7. Anti-sialyl-MUC1 monoclonal antibody, MY.1E12 was generated by the group of Dr. Irimura (University of Tokyo). This antibody recognizes the epitope involving sialyl alpha 2-3galactosyl beta 1–3 *N*-acetylgalactosaminide linked to a distinct threonine residue in the MUC1 tandem repeat [29].
8. All lectin microarray slides used in this study were produced in-house (Fig. 5a).
9. All scan data were obtained at a camera gain of 110 and an exposure time of 199 ms.
10. Tissue slides are not washed after removing the excess blocking solution in this step.
11. Tissue fragments from an area of 3 mm^2 and with 5 μm thickness are needed for reliable analysis.
12. The amounts of serum sialyl-MUC1 differ between cholangiocarcinoma, benign disease patients, and normal controls. However, in our experience, 5 μL of the enriched sialyl-MUC1 is sufficient for differential glycan profiling in all groups.

Acknowledgments

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Branched-chain amino acids reduce hepatic iron accumulation and oxidative stress in hepatitis C virus polyprotein-expressing mice

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Keywords
hepatitis C virus hepatic mitochondrial dysfunction hepcidin 25 iron metabolic disorder reactive oxygen species

Abbreviations
BAP, biological antioxidant potential; BCAA, branched chain amino acids; BTR, ratio of BCAA relative to tyrosine; CHOP, CCAAT/enhancer binding protein homology protein; CPT I, carnitine palmitoyl transferase I; dROM, derivatives of reactive oxygen metabolites; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVTgM, transgenic mice expressing hepatitis C virus polyprotein; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; SREBP, sterol regulatory element binding protein.

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Abstract

Background & Aims: Branched-chain amino acids (BCAA) reduce the incidence of hepatocellular carcinoma (HCC) in patients with cirrhosis. However, the mechanisms that underlie these effects remain unknown. Previously, we reported that oxidative stress in male transgenic mice that expressed hepatitis C virus polyprotein (HCVTgM) caused hepatic iron accumulation by reducing hepcidin transcription, thereby leading to HCC development. This study investigated whether long-term treatment with BCAA reduced hepatic iron accumulation and oxidative stress in iron-overloaded HCVTgM and in patients with HCV-related advanced fibrosis. *Methods:* Male HCVTgM were fed an excess-iron diet that comprised either casein or 3.0% BCAA, or a control diet, for 6 months. *Results:* For HCVTgM, BCAA supplementation increased the serum hepcidin-25 levels and antioxidant status [ratio of biological antioxidant potential (BAP) relative to derivatives of reactive oxygen metabolites (dROM)], decreased the hepatic iron contents, attenuated reactive oxygen species generation, and restored mitochondrial superoxide dismutase expression and mitochondrial complex I activity in the liver compared with mice fed the control diet. After 48 weeks of BCAA supplementation in patients with HCV-related advanced fibrosis, BAP/dROM and serum hepcidin-25 increased and serum ferritin decreased compared with the pretreatment levels. *Conclusions:* BCAA supplementation reduced oxidative stress by restoring mitochondrial function and improved iron metabolism by increasing hepcidin-25 in both iron-overloaded HCVTgM and patients with HCV-related advanced fibrosis. These activities of BCAA may partially account for their inhibitory effects on HCC development in cirrhosis patients.

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1). New direct-acting antiviral treatments are expected to eliminate this virus in about 90% of patients (2), but therapies that could reduce disease progression in chronically infected individuals would be highly beneficial.

Valine, leucine and isoleucine are essential branched-chain amino acids (BCAA). A decreased ratio of serum BCAA relative to aromatic amino acids, a hallmark of cirrhosis, is caused by several factors, including reduced nutritional intake and ammonia detoxification in skeletal muscles (3). BCAA supplementation can improve the nutritional status and albumin synthesis by activating the mammalian target of rapamycin signalling cascade (4, 5) and glucose metabolism in skeletal muscles (6, 7). Long-term oral BCAA supplementation decreases the frequency of HCC in male obese patients with cirrhosis (8). BCAA also had an antihepatocarcinogenic activity in an animal model of insulin resistance (9, 10). In addition, glucose intolerance is closely linked to hepatocarcinogenesis. However, the mechanisms that underlie these effects remain unknown.

Hepatic oxidative stress and iron overload have been implicated in liver injury and hepatocarcinogenesis in HCV-associated chronic liver diseases (11, 12). The HCV core protein inhibits mitochondrial complex I and generates reactive oxygen species (ROS) *in vivo* (13). Previously, we reported that HCV-induced ROS increases the hepatic iron concentration by reducing hepcidin transcription in transgenic mice that express HCV polyprotein (14), where even modest iron supplementation in these mice resulted in the development of liver tumours, including HCC, because of mitochondrial injury (15). Thus, hepatic iron overload and oxidative stress via mitochondrial injury are critical during HCC pathogenesis.

In the present study, we examined whether long-term BCAA supplementation could prevent the development of hepatic iron accumulation and oxidative stress in HCV transgenic mice fed an excess-iron diet and in patients with HCV-related advanced fibrosis.

Materials and methods

Animals and experimental design

The pAlbSVPA-HCV transgene contains the full-length HCV polyprotein-coding region under the control of the murine albumin promoter/enhancer (16, 17). HCV polyprotein is processed into individual proteins in the liver and expressed at biologically relevant levels in FL-N/35 transgenic mice (HCVTgM) (17). In the present study, male HCVTgM (8 weeks old) were fed a normal rodent diet, including carbonyl iron (45 mg/kg; control, $n = 6$), or an excess-iron diet (carbonyl iron, 225 mg/kg) that contained either 3.0% BCAA (BCAA/iron; $n = 5$) or casein (casein/iron; $n = 7$). Six months later, the mice were sacrificed by CO_2 asphyxiation after a 12-h fast, according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Clinical chemistry tests

The serum concentrations of alanine aminotransferase, aspartate aminotransferase (AST), albumin, glucose, insulin, BCAA, tyrosine and hepcidin-25 were determined in blood samples collected from the inferior vena cava of sacrificed mice at 12 h after fasting. The blood glucose levels were periodically measured using a glucometer (OneTouch Ultra, Lifescan, Inc., Milpitas, CA, USA). The serum insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Morinaga Milk, Kanagawa, Japan). The serum hepcidin-25 levels were determined by LC/MS/MS (18).

Hepatic iron and triglyceride contents

The hepatic iron concentrations were measured by atomic absorption spectrometry, as described previously (15). The liver tissue was homogenized and the lipids were extracted (19), and the triglyceride levels were measured using a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan), according to the manufacturer's instructions. The protein concentrations were determined by the Lowry method (20) using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

In situ ROS detection

In situ liver ROS production was assessed by staining with dihydroethidium (Invitrogen Corp., Carlsbad, CA, USA), as described previously (14). Dihydroethidium is oxidized to ethidium bromide in the presence of ROS, which stains the nuclei bright red via DNA intercalation (21). The intensity of the fluorescence was quantified using the NIH Image analysis program in three randomly selected areas of the digital images for each mouse.

Derivatives of reactive oxygen metabolites and biological antioxidant potential levels

The derivatives of reactive oxygen metabolites (dROM) and biological antioxidant potential (BAP) levels were measured using a Free Radical Elective Evaluator (Wismerll Co. Ltd, Tokyo, Japan) (22, 23). The dROM measurements were determined based on the ability of transition metals to catalyse the formation of coloured free radicals (detection at 505 nm). The results were expressed in Cartelli units (U.CARR), where 1 U.CARR = 0.8 mg/L of H_2O_2. To obtain the BAP measurements, the blood samples were added to a solution containing $FeCl_3$ bound to a chromogenic substrate (AT, a derivative of thiocyanate). Fe^{3+} reduction to Fe^{2+} caused a chromatic change that was directly proportional to the plasma ROS reduction, which was measured at 505 nm using a photometer. Blood aliquots (10 μl) were mixed with the $FeCl_3$ solution and incubated for 5 min at 37°C before the photometric analysis.

Histological staining

Part of each liver sample was snap-frozen immediately in liquid nitrogen to determine the hepatic triglycerides

and iron concentration. The remaining liver tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin for use in the histological analysis. The liver sections were stained with haematoxylin and eosin.

Real time reverse transcriptase PCR

One-step real-time real-time reverse transcriptase-PCR (RT-PCR) was performed, as described previously (14), where the results were expressed as the hepcidin, interleukin 6 (IL6), BMP6 and superoxide dismutase 2 (SOD2) gene mRNA levels relative to β-actin mRNA.

Extraction of nuclear and histone deacetylase activity assay

For isolation of nuclear proteins from mice liver, Nuclear Extraction Kit 1 (Epigentek, Farmingdale, NY, USA) was used. Histone deacetylase (HDAC) activity was assessed using HDAC Activity/Inhibition Direct Assay Kit (Epigentek) according to the manufacturer's instruction.

Isolation of mitochondria and complex I activity determination

Liver mitochondria were isolated and the activity of complex I was assayed (at 25°C) as described previously (3, 24).

