ANTICANCER RESEARCH *40*: 2033-2042 (2020)
doi:10.21873/anticanres.14160

SRSF3 Depletion Leads to an Increase in SF3B4 Expression in SNU-368 HCC Cells

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Abstract. *Background: SF3B4, a critical component of U2 pre-mRNA spliceosomal complex, has been recently indicated as a potential oncogene in hepatocellular carcinoma (HCC). However, limited information exists on how SF3B4 expression is regulated in HCC. Materials and Methods: To determine the regulatory factor for SF3B4 expression, small interfering RNA (siRNA), real-time polymerase chain reaction (qRT-PCR) and western blotting assay were performed. The in vivo expression profiles of SRSF3 and SF3B4 were analyzed using public datasets and clinical samples. Results: Among 10 liver-specific splicing factors, only SRSF3 knockdown resulted in a significant increase in SF3B4 mRNA and consequently protein levels in SNU-368 HCC cells, probably via the retardation of SF3B4 mRNA decay rates. Using green fluorescent protein-SF3B4 fusion construct, the coding region of SF3B4 was found to be involved in SRSF3-mediated regulation of SF3B4 expression. Publicly available data from paired normal and tumor tissues in HCC and results from patients with HCC suggest that SRSF3 and SF3B4 possess an inverse relationship. Conclusion: SRSF3 is a key molecule for determining SF3B4 levels in HCC cells.*

The splicing factor 3B subunit 4 (SF3B4), also known as the spliceosome-associated protein (SAP) 49, is a core protein of the SF3B complex in the U2 small nuclear ribonucleoprotein (snRNP). This splicing factor also plays an important role in tethering U2 snRNP to the pre-mRNA region located at the branched point of the prespliceosome complex (1). Happloinsufficiency or mutations of SF3B4 gene have been implicated as a major cause of acrofacial dystosis such as Nager syndrome and Rodriguez syndrome, suggesting that an accurate mRNA processing is critical for craniofacial development (2-5). Recently, Ueno *et al*. clearly demonstrated that SF3B4 functions as a bridging factor to facilitate the assembly of polysomes on the endoplasmic reticulum, which confers an enhanced translation of secretory proteins including collagen A1s. This showed that the impairment in the biosynthesis of collagen is a molecular base leading to the poor craniofacial formation caused by SF3B4 mutation (6).

A large-scale systematic genomic analysis identified frequent alterations in the splicing regulatory components in diverse types of cancers (7). Mutations in pre-mRNA regulatory sequences including exonic enhancers, exon silencers or those in splice site in the oncogene or tumor suppressor gene might affect the rate and pattern of splicing in key cancer-associated genes, which in turn might contribute to the modulation of cancer phenotypes. In addition to the mutations in the pre-mRNA cis- elements, mutations in genes encoding the trans-acting splicing factors, which include SF3B1, U2AF1, SRSF2, and ZRSR2, have been recurrently found predominantly in hematological malignancies (8, 9). In solid tumors, the occurrence of mutations in genes encoding splicing factors is very low but the alteration of expression levels of several regulatory splicing factors such as SRSF1, SRSF2, SRSF6, hnRNP A1, hnRNP K, or PTB exists in diverse types of human cancers (7). On the other hand, there has been limited research on the association of SF3B4 and cancer. Research on the relationship between SF3B4 and cancer is only at the beginning stage, most of which is

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Key Words: SRSF3, SF3B4, mRNA stability, HCC, SNU-368.

reported in hepato-cellular carcinoma (HCC). In a meta-analysis covering five independent studies, SF3B4 was identified to be up-regulated in HCC, which was subsequently validated by quantitative real-time polymerase chain reaction (qRT-PCR) (10). Another group consequently reported that increased copy number of SF3B4 gene is related with poor prognosis in HCC (11). Recently, Shen *et al*. demonstrated that SF3B4 is one of early-state makers suggesting its role in driving the development of HCC based on integrative analysis of multistage HCC tissues and subsequent functional analysis performed in HCC cell lines and mouse experiments (12). Recently, Liu *et al*. showed that microRNA (miRNA)-133b and SF3B4 have an inverse relationship in HCC tissues (13). They also demonstrated that overexpression of miRNA-133b inhibits the expression of SF3B4 while miRNA-133b knockdown promotes SF3B4 expression in HCC cells. Based on the increased luciferase reporter activity from SF3B4 3'-UTR sequence, in which miRNA-133b binding region was mutated, miR-133b was shown to directly regulate SF3B4 expression *via* its 3'-UTR sequence. However, the mechanism by which SF3B4 is up-regulated in HCC is largely unknown.

The expression of many RNA-binding proteins is regulated through splicing of their own pre-mRNA, auto-regulation, as well as through cross regulation by other splicing factors (14). Recently, the expression of 10 splicing factors has been specifically implicated *in vivo* in liver disorders (15, 16). In the present study we, therefore, examined the effect of 10 liver-specific splicing factors on the expression of SF3B4 in HCC cells using a knockdown strategy. We identified that SRSF3 is a negative regulator for SF3B4 expression, probably through regulation of SF3B4 mRNA decay process.

