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Original Article

LncRNA GAS6-AS2 facilitates tumor growth and metastasis of hepatocellular carcinoma by activating the PI3K/AKT/FoxO3a signaling pathway

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Abstract: The morbidity and mortality of hepatocellular carcinoma (HCC) are growing yearly. Several reports emphasize the importance of long non-coding RNAs (lncRNAs) in HCC. This paper provides a molecular mechanism for the function of GAS6-AS2 in HCC. The expressions of GAS6-AS2, miR-493-5p and OTUB1 were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Cell proliferation, apoptosis, migration, and invasion were measured by cell counting kit-8 (CCK-8), flow cytometry and transwell assay, respectively. The interaction of miR-493-5p and GAS6-AS2 or OTU domain-containing Ubiquitin Aldehyde-binding Protein 1 (OTUB1) was analyzed by starBase v2.0 and verified by luciferase reporter assay. The protein level of OTUB1 as well as PI3K, p-PI3K, AKT, p-AKT, FoxO3a, p-FoxO3a and β-actin protein levels were distinguished by western blot. GAS6-AS2 was up-regulated in HCC tissues and cells. GAS6-AS2 knockdown inhibited proliferation, migration, and invasion but promoted apoptosis. MiR-493-5p, a target of GAS6-AS2, was down-regulated in HCC tissues and cells. Inhibition of miR-493-5p reversed the effects of GAS6-AS2 knockdown on HCC cells. OTUB1, a target of miR-493-5p, was up-regulated in HCC cells and its expression was modulated by miR-493-5p. Overexpression of OTUB1 recovered the positive effects of miR-493-5p enrichment or GAS6-AS2 knockdown on HCC cells. GAS6-AS2 knockdown impeded the activation of PI3K/AKT/FoxO3a signaling pathway, while this activation was reversed by miR-493-5p inhibition or OTUB1 overexpression. In conclusion, GAS6-AS2 knockdown suppressed proliferation, migration, and invasion but promoted apoptosis of HCC cells by impeding PI3K/AKT/FoxO3a signaling pathway through regulating the GAS6-AS2/miR-493-5p/OTUB1 axis.

Keywords: GAS6-AS2, miR-493-5p, OTUB1, PI3K/AKT/FoxO3a, hepatocellular carcinoma

Introduction

In the past few years, evidence suggests gradually a increasing morbidity of hepatocellular carcinoma (HCC) in different countries [1]. HCC is one of the most familiar malignancies throughout the world and is regarded as the 3th leading cause of cancer-related deaths, which is the main histologic subtype among primary liver cancers, accounting for over 70% of the overall liver cancer burden all over the world [2, 3]. Despite advances in diagnosis and treatment over the past few decades, the recurrence rate of HCC remains high, reaching 50% within 2 years [4]. Besides, long-term prognosis of HCC is weak due to distant metastasis [5]. Therefore, it is urgent to explore novel biomarkers of HCC and its potential mechanisms, providing effective strategies for target therapy and prognosis of HCC.

Long non-coding RNAs (LncRNAs), >200 nucleotides-long, are a cluster of RNA without protein coding ability, that function in many biologic processes, including transcriptional, post-transcriptional, and epigenetic gene regulation [6]. Gradually, research on lncRNAs has mentioned the treatment of diseases, including cancer [7]. Several lncRNAs involved in HCC have been reported over the past few years. For example, lncRNA small nucleolar RNA host gene 10 (SNHG10) promoted malignant behaviors and epithelial-mesenchymal transition of HCC cells [8]. LncRNA AGAP2-AS1 functioning in an onco-

genic role in HCC, promotes proliferation and metastasis [9]. Growth arrest-specific 6 antisense RNA 2 (GAS6-AS2), one of lncRNAs, was primarily identified in our research. Before this, its roles and underlying molecular mechanisms in HCC remained unclear.

LncRNAs can interact with microRNAs (miRNAs) by acting as competing endogenous RNAs (ceRNAs) or "sponges" to play their roles [10]. MiRNAs are well known as short, endogenous non-coding RNA molecules (18-24 nucleotides). Generally, miRNAs function in the regulation of mRNA translation and degradation through combining with the 3' untranslated region (3' UTR) of the target messenger RNAs (mRNAs) [11]. Thereby, miRNAs modulate extensive biologic process such as cellular proliferation, differentiation, progression, and apoptosis. MiR-493-5p was confirmed to be a target of GAS6-AS2. The character of miR-493-5p has been reported in numerous cancers, such as human breast cancer [12], osteosarcoma [13], and ovarian cancer [14]. Unfortunately, the reports involving in the detailed roles of miR-493-5p in HCC remain limited.

OTU domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) is a deubiquitinating (DUB) enzyme, belonging to the ovarian tumor (OTU) domain protease superfamily [15]. DUB enzyme regulates multiple physiologic and pathologic processes, including tumor occurrence, development, and metastasis [16]. Recent reports present that OTUB1 is associated with cancers of different types. For instance, OTUB1 was overexpressed in prostate cancer cells and promoted tumorigenesis and cell invasion *in vitro* and *in vivo* [17]. In colorectal cancer (CRC), high OTUB1 expression was always involved in metastasis and associated with weak overall survival [18]. A similar functional role of OTUB1 existed in gastric adenocarcinoma [19]. From these results, it was quite possible that OTUB1 is an oncogene in different cancers. Nevertheless, related research on OTUB1 in HCC was minimal.

In our present study, the abundances of GAS6-AS2, miR-493-5p, and OTUB1 were measured in HCC tissues and cells. Gain-of-function and loss-of-function experiments were conducted to diagnose their functional roles in HCC cells. The interaction of miR-493-5p and GAS6-AS2 or OTUB1 was predicted and verified here. The purpose of this paper was to explore the underlying mechanisms of GAS6-AS2 in tumorigenesis and metastasis in HCC and its suitability as a therapeutic target for HCC.

Materials and methods

Sample tissues and cell culture

A total of thirty pairs of HCC tissues and paired normal tissues were acquired from patients who underwent surgical resection in The Second Affiliated Hospital of Xinjiang Medical University. The tissues were immediately placed in liquid nitrogen after removal from bodies and then stored at -80°C condition until use. This study was approved by The Second Affiliated Hospital of Xinjiang Medical University and all subjects had ratified informed consent forms prior to clinical operation.