Protein extraction and Western blotting

The liver lysate and mitochondrial lysate proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. These proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocked overnight at 4°C with 1–3% skim milk and 0.1% Tween 20 in Tris-buffered saline, which was followed by incubation at room temperature for 1 h with a primary antibody. Anti-rabbit carnitine palmitoyl transferase I (CPT I), anti-rabbit CPT II (Alpha Diagnostic International, San Antonio, TX, USA), anti-rabbit SREBP1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or anti-bacterially expressed mouse CCAAT/enhancer-binding protein homology protein (CHOP) fusion protein (Abcam, Cambridge, England) were used for the liver lysate proteins. Anti-SOD2 (Abcam), anti-Grp75 (mitochondrial heat shock protein70; Abcam), or anti-NDUFB8 (mitochondrial complex I) antibody (Abcam) were used for the mitochondrial lysates. The proteins were blocked for 1 h at room temperature and then incubated overnight at 4°C with a Phospho-stat3 (pSTAT3) antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and a Phospho-Smad1/Smad5/Smad8 (pSMAD1/5/8) antibody (Cell Signaling Technology Inc.).The anti-acetyl-histoneH3K9 and anti-histoneH3 (Cell Signaling Technology Inc,) were used for the nuclear lysates.

Human BCAA supplementation study design

We screened 68 HCV RNA-positive patients who were aged >65 years (Fig. S1). We enrolled 25 patients with HCV-related advanced fibrosis who satisfied the following criteria: serum albumin = 3.5–4.2 g/dl; platelet counts $<15 \times 10^4/\mu l$; amino acid imbalance [based on the ratio of BCAA relative to tyrosine (BTR) <4.40, which was lower than the normal limits]; and no HCC or symptoms of chronic liver failure such as ascites, varices or hepatic encephalopathy. Advanced fibrosis defined liver specimens (METAVIR fibrosis staging: >F3,4) or Fib-4 index (>3.25). The patients were assigned randomly to receive BCAA supplementation (BCAA group; $n = 12$) or follow-up without treatment (non-BCAA group; $n = 13$). BCAA group were given a 4 g BCAA preparation (LIVACT Granules; Ajinomoto, Tokyo, Japan) administered orally three times daily after meals. We measured the plasma oxidized/reduced albumin and serum dROM and BAP as oxidative stress markers at 12, 24 and 48 weeks after starting the treatment. We also measured the levels of serum iron, ferritin, transferrin saturation (TSAT) and hepcidin-25 to evaluate the oxidative stress-associated iron metabolism. Moreover, type IV collagen 7s, type III procollagen peptide (PIIP) and Fib-4 index were measured to confirm the degree of hepatic fibrosis.

Written informed consent was obtained from each study participant. This study was conducted in accordance with the provisions of the 1975 Declaration of Helsinki and it was approved by the Institutional Ethics Committee of Kawasaki Medical School.

Statistical analysis

The results were expressed as mean ± SD. The group results were compared using Levene's or Welch's tests. The changes in the levels of the iron metabolism and oxidative stress markers between the BCAA and the non-BCAA groups were analysed using Wilcoxon rank-sum tests. Pearson's product moment correlation coefficient was used to assess associations between the dihydroethidium-positive areas and the BAP and dROM ratios. Differences were considered statistically significant at $P < 0.05$. The statistical analyses were performed using SPSS software (IBM SPSS Statistics 20.0 for Windows).

Results

AST, fasting blood sugar, plasma BCAA and tyrosine levels in HCVTgM

The dietary intake and body weight did not differ significantly between the three groups of mice. BCAA administration for 6 months significantly reduced the serum

AST ($P < 0.05$) and fasting blood sugar (FBS) levels ($P < 0.05$) compared with HCV TgM fed the excess-iron diet with casein (casein/iron group) (Table 1). However, the FBS levels remained higher in the HCVTgM fed the excess-iron diet with BCAA (BCAA/iron group) compared with the HCV TgM fed a normal rodent diet (control group) ($P < 0.05$). The casein/iron group had significantly lower plasma BCAA and the ratio of BCAA relative to tyrosine (BTR) levels ($P < 0.05$) compared with the BCAA/iron and control groups (Table 1). The tyrosine levels were significantly higher in the casein/iron group than the control group ($P < 0.05$).

Hepatic iron contents and hepcidin 25 levels in HCVTgM

The hepatic iron contents of HCVTgM fed the excess-iron diet with casein were significantly higher than those of HCVTgM fed an excess-iron diet with BCAA or a control diet at 6 months after the treatment commenced (Fig. 1A). The hepcidin levels of HCVTgM fed the excess-iron diet with BCAA were significantly higher than those of HCVTgM fed the excess-iron diet with casein or the control diet (Fig. 1A). The serum hepcidin to ferritin ratio was lower in patients with HCV (25). The serum hepcidin-25 to hepatic iron ratio was significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein or the control diet.

ROS generation

BCAA administration resulted in significantly lower dROM levels and an increased BAP to dROM ratio (BAP/dROM) compared with casein administration ($P < 0.05$; Fig. 1B). Hepatic ROS production, which was determined by dihydroethidium staining, was significantly higher in HCVTgM fed the excess-iron diet with casein compared with those fed the excess-iron diet with BCAA or the control diet (Fig. 1C). The BAP/dROM ratios were negatively correlated with hepatic ROS production in all mice ($r = 0.8985$; $n = 15$; $P < 0.01$; Fig. 1D).

Table 1. Effects of casein/iron and branched chain amino acids (BCAA)/iron diets on the liver to body weight ratios and blood chemistry results in hepatitis C virus transgenic mice

	Control	Casein/iron	BCAA/iron
Mice (*N*)	6	7	5
Liver weight/ Body weight (%)	3.32 ± 0.30	3.50 ± 0.60	2.97 ± 0.28
AST (IU/L)	61 ± 23	92 ± 47	39 ± 7‡
ALT (IU/L)	14 ± 4	61 ± 60	16 ± 4
FBS (mg/dl)	115 ± 11	299 ± 49†	184 ± 47†‡
Insulin (ng/ml)	0.89 ± 0.36	1.19 ± 0.20	0.93 ± 0.39
Albumin (g/dl)	2.82 ± 0.04	2.77 ± 0.15	2.96 ± 0.15
BCAA (nmol/ml)	313 ± 22	275 ± 31†	318 ± 35‡
Tyrosine (nmol/ml)	63 ± 5	82 ± 11†	69 ± 11
BTR	5.01 ± 0.20	3.41 ± 0.40†	4.67 ± 0.40‡

*Results are mean ± SD.

†$P < 0.05$ vs. transgenic mice expressing hepatitis C virus polyprotein (HCVTgM) on control diet for 6 months.

‡$P < 0.05$ vs. HCVTgM on excess iron diet with casein for 6 months.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBS, fasting blood sugar.

Factors that affected hepcidin upregulation

HCV-induced ROS production downregulates hepcidin transcription by inhibiting the C/EBPα DNA-binding activity of CHOP (14). Thus, we examined CHOP expression and the hepcidin mRNA levels. Hepatic CHOP expression was significantly lower and hepatic hepcidin expression was significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with the levels in HCVTgM fed the excess-iron diet plus casein (Fig. 2A,B). The IL-6-gp130/signal transducer and activator of transcription are involved in the regulation of hepcidin transcription (26). Another pathway that regulates hepcidin expression involves the TGF-β/bone morphogenetic protein superfamily (27, 28). Thus, we examined the STAT-IL6 and SMAD-BMP signalling pathways. There were no differences in the phosphate STAT3, IL6, phosphor- SMAD1/5/8 and BMP6 expression levels between the BCAA and casein groups (Fig. 2C). In addition, HCV-induced oxidative stress inhibited hepcidin expression through increased histone deacetylase (HDAC) activity in cell culture system (29). HDAC activity of HCV TgM fed the excess-iron diet with BCAA was significantly lower than those of HCVTgM fed the excess-iron diet with casein or the control diet (Fig. S2). These results suggested that BCAA induced the upregulation of hepatic hepcidin by enhancing the antioxidant potential.

Hepatic steatosis and CPT1 expression

HCVTgM fed the excess-iron diet developed severe steatosis, including the centrilobular microvesicular type (15, 17). Previous studies showed that the antioxidant drugs N-acetylcysteine (NAC) and Stronger Neo-Minophagen C (SNMC) reduce the hepatic triglyceride levels in a dose-dependent manner (30, 31). In the present study, BCAA administration tended to reduce the hepatic triglyceride levels ($P = 0.055$; Fig. 3A).