Materials and Methods

Cell lines and culture. The SNU-368 human HCC cell line was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and Hep3B was acquired from ATCC (American Type Culture Collection, CEM, Manassas, VA, USA). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, GeneDEPOT Barker, TX, USA) and penicillin/streptomycin (Biowest, Nuaillé, France). All cells were maintained at 37˚C in a humidified incubator with 5% CO_2.

Small interfering RNA (siRNA), recombinant plasmid construction and transfection. Knock-down of 10 liver-specific splicing factors as well as SF3B4 in SNU-368 cells was carried out with the small interfering RNA (siRNA) strategy using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). Target-specific sequences of siRNA for each splicing factor were provided by Bioneer (Daejeon, South Korea) and are listed in Table I. To prepare green fluorescent protein (GFP)-SF3B4 fusion construct, the sequence of GFP in pEGFP C1 (Promega, Madison, WI, USA) was transferred to pcDNA 3.1 (Thermo Fisher Scientific) using Nhe I and BamH I sites. The open reading frame of SF3B4 was prepared by PCR from cDNA of SNU-368 cells with the following primer pair: forward, 5'-TATGGATCCATGGCTGCCGGGCCGA-3' and reverse, 5'-TCGGATCCTTACTGAGGGAGAGGG-3'. After verification of the sequence, the PCR product was subcloned into BamH I sites of GFP/pcDNA 3.1 vector and the construct was named GFP-SF3B4. After transfection of GFP-SF3B4 into SNU-368 cells, SRSF3 expression was suppressed by siRNA. The exogenous SF3B4 mRNA levels were then determined by reverse-transcription and PCR amplification with GFP primers as well as the primer sets recognizing GFP and SF3B4, respectively, and subsequent agarose gel electrophoresis (Table II).

Table I. *siRNA sequences used in this study.*

Gene	Sequence (5'→3')
Control	CCUACGCCACCAAUUUCGU
SRSF1	GCAGAUGAACUCGGGAUG
SRSF3	GAGUGGAACUGUCGAAUGG
SRSF6	CGUUCUAGAUCUCGUUCAATT
SRSF7	CGACGUCCCUUUGAUCCAATT
hnRNP A2B1	GGAUUAUUUAAUAACAUUAT
hnRNP H	GGUAUAUUGAAAUCUUUAAT
SFPQ	GAUAUCACGGAGGAUGAAU
NONO	GUCCAACGAACUGCUGGAA
SSB	GACUUCGUCAGAGGAGCAA
SF1	ACAACCUCAUCACAGAGAU

Reverse transcription and quantitative real-time PCR (qRT-PCR). Total RNA was isolated using RNAiso Plus (TaKara Biotechnology, Shiga, Japan) and Ribospin kit (GeneAll, Seoul, Korea). And cDNA was synthesized by PrimeScript™ RT Master Mix (TaKara Biotechnology) according to the manufacturer's instructions. To quantify the expression levels of target mRNA, quantitative real-time PCR (qRT-PCR) was performed using TB Green Premix Ex Taq™ (TaKara Biotechnology) on an Applied Biosystems 7300 instrument (Applied Biosystems, Carlsbad, CA, USA). The relative values for the target mRNAs were calculated using the $2^{-\Delta\Delta CT}$ method after normalization to the Ct value for β-actin. The specific primers for each mRNA are shown in Table III. The change in target mRNA expression levels was presented as a fold change compared to the control cells. To study the degradation rate of SF3B4 mRNA, 5 μg/ml of actinomycin D (Sigma Aldrich, St. Louis, MO, USA) was added to SNU-368 cells for indicated times before RNA was extracted.

Western blot analysis. Whole-cell proteins were extracted using lysis buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.5] and protease inhibitor cocktail (1:50 dilution; Roche, Penzberg, Germany). Equal amounts of proteins were separated by 10% or 15% sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis, and then electro-transferred onto the nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking in 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature, the membranes were then incubated with the following primary antibodies: anti-SF3B4 (Abcam, Cambridge, UK), anti-SRSF3 (Santa Cruz Biotechnology, CA, USA), anti-beta actin (Sigma Aldrich), followed by incubation with the secondary anti-bodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The immunoreactive bands were detected with the

Table II. *Primer sequences for qRT-PCR in this study.*

Gene	Forward (5'→3')	Reverse (5'→3')
SF3B4	CTATTCCTTGGACCAATCAG	GGACCAACATAAAAAAGAAA
SRSF3	GCATCGTGATTCCTGTCCAT	CGGAGTGGTCCATAGTAGCC
NONO	ATATGCCACTCCGTGGAAAG	TCCTCGATCATCCACAATGA
SSB	GCAAATCCAAGGCAGAACTCA	GCATCAGTTGGGAAGCCTTTA
SFPQ	TGTTGGGAATCTACCTGCTGA	TTTGGCAATTTCAGCCAAAGC
SF1	GTGAAAGAAGGGAAGGTTGGG	AGTCTCGATACCCTGCTTCAG
SRSF1	GAGATGGCACTGGTGTCGTG	TGCGACTCCTGCTGTTGCTTC
SRSF6	GTGGATACAGCAGTCGG	CTGGATCTGCTTCCAGAG
SRSF7	GGTCTAGATCACATTCTCG	GGTCTAGATCACATTCTCG
hnRNP A2B1	AGCTTTGAAACCACAGAAGAA	TTGATCTTTTGCTTGCAGGA
hnRNP H	GTGCAGTTTGCTTCACAGGA	CCCCAGGTCTGTCATAAGGA
β-Actin	AGTACTCCGTGTGGATCGGC	GCTGATCCACATCTGCTGGA

