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

3

4 **Short title:** Serum FABP5 in HCC progression

5

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19

1 **Abstract**

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

19

20 **Summary**

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

24

1 **Introduction**

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

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

12 Although FABPs are intracellular proteins, some reports have shown a relationship
13 between circulating FABPs in blood and metabolic syndrome (8,9) and some cancers.
14 Especially, circulating FABP4 and FABP5 have been studied in patients with breast cancer
15 while circulating FABP1 has been studied in patients with HCC (5,10). Moreover, some *in*
16 *vitro* studies have demonstrated the secretion of FABP5 from cells into the culture medium
17 (11,12). However, although we have made a thorough database search, we could not find
18 scholarly evidence regarding the relationship between circulating FABP5 and HCC. In this
19 study, we aimed to clarify the relationship between serum FABP5 (sFABP5) and HCC by
20 analyzing the potential of sFABP5 as a tumor marker in HCC and its clinical significance in
21 HCC progression.

22

23 **Materials and methods**

24 ***Patients***

25 Patients who underwent hepatic resection for HCC at Hokkaido University

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

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

1

2 ***Measurement of the sFABP5 concentration***

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

16

17 ***Measurement of the FABP5 expression level in resected liver specimen***

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

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

20

21 ***Cell culture***

22 Human HCC-derived cell lines, PLC/PRF/5 and KIM-1, were cultured in
23 Dulbecco's modified Eagle medium (DMEM; Nacalai, Kyoto, Japan) supplemented with
24 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Nacalai) in an
25 incubator containing 5% CO₂ at 37°C. PLC/PRF/5 was purchased from the Japanese

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

9

10 ***Establishment of FABP5 overexpressing (OE) cell lines***

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

18

19 ***Establishment of FABP5 knockout (KO) cell lines***

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

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

6 ***Western blotting for cell protein extract***

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

14 ***PCR***

15 We checked the knockout of *FABP5* using PCR. Whole genome from cells was
16 extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the
17 manufacturer's protocol. The sequences of the oligonucleotides used to check *FABP5*
18 knockout were 5'-TGGCCAGAGCTCAGATTTGG-3' for the forward primer and 5'-
19 CCACTCCTGTAGAATGTTAAGTCATG-3' for the reverse primer, and the product size was
20 349 bp. PCR products were subjected to electrophoresis, followed by soaking in ethidium
21 bromide solution and visualized using a UV transilluminator.

23 ***Analysis of FABP5 efflux from cells***

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

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

11

12 ***Cell proliferation assay***

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

19

20 ***Cell migration assay***

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

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

7

8 ***Cell viability assay under glucose-depleted condition***

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

24

25 ***Immunocytochemistry***

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

17

18 ***Statistical analysis***

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

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

4 **Results**

6 ***sFABP5 concentration in the healthy control group (preliminary study)***

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

12 ***The relationship between sFABP5 and the presence of HCC***

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

19 In general, sFABP5 concentration is affected by some clinical conditions related to
20 metabolic syndrome, such as diabetes mellitus (DM), hypertension (HT) and cardiovascular
21 diseases (i.e., atherosclerosis of the carotid and coronary arteries) (8,9). In addition,
22 dyslipidemia (DL) and high body mass index may also affect serum FABP4 concentration
23 (17-20). Therefore, these factors were considered in the following analyses.

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

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

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

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

20

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

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

1 significantly higher level of FABP5 in HCC than in adjacent normal liver tissues ($P <$
2 0.0001), which was consistent with our previous findings (6). This finding was not altered
3 when stratified by viral status. We also checked the correlation of FABP5 expression levels in
4 HCC to adjacent normal liver tissue, but no correlation was observed (Supplementary Figure
5 2).

6 We analyzed the correlation between the tissue FABP5 and sFABP5 levels (Figure
7 1D). There was no correlation between tissue FABP5 expression and sFABP5 levels in HCC
8 and adjacent normal liver tissues. Moreover, in simple and multiple linear regression
9 analyses, no relationship between FABP5 expression in tissues and sFABP5 was observed.
10 DM was significantly correlated with sFABP5 in simple linear regression and tended to be
11 correlated to sFABP5 in multiple linear regression; however, the relationship was not
12 significant (Supplementary Table 5).

