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Original Article

The biologic properties of HLJ1 in hepatocellular carcinoma cells

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Abstract: Hepatocellular carcinoma (HCC), a common malignant tumor with poor prognosis and a higher recurrence post operation, is the third leading cause of the lethal cancer systems worldwide. Proliferation and migration of HCC are the key steps of hepatocellular invasion and metastasis. DnaJ-like heat shock protein (HLJ1), a member of the heat shock protein-40 chaperone family, in HCC remains unclear. The aim of this study was to determine the biofunctional changes of HLJ1 in HCC in vitro. Both quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were used to detect the expression of HLJ1 in HCC cells, while, MTS, colony formation and apoptosis assay in vitro were performed to determine the effect of HLJ1 on cell proliferation and apoptosis. Moreover, transwell and wound healing assay were used to detect the invasion and migration of HCC cells. In this study, we found HLJ1 was aberrantly low expressed in HCC cells. Overexpression of HLJ1 suppressed HCC cell proliferation, invasion and migration. In contrast, HLJ1 knockdown promoted them. Furthermore, we searched for some potential downstream genes, such as VEGF, MMP2 and MMP9. These findings demonstrate a functional role of HLJ1 in HCC occurrence and development as a tumor suppressor.

Keywords: HLJ1, HCC, proliferation, apoptosis, invasion, migration

Introduction

Hepatocellular carcinoma (HCC), one of the most common malignant tumors with higher invasion and metastastic potential, leads to a poor prognosis and a higher recurrence [1]. Almost 30-100 million of new cases of HCC and approximately 600,000 deaths occur annually [2]. Accumulating studies have been done to investigate the mechanism of the pathogenesis of HCC. However, the precise molecular mechanisms underlying liver carcinogenesis and aggressiveness are unclear [3, 4].

HLJ1, a member of the heat shock protein-40 chaperone family, is located on human chromosome 1p31.1. HLJ1 consists of 337 amino acid, and the relative molecular mass is 38 KD [5]. Previous studies revealed that HLJ1 is a new tumor suppressor, which has the function of inhibiting cell growth, proliferation, invasion, migration, promoting apoptosis, and participating in cell cycle regulation [6]. Furthermore, HLJ1 is closely related to tumor recurrence and metastasis. However, most of the studies have focused on the molecular biological effects of the non-small cell lung cancer and colorectal cancer. The underlying mechanisms of the pathogenesis of HCC are rarely reported.

In this study, we explored the association between HLJ1 and HCC. The results elucidated that HLJ1 might act as a tumor suppressor in HCC, which could inhibit HCC proliferation, invasion and migration, while promote cell apoptosis.

Materials and methods

Cell lines culture and transfection

All the cell lines used for this study were routinely preserved in our lab, including human normal hepatocytes L-02, human hepatomacell

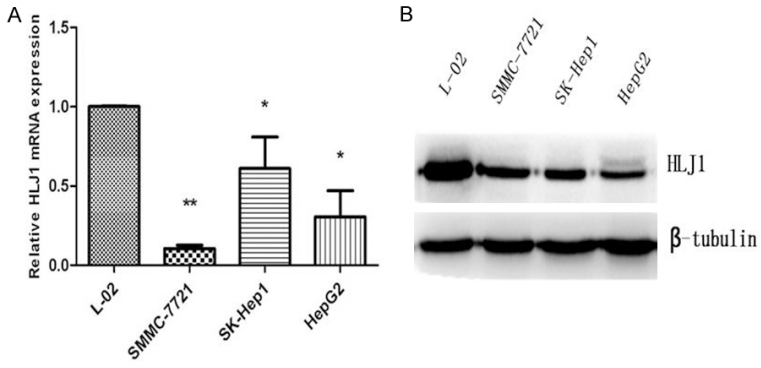

Figure 1. Low expression of HLJ1 in HCC cells. HLJ1 mRNA and protein expression in LO2 and the HCC cell lines (SMMC-7721, SK-Hep1 and HepG2). B-tubulin was used as the endogenous control. *P < 0.05, **P < 0.01.

lines SMMC-7721, SK-Hep1, HepG2. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were all maintained in a humidified incubator at 37°C with 5% CO_2.

