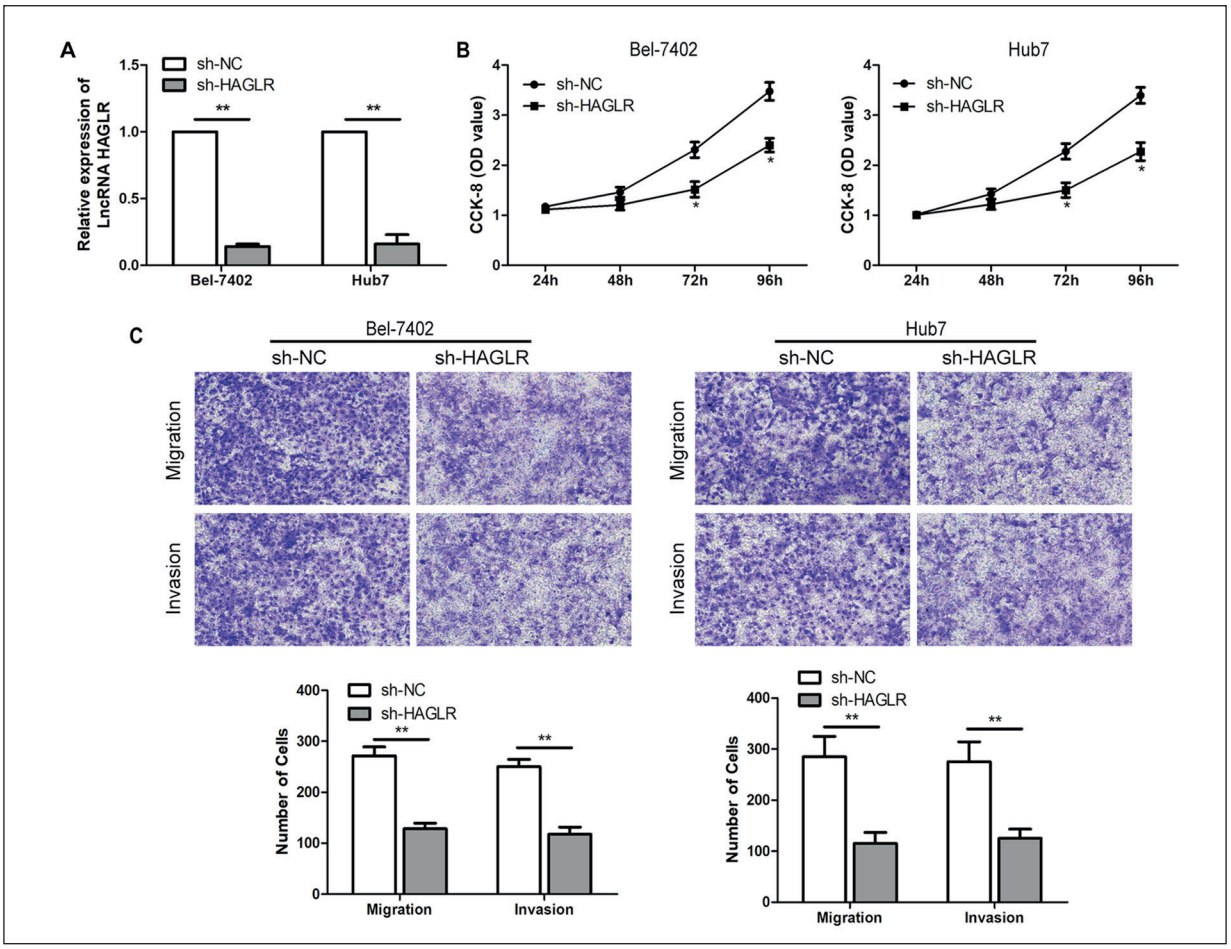

Figure 2. HAGLR stimulates proliferative and metastatic potentials in HCC. **A,** Transfection efficacy of sh-HAGLR in Bel-7402 and Hub7 cells. **B,** Viability in Bel-7402 and Hub7 cells transfected with sh-NC or sh-HAGLR. **C,** Migration and invasion in Bel-7402 and Hub7 cells transfected with sh-NC or sh-HAGLR (magnification: 40×). Data are expressed as mean ± SD. **p*<0.05, ***p*<0.01.

