

GPAA1 promotes the proliferation, invasion and migration of hepatocellular carcinoma cells by binding to RNA-binding protein SF3B4

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Abstract. It has previously been reported that glycosylphosphatidylinositol anchor attachment 1 (GPAA1) is overexpressed in hepatocellular carcinoma (HCC); however, its role in regulating the development of HCC remains unknown. The present study aimed to examine the potential role of GPAA1 in HCC and to characterize the associated mechanism. The expression of GPAA1 was first examined using the Gene Expression Profiling Interactive Analysis 2 database, and was then determined using reverse transcription-quantitative PCR and western blotting. The effects of GPAA1 silencing on the proliferation, colony formation, migration and invasion of HuH-7 cells were measured using Cell Counting Kit-8, colony formation, wound healing and Transwell assays, respectively. The interaction between splicing factor (SF)3B4 and GPAA1 was predicted by starBase and confirmed using RNA immunoprecipitation. The results of the present study demonstrated that GPAA1 was upregulated in HCC cells, and silencing GPAA1 markedly inhibited the proliferation, migration and invasion of HCC cells, which was accompanied by reduced levels of MMP2 and MMP9. In addition, it was observed that SF3B4 could bind to GPAA1. Furthermore, to confirm whether SF3B4 binds to GPAA1 to modulate HCC cell behavior, GPAA1 was knocked down and SF3B4 was overexpressed. Overexpression of SF3B4 reversed the effects of GPAA1 knockdown on the proliferation, migration and invasion of HCC cells. In conclusion, SF3B4 may promote the proliferation, invasion and migration of HCC cells by binding to GPAA1. The present study provided novel insight into the pathogenesis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent types of cancer and the second leading cause of cancer-related death worldwide (1). The independent risk factors for the occurrence and development of HCC include excessive intake of alcohol, smoking and obesity (2). Surgery is the preferred option for the treatment of HCC, although it is associated with a poor prognosis and a low 5-year survival rate (3). Furthermore, most patients are diagnosed at an advanced stage, which increases the complexity of surgery (4). Therefore, there is an urgent need to understand the pathogenesis of HCC in order to develop effective therapies against this disease.

Glycosylphosphatidylinositol (GPI) anchor attachment 1 (GPAA1) is one of the subunits of the GPI transferase complex, which serves as a link between GPI anchor sites and proteins (5). It has been reported that GPAA1 is upregulated in various types of cancer and that GPAA1 promotes disease progression by regulating C-Myc in childhood acute lymphoblastic leukemia (6). Moreover, overexpression of GPAA1 has been shown to promote tumorigenicity and invasiveness of breast cancer cells in nude mice (7). GPAA1 may also promote the metastasis and invasion of gastric cancer (5). Although GPAA1 has been reported to be upregulated in HCC, in-depth studies on its potential role in HCC are still lacking (8).

The starBase database suggested that the RNA-binding protein splicing factor (SF)3B4 may interact with GPAA1. Alternative splicing is an important step during gene transcription that allows the generation of multiple mRNA transcripts from one specific gene (9). Alternative splicing factors, which have been extensively studied in a wide variety of disorders and tumors, serve an essential role in the progression of cancer and the occurrence of chemoresistance (10-12). RNA splicing is modulated by U2 and U12 small nuclear ribonucleoprotein (snRNP)-dependent spliceosomes, and the U2 snRNP consists of U2 snRNA and the SF3A/SF3B complex (13). It has been identified that, among the six subunits of the SF3B complex, SF3B4 is upregulated in patients with HCC (14). SF3B4 can also be used as a diagnostic marker for HCC. Compared with the current diagnostic markers used for HCC (GPC3, GS and HSP70), SF3B4 combined with BANF1 and PLOD3 has been reported to exhibit stronger diagnostic efficacy for early HCC (15). Furthermore, this protein serves an oncogenic role in other types of cancer, such as pancreatic cancer and

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esophageal squamous cell carcinoma (16,17). Therefore, it was hypothesized in the present study that the upregulation of GPAA1, which may be regulated by SF3B4, could promote the progression of HCC.

Materials and methods

Cell culture. The normal human liver MIHA cell line, and HCC Hep10, HuH-7, SNU-387 and Hep 3B2.1-7 cell lines were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and streptomycin in a humidified atmosphere at 37°C with 5% CO₂.

