Original Article

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SNRPA promotes hepatocellular carcinoma proliferation and lenvatinib resistance *via* B7-H6-STAT3/AKT axis by facilitating B7-H6 pre-mRNA maturation

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SUMMARY: The pre-mRNAs splicing is important mechanisms of hepatocellular carcinoma (HCC) progression. Hence, this study aimed to explore the function and corresponding mechanisms of small nuclear ribonucleoprotein polypeptide A (SNRPA), a vital RNAs splicing molecule, in HCC. Here, the University of Alabama at Birmingham CANcer data analysis portal (UALCAN), western blotting, and immunohistochemistry indicated that SNRPA levels were elevated in HCC tissues. Moreover, high expression of SNRPA was correlated with unfavorable clinicopathologic features and poor survival in HCC patients. A series of *in vitro* and *in vivo* gain/loss-of-function experiments reported that SNRPA promoted the proliferation of HCC cells. Integrated nanopore full-length cDNA sequencing and RNA-binding protein immunoprecipitation sequencing revealed that B7 homologue 6 (B7-H6) was a potential target of SNRPA. Subsequently, western blotting and flow cytometry showed that SNRPA activated B7-H6-STAT3/AKT signaling axis in HCC cells with promotion of G1-S transition in the cell cycle and inhibition of cell apoptosis. Mechanistically, RNA-binding protein immunoprecipitation and polymerase chain reaction with using exon-exon and exon-intron junction primers revealed that SNRPA facilitated B7-H6 pre-mRNA maturation by binding to it directly and contributing to its intron 2 splicing. Moreover, drug sensitivity test found that SNRPA induced HCC cell resistance to lenvatinib. Finally, restoration experiments demonstrated that the effects of SNRPA on HCC cells relied on B7-H6 expression. Taken together, SNRPA promotes HCC growth and lenvatinib resistance *via* B7-H6-STAT3/AKT axis through facilitating B7-H6 pre-mRNA maturation by maintaining its intron 2 splicing. Thus, SNRPA may be a promising target for HCC therapy and lenvatinib resistance reversion.

Keywords: hepatocellular carcinoma, SNRPA, B7-H6, pre-mRNA maturation, lenvatinib resistance

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent type of cancer, and the third leading cause of cancer-related death worldwide (*1*). For the past several decades, surgery (including liver resection and transplantation) remains a primary treatment modality for patients with early-stage HCC (*2,3*). Although numerous molecular target drugs, such as tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs), have been utilized for the treatment of patients with advanced-stage HCC, their effects were limited (*4,5*). This is mainly due to the heterogeneity in the etiology of HCC and various factors resulting in drug resistance (*6,7*). Therefore, there is an urgent need to discover novel molecular targets for HCC therapy and reversion of drug resistance.

The splicing and removal of the introns of pre-mRNAs is an important process in pre-mRNA maturation. In this process, normal and abnormal splicing are regulated to produce various transcripts with different functions (alternative splicing), thereby enriching the genetic diversity (*8-10*). In recent years, accumulating evidence indicates that both normal and abnormal pre-mRNA splicing play an essential role in tumor progression, therapeutic resistance, and adaptation to harsh microenvironments (*11-14*). The small nuclear ribonucleoprotein polypeptide A (SNRPA) gene coding the U1A protein is a major component of the spliceosome U1 small nuclear ribonucleoprotein, which is intimately associated with RNA splicing, modification, and decay (*15-17*). Several studies have reported that SNRPA enhanced tumor progression in gastric cancer and colorectal cancer (*18-20*). Using bioinformatic analysis,

Zhang *et al*. found that elevated SNRPA expression was associated with poor survival in HCC patients (*21*). In addition, it was recently reported that SNRPA promoted HCC metastasis with microvascular invasion (*22*). However, above studies have not demonstrated that SNRPA aggravates HCC and other cancers *via* pre-mRNA splicing, which is the most essential and direct function of SNRPA. Thus, in this study, we sought to identify the mechanism of pre-mRNA splicing driven by SNRPA in HCC progression.

Coded by the NCR3LG1 gene, B7 homologue 6 (B7-H6) protein is a new member of the B7 family discovered by Brandt *et al*. in 2009 using bioinformatics and mass spectrometry (*23*). Human B7-H6 is rarely detected in normal human tissues; nevertheless, it was frequently present in various human tumors (*23,24*), including HCC, breast cancer, and gastric cancer (*25-27*). As a transmembrane protein, B7-H6 plays an important role in tumorigenesis *via* an immunological mechanism (*28-30*). In recent years, an increasing number of studies find that B7-H6 promotes tumor progression by regulating cell cycle and apoptosis *via* a non-immunological action. This process primarily includes the activation of signal transducer and activator of transcription 3 (STAT3), protein kinase B (AKT), and extracellular signal-regulated kinase (ERK) signaling pathways (*27,31-33*). Considering the absence of B7-H6 in normal tissues and its relative abundance among tumor tissues, its expression may be a response to tumorigenesis. Naturally, the study of the mechanism underlying the expression of B7-H6 in tumors may provide a novel treatment modality for patients with HCC.

In this study, we identified that SNRPA-B7-H6-STAT3/AKT axis plays a critical role in HCC cell proliferation and lenvatinib resistance through a mechanism of B7-H6 pre-mRNA maturation facilitated by SNRPA. Collectively, SNRPA may be a promising target molecule for HCC therapy and reversal of resistance to lenvatinib in HCC patients.

2. Materials and Methods

2.1. Human tissue samples

Initially, 12 pairs of fresh HCC tissues and matched para-tumor tissues from the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China) were collected to detect the SNRPA expression through western blotting. A total of 85 and 97 pairs of HCC tissues and corresponding adjacent tumor tissues, respectively, were obtained from the Second Affiliated Hospital of Chongqing Medical University and OUTDO BIOTECH (cat.no. HLivH180Su31; OUTDO BIOTECH, Shanghai, China). The tissues were utilized in immunohistochemistry staining to detect the SNRPA protein levels and analyze the correlation of SNRPA expression and the survival of patients with HCC. The selection criteria were as follows. Inclusion criteria: 1) patients were diagnosed as HCC by biopsy after surgery, 2) patients had no immunodeficiency disease; exclusion criteria: 1) patients suffered from other cancers, 2) patients had received chemotherapy, radiofrequency ablation, or molecular targeted therapy before liver resection. This research was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval number RER2022-637A). All patients provided informed consent.

2.2. Xenograft models

Indicated HCC cells (2.5×10^6/mice) were suspended in cool phosphate buffer saline and subsequently injected into the right hindlimb of nude mice (male, 4-week-old) subcutaneously. Additionally, we sought to investigate the impact of SNRPA on the sensitivity of HCC cells to lenvatinib *in vivo*. For this purpose, 10 days after the transplantation of indicated cells, the nude mice received treatment with lenvatinib once daily (10 mg/kg) *via* oral gavage. Tumor size was measured with a caliper every 3 days; the formula for the size calculation was as follows: volume = (length × width^2)/2 cm^3. Three weeks after implantation, the nude mice were killed by cervical dislocation and the tumors were removed. These experiments were approved by the Animal Ethics Committee of Chongqing Medical University (approval number RER2021-136X).

2.3. Other Methods

Detailed materials and methods including western blotting, immunohistochemistry, cell culture, lentivirus infection, cell counting Kit-8 test, EdU assay, colony formation assay, scratch wound healing assay, nanopore full-length cDNA sequencing, RNA-binding protein immunoprecipitation, flow cytometry for cell cycle analysis, flow cytometry for cell apoptosis detection, small-interfering RNA transfection, reverse transcription-polymerase chain reaction, quantitative real-time polymerase chain reaction, bioinformatic analysis, and statistical analysis are described in Supplemental Data. Additionally, antibodies, primers, and targeted sequences used in this project can be found in Supplemental Tables S1-S5 (*https://www.biosciencetrends.com/supplementaldata/253*).