Human HCC cell lines including Hep3B, HuH-7, Li-7 and CSQT-2, and normal liver cells HL-7702 were all purchased from BeNa Culture Collection (BNCC, Suzhou, China). Cells were maintained in 90% Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at a 37°C humidified atmosphere with 5% CO_2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the product's protocols. cDNAs were synthesized using Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) for GAS6-AS2 and OTUB1, and mirVana™ qRT-PCR miRNA Detection Kit (Invitrogen) for miR-493-5p. Quantitative analysis was carried out using Maxima SYBR Green qPCR Master Mix (Thermo) on BioRad CFX96™ (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and small nuclear RNA U6 acted as housekeeping genes to normalize the expression levels. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used were listed as follows: GAS6-AS2, forward: 5'-AAGGAGGACGCAATACC-3' and reverse: 5'-ATCCTGGCTAACACGGT-3'; OTUB1, forward: 5'-CAGC AAGAGATTGACTGTGAG-3' and reverse: 5'-CTTGATCTTCTGTTGATAGATGT-TGTC-3'; GAPDH, forward: 5'-AACGTGTCAGTG-

GTGGACCTG-3' and reverse: 5'-AGTGGGTGTC-GCTGTTGAAGT-3'. The primers of miR-493-5p and U6 were directly purchased from Ribobio (Guangzhou, China).

Cell transfection

Small interference RNA against GAS6-AS2 (si-GAS6-AS2) and its corresponding control (si-con) were obtained from Geneseed Biotech (Guangzhou, China). MiR-493-5p mimics (miR-493-5p) and its corresponding control (miR-con) together with miR-493-5p inhibition (anti-miR-493-5p) and its corresponding control (anti-miR-con) were all purchased from Ribobio. Overexpression of GAS6-AS2 or OTUB1 was accomplished by overexpressed fusion vector pcDNA-GAS6-AS2 or pcDNA-OTUB1; empty vector (pcDNA) as control was constructed by Geneseed Biotech. All transfection was processed using Lipofectamine 2000 Reagent (Invitrogen) in agreement with instructions. Following experiments were carried out at 48 h after transfection.

Cell counting kit-8 (CCK-8) assay

CCK-8 (Beyotime, Shanghai, China) was used to assess cell proliferation. Briefly, after 48-h transfection, Hep3B and HuH7 cells (5×10^3) were planted into 96-well plates (Corning Costar, Corning, NY, USA) incubated for 24, 48 and 72 h. Cells were then treated with CCK-8 solution for 2 h. The absorbance of optical density (OD) was measured at 490 nm using a Multiskan Ascent 354 microplate reader (Thermo Fisher Scientific).

Flow cytometry assay

This assay was adapted to analyze apoptosis using an Annexin V-FITC/PI kit (Yesen, Shanghai, China). Briefly, HCC cells were collected at 48 h post transfection, washed with pre-cooled phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO, USA), digested with trypsin, and fixed in 70% pre-cooled ethanol. Next, cells were stained with FITC-Annexin V and PI based on theinstructions. Apoptotic cells were detected by CytoFLEX (Beckman Coulter, Miami, FL, USA) and analyzed by Flowjo software (Tree Star Corp, San Carlos, CA, USA).

Transwell assay

Transwell assay was performed to evaluate migration and invasion. In brief, the cells incubated 48 h after transfection were suspended in 200 μL serum free medium and placed into the top well of chambers (Corning) for migration, and placed into the upper well of chambers pre-coated with matrigel (Corning) for invasion. Meanwhile, the bottom chambers were covered by DMEM supplemented with 10% FBS. After incubating for 12 h, the cells underside of membrane were immobilized with pre-cooled methanol and stained with crystal violet (1%) for 15 min. Finally, cells were distinguished by using a light microscope (Olympus, Tokyo, Japan).

Bioinformatics analysis and luciferase reporter analysis

Bioinformatics online tool starBase v2.0 was used to screen potential target genes and analyze putative binding sites. Luciferase reporter assay was performed to verify the relationship between miR-493-5p and GAS6-AS2 or OTUB1. In brief, GAS6-AS2 (wild type and mutant) sequences and 3' UTR of OTUB1 sequences (wild type and mutant) containing the binding sites with miR-493-5p were amplified by qRT-PCR and respectively constructed into PGL4 luciferase reporter vectors by Geneseed Biotech, named WT-GAS6-AS2, MUT-GAS6-AS2, WT-OTUB1 and MUT-OTUB1, respectively. Afterwards, the WT (or MUT)-GAS6-AS2 or WT (or MUT)-OTUB1 and miR-493-5p mimics or miR-con were co-transfected into Hep3B and HuH7 cells. The luciferase activity was detected by using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Western blot analysis

The protein was dissociated from cells using RIPA cell lysis buffer (Beyotime). After measuring the concentration of protein, equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% skim milk at room temperature for 1.5 h and were incubated with the primary antibodies against OTUB1 (Abcam, Cambridge, MA, USA, 1:2000, ab175200), PI3K (1:2000, ab5451; Abcam), p-PI3K (1:2000, ab182651; Abcam), AKT (1:2000, ab126811; Abcam), p-AKT (1:2000, ab18206; Abcam), Fox03a (1:2000, ab23683; Abcam), p-Fox03a (1:2000, ab47285; Abcam) and β-actin (1:2000, ab82-

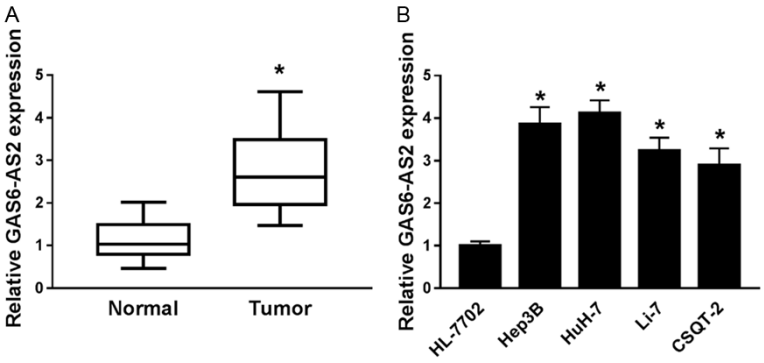


Figure 1. Expression of GAS6-AS2 was measured in HCC tissues and cell lines by qRT-PCR. GAS6-AS2 was upregulated in HCC tissues (A) compared with normal tissues, and upregulated in HCC cell lines (B) compared with normal liver cells. $^{*}P<0.05$.

26; Abcam) at 4°C overnight. Next day, the membranes were washed with PBS and incubated with horseradish peroxidase-labeled secondary antibody (1:5000, Abcam) at room temperature for 2 h. Then, the protein bands were visualized enhanced chemiluminescent ECL™ Detection Reagents (Sigma-Aldrich).

