Increased Copy Number of the Gene Encoding SF3B4 Indicates Poor Prognosis in Hepatocellular Carcinoma

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Abstract. *Background/Aim: Defects in alternative splicing contribute to carcinogenesis, cancer progression and chemoresistance. The spliceosome pathway, including SF3B4, a component of spliceosomal complex is suggested to play a role in progression of hepatocellular carcinoma (HCC); however, the clinical relevance of SF3B4 in HCC remains unknown. Patients and Methods: SF3B4 expression was evaluated by real-time reverse transcription polymerase chain reaction in 72 HCC samples and non-cancerous liver samples. The relationship between the DNA copy number and SF3B4 expression levels was investigated using TCGA datasets. Results: SF3B4 expression was significantly higher in cancerous than in non-cancerous tissues and positively correlated with SF3B4 DNA copy number. High SF3B4 expression is significantly associated with intrahepatic metastasis and poor prognosis. These results were consistent with data from the public datasets. Conclusion: Overexpression of SF3B4, that is due to DNA copy number increase, is suggested to play a role in progression of HCC.*

Hepatocellular carcinoma (HCC), a major histological subtype of liver cancer, is one of the most common solid cancers worldwide. Despite advances in diagnostic and surgical approaches, HCC is the second leading cause of cancer-related death because of a high incidence of recurrence (1, 2). Therefore, there is an urgent need to establish novel therapeutic strategies for treating patients with advanced HCC based on molecular information; however, the molecular and genetic mechanisms underlying HCC progression remain unclear.

Splicing is an important step during gene transcription, wherein intron sequences are removed from pre-mRNA and exon sequences are joined, followed by production of mature mRNA. The spliceosome is composed of 5 small nuclear ribonucleoproteins (snRNPs) – U1, U2, U4, U5, and U6 – and multiple other proteins (3, 4). Alternative splicing factors have been reported in various disorders, including cancers (5), leading to defective alternative splicing, abnormal production of specific splicing variants promoting carcinogenesis, progression and chemoresistance (6-8). Recently, bioinformatics studies have shown that the spliceosomal pathway is involved in the progression of HCC (9-12).

It has been known that mutant forms of SF3B4 (Splicing factor B, subunit 4), a component of the U2 pre-mRNA spliceosomal complex, is the major cause of Nager syndrome (13-15). The *SF3B4* gene may also act as an oncogene. Terada *et al*. reported that abolishing the function of the SF3B2–SF3B4 complex activates cell cycle check points and induces G2 arrest (16). Also, recent comprehensive analysis of HCC indicated that many spliceosome pathway-related genes, including *SF3B4*, are up-regulated in HCC (12). Thus, the aim of this study was to clarify the clinical significance of *SF3B4* expression in HCC.

Patients and Methods

Patients. Between August 2000 and July 2004, 113 patients underwent hepatic resection and were diagnosed histologically to have HCC at our Institute and our affiliated hospitals. Of 113 patients with HCC, 72 providing HCC tissue and matched non-cancerous tissue were enrolled in this study. The mean follow-up after initial surgery was 3.5±1.7 years (median=4.9 years). Any postoperative survival or recurrence was entered into the database immediately when a patient died or a recurrence was strongly suspected following standard surveillance. All clinicopathological

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Key Words: SF3B, copy number alteration, hepatocellular carcinoma, progression, prognosis.