3. Results

3.1. SNRPA is frequently elevated in HCC tissues, and its elevation predicts poor survival in HCC patients.

Analysis using the UALCAN revealed that both the mRNA (Figure 1A) and protein (Figure 1B) levels of SNRPA were increased in HCC tissues compared

with normal liver tissues. In addition, the expression of SNRPA was higher in patients with stages 2–3 HCC than in those with stage 1 HCC (Figure 1C). Subsequently, genes positively associated with SNRPA were subjected to KEGG analysis by DAVID; among the enriched KEGG pathways, "cell cycle" was highly related to HCC progression (Figure 1D). Furthermore, western blotting demonstrated that SNRPA protein expression was significantly upregulated in HCC tissues compared with paired para-tumor tissues (Figure 1E). Moreover, immunohistochemistry assay indicated that SNRPA were frequently elevated both in the Chongqing cohort of 85 HCC patients and OUTDO BIOTECH cohort of 97 HCC patients (Figure 1F). The relationship between SNRPA levels and clinicopathological features was analyzed with the chi-squared test, and the results showed that SNRPA levels were positively correlated with tumor size in HCC patients from the above-mentioned cohorts (Supplemental Tables S6, S7, *https://www.biosciencetrends.com/supplementaldata/253*). Notably, SNRPA expression was positively related to tumor TNM stage only in HCC patients from the Chongqing cohort (Supplemental Tables S6, S7, *https://www.biosciencetrends.com/supplementaldata/253*). Subsequently, Kaplan–Meier analysis, as well as univariate and multivariate Cox proportional hazards regression models demonstrated that overall survival was poorer in HCC patients with high levels of SNRPA than in those with low levels of SNRPA in both the Chongqing and OUTDO BIOTECH cohorts. In addition, high expression of SNRPA was an independent predictor of the poor overall survival in HCC patients from the OUTDO BIOTECH cohort (Figure 1G, H and Supplemental Figure S1A, B, *https://www.biosciencetrends.com/supplementaldata/253*). Meanwhile, in the Chongqing cohort, HCC patients with high levels of SNRPA suffered from a shorter disease-free survival versus those with low levels of SNRPA. Notably, high SNRPA expression was an independent risk factor for tumor recurrence in HCC (Figure 1I and Supplemental Figure S1C, *https://www.biosciencetrends.com/supplementaldata/253*). Moreover, we used the Gene Expression Profiling Interactive Analysis (GEPIA) to analyze TCGA-LIHC samples, the results further showed that high expression of SNRPA was closely associated with a poor prognosis in HCC patients (Supplemental Figure S2A, B, *https://www.biosciencetrends.com/supplementaldata/253*). Collectively, these data indicate that SNRPA is elevated in HCC, and its elevation is related to unfavorable clinicopathologic features and poor survival in HCC patients. It is therefore likely that SNRPA plays a vital role in HCC progression.

3.2. SNRPA promotes HCC cell proliferation both *in vitro* and *in vivo*.

Western blotting revealed that SNRPA was highly expressed in Huh-7 and SK-Hep-1 cells, whereas it was lowly expressed in Hep 3B cells (Supplemental Figure S3A, *https://www.biosciencetrends.com/supplementaldata/253*). Based on these results, we silenced SNRPA expression in Huh-7 and SK-Hep-1 cells, and overexpressed SNRPA in Hep 3B cells through lentivirus infection. The efficiency of SNRPA knockdown (Supplemental Figure S3B, *https://www.biosciencetrends.com/supplementaldata/253*) and overexpression (Supplemental Figure S3C, *https://www.biosciencetrends.com/supplementaldata/253*) was identified by western blotting. Knockdown of SNRPA significantly suppressed the proliferation of Huh-7 and SK-Hep-1 cells, whereas SNRPA overexpression significantly promoted the proliferation of Hep 3B cells, as demonstrated by CCK-8, EdU, and colony formation assays (Figure 2A-F). However, wound healing assay demonstrated that SNRPA knockdown or overexpression did not affect the migration of HCC cells (Supplemental Figure S4A, B, *https://www.biosciencetrends.com/supplementaldata/253*). We also established nude mouse subcutaneous xenograft models and found that SNRPA silencing could significantly inhibit the growth of Huh-7 and SK-Hep-1 cells *in vivo* (Figure 2G, H and Supplemental Figure S5A, B, *https://www.biosciencetrends.com/supplementaldata/253*). Furthermore, immunohistochemistry staining of xenograft tumor tissues illustrated that Ki-67 expression was decreased in tumor tissues from the SNRPA knockdown groups compared with the negative control groups (Supplemental Figure S5C, D, *https://www.biosciencetrends.com/supplementaldata/253*). Overall, the above data suggest that SNRPA promotes HCC cell proliferation both *in vitro* and *in vivo*.

3.3. SNRPA activates the B7-H6-STAT3/AKT signaling axis in HCC cells with promotion of G1-S transition in the cell cycle and inhibition of cell apoptosis.

Firstly, we detected significantly changed transcripts after SNRPA silencing using nanopore full-length cDNA sequencing in Huh-7 cells. The sequencing identified 348 transcripts with significant difference ($|\text{fold change}| \geq 2$; $P < 0.05$) between the SNRPA knockdown and negative control groups (Supplemental Figure S6A, B, *https://www.biosciencetrends.com/supplementaldata/253*). Moreover, RIP-sequencing with using anti-SNRPA antibody identified numerous putative SNRPA binding peaks in Huh-7 cells (Supplemental Figure S6C, D, *https://www.biosciencetrends.com/supplementaldata/253*). RIP-sequencing assay showed that on both single-transcript and whole-transcriptome levels, SNRPA always dominantly combined with the CDS zones (Supplemental Figure S6E, F, *https://www.biosciencetrends.com/supplementaldata/253*). This observation suggested that SNRPA plays an essential