Statistical analysis

All data were analyzed by GraphPad Prism 5.0 (Version X, La Jolla, CA, USA) and presented as the mean ± standard deviation (SD). Every experiment was repeated at least three times. The two-tailed Student's *t*-test or one-way analysis of variance were used to compare means between two groups or multiple groups, respectively. Spearman' correlation analyses were used to analyze correlations between miR-493-5p and GAS6-AS2 expression levels. $P<0.05$ was considered significant.

Results

GAS6-AS2 was significantly up-regulated in HCC tissues and cell lines

To detect whether GAS6-AS2 was involved in HCC, the expression of GAS6-AS2 was measured in HCC tissues and cell lines by qRT-PCR. As in **Figure 1A**, the expression of GAS6-AS2 was drastically increased in HCC tumor tissues compared with adjacent normal tissues. GAS6-AS2 expression was also obviously enhanced in HCC cell lines including Hep3B, HuH-7, Li-7 and CSQT-2 compared with that in normal liver cells HL-7702 (**Figure 1B**). The data suggested that GAS6-AS2 might play a key role in the progress of HCC.

Downregulation of GAS6-AS2 inhibited proliferation, invasion, and migration but promoted apoptosis of HCC cells

To investigate the potential role of GAS6-AS2 in HCC cell lines, the expression of GAS6-AS2 was down-regulated in Hep3B and HuH-7. First, the interference efficiency of GAS6-AS2 was checked and the result showed that the expression of GAS6-AS2 was sharply reduced both in Hep3B and HuH7 transfected with si-GAS6-AS2 compared to control or si-con (**Figure 2A**). As shown in **Figure 2B** and **2C**, the proliferative ability of both Hep3B and HuH7 cell lines was markedly inhibited after GAS6-AS2 knockdown compared with control or si-con, while cell apoptosis rate was notably strengthened (**Figure 2D**). Transwell assay revealed that the knockdown of GAS6-AS2 could suppress the migration and invasion of Hep3B and HuH7 cells compared with control or si-con (**Figure 2E** and **2F**). In conclusion, these data proved that knockdown of GAS6-AS2 inhibited proliferation, invasion, and migration but promoted apoptosis in HCC cells, suggesting GAS6-AS2 may act as an oncogene in HCC.

GAS6-AS2 functioned as a ceRNA and played its role by regulating the expression of miR-493-5p

To explore the mechanisms of GAS6-AS2 involved in breast cancer progression, the direct interaction of GAS6-AS2 and miR-493-5p was verified. Bioinformatics prediction by starBase showed that GAS6-AS2 held several binding sites to miR-493-5p (**Figure 3A**). Then dual luciferase reporter assay was performed in HEK293T cells cotransfected with WT-GAS6-AS2 and miR-493-5p mimics, using MUT-GAS6-AS2 and miR-con as controls. As shown in **Figure 3B** and **3C**, with the transfection of miR-493-5p mimics, luciferase activities both in Hep3B and HuH7 cells containing WT-GAS6-AS2 transfection was significantly decreased while the luciferase activities in MUT-GAS6-AS2