Thus, we examined the effects of BCAA on CPT1 and CPT2, which are proteins that regulate long-chain fatty acid oxidation in mitochondria, and SREBP1 expression, which is a transcription factor that activates genes required for lipogenesis. Our previous study indicated that decreased CPT1 and increased SREBP1 expression contribute to the development of hepatic steatosis in HCVTgM fed an excess-iron diet (30). In the present study, CPT1 expression increased significantly in HCVTgM fed the excess-iron diet with BCAA after 6 months ($P < 0.05$, Fig. 3C), whereas CPT2 expression

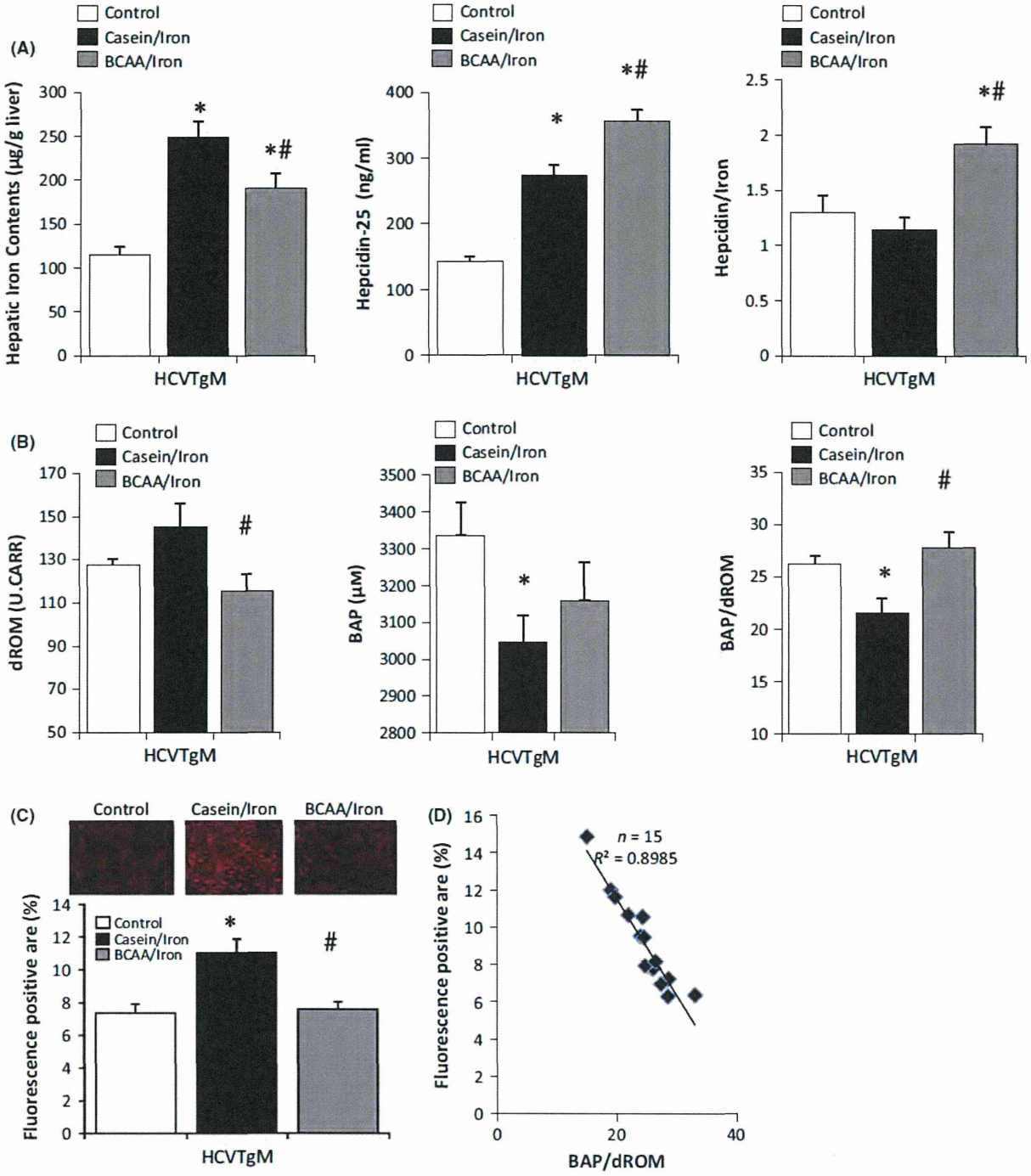

Fig. 1. (A) Hepatic iron contents, hepcidin 25 levels, and hepcidin 25 to iron content ratios (hepcidin/iron). (Left) Hepatic iron contents in mice at 6 months after starting treatment for the control (n 6), casein/iron (n 7) and BCAA/iron groups (n 5). (Centre) Serum hepci din 25 levels. (Right) The hepcidin/iron ratios were used as an index of the sensitivity of hepcidin upregulation against iron overload. (B) Oxi dative stress markers in serum. (Left) dROM and (centre) BAP were measured at 6 months after starting treatment. (Right) The antioxidant status was determined as the BAP to dROM ratio. (C) Dihydroethidium fluorescence intensity was quantified for three randomly selected areas in digital images for the control (n 3), casein/iron (n 7), and BCAA/iron groups (n 5) at 6 months after starting treatment. (D) Correlations between the BAP/dROM ratios and fluorescence positive areas in liver. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs casein/iron group.

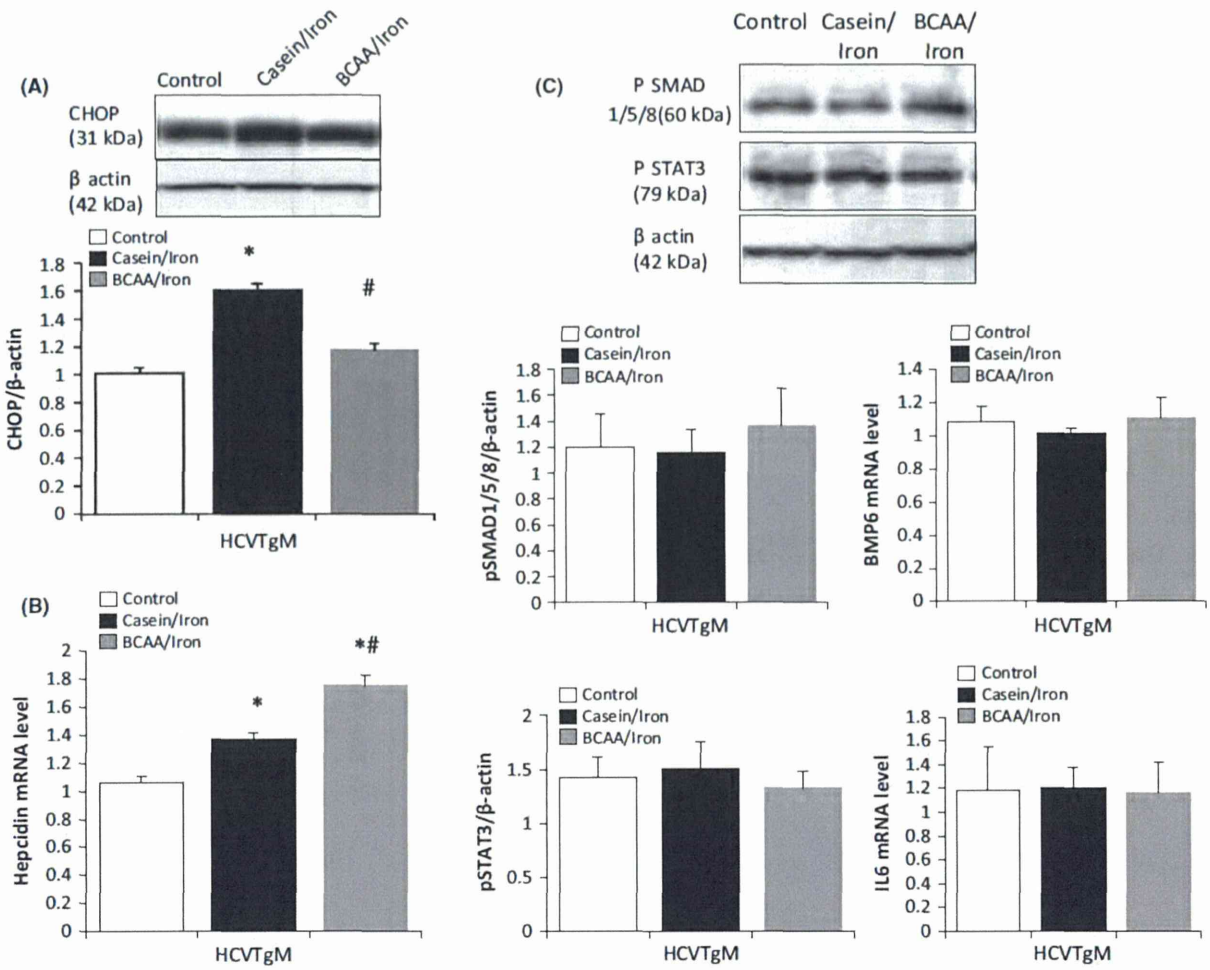

Fig. 2. (A) Immunoblots for CHOP after 6 months of treatment. (B) Liver hepcidin expression was determined for four mice in each group (C) Immunoblots for p SMAD1/5/8 and (C) p STAT3 after 6 months of treatment. (C) BMP6and (C) IL6 mRNA expression was determined for four mice in each group. The protein expression levels were normalized against that of β actin. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs casein/iron group.

did not increase significantly. However, SREBP1 expression did not decrease in HCVTgM fed the excess-iron diet with BCAA ($P = 0.082$; Fig. 3B). These results suggest that the administration of BCAA was insufficient to prevent iron-induced steatosis in HCVTgM because BCAA failed to reduce SREBP1 expression.

SOD2 expression and mitochondrial complex I activity

CPT1 is localized to the mitochondrial outer membrane. Decreased CPT1 expression may be related to the HCV core protein's association with the mitochondrial outer membrane. The HCV core protein interacts with mitochondria complex I, which generates ROS (13). Alterations in the mitochondrial ultrastructure were observed in HCVTgM fed the excess-iron diet after 6 months, as described previously (15, 30). We examined whether BCAA supplementation reduced iron- and HCV-induced mitochondrial injury.