Table III. *PCR primer sequences for agarose gel electrophoresis.*

Gene	Forward (5'→3')	Reverse (5'→3')
GFP	AGCTGACCCTGAAGTTCATCTG	GAACTCCAGCAGGACCATGT
GAPDH	CCATGTTCGTCATGGGTGTGAACCA	GCCAGTAGAGGCAGGGATGATGTTC
GFP-SF3B4	GCATGGACGAGCTGTACAAG	CCGTATTGGCTTCCCATAGA
*SF3B4**	CTCCGAGCGGAATCAGGATG	CCTCGAGGAAGGAAAATGTGAATTTA
SRSF3	AGGAAAGCGGGAAGACTCAT	GGACGGCTTGTGATTTCTCT

*Covering whole exons.

chemiluminescence detection system (Thermo Fisher Scientific). Densitometric analysis of the bands was performed with ImageJ version 1.51 (NIH, Bethesda, MD, USA).

Public data and clinical samples. The public data for SRSF3 and SF3B4 mRNA levels in HCC and mouse liver samples were obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database portal (http://www.ncbi.nlm.nih.gov/geo/, Accession Number: GSE62043, GSE35686, and GSE74656). Human HCC tissues were provided by Catholic Central Biobank. All experimental protocols were approved by the Ethics Committee at the Songeiu Campus of the Catholic University of Korea.

Statistics. All of the experiments were performed at least three times and the results are expressed as mean±standard error (SE). The Student's *t*-test was used to compare data between two groups. *p*-Value of less than 0.05 was considered statistically significant.

Results

SRSF3 knockdown increases SF3B4 expression in SNU-368 HCC cells. SF3B4 plays an important role in tumorigenesis especially in HCC (12, 13). It has recently been reported that alteration in the expression of several splicing factors has been implicated in the pathogenesis of chronic liver diseases as well as hepatic cancer (15, 16). In the present study we, therefore, investigated if SF3B4 expression in SNU-368 HCC cells is regulated by other splicing factors or RNA-binding proteins, which are associated with liver pathogenesis. The expression of 10 liver-specific splicing factors was efficiently suppressed using specific siRNA, and SF3B4 expression was then examined using quantitative mRNA analysis. Among 10 liver-specific splicing factors, only the knockdown of SRSF3 notably increased the expression of SF3B4 in SNU-368 cells (Figure 1A). Time-dependent analysis revealed that SRSF3 depletion increased SF3B4 mRNA levels, as early as 9 h following transfection (Figure 1B). To examine the possibility that an alternative spliced form of SF3B4 is increased by SRSF3 depletion, we performed RT-PCR with the primer sets to cove the sequence from exon 1 to exon 6 of SF3B4. As shown in Figure 1C, the PCR product representing whole exons of SF3B4 was increased following SRSF3 depletion, and no significant difference in the length of PCR product of SF3B4 was observed between control cells and cells treated with SRSF3 siRNA. In addition to mRNA levels, SRSF3 knockdown significantly increased SF3B4 expression at the protein level by 1.5-fold as assessed by western blotting assay (Figure 1D). Therefore, SRSF3 depletion-mediated accumulation of SF3B4 mRNA was not attributable to blocking in translational process. Consistent with the results of RT-PCR, no truncated form of SF3B4 protein was detected in western blots following SRSF3