13 We divided the patients into two groups according to the FABP5 expression levels
14 in HCC tissues (tFABP5) (i.e., high and low tFABP5 groups). Especially, to determine the
15 cut-off value of tFABP5, we used a ROC curve with a positive outcome of 2-year mortality
16 whose area under the curve (AUC) had the highest value among 2- to 5-year mortality, and its
17 95% confidence interval (95% CI) was not < 0.5 (Supplementary Figure 3A). The
18 clinicopathological characteristics of low and high tFABP5 groups are described in Table 3.
19 The two groups had significant differences in age, α -fetoprotein (AFP), differentiation and
20 Union for International Cancer Control (UICC) stage. We further analyzed the pathological
21 factors adjusting for the uneven clinical factors between the high and low tFABP5 groups. We
22 excluded the UICC stage from this analysis because of multicollinearity. We found that AFP
23 and differentiation were positively correlated with tFABP5, while the tumor size was
24 negatively correlated with tFABP5 (Table 4).

25

1 *Analysis of sFABP5 concentration and oncological factors*

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

12

13 *Survival analysis*

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

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

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

3

4 ***Establishment of FABP5-OE and -KO cells***

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

9

10 ***Efflux of FABP5 from HCC cells***

11 Our results from the analyses of sFABP5 indicated that FABP5 was not released
12 from HCC cells into the systemic circulation. We confirmed this finding using a cell-based
13 assay (Figure 2B). Especially, we detected FABP5 in cell lysates from PLC/PRF/5 and KIM-
14 1 and did not detect FABP5 in DMEM without FBS, which were used as positive and
15 negative controls, respectively. Regarding the culture supernatant, we could not detect
16 FABP5 except for FABP5-OE KIM-1. Although we detected FABP5 in the FABP5-OE KIM-
17 1 culture supernatant, the level was low (mean, 0.22 ng/mL).

18

19 ***Cell proliferation and migration***

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

1

2 ***Cell viability under glucose-depleted condition***

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

8

9 ***Impact of exFABP5 on cell viability***

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

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

20

21 **Discussion**

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

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

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

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

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

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

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

1 7.35 ng/mL in this study, which was higher than that reported previously (1.5 ng/mL) (9).
2 This variability could be attributed to differences in the commercial ELISA kits used to
3 measure sFABP5. Therefore, the sFABP5 value obtained in this study may not apply to other
4 studies. Third, measurement of the tissue FABP5 expression level was achieved by western
5 blotting instead of immunohistochemistry because it is easier to quantify the FABP5 levels
6 using the former than the latter. For this analysis, we extracted proteins from small pieces of
7 resected specimens. However, cancer tissues are heterogenous, and these resected pieces
8 might not represent the entire HCC tissue, especially the multinodular tumor. This may be the
9 reason for the lack of differences in the DFS rates between the low and high tFABP5 groups,
10 which we had previously reported (7). Additionally, the cut-off values of sFABP5 and
11 tFABP5 used in this study might not have been appropriate. Especially since we determined
12 the cut-off values using the ROC curve. However, when we used the median split in
13 determining the tFABP5 cut-off value, the low tFABP5 group tended to have a higher DFS
14 rate than the high tFABP5 group (data not shown), highlighting the need to perform larger
15 studies to determine the appropriate cut-off values.

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

21

22 **Supplementary material**

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

24

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2 Development (AMED) under the Grant numbers JP20fk0310111, JP20fk0210072 and
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4

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8

9 *Conflict of Interest Statement:* The authors have no conflicts of interest to disclose.

10

11

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- 25

1 **TABLES AND FIGURE LEGENDS**2 **Table 1.** Clinical characteristics and sFABP5 concentration of the HCC and hepatitis groups

Factors	HCC group (<i>n</i> = 128)	Hepatitis group (<i>n</i> = 89)	<i>P</i>
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

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

4 FABP5, fatty acid-binding protein 5; sFABP5, serum FABP5; HCC, hepatocellular carcinoma;

5 DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT, hypertension; HBV,

6 hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C hepatitis; SD, standard

1 deviation

2

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

Variables	Simple linear regression			Multiple linear regression		
	Coefficient	SE	<i>P</i>	Coefficient	SE	<i>P</i>
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

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

6

7

1 **Table 3.** Clinicopathological characteristics stratified by tFABP5 or sFABP5

