Decreased Expression of Fructose-1,6-bisphosphatase Associates with Glucose Metabolism and Tumor Progression in Hepatocellular Carcinoma

Hidenari Hirata1,2,3, Keishi Sugimachi1, Hisateru Komatsu1, Masami Ueda1, Takaaki Masuda1, Ryutaro Uchi1, Shotaro Sakamura1, Sho Nambara1, Tomoko Saito1, Yoshiaki Shinden1, Tomohiro Iguchi1, Hidetoshi Eguchi1, Shuhei Ito1, Kotaro Terashima2, Katsumi Sakamoto2, Masakazu Hirakawa2, Hiroshi Honda3, and Koshi Mimori1

Abstract

Fructose-1,6-bisphosphatase (FBP1), the rate-limiting enzyme in gluconeogenesis, is reduced in expression in certain cancers where it has been hypothesized to act as a tumor suppressor, including in hepatocellular carcinoma (HCC). Here, we report functional evidence supporting this hypothesis, providing a preclinical rationale to develop FBP1 as a therapeutic target for HCC treatment. Three independent cohorts totaling 594 cases of HCC were analyzed to address clinical significance. Lower FBP1 expression associated with advanced tumor stage, poor overall survival, and higher tumor recurrence rates. In HCC cell lines, where endogenous FBP1 expression is low, engineering its ectopic overexpression inhibited tumor growth and intracellular glucose uptake by reducing aerobic glycolysis. In patient specimens, promoter methylation and copy-number loss of FBP1 were independently associated with decreased FBP1 expression. Similarly, FBP1 downregulation in HCC cell lines was also associated with copy-number loss. HCC specimens exhibiting low expression of FBP1 had a highly malignant phenotype, including large tumor size, poor differentiation, impaired gluconeogenesis, and enhanced aerobic glycolysis. The effects of FBP1 expression on prognosis and glucose metabolism were confirmed by gene set enrichment analysis. Overall, our findings established that FBP1 downregulation in HCC contributed to tumor progression and poor prognosis by altering glucose metabolism, and they rationalize further study of FBP1 as a prognostic biomarker and therapeutic target in HCC patients. Cancer Res; 76(11); 3265–76. ©2016 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide and is associated with a high mortality rate (1). Therefore, identification of useful biomarkers predicting clinical outcomes and molecular targets for treatment is required. Altered glucose metabolism, particularly aerobic glycolysis, is one of the hallmarks of cancers, including HCC (2), supporting the notion that the molecules involved in altered glucose metabolism may be potential biomarkers and therapeutic targets. Many studies have examined aerobic glycolysis in various cancers; however, gluconeogenesis has not been studied extensively. Gluconeogenesis is also thought to play an important role in tumor progression in HCC because gluconeogenesis mainly occurs in normal hepatocytes (3). A recent study reported the loss of gluconeogenic capacity in HCC (4). Moreover, the switch from aerobic glycolysis to gluconeogenesis may be an effective treatment in patients with HCC (5). Nevertheless, the role of impaired gluconeogenesis in HCC progression is still poorly understood.

The tumor-suppressive function of the fructose-1,6-bisphosphatase (FBP1) gene, which encodes a rate-limiting gluconeogenic enzyme, has been reported in certain cancers. Loss of FBP1 expression has been shown to promote tumor progression by enhancing aerobic glycolysis, thereby resulting in poor prognosis in patients with clear cell renal cell carcinoma (cRCC; ref. 6) and breast cancer (7). With regard to HCC, in vitro experiments have shown that FBP1 expression is silenced by promoter methylation and that ectopic FBP1 expression inhibits anchorage-dependent growth of a cell line through cell-cycle arrest (8). However, the role of FBP1 in altered glucose metabolism has not been assessed in HCC. Furthermore, no studies have investigated whether aberrant FBP1 expression affects clinical outcomes in patients with HCC.

Therefore, the objective of this study was to examine the function and clinical significance of FBP1 expression in HCC. Through analysis of three independent large cohorts of HCC cases, the present study evaluated whether aberrant FBP1 expression was associated with tumor progression and prognosis in patients with HCC. We also elucidated the genetic and epigenetic mechanisms regulating FBP1 expression and investigated the role of FBP1 expression in altered glucose metabolism.
Materials and Methods

Patients and sample collection

One hundred eighteen patients with HCC who underwent hepatic resection at Kyushu University Beppu Hospital and affiliated hospitals between 2000 and 2004 were enrolled in this study. Resected tumor tissues and paired normal liver tissues were immediately stored in RNAlater (Ambion), frozen in liquid nitrogen, and kept at -80°C until RNA extraction. A 5-year follow-up was conducted after operation. The median follow-up for the 118 patients was 59.5 months (range, 3.0–60 months). Patients were staged according to the seventh edition of the International Union against Cancer TNM classification system. Of the 118 HCC patients, paired normal liver tissues were available for 115 patients. All protocols were approved by the Ethics and Indications Committee of Kyushu University. Written informed consent was obtained from all patients.