Cell transfection. To knock down the expression of GPAA1 and SF3B4, small interfering RNA (siRNA) molecules targeting GPAA1 [si-GPAA1#1 (siG000008733A-1-5) and si-GPAA1#2 (siG000008733B-1-5)] and SF3B4 [si-SF3B4#1 (siG000010262A-1-5) and si-SF3B4#2 (siG000010262B-1-5)] were purchased from Guangzhou RiboBio Co., Ltd. In addition, a scrambled siRNA negative control (si-NC; cat. no. A06001) was designed and synthesized by Shanghai GenePharma Co., Ltd. The SF3B4 overexpression (oe-SF3B4) plasmid was constructed by cloning the full length of the SF3B4 sequence into the pcDNA3.1 vector obtained from Shanghai GenePharma Co., Ltd. The empty vector pcDNA3.1 is referred to as the control (oe-NC) plasmid. HuH-7 cells were plated in 24-well dishes at a density of 1x10⁶ cells/well, and plasmid transfection was performed at a concentration of 50 ng/ml using Lipofectamine™ 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37°C according to the manufacturer's protocols. The transfection efficiency was detected 48 h post-transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from MIHA, Hep10, HuH-7, SNU-387 and Hep3B cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using the PrimeScript™ RT MasterMix kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using the LightCycler 480 Probes Master kit (Roche Applied Science) on a LightCycler 480 system (Roche Applied Science). The following thermocycling conditions were used for qPCR: 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 60 sec. The primer sequences were as follows: GPAA1, forward 5'-CTCCCGCTTCGTCTCCATC-3' and reverse 5'-CACTGGCAGGACATAGAGGG-3'; SF3B4, forward 5'-AGACGGCGGGATCTCTTT-3' and reverse 5'-CACGTACACAGTGGCATCT-3'; and GAPDH, forward 5'-CATCACTGCCACCCAGAAGA-3' and reverse 5'-CCACCTGGTGTCTAGTGTAG-3'. Target mRNA expression was calculated using the 2^{-ΔΔC_q} method and normalized to GAPDH levels (18).

Western blotting. MIHA, Hep10, HuH-7, SNU-387 and Hep3B cells were lysed in cell lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), and protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific,

Inc.). The cell lysates containing equal amounts of protein (30 μg per lane) were resolved by 10% SDS-PAGE and were then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skimmed milk powder for 2 h at room temperature in 0.1% TBS-Tween (TBST) buffer, then incubated with the primary antibodies against GPAA1 (cat. no. PA5-100548; dilution, 1:1,000; Thermo Fisher Scientific, Inc.), MMP2 (cat. no. ab92536; dilution, 1:1,000; Abcam), MMP9 (cat. no. ab76003; dilution, 1:1,000; Abcam), SF3B4 (cat. no. ab157117; dilution, 1:1,000; Abcam) and GAPDH (cat. no. ab9485; dilution, 1:2,500; Abcam) at 4°C overnight. After washing in TBST three times, the membranes were incubated with a secondary antibody (cat. no. ab6721; dilution, 1:2,000; Abcam) at room temperature for 1 h. GAPDH was used as the loading control. The protein bands were developed using a chemiluminescence detection kit (Cytiva) and the band densities of the target proteins were semi-quantified using ImageJ 1.51 (National Institutes of Health).

Cell proliferation. To examine proliferation, HuH-7 cells were seeded at a density of 4x10³ cells/well in 24-well plates. After 24, 48 and 72 h of culture, the cells were incubated with 10 μl Cell Counting Kit-8 reagent (Thermo Fisher Scientific, Inc.) at 37°C for 4 h. Subsequently, the absorbance was measured at 450 nm using a microplate reader. The cell proliferation rate (%) was calculated via optical density (OD) using the following formula: (Experimental OD-control OD)/control OD x100.

Colony formation assay. The transfected HuH-7 cells were collected and inoculated into 24-well dishes at a density of 4x10³ cells/well. The medium was replaced every 4 days. After 2 weeks, the colonies that had formed were washed in PBS three times, fixed with 1% paraformaldehyde for 15 min at room temperature, then stained with 0.1% crystal violet for 30 min at room temperature. After washing and drying the colonies, images were captured using a light microscope.

Cell migration. HuH-7 cells were seeded at a density of 2x10⁴ cells/well into 6-well plates. After 24 h of cell culture, when cells were cultured to 100% confluence, a wound was made in the cell monolayer using a 200-μl pipette tip. After washing, the medium was replaced with serum-free medium. Images were captured under an inverted light microscope at 0 and 24 h using the following equation: (Initial width at 0 h-final width at 24 h)/initial width at 0 h.

Cell invasion. The invasion of HuH-7 cells was determined using 24-well Transwell chambers with 8-μm pores (Corning, Inc.) coated with Matrigel® (BD Biosciences) at room temperature for 24 h. HuH-7 cells were suspended in serum-free DMEM at a density of 2x10⁴ cells/well in the upper chamber of the Transwell. The lower chamber contained DMEM supplemented with 10% FBS. After 24 h of incubation at 37°C, the cells that had invaded to the lower surface of the membranes were fixed with 4% paraformaldehyde for 10 min at room temperature, then stained with 0.2% crystal violet at room temperature for 30 min. Images were captured under an inverted light microscope (Olympus CX23; Olympus Corporation).