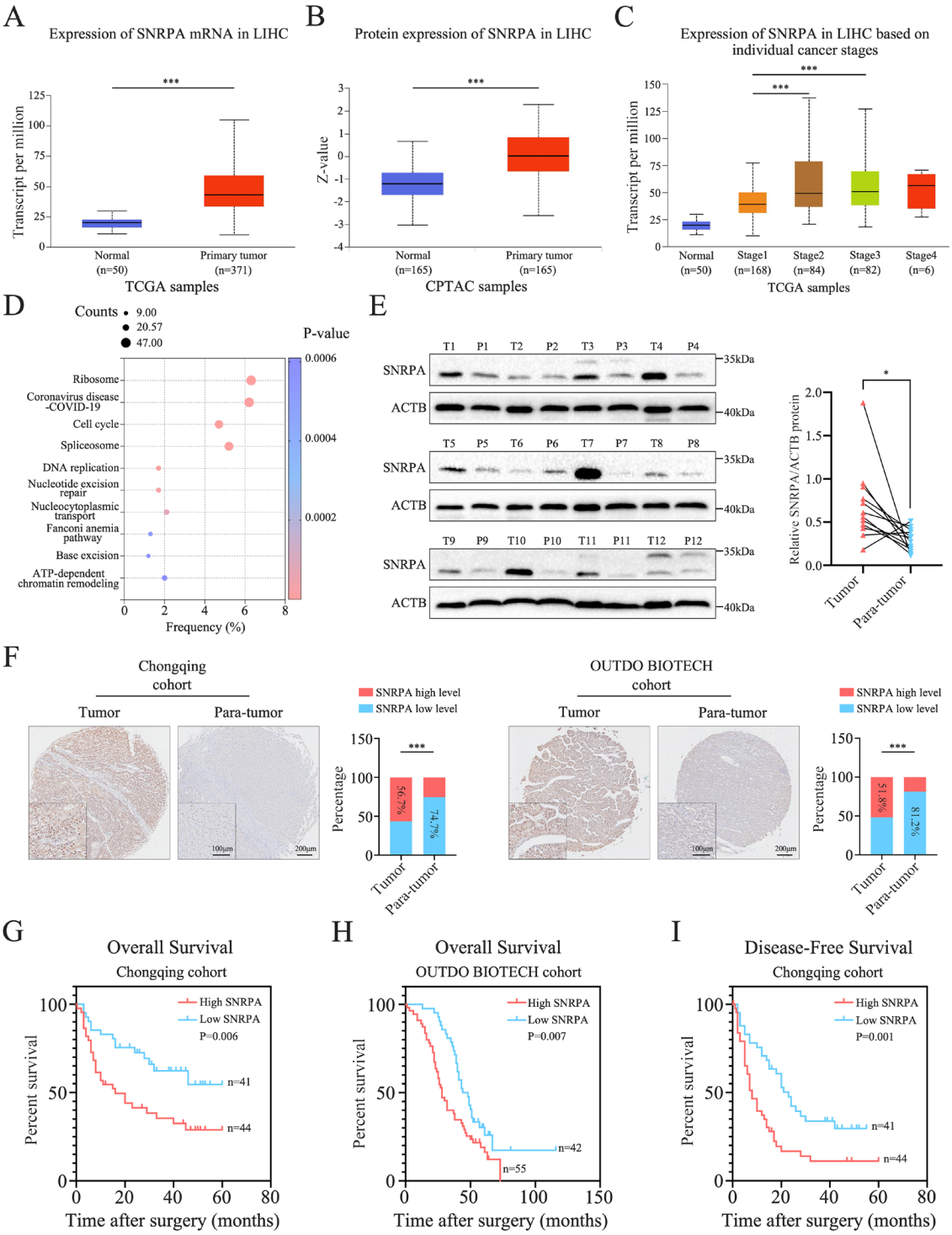

Figure 1. SNRPA is frequently elevated in HCC tissues, which predicts a poor survival in HCC patients. (A) UALCAN analysis of SNRPA mRNA levels in HCC tissues and normal liver tissues from TCGA samples. **(B)** SNRPA protein expression in HCC tissues and normal liver tissues from CPTAC samples acquired by UALCAN analysis. **(C)** UALCAN analysis showing SNRPA mRNA expression at different cancer stages. **(D)** Enriched KEGG pathways of SNRPA positively correlated genes acquired from DAVID analysis. **(E)** Western blot showing SNRPA levels in HCC tissues and matched para-tumor tissues. **(F)** IHC stanning analysis of SNRPA expression in HCC tissues and paired para-tumor tissues from Chongqing (left panel) and OUTDO BIOTECH (right panel) cohorts. **(G and H)** Kaplan-Meier analysis showing the association between SNRPA levels and overall survival of HCC patients from Chongqing **(G)** and OUTDO BIOTECH **(H)** cohorts. **(I)** Kaplan-Meier analysis showing the correlation between SNRPA expression and disease-free survival of patients with HCC from Chongqing cohort. Continuous data were shown as the mean ± standard deviation (SD). $^{*}P < 0.05$, $^{***}P < 0.001$.

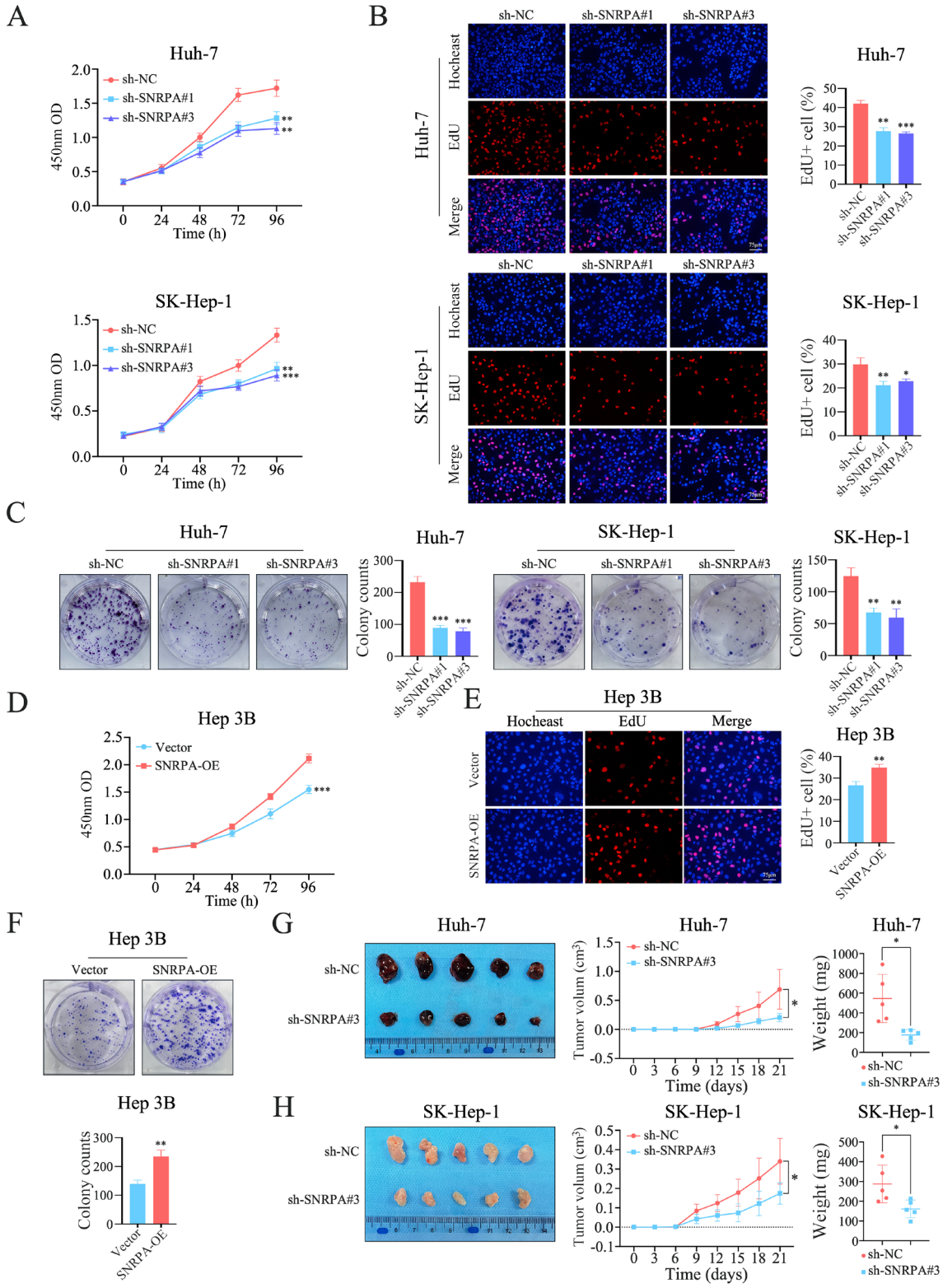

Figure 2. SNRPA promotes HCC cell proliferation both *in vitro* and *in vivo*. **(A)** CCk-8 test, **(B)** EdU staining, and **(C)** Colony formation assay to detect the proliferative ability of Huh-7 and SK-Hep-1 cells after SNRPA silencing. **(D)** CCk-8 assay, **(E)** EdU test, and **(F)** Colony formation assay to analyze Hep 3B cell proliferative activity after SNRPA overexpression. **(G and H)** Subcutaneous xenograft models of Huh-7 (**G**) and SK-Hep-1 (**H**) cells to assess the influence of SNRPA knockdown on tumor growth. Continuous data were presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