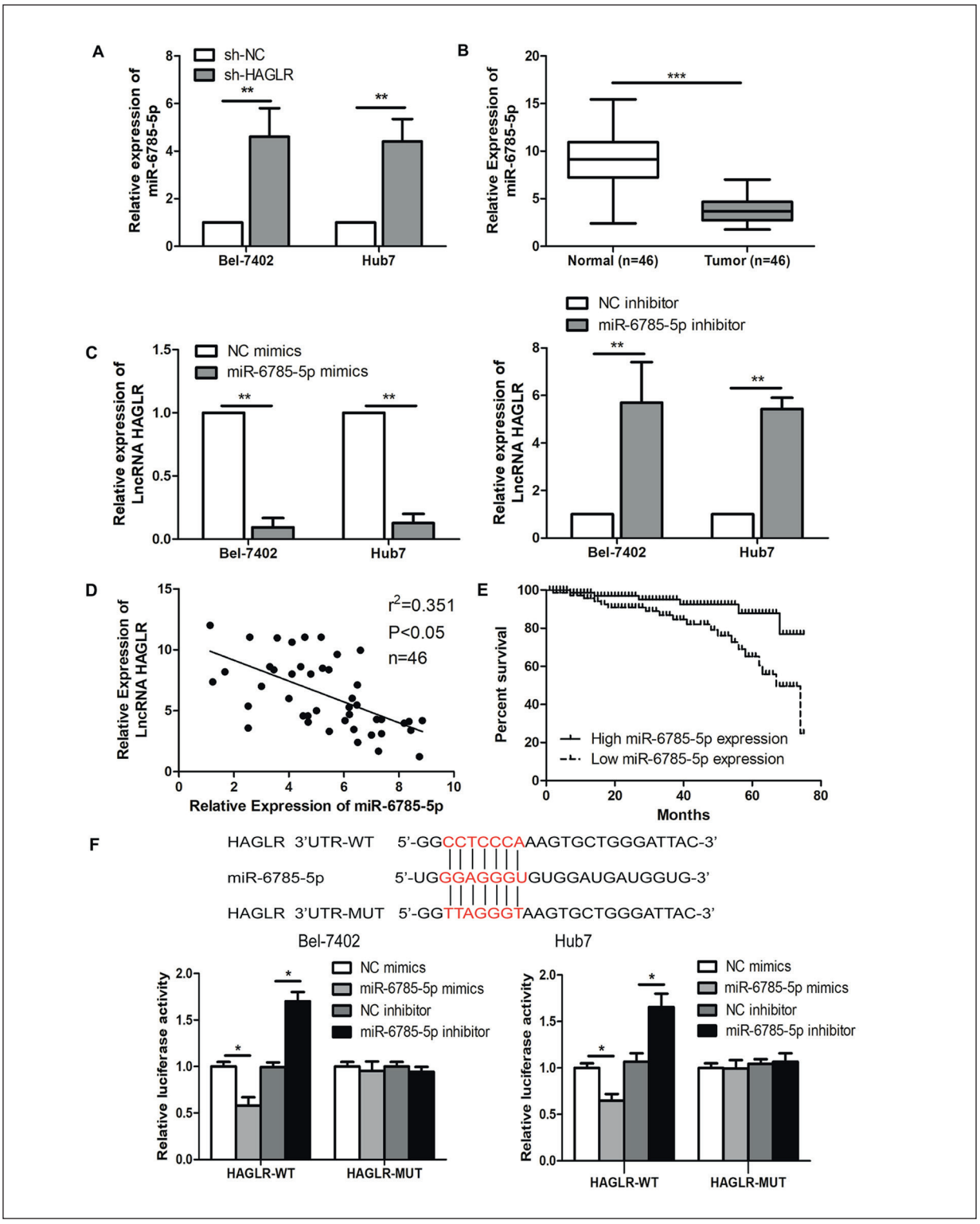

Figure 3. Interaction between HAGLR and miR-6785-5p. **A,** MiR-6785-5p level in Bel-7402 and Hub7 cells transfected with sh-NC or sh-HAGLR. **B,** MiR-6785-5p levels in HCC tissues (n=46) and paracancerous tissues (n=46). **C,** HAGLR level in Bel-7402 and Hub7 cells regulated by miR-6785-5p. **D,** A negative correlation between expression levels of HAGLR and miR-6785-5p in HCC species. **E,** Kaplan-Meier curves in HCC patients with a high or low level of miR-6785-5p. **F,** Binding sequences in the 3'UTR of HAGLR and miR-6785-5p (upper). Luciferase activity in co-transfected Bel-7402 and Hub7 cells. Data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