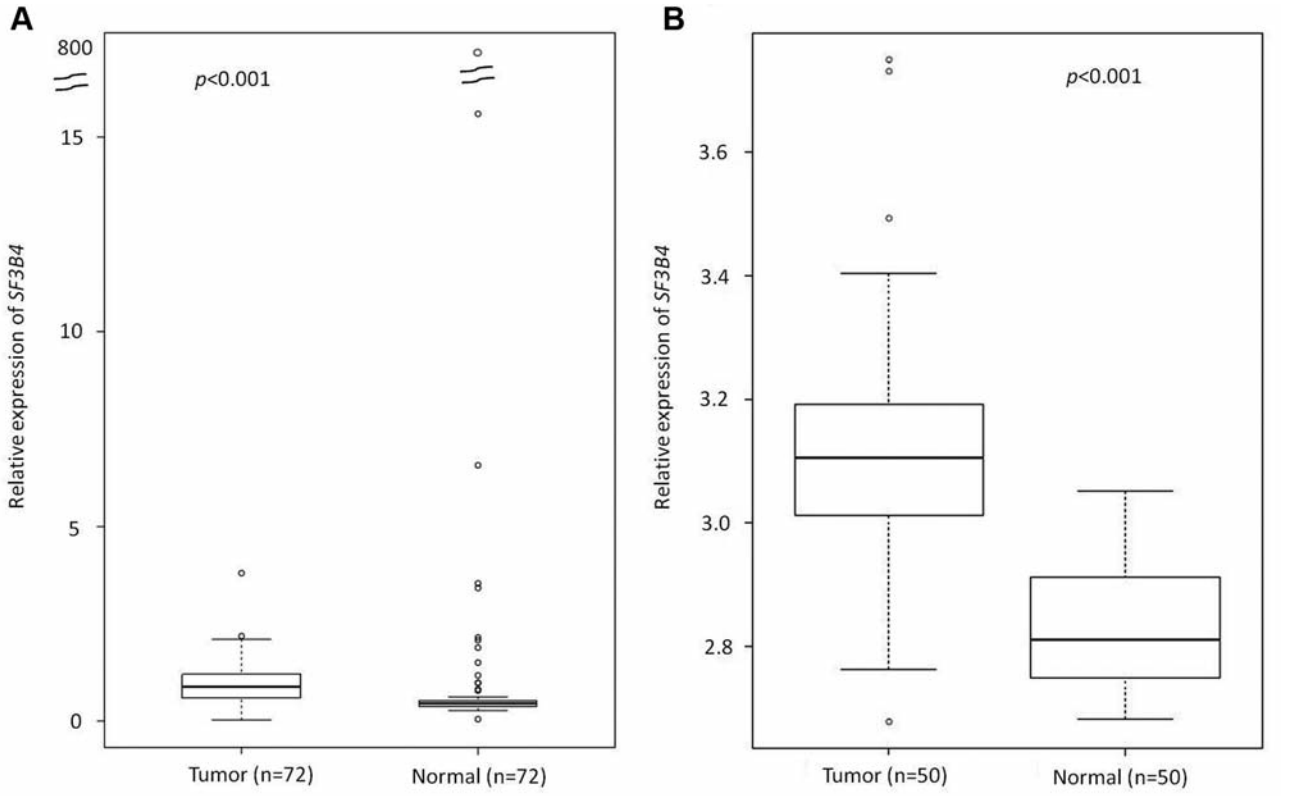

Figure 1. *Comparison of SF3B4 expression between HCC and non-cancerous tissue. SF3B4 expression in HCC was significantly higher compared to non-cancerous tissue in our cohort (A) and TCGA (B).*

data, including patient's age, sex, etiology, Child-Pugh classification, alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP), maximum tumor size, invasion to fibrous capsule, portal venous invasion, hepatic venous invasion, bile ductal invasion, intrahepatic metastasis and Edmondson classification were obtained from the database. Informed consent was obtained from each patient included in the study. All resected HCC and adjacent non-cancerous liver tissue samples were immediately collected, frozen in liquid nitrogen and stored at –80˚C until RNA extraction.

RNA preparation and reverse transcription (RT) reaction. Total RNA was extracted from frozen HCC and non-cancerous tissue samples using ISOGEN (Nippon Gene, Tokyo, Japan). RT was performed according to the manufacturer's protocol. cDNA was generated from 8 μg total RNA with M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA).