role in pre-mRNA splicing. Finally, we intersected the transcripts whose levels showed significant changes (|fold change| ≥ 2; *P*-value < 0.05) after SNRPA knockdown according to nanopore full-length cDNA sequencing with the transcripts that were annotated by significantly enriched SNRPA binding peaks (fold enrichment ≥ 5; $P < 0.05$) identified through RIP sequencing. Surprisingly, the ENST00000338965 transcript of the NCR3LG1 gene encoding B7-H6 protein was downregulated after SNRPA knockdown, and it combined directly with SNRPA (Figure 3A). It has been reported that B7-H6 promotes tumor growth, inhibits cell apoptosis, and accelerates G1-S transition in the cell cycle in several types of cancer, also including HCC among them; these effects are driven by ERK, AKT, and STAT3 signaling pathways (*27, 31, 33*). Subsequently, western blotting was performed to detect the B7-H6 related pathway activity after SNRPA silencing or overexpression in HCC cells. The results revealed that SNRPA knockdown decreased the B7-H6 expression, as well as the relative levels of phosphorylated-STAT3 (p-STAT3) and p-AKT in Huh-7 and SK-Hep-1 cells. In contrast, SNRPA overexpression significantly upregulated the B7-H6 expression and the phosphorylation levels of STAT3 and AKT in Hep 3B cells (Figure 3B). However, there were no apparent changes in relative p-ERK levels after SNRPA knockdown or overexpression (Figure 3B). Immunohistochemistry staining also showed that the levels of B7-H6, p-STAT3, and p-AKT were markedly decreased in xenograft tumor tissues from the SNRPA silencing groups compared with xenograft tumor tissues from the negative control groups (Supplemental Figure S7A, B, *https://www.biosciencetrends.com/supplementaldata/253*). Moreover, as demonstrated by flow cytometry assays, SNRPA knockdown blocked G1-S transition in the cell cycle and induced apoptosis in Huh-7 and SK-Hep1 cells (Figure 3C and Supplemental Figure S8, *https://www.biosciencetrends.com/supplementaldata/253*). In contrast, SNRPA overexpression promoted G1-S transition (Figure 3D) and inhibited the apoptosis in Hep 3B cells (Figure 3E). Western blotting showed that the levels of cyclin dependent kinase 4 (CDK4) and CDK6 were evidently declined after SNRPA silencing in Huh-7 and SK-Hep-1 cells. Meanwhile, the levels of cleaved-caspase3 in Huh-7 and SK-Hep-1 cells were markedly elevated after SNRPA silencing, whereas SNRPA overexpression in Hep 3B cells exerted an opposite effect (Figure 3F), which was consistent with the results from flow cytometry. However, downregulation or upregulation of SNRPA expression did not significantly alter the levels of cyclin D1 (CCND1) (Figure 3F). Taken together, it is possible that SNRPA promotes HCC cell proliferation *via* B7-H6-STAT3/AKT axis-mediated inhibition of apoptosis and promotion of G1-S transition in the cell cycle.

3.4. SNRPA facilitates B7-H6 pre-mRNA maturation *via* its intron 2 splicing.

We analyzed the underlying SNRPA-binding sequences within the B7-H6 pre-mRNA based on the RIP-sequencing data. The results showed that there were three significantly enriched SNRPA binding peaks on B7-H6 pre-mRNA, namely Peak 1, Peak 2, and Peak 3 (Figure 4A). In detail, Peak 1 spanned the whole intron 2 of B7-H6 pre-mRNA, whereas Peak 2 and Peak 3 were located on exon 5 (Figure 4A). Interestingly, between the Peak 2 and Peak 3 zones, B7-H6 pre-mRNA was alternatively spliced into two transcripts, namely variant 1, which encodes B7-H6 protein, and variant 2, which is eliminated *via* the nonsense-mediated decay pathway (Figure 4B). According to the location of SNRPA binding peaks on B7-H6 pre-mRNA, we formulated two hypotheses regarding the mechanism of SNRPA involved in elevating B7-H6 protein expression. Firstly, SNRPA promotes the B7-H6 pre-mRNA maturation by facilitating its intron 2 splicing to increase the B7-H6 protein levels; Secondly, SNRPA enhances the transformation of variant 2 to variant 1 of B7-H6 to upregulate its protein levels. To determine these hypotheses, we firstly precipitated the endogenous SNRPA protein in wild-type Huh-7 cells and exogenous SNRPA protein in SNRPA overexpression Hep 3B cells with using anti-SNRPA and anti-FLAG antibodies, respectively (Figure 4C). Subsequently, analysis of the immunoprecipitated RNA through RT-PCR with agarose gel electrophoresis and qRT-PCR showed that both endogenous and exogenous SNRPA protein could combine with Peak 1, Peak 2, and Peak 3 of B7-H6 pre-mRNA (Figure 4D, E). Finally, we designed four pairs of specific exon–exon and exon–intron junction primers to study the effect of SNRPA on B7-H6 pre-mRNA fate (Figure 4F). The qRT-PCR results showed that SNRPA silencing significantly decreased the levels of B7-H6 mature mRNA in both Huh-7 and SK-Hep-1 cells, while an opposite result was obtained in Hep 3B cells after SNRPA overexpression. Nevertheless, the B7-H6 pre-mRNA levels increased moderately in Huh-7 cells after SNRPA silencing; whereas, they remained stable in SK-Hep-1 cells after SNRPA knockdown and Hep 3B cells after SNRPA overexpression (Figure 4G and Supplemental Figure S9A, *https://www.biosciencetrends.com/supplementaldata/253*). Overall, the above data indicated that SNRPA promoted the transformation of B7-H6 pre-mRNA to mature mRNA; however, this effect only altered the levels of B7-H6 mature mRNA and did not change the pre-mRNA levels in SK-Hep-1 and Hep 3B cells. This was probably caused by the coupling of splicing and transcription (*34*) or pre-mRNA decay after a splicing defect (*35*). On the other hand, SNRPA knockdown in Huh-7 and SK-Hep-1 cells inhibited both the B7-H6 variant 1 and variant 2 mRNA expression, while SNRPA