RNA extraction and qRT-PCR

Total RNA were isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, USA). For HLJ1, the first-strand cDNA was generated using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions (Takara). The primers used were 5'-AAAGGCTAAACGCTGATG-3' (forward) and 5'-AACAATGTCTGCTGGAAT-3' (reverse). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used for normalization, the primers for it were 5'-CGACCACTTTGTCAAGCTCA-3' (forward), 5'-AGGGGTCTACATGGCAACTG-3' (reverse). The others forward and reverse primers of qRT-PCR as follows: VEGF-F: TCCCGGTATAAGTCCTGGAG, VEGF-R: ACAAATGCTTTCTCCGCTCT. MMP2-F: GTGGATGATGCCTTTGCTC, MMP2-R: CAGGAGTCCGTCCTTACC. MMP9-F: GAGTTCCCGGAGTGAGTTGA, MMP9-R: AAAGGTGAGAAGAGAGGGCC. PAK6-F: CAGGTCTTCCCTCTCCTCAG, PAK6-R: TCTCAGGGCGTTTCTTCTTT. BCAT1-F: GAGCCTGGAAAGGTGGAACTG, BCAT1-R: GCTGACACCCATTATCTACTGCT. SESN2-F: CATCAGTCAGAGGCAATCA, SESN2-R: CCAGGAACGCTAACATAGA. All quantitative real-time polymerase chain reaction (qRT-PCR) samples were performed by using UltraSYBR mixture (Cwbio, China) and conducted using the CFX Connet TM real-time PCR system (Bio-Rad). The quantification analysis was analyzed by the 2-ΔΔCT method.

Western blot analysis

Cells were lysed in RIPA Buffer (Beyotime, China), supplementing with 1 mmol/L PMSF, and protein concentration was measured by the BCAAssay Kit (Beyotime, China). Proteins were separated on a 10-12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked with 5% milk, incubated overnight at 4°C with a primary rabbit antibody against HLJ1 (Cat # 13064-1-AP, 1:1000 dilution, Proteintech), washed three times in TBST, then the blots were incubated with a goat anti-rabbit or anti-mouse HRP secondary antibody (Bioword, USA, 1:1000 dilution). Finally, ECL Detection Reagent (Millipore, Billerica, MA) was used to detect the signal. The data were normalized to β-tubulin (Cat # 66240-1-Ig, 1:8000, Proteintech).

Plasmids, small interfering (si)RNA and transfection

The pcDNA5/FRT/TO V5 HLJ1 as overexpressived plasmid was purchased from Addgene, while pCMV sport6 was used as control. For HLJ1 inhibitor (si-HLJ1) and negative control (NC) were purchased from Biotend. Transfections were performed with a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were harvested 48-72 hours after transfection.

MTS assay

Cells (4000 cells/well) were seeded into 96 well plates after 24 h transfection, and measured at different time points (24, 48, 72, and 96 h) using the MTS kit (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega, USA), followed the manufacturer's protocol. The 490 nm wave-length absorption value was measured. All experiments were performed in triplicate and repeated 3 times.

Colony formation assay

Cells (1 × 10^5 cells/well) were seeded into 6-well plates after 24 h transfection and cultured for 3 days, 4% paraformaldehyde was used to fix with cells. Then, cells were stained witha crystal violet cell colony staining kit (Gen Med Scientifics) according to the manufacturer's instructions.

Apoptosis assay

Cells in 6-well plates were transfected for 24 h followed by serum deprivation for another 48 h. The cells were then harvested by trypsinization, washed with PBS, andresuspended in 1 ml PBS. Apoptosis in HCC cells was quantified by staining with fluorescein isothiocyanate (FITC)-AnnexinV and PI. The stained cells were immediately analyzed by flow cytometry.