Quantitative real-time PCR (qPCR). qPCR was performed in a LightCycler 480 instrument (Roche Applied Science, Basel, Switzerland) using a LightCycler 480 Probes Master kit (Roche Applied Science) according to the manufacturer's instructions. PCR primer sequences for human *SF3B4* were as follows: sense, 5'-AGACGGCGGGATCTCTTT-3'; antisense, 5'-CACGTACACAGTGG CATCCT-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers, which served as the internal control to normalize the expression level of *SF3B4*, were as follows: sense, 5'-TTGGTATCG TGGAAGGACTCTCA-3'; antisense, 5'-TGTCATATTTGGCAGGTT-3'. The amplification conditions were as follows: 10 min at 95˚C, followed by 45 cycles of 10 s at 95˚C and 30 s at 60˚C. The expression levels were expressed as the values relative to the expression levels of Human Universal Reference Total RNA (Clontech, Palo Alto, CA, USA).

Public clinical dataset. We obtained *SF3B4* expression profiles and data on prognosis of HCC cases from The Cancer Genome Atlas (TCGA) of the Broad Institute's Firehose (http:// gdac.broadinstitute.org/) and The National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (accession codes GSE14520). Copy number data for 370 cases were also obtained from TCGA.

Statistical analysis. x^2 test or Fisher's exact test was used for comparisons between SF3B4 expression and clinicopathological findings. Survival curves were calculated by the Kaplan-Meier method and differences between the curves were analyzed by the log-rank test. A comparison of *SF3B4* expression in HCC and non-cancerous tissue was evaluated using Mann-Whitney's *U*-test. These results were analyzed using JMP 9 software (SAS Institute, Cary, NC, USA) or R version 3.1.1 (R Core Team (2014). R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/). *p*-Values less than 0.05 were considered statistically significant.