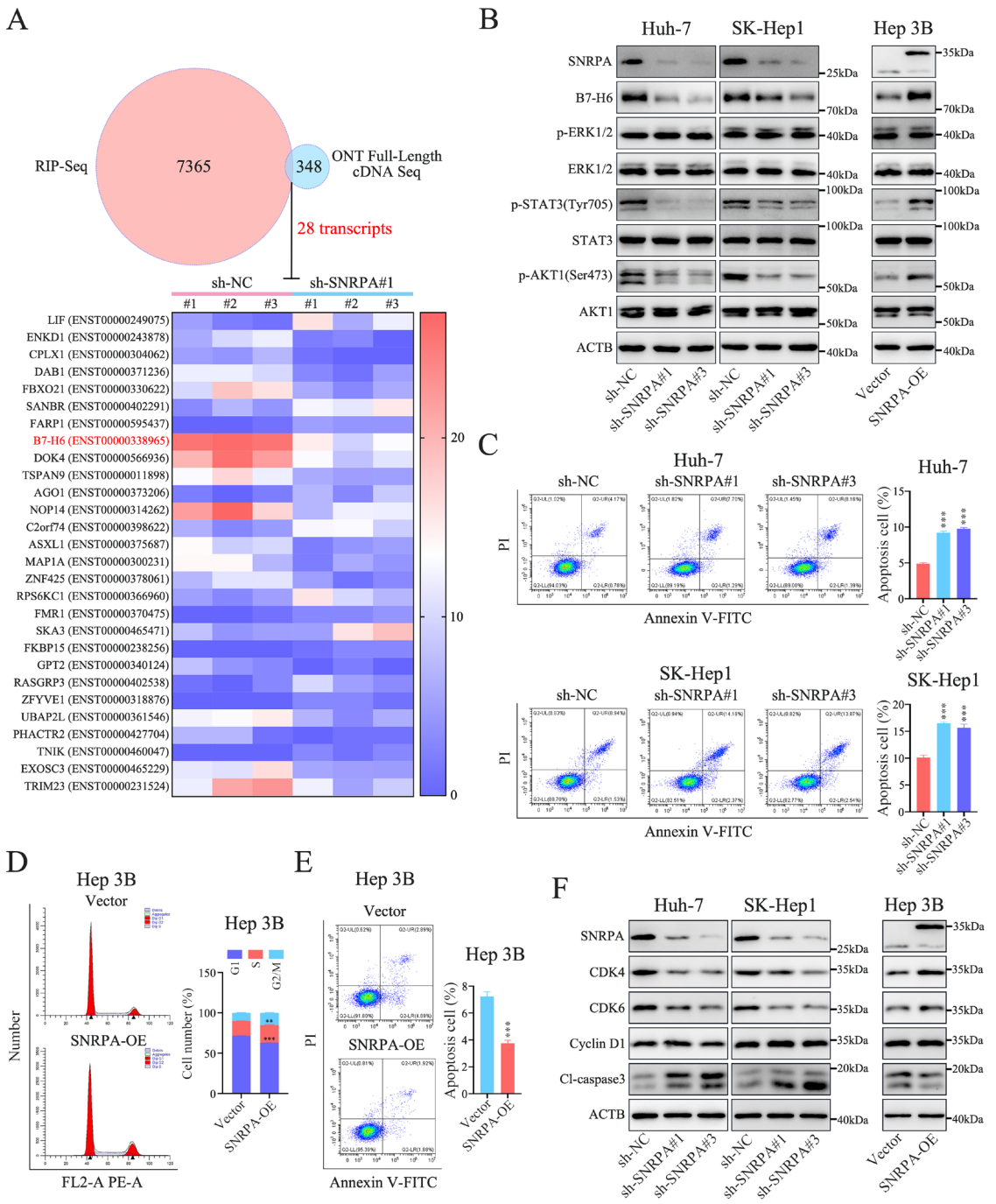

Figure 3. SNRPA activates B7-H6-STAT3/AKT signaling pathways in HCC cells. (A) Venn diagram and heatmap showing the intersection of transcripts whose levels changed significantly (|fold change| ≥ 2; *P*-value < 0.05) after SNRPA knockdown according to nanopore full-length cDNA sequencing with transcripts annotated by significantly enriched SNRPA binding peaks (fold enrichment ≥ 5; $P < 0.05$) identified through RIP sequencing. **(B)** Western blot to detect the effect of SNRPA on B7-H6-ERK/STAT3/AKT signaling pathways in HCC cells. **(C)** Flow cytometry analysis for cell apoptosis in Huh-7 and SK-Hep-1 cells after SNRPA silencing. **(D and E)** Flow cytometry analysis for the cell cycle (**D**) and apoptosis (**E**) in Hep 3B cells after SNRPA overexpression. **(F)** Western blot showing the affection of SNRPA on the cell cycle and apoptosis related gene expression in HCC cells. Continuous data were reported as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

overexpression had an opposite effect in Hep 3B cells (Figure 4H and Supplemental Figure S9B, *https://www.biosciencetrends.com/supplementaldata/253*). Collectively, the results above indicate that SNRPA increases B7-H6 protein levels in HCC cells at least in part by facilitating its pre-mRNA maturation *via* intron 2 splicing.

3.5. SNRPA enhances HCC cell resistance to lenvatinib.

The acquirement of resistance to TKIs in HCC treatment is partly due to the complementary activation of the STAT3 and AKT signaling pathways (*36-38*). Thus, we further investigated the impact of SNRPA on the sensitivity of HCC cells to lenvatinib. Using dose-