Wound healing assay

Transfected cells were plated in 6-well plates and incubated overnight, and scratched using a 200 ul pipette tip to create the wound. PBS was used to wash the cells and cells were further cultured in DMEM with 2% FBS. Then take photos after wounding at 0 h, 24 h and 48 h.

Transwell assays

After 24 h transfection, 3 × 10^5 cells suspending in 100 ul of serum-free DMEM were added to the 8 um transwell migration chambers (Costar). 800 ul of DMEM media containing 10% FBS was added to the lower chamber. After 24 h at 37°C in a 5% CO_2 incubator, the membrane was wiped off using PBS. Migrated cells were fixed with 4% paraformaldehyde for 30 min and stained with Giemsa. Then photographed, and valued according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means±standard deviation (SD). Statistical analysis was performed by X^2 analysis and Student's t test. Significance was assumed for $P < 0.05$ (*) and $P < 0.01$ (**).

Results

HLJ1 expression in HCC cell lines

QRT-PCR and Western blotting were done in HCC cell lines. The results showed that HLJ1 was down-regulated in HCC cell lines (SMMC-7721, SK-Hep1 and HepG2) compared with the hepatic immortal cell line L-02. HLJ1 protein levels were also markedly lower in HCC cell lines than in L-02 cell (**Figure 1**).

HLJ1 inhibits HCC cell proliferation in vitro

To determine the biological effect of HLJ1 in HCC cells, we constructed a pcDNA5/FRT/TO V5 HLJ1 vector that could overexpress HLJ1 and a HLJ1 siRNA. SMMC-7721 cells were transiently transfected with pcDNA5/FRT/TO V5 HLJ1 and pCMV sport6, while treated with siHLJ1 and NC. Over-expression and siRNA efficiency was measured with qRT-PCR and Western blot (**Figure 2A**, **2B**). MTS and colony formation assay in SMMC-7721 cells indicated that cell proliferation and colonies decreased in pcDNA5/FRT/TO V5 HLJ1 compared to pCMV-sport6, whereas HLJ1 inhibitor promoted cell proliferation, over a course of 4 days ($P < 0.05$) (**Figure 2C-E**). Thus, HLJ1 could repress HCC cell proliferation.

HLJ1 induces HCC cells apoptosis

Cell apoptosis assay was performed to analyze the function of HLJ1 in HCC cells. We found that over-expression HLJ1 enhanced apoptosis rate compared to control, and apoptosis rate was recovered in siHLJ1 (**Figure 3A**, **3B**). These results indicated that HLJ1 could promote apoptosis in the HCC.

HLJ1 represses invasion and migration of HCC cells

To further determine whether HLJ1 could affect the invasion and migration capacities of HCC cells, transwell assay and wound healing assay were performed. We found that HLJ1 overexpression inhibited the invasion activity and migration index while siHLJ1 promoted those of SMMC-7721 cells (**Figure 4A-D**). These findings indicated that HLJ1 inhibits the invasion and migration of HCC cells.

Identification of HLJ1 downstream genes

QRT-PCR and Western blotting analyses were performed to determine whether any genes were differentially expressed between overexpression HLJ1 and siHLJ1. We screened a series of related genes related to cell cycle, growth, invasion and migration. A total of 3