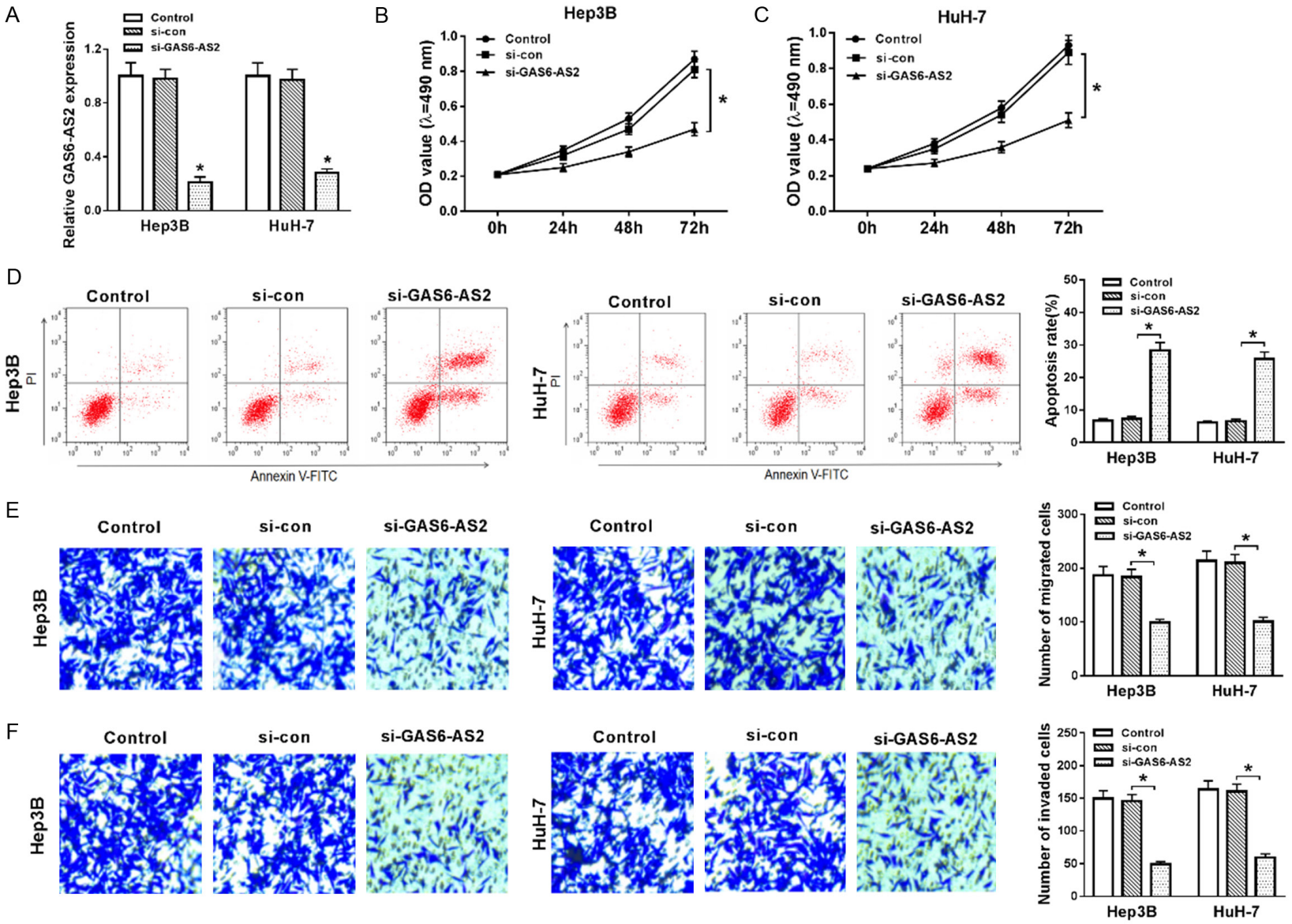


Figure 2. GAS6-AS2 knockdown inhibited the malignant behavior of the HCC cells. A. GAS6-AS2 level in HCC cells after transfection with si-GAS6-AS2 or si-con. B and C. Proliferation of Hep3B and HuH7 cells after transfection. D. Apoptosis rate after transfection in Hep3B and HuH7 cells. E. Migration of Hep3B and HuH7 cells after transfection. F. Invasion of Hep3B and HuH7 cells after transfection. $^{*}P<0.05$.

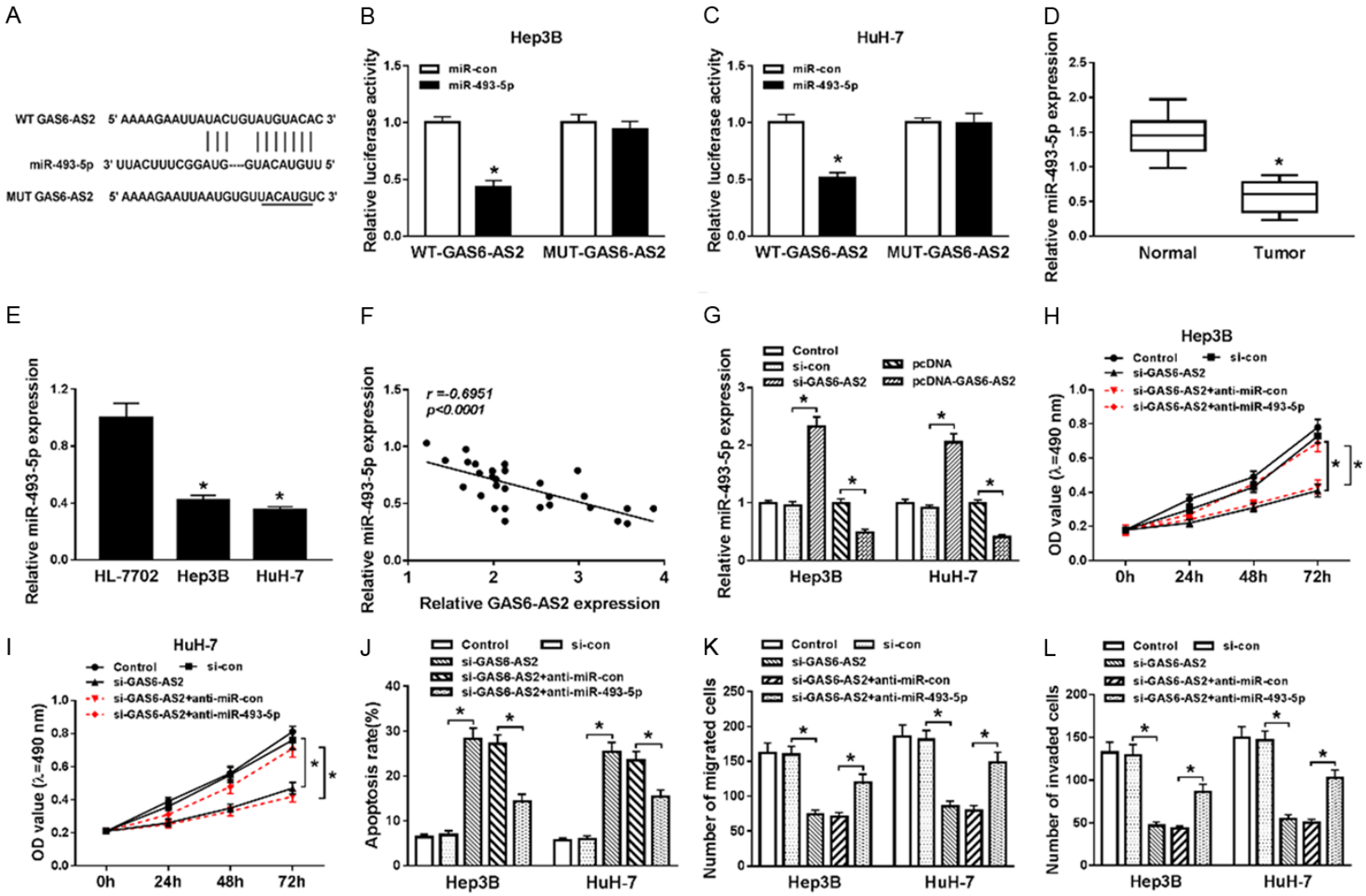


Figure 3. GAS6-AS2 knockdown inhibited the malignant behavior of the HCC cells by negative regulation of miR-124-3p. A. The binding sites between GAS6-AS2 and miR-493-5p were analyzed by bioinformatics tool starBase. B and C. Luciferase reporter assay was used to verify the interaction of GAS6-AS2 and miR-493-5p in Hep3B and HuH7 cells. D. MiR-493-5p expression in HCC tissues and normal tissues. E. MiR-124-3p expression in HCC cells and normal liver cells. F. Correlation between miR-124-3p expression and GAS6-AS2 expression. G. Effects of the changed GAS6-AS2 expression on the expression of miR-124-3p. Hep3B and HuH7 cells were transfected with si-GAS6-AS2 and miR-124-3p inhibitor. H and I. Proliferation of Hep3B and HuH7 cells after transfection. J. Apoptosis of Hep3B and HuH7 cells after transfection. K. Migration of Hep3B and HuH7 cells after transfection. L. Invasion of Hep3B and HuH7 cells after transfection. $^{*}P<0.05$.