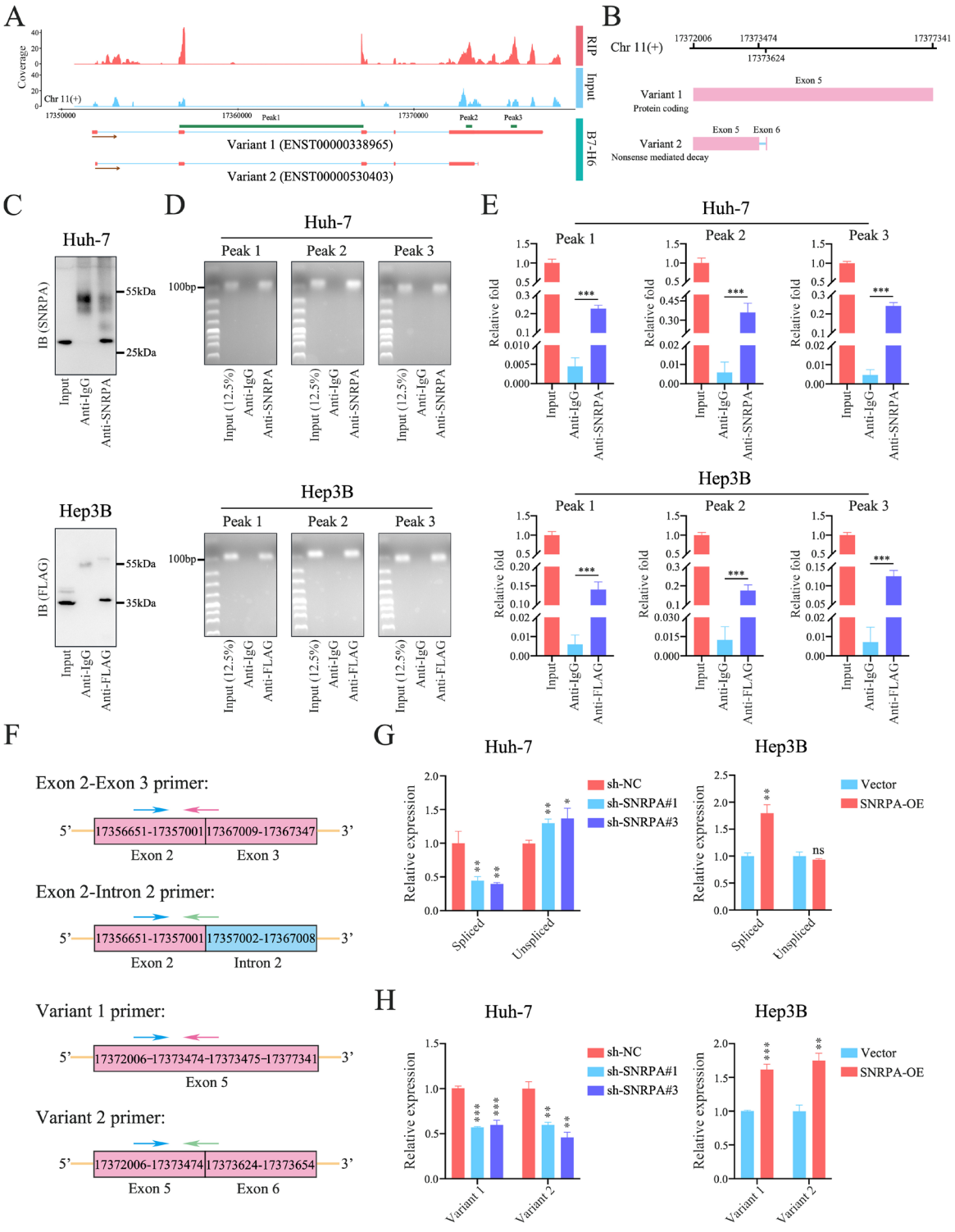

Figure 4. SNRPA promotes B7-H6 pre-mRNA maturation *via* its intron 2 normal splicing. (A) The coverage of SNRPA binding peak reads acquired from RIP-sequencing on B7-H6 transcripts. **(B)** The schematic representing alternative splicing modes of B7-H6 gene. **(C)** Western blot to detect the efficiency of SNRPA (upper panel) and FLAG (nether panel) immunoprecipitations. **(D)** RT-PCR with agarose gel electrophoresis assays to determine combining of endogenous (upper panel) and exogenous (nether panel) SNRPA with Peak 1, Peak 2, and Peak 3 of B7-H6 pre-mRNA. **(E)** qRT-PCR showing the binding of endogenous (upper panel) and exogenous (nether panel) SNRPA with Peak 1, Peak 2, and Peak 3 of B7-H6 pre-mRNA. **(F)** The schematic of specific primers to identify B7-H6 pre-mRNA or mature mRNA (upper panel), and B7-H6 variant 1 or variant 2 mRNA (nether panel). Specific primers represented by the two arrows, illustrating their approximate locations. **(G)** qRT-PCR to detect the B7-H6 pre-mRNA and mature mRNA levels in HCC cells after SNRPA knockdown or overexpression. **(H)** qRT-PCR to detect the B7-H6 variant 1 and variant 2 expression in HCC cells after SNRPA silencing or upregulating. Continuous data were demonstrated as the mean ± SD. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

response-inhibition test found that SNRPA knockdown significantly declined the IC_{50} of SK-Hep-1 cells to lenvatinib, whereas SNRPA overexpression markedly elevated the IC_{50} of Hep 3B cells to lenvatinib (Figure 5A). Consistently, colony formation assays showed that SNRPA silencing in SK-Hep-1 cells could enhance the inhibition of proliferation induced by treatment with lenvatinib, whereas SNRPA overexpression had an opposite effect in Hep 3B cells (Figure 5B). Moreover, as shown by flow cytometry assays, SNRPA silencing evidently aggravated the lenvatinib-induced apoptosis of SK-Hep-1 cells; in contrast, SNRPA upregulating in Hep 3B cells significantly relieved the apoptosis caused by treatment with lenvatinib (Figure 5C). Finally, xenograft models with treatment of lenvatinib found that SNRPA silencing could markedly enhance the sensitivity of SK-Hep-1 cells to lenvatinib *in vivo* (Figure 5D and Supplemental Figure S10, *https://www.biosciencetrends.com/supplementaldata/253*). Collectively, these results suggest that SNRPA induces HCC cell resistance to lenvatinib both *in vitro* and *in vivo*.

3.6. The effects of SNRPA on HCC cells relies on B7-H6-mediated activation of STAT3 and AKT.

We used RNAi to silence the expression of B7-H6 in Hep 3B cells and verified the silencing efficiency using western blotting (Figure 6A). As shown by CCK-8 and EdU assays, B7-H6 silencing significantly inhibited the proliferation of Hep 3B cells in the empty vector group and could abolish the proliferative ability enhanced by SNRPA overexpression in Hep 3B cells (Figure 6B, C). Similarly, the flow cytometry results demonstrated that B7-H6 silencing suppressed G1-S transition in the cell cycle and promoted apoptosis in the empty vector group Hep 3B cells. In addition, the promotion of G1-S transition in the cell cycle and inhibition of apoptosis in Hep 3B cells caused by SNRPA upregulating were partially offset by B7-H6 silencing (Figure 6D, E). Moreover, western blotting showed that B7-H6 silencing inhibited the phosphorylation of STAT3 and AKT, as well as the expression of CDK4 and CDK6 in empty vector group Hep 3B cells. However, Caspase3 activation in these cells was augmented after B7-H6 silencing. In addition, upregulation of p-STAT3, p-AKT, CDK4, and CDK6, as well as inhibition of Caspase3 activation resulted from SNRPA overexpression depended on B7-H6 expression (Figure 6F). Finally, the dose-response-inhibition test demonstrated that B7-H6 interference reversed lenvatinib resistance acquired by SNRPA overexpression in Hep 3B cells (Supplemental Figure S11, *https://www.biosciencetrends.com/supplementaldata/253*). Taken together, these results indicate that SNRPA promotes the proliferation and resistance to lenvatinib of HCC cells mainly through B7-H6-mediated activation of the STAT3 and AKT signaling pathways.

4. Discussion

Thus far, the number of available target therapeutics for HCC has been limited by an accumulative activation of multiple signaling pathways and reactivation of complementary signaling pathways (*7,36*). Increasing amount of evidence indicates that normal and abnormal pre-mRNA splicing participates in the tumor malignant process and the development of resistance to treatment (*11,12,39*). In this study, we revealed that the levels of splicing factor SNRPA were frequently elevated in HCC tissues; this increase was predictive of poor survival in patients with HCC. Additionally, SNRPA promoted HCC cell proliferation and resistance to lenvatinib. These findings suggested that SNRPA is an oncogene in HCC, as well as a potential target for therapy and overcoming resistance to lenvatinib in HCC patients.

Previous studies have demonstrated that SNRPA was upregulated in HCC tissues and promoted HCC cell metastasis *via* microvascular invasion (*21,22*). However, these investigations did not further explore the direct mechanism underlying the promotion of HCC progression by SNRPA through pre-mRNA splicing and the SNRPA resulting in development of resistance to treatment. Additionally, these studies did not employ sequencing to detect the transcript expression profile after SNRPA knockdown or overexpression. In the present study, utilizing nanopore full-length cDNA sequencing and RIP-sequencing assays, we hypothesized that SNRPA promotes HCC proliferation *via* B7-H6 pre-mRNA splicing. According to the sequences of B7-H6 pre-mRNA combined with SNRPA, we designed several pairs of specific exon–intron and exon–exon conjunction primers and performed qRT-PCR. The results revealed that SNRPA upregulated B7-H6 expression by promoting B7-H6 pre-mRNA maturation *via* its intron 2 normal splicing. Reports have shown that SNRPA more often upregulated the expression of transcripts directly bound by SNRPA on the whole-genome level; moreover, SNRPA promoted splicing at the 5' splice site in intron 5 of the mTOR gene to increase its expression (*17,40*), which further supported our results to a large extent. Additionally, owing to its superiority in detecting transcript levels compared with traditional sequencing (*41,42*), we utilized nanopore full-length cDNA sequencing to analyze changes in the SNRPA downstream targets. This is the first study exploring the mechanism through which SNRPA aggravates the malignant behaviors of HCC cells at the pre-mRNA splicing level.