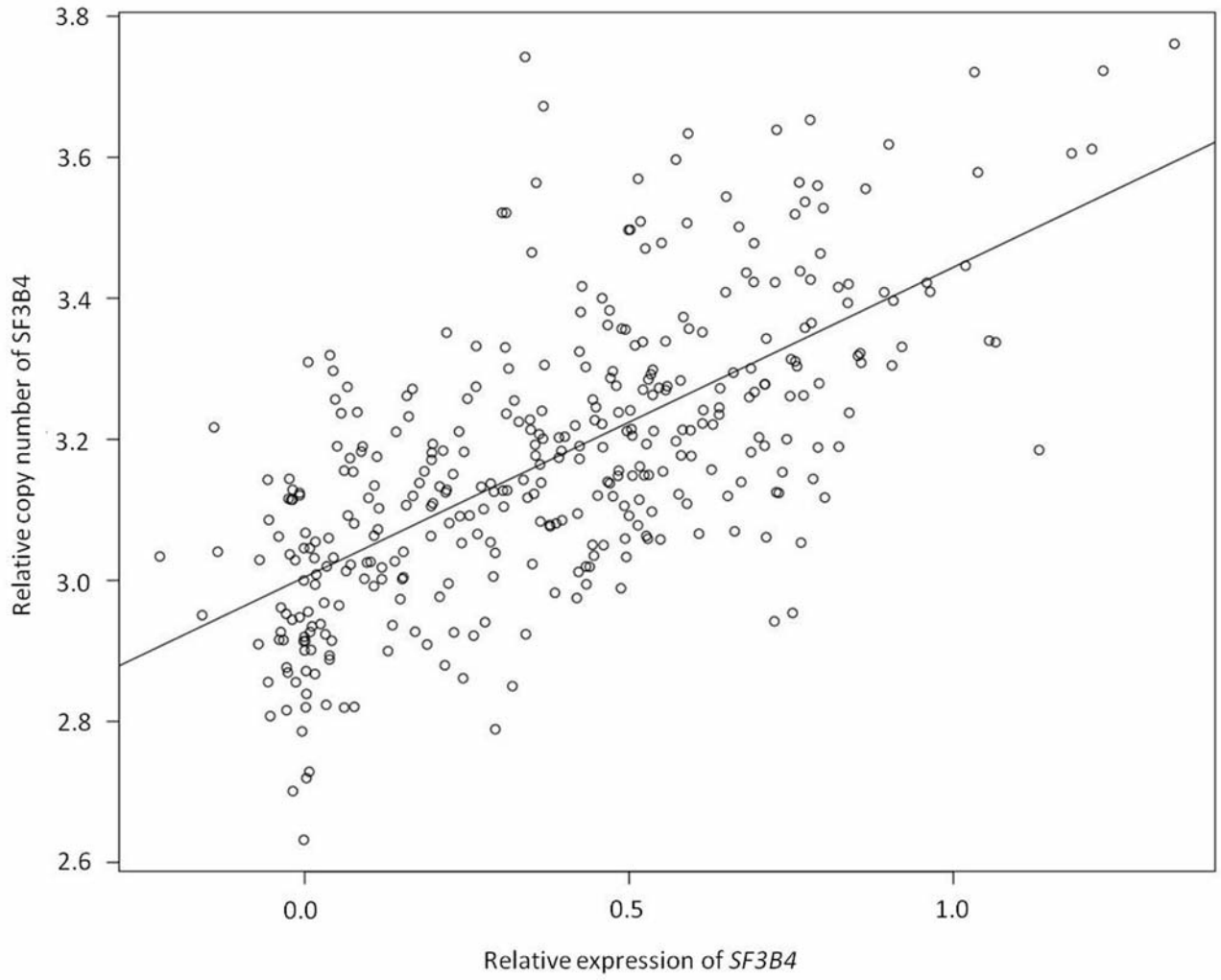

Figure 2. *Relationship between copy number and expression levels of SF3B4. SF3B4 expression was positively correlated with SF3B4 gene copy number (R=0.67, p<0.001).*

Results

SF3B4 expression was higher in HCC than in non-cancerous tissue. We compared *SF3B4* expression between HCC and adjacent non-cancerous tissue by RT-qPCR. *SF3B4* expression was higher in HCC than in the non-cancerous tissue ($p<0.001$; Figure 1A). In addition, it was consistent with the data from the public datasets, TCGA (Figure 1B).

Correlation between SF3B4 gene copy number variation and SF3B4 expression. To examine the influence of gene copy number variation on *SF3B4* mRNA expression, we examined the relationship between copy number and expression levels of *SF3B4* in the TCGA dataset. A strong correlation between them was observed in tumor tissues (R=0.67, $p<0.001$; Figure 2).

Up-regulated SF3B4 expression was associated with poor outcome in patients with HCC. We divided the 72 patients with HCC in our cohort into an *SF3B4* high-expression group (n=38) and a low-expression group (n=34) according to the ratio of *SF3B4* expression in HCC to the non-cancerous tissue by the minimum *p*-value approach for recurrence-free survival (RFS). RFS rates in patients with low *SF3B4* expression were 76.5%, 51.7% and 33.3% at 1, 3 and 5 years, respectively, while those in patients with high *SF3B4* expression were 54.0%, 29.3% and 18.6%, respectively. The analysis of RFS revealed that the *SF3B4* high-expression group had significantly poorer outcomes than the low-expression group ($p=0.046$; Figure 3A). However, no significant difference was found in overall survival (OS) between the two groups (data not shown). In addition, the public datasets also revealed that the *SF3B4* high-expression group had significantly poorer outcomes than the low-expression group for