Cell culture

HuH7 and HepG2 cells authenticated by short tandem repeat profiling using the PowerPlex 1.2 System (Promega) were obtained from the cell bank of RIKEN BioResource Center (Tsuchi, Japan) in 2015. Cells were used within 6 months of culturing after resuscitation. Details are provided in the Supplementary Materials and Methods.

construction of an FBP1 expression lentiviral vector

A full-length cDNA insert of human FBP1 was amplified by PCR. Lentiviruses were generated by transfection of HEK293T cells with pCMV- hsv-G-RSV-Rev, pCAG-HIVgp, and either CSII-CMV-FBP1 or CSII-CMV-MCS (empty) plasmid DNAs (5 μg each) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Forty-eight hours after transfection, the lentivirus-containing supernatant was collected and filtered through a 0.45-μm filter. Infections were then carried out by incubating cells with medium containing the lentiviral supernatant for 48 hours.

RNA preparation and quantitative real-time RT-PCR

Detailed methods are provided in the Supplementary Materials and Methods.

Analysis of public clinical datasets and gene set enrichment analysis

We obtained paired gene-expression and survival profiles of 242 HCC samples from the National Cancer for Biotechnology Information Gene Expression Omnibus (GEO) database (accession codes GSE14520; ref. 9). Expression profiles of 228 paired normal liver samples were available. Moreover, we obtained paired RNA sequencing and survival data of 234 HCC cases in The Cancer Genome Atlas (TCGA) from the Broad Institute’s Firehose (http://gdac.broadinstitute.org/runs/stddata__2014_10_17/data/LIHC/20141017/). Expression profiles of 48 paired normal liver samples were available. Data from methylation arrays, SNP arrays, and whole-exome sequencing were also obtained. Details are provided in the Supplementary Materials and Methods.

Analysis of the Cancer Cell Line Encyclopedia database

We obtained normalized mRNA expression and DNA copy-number data of human cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE; ref. 10) from cBioPortal For Cancer Genomics (http://www.cbioportal.org/index.do; ref. 11). Of the 967 human cancer cell lines in the CCLE database, FBP1 expression profiles were available for 27 liver cancer cell lines. A z-score threshold of 2 was used as the cutoff value. For the analysis of copy-number alterations, data were available for 23 liver cancer cell lines (Supplementary Table S1). Moreover, FBP1 promoter methylation status was determined using the results of methylation-specific PCR, as described in a previous report (8), or methylation array data from GEO database accession numbers GSE60753 (12) and GSE67485 (13). Data on promoter methylation profiles were available for 7 liver cancer cell lines (Supplementary Table S2).

Western blotting analysis

Proteins were detected using antibodies against FBP1 (SAB405798; Sigma-Aldrich), thioredoxin-interacting protein (TXNIP; K0204-3; Medical & Biological Laboratories), and b-actin (Santa Cruz Biotechnology). The methods are described in detail in the Supplementary Materials and Methods.

Sphere formation assays

Detailed methods are provided in the Supplementary Materials and Methods.

Xenograft studies

Five-week-old female BALB/c nu/nu mice were obtained from SLC, Inc. and maintained under specific pathogen-free conditions. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of Kyushu University. A total of 5 × 10^6 cells (empty vector control cells or HuH7 cells stably expressing FBP1) in 150 μl of phosphate-buffered saline were injected subcutaneously into both flanks of the mice. Tumor size was measured every 3 to 4 days with a digital caliper and calculated using the following formula: tumor volume = length × width^2 × 0.5.

Quantification of metabolites

Intracellular glucose uptake was determined by 2-N-(7-nitro-benzo-2-oxa-1,3-diazol-4-yl)aminoo)-2-deoxy-C-glucose (2-NBDG), a fluorescently-tagged glucose derivative, using a Glucose Uptake Cell-Based Assay Kit (Cayman Chemical). Lactate secretion was examined using a Lactate Assay Kit (BioVision). Isotopomer distribution analysis was performed by F-SCOPE package of Human Metabolome Technologies using capillary electrophoresis time-of-flight mass spectrometry (14–16). Cells were incubated in DMEM with glucose replaced by 0.1% [1-13C]glucose (Sigma-Aldrich) for 4 hours. All methods are described in detail in the Supplementary Materials and Methods.