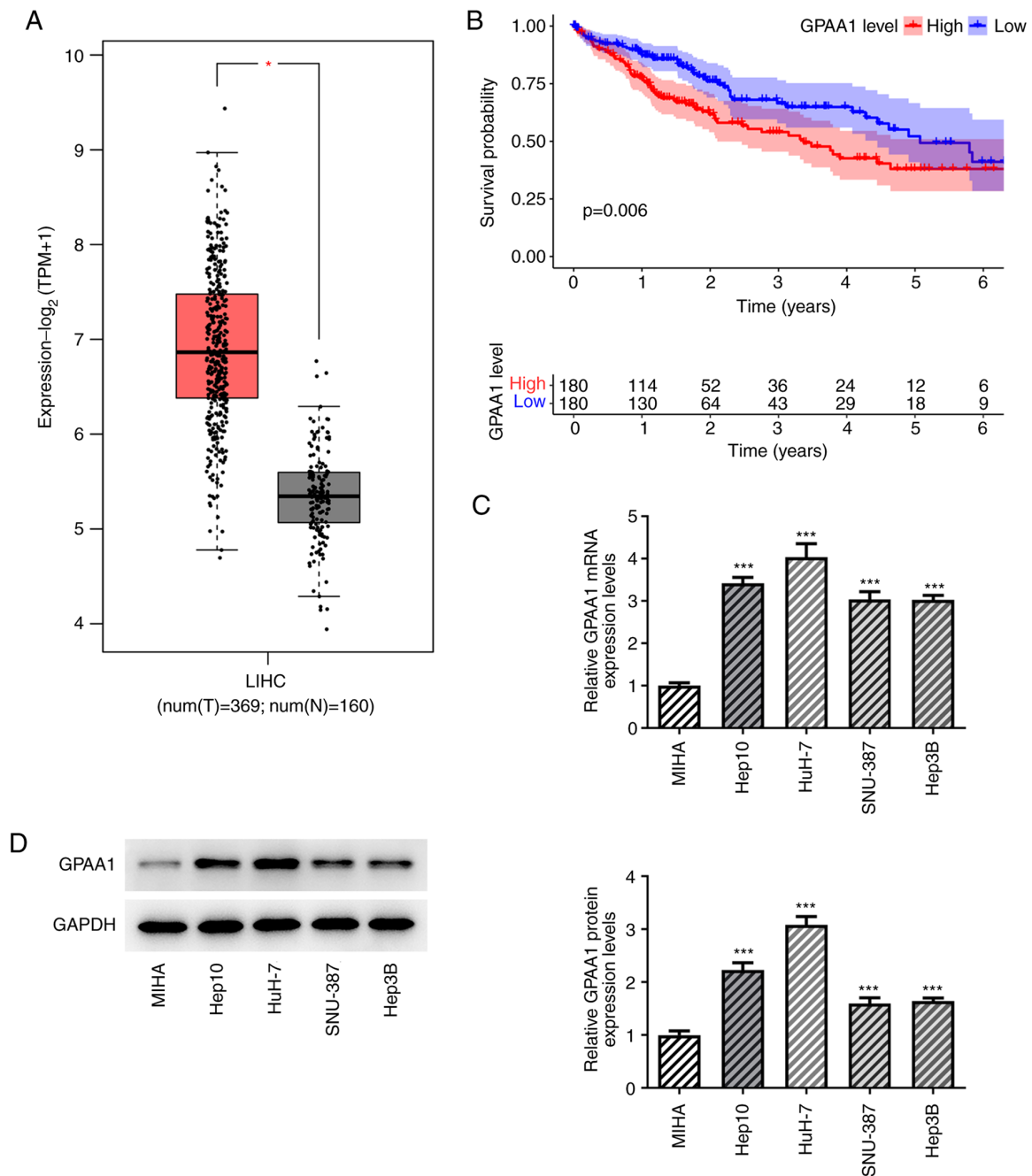


Figure 1. GPAA1 was upregulated in HCC cells. Prediction by the Gene Expression Profiling Interactive Analysis 2 database of (A) GPAA1 expression in HCC and (B) overall survival in high and low GPAA1 groups. * $P<0.05$. (C) mRNA and (D) protein expression levels of GPAA1 in HCC cells. *** $P<0.001$ vs. MIHA. LIHC, liver hepatocellular carcinoma; GPAA1, glycosylphosphatidylinositol anchor attachment 1; HCC, hepatocellular carcinoma.