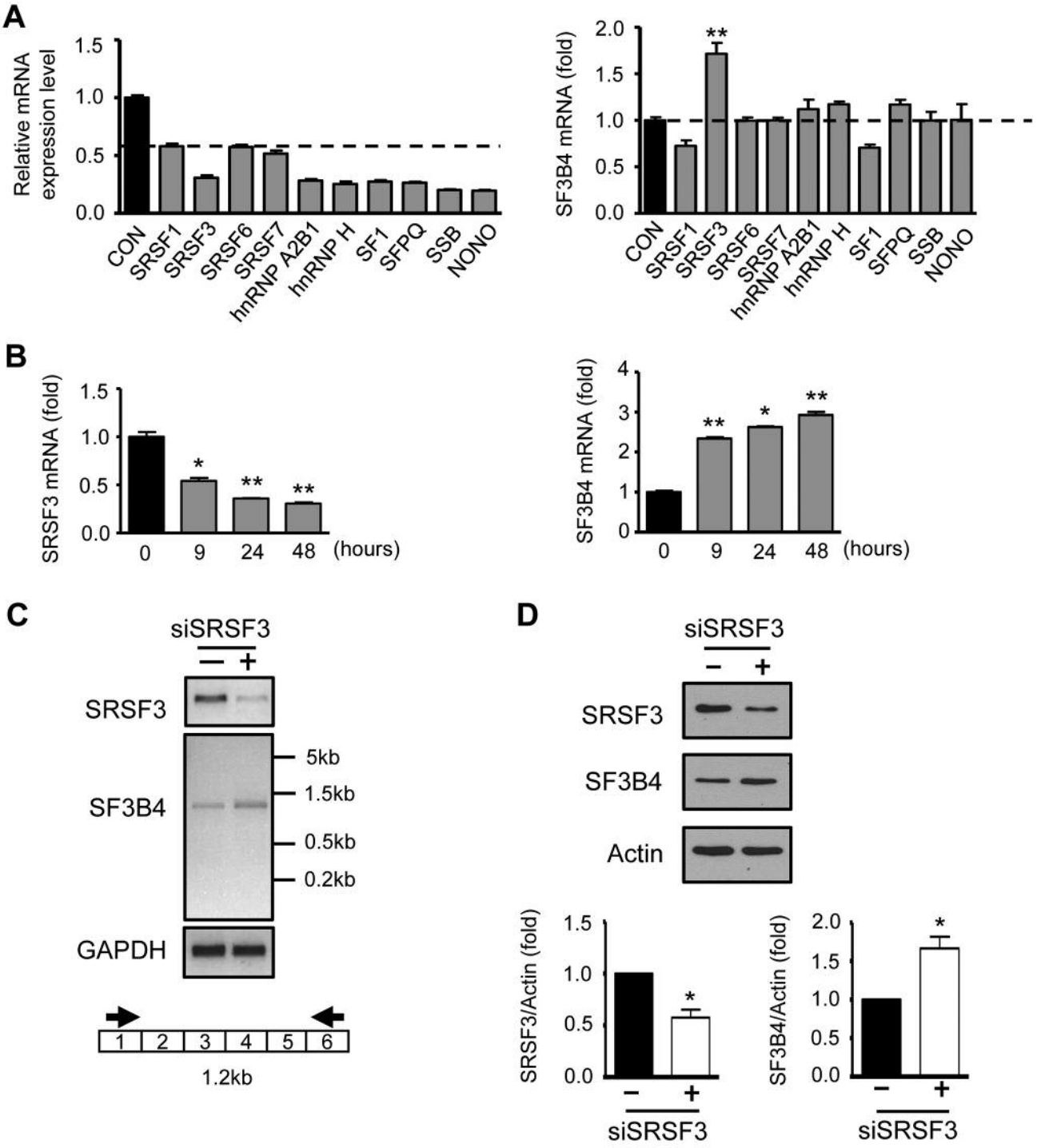

Figure 1. *SRSF3 knockdown increased SF3B4 expression in SNU-368 cells. (A) SNU-368 cells were treated with the indicated siRNAs (100 nM) targeting the liver-specific splicing factors for 48 h. The suppression efficiency on the expression of each splicing factor (left) and the consequent effect on mRNA expression levels of SF3B4 (right) were analyzed by qRT-PCR. Broken lines indicate 50% suppression of each mRNA (left) and control SF3B4 levels (right). (B) SNU-368 cells treated with SRSF3 siRNA (siSRSF3, 50 nM) at the indicated times and the expression of SRSF3 (left) and SF3B4 mRNA (right) were analyzed by qRT-PCR. (C) After treatment with siSRSF3, the expression of SRSF3, SF3B4, and GAPDH mRNA levels was analyzed by RT-PCR and 1.5% agarose electrophoresis (upper). The lower panel shows the schematic diagram of the exons of human SF3B4 mRNA and the position of primers (arrows). (D) The protein levels of SRSF3 and SF3B4 were analyzed by western blot assay. β-actin served as loading control (upper). Histogram summarized the quantitation of SRSF3 and SF3B4 proteins by densitometry (lower). Data are mean±S.E. of three independent experiments. *p<0.05, **p<0.01.*