Factors	tFABP5			sFABP5		
	Low (n = 66)	High (n = 62)	<i>P</i>	Low (n = 71)	High (n = 57)	<i>P</i>
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 \geq III	22 (33.3%)	37 (59.7%)	0.0027	29 (40.9%)	30 (52.6%)	0.18

- 1 Data are expressed as *n* (%) or means \pm SDs.
- 2 FABP5, fatty acid-binding protein 5; tFABP5, FABP5 expression level in HCC tissue; sFABP5,
- 3 serum FABP5; DM, diabetes mellitus; DL, dyslipidemia; BMI, body mass index; HT,
- 4 hypertension; AFP, α -fetoprotein; PIVKA II, protein induced by vitamin K absence or
- 5 antagonist II; mod, moderately; por, poorly; UICC, Union for International Cancer Control;
- 6 SD, standard deviation
- 7

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

Variables	For tFABP5			For sFABP5		
	Coefficient	SE	<i>P</i>	Coefficient	SE	<i>P</i>
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

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

6

7

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

2 (A) Flowchart of patient inclusion and exclusion. In total, 243 patients underwent

3 hepatectomy for HCC during the study period, but 115 cases were excluded, thus, retaining

4 128 cases in the HCC group. The hepatitis group comprised 92 hepatitis cases without HCC.

5 (B) The distribution of serum FABP5 concentration. Left and right panels present the

6 distribution before and after log-transformation, respectively. Three outliers were detected in

7 the hepatitis group and excluded from the analyses.

8 (C) The FABP5 expression levels in HCC and adjacent normal liver tissues are presented.

9 The HCC tissues had a significantly higher FABP5 level than the normal liver tissues, as

10 presented by the paired *t*-test. When cases were stratified by viral status, the difference was

11 not altered. * $P < 0.0001$.

12 (D) Correlation of the FABP5 expression level in tissue to sFABP5. In the upper panel, the

13 correlation analysis between the FABP5 expression level in the HCC tissue and sFABP5 is

14 presented; no significant correlation was observed ($R^2 < 0.0001$; $P = 0.94$). In the lower

15 panel, the correlation analysis between the FABP5 expression level in adjacent normal liver

16 tissue and sFABP5 is presented; no significant correlation was observed ($R^2 = 0.0010$; $P =$

17 0.72).

18 (E) The survival curves of the low and high tFABP5 groups are presented. The low tFABP5

19 group had a significantly higher OS rate, as presented by the log-rank and generalized

20 Wilcoxon tests ($P = 0.030$ and $P = 0.014$, respectively). However, there was no difference in

21 the DFS rate (log-rank, $P = 0.39$; Wilcoxon, $P = 0.16$).

22 (F) The survival curves of the low and high sFABP5 groups are presented. The low sFABP5

23 group had a significantly higher OS rate, as confirmed using the log-rank and generalized

24 Wilcoxon test ($P = 0.035$ and $P = 0.024$, respectively). We found the same result in the DFS

25 rate (log-rank, $P = 0.038$; Wilcoxon, $P = 0.021$)

1 (G) The survival curves of four groups stratified by tFABP5 and sFABP5 are presented. In
2 OS, the low-S low-T group had a better survival rate than the other three groups (Log-rank, P
3 = 0.041; Wilcoxon, P = 0.021).

4

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

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

9 (B) Analysis of FABP5 efflux from cells using ELISA. We detected FABP5 in cell lysate
10 from HCC cell lines, which was used as a positive control, and there was no detectable level
11 of FABP5 in DMEM without FBS, which was used as a negative control. Culture supernatant
12 from PLC/PRF/5 lacked a detectable level of FABP5, although FABP5 was overexpressed.
13 We detected FABP5 in culture supernatant from FABP5-OE KIM-1, but it was unexpectedly
14 low considering the supernatant was concentrated 10-fold by ultrafiltration (approximately,
15 0.022 ng/mL). ND, not detectable.

16 (C) Cell proliferation under nutrient-sufficient conditions. FABP5-OE cells showed slower
17 proliferation and FABP5-KO cells had faster proliferation than FABP5-WT cells. However,
18 after long-term incubation without changing the medium, FABP5-OE cells had good viability
19 while FABP5 WT and KO cells underwent relatively prompt death. * P < 0.05, ** P <
20 0.0001.

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

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

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

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

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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 .