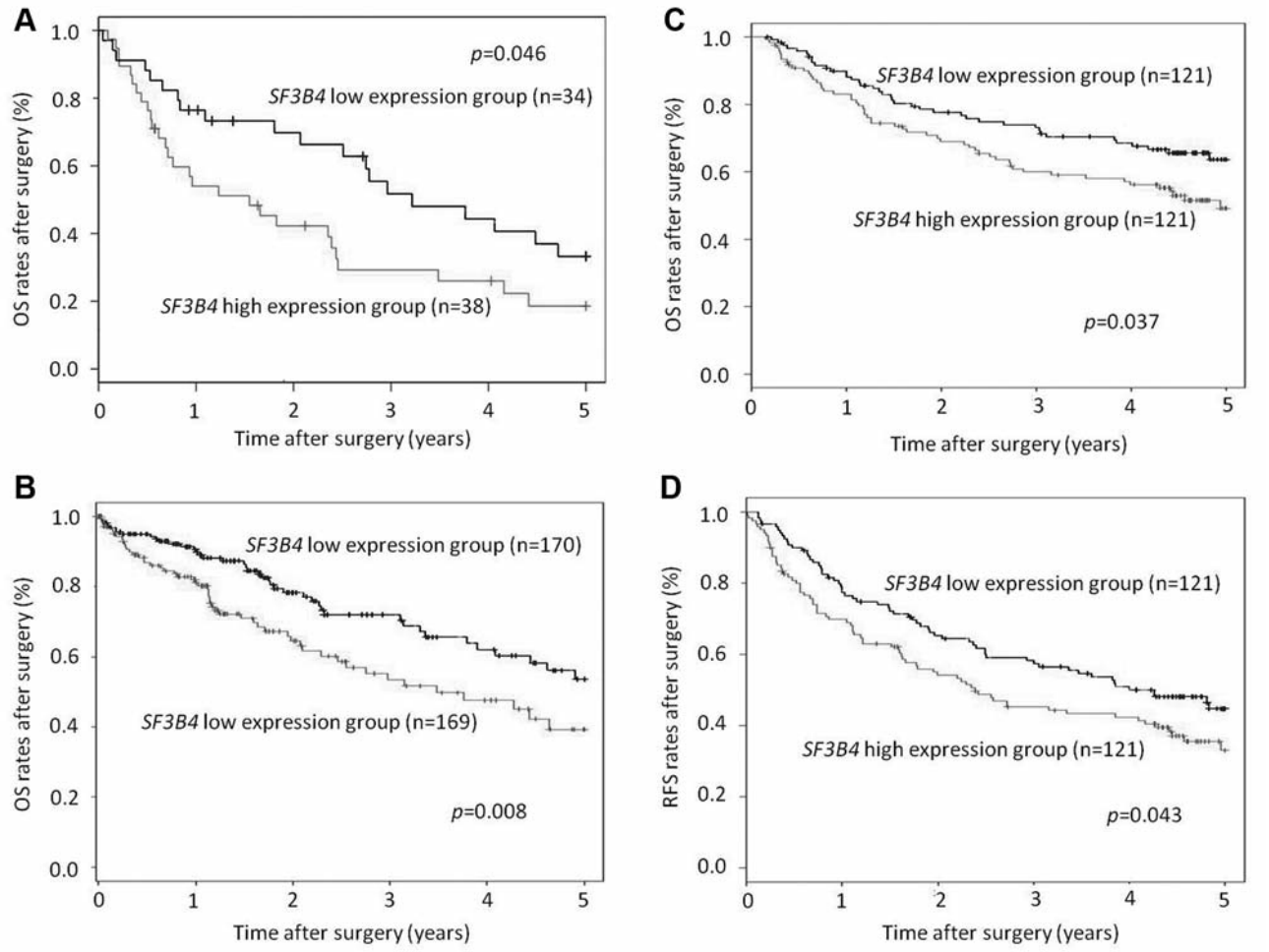

Figure 3. *Kaplan-Meier curves for SF3B4 high-expression group and SF3B4 low-expression group. The SF3B4 high-expression group had significantly poorer outcomes than the SF3B4 low-expression group for RFS of our cohort (A), OS of TCGA (B) and OS and DFS of GSE14520 (C, D) (p=0.046, p=0.008, p=0.037 and p=0.043, respectively). OS, Overall survival; RFS, recurrence-free survival.*

OS of TCGA and OS and DFS of GSE14520 (p=0.008, p=0.037 and p=0.043, respectively; Figure 3B, C, D).