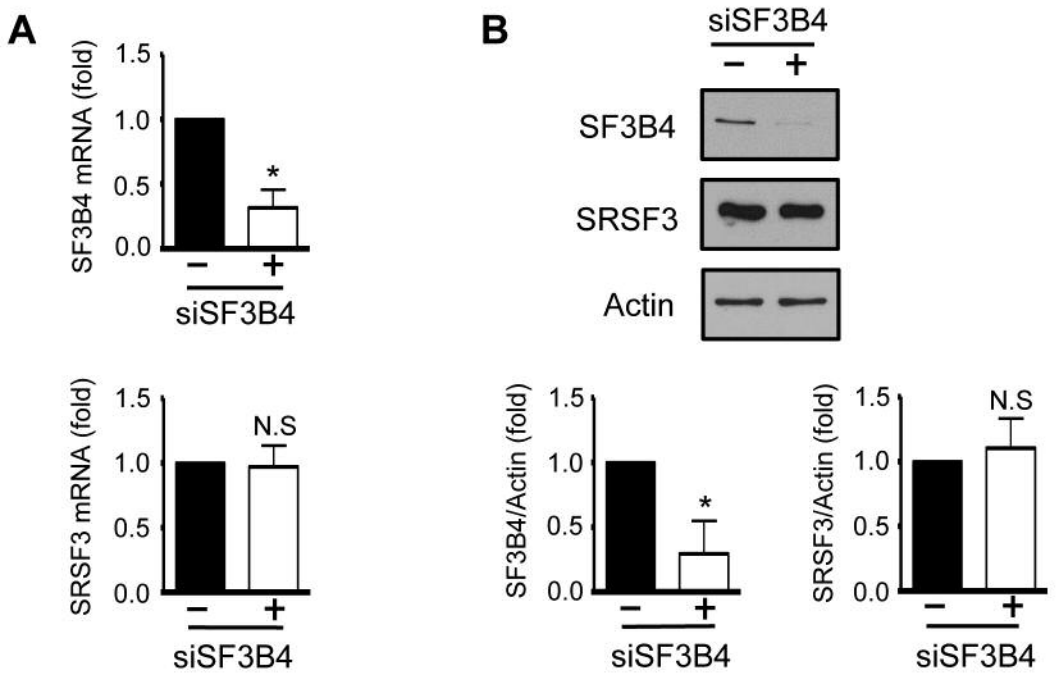

Figure 2. *SF3B4 knockdown does not alter the expression of SRSF3 in SNU-368 cells. SNU-368 cells were transfected with SF3B4 siRNA (siSF3B4) or control siRNA (100 nM, 48 h). (A) Relative mRNA levels for SF3B4 and SRSF3 were examined by qRT-PCR. The data represent mean±S.E from three independent experiments. (B) SRSF3 and SF3B4 expression was detected by western blot analysis (upper). Protein levels were determined by densitometry and expressed as fold change compared to control. Data are mean±S.E., of three independent experiments. *p<0.05.*

knockdown. Thus, the function of SRSF3 as a splicing factor does not seem to be involved in the regulation of SF3B4 expression.

SF3B4 did not affect SRSF3 expression. To investigate the possibility of cross-regulation between SRSF3 and SF3B4, SF3B4 expression was suppressed by siRNA and SRSF3 expression was determined at mRNA and protein levels. Figure 2A shows that SF3B4 was effectively down-regulated, but SRSF3 mRNA levels were not significantly changed. Western blot analysis also indicated that SRSF3 expression was not affected by SF3B4 depletion. In addition to SNU-368 cells, we also demonstrated that SRSF3 depletion notably increased SF3B4 expression both at mRNA and protein levels in Hep3B cells, another HCC cell line (17), whereas SF3B4 did not affect SRSF3 expression (Figure 3A and B). These findings indicate that SRSF3 is an upstream regulator to SF3B4 expression in HCC cells.

SRSF3 knockdown delayed the SF3B4 mRNA degradation involving the coding region in SF3B4 mRNA. We then investigated if SRSF3 depletion-induced accumulation of SF3B4 mRNA is due to the stabilization of SF3B4 mRNA using actinomycin D chase experiments. As shown in Figure 4A, the degradation rate of SF3B4 mRNA following actinomycin D treatment is noticeably retarded by SRSF3 knockdown. In has been previously shown that SF3B4 is regulated by miR-133b *via* 3'-UTR (13). Next, we prepared a construct including only the coding region of SF3B4 in a fusion of GFP in order to examine the requirement of UTR of SF3B4 mRNA for SRSF3-mediated its own expression. RT-PCR results performed with two pairs of primers, targeting GFP and GFP-SF3B4, respectively, showed that SRSF3 depletion clearly lead to an increase in the GFP-SF3B4 mRNA expression, which includes only coding region of SF3B4 (Figure 4B). Thus, the presence of 5' or 3'-UTR of SF3B4 is not involved in the SRSF3-mediated regulation of SF3B4 expression.

Expression profiles of SRSF3 and SF3B4 in human HCC tissues and in SRSF3-knockout mouse liver. The expression pattern of SRSF3 and SF3B4 was analyzed using three GEO public datasets. Figure 5A shows that the overall correlation between SRSF3 and SF3B4 expression levels in the HCC tissues from 100 HCC patients appeared weak (r^2=0.0019). Another GEO dataset of SRSF3-knockout mouse revealed that following SRSF3 knockdown, SF3B4 mRNA increased to about 2.3-fold compared to wild-type mice (Figure 5B). Moreover, the paired analysis between normal and HCC tissues from five patients revealed that SF3B4 mRNA levels

were increased and SRSF3 mRNA levels were decreased in cancer tissues in all the patients (Figure 5C). We also examined the SRSF3 and SF3B4 mRNA levels in HCC tissues from 10 patients by qRT-PCR. As shown in Figure 5D, of these patients, five (P1-P5) exhibited an inverse relationship in SRSF3 and SF3B4 mRNA levels, but the other five (P6-P10) showed similar expression levels of SRSF3 and SF3B4. Collectively, these *in vivo* data indicate that SRSF3 is an important regulator, but not the only factor for SF3B4 expression in HCC.

Discussion

The oncogenic role of SF3B4 in the development of HCC has been suggested by previous studies using extensive meta-analysis as well as integrative analysis of multistage HCC tissues (12, 13). However, limited information exists on how SF3B4 expression is regulated in HCC cells. In the present study, we demonstrated that SRSF3 depletion resulted in a significant increase in SF3B4 mRNA levels as well as in SF3B4 protein levels in SNU-368 and Hep3B HCC cell lines. Public data and clinical data suggest that SRSF3 and SF3B4 are inversely correlated in HCC, although the association is not strong. Thus, our results suggest that SRSF3 is a negative regulator for determining SF3B4 levels in HCC cells.