RNA immunoprecipitation (RIP) assay. The RIP assay was conducted using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (MilliporeSigma) according to the manufacturer's protocol. After transfection, HuH-7 cells (1×10^7) were inoculated and lysed in 100 μ l RIP lysis buffer. The cell lysate (100 μ l) was then incubated with 50 μ l magnetic beads coupled with anti-SF3B4 antibody (cat. no. ab157117; Abcam) or control IgG (cat. no. ab172730; Abcam) in RIP buffer. The expression of GPAA1 was analyzed by RT-qPCR as previously described.

Detection of RNA stability. Transfected HuH-7 cells (6×10^5 cells/well) were plated into 24-well plates and cultured

for 24 h. Subsequently, the cells were treated with 5 μ g/ml actinomycin D (MedChemExpress) at 37°C and collected after 20, 40 or 60 min. Total RNA was extracted using the miRNeasy Kit (Qiagen GmbH), and GPAA1 expression was analyzed using RT-qPCR and western blotting.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, Inc.). Data were generated from three independent experimental repeats. The results are presented as the mean \pm standard deviation. Differences between groups were compared using Student's t-test or using one-way ANOVA followed by Tukey's post hoc test. Mantel-Cox test was to determine the overall

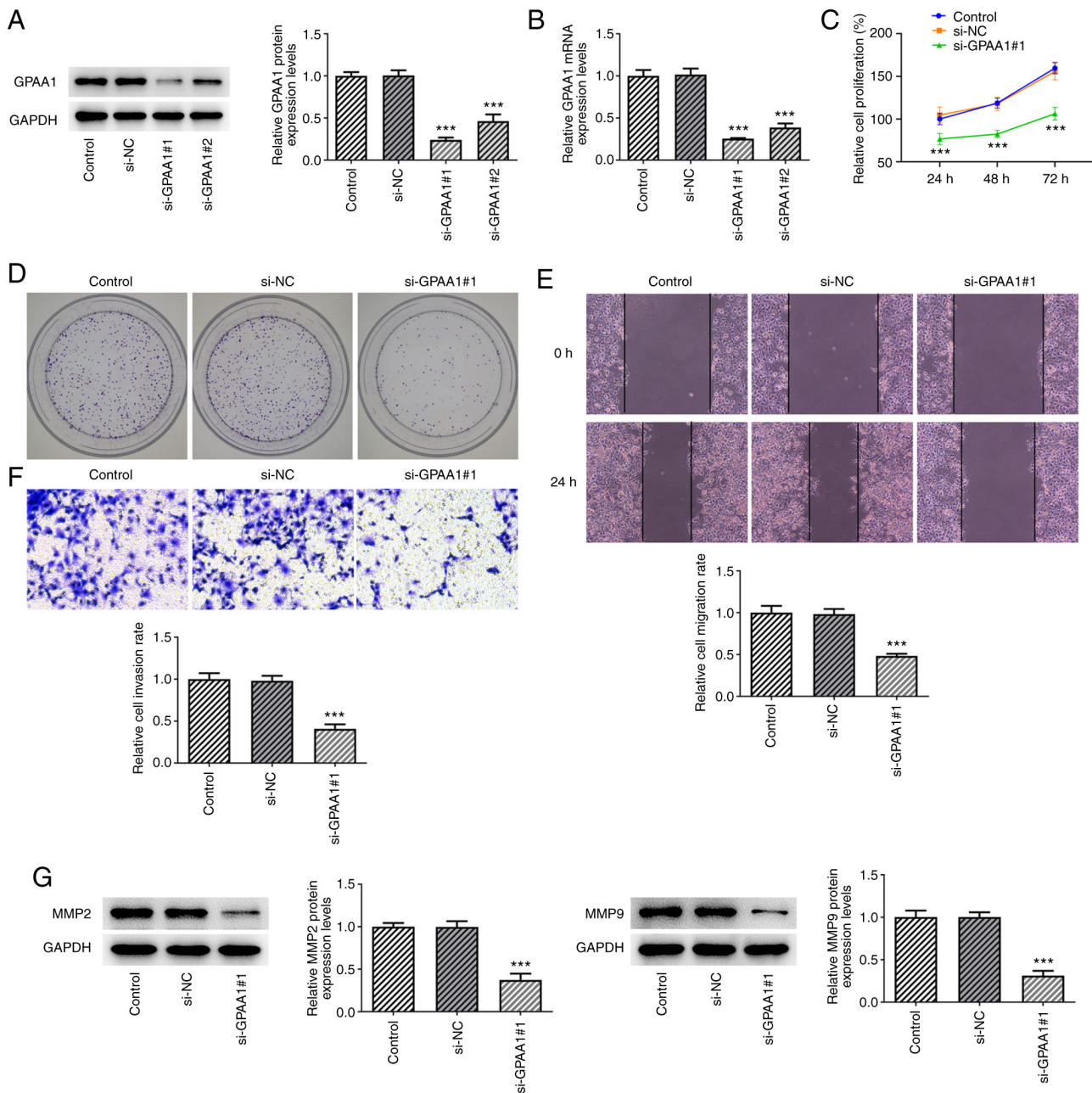


Figure 2. GPAA1 regulates the proliferation, migration and invasion of HCC cells. (A) Protein and (B) mRNA expression levels of GPAA1 in HCC cells transfected with si-GPAA1#1 and si-GPAA1#2. (C) Proliferation, (D) colony formation, (E) migration (x100 magnification), (F) invasion (x100 magnification), and (G) MMP2 and MMP9 expression in HCC cells transfected with si-GPAA1#1. *** $P < 0.001$ vs. Control. GPAA1, glycosylphosphatidylinositol anchor attachment 1; HCC, hepatocellular carcinoma; NC, negative control; si, small interfering.

survival rate of HCC patients. Pearson's correlation analysis was utilized to confirm the correlation between SF3B4 and GPAA1. $P < 0.05$ was considered to indicate a statistically significant difference.

Bioinformatics tools. GPAA1 and SF3B4 expression in HCC tissues, and the correlation between GPAA1 or SF3B4 expression and the overall survival rate of patients with HCC were analyzed based on Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database (<http://gepia2.cancer-pku.cn/#index>). The binding between SF3B4 and GPAA1 and the correlation between SF3B4 and GPAA1 in HCC was predicted by star-Base database (<https://starbase.sysu.edu.cn/>).

Results

GPAA1 is upregulated in HCC cells. To examine the role of GPAA1 in HCC, the expression of GPAA1 was examined using the GEPIA2 database. The results suggested that GPAA1 was upregulated in HCC, which may be associated with poor overall survival in patients with HCC (180 patients in high GPAA1 group and 180 patients in low GPAA1 group; divided using median expression value) (Fig. 1A and B). To validate this finding, RT-qPCR and western blotting were carried out on HCC cell lines. The expression level of GPAA1 was increased in HCC cells compared with that in the MIHA cell line, and the HuH-7 cell line exhibited