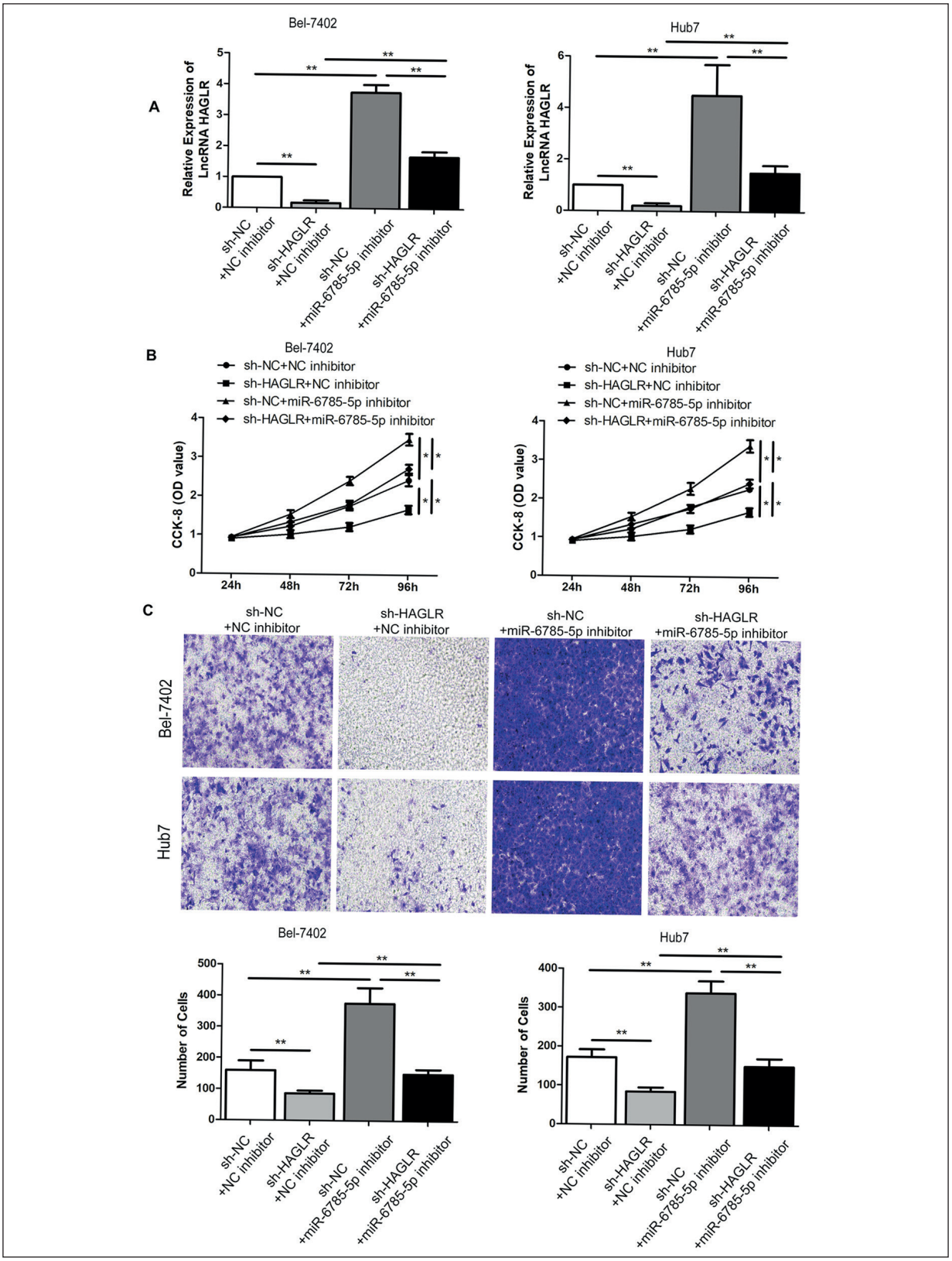

Figure 4. Knockdown of miR-6785-5p reverses the inhibitory effects of silenced HAGLR on the malignant development of HCC. **A,** HAGLR level in co-transfected Bel-7402 and Hub7 cells. **B,** Viability in co-transfected Bel-7402 and Hub7 cells. **C,** Migration and invasion in co-transfected Bel-7402 and Hub7 cells (magnification: 40×). Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$.

as well (Figure 3C). A negative correlation was identified between expression levels of HAGLR and miR-6785-5p in HCC species (Figure 3D). Kaplan-Meier curves revealed that a low level of miR-6785-5p was unfavorable to overall survival in HCC patients (Figure 3E). Bioinformatics analysis showed potential binding sequences in the 3'UTR of HAGLR and miR-6785-5p. Subsequently, the Luciferase assay demonstrated that the overexpression of miR-6785-5p decreased Luciferase activity in wild-type HAGLR, and knockdown of miR-6785-5p yielded the opposite trend (Figure 3F).

Knockdown of MiR-6785-5p Reversed the Inhibitory Effects of Silenced HAGLR on the Malignant Development of HCC

Rescue experiments were conducted to explore the involvement of miR-6785-5p in the malignant development of HCC. A lower level of HAGLR was observed in the cells co-transfected with sh-HAGLR and miR-6785-5p inhibitor compared with those transfected with only sh-HAGLR (Figure 4A). Notably, the suppressed viability (Figure 4B) and metastatic potential (Figure 4C) in HCC cells with HAGLR knockdown were partially reversed by co-transfection of miR-6785-5p inhibitor.

Discussion

Primary liver cancer is highly prevalent in the world, and over 90% cases are pathologically diagnosed with HCC[1-3]. Recurrence is the most pronounced feature of HCC, leading to the high 5-year survival rate of postoperative HCC up to 50%[7,8]. Therefore, clarifying the mechanisms of HCC recurrence contributes to improving its prognosis.

