Serum fatty acid-binding protein 5 is a significant factor in hepatocellular carcinoma progression independent of tissue expression level

Short title: Serum FABP5 in HCC progression

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Abstract

Fatty acid-binding protein 5 (FABP5) is highly expressed in hepatocellular carcinoma (HCC) tissues and is related to HCC progression. In this study, we analyzed the potential of serum FABP5 (sFABP5) as a tumor marker in HCC and its clinical significance in HCC progression. We compared the sFABP5 concentration in patients with HCC (HCC group) with that of patients with hepatitis without HCC (hepatitis group). Moreover, we measured the FABP5 expression levels in resected HCC tissues (tFABP5) and analyzed their relationship with sFABP5. We also performed cell-based assays using FABP5 knockout and overexpressing HCC cell lines to analyze the effect of extrinsic FABP5 on HCC cells. We showed that sFABP5 was not a useful tumor marker for HCC, as HCC and sFABP5 were not correlated. However, sFABP5 and tFABP5 significantly correlated with survival after surgery for HCC, while sFABP5 and tFABP5 were independent of each other. In cell-based assays, extrinsic FABP5 was taken up by HCC cell lines and positively affected cell survival under glucose-depleted conditions by complementing the endogenous FABP5 function. In conclusion, sFABP5 had a significant impact on HCC progression irrespective of tFABP5 by augmenting cell viability under glucose-depleted conditions. As tFABP5 and sFABP5 are important factors that are independent of each other in HCC progression, both of them should be considered independently in improving the prognosis of patients with HCC.

Summary

Serum FABP5 is not a useful tumor marker for HCC. However, it has a significant impact on the prognosis of patients with HCC, independent of FABP5 expression in HCC tissues, complementing the function of endogenous FABP5 in HCC cells.

Introduction

Fatty acid-binding protein (FABP) is an intracellular protein that was first identified in 1972 (1). In mammals, 10 isoforms of FABPs are expressed: FABP1, 2, 3, 4, 5, 6, 7, 8, 9 and 12, constituting the FABP family (2,3). FABPs, which are referred to as lipid chaperones, bind free fatty acids for intracellular transport. They are significant for lipid storage, signaling via fatty acids, fatty acid beta-oxidation and membrane synthesis (4).

The role of FABPs, especially that of FABP4 and FABP5, concerning metabolic syndrome and some cancers has been studied widely (4,5). We previously identified a higher expression of epidermal FABP (FABP5) in hepatocellular carcinoma (HCC) cells than in normal liver cells (6), and reported that high expression of FABP5 in HCC tissues was associated with tumor progression and worse prognosis (7).

Although FABPs are intracellular proteins, some reports have shown a relationship between circulating FABPs in blood and metabolic syndrome (8,9) and some cancers. Especially, circulating FABP4 and FABP5 have been studied in patients with breast cancer while circulating FABP1 has been studied in patients with HCC (5,10). Moreover, some *in vitro* studies have demonstrated the secretion of FABP5 from cells into the culture medium (11,12). However, although we have made a thorough database search, we could not find scholarly evidence regarding the relationship between circulating FABP5 and HCC. In this study, we aimed to clarify the relationship between serum FABP5 (sFABP5) and HCC by analyzing the potential of sFABP5 as a tumor marker in HCC and its clinical significance in HCC progression.

Materials and methods

Patients

Patients who underwent hepatic resection for HCC at Hokkaido University

Hospital between January 2012 and August 2018 were eligible for this study. The exclusion criteria were as follows: preoperative treatment history, such as chemotherapy, radiofrequency ablation and transarterial chemoembolization; distant metastasis at the time of operation; other types of malignant diseases apart from HCC; presence of obvious alcoholic hepatitis; autoimmune diseases related to the liver, such as autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC); death because of intercurrent diseases; inadequate amount of stored serum or resected HCC specimen; and lack of appropriate clinical data on our institutional medical records and database. For comparison, we analyzed patients with hepatitis who did not have HCC and attended our hospital regularly. Their etiology was limited to hepatitis B virus (HBV), hepatitis C virus (HCV) and non-B non-C (NBNC) hepatitis, other than AIH, PBC, PSC and alcoholic hepatitis. Active hepatitis B or C was defined as seropositivity for hepatitis B surface antigen or HCV RNA, respectively. Patients with seropositivity for hepatitis B core or surface antibody and seronegativity for hepatitis B surface antigen were categorized as having past HBV infection. Patients with seropositivity for hepatitis C antibody and seronegativity for HCV RNA were categorized as having past HCV infection. NBNC hepatitis was defined as seronegativity based on the aforementioned factors. We excluded patients with alcoholic hepatitis from this study, as it was a disease with many complicated factors, which could make the analysis challenging. Additionally, serum samples from healthy volunteers without hepatitis, HCC, or other malignant diseases were analyzed to determine the sFABP5 concentration in the general population.

This study was approved by the Institutional Review Board of the Graduate School of Medicine, Hokkaido University (No. 18-011, No. 14-015). All study participants provided written informed consent for the use of their samples and clinical data and were informed concerning their opportunity to opt out.

Measurement of the sFABP5 concentration

Serum samples from patients with HCC were collected immediately before surgery, and serum samples from patients with hepatitis were collected at some time during their follow-up. Whole blood samples from patients were incubated at room temperature for 30 min after collection and centrifuged at 1,500×*g* for 10 min. Then, the supernatants were collected in microtubes and stored at -80°C until measurement. We used commercial enzyme-linked immunosorbent assay (ELISA) kits for FABP5 (Biovendor, Brno, Czech Republic) and followed the manufacturer's protocol to measure the sFABP5 concentration in the samples. All samples were measured in duplicate, and the average values were used for the analyses. After the measurement, the values were logarithmically transformed to convert the data to a normal distribution. As we used stored serum and the measured values could have been influenced by the storage conditions, we excluded extreme outliers from the final analyses. Outliers were defined as values below the first quartile (Q_1) – 3 × interquartile range (IQR) or above the third quartile (Q_3) + 3 × IQR.

Measurement of the FABP5 expression level in resected liver specimen

We quantified the FABP5 expression levels in resected liver specimens using western blotting with total protein normalization. First, we extracted total protein from frozen HCC tissues and adjacent normal liver tissues by homogenization in lysis buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% sodium deoxycholate; 1 mM EDTA; 0.5% octylphenoxypolyethoxyethanol; 5 mM NaF; 5 mM sodium orthovanadate) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). After a 30-min incubation on ice, the samples were centrifuged at 12,000×*g* for 10 min, and the supernatants were collected as total protein extracts. The protein concentrations of the samples were determined by the

bicinchoninic acid assay using the Pierce BCA Protein Assay Kit (Thermo, Rockford, IL). Laemmli sample buffer (Bio-Rad, Hercules, CA) and 2-mercaptoethanol were added to these samples and boiled at 95°C for 5 min for denaturation. Each total protein extract (40 μg/lane) was subjected to SDS-PAGE and immunoblotting. Membrane blocking was performed using 3% non-fat dry milk (Fujifilm Wako, Osaka, Japan). Then, the membranes were incubated with mouse monoclonal anti-FABP5 antibody (dilution, 1:8000; Proteintech, Rosemont, IL) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG antibody (dilution, 1:5000; Cell Signaling Technology, Danvers, MA) at room temperature for 1 h. The target bands were visualized using Clarity Western ECL Substrate and ChemiDoc XRS Plus (Bio-Rad). The band intensity of FABP5 was measured using Image Lab software (Bio-Rad) and normalized by the intensity of all proteins in the lane, which was achieved using Stain-Free Technology (Bio-Rad). All samples were measured twice, and the average values were used for the analyses. As a control, we used the FABP5 band intensity of the total protein extract obtained from the HCC cell line, PLC/PRF/5. Therefore, the FABP5 expression levels of the samples were described as the FABP5 band intensity ratio of the samples to that of PLC/PRF/5, which was, then, logarithmically transformed to convert the data to a normal distribution. As we used frozen stored samples and the measured values might have been influenced by the storage conditions, we excluded extreme outliers from the final analyses, as described previously.