Coded by the natural killer cell cytotoxicity receptor 3 ligand 1 (NCR3LG1) gene, B7-H6 protein contributes to tumor progression *via* its two extracellular immunoglobulin domains mediating immune escape (*29,30*). This was identified as the primary immunological mechanism underlying the involvement of B7-H6 in tumorigenesis. However, at the non-

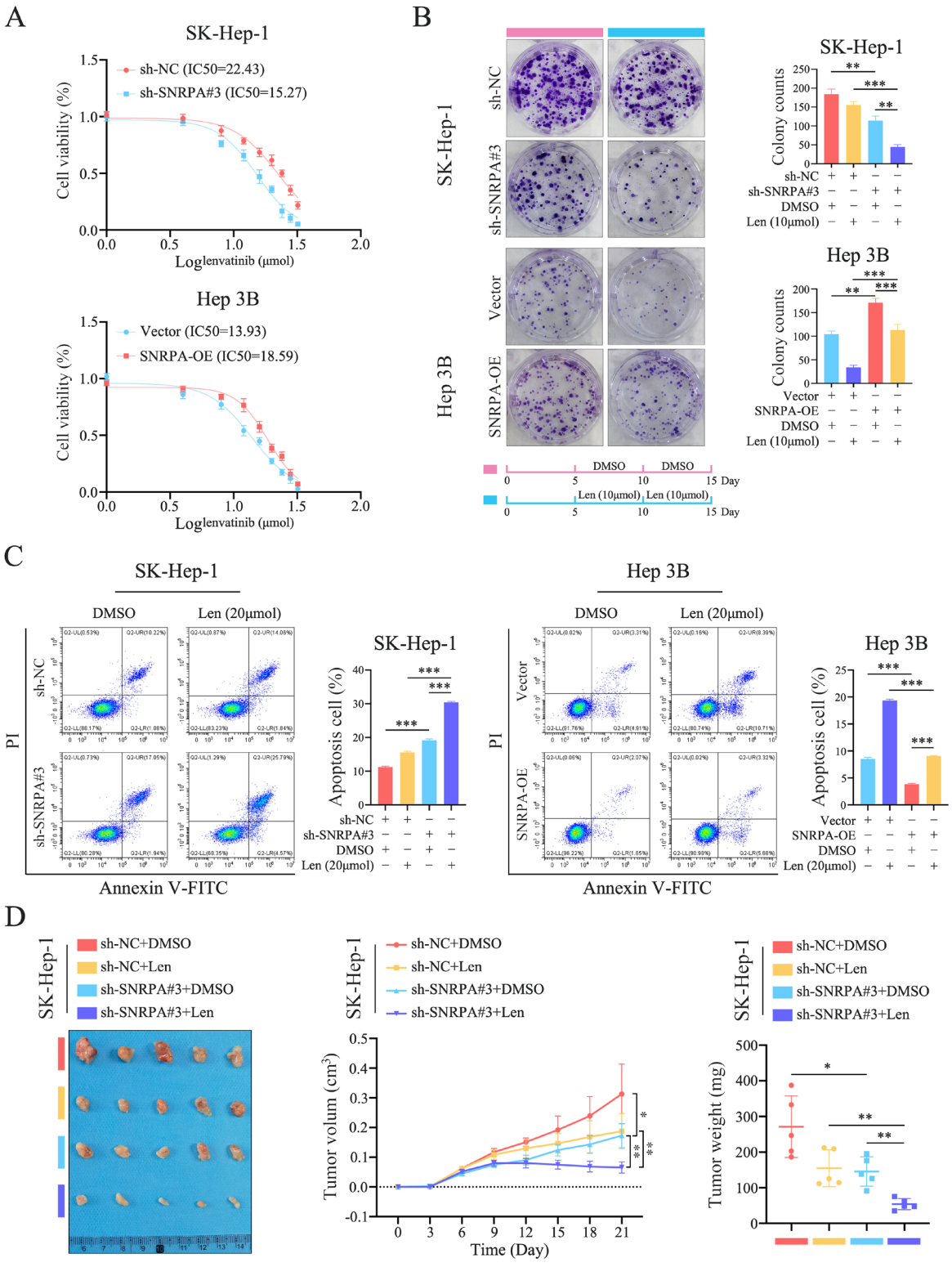

Figure 5. SNRPA enhances HCC cell resistance to lenvatinib. (A) The dose-response-inhibition curve of SK-Hep-1 cells after SNRPA knockdown (upper panel) and Hep 3B cells after SNRPA overexpression (nether panel) to lenvatinib. **(B)** Colony formation assay showing the effect of specific lenvatinib concentration on the proliferation of HCC cells after SNRPA silencing or upregulating. **(C)** Flow cytometry assay to detect the impact of specific lenvatinib concentration on apoptosis of HCC cells after SNRPA knockdown or overexpression. **(D)** Subcutaneous xenograft models of SK-Hep-1 cells to assess the affection of lenvatinib on HCC cell growth *in vivo* after SNRPA silencing. Continuous data were shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