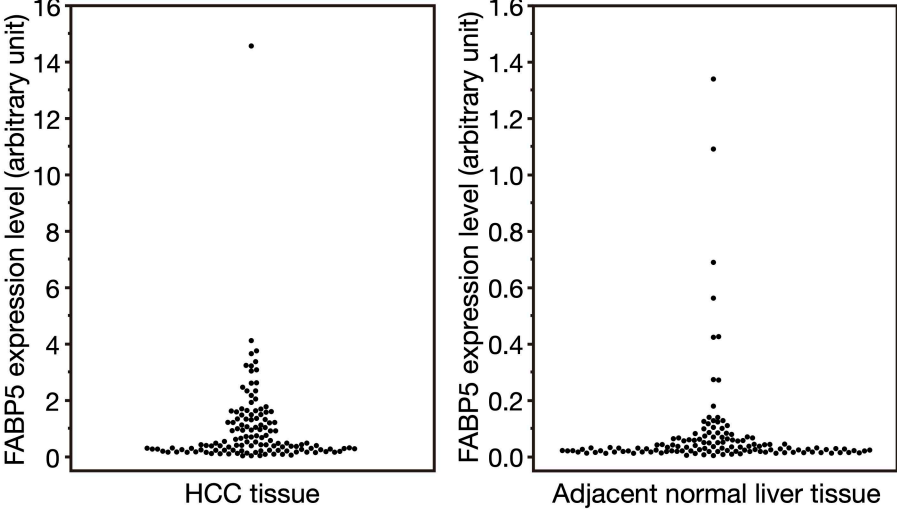
(A) ROC curve for determining the cut-off value of tFABP5. ROC-AUC for tFABP5 was the largest when the positive outcome was set as 2-year mortality (AUC = 0.764; 95% CI, 0.625–0.863), and the cut-off value was determined at -0.76684.

(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.

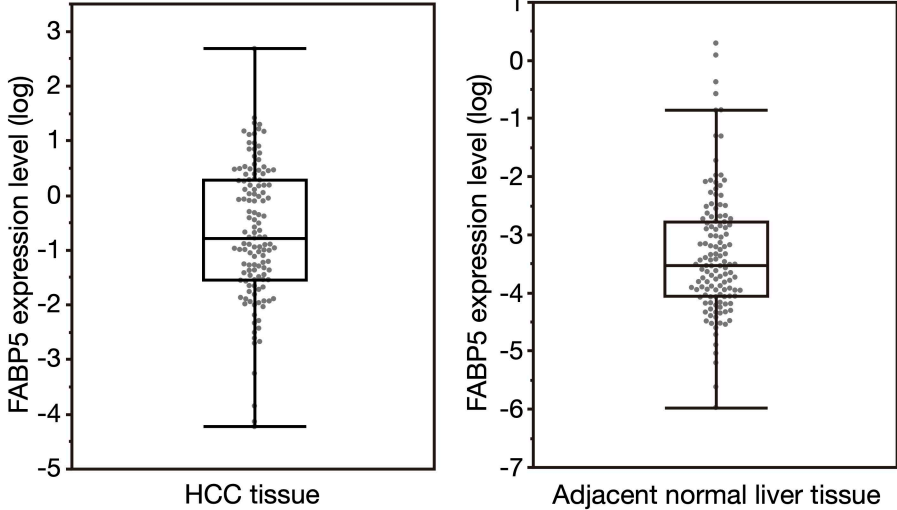
Supplementary Figure 4. Full-length images of western blotting. The GAPDH and FABP5 bands are presented on the 37-kDa and 15-kDa lines, respectively.

Supplementary Figure 1.