Cell culture

Human HCC-derived cell lines, PLC/PRF/5 and KIM-1, were cultured in Dulbecco's modified Eagle medium (DMEM; Nacalai, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Nacalai) in an incubator containing 5% CO_2 at 37°C. PLC/PRF/5 was purchased from the Japanese

Collection of Research Bioresources Cell Bank (JCRB Cell Bank) in 2013. KIM-1 was established at Kurume University in 1984 (13) and was kindly provided by the National Cancer Center Research Institute (Tokyo, Japan) in 2015. All cell lines were stocked in the vapor phase above liquid nitrogen immediately after acquisition and passaged for no longer than 6 months. Authentication of these cell lines was performed using short-tandem repeat profiling by the providers. None of the cell lines was tested or authenticated by the authors. The number of cells was counted using a Countess II Automated Cell Counter (Invitrogen, Carlsbad, CA).

Establishment of FABP5 overexpressing (OE) cell lines

We used the expression vector pcDNA3.1 (+) (Invitrogen) with the whole exon sequence of *FABP5* containing the Kozak sequence, GCCACC, to make cells with high FABP5 expression. The actual cloned sequence is presented in Supplementary Table 1. We transfected this expression vector into HCC cell lines using Lipofectamine 3000 (Invitrogen) and established stable clones under the selection pressure of G-418 Disulfate (Nacalai) for 14 days. Finally, we checked the FABP5 expression status of the stable clones using western blotting.

Establishment of FABP5 knockout (KO) cell lines

The *FABP5* gene was knocked out using the CRISPR/Cas9 system. Cas9 and guide RNA expression plasmid, pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230; http://n2t.net/addgene:42230; RRID:Addgene_42230) (14), and a donor vector was purchased from System Biosciences (PrecisionX; Palo Alto, CA). The sequence cloned for guide RNA in pX330 for *FABP5* knockout was 5'-GACTTAACATTCTACAGGAGT-3', designed using a web tool for CRISPR, CHOPCHOP

(15). pX330 and donor vector were co-transfected into cells using Lipofectamine 3000 (Invitrogen), and the successfully knocked-out cells were selected under the selection pressure of puromycin dihydrochloride (Sigma) for 10 days. Finally, we checked *FABP5* knockout using western blotting and polymerase chain reaction (PCR).

Western blotting for cell protein extract

The western blotting method for cell protein extract was performed as described in the previous section. Each total protein extract (12 μg/lane) was subjected to SDS-PAGE and immunoblotting. We used rabbit monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (dilution, 1:1000; Cell Signaling Technology) and horseradish peroxidase-conjugated anti-rabbit IgG antibody (dilution, 1:5000; Cell Signaling Technology), as a loading control antibody.

PCR

We checked the knockout of *FABP5* using PCR. Whole genome from cells was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The sequences of the oligonucleotides used to check *FABP5* knockout were 5'-TGGCCAGAGCTCAGATTTGG-3' for the forward primer and 5'-CCACTCCTGTAGAATGTTAAGTCATG-3' for the reverse primer, and the product size was 349 bp. PCR products were subjected to electrophoresis, followed by soaking in ethidium bromide solution and visualized using a UV transilluminator.

Analysis of FABP5 efflux from cells

We analyzed the efflux of FABP5 from cells by measuring the amount of FABP5 in the culture supernatant. First, 1.0×10^6 cells were seeded into a 3.5 cm dish, and after a 24-h

preincubation, we changed the medium to DMEM without FBS. After a 24-h incubation, the culture medium was collected and centrifuged at 500×*g* for 10 min to spin down the contaminating cells, and the supernatant was collected for the assay. The collected culture supernatant was concentrated to approximately 1/10 of the initial amount using an ultrafiltration membrane (Amicon Ultra-0.5; Merck, Darmstadt, Germany). FABP5 concentration was measured using a commercial ELISA kit (AssayMax ELISA Kit; Assaypro, St. Charles, MO) according to the manufacturer's protocol. We also measured FABP5 concentration in cell lysates from PLC/PRF/5 and KIM-1 with the total protein concentration adjusted to 100 μg/mL as positive controls and DMEM without FBS as a negative control. All samples were measured in triplicate and analyzed.

Cell proliferation assay

Cells were seeded into 96-well culture plates at a density of 5.0×10^3 cells/well. After a 12-h preincubation, the live cells were counted using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The time point was set as 0 h. The live cells were counted every 24 h after the first measurement, and the relative cell proliferation was calculated as the ratio of the number of live cells at each time point to the corresponding at 0 h. This assay was conducted in triplicate.

Cell migration assay

We checked the cell migration ability using an *in vitro* wound healing assay. Cells were seeded into 24-well culture plates at a density of 3.0×10^5 cells/well. After a 12-h preincubation, a single scratch wound was created in the center of the cell monolayer using a sterile plastic pipette tip. Then, the wells were washed with phosphate-buffered saline (PBS) to remove the detached cells, and the medium was changed to DMEM supplemented with 1%

FBS. Images of the wounds were obtained at 0, 24 and 48 h after making the wounds using a phase-contrast microscope (Keyence, Osaka, Japan), and the cell-free areas were measured using ImageJ software (NIH, Bethesda, MD) (16). The values of the cell-free areas were divided by the longitudinal length of the wounds to obtain the mean width of each wound. Then, the diminution of the wound width or the migration distance was calculated. This assay was conducted in quintuplicate.

Cell viability assay under glucose-depleted condition

Cells were seeded into 96-well culture plates at a density of 2.0×10^4 cells/well. After a 12-h preincubation, the live cells were counted using a Cell Counting Kit-8 (Dojindo). The time point was set as 0 h. At the same time, we changed the medium to glucose-depleted DMEM. This medium comprised glucose-free DMEM (Nacalai) supplemented with 10% FBS and 1% penicillin/streptomycin. The surviving cells were counted every 24 h after the first measurement, and the relative cell viability was determined as the ratio of the number of live cells at each time point to the corresponding at 0 h. In addition, the effect of extrinsic FABP5 (exFABP5) on cell viability was analyzed using the FABP5 recombinant protein (Cayman, Ann Arbor, MI). FABP5 recombinant protein was dissolved in PBS and added to each well at concentrations of 0, 10 and 100 ng/mL after 12-h preincubation. The maximum concentration of 100 ng/mL was determined in reference to the highest value of sFABP5 in this study (i.e., 83.47 ng/mL). Then, at 48 h after the addition of FABP5 recombinant protein, the surviving cells were counted, and the relative cell viability was determined as the ratio of the number of live cells at that time to the corresponding at 0 h. This assay was conducted in triplicate.

Immunocytochemistry

Cells were seeded into an 8-well chamber slide at a density of 5.0×10^4 cells/well. After a 24-h preincubation, the medium was changed, and FABP5 recombinant protein at 100 ng/mL concentration or PBS was added to the wells. At 6 h after the addition of the FABP5 recombinant protein or PBS, cells were washed thrice with PBS and fixed in 95% EtOH. Fixed cells were blocked with 3% bovine serum albumin for 30 min. Then, the cells were incubated with rabbit polyclonal anti-FABP5 antibody (dilution, 1:100; Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with Alexa Fluor 488-labeled anti-rabbit IgG (dilution, 1:250; Invitrogen). Finally, the cells were mounted with ProLong Diamond Antifade Mountant (Life Technologies, Eugene, OR). Images of stained cells were captured using a fluorescence microscope (BIOREVO BZ-9000; Keyence), and the intensity of fluorescence was measured using a BZ-II Analyzer (Keyence). The intensity of fluorescence was measured at five random points on each slide, and the average value was adopted as the intensity of the cell. The fluorescence intensity of the cell was calculated by dividing the total intensity by the number of nuclei, which stands for the number of cells in each field. This assay was conducted in triplicate, and the relative intensity was described as the ratio of the intensity of each cell to that of FABP5 wild-type (WT) cells treated with PBS.

Statistical analysis

Patient data (clinicopathological characteristics, laboratory data and survival data) were obtained from a prospectively maintained institutional database. Statistical analyses were performed using Student's or paired *t*-test to examine differences in continuous variable distributions, and the chi-square test was used for categorical variables. Simple and multiple linear regression models were used to analyze correlations between factors. The cut-off values were determined using the receiver operating characteristic (ROC) curve. Survival curves were analyzed using the Kaplan–Meier method and compared using the log-rank and

generalized Wilcoxon tests. Statistical significance was set at $P < 0.05$. All analyses were conducted using JMP (SAS Institute, Cary, NC) and R software (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

sFABP5 concentration in the healthy control group (preliminary study)

First, we checked the sFABP5 concentration of 23 volunteers without hepatitis, HCC and other malignant diseases (healthy control group). Clinical characteristics and data are summarized in Supplementary Table 2. The mean ± standard deviation (SD) of sFABP5 concentration in this group was 7.35 ± 2.27 ng/mL.