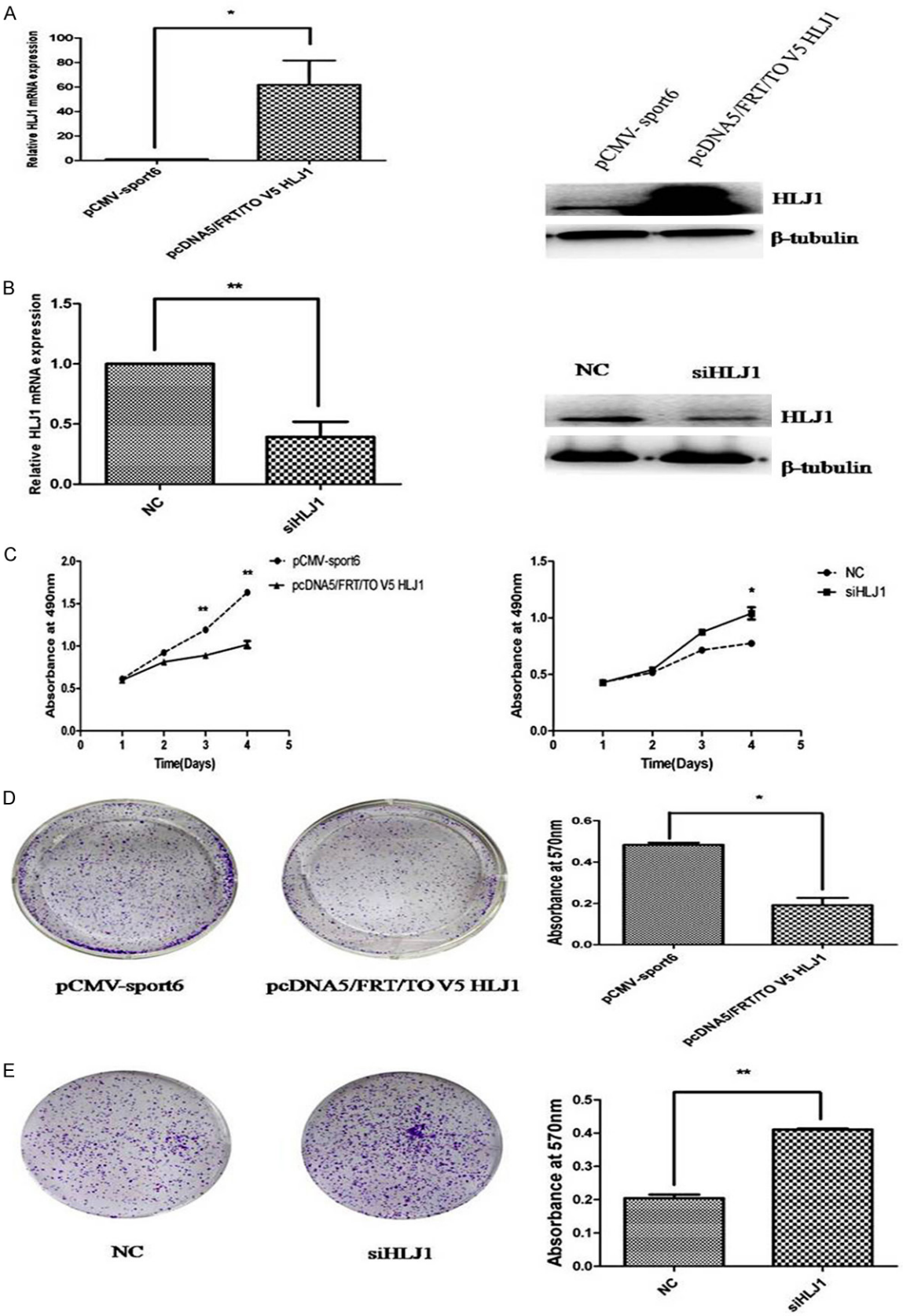
A
Relative HLJ1 mRNA expression
pCMV-sport6
pcDNA5/FRT/TO V5 HLJ1
HLJ1
β-tubulin
B
Relative HLJ1 mRNA expression
NC
siHLJ1
C
Absorbance at 490nm
Time(Days)
D
Absorbance at 570nm
E

Figure 2. HLJ1 inhibits HCC cell proliferation in vitro. A. HLJ1 mRNA and protein expression in SMMC7721 cells transfected with pcDNA5/FRT/TO V5 HLJ1 and pCMV-sport6 as control. B. HLJ1 mRNA and protein expression in SMMC7721 cells transfected with siHLJ1 and NC. *P < 0.05, **P < 0.01. C. Cell growth was measured at different times (24, 48, 72, and 96 h, respectively) by MTS assay in SMMC7721 cells. D. Representative results of colony formation of pcDNA5/FRT/TO V5 HLJ1 transfected SMMC7721 cells, compared with the controls. Colonies were valued as reported. E. Representative results of colony formation of siHLJ1 and NC transfected in SMMC7721 cells. *P < 0.05, **P < 0.01.