SRSF3, also called SRp20, is the smallest member of the SR protein family (18). In addition to the role in the regulation of splicing, SRSF3 has been involved in various RNA metabolism including transcription, polyadenylation, RNA export, and translation (19-23). In the present study, SF3B4 protein expression was shown to be increased by SRSF3 depletion (Figure 1D), excluding the possibility for involvement of SRSF3 in the translation inhibition of SF3B4. Actinomycin D chase experiments revealed that SRSF3 depletion delayed the degradation of SF3B4 mRNA in SNU-368 cells. Moreover, the mRNA from GFP-SF3B4 construct, including only coding region, also increased following SRSF3 knockdown (Figure 4). Thus, SRSF3-mediated regulation of SF3B4 mRNA is attributed to the regulation on the degradation rate rather than transcription rate of SF3B4 mRNA. In addition, this mechanism is different from that by miR-133b, which targets 3'-UTR of SF3B4 (13). Although the involvement of SRSF3 in mRNA decay has not been widely known, Mure *et al*. previously reported that SRSF3 makes a specific contribution to the destabilization of intronless viral mRNA decays through recruiting RNA exosome (24). Therefore, in HCC, SF3B4 might be a possible target for SRSF3 to link RNA exosome-mediated degradation, but the specific nuclear milieu in which SF3B4 mRNA should be degraded remains to be defined.

Recently, pro-oncogenic activity of SRSF3 has been proposed on the basis of its increased expression in multiple human cancers (25-28), and its ability to induce tumor formation in nude mice when overexpressed in rat fibroblasts, *via* promoting progression of the G2/M transition (28). Moreover, down-regulation of SRSF3 was shown to lead to G1 arrest and apoptosis, promotion of senescence, retardation of migratory activity and impairment of DNA repair in various cancer cell lines (29-34). These findings imply that SRSF3 promotes tumor progression and gain of malignant phenotypes. Considering the potential role of SF3B4 as an oncogenic driver of HCC in previous studies (12, 13), our results, which show the negative regulatory role of SRSF3 for SF3B4 expression, suggest that SRSF3 depletion may be rather favorable for tumor phenotypes of HCC, which is inconsistent with previous studies for other types of cancer cells. However, the specific role of SRSF3 in preventing chronic liver pathology has been reported by recent studies using hepatocyte-specific Srsf3-knockout (SRSF HKO) mice. The SRSF3 HKO mice at 1 month of age revealed impaired hepatocyte maturation with alteration in glucose and lipid homeostasis, indicating that SRSF3 is required for the morphological and functional differentiation of hepatocytes (35). Subsequently, the same group reported that, with aging, SRSF3 HKO mice develop spontaneous HCC following chronic liver diseases with progressive steatosis and fibrosis (36). These data indicate that SRSF3 is critical for the normal physiology of liver and functional loss of SRSF3 ultimately leads to the development of HCC. Wang *et al*. also demonstrated the protective role of SRSF3 in the prevention of HCC, showing that 5-year survival rate of HCC patients was higher in the SRSF3-high group than in the SRSF3-low group (16). Thus, our results, which show the negative regulatory function of SRSF3 in SF3B4 expression, might reinforce the protective role played by SRSF3 in the progression of HCC. Furthermore, accumulation of SF3B4 could also contribute to the development of HCC driven by SRSF3 knockout in addition to the previously proposed events, such as the abnormal splicing of epithelial-mesenchymal transition genes and increased expression of insulin-like growth factor and c-myc (16).

Even though we demonstrated that SF3B4 expression is specifically regulated by SRSF3 in SNU-368 HCC cells, the association of SRSF3 and SF3B4 mRNA levels was not strong in HCC tissue samples in public datasets as well as in our study (Figure 5). A possible explanation for this discrepancy is that the association between them might be dependent on clinical stages, early or late, as well as on the presence of other factors that determine SF3B4 expression. In addition, it might be due to the auto-regulatory function of SRSF3, which means that SRSF3 protein levels do not always correlate with SRSF3 mRNA levels (37). In line with this, the functional loss of SRSF3 in human liver diseases such as non-alcoholic fatty liver and steatohepatitis or