The relationship between sFABP5 and the presence of HCC

In total, 128 and 92 patients with HCC (HCC group) and hepatitis (hepatitis group), respectively, met the inclusion criteria. A flowchart indicating the patient inclusion and exclusion criteria is presented in Figure 1A. The sFABP5 concentration of each group before and after logarithmic transformation is shown in Figure 1B. We detected three outliers in the hepatitis group and excluded them from the analyses. Thus, the final analyses included 128 patients in HCC group and 89 patients in hepatitis group.

In general, sFABP5 concentration is affected by some clinical conditions related to metabolic syndrome, such as diabetes mellitus (DM), hypertension (HT) and cardiovascular diseases (i.e., atherosclerosis of the carotid and coronary arteries) (8,9). In addition, dyslipidemia (DL) and high body mass index may also affect serum FABP4 concentration (17-20). Therefore, these factors were considered in the following analyses.

A summary of the clinical characteristics and sFABP5 concentrations of each group is described in Table 1. There were significant differences in sex, age, HT, cardiovascular

disease and etiology between the HCC and hepatitis groups. There was no significant difference in the sFABP5 levels between the HCC and hepatitis groups in this unadjusted analysis.

A summary of the clinical characteristics and sFABP5 stratified by etiology is presented in Supplementary Table 3. The patients in the past virus infection (past HBV, past HCV and combination of both) and NBNC groups tended to have a higher prevalence of metabolic syndrome symptoms than those in the active viral hepatitis groups (HBV, HCV and coinfection of both). Therefore, we divided our cohort into two groups: viral hepatitis comprising HBV, HCV and coinfection of both (HBV and HCV) and non-viral hepatitis comprising past HBV, HCV, HBV and HCV, and NBNC, to simplify the analyses. The non-viral hepatitis group had a significantly higher prevalence of DM, DL, obesity, HT and cardiovascular disease. From this perspective and according to previous reports, the non-viral hepatitis group should have higher sFABP5 concentrations; however, there was no difference in the sFABP5 levels between these two groups (Supplementary Table 4).

Table 2 shows simple and multiple linear regression analyses of clinical factors for sFABP5. In simple linear regression, there was a significant correlation between DM and sFABP5 ($P = 0.035$). However, in multiple linear regression, the correlation between DM and sFABP5 was not significant. The presence of HCC did not correlate with sFABP5 in simple and multiple linear regression analyses.

Analysis of the FABP5 expression level in HCC tissues and oncological factors

The distribution of FABP5 expression levels in HCC and adjacent normal liver tissues before and after logarithmic transformation is presented in Supplementary Figure 1. There was no outlier to be excluded. The comparison of the FABP5 expression levels in HCC and adjacent normal liver tissues is presented in Figure 1C. A paired *t*-test showed a

significantly higher level of FABP5 in HCC than in adjacent normal liver tissues ($P < 0.0001$), which was consistent with our previous findings (6). This finding was not altered when stratified by viral status. We also checked the correlation of FABP5 expression levels in HCC to adjacent normal liver tissue, but no correlation was observed (Supplementary Figure 2).

We analyzed the correlation between the tissue FABP5 and sFABP5 levels (Figure 1D). There was no correlation between tissue FABP5 expression and sFABP5 levels in HCC and adjacent normal liver tissues. Moreover, in simple and multiple linear regression analyses, no relationship between FABP5 expression in tissues and sFABP5 was observed. DM was significantly correlated with sFABP5 in simple linear regression and tended to be correlated to sFABP5 in multiple linear regression; however, the relationship was not significant (Supplementary Table 5).

We divided the patients into two groups according to the FABP5 expression levels in HCC tissues (tFABP5) (i.e., high and low tFABP5 groups). Especially, to determine the cut-off value of tFABP5, we used a ROC curve with a positive outcome of 2-year mortality whose area under the curve (AUC) had the highest value among 2- to 5-year mortality, and its 95% confidence interval (95% CI) was not < 0.5 (Supplementary Figure 3A). The clinicopathological characteristics of low and high tFABP5 groups are described in Table 3. The two groups had significant differences in age, α-fetoprotein (AFP), differentiation and Union for International Cancer Control (UICC) stage. We further analyzed the pathological factors adjusting for the uneven clinical factors between the high and low tFABP5 groups. We excluded the UICC stage from this analysis because of multicollinearity. We found that AFP and differentiation were positively correlated with tFABP5, while the tumor size was negatively correlated with tFABP5 (Table 4).

Analysis of sFABP5 concentration and oncological factors

First, we determined the cut-off value of sFABP5 concentration to analyze its relationship with oncological factors. The cut-off value of log(sFABP5) was determined as 2.293 using the ROC curve with a positive outcome of 3.5-year mortality, whose AUC had the highest value among 2- to 5-year mortality. Moreover, its 95% CI was not < 0.5 (Supplementary Figure 3B). The clinicopathological characteristics of the low and high sFABP5 groups are indicated in Table 3. There were no significant differences between the factors in the two groups. We further analyzed the oncological factors between the low and high sFABP5 groups adjusted for the presence of DM, which was related to sFABP5 in the prior analysis (Table 4). However, the oncological factors had no significant differences even with the adjustment.

Survival analysis

Survival curves of the low and high tFABP5 groups are presented in Figure 1E. The low tFABP5 group had a significantly higher overall survival (OS) rate than the high tFABP5 group, which was consistent with our previous report (7). However, no significant difference in the disease-free survival (DFS) rates was observed between the two groups. Next, we checked the survival rates of the low and high sFABP5 groups. The low sFABP5 group showed a significantly higher survival rate than the high sFABP5 group in the OS and DFS rates (Figure 1F).

These findings showed that tFABP5 and sFABP5 were not correlated, but had some impact on prognosis in patients with HCC. Therefore, we divided our cohort into four groups, taking sFABP5 and tFABP5 into consideration: the low sFABP5 and low tFABP5 (low-S low-T), high sFABP5 and low tFABP5 (high-S low-T), low sFABP5 and high tFABP5 (low-S high-T), and high sFABP5 and high tFABP5 (high-S high-T) groups. The survival curves of

the four groups are presented in Figure 1G. We found that the low-S low-T group had a higher OS rate than the other three groups.

Establishment of FABP5-OE and -KO cells

We successfully established FABP5-OE and -KO cells, which were confirmed by western blotting and PCR. The morphology of PLC/PRF/5 and KIM-1 was not affected by the FABP5 status (Figure 2A). The full-length image of the western blotting is presented in Supplementary Figure 4.

Efflux of FABP5 from HCC cells

Our results from the analyses of sFABP5 indicated that FABP5 was not released from HCC cells into the systemic circulation. We confirmed this finding using a cell-based assay (Figure 2B). Especially, we detected FABP5 in cell lysates from PLC/PRF/5 and KIM-1 and did not detect FABP5 in DMEM without FBS, which were used as positive and negative controls, respectively. Regarding the culture supernatant, we could not detect FABP5 except for FABP5-OE KIM-1. Although we detected FABP5 in the FABP5-OE KIM-1 culture supernatant, the level was low (mean, 0.22 ng/mL).

Cell proliferation and migration

The cell proliferation rate was higher in FABP5-KO than in FABP5-WT cells after short-term incubation under nutrient-sufficient conditions, and FABP5-OE cells showed the opposite result (Figure 2C). However, long-term incubation without medium change showed lower and higher viability of FABP5-KO and FABP5-OE cells, respectively, than of FABP5-WT cells. Concerning cell migration, the FABP5 expression level was negatively correlated with the migration distance (Figure 2D).

Cell viability under glucose-depleted condition

Considering the cell proliferation results, we hypothesized that nutrition was a key factor for the relationship between the FABP5 expression level and HCC progression. Thus, we checked the cell viability under glucose-depleted conditions using glucose-free DMEM. In this assay, cell viability was positively correlated with the FABP5 expression levels (Figure 2E).