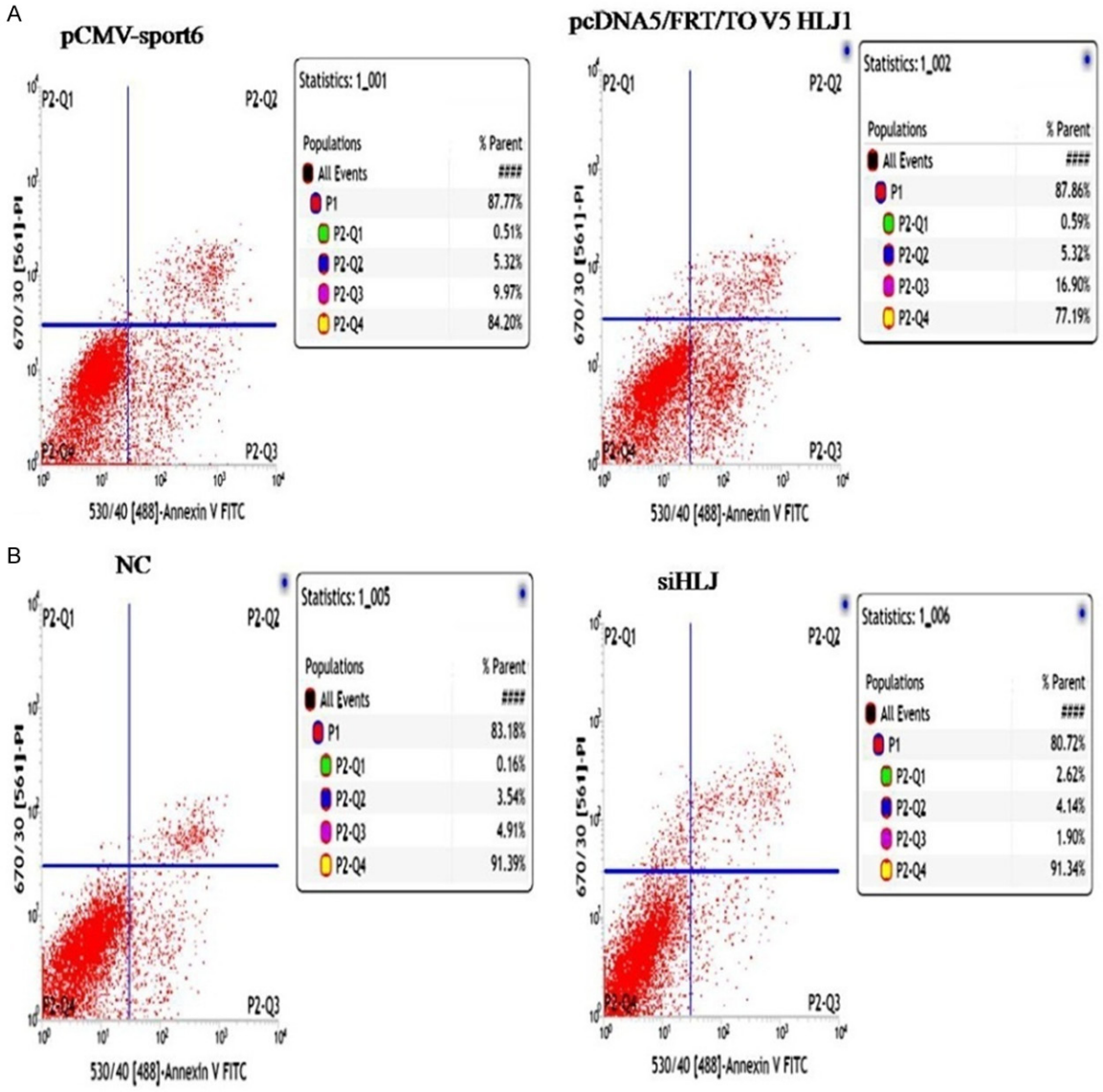

Figure 3. HLJ1 induces HCC cells apoptosis. A. HLJ1 overexpression in SMMC-7721 cells apoptosis was analyzed. B. SiHLJ1 and NC in SK-Hep1 cells apoptosis were analyzed by flow cytometry.

genes showed remarkable changes in expression levels between pcDNA5/FRT/TO V5 HLJ1 and siHLJ1 in SMMC-7721 cells. The VEGE, MMP2, MMP9 genes were suppressed in overexpression HLJ1 in HCC cells, whereas those were stimulated by HLJ1 knockdown (**Figure 5A**, **5B**).

Discussion

HCC is one of the most common causes of cancer-related death with high incidence and mortality rates, affecting certain of carcinogenic-related genes by genetic or epigenetic changes [7]. With the development of molecular biology,