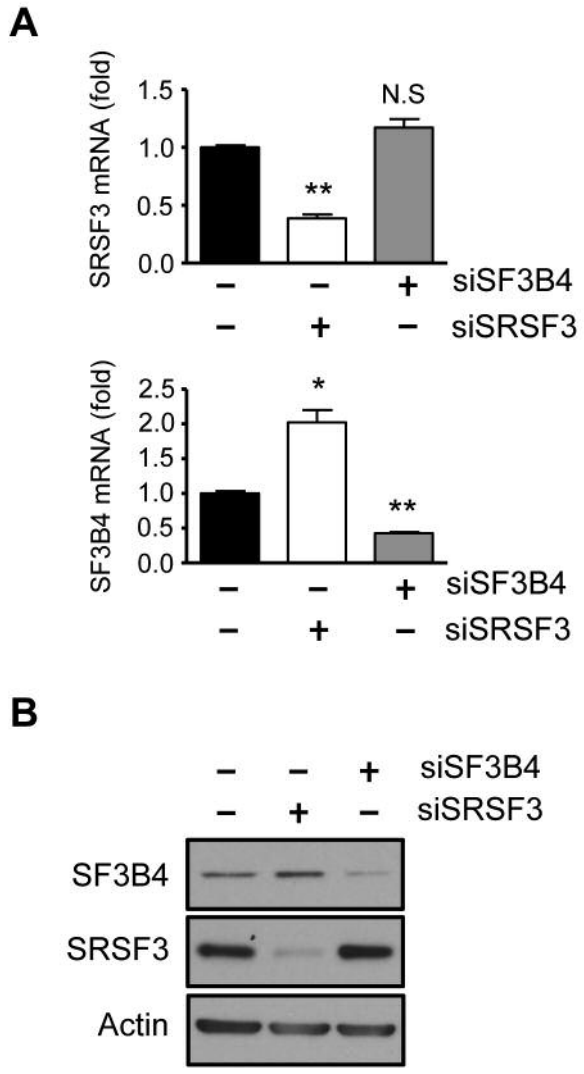

Figure 3. *Effect of SRSF3 or SF3B4 knockdown on the expression of SF3B4 or SRSF3, respectively in Hep3B cells. Hep3B cells were transfected with control, SRSF3 or SF3B4 siRNA (50 nM, 48 h). (A) SRSF3 and SF3B4 levels mRNA were analyzed with qRT-PCR and expressed as fold change compared to control. Data are mean±S.E, of three independent experiments. (B) The expression of SRSF3 and SF3B4 were detected by western blot analysis. *p<0.05, **p<0.01.*

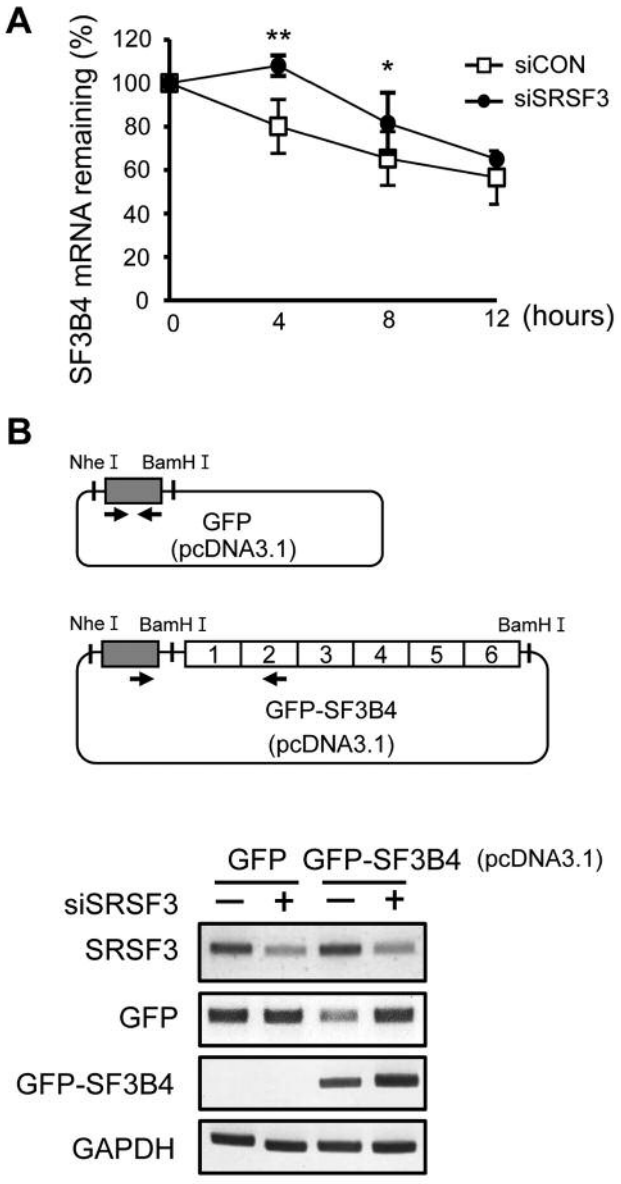

Figure 4. *SRSF3 regulates SF3B4 mRNA stability involving the coding region of SF3B4. (A) Degradation rate of SF3B4 mRNA in SRSF3-depleted SNU-368 cells was measured by actinomycin D chase assay. SF3B4 mRNA levels were quantified by qRT-PCR at the indicated times after treatment actinomycin D (5.0 μg/ml) and the remaining levels were presented as percentage of control at 0 h time. Data are mean±S.E, of three independent experiments. *p<0.05, **p<0.01 vs. the value of control at the same time. (B) Schematic diagram of GFP and GFP-SF3B4 fusion constructs (upper). The mRNA levels from GFP-SF3B4 fusion construct including full length of coding region of SF3B4 was analyzed using RT-PCR and visualized by 1.5% agarose electrophoresis (lower).*

cirrhosis as well as in mouse models, is attributable to the neddylation-dependent proteasomal degradation, which occurs independently of mRNA expression (38).

