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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

High-sensitivity *Lens culinaris* agglutinin-reactive alpha-fetoprotein assay predicts early detection of hepatocellular carcinoma

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Abstract
Background Prognosis of patients with hepatocellular carcinoma (HCC) remains poor because HCC is frequently diagnosed late. Therefore, regular surveillance has been recommended to detect HCC at the early stage when curative treatments can be applied. HCC biomarkers, including *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3), are widely used for surveillance in Japan. A newly developed immunoassay system measures AFP-L3 % with high sensitivity. This retrospective study aimed to evaluate clinical utility of high-sensitivity AFP-L3 (hs-AFP-L3) as a predictor of early stage HCC in surveillance at a single site.
Methods Of consecutive 2830 patients in the surveillance between 2000 and 2009, 104 HCC-developed and 104 non-HCC patients were selected by eligibility criteria and propensity score matching. Samples were obtained from the HCC patients who had blood drawn annually for 3 years prior to HCC diagnosis.
Results In the present study, hs-AFP-L3 was elevated 1 year prior to diagnosis in 34.3 % of patients. The survival rate of patients with the hs-AFP-L3 $\geq$ 7 % at 1 year prior to diagnosis was significantly lower than that of patients with hs-AFP-L3 $<$ 7 %.
Conclusions Elevation of hs-AFP-L3 was early predictive of development of HCC even at low AFP levels and in absence of ultrasound findings of suspicious HCC. The hs-AFP-L3 should be added to surveillance programs with US because elevated hs-AFP-L3 may be a trigger to perform enhanced imaging modalities for confirmation of HCC.

Keywords Surveillance · A propensity score analysis · High-sensitivity AFP-L3 · DCP · HCC

Abbreviations

HCC	Hepatocellular carcinoma
AFP	Alpha-fetoprotein
AFP-L3	*Lens culinaris* agglutinin-reactive fraction of AFP
hs-AFP-L3	High-sensitivity AFP-L3
US	Ultrasound
DCP	Des-gamma-carboxy prothrombin
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
ALT	Alanine aminotransferase
MRI	Magnetic resonance imaging

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer worldwide [1], and poor prognosis is reported because HCC is frequently diagnosed at late stages and is often untreatable. Therefore, surveillance for HCC has been advocated to detect HCC at

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early stages when curative treatments can be applied [2, 3]. Global liver associations, including the American Association for the Study of Liver Disease (AASLD), the European Association for the Study of the Liver (EASL), and the Asian Pacific Association for the Study of the Liver (APASL), recommend regular surveillance on patients at high risk for HCC [4–6]. The most common tests used for surveillance are alpha-fetoprotein (AFP) tests and ultrasound (US). EASL and APASL adopt AFP and US in their guidelines, while AASLD recommends only US. Interpretation of US can be challenging when routine screening and comparison to previous imaging results are impossible or when US are performed by different institutes or instruments, whereas HCC biomarker values can be used independently with appropriate cutoff values. The Japan Society of Hepatology (JSH) has recommended not only US but also assays of three biomarkers: AFP, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3), and des-gamma-carboxy prothrombin (DCP) [7].

However, AFP levels are often elevated even in patients with benign liver diseases. The low specificity of AFP has been a cause of concern for use as a HCC marker [8–10]. In contrast, a rate of AFP-L3 in total AFP (AFP-L3 %) has been reported to be highly specific for HCC in many studies [11–13]; however, accurate measurements of AFP-L3 % have been limited to patients having AFP >20 ng/mL by insufficient analytical sensitivity on a conventional assay system that is a liquid-phase binding assay (LiBASys) [14]. Recently, a micro-total analysis system (μTAS) based lectin-affinity electrophoresis using microfluidics technology has enabled accurate measurements of AFP-L3 % even at low AFP [15]. The high-sensitivity AFP-L3 (hs-AFP-L3) assay has demonstrated improvement in clinical sensitivity and predicting of prognosis in HCC patients with AFP < 20 ng/mL [16–18]. The Liver Cancer Study Group of Japan has reported that 37 % of HCC patients had low AFP (<15 ng/mL) at the HCC diagnosis [19]. They also show that 34 % of patients had tumors with maximum diameter of <2 cm. Early HCC is a distinct clinical entity with a high rate of surgical cure and detection of early HCC results in long-term survival [20]. However, elevated AFP is not always observed in patients with such small tumors. Therefore, the hs-AFP-L3 assay which can measure serum levels at low AFP is expected to improve detection of HCC at the early stage. Moreover, lower cutoff values for hs-AFP-L3 has been considered to improve clinical sensitivity [16–18].