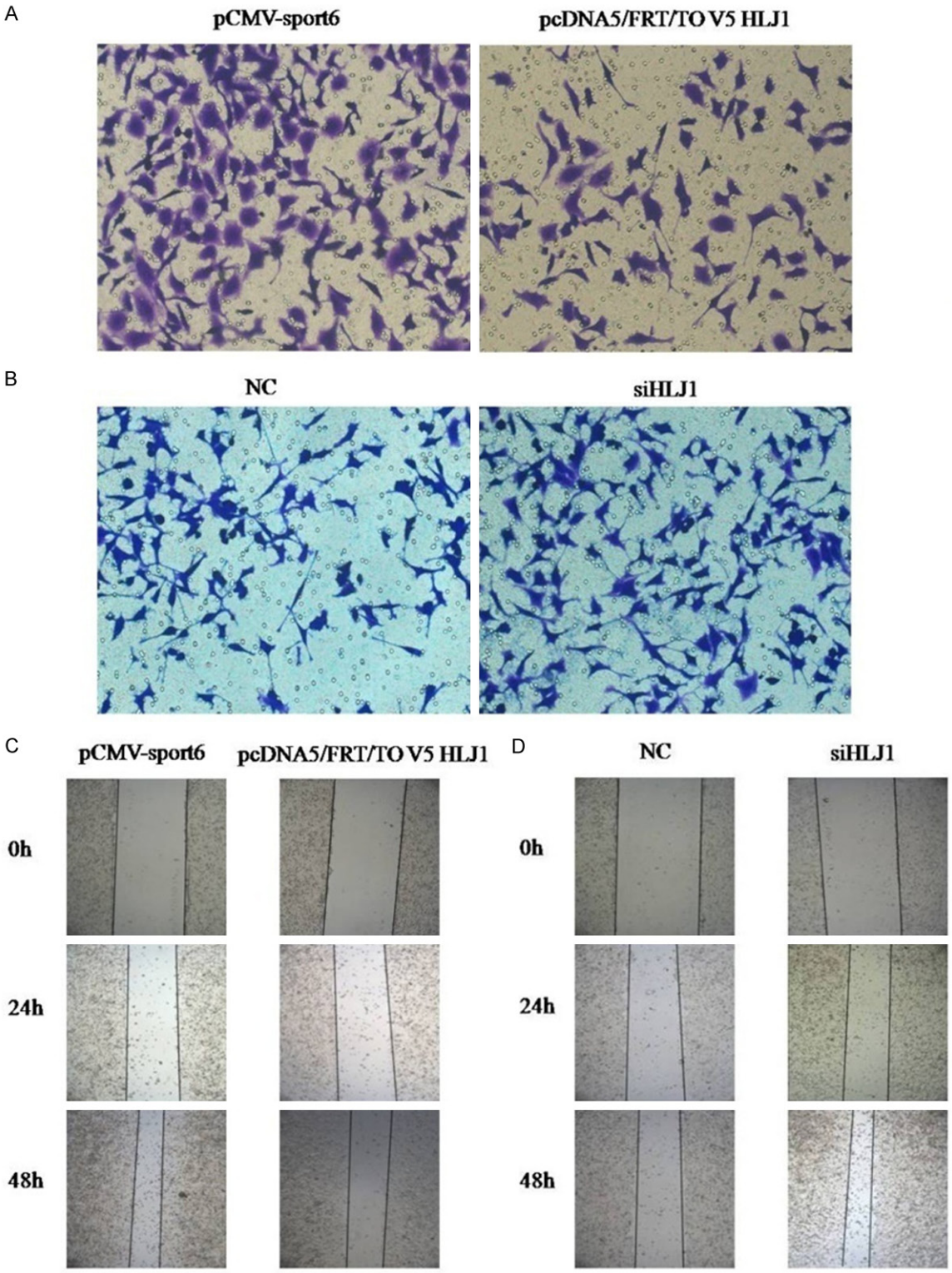

Figure 4. HLJ1 represses invasion and migration of HCC cells. A, B. Cell migration was evaluated in SMMC-7721 cells over-expressing HLJ1, siHLJ1 and their controlby using Matrigel migration chamber. Cells that migrated through the pores were fixed and stained with crystal violet after incubation (magnification × 400). C, D. Wound healing in SMMC-7721 cells transfected over-expressing HLJ1, siHLJ1 and their control.

the studies of focusing on HCC metastasis and recurrence prediction become the hotspot in the recent years. Further studies showed that proliferation and metastasis genes are abnor-

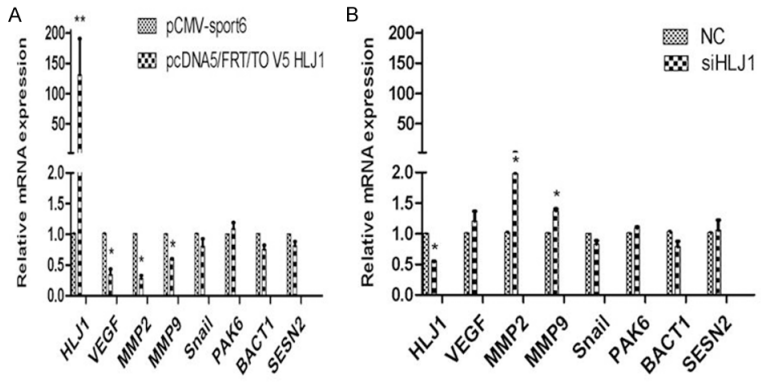

Figure 5. Identification of HLJ1 downstream genes. A. The VEGE, MMP2 and MMP9 genes were suppressed in overexpression HLJ1 by qRT-PCR. B. The expression of VEGE, MMP2 and MMP9 genes were recovered in siHLJ1 by qRT-PCR.

mal expression or gene deletion. To understand the molecular mechanism on the occurrence and development of HCC is benefit for early diagnosis and treatment of HCC, which could provide a new method for the treatment of individual patients according to the characteristics of biological effect.