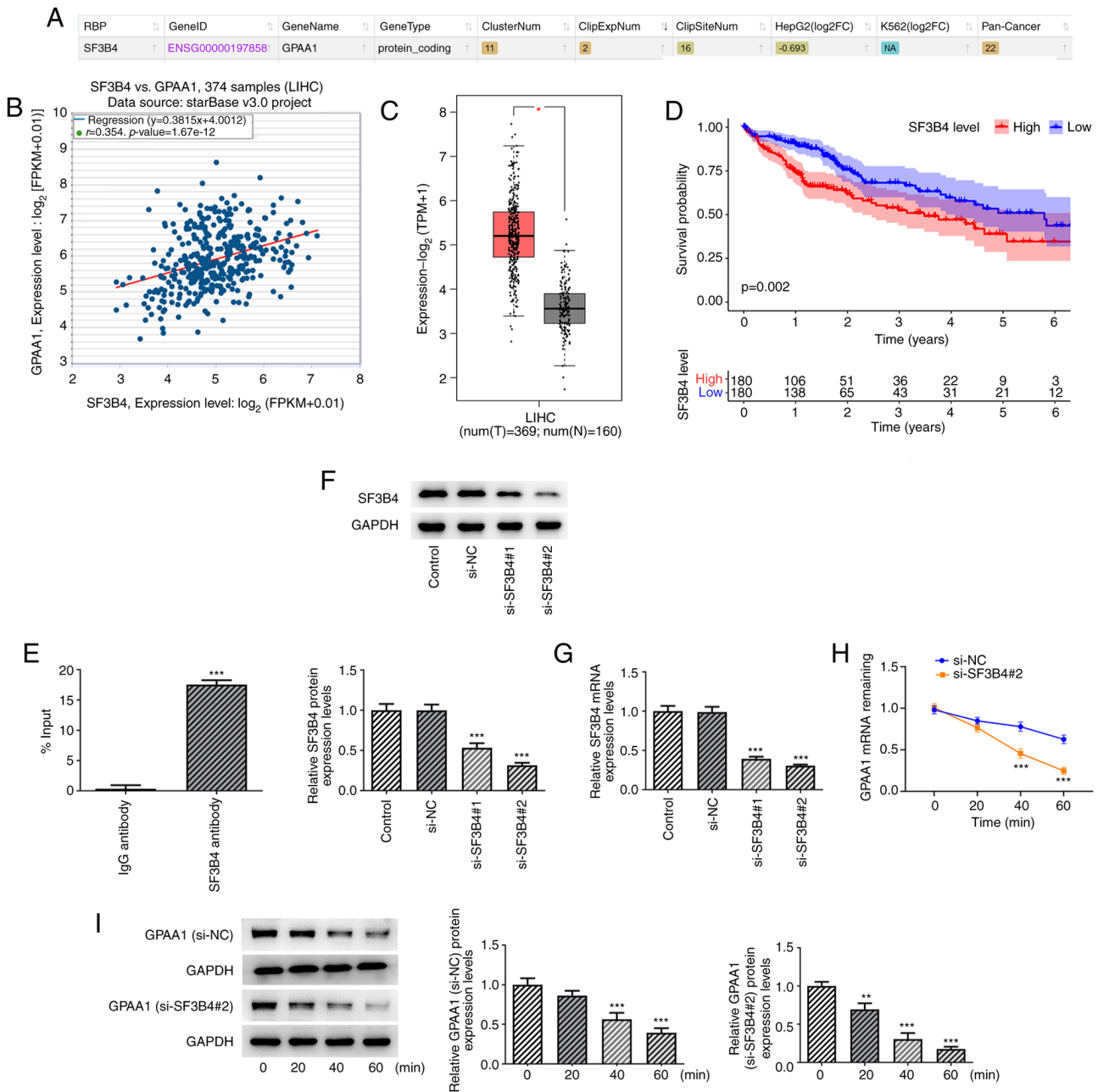


Figure 3. SF3B4 binds to and stabilizes GPAA1 mRNA. (A and B) It was predicted that SF3B4 could combine with GPAA1, and SF3B4 had a positive correlation with GPAA1 in HCC. (C) Expression of SF3B4 in HCC samples and (D) the overall survival rate of patients with HCC in high SF3B4 level and low SF3B4 level groups. *P<0.05. (E) Binding relationship between SF3B4 and GPAA1 was confirmed by RNA immunoprecipitation. ***P<0.001 vs. IgG antibody. (F) Protein and (G) mRNA expression levels of SF3B4 after transfection with si-SF3B4#1 and si-SF3B4#2. ***P<0.001 vs. si-NC. (H) mRNA stability of GPAA1 following exposure to actinomycin D. ***P<0.001 vs. si-NC. (I) Protein stability of GPAA1 following exposure to actinomycin D. **P<0.01 and ***P<0.001 vs. 0 min. LIHC, liver hepatocellular carcinoma; GPAA1, glycosylphosphatidylinositol anchor attachment 1; HCC, hepatocellular carcinoma; NC, negative control; SF3B4, splicing factor 3B4; si, small interfering.

the highest mRNA and protein expression levels of GPAA1 (Fig. 1C and D). Therefore, HuH-7 cells were used in subsequent experiments.

GPAA1 regulates the proliferation, migration and invasion of HCC cells. As GPAA1 was upregulated in HCC cells, its expression was knocked down by transfecting siRNA targeting GPAA1 into these cells. The expression levels of GPAA1 were significantly downregulated after transfection of si-GPAA1#1/2 plasmids, and the interference efficiency of si-GPAA1#1 was greater than that of si-GPAA1#2; thus, si-GPAA1#1 was used

in subsequent experiments (Fig. 2A and B). In addition, the proliferation and colony formation abilities of HuH-7 cells were reduced following si-GPAA1#1 transfection (Fig. 2C and D). Furthermore, transfection with si-GPAA1#1 inhibited the migration and invasion of HuH-7 cells, which was accompanied by MMP2 and MMP9 downregulation (Fig. 2E-G). These findings indicated that GPAA1 could regulate the proliferation, migration and invasion of HCC cells.

SF3B4 binds to and stabilizes GPAA1 mRNA. As predicted by starBase, GPAA1 was predicted to bind to SF3B4 and