Statistical analysis

For continuous variables, statistical analyses were performed using Student t tests or Welch tests, and the degree of linearity was estimated by Pearson’s correlation coefficient. Differences among multiple groups were examined by one-way ANOVA followed by Tukey–Kramer tests. Categorical variables were compared using χ^2 tests or Fisher’s exact tests. Overall survival (OS) and recurrence-free survival (RFS) were estimated using the Kaplan–Meier method, and survival curves were compared using log-rank tests. Multivariable analysis was performed using the Cox proportional hazards model.
affected glucose metabolism in HCC. By estimating the to elucidate whether FBP1 suppressed tumor progression and improved altered hazards model to determine the prognostic value of FBP1 expression. Differences or correlations with P values of less than 0.05 were considered significant. Data analyses were performed using R software version 3.1.1 (The R Foundation).

Results

FBP1 repressed tumor progression and improved altered glucose metabolism in HCC cells

Initially, we performed functional analysis using HCC cell lines to elucidate whether FBP1 suppressed tumor progression and affected glucose metabolism in HCC. By estimating the FBP1 expression profile across 967 cancer cell lines from the CCLE (10), we found that none of the 27 available liver cancer cell lines showed high expression of FBP1 (Fig. 1A). Notably, 35% of liver cancer cell lines from the CCLE database harbored FBP1 copy-number loss, with markedly reduced expression of FBP1 (Fig. 1B; Supplementary Table S1). Copy-number loss, promoter methylation, or both contributed to downregulation of FBP1 expression (Supplementary Table S2). Therefore, because FBP1 expression was low in liver cancer cell lines, we used overexpression experiments rather than knockdown experiments. We established HCC cell lines (HuH7 and HepG2) stably expressing FBP1 or empty vector control. Overexpression of FBP1 was confirmed by RT-PCR and Western blotting (Fig. 1C). Consistent with previous reports in ccRCC cells (6) and breast cancer cells (7), FBP1 inhibited anchorage-independent tumor cell growth in vitro (Fig. 1D) and xenograft tumor growth (Fig. 1E) in HCC cells.

Because the FBP1 gene encodes a rate-limiting gluconeogenic enzyme, we further investigated the effects of FBP1 on glucose metabolism in HCC cells. Intracellular glucose uptake and lactate secretion were significantly decreased in FBP1-overexpressing HCC cells as compared with those in vector control cells (Fig. 2A and B). We also examined glucose-induced TXNIP expression, which is commonly used as an intracellular glucose sensor (7, 17–19). TXNIP was robustly induced in vector control cells following glucose stimulation. In contrast, the induction of TXNIP was suppressed in FBP1-overexpressing HCC cells (Fig. 2C), supporting the results showing that FBP1 inhibited intracellular glucose uptake in HCC cells. Interestingly, the expression levels of hexokinase2 (HK2; refs. 20, 21) and phosphofructokinase-1 (PFK1, also known as PFKM; refs. 22, 23), key regulators of aerobic glycolysis in cancer cells, were significantly decreased in FBP1-overexpressing HCC cells as compared with those in vector control cells after glucose stimulation (Fig. 2D). To further estimate the effect of FBP1 on aerobic glycolysis, we performed isotopomer distribution analysis using [U-13C]glucose, which directly produces glycolytic intermediates containing six or three 13C atoms (M6/M3 species), and the first turn of the tricarboxylic acid (TCA) cycle intermediates containing two 13C atoms (M2 species; Supplementary Fig. S1A).

M6 enrichment of lactate was significantly inhibited when FBP1 was overexpressed (Fig. 2E), and glycolytic intermediates such as fructose-1,6-biphosphate (F-1,6-BP; M6 species; Fig. 2F), dihydroyxacetone phosphate (M3 species; Supplementary Fig. S1B), and glucose-6-phosphate (Supplementary Fig. S1C) were decreased in FBP1-overexpressing cells, supporting the view that FBP1 suppressed glucose uptake via inhibition of aerobic glycolysis. Similar to ccRCC cells (6), ectopic FBP1 also tended to inhibit M2 enrichment of TCA cycle intermediates such as malate and citrate (Fig. 2G; Supplementary Fig. S1D and S1E). Furthermore, isotopomer distribution analysis showed that M5 enrichment of adenosine triphosphate (ATP) and adenosine diphosphate were decreased in FBP1-overexpressing cells (Supplementary Fig. S1F and S1G), suggesting that FBP1 suppressed de novo nucleic synthesis through the pentose phosphate pathway (PPP) in HCC cells (five 13C atoms were preserved through the PPP). Total ATP synthesis was also decreased when FBP1 was overexpressed (Supplementary Fig. S1H), consistent with the results of reduced aerobic glycolysis and TCA cycle in FBP1-overexpressing cells. Overall, the results of functional analysis suggested that FBP1 acted as a tumor suppressor through inhibition of glucose uptake and aerobic glycolysis, which encouraged us to further explore the significance of FBP1 in HCC.