In recent years, HLJ1 was found to be a new tumor suppressor, which could regulate cell proliferation and migration. HLJ1 can inhibit cell division and proliferation by up-regulating $P21^{WAF1}$ and down-regulating Cyclin D in a P53-independent manner [8, 9]. Furthermore, HLJ1 can inhibit cell migration by up-regulating Cadherin E. The enhancer-binding AP-1 and promoter-binding YY1 through DNA bending upregulates the expression of HLJ1, and a correlation of the increase in HLJ1 with E-cadherin expression was detected [10]. In our earlier experiments, we demonstrated that HBV induced the expression of transcription factor YY1, which resulted in activation of the HLJ1 promoter and subsequently upregulation of the HLJ1 expression in HCC [11]. In addition, Tsai, et al. concluded that HLJ1 is a novel tumor suppressor in Non-Small-Cell Lung Carcinoma (NSCLC), and high HLJ1 expression is associated with reduced cancer recurrence and prolonged survival of NSCLC patients. HLJ1 is a novel substrate of caspase-3 during the UV-induced apoptotic process in NSCLC [12]. Moreover, HLJ expression and metastasis were associated with an increased risk of death from CRC, HLJ1 expression was singled out as one significant and independent prognostic factor relative to overall survival on multivariate analysis [13]. Until now, the role of HLJ1 in HCC remains controversial.