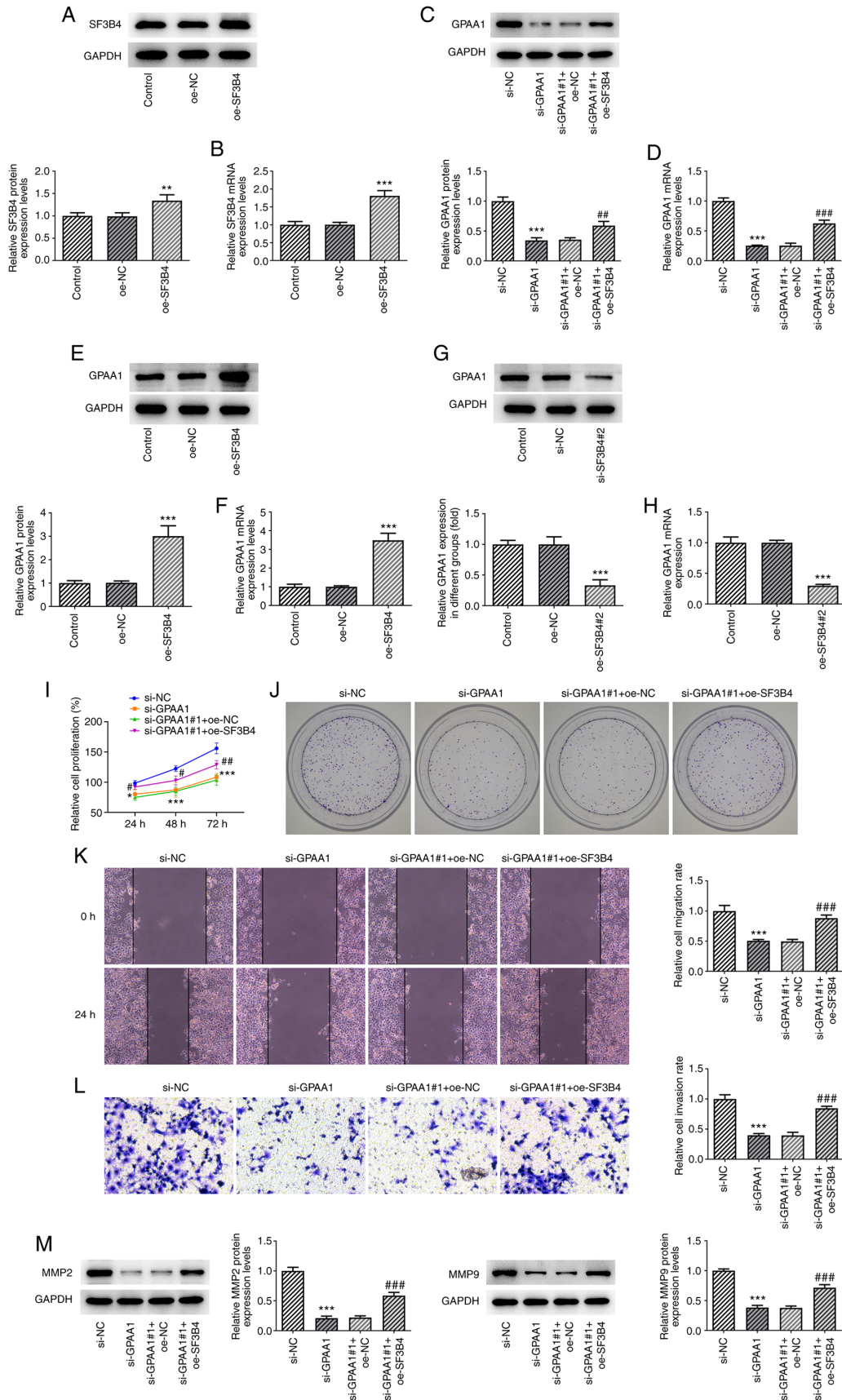


Figure 4. Overexpression of SF3B4 reverses the effects of GPAA1 knockdown on the proliferation, migration and invasion of HCC cells. (A) Protein and (B) mRNA expression levels of SF3B4 after transfection with oe-SF3B4. (C) Protein and (D) mRNA expression levels of GPAA1 after transfection with si-GPAA1 and oe-SF3B4. (E) Protein and (F) mRNA expression levels of GPAA1 after transfection with oe-SF3B4. (G) Protein and (H) mRNA expression levels of GPAA1 after transfection with si-SF3B4#2. (I) Proliferation, (J) colony formation, (K) migration (x100 magnification), (L) invasion (x100 magnification), and (M) MMP2 and MMP9 expression in HCC cells transfected with si-GPAA1 and oe-SF3B4. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. si-NC; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. si-GPAA1 + oe-NC. GPAA1, glycosylphosphatidylinositol anchor attachment 1; HCC, hepatocellular carcinoma; NC, negative control; oe, overexpression; SF3B4, splicing factor 3B4; si, small interfering.

the expression levels of GPAA1 were positively correlated with those of SF3B4 (Fig. 3A and B). Further analysis using GEPIA2 indicated that SF3B4 displayed high expression in HCC tissues and high expression levels of SF3B4 were associated with poor overall survival in patients with HCC (180 patients in high SF3B4 group and 180 patients in low SF3B4 group) (Fig. 3C and D). Thus, it was hypothesized that SF3B4 may modulate the progression of HCC by interacting with GPAA1. The results of the RIP assay confirmed that these two proteins could interact with each other (Fig. 3E). Subsequently, si-SF3B4 was transfected into HCC cells. The expression levels of SF3B4 were lowest in the si-SF3B4#2 group; therefore, this siRNA was used for subsequent experiments (Fig. 3F and G). Following treatment with actinomycin D, si-SF3B4 transfection reduced the mRNA and protein stability of GPAA1 (Fig. 3H and I). These findings suggested that SF3B4 could bind to and stabilize GPAA1 mRNA.