The role of lncRNAs in tumor metastasis has been highlighted in recent years[7,9]. LncRNAs are considered as novel tumor biomarkers and therapeutic targets, displaying a promising application in clinical treatment of tumor diseases[17,18]. Through analyses on online bioinformatics websites, HAGLR was identified to be differentially expressed in HCC patients. Subsequently, the findings of this study further validated that HAGLR was upregulated in HCC species and cell lines. Its level was linked to metastasis and poor prognosis in HCC patients, suggesting that HAGLR may be an oncogene in the malignant development of HCC. *In vitro* experiments indicated that HAGLR stimulated proliferative and metastatic potentials in HCC cells.

Abnormally expressed lncRNAs are vital regulators in tumor diseases[17,19]. Through transcriptionally or post-transcriptionally regulating expressions and functions of downstream genes by cis or trans mediation, lncRNAs are capable of influencing tumor development[20-24]. Here, miR-6785-5p was indicated as the target gene binding to HAGLR. The results displayed that miR-6785-5p was downregulated in HCC and negatively linked to HAGLR level. Notably, miR-6785-5p was able to partially abolish the regulatory effects of HAGLR on proliferative and metastatic potentials in HCC cells. To sum up, the negative feedback loop HAGLR and miR-6785-5p exacerbated the development of HCC.

Conclusions

Shortly, lncRNA HAGLR stimulates proliferative and metastatic potentials in HCC *via* negatively regulating miR-6785-5p, thus exacerbating the development of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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