The mitochondrial SOD2 mRNA levels were significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein or the control diet. The SOD2 expression levels in mice fed the excess-iron diet with casein were significantly lower than those fed the control diet. However, the SOD2 expression levels were restored by BCAA supplementation (Fig. 4A). After 6 months, the mitochondrial complex I expression levels were significantly lower in mice fed the excess-iron diet with casein compared with those fed the control diet. Similar to SOD2, the mitochondrial complex I expression levels were restored by BCAA supplementation (Fig. 4B).

The enzymatic activity of mitochondrial complex I was significantly lower in mice fed the excess-iron diet

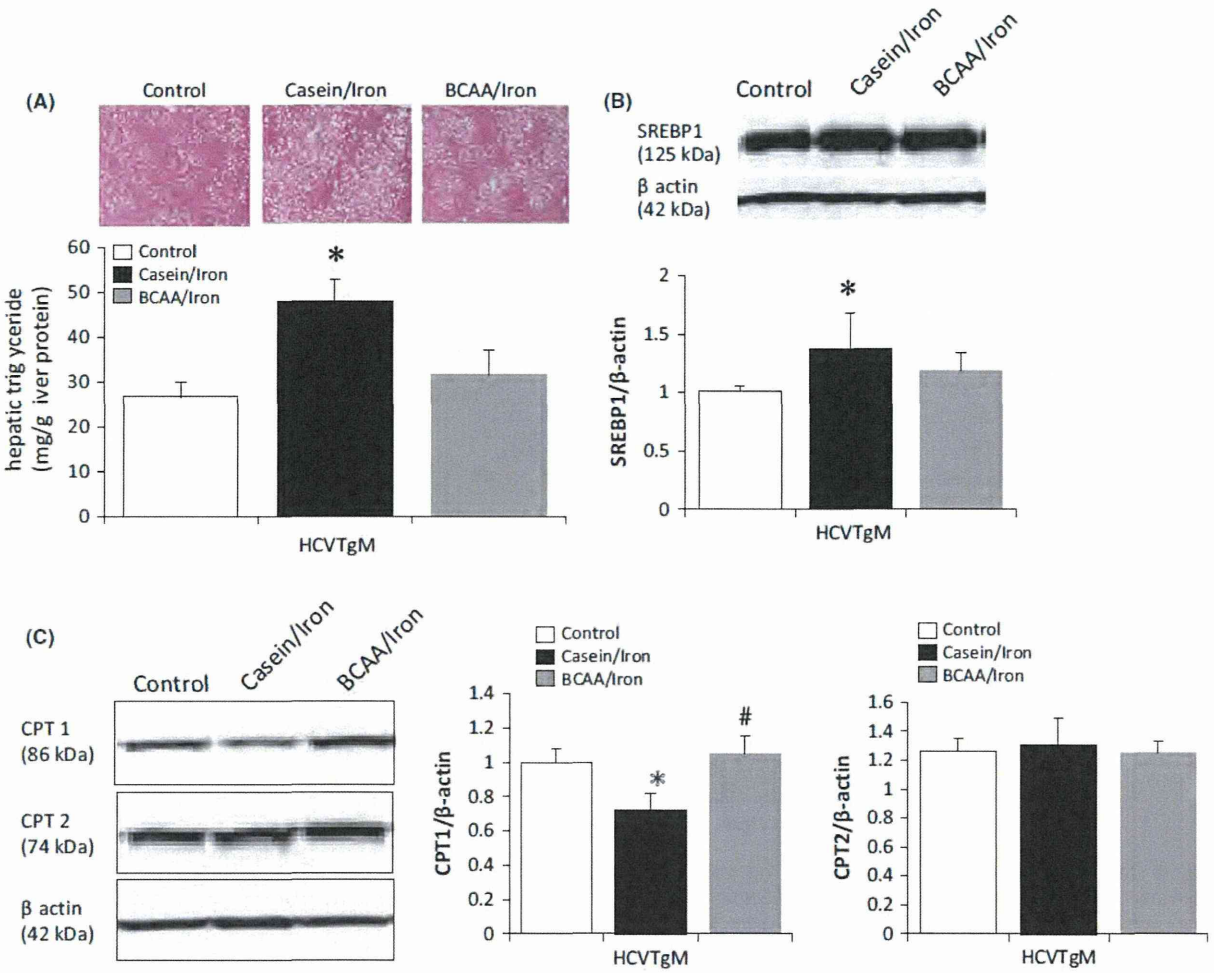

Fig. 3. (A) Hepatic steatosis in HCVTgM fed the excess iron diet with BCAA and HCVTgM fed the excess iron diet with casein after treat ment for 6 months (haematoxylin and eosin, original magnification × 100). The hepatic triglyceride levels were determined. (B) Immunoblots of SREBP1, (C) CPT1, and (C) CPT2 in the livers of three mice from each group. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs casein/iron group.

with casein compared with those fed the control diet. The activity was restored by BCAA supplementation (Fig. 4C). Thus, these improvements in the mitochondrial complex I activity and CPT1, SOD2, and mitochondrial complex I expression indicate that BCAA may protect against the mitochondrial injury induced by HCV proteins and iron overload.

Antioxidant effects of BCAA supplementation in patients with HCV related severe fibrosis

Next, we determined whether oral BCAA supplementation reduced oxidative stress and affected iron metabolism in patients with HCV-related advanced liver fibrosis. We assigned 25 patients to receive either BCAA supplementation (BCAA group; $n = 12$) or follow-up without treatment (non-BCAA group; $n = 13$). There were no differences in the clinical characteristics, oxidative stress markers, or iron metabolic markers at baseline between these groups (Table 2). Serum albumin and AST levels in BCAA group tended to be lower than those in non-BCAA group, although these differences were not statistically significant ($P = 0.071$ and $P = 0.074$ respectively).

The dROM levels increased significantly at weeks 24 and 48 in the non-BCAA group, whereas they did not in the BCAA group. The BAP levels also increased at weeks 12 and 24 in the non-BCAA group, and at weeks 12, 24 and 48 in the BCAA group (Table 3). The BAP/dROM ratio, an indicator of antioxidant potential, decreased significantly at week 48 in the non-BCAA group, but increased at weeks 24 and 48 in the BCAA group. This suggests that the BAP levels of the non-BCAA group increased in response to oxidative stress, while the increased BAP levels in the BCAA group indicated enhanced antioxidant potential.

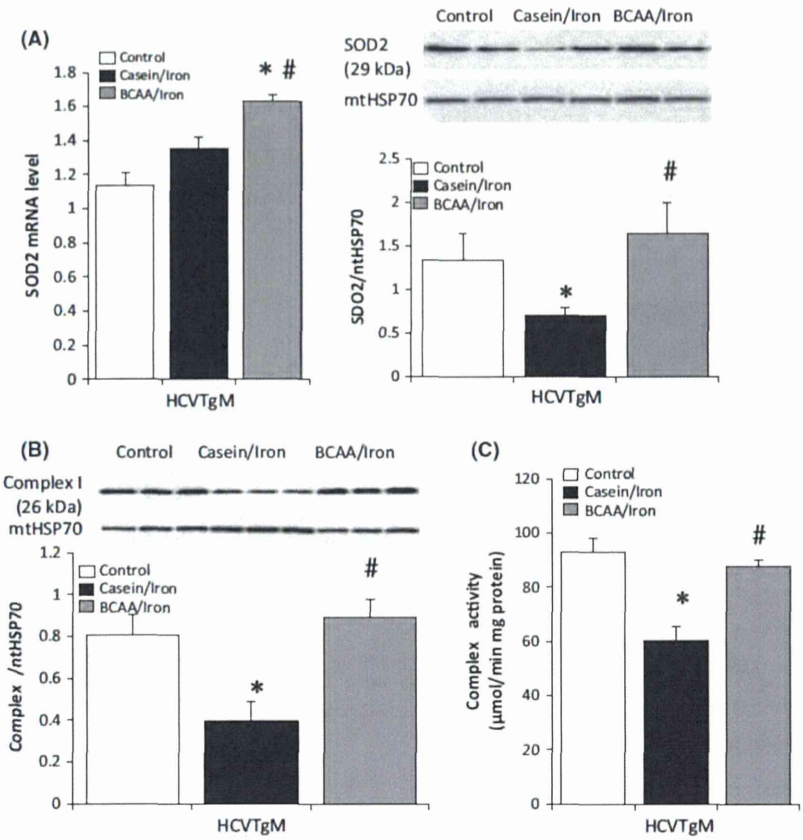

Fig. 4. (A) SOD2 mRNA expression in the livers of four mice from each group. (B) Immunoblots of SOD2 and mitochondrial complex I in the mitochondria of four mice from each group after treatment for 6 months. The protein expression levels were normalized against that of mitochondrial heat shock protein 70. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs casein/iron group.

In agreement with the antioxidant status, the serum ferritin levels were significantly lower after week 48 of BCAA supplementation (137 ± 109 mg/dl; $P < 0.05$) compared with those before treatment (Table 3). BCAA supplementation significantly increased the serum hepcidin-25 levels at week 48 (20.2 ± 14.5 mg/dl; $P < 0.05$). In addition, we determined the level of albumin synthesis after BCAA supplementation, because the oxidized albumin to total albumin ratio increases with cirrhosis progression and it is related to oxidative stress (32,33). In the present study, there were no differences in the total albumin changes in the non-BCAA or BCAA groups (Table 4). However, the amount of albumin present in the reduced form increased significantly in the BCAA group at week 48 compared with that before the study. By contrast, the level of reduced albumin decreased significantly at week 48 in the non-BCAA group. This suggests that long-term BCAA supplementation reduced iron overload by upregulating antioxidant potential and this improved the albumin status in patients without hypoalbuminaemia and chronic liver failure.