Lower FBP1 expression promoted tumor progression in HCC cases

Next, to clarify the clinical significance of FBP1 expression in HCC, we analyzed FBP1 expression in tumor tissues and normal liver tissues from three independent cohorts of HCC cases (Fig. 3A). For the analysis of patients who underwent resection of primary HCC at our institution (Kyushu HCC set), FBP1 expression in tumors was significantly lower than that in normal liver tissues (P < 0.001). Given the observation that FBP1 was expressed at low levels in both tumor tissues and liver cancer cell lines, these data implied that downregulation of FBP1 expression was essential for HCC tumorigenesis. Furthermore, decreased FBP1 expression was significantly associated with advanced tumor stage (stage I vs. stage II, P = 0.017; stage I vs. stage III, P = 0.005). The median expression levels of FBP1 in each tumor stage (stage I, stage II, and stage III) relative to those in normal liver tissues were 0.427, 0.179, and 0.059, respectively. To confirm the results of our series, we analyzed the public datasets of HCC cases from the GSE14520 and the TCGA datasets. In accordance with our cases, FBP1 expression in tumors was significantly lower than that in normal liver tissues (P < 0.001 and P < 0.001, respectively). Moreover, the significant association between lower FBP1 expression and advanced tumor stage was validated in the GSE14520 (stage I vs. stage III, P < 0.001; stage II vs. stage III, P < 0.009) and the TCGA (stage I vs. stage II, P < 0.001; stage I vs. stage III, P < 0.001) datasets. The median expression levels of FBP1 in each tumor stage (stage I, stage II, and stage III) relative to those in normal liver tissues were 0.262, 0.202, and 0.059, respectively, in the GSE14520 dataset and 0.280, 0.143, and 0.153, respectively, in the TCGA dataset. These results suggested that the loss of FBP1 expression was associated with tumor progression in HCC cases.