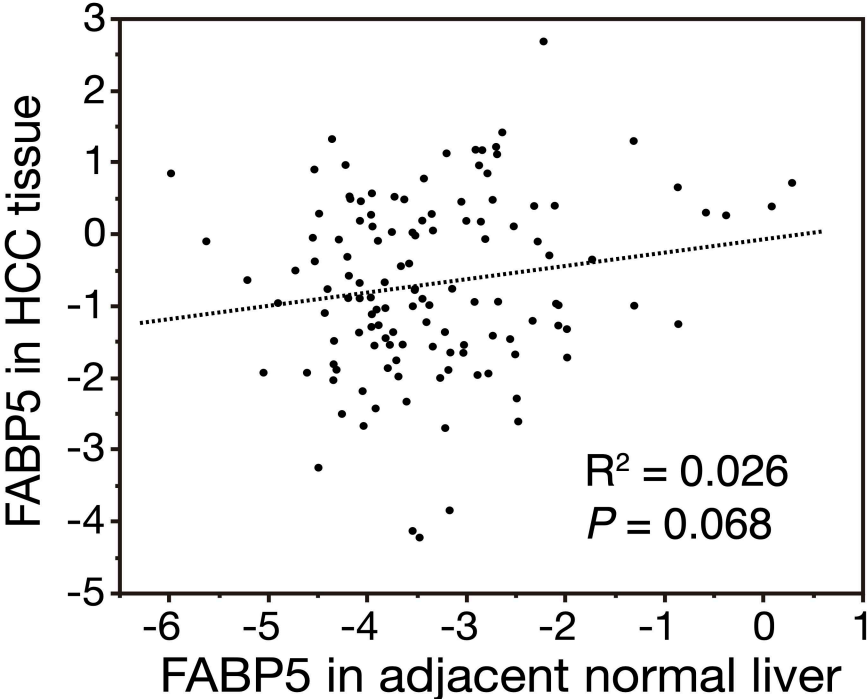
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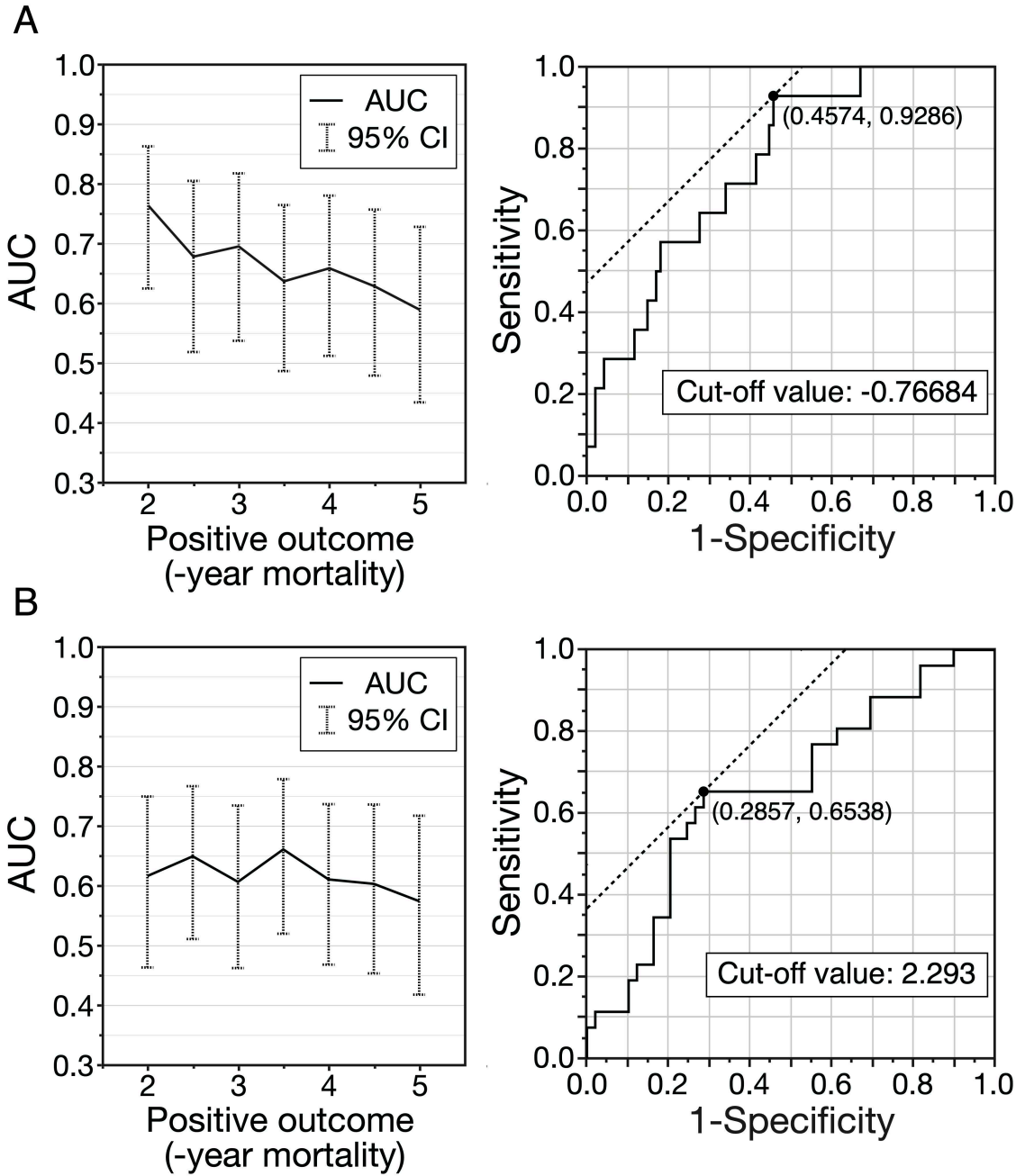
B