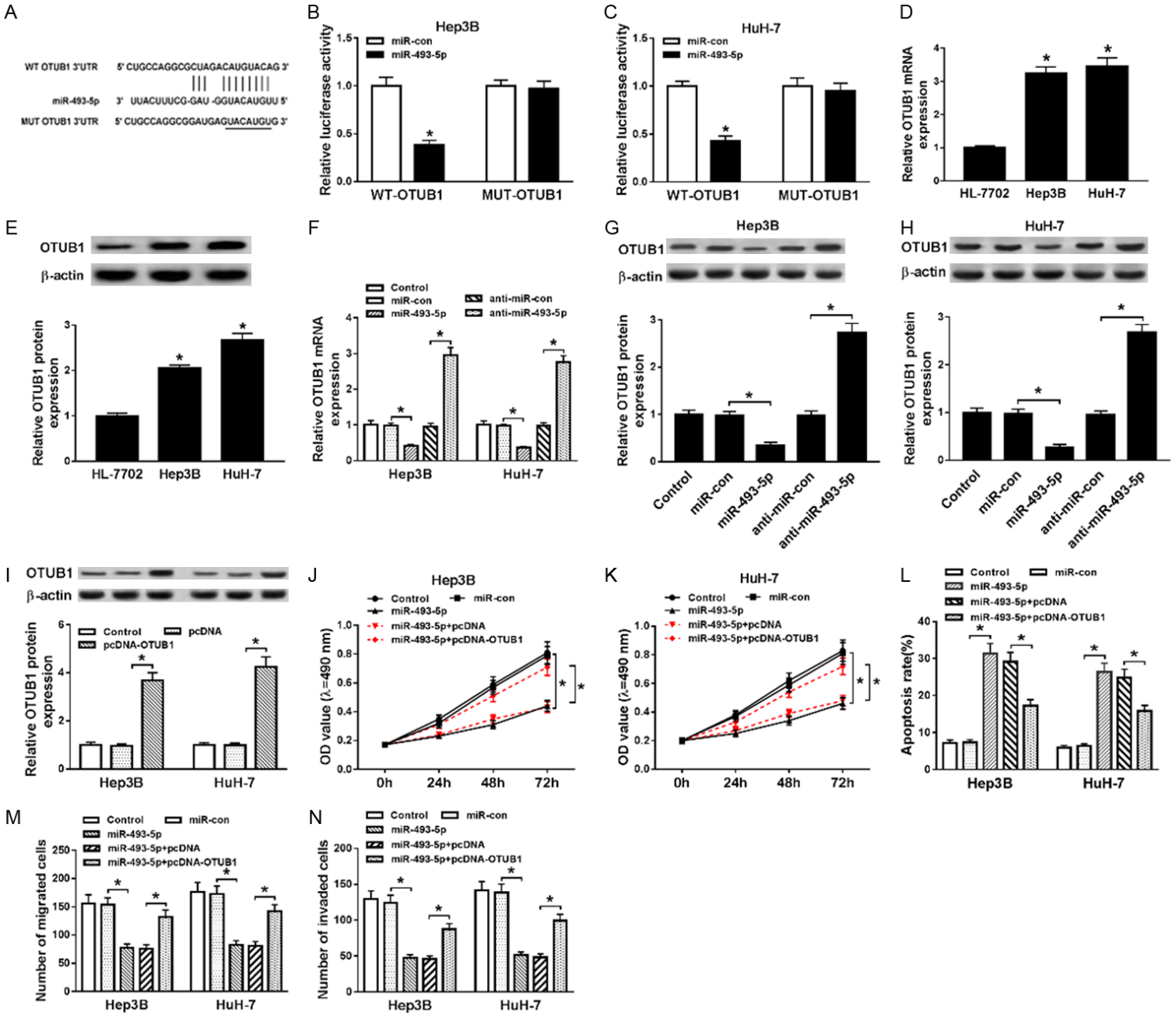
group showed no observable difference. Next, qRT-PCR analysis exhibited that the expression of miR-493-5p was prominently decreased both in HCC tumor tissues and cell lines compared with normal tissues and cell lines (**Figure 3D** and **3E**). Moreover, we further analyzed the expression correlation between GAS6-AS2 and miR-493-5p and we found miR-493-5p expression was negatively correlated with GAS6-AS2 (**Figure 3F**). qRT-PCR analysis presented that overexpression of GAS6-AS2 inhibited miR-493-5p expression, while underexpression of GAS6-AS2 improved miR-493-5p expression (**Figure 3G**). To figshow the effects of GAS6-AS2 and miR-493-5p interaction on cell proliferation, apoptosis, migration, and invasion, si-GAS6-AS2+anti-miR-493-5p and si-GAS6-AS2+anti-miR-con were transfected into Hep3B and HuH7 cells, respectively. MTT assay indicated that si-GAS6-AS2+anti-miR-493-5p transfection could reinforce proliferation blocked by si-GAS6-AS2 compared with si-GAS6-AS2+anti-miR-con transfection (**Figure 3H** and **3I**). On the contrary, the high apoptosis rate caused by si-GAS6-AS2 could be weakened by si-GAS6-AS2+anti-miR-493-5p transfection (**Figure 3J**). Transwell assay implied that the low number of migrated and invaded cells following GAS6-AS2 down-regulation could be enhanced by miR-493-5p synchronous inhibition (**Figure 3K** and **3L**). The above data suggested that miR-493-5p was indeed a target of GAS6-AS2; and GAS6-AS2 regulated cell proliferation, apoptosis, migration and invasion by targeting miR-493-5p.

MiR-493-5p functioned in HCC cells by binding to the 3' UTR of OTUB1

To explore the potential mechanism of miR-493-5p function in HCC, we sought todetermine the target genes of miR-493-5p. As shown in **Figure 4A**, starBase tool predicted that OTUB1 was a target of miR-493-5p with several binding sites between them. Furthermore, luciferase reporter assay manifested that the luciferase activity was reduced in Hep3 and HuH-7 cells transfected with WT-OTUB1 and miR-493-5p mimics compared with miR-con, while the luciferase activity had no change in two cells with MUT-OTUB1 and miR-493-5p mimics (**Figure 4B** and **4C**). The qRT-PCR and western blot analysis elucidated that the expression of OTUB1 was accelerated in Hep3B and HuH-7 cells both from mRNA and protein levels (**Figure 4E**). We found the expression of OTUB1 was regulated by miR-493-5p expression. To be specific, OTUB1 was down-regulated when Hep3B and HuH-7 cells were transfected with miR-493-5p mimics compared with miR-con, while its expression soared when the 2 cell lines were transfected with anti-miR-493-5p (compared with anti-miR-con) at both the mRNA (**Figure 4F**) and protein levels (**Figure 4G** and **4H**). To define the effect of the interactions of miR-493-5p and OTUB1 on HCC cells, miR-493-5p mimics+pcDNA-OTUB and miR-493-5p mimics+pcDNA were transfected into Hep3B and HuH-7 cells, respectively. Before this, the efficiency of OTUB1 overexpression was determined and the result showed that OTUB1 was strikingly up-regulated in cells with pcDNA-OTUB1 transfection compared to that with pcDNA (**Figure 4I**). Afterwards, an MTT assay showed that miR-493-5p mimics+pcDNA-OTUB transfection reversed the inhibition of proliferation caused by miR-493-5p mimics both in Hep3B and HuH-7 cells. Flow cytometry showed that miR-493-5p mimics+pcDNA-OTUB transfection abated the higher apoptosis rate caused by miR-493-5p mimics. Transwell assay disclosed that overexpression of OTUB1 reversed the migration and invasion inhibition caused by miR-493-5p mimics in Hep3B and HuH-7 cells. These data indicated that miR-493-5p executed its role in HCC cells by binding to 3' UTR of OTUB1.