Impact of exFABP5 on cell viability

First, we checked the uptake of FABP5 by HCC cells using immunocytochemistry (Figure 2F). The intensity of fluorescence in cells was higher when cells were treated with exFABP5 than when treated with vehicle. This means that exFABP5 is taken up by HCC cells, but the amount is relatively smaller than endogenous FABP5, given the change in fluorescence.

Next, we checked the effect of exFABP5 on cell viability under glucose-depleted conditions, considering the higher viability of FABP5-OE cells in prior experiments. Especially, exFABP5 had a positive effect on the survival of FABP5-KO cells under glucose-depleted conditions, although the effect was relatively small. In contrast, the viability of FABP5-WT cells did not change with exFABP5 treatment (Figure 2G).

Discussion

This study was the first to analyze the relationship between sFABP5 expression and HCC progression. We showed that the HCC and hepatitis groups had no difference in the sFABP5 levels. In addition, sFABP5 and tFABP5 were not correlated. Furthermore, the amount of FABP5 secreted by cells was very small according to our *in vitro* experiments.

These results indicated that FABP5, an intracellular protein, hardly leaks out of HCC cells into the circulation. This means that sFABP5 is not a useful tumor marker in HCC. However, sFABP5 had a significantly negative impact on the prognosis of patients with HCC following surgery, irrespective of tFABP5. In cell-based assays, we observed a slower proliferation rate and smaller migration ability in FABP5-OE HCC than in FABP5-KO HCC cells. This finding was consistent with the negative correlation observed between tFABP5 and tumor size. However, FABP5-OE HCC cells had higher viability under glucose-depleted conditions. Moreover, exFABP5 had positive effect on cell survival under glucose-depleted conditions for FABP5-KO HCC cells.

We found that sFABP5 was not affected by the presence of HCC, tFABP5, etiology of hepatitis, or other clinicopathological factors associated with HCC. According to some reports, sFABP5 levels are elevated because of metabolic syndromes, such as DM, HT, and atherosclerosis (8,9). Among them, DM was positively correlated with sFABP5 levels in our cohort. Therefore, we can state that the sFABP5 levels fluctuate because of factors other than HCC and hepatitis. Thus, controlling metabolic syndromes, such as DM, which is an important factor in sFABP5 elevation, could improve the prognosis of patients with HCC after surgery.

Our findings showed that controlling sFABP5 may improve HCC prognosis. To achieve this goal, the main source of sFABP5 in the human body should be identified. FABP4, the adipocyte-type FABP, is secreted into the bloodstream by adipocytes, although it lacks a secretory signal peptide (21). Similarly, FABP5 lacks a secretory signal peptide and is secreted into the bloodstream by adipocytes (12). Adipose tissue mainly expresses FABP4 and has a lower level of FABP5, according to the Human Protein Atlas (http://www.proteinatlas.org) (22). However, FABP4 and FABP5 are unevenly distributed between subcutaneous and visceral fat. Especially, compared with the subcutaneous fat, the

visceral fat has a higher ratio of FABP5 to FABP4 expression (23). Therefore, the main source of sFABP5 may be the visceral fat tissue. The significant correlation between sFABP5 and DM is consistent with the existing evidence, indicating an association between visceral fat and DM (24). However, in the FABP family, FABP5 has the broadest distribution in the human body; hence, other possible sources of FABP5 should be analyzed (25,26).

Most likely, sFABP5 plays a role in HCC cells as an extrinsic factor; therefore, we evaluated the effect of exFABP5 on cell functions using cell-based assays. The relationship between extrinsic FABPs and cancer progression has been studied previously. Extrinsic FABP4 was reported to be a tumor-progressive factor in HCC, breast cancer, and prostate cancer (27-29). However, we could not find scholarly evidence regarding the effect of exFABP5 on cancer progression. The exFABP5 assay results of the current study suggested that sFABP5 is incorporated into HCC cells with low FABP5 expression levels and complement the function of endogenous FABP5, thus, improving the viability under glucose-depleted conditions. However, the amount of exFABP5 taken up by HCC is small relative to endogenous FABP5; hence, its effect on HCC with adequate endogenous FABP5 may not be significant. In this study, we did not analyze the molecular biological mechanisms of exFABP5 in HCC progression; thus, mechanistic studies on the function of exFABP5 in improving cell viability should be conducted in future. Moreover, considering that the effect of exFABP5 on cell viability was relatively small, the role of exFABP5 may vary between *in vitro* and *in vivo* experiments. Therefore, *in vivo* experiments are needed to evaluate the actual effect of exFABP5 in the human body.

This study had several limitations. First, we excluded considerable cases from this study to simplify the analyses. Hence, we cannot adapt the results to the excluded cohorts. In addition, we used stored samples for analysis, although storage conditions may affect some parameters. Second, the sFABP5 concentration in the general population was approximately

7.35 ng/mL in this study, which was higher than that reported previously (1.5 ng/mL) (9). This variability could be attributed to differences in the commercial ELISA kits used to measure sFABP5. Therefore, the sFABP5 value obtained in this study may not apply to other studies. Third, measurement of the tissue FABP5 expression level was achieved by western blotting instead of immunohistochemistry because it is easier to quantify the FABP5 levels using the former than the latter. For this analysis, we extracted proteins from small pieces of resected specimens. However, cancer tissues are heterogenous, and these resected pieces might not represent the entire HCC tissue, especially the multinodular tumor. This may be the reason for the lack of differences in the DFS rates between the low and high tFABP5 groups, which we had previously reported (7). Additionally, the cut-off values of sFABP5 and tFABP5 used in this study might not have been appropriate. Especially since we determined the cut-off values using the ROC curve. However, when we used the median split in determining the tFABP5 cut-off value, the low tFABP5 group tended to have a higher DFS rate than the high tFABP5 group (data not shown), highlighting the need to perform larger studies to determine the appropriate cut-off values.

In conclusion, sFABP5 was not a useful tumor marker for HCC but had a significant impact on HCC progression by improving cell viability under glucose-depleted conditions, thus, resulting in a worse prognosis. As tFABP5 and sFABP5 are independently significant in HCC progression, they should be considered independently in improving the prognosis of these patients.

Supplementary material

Supplementary Figures and Tables can be found at http://carcin.oxfordjournals.org/

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References

1. Ockner, R.K. *et al.* (1972) A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science*, **177**, 56-58.
2. McKillop, I.H. *et al.* (2019) Role of fatty acid binding proteins (FABPs) in cancer development and progression. *Cell Signal*, **62**, 109336.
3. Yamamoto, T. *et al.* (2009) Classification of FABP isoforms and tissues based on quantitative evaluation of transcript levels of these isoforms in various rat tissues. *Biotechnol Lett*, **31**, 1695-1701.
4. Furuhashi, M. *et al.* (2008) Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov*, **7**, 489-503.
5. Guaita-Esteruelas, S. *et al.* (2018) The peritumoural adipose tissue microenvironment and cancer. The roles of fatty acid binding protein 4 and fatty acid binding protein 5. *Mol Cell Endocrinol*, **462**, 107-118.
6. Fujii, K. *et al.* (2005) Proteomic study of human hepatocellular carcinoma using two-dimensional difference gel electrophoresis with saturation cysteine dye. *Proteomics*, **5**, 1411-1422.
7. Ohata, T. *et al.* (2017) Fatty acid-binding protein 5 function in hepatocellular carcinoma through induction of epithelial-mesenchymal transition. *Cancer Med*, **6**, 1049-1061.
8. Yeung, D.C. *et al.* (2008) Epidermal fatty-acid-binding protein: a new circulating biomarker associated with cardio-metabolic risk factors and carotid atherosclerosis. *Eur Heart J*, **29**, 2156-2163.
9. Furuhashi, M. *et al.* (2017) Serum FABP5 concentration is a potential biomarker for residual risk of atherosclerosis in relation to cholesterol efflux from macrophages. *Sci Rep*, **7**, 217.