SF3B4 overexpression reverses the effects of GPAA1 knockdown on the proliferation, migration and invasion of HCC cells. To investigate whether SF3B4 exerted effects on the progression of HCC cells by binding to GPAA1, SF3B4 was overexpressed in HCC cells; the transfection efficiency of oe-SF3B4 was confirmed by western blotting and RT-qPCR (Fig. 4A and B). As shown in Fig. 4C and D, the si-GPAA1-induced inhibition of GPAA1 was abrogated by oe-SF3B4. In addition, SF3B4 overexpression promoted the expression levels of GPAA1 (Fig. 4E and F), whereas SF3B4 knockdown suppressed the expression levels of GPAA1 (Fig. 4G and H). Moreover, the reduction in proliferation and colony formation of HuH-7 cells mediated by si-GPAA1 was abolished by SF3B4 overexpression (Fig. 4I and J). Additionally, GPAA1 knockdown reduced the migration and invasion of HuH-7 cells, which was reversed following oe-SF3B4 transfection (Fig. 4K and L). The expression levels of MMP2 and MMP9 were also suppressed following si-GPAA1 transfection, which was reversed by oe-SF3B4 transfection (Fig. 4M). These findings indicated that the overexpression of SF3B4 reversed the effects of GPAA1 knockdown on the proliferation, migration and invasion of HCC cells.

Discussion

Several studies have focused on GPAA1, due to its functional role in cancer development (8). It has been proposed that GPAA1 may increase the activity of the GPI transamidase complex, which mediates the transfer of a GPI anchor to the C-terminus of target proteins without transmembrane domain proteins (19,20). The regulatory role of GPAA1 in the progression of numerous types of cancer has been well documented in previous years. For example, GPAA1 has been reported to regulate the expression of GPI-anchored proteins and promote the ERBB signaling pathway, thus contributing to tumor growth in gastric cancer (5). Moreover, the upregulation of GPAA1 in patients with colorectal cancer has highlighted its potential significance in regulating the proliferation, invasion and metastasis of this type of cancer (21). It has been previously reported that GPAA1 is expressed at high levels in HCC compared with in matched adjacent non-tumor tissue samples, suggesting that GPAA1 expression may be associated with HCC progression

and poor survival rate (8). In the present study, it was predicted by the GEPIA2 database and further confirmed by subsequent experiments that GPAA1 was highly expressed in HCC cells. Transfection with si-GPAA1 resulted in significantly reduced cell proliferation, fewer numbers of colonies, and decreased migratory and invasive capacities in HuH-7 cells.

It is well established that alternative splicing of pre-mRNA is a common phenomenon that governs the diversity of the proteome (22). Dysregulation of alternative splicing, which is usually seen in tumor cells, can regulate the malignant behavior of cells, including proliferation, angiogenesis, invasion and metastasis (23). Alternative splicing is one of the most important processes that can affect cellular functions; it is mediated by splicing factors, which are regulatory proteins expressed intracellularly (23). A previous study also indicated the importance of alternative splicing as a source of HCC prognostic markers (24). SF3B4 has been demonstrated to serve an oncogenic role in various tumor types and is associated with a poor prognosis (13,14,16). In the present study, it was demonstrated that the expression of GPAA1 was positively correlated with that of SF3B4, and was associated with a poor prognosis in patients with HCC. RIP experiments confirmed the interaction between GPAA1 and SF3B4. Furthermore, SF3B4 knockdown reduced the mRNA stability of GPAA1. Comprehensive meta-analyses on gene profiles have suggested that upregulation of SF3B4 in HCC may be linked to a poor prognosis in patients with HCC, consistent with previous findings regarding the role of GPAA1 in HCC (25). Thus, it was hypothesized that SF3B4 may exert its effects on HCC cells by binding to GPAA1. In comparison to the HuH-7 cells transfected with si-GPAA1 alone, co-transfection with si-GPAA1 and oe-SF3B4 resulted in increased mRNA and protein expression levels of GPAA1, demonstrating the participation of SF3B4 in the mechanism underlying the pathogenesis of HCC. As anticipated, the proliferation, migration and invasion of HuH-7 cells, which were significantly inhibited by GPAA1 knockdown, were increased following SF3B4 overexpression.

In conclusion, SF3B4 may promote the proliferation, invasion and migration of HCC cells by binding to GPAA1. This finding provides novel insight into the pathogenesis of HCC. Further studies are required to confirm this conclusion in *in vivo* models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

SG and XY conceptualized and designed the present study. SG and QZ acquired, analyzed and interpreted data. SG drafted

the manuscript and XY revised it critically for important intellectual content. SQ, QZ and XY confirm the authenticity of all the raw data. All authors approved the final manuscript for submission.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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