Discussion

Hepatic iron overload and ROS production are both pathophysiological features of HCV-associated chronic liver disease (34) and risk factors for HCC development (35). The reduced hepatic oxidative stress observed after oral BCAA supplementation may be related to changes in the albumin redox state (32, 36). However, previous studies did not determine how BCAA affects iron metabolism and ROS generation.

The mouse model used in the present study shared similarities with the patients who had HCV-associated chronic liver disease in terms of hepatic ROS production and steatosis (14) at 6 months after treatment, followed by hepatocarcinogenesis (15). Furthermore, the hepatic iron concentrations in HCVTgM fed the excess-iron diet were comparable to those of a large number of patients with chronic hepatitis C (30, 37, 38). Thus, HCVTgM fed the excess-iron diet is a suitable model for assessing the effects of long-term supplementation with BCAA on disordered iron metabolism and ROS production in HCV infection.

Table 2. Patient baseline characteristics

	non BCAA	BCAA	*P* value
Patients (*N*)	13	12	N.S.
Age (years)	73.5 (65 87)	74.9 (65 83)	N.S.
Sex (male/female)	6/7	6/6	N.S.
White blood cell count ($\times 10^2/mm^3$)	46.2 (30.1 63.4)	45.1 (27.1 84.5)	N.S.
Haemoglobin concentration (g/dl)	13.3 (10.6 16.3)	13.2 (11.4 15.7)	N.S.
Platelet counts ($\times 10^4/mm^3$)	12.2 (4.9 15)	10.5 (3.7 15)	N.S.
Total bilirubin (mg/dl)	0.7 (0.5 1.1)	1.0 (0.3 1.7)	N.S.
Albumin (g/dl)	4.0 (3.5 4.2)	3.9 (3.5 4.2)	N.S.
ALT (IU/L)	33 (23 47)	41 (21 55)	N.S.
AST (IU/L)	43 (32 54)	48 (32 54)	N.S.
ALP (IU/L)	286 (158 435)	302 (145 491)	N.S.
GTP (IU/L)	46 (15 177)	53 (16 137)	N.S.
FBS (mg/dl)	94 (70 130)	107 (72 158)	N.S.
Insulin (μIU/ml)	14 (5.3 39)	14 (6.3 28)	N.S.
Tyrosine (nmol/ml)	104 (76 123)	103 (63 149)	N.S.
BCAA (nmol/ml)	424 (319 606)	401 (269 617)	N.S.
BTR	4.1 (2.6 4.9)	4.0 (2.7 4.4)	N.S.
AFP (ng/dl)	11 (2 61)	18 (2 95)	N.S.
Serum iron (μg/ml)	134 (50 255)	136 (37 256)	N.S.
TSAT (%)	38 (11 70)	44 (12 88)	N.S.
Ferritin (ng/ml)	120 (30 429)	190 (30 346)	N.S.

Results are mean (range). Comparisons between branched chain amino acids (BCAA) and non BCAA groups were made using Levene's or Welch's tests. AFP, α foetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; BTR, the ratio of BCAA relative to tyrosine; FBS, fasting blood sugar; N.S., Not significant; TSAT, transferrin saturation.

BCAA supplementation improves the nutritional status, prognosis and quality of life for patients with cirrhosis (39, 40). A randomized, controlled trial demonstrated that BCAA supplementation reduced the frequency of HCC in obese male patients with cirrhosis and HCV infection (18). BCAA treatment also reduced the hepatocarcinogenic activity in obese diabetic animals with insulin resistance (9, 10). Insulin resistance promotes hepatocarcinogenesis by activating the mitogen-activated protein kinase (MAPK) pathway and insulin-like growth factor 1 (IGF-1) receptors, which further activates the Raf/MAPK kinase/MAPK cascade (41, 42). BCAA suppress the IGF/IGF-1R axis by down-regulating IGF-1, IGF-2 and IGF-1R mRNA expression, thereby leading to the inhibition of mitosis and cell growth (9). BCAA reduce HCC development by inhibiting insulin resistance (43).

In the present study, the FBS levels of HCVTgM fed the excess-iron diet with casein increased after 6 months. BCAA supplementation reduced the iron overload-induced elevation of the FBS. There was no intrahepatic inflation or fibrosis in the HCVTgM fed the excess-iron diet, but those fed the excess-iron diet with casein had significantly lower plasma BCAA levels and a lower BTR compared with those fed excess-iron with BCAA and the control diet. An amino acid imbalance, which is indicated by a lower BTR, has been observed in patients with compensated cirrhosis or chronic hepatitis (44, 45). This suggests that BCAA might potentially reduce hepatic iron accumulation and ROS in patients with HCV-related advanced fibrosis.

Table 3. Changes in oxidative stress and iron metabolism markers during branched chain amino acids (BCAA) administration*

	Week 0	Week 12	Week 24	Week 48
Hepcidin (ng/ml)				
non BCAA	11.6 ± 7.9	10.4 ± 9.8	11.8 ± 9.5	10.5 ± 8.8
BCAA	9.5 ± 8.7	9.2 ± 9.5	11.0 ± 9.1	20.2 ± 14.5†
Ferritin (ng/ml)				
non BCAA	120 ± 121	112 ± 105	100 ± 108	118 ± 120
BCAA	190 ± 135	164 ± 129	163 ± 130	137 ± 109†
Serum iron (μg/ml)				
non BCAA	134 ± 52	143 ± 60	133 ± 55	142 ± 38
BCAA	136 ± 64	131 ± 63	134 ± 68	117 ± 57
TSAT (%)				
non BCAA	38 ± 14	42 ± 19	39 ± 15	42 ± 19
BCAA dROM (U.CARR)	45 ± 29	42 ± 24	35 ± 19†	33 ± 16†
non BCAA	342 ± 64	405 ± 84	431 ± 76†	455 ± 96†
BCAA	360 ± 113	372 ± 80	361 ± 118	359 ± 65
BAP (uM)				
non BCAA	2369 ± 386	2772 ± 487†	2798 ± 337†	2630 ± 64
BCAA	2139 ± 587	2516 ± 678†	2601 ± 647†	2758 ± 413†
BAP/dROM				
non BCAA	7.0 ± 1.0	7.1 ± 1.7	6.6 ± 0.7	6.0 ± 1.0†
BCAA	6.1 ± 1.3	6.8 ± 1.5	7.5 ± 1.6†	7.8 ± 1.5†

*Results are mean ± SD.

†$P < 0.05$ vs. before BCAA treatment, Wilcoxon rank sum test; U.CARR, Cartelli Units (1 U.CARR 0.8 mg/L of H_2O_2), TSAT, transferrin saturation.

Table 4. Changes in the serum albumin characteristics during branched chain amino acid (BCAA) administration*

	Week 0	Week 12	Week 24	Week 48
Albumin (g/dl)				
non BCAA (13)	4.0 ± 0.2	4.0 ± 0.2	4.0 ± 0.2	4.0 ± 0.2
BCAA (12)	3.9 ± 0.3	3.8 ± 0.4	3.9 ± 0.3	4.0 ± 0.3
Reduced albumin (%)				
non BCAA (10)	66 ± 4.5	66 ± 5.3	66 ± 3.9	63 ± 4.9†
BCAA (10)	66 ± 3.9	68 ± 2.8	68 ± 5.2	70 ± 3.2†
Type IV collagen 7s (U/ml)				
non BCAA (13)	5.8 ± 1.7	6.1 ± 2.5	6.0 ± 2.2	6.1 ± 2.1
BCAA (12)	6.8 ± 2.1	7.1 ± 1.8	7.0 ± 2.1	6.7 ± 2.0
P III P (U/ml)				
non BCAA (13)	0.89 ± 0.19	0.83 ± 0.19	0.91 ± 0.24	0.83 ± 0.15
BCAA (12)	0.88 ± 0.23	0.90 ± 0.16	0.88 ± 0.18	0.86 ± 0.22
Fib4 index				
non BCAA (13)	5.0 ± 2.7	5.0 ± 2.5	5.4 ± 2.9	5.5 ± 3.7
BCAA (12)	6.8 ± 4.2	7.2 ± 3.7	6.4 ± 4.0	6.3 ± 4.0

*Results are mean ± SD.

†$P < 0.05$ vs. before treatment, Wilcoxon rank sum test, P III P: Type III procollagen peptide.

Our previous study indicated that the antioxidant N-acetylcysteine (NAC) almost completely blocked ROS production and abrogated the hepatic steatosis induced by HCV proteins and iron (30). In the present study, the hepatic triglyceride levels tended to be lower in mice fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein, although these differences were not statistically significant ($P = 0.055$). This may have been because BCAA reduce ROS production to a lesser degree than NAC, or because BCAA supplementation did not completely inhibit the ROS-associated unfolded protein response or improve glucose intolerance compared with the control diet. SREBP1 expression is positively regulated by insulin signalling pathways (46). Therefore, further studies are needed to determine whether BCAA reduce hepatic iron accumulation without affecting hepatic steatosis.