Promoter methylation and copy-number loss inhibited FBP1 expression in HCC cases

To investigate the mechanisms suppressing FBP1 expression in HCC cases, promoter methylation and copy-number profiles of FBP1 were analyzed in the TCGA dataset (Fig. 3B). We found that the FBP1 promoter exhibited significantly higher methylation in tumors than in normal liver tissues (P < 0.001; Fig. 3C). In addition, FBP1 expression was inversely correlated with the degree of FBP1 methylation in tumors (Pearson correlation coefficient = −0.437; P < 0.001; Fig. 3D), indicating that FBP1 promoter methylation suppressed the transcriptional activity of FBP1 in HCC. Similarly, copy-number loss of FBP1 was also significantly associated with lower FBP1 expression (the median expression level in HCCs harboring copy-number loss relative to that in HCCs
harboring copy-number neutral/gain was 0.519; Fig. 3E). For an integrated assessment of the influence of both promoter methylation and copy-number alterations on FBP1 expression levels, multiple linear regression analysis was performed. The standardized effect sizes (t values) for promoter methylation and copy-number alterations were -7.55 (P < 0.001) and 3.48 (P < 0.001; Fig. 3B), respectively. These findings suggested that promoter methylation and copy-number loss were independently associated with downregulation of FBP1 expression. We did not find any correlations between FBP1 mutations and FBP1 expression because only two nonsynonymous mutations were observed in the TCGA dataset (Supplementary Fig. S2).
Figure 2.
FBP1 improved altered glucose metabolism in HCC cells. A, intracellular glucose uptake was evaluated by 2-NBDG, a fluorescently tagged glucose derivative, in HCC cells with or without ectopic FBP1 expression. B, lactate secretion was significantly decreased when FBP1 was overexpressed. C, the expression of glucose-induced TXNIP was used to sense intracellular glucose uptake. Cells were incubated in glucose-free medium for 12 hours, followed by glucose stimulation for an additional 3 hours. D, the expression levels of genes encoding enzymes involved in aerobic glycolysis, that is, HK2, PFK1, and PKM2, were assessed by RT-PCR after glucose stimulation, as described in C. E–G, isotopomer distribution analysis in vector control or FBP1-overexpressing HuH7 cells labeled with [U-13C]glucose for 4 hours. M3 enrichment of lactate (E), M6 enrichment of 1,6-diphosphate (F-1,6-BP; F), and M2 enrichment of malate and citrate (G) were quantified. * in the analysis of F-1,6-BP, the metabolite concentrations of one experiment in the vector control and all three experiments in FBP1-expressing cells were below the detection limit. P values were calculated using Welch’s t test. Values represent the means ± SDs of triplicate experiments. EV, empty vector control cells; ND, not detectable.
Figure 3.
Downregulation of FBP1 expression via promoter methylation and copy-number loss was associated with tumor progression in HCC cases. A, box plots of FBP1 expression in normal liver and tumor tissues grouped into stages I to III in three independent cohorts of HCC cases. Information on tumor stage was available for 114 of 118 patients from the Kyushu HCC set (left), 225 of 242 cases from GSE14520 (middle), and 214 of 234 cases from TCGA (right). Five patients with stage IV cancer were excluded from the analysis of TCGA data. B, an integrative view of expression, promoter methylation, and copy-number profiles of FBP1 across 234 HCC cases in TCGA. The samples are sorted according to FBP1 expression levels. C, box plots of FBP1 methylation status in normal liver and tumor tissues from TCGA (data were available for 46/48 normal tissues). D, FBP1 expression levels in tumor samples were inversely correlated with the degree of FBP1 methylation in TCGA. E, box plots of FBP1 expression based on FBP1 copy-number alterations in TCGA (data were available for 229/234 cases). F, box plots of FBP1 expression based on tumor size in GSE14520 (data were available for 241/242 cases). Information on tumor size was not available in TCGA. G, box plots of FBP1 expression based on tumor differentiation in TCGA (data were available for 230/234 cases, and one anaplastic HCC was excluded). Information on tumor differentiation was not available in GSE14520. NS, not significant.
Table 1. FBP1 expression and clinicopathologic factors in patients in the Kyushu HCC set (n = 118)

<table>
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<th>Low expression (n = 50)</th>
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<td>22 (73.3)</td>
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<td>4 (13.3)</td>
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<td>C</td>
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</table>

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; NA, not available.

*WHO grades 1/2 or Edmondson-Steiner grades 1/2 were classified as well/moderately; WHO grades 3/4 or Edmondson-Steiner grades 3/4 were classified as poorly/differenated.

FBP1 expression levels predicted survival in patients with HCC

To further investigate the clinical significance of FBP1 expression, we assessed survival rates in three independent datasets of HCC. On the basis of the FBP1 expression level, cases were divided into two groups using the minimum P value approach, which is a comprehensive method to identify the optimal risk separation cutoff point in continuous gene-expression measurements for survival analysis in multiple datasets (24). The cutoff values for high and low FBP1 expression groups were 0.6 (FBP1/GAPDH expression) in the Kyushu HCC set, 6.8 (log2 expression) in the GSE14520 dataset, and 13.0 (log2 expression) in the TCGA dataset. For the analysis of 118 patients in the Kyushu HCC set, clinicopathologic factors were compared between the groups (Table 1). Notably, the low FBP1 expression group exhibited larger tumor size (P = 0.010) and poorer differentiation grade (P < 0.001) than the high FBP1 expression group. These findings were validated with the available public data. Lower FBP1 expression was significantly associated with larger tumor size (GSE15420: ≤ 5 cm vs. > 5 cm; P < 0.001; Fig. 3F) and poorly differentiated HCC (TCGA: poorly vs. well; P = 0.003; Fig. 3G), suggesting that highly malignant HCC expressed low levels of FBP1. For the analysis of OS (Fig. 4A), the low FBP1 expression group had significantly poorer OS than the high FBP1 expression group (P = 0.005). Consistent with our cohort, lower FBP1 expression was significantly associated with poorer OS in the GSE14520 (P < 0.001) and TCGA (P = 0.010) datasets. In the multivariate analysis that excluded tumor stage as a confounding variable, the association between lower FBP1 expression and poorer OS remained significant in the Kyushu HCC (HR, 2.49; 95% confidence interval [CI], 1.22–5.06; P = 0.012) and TCGA (HR, 2.84; 95% CI, 1.22–6.64; P = 0.016) datasets (Table 2).