Our study provides a novel understanding of the relationship between HLJ1 and HCC. We have showed that HLJ1 was aberrantly low expressed in HCC cells, suppressed cell proliferation, invasion and migration, while, promoted cell apoptosis. We found that the potential downstream genes associated with HLJ1. The following task is to clarify the regulatory mechanism of HLJ1 in HCC occurrence and development, which may facilitate the development of personalized therapy by inhibiting HCC proliferation, angiogenesis, and metastasis.

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Disclosure of conflict of interest

None.

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References

[1] Cao H, Phan H, Yang LX. Improved chemotherapy for hepatocellular carcinoma. Anticancer Res 2012; 32: 1379-1386.

[2] Cervello M, McCubrey JA, Cusimano A, Lampiasi N, Azzolina A, Montalto G. Targeted therapy for hepatocellular carcinoma: novel agents on the horizon. Oncotarget 2012; 3: 236-260

[3] El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365: 1118-1127.

[4] Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-

associated hepatocellular carcinoma. Nat Rev Cancer 2013; 13: 123-135.
[5] Hoe KL, Won M, Chung KS, Jang YJ, Lee SB, Kim DU, Lee JW, Yun JH, Yoo HS. Isolation of a new member of DnaJ-like heat shock protein 40(Hsp40) from human liver. Biochim Biophys Acta 1998; 1383: 4-8.
[6] Tsai MF, Wang CC, Chang GC, Chen CY, Chen HY, Cheng CL, Yang YP, Wu CY, Shih FY, Liu CC, Lin HP, Jou YS, Lin SC, Lin CW, Chen WJ, Chan WK, Chen JJ, Yang PC. A new tumor suppressor Dna J-like heat shock protein, HLJ1, and survival of patients with non-small-cell lung carcinoma. J Natl Cancer Inst 2006; 98: 825-38.
[7] Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. Gut 2014; 63: 844-855.
[8] Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin dependent kinase inhibitor suppresser tumorigenicity in vivo. Nat Med 1995; 1: 1052-6.
[9] Baldin V, Lukas J, Marcote MJ, Pagano M, Draette G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 1993; 7: 812-21.
[10] Wang CC, Tsai MF, Hong TM, Chang GC, Chen CY, Yang WM, Chen JJ, Yang PC. The transcriptional factor YY1 upregulates the novel invasion suppressor HLJ1 expression and inhibits cancer cell invasion. Oncogene 2005; 24: 4081-93.
[11] Zhang L, Cai X, Chen K, Wang Z, Wang L, Ren M, Huang A, Tang H. Hepatitis B virus protein up-regulated HLJ1 expression via the transcription factor YY1 in human hepatocarcinoma cells. Virus Res 2011; 157: 76-81.
[12] Lin SY, Hsueh CM, Yu SL, Su CC, Shum WY, YehKC, Chang GC, Chen JJ. HLJ1 is a novel caspase-3 substrate and its expression enhances UV-induced apoptosis in non-small cell lung carcinoma. Nucleic Acids Res 2010; 38: 6148-58.
[13] Liu Y, Zhou J, Zhang C, Fu W, Xiao X, Ruan S, Zhang Y, Luo X, Tang M. HLJ1 is a novel biomarker for colorectal carcinoma progression and overall patient survival. Int J Clin Exp Pathol 2014; 7: 969-977.