Correlations between the expression level of SF3B4 and clinicopathological factors. We compared the clinicopathological findings of patients with high and low *SF3B4* expression in our cohort (Table I). Intrahepatic metastasis was more frequently observed in patients in the *SF3B4* high expression group than in patients in the *SF3B4* low expression group (p=0.076). However, no significant differences were found in other clinicopathological factors.

Discussion

Splicing is affected by point-mutations, histone modifications, non-coding RNA and the transcription machinery (17). SF3B, a multiprotein complex, is an essential component of the spliceosome for mature mRNA processing and its genetic aberration has been reported in several cancers (18-21). For example, *SF3B1* mutation has been well documented in solid cancers, such as breast cancer, pancreatic cancer and uveal melanoma (18-20). *SF3B3* overexpression is also associated with prognosis and endocrine resistance in breast cancer (21). A recent study showed that *SF3B4* was up-regulated in HCC relative to non-cancerous tissue (12). Herein we provided the first description of the clinicopathological role of *SF3B4* in HCC through analysis of our cohort and public data.

Aberrant expression of splicing factors induces malignant transformation (22). Recently, the involvement of the spliceosome pathway was reported in the development of HCC from cirrhosis due to HCV (9). Additionally, *SF3B4*

Table I. *Comparative analysis of clinicopathological findings between the SF3B4 low-expression group and the SF3B4 high-expression group.*

	SF3B4 low expression group (n=34)		SF3B4 high expression group (n=38)		*p*-Value
	n	%	n	%	
Age (years)					
<70	19	61.3	18	56.3	0.7994
>71	12	38.7	14	43.7	
Gender					
Male	22	71	24	75	0.7816
Female	9	29	8	25	
Etiology					
HBV	7	20.6	11	28.9	0.7158
HCV	22	64.7	22	57.9	
NBNC	5	14.7	5	13.2	
Child-Pugh					
A	31	91.2	33	86.8	0.714
B	3	8.8	5	13.2	
AFP					
<100	9	27.3	16	43.2	0.2139
>100	24	72.7	21	56.8	
DCP					
<200	11	33.3	13	35.1	0.999
>200	21	66.7	24	64.9	
Maximum tumor size					
<3cm	14	41.1	18	47.4	0.6409
>3cm	20	58.8	20	52.6	
Invasion to fibrous capsule					
Absent	9	31	10	32.3	0.999
Present	20	69	21	67.7	
Portal venous invasion					
Absent	20	58.8	22	57.9	0.999
Present	14	41.2	16	42.1	
Hepatic venous invasion					
Absent	22	66.7	22	57.9	0.4736
Present	11	33.3	16	42.1	
Bile ductal invasion					
Absent	32	94.1	34	89.5	0.6769
Present	2	5.9	4	10.5	
Intrahepatic metastasis					
Absent	27	79.4	22	57.9	0.076
Present	7	20.6	16	42.1	
Edmondson classification					
Grade I	6	17.6	3	7.9	0.2582
Grade II	21	61.8	30	78.9	
Grade III	7	20.6	5	13.2	

HBV, Hepatitis B virus; HCV, hepatitis C virus; NBNC, non-viral infection; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

was significantly up-regulated in HCC compared to adjacent liver tissue (12). Consistent with these previous reports, *SF3B4* expression was found to be significantly higher in HCC than in the non-cancerous tissue in our cohort in Japan and the public datasets. The major etiology of HCC in Japan remains HCV, differing from that worldwide (23). These findings suggest that *SF3B4* may contribute to hepatocarcinogenesis regardless of etiology.

However, the question of how *SF3B4* expression is regulated has been elusive. Providing valuable insight into this question, we showed that *SF3B4* expression was positively correlated with DNA copy number. This corresponds with previous reports that up-regulated SF2/ASF, a splicing factor, functions as a proto-oncogene due to amplification of its gene (22), and a localized duplication at 1q21.2, in which SF3B4 is located, were identified by FISH in acute lymphoblastic leukemia and Burkitt lymphoma (24).