10. Eguchi, A. *et al.* (2019) Serum Liver-Type Fatty Acid-Binding Protein Is a Possible Prognostic Factor in Human Chronic Liver Diseases From Chronic Hepatitis to Liver Cirrhosis and Hepatocellular Carcinoma. *Hepatol Commun*, **3**, 825-837.
11. Pepaj, M. *et al.* (2016) Proteomic analysis of the INS-1E secretome identify novel vitamin D-regulated proteins. *Diabetes Metab Res Rev*, **32**, 514-521.
12. Yamamoto, T. *et al.* (2016) Transcriptome and Metabolome Analyses in Exogenous FABP4- and FABP5-Treated Adipose-Derived Stem Cells. *PLoS One*, **11**, e0167825.
13. Murakami, T. (1984) Establishment and characterization of human hepatoma cell line (KIM-1). *Act Hepatol Jpn*, **25**, 532-539.
14. Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819-823.
15. Labun, K. *et al.* (2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res*, **47**, W171-W174.
16. Schneider, C.A. *et al.* (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*, **9**, 671-675.
17. Xu, A. *et al.* (2006) Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. *Clin Chem*, **52**, 405-413.
18. Cabre, A. *et al.* (2007) Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients. *Atherosclerosis*, **195**, e150-158.
19. Cabre, A. *et al.* (2008) Plasma fatty acid-binding protein 4 increases with renal dysfunction in type 2 diabetic patients without microalbuminuria. *Clin Chem*, **54**, 181-187.
20. Coll, B. *et al.* (2008) The fatty acid binding protein-4 (FABP4) is a strong biomarker of metabolic syndrome and lipodystrophy in HIV-infected patients. *Atherosclerosis*,

199, 147-153.

21. Xu, A. *et al.* (2007) Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: a 5-year prospective study. *Circulation*, **115**, 1537-1543.

22. Uhlen, M. *et al.* (2015) Proteomics. Tissue-based map of the human proteome. *Science*, **347**, 1260419.

23. Fisher, R.M. *et al.* (2001) Fatty acid binding protein expression in different adipose tissue depots from lean and obese individuals. *Diabetologia*, **44**, 1268-1273.

24. Lebovitz, H.E. *et al.* (2005) Point: visceral adiposity is causally related to insulin resistance. *Diabetes Care*, **28**, 2322-2325.

25. Amiri, M. *et al.* (2018) Diverse roles of fatty acid binding proteins (FABPs) in development and pathogenesis of cancers. *Gene*, **676**, 171-183.

26. Storch, J. *et al.* (2010) Tissue-specific functions in the fatty acid-binding protein family. *J Biol Chem*, **285**, 32679-32683.

27. Thompson, K.J. *et al.* (2018) Altered fatty acid-binding protein 4 (FABP4) expression and function in human and animal models of hepatocellular carcinoma. *Liver Int*, **38**, 1074-1083.

28. Guaita-Esteruelas, S. *et al.* (2017) Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins. *Mol Carcinog*, **56**, 208-217.

29. Uehara, H. *et al.* (2014) Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. *Int J Cancer*, **135**, 2558-2568.

TABLES AND FIGURE LEGENDS

Table 1. Clinical characteristics and sFABP5 concentration of the HCC and hepatitis groups

Factors	HCC group (n = 128)	Hepatitis group (n = 89)	P
Male sex	107 (83.6%)	37 (41.6%)	< 0.0001
Age (year)	68.7 ± 10.7	59.6 ± 14.6	< 0.0001
DM	61 (47.7%)	26 (29.2%)	0.060
DL	44 (34.4%)	27 (30.3%)	0.53
BMI > 25 kg/m^2	45 (35.2%)	38 (42.7%)	0.26
HT	79 (61.7%)	29 (32.6%)	< 0.0001
Cardiovascular disease	25 (19.5%)	8 (9.0%)	0.029
Etiology			< 0.0001
HBV	34 (26.6%)	31 (34.8%)	
HCV	12 (9.4%)	29 (32.6%)	
HBV and HCV	1 (0.8%)	0 (0%)	
Past HBV	17 (13.3%)	0 (0%)	
Past HCV	11 (8.6%)	0 (0%)	
Past HBV and HCV	6 (4.7%)	0 (0%)	
NBNC	47 (36.7%)	29 (32.6%)	
sFABP5 (ng/mL)	11.3 ± 7.4	10.9 ± 8.9	0.70
log(sFABP5)	2.28 ± 0.53	2.24 ± 0.51	0.61

Data are expressed as n (%) or means ± SDs.

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; HCC, hepatocellular carcinoma; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C hepatitis; SD, standard

deviation

Table 2. Correlation between sFABP5 concentration and clinical factors, including the presence of HCC

	Simple linear regression			Multiple linear regression		
Variables	Coefficient	SE	*P*	Coefficient	SE	*P*
Male sex	0.0611	0.0375	0.10	0.0623	0.0426	0.15
Age	-0.0016	0.0027	0.56	-0.0035	0.0031	0.26
DM	0.0764	0.0360	0.035	0.0755	0.0393	0.056
DL	0.0235	0.0380	0.54	0.0226	0.0384	0.56
BMI > 25 kg/m^2	0.00003	0.0367	1	-0.0235	0.0378	0.53
HT	0.0478	0.0355	0.18	0.0445	0.0399	0.27
Cardiovascular disease	-0.0147	0.0496	0.77	-0.0351	0.0517	0.50
HCC	0.0186	0.0362	0.61	0.0173	0.0436	0.69

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; SE, standard error; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; HCC, hepatocellular carcinoma

Table 3. Clinicopathological characteristics stratified by tFABP5 or sFABP5

Factors	tFABP5			sFABP5		
	Low (n = 66)	High (n = 62)	P	Low (n = 71)	High (n = 57)	P
Male sex	59 (89.4%)	48 (77.4%)	0.066	59 (83.1%)	48 (84.2%)	0.87
Age (year)	70.8 ± 10.2	66.5 ± 11.0	0.022	68.8 ± 10.8	68.6 ± 10.8	0.93
DM	34 (51.5%)	27 (43.6%)	0.37	30 (42.3%)	31 (54.4%)	0.17
DL	21 (31.8%)	23 (37.1%)	0.53	23 (32.4%)	21 (36.8%)	0.60
BMI > 25 kg/m^2	22 (33.3%)	23 (37.1%)	0.66	27 (38.0%)	18 (31.6%)	0.45
HT	41 (62.1%)	38 (61.3%)	0.92	41 (57.8%)	38 (66.7%)	0.30
Cardiovascular disease	14 (21.2%)	11 (17.7%)	0.62	15 (21.1%)	10 (17.5%)	0.61
AFP > 20 ng/mL	16 (24.2%)	34 (54.8%)	0.0003	24 (33.8%)	26 (45.6%)	0.17
PIVKA II > 40 mAU/mL	49 (74.2%)	51 (82.3%)	0.27	54 (76.1%)	46 (80.7%)	0.53
Tumor size > 5 cm	32 (48.5%)	25 (40.3%)	0.35	28 (39.4%)	29 (50.9%)	0.20
Differentiation			0.040			0.087
Well	17 (25.8%)	8 (12.9%)		16 (22.5%)	9 (15.8%)	
Mod	40 (60.6%)	36 (58.1%)		45 (63.4%)	31 (54.4%)	
Por	9 (13.6%)	18 (29.0%)		10 (14.1%)	17 (29.8%)	
Microvascular	22 (33.3%)	28 (45.2%)	0.17	23 (32.4%)	27 (47.4%)	0.084

invasion						
Multinodular tumor	15 (22.7%)	21 (33.9%)	0.16	21 (29.6%)	15 (26.3%)	0.68
UICC Stage ≥ III	22 (33.3%)	37 (59.7%)	0.0027	29 (40.9%)	30 (52.6%)	0.18

Data are expressed as *n* (%) or means ± SDs.

FABP5, fatty acid-binding protein 5; tFABP5, FABP5 expression level in HCC tissue; sFABP5, serum FABP5; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; AFP, α-fetoprotein; PIVKA II, protein induced by vitamin K absence or antagonist II; mod, moderately; por, poorly; UICC, Union for International Cancer Control; SD, standard deviation

Table 4. Multiple linear regression analyses for the correlation between tFABP5 or sFABP5 and pathological factors adjusted for the clinical factors uneven between the high and low groups

	For tFABP5			For sFABP5		
Variables	Coefficient	SE	*P*	Coefficient	SE	*P*
Male sex	-0.2822	0.1304	0.033			
Age	-0.0062	0.0091	0.50			
DM				0.0984	0.0476	0.041
log(AFP)	0.1452	0.0351	< 0.0001			
log(Tumor size)	-0.5736	0.1583	0.0004	0.1121	0.0771	0.15
Differentiation	0.3290	0.1590	0.041	0.0362	0.0786	0.65
Microvascular invasion	0.1446	0.1057	0.17	0.0122	0.0525	0.82
Multinodular tumor	0.1395	0.1071	0.20	-0.0180	0.0529	0.73

FABP5, fatty acid-binding protein 5; tFABP5, FABP5 expression level in HCC tissue; sFABP5, serum FABP5; SE, standard error; DM, diabetes mellitus; AFP, α-fetoprotein

Figure 1. Analyses of samples from patients with HCC and those with hepatitis.