To validate the effects of FBP1 expression on survival in patients with HCC, we applied gene set enrichment analysis (GSEA; ref. 25) on the GSE14520 and TCGA datasets using two gene sets according to OS following resection of HCC (26). GSEA revealed that upregulation of FBP1 was significantly correlated with better survival in the GSE14520 (P < 0.001) and TCGA (P < 0.001) datasets (Fig. 4B). Conversely, downregulation of FBP1 was significantly correlated with worse survival in the GSE14520 (P < 0.001) and TCGA (P < 0.001) dataset (Fig. 4C). Collectively, survival analysis with Kaplan–Meier curves and GSEA demonstrated that lower FBP1 expression was significantly associated with poor survival in patients with HCC.

Higher FBP1 expression was associated with a lower risk of recurrence in HCC

Subsequently, we assessed the effects of FBP1 expression on recurrence following resection of primary HCC (Fig. 4D). In our analysis of the Kyushu HCC set, RFS in the high FBP1 expression group was significantly better than that in the low FBP1 expression group (P = 0.016). Consistent with the results of our series, higher FBP1 expression was also significantly associated with better RFS in the available public data (GSE14520, P < 0.001). The association between lower FBP1 expression and poorer RFS remained significant after excluding tumor stage as a confounding variable in the GSE14520 dataset (HR, 1.49; 95% CI, 1.01–2.20; P = 0.045; Table 2).

We then performed GSEA to determine whether the gene sets associated with HCC recurrence were differentially regulated depending on the expression levels of FBP1 in HCC. GSEA showed that higher FBP1 expression was significantly correlated with a lower risk of early recurrence (27) in the GSE14520 and TCGA datasets (P < 0.001 and P < 0.001, respectively; Fig. 4E), and with that of late recurrence (28) in the GSE14520 and TCGA datasets (P < 0.001 and P < 0.001, respectively; Fig. 4F). These results demonstrated that higher FBP1 expression was associated with a lower risk of recurrence.

FBP1 expression levels reflected altered glucose metabolism in HCC

To further investigate the role of FBP1 in HCC progression, we focused on glucose metabolism. First, FBP1 expression and gluconeogenic activity were compared using genome-wide gene-expression profiles of the GSE14520 and TCGA datasets. To comprehensively analyze gluconeogenic activity, we defined the gluconeogenic module composed of genes specific to gluconeogenesis (4–6). This module included genes encoding enzymes involved in irreversible steps of gluconeogenesis, for example,
Figure 4.
FBP1 expression levels predicted survival and recurrence in patients with HCC. A, Kaplan–Meier OS curve based on FBP1 expression in three independent datasets of HCC. Left, Kyushu HCC set; middle, GSE14520; right, TCGA. B and C, gene set enrichment analysis of the GSE14520 and TCGA datasets showed that higher FBP1 expression was significantly correlated with better survival in HCC (B). In contrast, lower FBP1 expression was significantly correlated with poorer survival in HCC (C). D, Kaplan–Meier recurrence-free survival in patients with HCC based on FBP1 expression. Left, Kyushu HCC set; middle, GSE14520; right, information on recurrence status was not available in TCGA. E and F, gene set enrichment analysis of the GSE14520 and TCGA datasets revealed that upregulation of FBP1 expression was significantly correlated with a lower risk of early (E) and late (F) recurrence. ES, enrichment score.
Among these three enzymes, we focused on the glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase. Three enzymes are involved in catalyzing the irreversible steps of aerobic glycolysis in cancer cells; therefore, an aerobic glycolytic module containing these three genes was deleted. Contrary with glucogenesisis, significant inverse correlations were observed between FBP1 expression and aerobic glycolytic module activity in the GSE14520 (Pearson correlation coefficient = −0.719; P < 0.001) and TCGA (Pearson correlation coefficient = −0.716; P < 0.001) datasets (Fig. 5B). Our findings suggested that hepatic glucogenesisis was impaired in HCCs expressing low levels of FBP1.

Subsequently, we evaluated aerobic glycolysis in HCC cases. Three enzymes are involved in catalyzing the irreversible steps of glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase (Fig. 5A). Among these three enzymes, we focused on the HK2 (20, 21), PFK1 (22, 23), and pyruvate kinase M2 (PKM2; refs. 30, 31) genes, which have been reported to play a pivotal role in promoting aerobic glycolysis in cancer cells; therefore, an aerobic glycolytic module containing these three genes was defined. Contrary with glucogenesisis, significant inverse correlations were observed between FBP1 expression and aerobic glycolytic module activity in the GSE14520 (Pearson correlation coefficient = −0.458; P < 0.001) and TCGA (Pearson correlation coefficient = −0.457; P < 0.001) datasets (Fig. 5B). Our findings suggested that hepatic glucogenesisis was impaired in HCCs expressing low levels of FBP1.