This study revealed that *SF3B4* expression is involved in intrahepatic metastasis and poor prognosis in HCC. Alternative splicing variants of specific genes could affect invasiveness, proliferation, anti-apoptosis, angiogenesis and survival (6, 25). Aberrant *SF3B4* expression may likewise contribute to several pathways involved in the progression of HCC. Further examination is needed to confirm the molecular mechanisms of *SF3B4* in cancer progression.

In conclusion, our study showed that *SF3B4* plays an oncogenic role in progression of HCC. *SF3B4* could be a therapeutic target, as well as a novel prognostic factor in HCC.

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References

1 GLOBOCAN 2012; http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx
2 Bruix J, Gores GJ and Mazzaferro V: Hepatocellular carcinoma: clinical frontiers and perspectives. Gut *63*: 844-855, 2014.
3 Maniatis T and Tasic B: Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature *418*: 236-243, 2002.

4 Matera AG and Wang Z: A day in the life of the spliceosome. Nat Rev Mol Cell Biol *15*: 108-121, 2014.
5 Wang GS and Cooper TA: Splicing in disease: disruption of the splicing code and the decoding machinery. Nat Rev Genet *8*: 749-761, 2007.
6 Grosso AR, Martins S and Carmo-Fonseca M: The emerging role of splicing factors in cancer. EMBO Rep *9*: 1087-1093, 2008.
7 Matlin AJ, Clark F and Smith CW Understanding alternative splicing: towards a cellular code. Nat Rev Mol Cell Biol *6*: 386-398, 2005.
8 Kim E, Goren A and Ast G: Insights into the connection between cancer and alternative splicing. Trends Genet *24*: 7-10, 2008.
9 Wang Y, Li J, Chen J, Liu L, Peng Z, Ding J and Ding K: From cirrhosis to hepatocellular carcinoma in HCV-infected patients: genes involved in tumor progression. Eur Rev Med Pharmacol Sci *16*: 995-1000, 2012.
10 Wong YH, Wu CC, Lin CL, Chen TS, Chang TH and Chen BS: Applying NGS Data to Find Evolutionary Network Biomarkers from the Early and Late Stages of Hepatocellular Carcinoma. Biomed Res Int *2015*: 391475, 2015.
11 Tian M, Cheng H, Wang Z, Su N, Liu Z, Sun C, Zhen B, Hong X, Xue Y and Xu P: Phosphoproteomic analysis of the highly-metastatic hepatocellular carcinoma cell line, MHCC97-H. Int J Mol Sci *16*: 4209-4225, 2015.
12 Xu W, Huang H, Yu L and Cao L: Meta-analysis of gene expression profiles indicates genes in spliceosome pathway are up-regulated in hepatocellular carcinoma (HCC). Med Oncol *32*: 96, 2015.
13 Bernier FP, Caluseriu O, Ng S, Schwartzentruber J, Buckingham KJ, Innes AM, Jabs EW, Innis JW, Schuette JL, Gorski JL, Byers PH, Andelfinger G, Siu V, Lauzon J, Fernandez BA, McMillin M, Scott RH, Racher H; FORGE Canada Consortium, Majewski J, Nickerson DA, Shendure J, Bamshad MJ and Parboosingh JS: Haploinsufficiency of SF3B4, a component of the pre-mRNA spliceosomal complex, causes Nagersyndrome. Am J Hum Genet *90*: 925-933, 2012.
14 Lehalle D, Wieczorek D, Zechi-Ceide RM, Passos-Bueno MR, Lyonnet S, Amiel J and Gordon CT: A review of craniofacial disorders caused by spliceosomal defects. Clin Genet *88*: 405-415, 2015.
15 Czeschik JC, Voigt C, Alanay Y, Albrecht B, Avci S, Fitzpatrick D, Goudie DR, Hehr U, Hoogeboom AJ, Kayserili H, Simsek-Kiper PO, Klein-Hitpass L, Kuechler A, López-González V, Martin M, Rahmann S, Schweiger B, Splitt M, Wollnik B, Lüdecke HJ, Zeschnigk M and Wieczorek D: Clinical and mutation data in 12 patients with the clinical diagnosis of Nager syndrome. Hum Genet *132*: 885-898, 2013.