GAS6-AS2 exerts an effect through up-regulating OTUB1 by sponging miR-493-5p

To further explain the mechanism of GAS6-AS2 function, Hep3B and HuH-7 cells were transfected with different transfection. First, the expression of OTUB1 was detected and western blot analysis exhibited that the expression of OTUB1 was inhibited by GAS6-AS2 knockdown compared with si-con, but was recovered by miR-493-5p simultaneous inhibition (**Figure 5A** and **5B**). Additionally, MTT assay confirmed that si-GAS6-AS2+pcDNA-OTUB1 transfection could recover the proliferation suppression caused by si-GAS6-AS2 both in Hep3B and HuH-7 cells (**Figure 5C** and **5D**). Flow cytometry revealed that the high apoptosis rate caused by si-GAS6-AS2 was depleted by si-GAS6-AS2+pcDNA-OTUB1 (**Figure 5E**). Transwell assay confirmed that si-GAS6-AS2+pcDNA-


A
WT OTUB1 3'UTR 5' CUGCCAGGCGCUAGACAUGUACAG 3'
miR-493-5p 3' UUACUUUCG-GAU -GGUACAUGUU 5'
MUT OTUB1 3'UTR 5' CUGCCAGGCGGAUGAGUACAUGUG 3'
B
Hep3B
miR-con
miR-493-5p
Relative luciferase activity
WT-OTUB1
MUT-OTUB1
C
HuH-7
miR-con
miR-493-5p
Relative luciferase activity
WT-OTUB1
MUT-OTUB1
D
Relative OTUB1 mRNA expression
HL-7702
Hep3B
HuH-7
E
OTUB1
β-actin
Relative OTUB1 protein expression
HL-7702
Hep3B
HuH-7
F
Control
miR-con
miR-493-5p
anti-miR-con
anti-miR-493-5p
Relative OTUB1 mRNA expression
Hep3B
HuH-7
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Control
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β-actin
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pcDNA
pcDNA-OTUB1
Relative OTUB1 protein expression
Hep3B
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Hep3B
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miR-con
miR-493-5p
miR-493-5p+pcDNA
miR-493-5p+pcDNA-OTUB1
OD value (λ=490 nm)
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HuH-7
Control
miR-con
miR-493-5p
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miR-493-5p+pcDNA-OTUB1
OD value (λ=490 nm)
0h
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48h
72h
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Control
miR-con
miR-493-5p
miR-493-5p+pcDNA
miR-493-5p+pcDNA-OTUB1
Apoptosis rate(%)
Hep3B
HuH-7
M
Control
miR-con
miR-493-5p
miR-493-5p+pcDNA
miR-493-5p+pcDNA-OTUB1
Number of migrated cells
Hep3B
HuH-7
N
Control
miR-con
miR-493-5p
miR-493-5p+pcDNA
miR-493-5p+pcDNA-OTUB1
Number of invaded cells
Hep3B
HuH-7

Figure 4. OTUB1 was verified as a functional target of miR-493-5p and miR-493-5p that exerts its influence on HCC cells through targeting OTUB1. (A) Predicted binding sequence between miR-493-5p and OTUB1 3' UTR. (B and C) Luciferase reporter assay shows that OTUB1 3' UTR could be targeted by miR-493-5p both in Hep3B and HuH7 cells. (D) Expression of OTUB1 at the mRNA level in HCC cells and normal cells. (E) Expression of OTUB1 at the protein level in HCC cells and normal cells. Effects of altered miR-493-5p expression (overexpression and underexpression) on the expression of OTUB1 at the mRNA level (F) and protein level (G and H) in Hep3B and HuH7 cells. (I) The efficiency of OTUB1 overexpression was detected by western blot. Hep3B and HuH7 cells were transfected with miR-493-5p+pcDNA-OTUB1 or miR-493-5p+pcDNA. (J and K) Proliferation of Hep3B and HuH7 cells after transfection. (L) Apoptosis of Hep3B and HuH7 cells after transfection. (M) Migration of Hep3B and HuH7 cells after transfection. (N) Invasion of Hep3B and HuH7 cells after transfection. $^{*}P<0.05$.

OTUB1 transfection accelerated migration and invasion inhibited by si-GAS6-AS2 (**Figure 5F** and **5G**). In summary, GAS6-AS2 promoted proliferation and metastasis but inhibited apoptosis of HCC cells by regulating the GAS6-AS2/miR-493-5p/OTUB1 axis.

GAS6-AS2 regulates the activation of PI3K/AKT/FoxO3a pathway through miR-493-5p/OTUB1 axis

To test whether GAS6-AS2 regulated cell proliferation, apoptosis, and metastasis through activation of the PI3K/AKT/FoxO3a signaling pathway, the related protein expression was detected in Hep3B and HuH-7 cells. The results showed that low levels of p-PI3K, p-AKT, and p-FoxO3a were observed in Hep3B (**Figure 6A**) and HuH-7 (**Figure 6B**) cells transfected with si-GAS6-AS2 compared with the si-con group, which was strengthened in Hep3B and HuH-7 cells transfected with si-GAS6-AS2+anti-miR-493-5p compared with si-GAS6-AS2+anti-miR-con transfection. Additionally, the expression levels of p-PI3K, p-AKT, and p-FoxO3a were enhanced in Hep3B and HuH-7 cells transfected with si-GAS6-AS2+pcDNA-OTUB1 compared with si-GAS6-AS2+pcDNA. These data demonstrated that activation of the PI3K/AKT/FoxO3a pathway may be a mechanism mediating the function of GAS6-AS2/miR-493-5p/OTUB1 axis in HCC cells.