(A) Flowchart of patient inclusion and exclusion. In total, 243 patients underwent hepatectomy for HCC during the study period, but 115 cases were excluded, thus, retaining 128 cases in the HCC group. The hepatitis group comprised 92 hepatitis cases without HCC.

(B) The distribution of serum FABP5 concentration. Left and right panels present the distribution before and after log-transformation, respectively. Three outliers were detected in the hepatitis group and excluded from the analyses.

(C) The FABP5 expression levels in HCC and adjacent normal liver tissues are presented. The HCC tissues had a significantly higher FABP5 level than the normal liver tissues, as presented by the paired *t*-test. When cases were stratified by viral status, the difference was not altered. * $P < 0.0001$.

(D) Correlation of the FABP5 expression level in tissue to sFABP5. In the upper panel, the correlation analysis between the FABP5 expression level in the HCC tissue and sFABP5 is presented; no significant correlation was observed ($R^2 < 0.0001$; $P = 0.94$). In the lower panel, the correlation analysis between the FABP5 expression level in adjacent normal liver tissue and sFABP5 is presented; no significant correlation was observed ($R^2 = 0.0010$; $P = 0.72$).

(E) The survival curves of the low and high tFABP5 groups are presented. The low tFABP5 group had a significantly higher OS rate, as presented by the log-rank and generalized Wilcoxon tests ($P = 0.030$ and $P = 0.014$, respectively). However, there was no difference in the DFS rate (log-rank, $P = 0.39$; Wilcoxon, $P = 0.16$).

(F) The survival curves of the low and high sFABP5 groups are presented. The low sFABP5 group had a significantly higher OS rate, as confirmed using the log-rank and generalized Wilcoxon test ($P = 0.035$ and $P = 0.024$, respectively). We found the same result in the DFS rate (log-rank, $P = 0.038$; Wilcoxon, $P = 0.021$)

(G) The survival curves of four groups stratified by tFABP5 and sFABP5 are presented. In OS, the low-S low-T group had a better survival rate than the other three groups (Log-rank, $P = 0.041$; Wilcoxon, $P = 0.021$).

Figure 2. Cell-based assays using HCC cell lines, PLC/PRF/5, and KIM-1.

(A) We established FABP5-OE and -KO cells. FABP5-OE and -KO were confirmed using western blotting. Genomic PCR was used to check the disruption of *FABP5* gene. The FABP5 status did not affect the cell morphology in PLC/PRF/5 and KIM-1 based on the cell pictures.

(B) Analysis of FABP5 efflux from cells using ELISA. We detected FABP5 in cell lysate from HCC cell lines, which was used as a positive control, and there was no detectable level of FABP5 in DMEM without FBS, which was used as a negative control. Culture supernatant from PLC/PRF/5 lacked a detectable level of FABP5, although FABP5 was overexpressed. We detected FABP5 in culture supernatant from FABP5-OE KIM-1, but it was unexpectedly low considering the supernatant was concentrated 10-fold by ultrafiltration (approximately, 0.022 ng/mL). ND, not detectable.

(C) Cell proliferation under nutrient-sufficient conditions. FABP5-OE cells showed slower proliferation and FABP5-KO cells had faster proliferation than FABP5-WT cells. However, after long-term incubation without changing the medium, FABP5-OE cells had good viability while FABP5 WT and KO cells underwent relatively prompt death. * $P < 0.05$, ** $P < 0.0001$.

(D) Cell migration assay. The expression level of FABP5 was negatively correlated with cell migration ability. The migration distance of FABP5-KO cells was longer than that of FABP5-WT cells, and FABP5-OE cells showed opposite results. * $P < 0.05$, ** $P < 0.0001$.

(E) Cell viability under glucose-depleted condition. FABP5-OE cells showed better viability than FABP5-WT and -KO cells. * $P < 0.05$, ** $P < 0.0001$.

(F) Evaluating FABP5 uptake by HCC cell lines using immunocytochemistry. FABP5-KO cells treated with 100 ng/mL exFABP5 showed significantly higher fluorescence in their cytoplasm than FABP5-KO cells treated with vehicle. There was a small rise in the fluorescence intensity in FABP5-WT cells when treated with exFABP5, although it was not significant. FABP5-KO cells showed some fluorescence, which was attributed to nonspecific binding of primary antibody to the cells. * $P < 0.05$.

(G) The effect of exFABP5 on cell viability under glucose-depleted condition. In PLC/PRF/5 and KIM-1, FABP5-KO cells showed lower viability than FABP5-WT cells when treated with vehicle. However, FABP5-KO cells showed improvement in cell viability when supplemented with exFABP5, while FABP5-WT cells did not present such effect. The viability of FABP5-KO PLC/PRF/5 improved up to the viability level of FABP5-WT cells when treated with 100 ng/mL of exFABP5. * $P < 0.05$.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Distribution of FABP5 expression levels in HCC and adjacent normal liver tissues.

(A) Before logarithmic transformation.

(B) After logarithmic transformation. No outlier was identified.

Supplementary Figure 2. Correlation between FABP5 expression levels in adjacent normal liver and HCC tissues. No correlation between them was observed ($R^2 = 0.026$; $P = 0.068$).

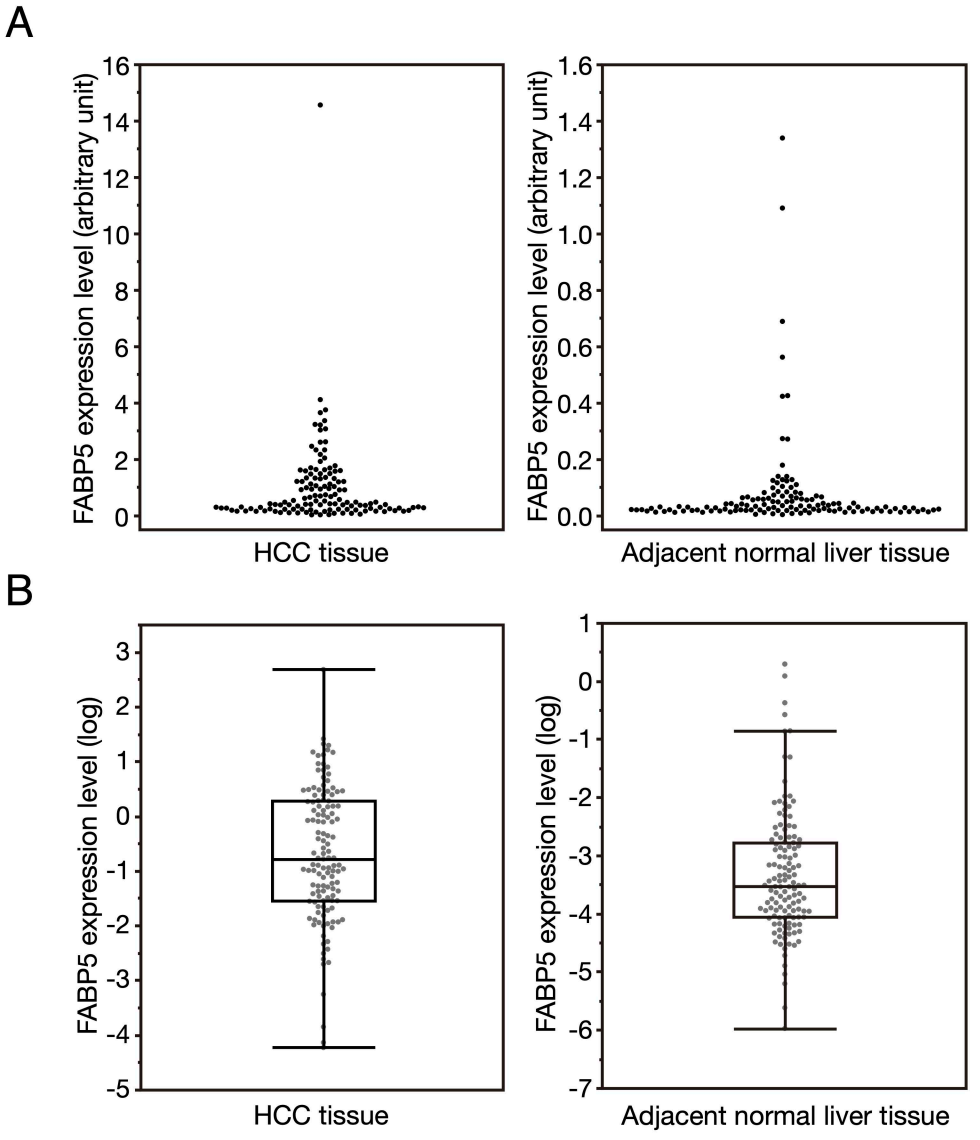
Supplementary Figure 3. Fluctuation of ROC-AUC according to the change in positive outcomes among 2- to 5-year mortality and the actual ROC curve for determining the cut-off value of tFABP5 and sFABP5. The positive outcome for drawing the ROC curve was set as the value with the largest ROC-AUC, and its 95% CI was not < 0.5.