In summary, our results indicate that SRSF3 is a novel regulator for SF3B4 mRNA levels in HCC cells, suggesting the possibility that inhibition of SRSF3 degradation is an important strategy for preventing the progression of HCC. The results provide an important link between the opposite roles played by SRSF3 and SF3B4 in the progression of HCC, *i.e.*, protective and oncogenic function, respectively.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

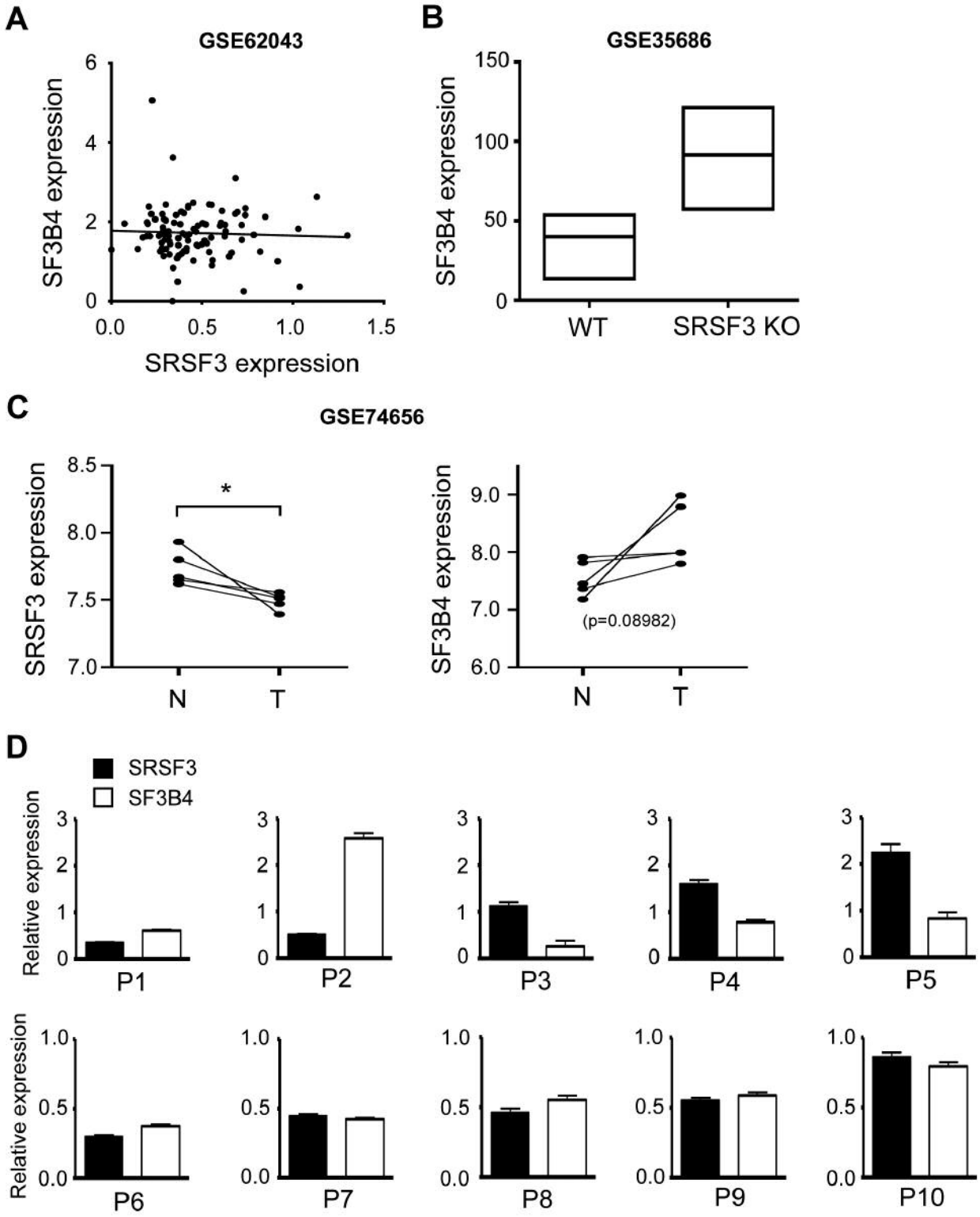

Figure 5. *In vivo profile of SRSF3 and SF3B4 expression in human HCC tissues and SRSF3-knockout mice. (A) The association of SRSF3 and SF3B4 levels was analyzed in HCC patients by scatter plotting (n=100). (B) SF3B4 mRNA levels in the liver from wild-type (WT) and SRSF3-knockout (KO) mice. (C) The paired expression of SRSF3 and SF3B4 mRNA levels in adjacent normal (N) tissues and HCC (T) tissues (n=5), *p<0.05. (D) The relative expression levels of SRSF3 and SF3B4 mRNAs in 10 HCC patients (P: patient). The SRSF3 or SF3B4 mRNA levels in SNU-368 cells were designated as 1.0.*

Authors' Contributions

JL and GS conducted the experiments. JL drafted the manuscript under the supervision of JHL. JHL conceived the idea for this study, wrote and edited the manuscript. WH and SKY analyzed the clinical data. SWN interpreted the public data. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by Basic Science Research Programs though the National Research Foundation of Korea (NRF), funded by the Minister of Education, Science and Technology (2017R1A2B2005508 and 2019R1A2C1086949).

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Received March 3, 2020
Revised March 11, 2020
Accepted March 17, 2020