Discussion

Currently, HCC is still a devastating cancer with limited treatment options [20]. The occurrence and metastasis of HCC is a complex biologic process, involving abnormal expression of diverse genes and dysregulation of several signaling pathways [21]. In the present study, our data revealed that GAS6-AS2 was substantially up-regulated in HCC tissues and cells. Functional analysis revealed that a low level of GAS6-AS2 expression was associated with poor proliferation, anabatic apoptosis, limited migration, and invasion. miR-493-5p was a target of GAS6-AS2, and it directly bound to the 3' UTR of OTUB1. Moreover, we found that GAS6-AS2 regulated the growth and metastasis of HCC by activating the PI3K/AKT/FoxO3a pathway through mediating the GAS6-AS2/miR-493-5p/OTUB1 axis. This may provide valuable clues to understand the molecular mechanism of development of HCC.

Growing evidence shows that lncRNAs participate in the progress of HCC. However, the role of GAS6-AS2 was first investigated in HCC in the current paper. A previous study claimed that GAS6-AS2 was substantially up-regulated in tissues and cells of melanoma, and a lack ofof GAS6-AS2 suppressed proliferation but promoted apoptosis [22]. Another study mentioned that the expression of GAS6-AS2 was also elevated in tissues and cells of bladder cancer, and it induced proliferative activity and promoted metastatic capacity of bladder cancer cells [23]. Consistent with these former studies, our results showed that the expression of GAS6-AS2 was enhanced in HCC specimen tissues and cell lines, and, GAS6-AS2 facilitated HCC cell growth and metastasis. These data suggested that GAS6-AS2 may act as an oncogene in most types of tumors.

We established that GAS6-AS2 functions as a ceRNA by competitively targeting of miR-493-5p in this paper. MiR-493-5p serving as a tumor suppressor has been already identified in HCC. Gailhouste *et al.* held the view that miR-493-5p enrichment inhibited cell proliferation and migration in HepG2 and HuH7 cells *in vitro*, and impeded tumor growth *in vivo* [24]. Zhao *et al*. declared that miR-493-5p expression was remarkably decreased in HCC tissues, and accumulation of miR-493-5p could lower cell proliferation and induce apoptosis [21]. In

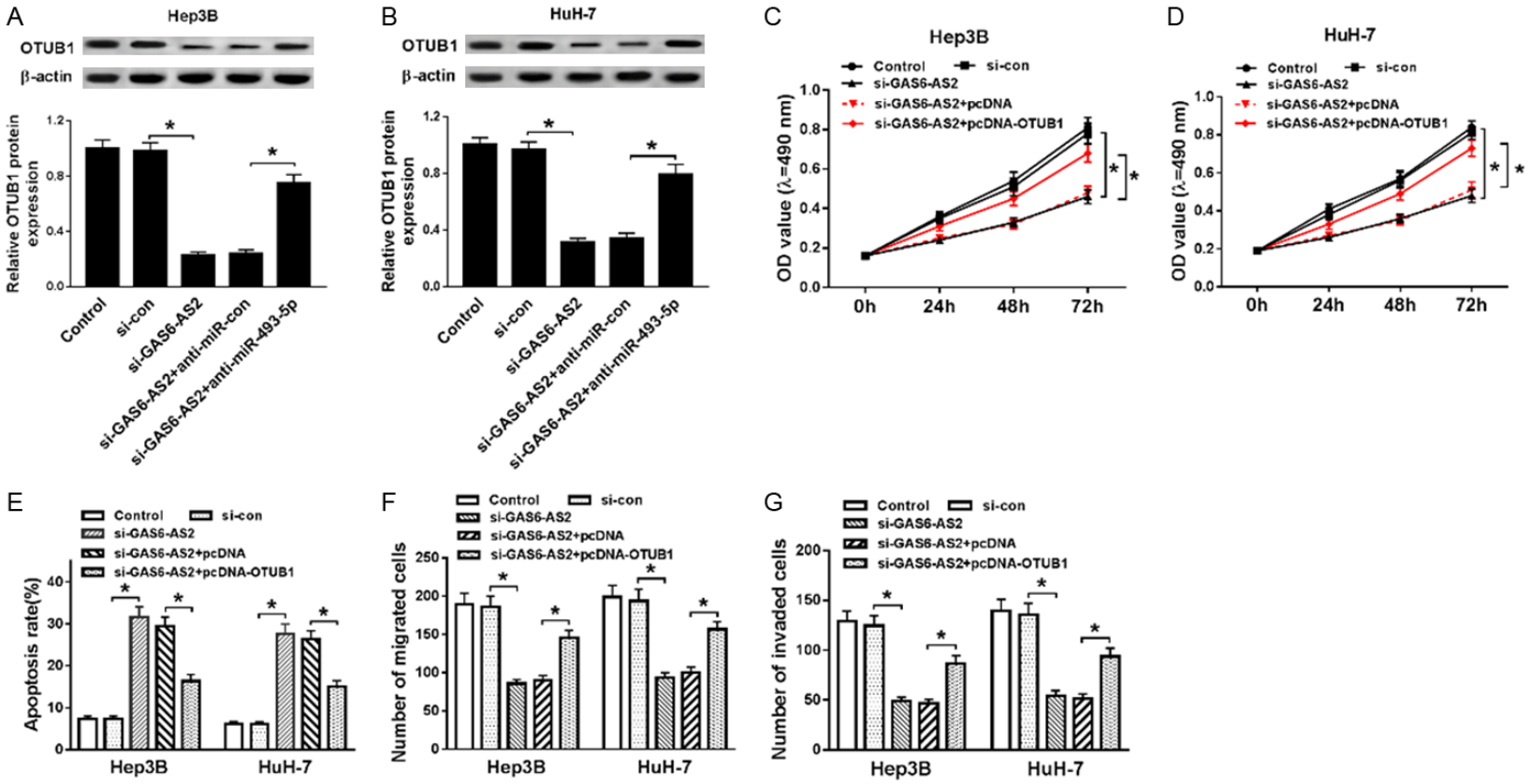

Figure 5. GAS6-AS2 knockdown regulated proliferation, apoptosis, migration, and invasion by inhibiting the expression of OTUB1. A and B. The expression of OTUB1 was assessed in Hep3B and HuH7 cells transfected with si-GAS6-AS2 or si-GAS6-AS2+anti-miR-493-5p and their corresponding control si-con and si-GAS6-AS2+anti-miR-con. C and D. Cell proliferation was measured in Hep3B and HuH7 cells transfected with si-GAS6-AS2 or si-GAS6-AS2+pcDNA-OTUB1 and their corresponding control si-con and si-GAS6-AS2+pcDNA. E. Cell apoptosis after transfection. F. Cell migration after transfection. G. Cell invasion after transfection. $^{*}P<0.05$.