(A) ROC curve for determining the cut-off value of tFABP5. ROC-AUC for tFABP5 was the largest when the positive outcome was set as 2-year mortality (AUC = 0.764; 95% CI, 0.625–0.863), and the cut-off value was determined at -0.76684.

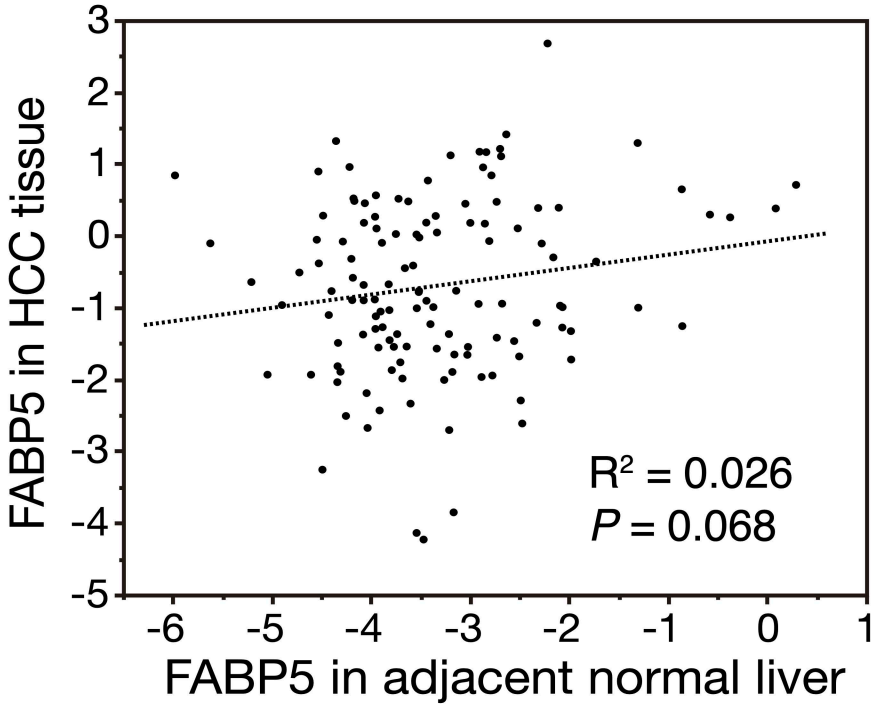
(B) ROC curve for determining the cut-off value of sFABP5. ROC-AUC for sFABP5 was the largest when the positive outcome was set as 3.5-year mortality (AUC = 0.660; 95% CI, 0.519–0.778), and the cut-off value was determined at 2.293.

Supplementary Figure 4. Full-length images of western blotting. The GAPDH and FABP5 bands are presented on the 37-kDa and 15-kDa lines, respectively.

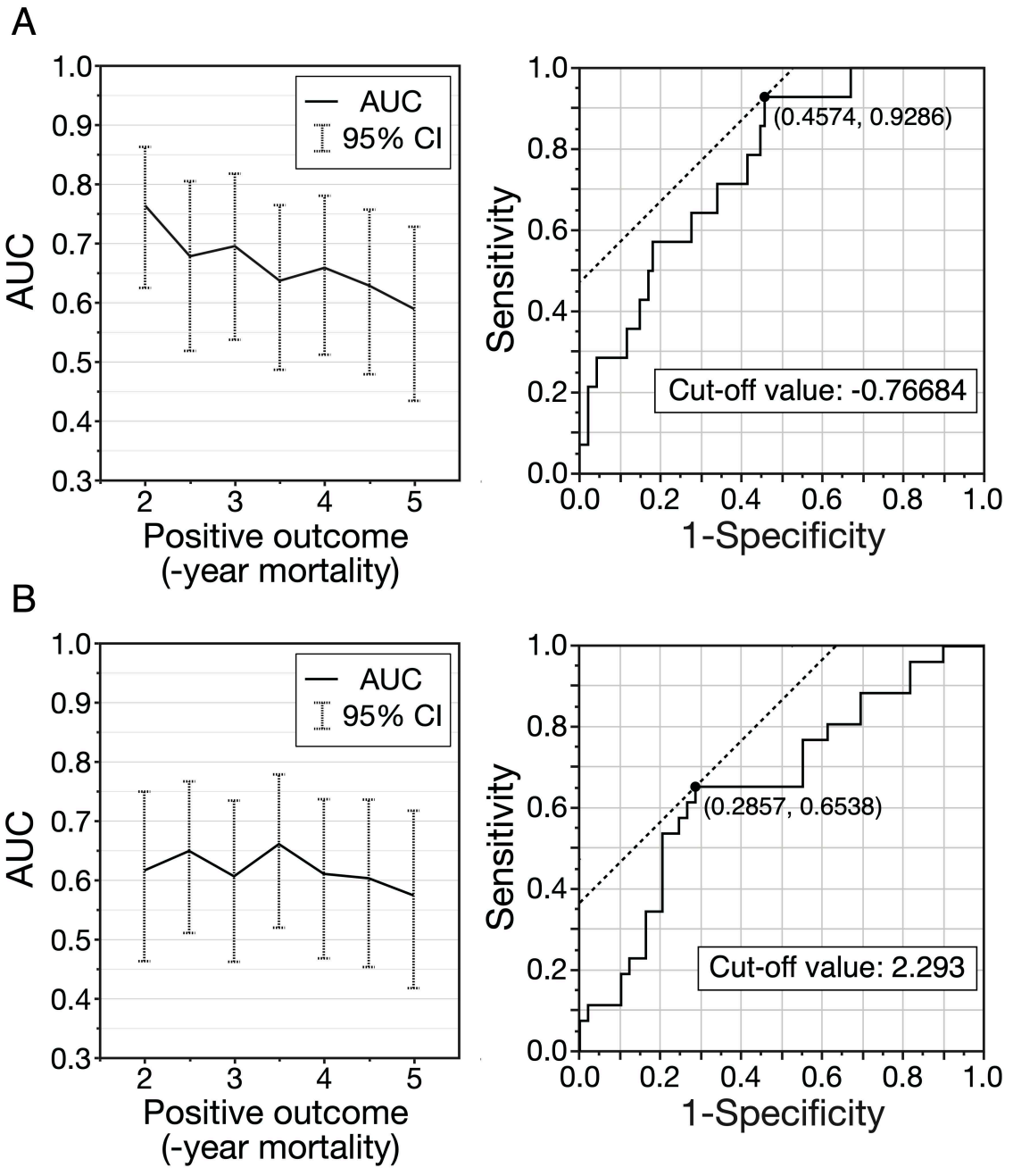
Supplementary Figure 1.