16 Terada Y and Yasuda Y: Human immunodeficiency virus type 1 Vpr induces G2 checkpoint activation by interacting with the splicing factor SAP145. Mol Cell Biol *26*: 8149-8158, 2006.
17 Luco RF and Misteli T: More than a splicing code: integrating the role of RNA, chromatin and non-coding RNA in alternative splicing regulation. Curr Opin Genet Dev *21*: 366-372, 2011.
18 Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, Song S, Harliwong I, Idrisoglu S, Nourse C, Nourbakhsh E, Manning S, Wani S, Gongora M, Pajic M, Scarlett CJ, Gill AJ, Pinho AV, Rooman I, Anderson M, Holmes O,Leonard C, Taylor D, Wood S, Xu Q, Nones K, Fink JL, Christ A, Bruxner T, Cloonan N, Kolle G, Newell F, Pinese M, Mead RS, Humphris JL, Kaplan W, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chou A, Chin VT, Chantrill LA, Mawson A, Samra JS, Kench JG, Lovell JA, Daly RJ, Merrett ND, Toon C, Epari K,Nguyen NQ, Barbour A, Zeps N; Australian Pancreatic Cancer Genome Initiative, Kakkar N, Zhao F, Wu YQ, Wang M, Muzny DM, Fisher WE, Brunicardi FC,Hodges SE, Reid JG, Drummond J, Chang K, Han Y, Lewis LR, Dinh H, Buhay CJ, Beck T, Timms L, Sam M, Begley K, Brown A, Pai D, Panchal A, Buchner N,De Borja R, Denroche RE, Yung CK, Serra S, Onetto N, Mukhopadhyay D, Tsao MS, Shaw PA, Petersen GM, Gallinger S, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Schulick RD, Wolfgang CL, Morgan RA, Lawlor RT, Capelli P, Corbo V, Scardoni M, Tortora G, Tempero MA, Mann KM, Jenkins NA, Perez-Mancera PA, Adams DJ, Largaespada DA, Wessels LF, Rust AG, Stein LD, Tuveson DA, Copeland NG, Musgrove EA, Scarpa A, Eshleman JR, Hudson TJ, Sutherland RL,Wheeler DA, Pearson JV, McPherson JD, Gibbs RA and Grimmond SM: Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature *491*: 399-405, 2012.
19 Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature *490*: 61-70, 2012.
20 Harbour JW, Roberson ED, Anbunathan H, Onken MD, Worley LA and Bowcock AM: Recurrent mutations at codon 625 of the splicing factor SF3B1 in uveal melanoma. Nat Genet *45*: 133-135, 2013.
21 Gökmen-Polar Y, Neelamraju Y, Goswami CP, Gu X, Nallamothu G, Janga SC and Badve S: Expression levels of SF3B3 correlate with prognosis and endocrine resistance in estrogen receptor-positive breast cancer. Mod Pathol *28*: 677-685, 2015.
22 Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D and Krainer AR: The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nat Struct Mol Biol *14*: 185-193, 2007.
23 Higuchi M, Tanaka E and Kiyosawa K: Epidemiology and clinical aspects on hepatitis C. Jpn J Infect Dis *55*: 69-77, 2002.
24 La Starza R, Crescenzi B, Pierini V, Romoli S, Gorello P, Brandimarte L, Matteucci C, Kropp MG, Barba G, Martelli MF and Mecucci C: A common 93-kb duplicated DNA sequence at 1q21.2 in acute lymphoblastic leukemia and Burkitt lymphoma. Cancer Genet Cytogenet *175*: 73-76, 2007.
25 Ghigna C, Giordano S, Shen H , Benvenuto F, Castiglioni F, Comoglio PM, Green MR, Riva S and Biamonti G: Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. Mol Cell *20*: 881-890, 2005.

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