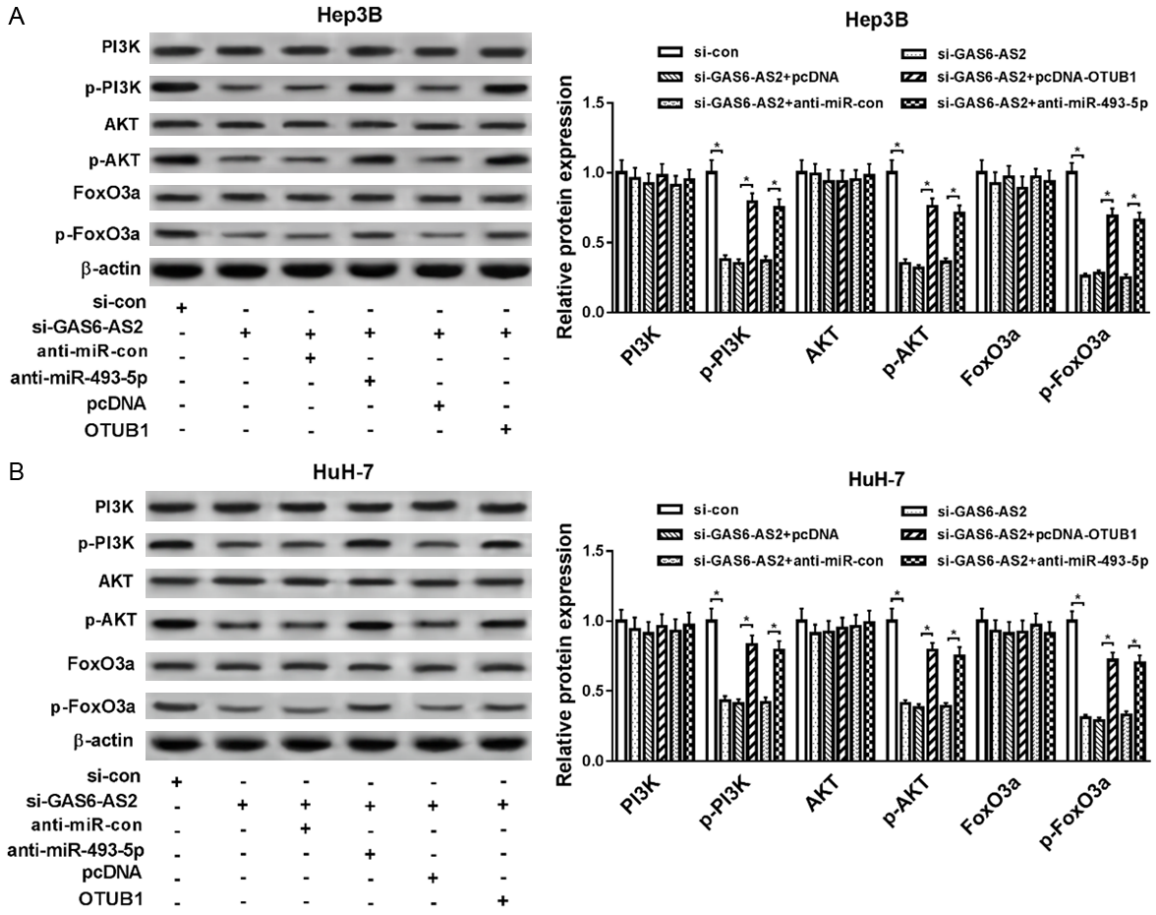

Figure 6. OGFRP1 regulates the activation of PI3K/AKT/GSK-3b pathway by mediating the miR-493-5p/OTUB1 axis. Western blot showing the expression of p-PI3K, p-AKT and p-FoxO3a both in (A) Hep3B and (B) HuH7 cells after different transfections including si-GAS6-AS2, si-GAS6-AS2+pcDNA-OTUB1 or si-GAS6-AS2+anti-miR-493-5p and their corresponding control si-con, si-GAS6-AS2+pcDNA or si-GAS6-AS2+anti-miR-con. *P<0.05.

agreement with these studies, our analyses exhibited that miR-493-5p expression was weakened in HCC tissues, and could antagonize the most malignant behaviors of HCC cells.

Further probing the functional mechanism of GAS6-AS2/miR-493-5p axis, we noticed that miR-493-5p indeed interacted with OTUB1 and restrained the expression of OTUB1 both from mRNA and protein levels. OTUB1 has been emphasized to play crucial roles in the progress of many types of cancers, particularly in HCC. One study asserted that the disruption of OTUB1 expression suppressed proliferation, migration, and invasion in HCC [25]. In accordance with the previous study, our paper found that overexpression of OTUB1 reversed the function of miR-493-5p enrichment or GAS6-AS2 knockdown, and allowed tumor growth and metastasis of HCC.

Additionally, the prior study illustrated that the PI3K/AKT signaling pathway was associated with the occurrence and metastasis of various cancers, including HCC [26-28]. FoxO3a is a member of the forkhead box O (FoxO) family and plays its role downstream of PI3K/AKT pathway, generally serving as a tumor suppressor in various cancers [29]. Activation of the PI3K/AKT pathway phosphorylates FoxO3a, leading to the loss of FoxO3a transcriptional activity [30]. Therefore, FoxO3a can be activated by weakening activity of the PI3K/AKT signaling pathway [31, 32]. In our study, GAS6-AS2 knockdown reduced the activity of PI3K/AKT, thereby preventing the phosphorylation of FoxO3a, stimulating the expression activity of FoxO3a, and presenting inhibition of tumor growth *in vitro*. Thus, increasing of FoxO3a activity may be an important cancer therapeutic strategy. Nevertheless, the above effects

could be reversed by miR-493-5p inhibition or OTUB1 overexpression. The result implied that PI3K/AKT/ FoxO3a signaling pathway was regulated by the GAS6-AS2/miR-493-5p/OTUB1 axis.

Taken together, we discovered that lncRNA GAS6-AS2 was up-regulated in HCC tissues and cells, and contributed to tumor growth and metastasis *in vitro*. We further revealed a potential mechanism by which GAS6-AS2 promoted tumorigenesis and metastasis of HCC by activating PI3K/AKT/FoxO3a signaling pathway through modulating GAS6-AS2/miR-493-5p/OTUB1 regulatory axis. Our investigation provides a biologic target for clinical treatment and prognosis of HCC.

Disclosure of conflict of interest

None.

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