CPT1, a transmembrane enzyme in the mitochondrial outer membrane, is negatively regulated at the transcriptional level by malonyl-CoA, which is an intermediate product of fatty acid synthesis (47). Decreased CPT1 expression may be related to the HCV core protein, which is also located in the mitochondrial outer membrane and it generates mitochondrial ROS production indirectly (13). BCAA enhanced protection against mitochondrial injury by restoring the mitochondrial antioxidant potential and mitochondrial complex I activity. Thus, how does BCAA protect from HCV-induced ROS production and mitochondrial injury? The hepatic ROS production increased more in the HCVTgM fed the control diet compared with non-transgenic mice (14), but we did not test whether BCAA supplements reduced ROS production in HCVTgM without the excess-iron diet. However, there were no differences in the liver enzyme, glucose, insulin, BCAA and tyrosine levels of HCVTgM and non-transgenic mice. Furthermore, HCVTgM without the excess-iron diet did not develop severe steatosis and HCC. This indicates that HCVTgM without the excess-iron diet are not a suitable model for long-term treatments with BCAA.

BCAA supplementation increases the reduced form of albumin, which is a predictor of the cirrhosis prognosis (32, 33), while it also improves oxidative stress and iron metabolism in patients with decompensated cirrhosis (36), and in rats exposed to a fibrogenic agent (48). This suggests that the antioxidant effects of BCAA may be related to qualitative changes in serum albumin or the upregulation of albumin synthesis (4, 5, 9, 49). BCAA itself activates the mammalian target of rapamycin, which subsequently upregulates the downstream molecules, eukaryotic initiation factor 4E-binding protein-1 and 70-kDa ribosomal protein S6 kinase, thereby regulating mRNA translation and synthesis (50). In the present clinical study, we confirmed that long-term BCAA supplementation increased the BAP/dROM ratios and serum hepcidin-25 levels, whereas it decreased the serum ferritin levels in patients with HCV-related advanced fibrosis. Moreover, we found that BAP continued to increase from week 12 to week 48, and the level of the reduced albumin form increased at week 48, but without changes in the serum albumin levels, in our BCAA group. The mechanism that allows BCAA to protect against HCV and iron-induced oxidative stress remains uncertain, but BCAA may improve iron metabolism by upregulating the antioxidant potential in patients without decompensated cirrhosis.

In addition, amino acid imbalance is a risk factor for HCC development in patients without hypoalbuminaemia (40), which suggests that BCAA supplementation should be recommended to patients with amino acid imbalances with advanced fibrosis, who may have a decreased antioxidant potential and reduced albumin level. In this study, we could not show any effect by

which BCAA supplementation prevented fibrotic progression (Table 4). However, Fib-4 index in BCAA group at 48 weeks tended to be decreased compared with those at initial point, although these differences were not statistically significant ($P = 0.061$). Long-term BCAAs treatment might inhibit hepatic fibrosis in HCV patients with advanced fibrosis.

Our clinical study had some limitations, including a higher number of older patients who had higher serum albumin and ferritin levels than those in the cohorts reported in other studies, although they used small sample sizes and were not randomized. Further studies should use large cohorts to clarify these effects.

In conclusion, we demonstrated that BCAA administration reduced the hepatic iron contents and ROS levels, which were induced by HCV proteins and iron overloading in mice, probably by protecting the function of mitochondrial complex I. Furthermore, we confirmed that BCAA supplementation improved disordered iron metabolism and the antioxidant status in patients with HCV-related advanced fibrosis. These effects of BCAA may partially account for their inhibitory effects on HCC development in patients with cirrhosis.

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Conflict of interest: The authors do not have no any disclosures to report.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Twenty five patients with HCV-related advanced fibrosis enrolled Human BCAA supplementation study. Advanced fibrosis defined liver specimens (METAVIR fibrosis staging: >F3,4) or Fib-4 index (>3.25).

Fig. S2. HDAC activity of HCV TgM fed the excess-iron diet with BCAA was significantly lower than those of HCVTgM fed the excess-iron diet with casein or the control diet. All samples were nuclear which was extracted from liver tissue.

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Elevated Serum Levels of *Wisteria floribunda* Agglutinin-Positive Human Mac-2 Binding Protein Predict the Development of Hepatocellular Carcinoma in Hepatitis C Patients

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The *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was recently shown to be a liver fibrosis glycobiomarker with a unique fibrosis-related glycoalteration. We evaluated the ability of WFA^+-M2BP to predict the development of hepatocellular carcinoma (HCC) in patients who were infected with the hepatitis C virus (HCV). A total of 707 patients who had been admitted to our hospital with chronic HCV infection without other potential risk factors were evaluated to determine the ability of WFA^+-M2BP to predict the development of HCC; factors evaluated included age, sex, viral load, genotypes, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, alpha-fetoprotein (AFP), WFA^+-M2BP, and the response to interferon (IFN) therapy. Serum WFA^+-M2BP levels were significantly increased according to the progression of liver fibrosis stage ($P<0.001$). In each distinctive stage of fibrosis (F0-F1, F2, F3, and F4), the risk of development of HCC was increased according to the elevation of WFA^+-M2BP. Multivariate analysis identified age >57 years, F4, AFP >20 ng/mL, WFA^+-M2BP $\geq$4, and WFA^+-M2BP 1-4 as well as the response to IFN (no therapy vs. sustained virological response) as independent risk factors for the development of HCC. The time-dependent areas under the receiver operating characteristic curve demonstrated that the WFA^+-M2BP assay predicted the development of HCC with higher diagnostic accuracy than AFP. *Conclusion:* WFA^+-M2BP can be applied as a useful surrogate marker for the risk of HCC development, in addition to liver biopsy. (HEPATOLOGY 2014;60:1563-1570)

The annual incidence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-related cirrhosis ranges from 1% to 7%.[1,2] Therefore, reliable methods for the early identification of liver fibrosis progression and compensated liver cirrhosis are an essential part of an efficient surveillance program for the detection of HCC.[3]

Until recently, liver biopsy was considered the gold standard for assessing the severity of liver fibrosis and cirrhosis.[4,5] Although liver biopsy is generally accepted

Abbreviations: Ab, antibody; AFP, alpha-fetoprotein; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUROC, area under the ROC; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MRI, magnetic resonance imaging; Peg-IFN, pegylated IFN; RBV, ribavirin; ROC, receiver operating characteristic; RT-PCR, reverse-transcriptase polymerase chain reaction; SVR, sustained virological response; US, ultrasound; WFA^+-M2BP, Wisteria floribunda *agglutinin-positive human Mac-2-binding protein.*

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to be a safe procedure, it can cause discomfort and carries a small risk of life-threatening complications.[6,7] Recently, an assay for *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was reported as a novel, noninvasive, and rapid bedside method to assess liver fibrosis.[8] M2BP has been shown to have multibranching and sialylated N-glycans. WFA is considered to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure. Currently, we are analyzing the glycan structures of WFA^+-M2BP in detail using mass spectrometry–based technology.[9] Glycans can reflect the differentiation stage of cells, but not necessarily the level of cellular damage, and therefore they can be very effective markers for chronic disease. In the case of hepatitis, glycans are considered to reflect the progression of fibrosis more specifically than viral load. Several reports have identified M2BP as a potential marker of fibrosis progression in proteome study.[10-13] Kuno et al. were the first to report that a rapid, simple glycan-based immunoassay for WFA^+-M2BP can quantify fibrosis.[8,14]

On the other hand, we reported that alpha-fetoprotein (AFP) is a noninvasive predictive marker for the development of HCC in patients infected with HCV, which can be used to complement the information of fibrosis stage.[15]

In this report, we evaluated the utility of WFA^+-M2BP to predict the development of HCC in patients who were infected with HCV.

Patients and Methods

Patients. Between January 1992 and December 2003, 832 patients were determined to be positive for both anti-HCV by a second- or third-generation enzyme-linked immunoadsorbent assay and HCV RNA by polymerase chain reaction (PCR). They underwent liver biopsy guided by ultrasonography at the National Hospital Organization, Nagasaki Medical Center (Omura, Japan). Among them, 125 (15.0%) patients were excluded from enrollment in this retrospective analysis for the following reasons: (1) positivity for hepatitis B surface antigen (n = 12); (2) a heavy habitual drinking habit defined by an average daily consumption of >100 g of ethanol (n = 26); (3) autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension (n = 8); (4) positive antinuclear antibody (Ab; defined as titer >320×) without the diagnosis of AIH (n = 8); or (5) a short follow-up period <180 days (n = 71). The remaining 707 patients were analyzed retrospectively for the incidence of HCC.

For all patients in our cohort, a blood sample was taken on the day of the liver biopsy at our hospital. All samples were preceded to separate serum and stored at −20°C. At the time of blood withdrawal, all patients underwent liver biopsy. Their medical histories had been recorded, along with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of ultrasound (US)-guided liver biopsy and at regular intervals thereafter. Complete blood cell counts and biochemical tests were performed using automated procedures in the clinical pathological laboratories of our hospital.