In this study, clinical utility in early prediction of development of HCC in our study cohort under surveillance using hs-AFP-L3 and analyzed retrospectively is reported.

Patients and methods

Patients

The study protocol was approved by the Institutional Ethics Committee of Ogaki Municipal Hospital in January 2009 and was in compliance with the Declaration of Helsinki. Written informed consent for use of stored serum samples for the study was obtained from the enrolled patients.

Between 2000 and 2009, a total of consecutive 2830 patients positive for hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody who visited the Department of Gastroenterology and Hepatology at Ogaki Municipal Hospital were prospectively enrolled in our HCC surveillance. Of the 2830, 1214 patients met eligibility criteria: HBsAg- or HCV RNA-positive for more than 6 months, follow-up period of >3 years before HCC diagnosis, availability of sera sampled at least twice at 12-month intervals, maximal tumor diameter <3 cm and 3 nodules or less at diagnosis, and no oral intake of warfarin which is a DCP-inducing agent.

Of these 1214 patients, 114 patients had HCC and 1100 patients had no evidence of HCC during follow-up period. To reduce the confounding effects of covariates between HCC and control patients, we selected patients using propensity score matching. Six covariates including age, gender, etiology (HBV or HCV), Child-Pugh classification, platelet number, and alanine aminotransferase (ALT) except tumor markers were used. We computed the propensity score by using logistic regression with the independent variable including age (<65 years or ≤65 years), sex (female or male), etiology (HBV or HCV), Child-Pugh classification (A, B, or C), platelet count (>150 × $10^3/m^3$ or ≤150 × $10^3/m^3$), and ALT activity (≤40 IU/mL or >40 IU/mL) as shown in previous reported cut-off values according to the previous reports [21, 22]. This model yielded a *c* statistic of 0.832 (95 % confidence interval [CI], 0.797–0.866), indicating a strong ability to differentiate between HCC and control patients. Calibration was assessed using the Hosmer–Lemeshow goodness-of-fit test [23]. The *P* value of the calculated propensity score was 0.647 based on the Hosmer–Lemeshow test and showed an absence of bias. We were able to match 104 HCC developed patients to 104 non-HCC developing patients. Table 1 shows demographics of HCC and non-HCC groups. The median of tumor size was 1.9 cm. The 69 % of HCC patients had single tumor and the 86 % of HCC patients were at TNM stage I and II.

Surveillance and diagnosis

According to Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan [7], we performed US and three

Table 1 Demographics and propensity score matching

Characteristics		HCC ($n = 104$)	Non-HCC ($n = 104$)	P value
Age (years)	Median (range)	67 (37–81)	68 (14–84)	0.980
Gender	Male/female	58 (56 %)/46 (44 %)	58 (56 %)/46 (44 %)	0.889
Etiology	B/C/B + C	14 (13 %)/89 (86 %)/1 (1 %)	14 (13 %)/89 (86 %)/1 (1 %)	1.000
Child-Pugh classification	A/B/C	82 (79 %)/18 (17 %)/4 (4 %)	84 (81. %)/17 (16 %)/3 (3 %)	0.907
ALT (IU/L)	Median (range)	49 (7–361)	46 (12–321)	0.582
Platelet ($\times 10^4/mm^3$)	Median (range)	10.1 (3.2–34.0)	12.1 (2.1–41.4)	0.150
Tumor size (cm)	Median (25 %, 75 % quartile)	1.9 (1.5, 2.3)	NA	NA
Tumor number	Single/Multiple	72 (69 %)/32 (31 %)	NA	NA
TNM stage	I/II/III	49 (47 %)/41 (39 %)/14 (14 %)	NA	NA