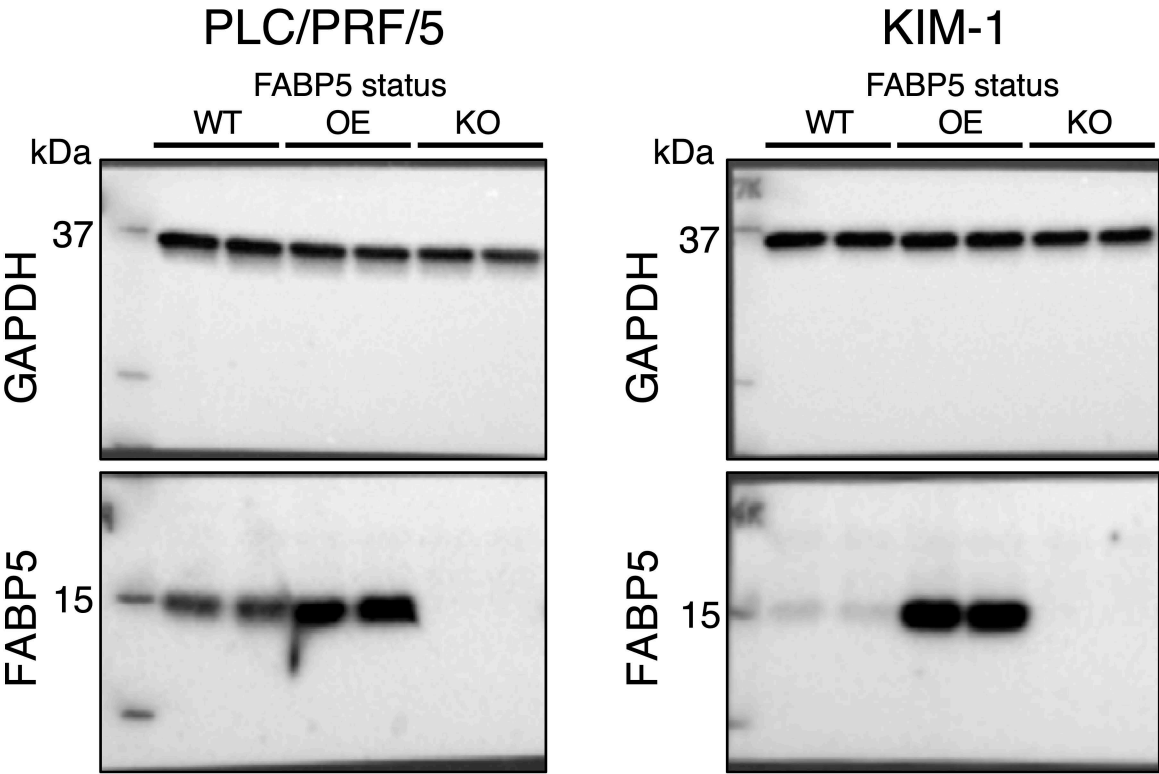
Supplementary Figure 2.



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
AGAAAACTCAGACTGTCTGCAACTTTACAGATGGTGCATTGGTTCAGCA
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 (<i>n</i> = 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 (<i>n</i> = 65)	HCV (<i>n</i> = 41)	HBV and HCV (<i>n</i> = 1)	Past HBV (<i>n</i> = 17)	Past HCV (<i>n</i> = 11)	Past HBV and HCV (<i>n</i> = 6)	NBNC (<i>n</i> = 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 (<i>n</i> = 107)	Non-viral hepatitis (<i>n</i> = 110)	<i>P</i>
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

Factor	Simple linear regression			Multiple linear regression		
	Coefficient	SE	<i>P</i>	Coefficient	SE	<i>P</i>
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