Staging of Hepatic Fibrosis. Liver biopsies were taken by fine-needle aspiration (16G or 18G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al.[16]

Measurement of WFA^+-M2BP. WFA^+-M2BP quantification was measured based on a lectin-Ab sandwich immunoassay using the fully automatic immunoanalyzer, HISCL-2000i (Sysmex Co., Hyogo, Japan).[8] The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

$$\text{Cutoff index (COI)} = \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{sample}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right) / \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{PC}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right)$$

where $[WFA^+\text{-M2BP}]_{sample}$ is the WFA^+-M2BP count of serum sample, PC is positive control, and NC is negative control. The positive control was supplied as

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a calibration solution preliminarily standardized to yield a COI value of 1.0.[14]

HCV RNA, HCV Core Antigen, and HCV Genotypes. HCV RNA was determined by reverse-transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the Lumispot Eiken HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified into low and high with a cutoff at 1,000 fmol/mL.[17] Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan).[18]

Interferon Therapy. During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (Peg)-IFN monotherapy, or combination therapy with IFN plus ribavirin (RBV) or Peg-IFN plus RBV. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of 6 months or more of treatment, whereas patients who failed to meet these criteria were judged as having non-SVR. There was no relapse of viremia after 6 months among the SVR patients.

Diagnosis of HCC. Patients were followed up by hematological and biochemical tests at an interval of 1-12 months. Diagnostic imaging by US, computed tomography (CT), and magnetic resonance imaging (MRI) were performed in most patients. HCC was diagnosed by typical vascular patterns on CT, MRI, and angiography or by fine-needle biopsy of space-occupying lesions detected in the liver.

Ethical Considerations. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the institution's human research committee.

Statistical Analysis. Continuous variables (platelet counts, albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT], AFP, HCV core antigen, and WFA^+-M2BP) were dichotomized with respect to the median value or clinically meaningful values in the multivariate analysis. To estimate the cumulative risk of developing HCC, Kaplan-Meier's method and the log-rank test were used. Cox's proportional hazards regression analysis was performed to evaluate risk factors for HCC. Regression analysis was performed to calculate Spearman's rank-correlation coefficient. Kruskal-Wallis' analysis of variance (ANOVA), followed by the Games-Howel's posthoc test, was used to assess whether there were any significant differences in terms of fibrosis stages (F0-F1, F2, F3, and F4). The diagnostic performances of WFA^+-M2BP and AFP for censored development of HCC were assessed by using time-dependent receiver operating characteristic (ROC) curves by examining the area under the ROC (AUROC).[19] Inclusion of variables was assessed using a step-wise selection method. Cochran-Armitage's test for trend was used in the categorical data analysis to assess for the presence of an association between a variable with two categories and a variable with more than three categories. A P value of 0.05 was considered statistically significant. Data analysis was performed with SPSS statistical software (version 22.0; (SPSS, Inc., Chicago, IL) and JMP 10 (SAS Institute Inc., Cary, NC).

Table 1. Demographic, Clinical, and Virological Characteristics of the 707 Patients Persistently Infected With HCV

Age, years	57.0 (19 79)
Male, N (%)	351 (49.6)
Observation period, years	8.2 ± 4.4*
IFN therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Pathological findings	
Fibrosis (N) 0 1/2/3/4	274/193/120/120
Activity (N) 0 1/2/3	199/365/143
Platelet count, $\times 10^4/mm^3$	15.6 (3.0 39.1)
Albumin, g/dL	4.2 (2.7 5.3)
Bilirubin, mg/dL	0.7 (0.1 2.5)
AST, IU/mL	53 (11 422)
ALT, IU/mL	82 (1 1,057)
AFP, ng/mL	6 (0.7 510)
HCV core antigen ≥1,000 fmol/L (%)	539 (76.2)
HCV genotype, N (%) 1b	510 (72.1)
2a/2b	195 (27.6)
Unknown	2 (0.3)
WFA^+ M2BP	1.9 (0.2 19.2)

Values are the medians with ranges in parentheses.
*Results are expressed as the mean ± standard deviation.

Results

Characteristics at Enrollment. The baseline characteristics of the 707 patients at enrollment are summarized in Table 1. Median age was 57.0 years; 120 (17.0%) patients were diagnosed histologically with liver cirrhosis (fibrosis stage F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The median value of WFA^+-M2BP was 1.9 (range, 0.2-19.2). The average follow-up period was 8.2 years.

WFA^+-M2BP Value and Fibrosis Stage. The average values (mean ± 1 standard error) for each fibrosis stage were 1.3 ± 0.1 in F0-F1 (n = 274), 2.2 ± 0.1 in F2 (n = 193), 3.3 ± 0.2 in F3 (n = 120),

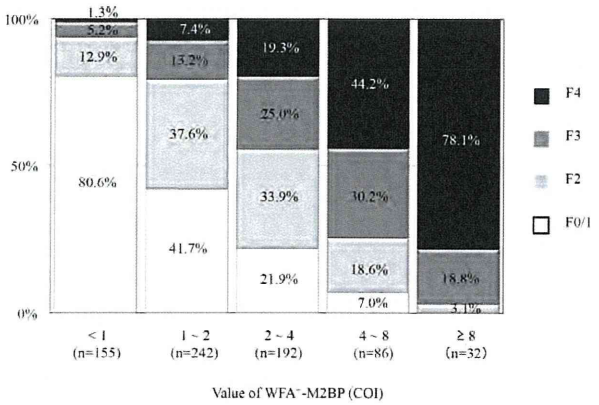

Fig. 1. Proportions of patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage. The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test), whereas that with F4 was increased ($P < 0.0001$; Cochran-Armitage's trend test).

and 5.2 ± 0.3 in F4 (n = 120). The degree of fibrosis was positively correlated with the median value of WFA$^+$-M2BP ($P < 0.001$) by a nonparametric method (Kruskal-Wallis' one-way ANOVA). Games-Howel's test confirmed that the WFA$^+$-M2BP value increased significantly with increasing stage of liver fibrosis: $P < 0.0001$ (F0-F1, compared with F2, F3, and F4); $P < 0.0001$ (F2, compared with F3 and F4); and $P < 0.0001$ (F3, compared with F4).

We estimated the diagnostic accuracy of WFA$^+$-M2BP for detecting stage F3-F4 disease. The AUROC in the prediction of ≥F3 was 0.815 (range, 0.782-0.842). The desired specificity level of 95% was achieved for a 4.0 threshold, and the sensitivity was 40.0%.

We analyzed the proportions of the patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage (Fig. 1). The proportion of patients with F1 was 125 cases (80.7%) in WFA$^+$-M2BP <1 (n = 155), 101 cases (41.7%) in WFA$^+$-M2BP ≤1 and <2 (n = 242), 42 cases (21.9%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 6 cases (7.0%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 0 cases (0.0%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test). Conversely, the proportion of patients with F4 was 2 cases (1.3%) in WFA$^+$-M2BP <1 (n = 155), 18 cases (7.4%) in WFA$^+$-M2BP ≤1, and <2 (n = 242), 37 cases (19.3%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 38 cases (44.2%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 25 cases (78.1%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F4 was increased with increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test).

Table 2. Step-wise Multiple Linear Regression Model to Identify Significant Independent Factors Affecting Serum WFA$^+$-M2BP Level

Final Fitted Model	Adjusted R^2	Standardized Coefficient β	P Value
Fibrosis stage		0.258	<0.001
AFP		0.187	<0.001
Albumin		−0.202	<0.001
AST (1: <53 IU/L; ≥2: 53 IU/L)		0.186	<0.001
Platelet	0.501	−0.147	<0.001
Sex (1: male; 2: female)		0.111	<0.001
HCV core antigen		−0.098	<0.001
Total bilirubin		0.091	0.001
Age		0.071	0.014

Relationship Between the WFA$^+$-M2BP Value and Baseline Biochemical Markers. To determine whether the WFA$^+$-M2BP value was associated with fibrosis stage, age, gender, platelet count, albumin, bilirubin, AST, ALT, AFP, HCV core antigen, HCV genotype, or histological grading, a step-wise multiple linear regression analysis was performed. Our results showed that independent variables, except for ALT, genotype, and histological grading, remained in the final equation (Table 2), suggesting that fibrosis stage was most closely associated with serum WFA$^+$-M2BP value (coefficient β, 0.258; $P < 0.001$).

Risk Factors for HCC. Cox's regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, biochemical and virological parameters, and serum WFA$^+$-M2BP level. The following factors were identified as posing an increased risk for HCC by the univariate analysis: age; response to IFN therapy (no therapy vs. SVR; $P < 0.001$); fibrosis stage (F3 and F4 vs. F0-F1; $P < 0.001$); platelet count ($<15 \times 10^4/mm^3$ vs. $\geq 15 \times 10^4/mm^3$; $P < 0.001$); albumin (<4.2 vs. ≥4.2 g/mL; $P < 0.001$); AST (<53 vs. ≥53 IU/mL; $P < 0.001$), ALT (<82 vs. ≥82 IU/mL; $P = 0.035$), and AFP levels (≥20 and 6-20 vs. <6 ng/mL; $P < 0.001$); HCV genotype (1b vs. non-1b; $P = 0.025$); and serum WFA$^+$-M2BP level (≥4 and 1-4 vs. <1; $P < 0.001$). Multivariate analysis was performed on these factors (Table 3) and the following were identified as independent risk factors: fibrosis stage (F4); AFP (≥20 ng/mL); age (≥57 years); response to IFN therapy (no therapy vs. SVR); and WFA$^+$-M2BP (1-4 and ≥4).