To confirm whether FBP1 expression levels were associated with altered glucose metabolism in HCC, GSEA was performed. Predefined gene sets involved in normal glucose metabolism were significantly enriched in HCCs expressing high levels of FBP1, as follows: KEGG_GLYCOLYSIS_GLUONEOGENESIS in the GSE14520 (P < 0.001) and TCGA (P < 0.001) datasets (Fig. 5D); and REACTOME_GLYCOGENESIS_METABOLISM in the GSE14520 (P < 0.001) and TCGA (P = 0.023) datasets (Fig. 5E). The results of GSEA indicated that normal glucose metabolism was significantly impaired in HCCs expressing low levels of FBP1 compared with that in HCCs expressing high levels of FBP1.

Finally, we assessed the effects of the other genes involved in glucogenesisis/aerobic glycolysis on prognosis in patients with HCC. Lower G6PC and PGC-1α expression and higher PKM2 expression were significantly associated with poor OS in both the GSE14520 and TCGA datasets (Supplementary Table S3). Consistent with these observations, higher expression levels of genes specific to glucogenesisis, particularly those involved in maintenance of normal glucose metabolism (eleved glucogenesisis and decreased aerobic glycolysis), were significantly associated with better OS (Supplementary Fig. S4A–S4C), suggesting that the glucose metabolic shift affected prognosis. Together with the results of functional analysis and survival analysis, our results showed that FBP1 was not only one of the key regulators of HCC progression but a promising biomarker-predicting prognosis in patients with HCC. Among genes involved in glucogenesisis, downregulation of FBP1 may contribute to a vicious cycle of altered glucose metabolism in HCC considering the observation that loss of FBP1 directly enhanced tumor progression and aerobic glycolysis. Overall, we found that lower FBP1 expression was significantly associated with altered glucose metabolism, which contributed to tumor progression in HCC.

**Discussion**

We demonstrated the function and clinical significance of FBP1 expression in HCC. Promoter methylation and copy-number loss

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**Table 2. Effect of FBP1 expression on OS and recurrence-free survival in the multivariate analysis that excluded tumor stage as a confounding variable**

<table>
<thead>
<tr>
<th>Factor</th>
<th>OS Kyushu HCC set</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Univariate analysis</td>
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<td>Multivariate analysis</td>
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<tr>
<td></td>
<td>HR 95% CI</td>
<td>P</td>
<td>HR 95% CI</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBP1 expression (low/high)</td>
<td>2.47 (1.28–4.77)</td>
<td>0.007b</td>
<td>2.49 (1.22–5.06)</td>
<td>0.012b</td>
<td></td>
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<tr>
<td>TNM stage (II/III)</td>
<td>0.97 (0.45–2.10)</td>
<td>0.939</td>
<td>0.96 (0.44–2.07)</td>
<td>0.910</td>
<td></td>
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<tr>
<td>GSE14520</td>
<td>2.12 (0.75–5.95)</td>
<td>0.156</td>
<td>1.37 (0.45–4.07)</td>
<td>0.577</td>
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<tr>
<td>FBP1 expression (low/high)</td>
<td>2.08 (1.37–3.14)</td>
<td>&lt;0.00b</td>
<td>1.45 (0.91–2.29)</td>
<td>0.116</td>
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<tr>
<td>TNM stage (II/III)</td>
<td>2.09 (1.20–3.64)</td>
<td>0.009b</td>
<td>1.99 (1.14–3.47)</td>
<td>0.016b</td>
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<tr>
<td>GSE14520</td>
<td>5.30 (3.05–9.20)</td>
<td>&lt;0.00b</td>
<td>4.64 (2.60–8.29)</td>
<td>&lt;0.00b</td>
<td></td>
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</tr>
<tr>
<td>FBP1 expression (low/high)</td>
<td>2.68 (1.23–5.85)</td>
<td>0.035b</td>
<td>2.84 (1.22–6.64)</td>
<td>0.016b</td>
<td></td>
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<tr>
<td>TNM stage (II/III)</td>
<td>0.64 (0.31–1.29)</td>
<td>0.210</td>
<td>0.55 (0.27–1.13)</td>
<td>0.102</td>
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<tr>
<td>FBP1 expression (low/high)</td>
<td>1.44 (0.83–2.50)</td>
<td>0.196</td>
<td>1.35 (0.77–2.34)</td>
<td>0.292</td>
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</table>

Abbreviation: CI, confidential interval.