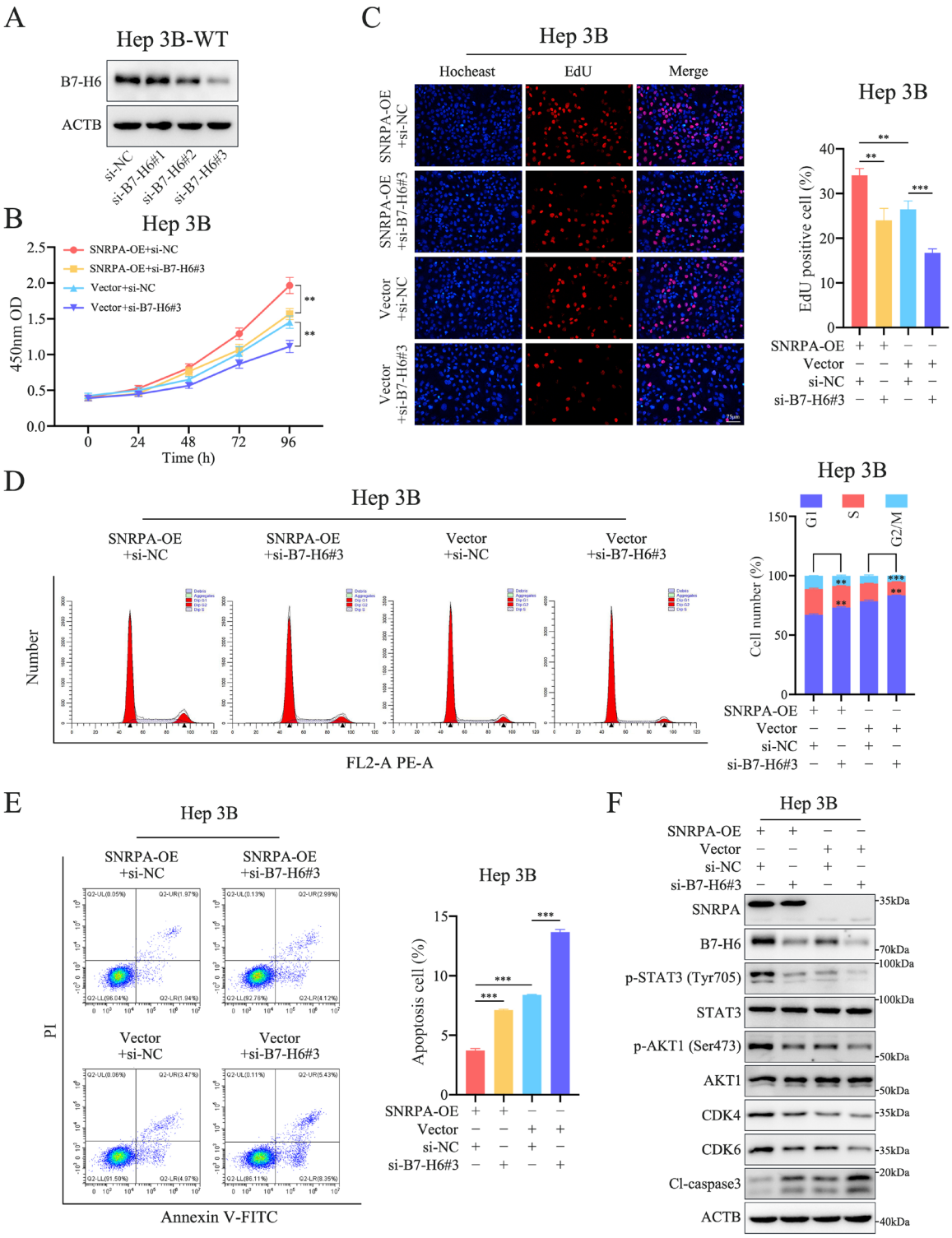

Figure 6. SNRPA promotes HCC cell proliferation relying on B7-H6 expression. (A) Western blot to detect the efficiency of B7-H6 interference in Hep 3B cells after treatment of siRNAs. **(B)** CCk-8 test, **(C)** EdU assay, **(D)** flow cytometry analysis for the cell cycle, and **(E)** flow cytometry analysis for cell apoptosis to determine the effect of SNRPA overexpression on proliferation, cell cycle, and apoptosis of Hep 3B cells in shortage of B7-H6. **(F)** Western blot to detect the impact of SNRPA upregulating on STAT3/AKT signaling pathway activation, as well as the cell cycle and apoptosis related gene expression of Hep 3B cells in context of B7-H6 interference. Continuous data were shown as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

immunological level, accumulating evidence revealed that B7-H6 activated the STAT3, AKT, and ERK signaling pathways in the development of cancer (*27,31*). This function may be associated with its ITIM/SH2/SH3 domains, which possess protein tyrosine kinase activity (*23,43*). Other studies showed that, by accelerating G1-S transition and inhibiting apoptosis, B7-H6 played an important role in the progression of several tumor types

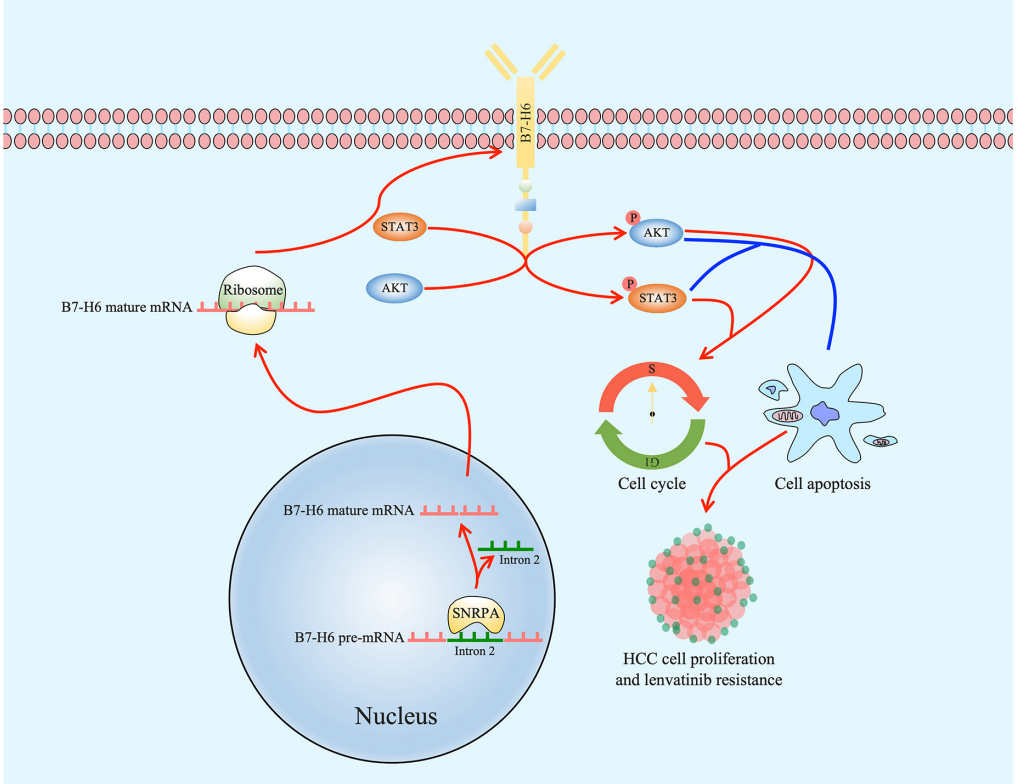

Figure 7. Schematic depicting the molecular mechanism of SNRPA contributing to HCC progression.

(*32,44,45*). In our study, B7-H6 only upregulated the phosphorylation levels of STAT3 and AKT, but not those of ERK, in HCC cells. B7-H6 was abundantly expressed in various tumor tissues, whereas it was rarely detected in normal tissues (*23-26*). It was previously revealed that, as an oncogene overexpressed in certain tumors, Myc transcriptionally drives B7-H6 expression in tumor cells (*30*). In addition, it was hypothesized that some post-transcriptional modifications, including ubiquitin or SUMOylation, may block B7-H6 expression in normal tissues by another study (*46*). Interestingly, our study is the first to demonstrate that B7-H6 pre-mRNA normal splicing is a mechanism involved in maintaining B7-H6 expression in tumor tissues. Notably, there is a lack of the full-length B7-H6 gene sequence and only a short region corresponding to the first exon of human B7-H6 in the mouse genome (*23*). Consequently, we did not further investigate the relationship of SNRPA with B7-H6 to promote tumorigenesis in a DEN/CCL4-induced mouse HCC model using a gene knockout technique.

Lenvatinib is currently the first-line treatment for patients with advanced HCC (*47*); however, according to clinical data, only a limited number of patients with HCC benefit from treatment with lenvatinib (*48*). This was attributed to extensive resistance to lenvatinib in patients with HCC, while the specific factors driving this resistance remain unclear to a large extent. Hu *et al.* revealed that epidermal growth factor receptor (EGFR) induced resistance to lenvatinib in HCC by STAT3-ATP binding cassette subfamily B member 1 (STAT3-ABCB1) signaling (*36*). In addition, another study demonstrated that chromobox 1 (CBX1) increased resistance to TKIs (*i.e.*, sorafenib and lenvatinib) in HCC *via* the insulin like growth factor 1 receptor/AKT/SNAIL (IGF-1R/AKT/SNAIL) signaling axis (*49*). Collectively, the evidence indicated that the complementary reactivation of the STAT3 and AKT signaling pathways plays an essential role in the development of resistance to lenvatinib in patients with HCC. In the present study, we showed that SNRPA activated the STAT3 and AKT signaling pathways in HCC. Furthermore, pre-mRNA splicing has been identified as an important mechanism of resistance to therapy in cancer (*50*). Thus, we further explored whether SNRPA affected HCC cell resistance to lenvatinib. Based on *in vitro* and *in vivo* functional experiments, we showed that SNRPA induced significant resistance to lenvatinib in HCC cells. Our results suggested that pre-mRNA splicing is also involved in the development of HCC cell resistance to lenvatinib.

In conclusion, the present study revealed that SNRPA promotes HCC cell proliferation and resistance to lenvatinib. This effect is supported through the binding of SNRPA to B7-H6 pre-mRNA; this binding increases B7-H6 expression by facilitating B7-H6 pre-mRNA maturation *via* its intron 2 splicing and thus activating the STAT3 and AKT signaling pathways (Figure 7). These results indicate that SNRPA is a promising target for the treatment of HCC and overcoming resistance to

lenvatinib in patients with this disease.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Ethics approval and consent to participate: This study was approved by Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval number RER2022-637A) and conformed to Helsinki Declaration. Additionally, the patients involved in this study provided their written informed consent. The animal study was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval number RER2021-136X).

Availability of data and materials: All original data can be available from the corresponding authors based on the reasonable request.

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