Development of HCC. During the follow-up period, HCC developed in 110 (15.6%) patients. Of

Table 3. Factors Associated With Risk for HCC*

Features		HR (95% CI)	P Value
Fibrosis	F0 F1	1	
	F2	0.883 (0.411 1.897)	0.749
	F3	1.347 (0.624 2.906)	0.448
	F4	3.133 (1.536 6.390)	0.002
AFP	<6 ng/mL	1	
	6 20 ng/mL	1.710 (0.963 3.038)	0.067
	≥20 ng/mL	3.417 (1.807 6.460)	<0.001
Age	<57 years	1	
	≥57 years	2.039 (1.278 3.252)	0.003
IFN therapy	No therapy	1	
	Non SVR	0.729 (0.467 1.137)	0.163
	SVR	0.089 (0.027 0.288)	<0.001
WFA^+ M2BP	<1	1	
	1 4	5.155 (1.180 – 22.500)	0.029
	≥4	8.318 (1.784 – 38.791)	0.007

Abbreviations: HR, hazard ratio; CI, confidence interval.
*Determined by multivariate analysis.

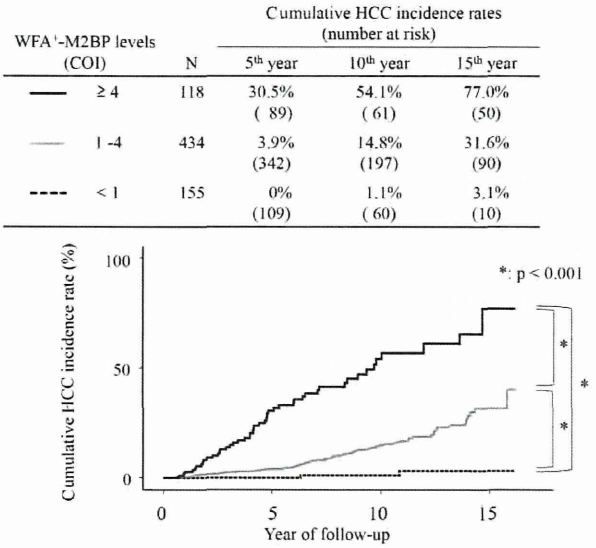

Fig. 2. Cumulative incidence of HCC according to WFA^+-M2BP level. Cumulative incidences of HCC according to the WFA^+-M2BP level were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA^+-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Figure 2 shows the relation between Kaplan-Meier's estimates of the cumulative risk of HCC and the different WFA^+-M2BP levels at entry. The 10-year cumulative risk of HCC was 1.1% in the patients with WFA^+-M2BP <1 at entry, 14.8% among the patients with WFA^+-M2BP 1-4, and 54.1% in patients with WFA^+-M2BP >4. The incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

Figure 3 shows the relation between the cumulative incidence of HCC and WFA^+-M2BP levels, stratified by the fibrosis stage. In patients with fibrosis stage F0-F1, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of <1 ($P < 0.01$) and between those with WFA^+-M2BP levels of ≥4 and those with levels of <1 ($P < 0.01$). In patients with fibrosis stage F2-F3, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of ≤1 and those with levels of >4 ($P < 0.01$) and between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.001$). In patients with fibrosis stage F4, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.05$). As with WFA^+-M2BP levels, incidence rates increased with fibrosis stage, and the change in incidence was significant for each fibrosis stage.

Predictive Accuracy of Cumulative Incidence of HCC Compared With WFA^+-M2BP and AFP. AUROC analyses for prediction of the development of HCC at 1, 2, 3, 5, 7, and 10 years (range) were 0.762 (0.553-0.971), 0.792 (0.669-0.915), 0.832 (0.751-0.914), 0.858 (0.805-0.911), 0.821 (0.767-0.876), and 0.800 (0.745-0.855) in WFA^+-M2BP and 0.791 (0.684-0.898), 0.790 (0.723-0.857), 0.772 (0.693-0.850), 0.800 (0.741-0.858), 0.796 (0.745-0.848), and 0.821 (0.773-0.868) in AFP, respectively. The WFA^+-M2BP assay was superior to AFP for predicting the development of HCC at 3, 5, and 7 years.

Discussion

Liver biopsy has long been considered the gold standard for assessment of hepatic fibrosis,[20-23] and the Metavir[24] and Desmet et al.[16] staging systems are most commonly used. A higher degree of liver fibrosis is known to be the strongest risk factor for hepatocarcinogenesis in hepatitis C patients.[1,20] However, it also has its limitations for the staging of fibrosis because of the heterogeneous distribution of fibrosis in the liver,[25] and liver biopsy is an invasive procedure with

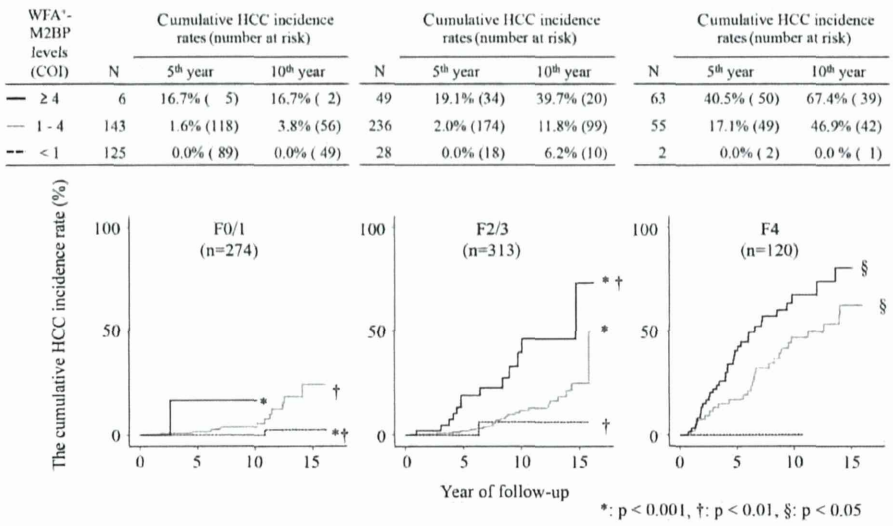

Fig. 3. Cumulative incidence of HCC according to WFA$^+$-M2BP levels, stratified by the fibrosis stage. Cumulative incidences of HCC, according to the WFA$^+$-M2BP level, stratified by the fibrosis stage were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA$^+$-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rates increased in accord with WFA$^+$-M2BP level.

associated morbidity (pain, bleeding, or hemobilia).[26] For these reasons, patients are often reluctant to undergo this invasive procedure and instead choose one of several noninvasive methods available for assessing the degree of liver fibrosis.

Nevertheless, in the past, no significant progress was made in the development of noninvasive biomarkers to guide clinical usage. WFA$^+$-M2BP was recently validated as a liver fibrosis glycobiomarker with a fully automated immunoassay.[8] In the present study, we assessed the performance of the WFA$^+$-M2BP assay in comparison with liver fibrosis stage and several serum markers, and, based on the results, we estimated whether WFA$^+$-M2BP is a useful predictor of the development of HCC as well as liver biopsy stage.

The first main finding of our study was that there was a significant correlation between the WFA$^+$-M2BP value and the fibrosis stage (Fig. 1). Moreover, step-wise multiple linear regression analysis showed that liver fibrosis stage was most closely associated with serum WFA$^+$-M2BP level. In addition, the degree of necroinflammation had no apparent effect on the WFA$^+$-M2BP value. Based on these results, we proposed a clinical management algorithm using a WFA$^+$-M2BP assay to predict the fibrosis stage. This approach could be used reliably for the first-line pretherapeutic evaluation of fibrosis in HCV-infected patients. On the other hand, the most widely used noninvasive techniques have recently shifted to physical measurements, such as FibroScan,[27-30] acoustic radiation force impulse, and real-time strain elastography. FibroScan has the advantages of being rapid and technically simple; however, operator skill affects its diagnostic success rate. Also, stiffness measurements can be difficult to obtain in obese patients and impossible in patients who have ascites. This is regarded as a limitation of transient elastography.[27,28] Therefore, we suggest that FibroScan, in conjunction with an assay of serum fibrosis biomarkers, would improve the diagnostic accuracy.

The second main finding of our study was the significant association between the WFA$^+$-M2BP level and the risk of HCC development in hepatitis C patients (Figs. 2 and 3). The diagnostic performance of WFA$^+$-M2BP, based on the AUROC values, was superior to that of AFP for predicting the development of HCC at 3, 5, and 7 years. The WFA$^+$-M2BP value can be used as a noninvasive predictor of HCC development and can be considered a surrogate marker for liver fibrosis. Various risk factors have been reported for HCC development among patients with HCV, including older age,[1] male sex,[1] heavy alcohol consumption,[31] obesity,[32] cirrhosis,[1,33] lower platelet count,[34-36] high serum AFP level,[15,36-44] low serum albumin level,[31] and high serum ALT and AST level.[45-47] Our results were consistent with these findings. Among them, liver fibrosis stage was the strongest prognostic indicator of chronic hepatitis. However, liver biopsy has several disadvantages. In our study, we have shown that the WFA$^+$-M2BP value is also a significant risk factor of HCC development independent of these factors. However, even though WFA$^+$-M2BP can be considered a surrogate marker for liver fibrosis, a distinct advantage of WFA$^+$-M2BP over liver biopsy is its wider dynamic range for the evaluation of liver cirrhosis. In the Metavir and Desmet et al. scoring systems, cirrhosis is represented by a single category (F4). However, the degree of fibrosis may vary widely