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
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’- AGACGCGGAGCTTTTCTCCTTTC-3’; antisense, 5’-CACGTACACAGTGGCATCCT-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’-TTGGTATCGTGGAAGCTCA-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.** $\chi^2$ 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.

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**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).
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
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
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

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