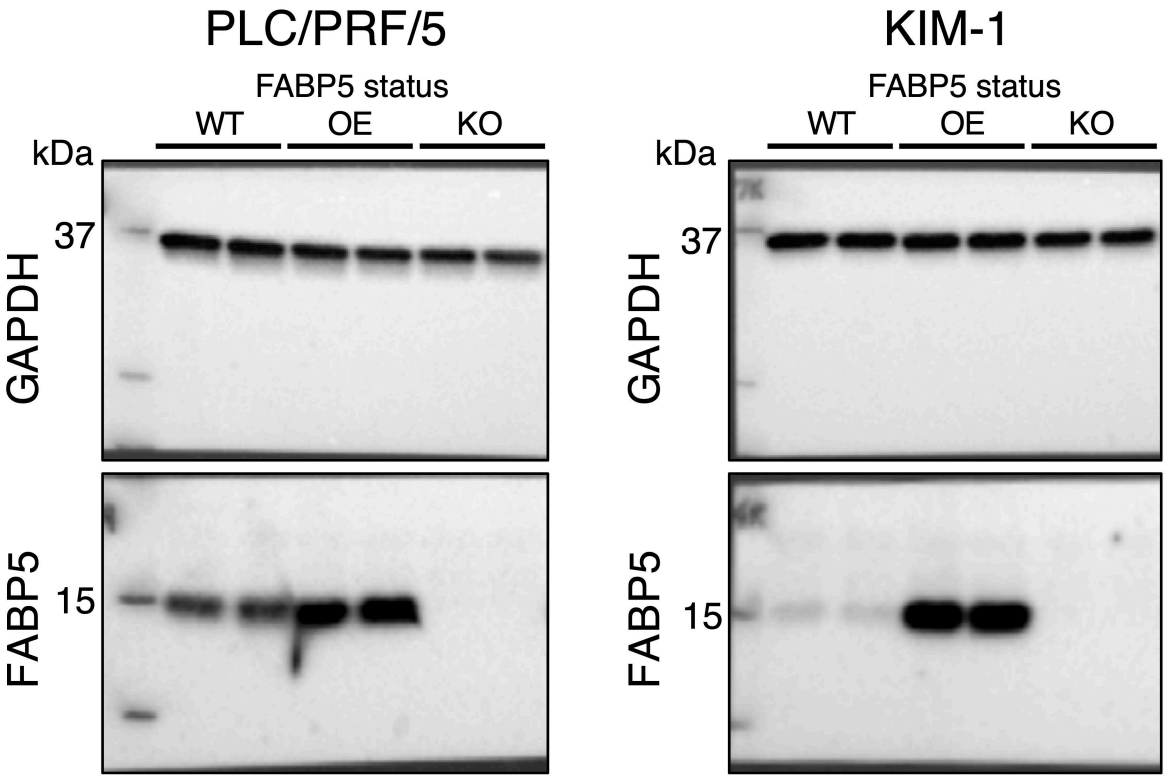
Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.



SUPPLEMENTARY TABLES

Supplementary Table 1. Actual sequence that was cloned into an expression vector to establish the FABP5-OE cells

Whole exon sequence of FABP5 with Kozak sequence
5'-GCCACCATGGCCACAGTTCAGCAGCTGGAAGGAAGATGGCGCCTGGTG
GACAGCAAAGGCTTTGATGAATACATGAAGGAGCTAGGAGTGGGAATAG
CTTTGCGAAAAATGGGCGCAATGGCCAAGCCAGATTGTATCATCACTTGT
GATGGTAAAAACCTCACCATAAAAACTGAGAGCACTTTGAAAACAACAC
AGTTTTCTTGTACCCTGGGAGAGAAGTTTGAAGAAACCACAGCTGATGGC
AGAAAAACTCAGACTGTCTGCAACTTTACAGATGGTGCATTGGTTCAGCA
TCAGGAGTGGGATGGGAAGGAAAGCACAATAACAAGAAAATTGAAAGAT
GGGAAATTAGTGGTGGAGTGTGTCATGAACAATGTCACCTGTACTCGGAT
CTATGAAAAAGTAGAATAA-3'

FABP5, fatty acid-binding protein 5; OE, overexpressing

Supplementary Table 2. Clinical characteristics and sFABP5 concentration in the healthy control group

Factors	Healthy control (n = 23)
Male sex	17 (73.9%)
Age (year)	46.9 ± 2.7
DM	0 (0%)
DL	16 (69.6%)
BMI > 25 kg/m^2	8 (34.8%)
HT	3 (13.0%)
Cardiovascular disease	1 (4.3%)
sFABP5 (ng/ml)	7.35 ± 2.27
log(sFABP5)	1.94 ± 0.37

Data are expressed as n (%) or means ± SDs.

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; SD, standard deviation

Supplementary Table 3. Clinical characteristics and sFABP5 concentration stratified by etiology

Factors	HBV (n = 65)	HCV (n = 41)	HBV and HCV (n = 1)	Past HBV (n = 17)	Past HCV (n = 11)	Past HBV and HCV (n = 6)	NBNC (n = 76)
Male sex	42 (64.6%)	20 (48.8%)	1 (100%)	15 (88.2%)	8 (72.7%)	5 (83.3%)	53 (69.7%)
Age (year)	60.8 ± 12.9	64.1 ± 12.8	49	69.7 ± 10.1	73.5 ± 9.8	71.7 ± 13.1	66.4 ± 13.8
DM	13 (20.0%)	9 (22.0%)	1 (100%)	9 (52.9%)	4 (36.4%)	3 (50.0%)	48 (63.2%)
DL	12 (18.5%)	9 (22.0%)	0 (0%)	3 (17.7%)	6 (54.6%)	3 (50.0%)	38 (50.0%)
BMI > 25 kg/m^2	21 (32.3%)	6 (14.6%)	1 (100%)	7 (41.2%)	2 (18.2%)	2 (33.3%)	44 (57.9%)
HT	25 (38.5%)	15 (36.6%)	1 (100%)	11 (64.7%)	10 (90.9%)	4 (66.7%)	42 (55.3%)
Cardiovascular disease	6 (9.2%)	4 (9.8%)	0 (0%)	1 (5.9%)	5 (45.5%)	1 (16.7%)	16 (21.1%)
sFABP5 (ng/mL)	10.1 ± 4.6	12.1 ± 12.6	54.7	12.7 ± 7.4	10.4 ± 5.3	5.9 ± 2.8	11.2 ± 6.1
log(sFABP5)	2.22 ± 0.42	2.26 ± 0.63	4.00	2.39 ± 0.55	2.23 ± 0.49	1.69 ± 0.46	2.29 ± 0.49

Data are expressed as n (%) or means ± SDs.

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C hepatitis; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; SD, standard deviation

Supplementary Table 4. Clinical characteristics and sFABP5 concentration stratified by the viral status

Factors	Viral hepatitis (n = 107)	Non-viral hepatitis (n = 110)	P
Male sex	63 (58.9%)	81 (73.6%)	0.021
Age (year)	62.0 ± 12.9	67.9 ± 13.0	< 0.001
DM	23 (21.5%)	64 (58.2%)	< 0.0001
DL	21 (19.6%)	50 (45.5%)	< 0.0001
BMI > 25 kg/m^2	28 (26.2%)	55 (50.0%)	0.0003
HT	41 (38.3%)	67 (60.9%)	< 0.001
Cardiovascular disease	10 (9.35%)	23 (20.9%)	0.016
sFABP5 (ng/mL)	11.3 ± 9.6	11.2 ± 6.2	0.85
log(sFABP5)	2.25 ± 0.54	2.27 ± 0.51	0.80

Data are expressed as n (%) or mean ± SD.

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; SD, standard deviation

Supplementary Table 5. Correlation between sFABP5 and factors related to metabolic syndrome, including tissue FABP5 expression levels

	Simple linear regression			Multiple linear regression		
Factor	Coefficient	SE	*P*	Coefficient	SE	*P*
DM	0.1000	0.0464	0.033	0.0993	0.0519	0.058
DL	0.0124	0.0497	0.80	-0.0168	0.0517	0.75
BMI > 25 kg/m^2	-0.0048	0.0495	0.92	-0.0350	0.0513	0.50
HT	0.0619	0.0483	0.20	0.0418	0.0515	0.42
Cardiovascular disease	0.0280	0.0595	0.64	0.0143	0.0622	0.82
FABP5 in HCC tissue	0.0030	0.0389	0.94	0.0120	0.0401	0.77
FABP5 in adjacent normal liver	0.0159	0.0445	0.72	0.0127	0.0464	0.78

FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; SE, standard error; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; HCC, hepatocellular carcinoma