*Information on recurrence status was not available in the TCGA dataset.

bP < 0.05.
were responsible for the decreased expression of FBP1, which contributed to tumor progression, poor prognosis, and altered glucose metabolism. Our data indicated that FBP1 acted as a tumor suppressor by restoring altered glucose metabolism, and implied that FBP1 was a more central regulator of the glucose metabolic pathway in HCC.

In the present study, we validated the prognostic impact of FBP1 expression using three independent large cohorts, and the results were reproducible. For either OS or RFS in each cohort, this prognostic impact persisted even after adjusting for tumor stage. Moreover, our data indicated that decreased expression of FBP1 promoted tumor progression, thereby affecting the clinical
outcomes and clinicopathologic characteristics of patients with HCC. Therefore, FBP1 expression could be a dependable biomarker for predicting prognosis and provide additional prognostic information beyond tumor stage in patients with HCC.

The proposed mechanism underlying the tumor-suppressive properties of FBP1 involves the inhibition of aerobic glycolysis (6, 7). Our results were consistent with this proposal and suggested that the reduced transcriptional activity of HK2 and PFK1 induced by ectopic FBP1 played a role in the decreased glucose-6-phosphate and F-1,6-BP levels. One of the limitations of this study was that glucose metabolism was mainly examined by transcriptional profiling of genes specific to gluconeogenesis or glycolysis in clinical cases. Enzyme activity can also be regulated by protein structure and expression. Indeed, FBP1 is involved in the posttranslational modification of PKM2 in the glycolytic pathway (30, 31). The loss of FBP1 directly increases the level of F-1,6-BP, which enhances PKM2 activity through dynamic structural switching from the less active dimer to the active tetramer. Furthermore, the effect of FBP1 knockdown on altered glucose metabolism was not evaluated because FBP1 was expressed at low levels in all available liver cancer cell lines from the CCLE. Despite these limitations, our findings provide a reasonable explanation for the effects of FBP1 expression on altered glucose metabolism in HCC. In addition to the enzymatic activity of FBP1 in glucose metabolism, Li and colleagues (6) reported that the direct binding of FBP1 to hypoxia-inducible factor (HIF) inhibits ccRCC progression by reducing HIF activity. Our data showed that the effects of FBP1 on malate, citrate, and other metabolites were not as robust as that seen in ccRCC cells, likely due to the fact that ccRCC cells have constitutive HIF activity and HCC cells do not in normoxic conditions. Considering these diverse functions of FBP1, further studies are needed to clarify the detailed mechanisms through which FBP1 suppresses cancer progression in HCC.

In HCC, FBP1 promoter methylation has been reported only in cell lines and a few clinical cases (8). However, in our larger analysis in this study, we found that copy-number loss and promoter methylation could explain the decreased expression of FBP1. FBP1 promoter methylation downregulated FBP1 expression in patients with basal-like breast cancer (7), gastric cancer (32), and small intestinal neuroendocrine tumor (33). Copy-number loss of FBP1 was also observed in ccRCC cases (34). Thus, our data indicated that genomic alterations as well as epigenetic modifications were responsible for inhibiting FBP1 expression in HCC.

In summary, we demonstrated the clinical significance of FBP1 expression in HCC using three independent clinical datasets. Downregulation of FBP1 via promoter methylation and copy-number loss promoted tumor progression by altering glucose metabolism. FBP1 could be a useful biomarker for predicting the prognosis of patients with HCC and may represent a novel therapeutic target for the treatment of HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Hirata, K. Sugimachi, K. Mimori
Development of methodology: H. Hirata, H. Komatsu, M. Ueda, T. Masuda, R. Uchi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Hirata, K. Sugimachi, H. Komatsu, H. Eguchi, S. Ito
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Hirata, K. Sugimachi, H. Komatsu, M. Ueda, T. Masuda, R. Uchi, S. Sakimura, S. Nambara, T. Saito, M. Hirakawa
Writing, review, and/or revision of the manuscript: H. Hirata, K. Sugimachi, T. Iguchi, K. Mimori
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Terashima, K. Sakamoto
Study supervision: Y. Shinder, H. Honda, K. Mimori

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Hidenari Hirata, Keishi Sugimachi, Hisateru Komatsu, et al.


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