biomarker studies (AFP, AFP-L3, and DCP) every 3–4 months and dynamic magnetic resonance imaging (MRI) every 12 months for cirrhosis patients under surveillance. For patients with chronic hepatitis, we performed US and three biomarker studies every 6 months. For diagnostic confirmation of HCC, patients had a dynamic MRI when US suggested progression in nodular lesion, change of echo pattern in nodules, or increased biomarkers: continuous elevation of AFP or increase to AFP 200 ng/mL or more, AFP-L3 15 % or more, or DCP 40 mAU/mL or more. The hs-AFP-L3 assay was not available for the surveillance of those days.

Forty-five patients were diagnosed as HCC histologically (surgical specimen, 39 patients; US-guided needle biopsy specimens, 6 patients). The remaining 59 patients were diagnosed as HCC as typical findings of dynamic MRI including hypervascular in the arterial phase with washout in the portal venous or delayed phase [4].

Treatments

Individual decisions for a primary treatment were generally made on the basis of the guidelines for HCC in Japan [7]. Patients were initially assessed for eligibility for resection. When patients declined or were deemed ineligible for resection, they underwent locoregional ablative therapy (LAT) as a second option or transcatheter arterial chemoembolization (TACE) as a third one. Of the enrolled 104 patients, 99 patients underwent resection ($n = 39$), LAT ($n = 23$), or TACE ($n = 37$: including patients with both LAT and TACE). Five patients did not receive any treatment for HCC. No patient underwent liver transplantation.

Imaging modalities

B-mode US was performed with an Aplio XV or XG ultrasound system (Toshiba Medical System, Tokyo, Japan) equipped with a convex probe (PUT-375BT). MR imaging was performed using a superconducting scanner operating at 1.5 T (Signa Twin Speed; General Electric Medical Systems, Milwaukee, WI). MR images were obtained in the axial plane with a phased-array multicoil for the body. To scan whole livers, the section thickness was 8–10 mm with 2- and 3-mm intersectional gaps, depending on liver size. Breath-hold T1-weighted in-phase and out-of-phase fast spoiled gradient-recalled echo (SPGR, 200/dual echo [4.3/2.1] [TR/TE], 80° flip angle, one signal averaged) MR images were obtained with a field of view of 36–42 cm and a 256 × 192 matrix during a 22-s acquisition time. T2-weighted fat suppression fast spin-echo (2000/85 [TR/TE], two signal averaged) MR images with respiratory synchronization were obtained with a field of view of 36–42 cm and a 352 × 256 matrix. Breath-hold double arterial dynamic fast SPGR images (115/1.2 [TR/TE], 70° flip angle, one signal averaged) were obtained with a field of view of 36–42 cm and 512 × 192 matrix during a 12-s acquisition time. Dynamic MR images were obtained before and after an antecubital intravenous bolus injection of 0.1 mmol/kg of gadopentetate dimeglumine (Magnevist; Bayer in Japan, Tokyo, Japan) followed by 15–20 ml of a sterile normal saline flush. The optimum timing of start of scanning was decided for each case after 1 ml test injection of gadopentetate dimeglumine. The scan times were about 25, 40, and 60 s, and 2–2.5 min after initiation of the contrast injection, representing the early hepatic artery, late hepatic artery, portal vein, and equilibrium phase, respectively. All MR images except T2-weight MR images were obtained using array spatial sensitivity encoding technique (ASSET).

Assays of hs-AFP-L3, AFP, and DCP

For this retrospective study, the measurements of hs-AFP-L3, AFP, and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on μTASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd.) [16]. Analytical sensitivity of the μTAS is 0.3 ng/mL AFP, and percentage of AFP-L3 can be

measured when AFP-L3 is over 0.3 ng/mL. Analytical sensitivity of LiBASys is 0.8 ng/mL AFP, but AFP-L3 % can not be calculated at AFP < 10 ng/mL.

Samples were obtained from 104 HCC patients who had blood drawn annually for 3 years prior to the HCC diagnosis and stored at −80 °C until the measurements. In the HCC patients, stored serum samples at −3 years (over 30 months before, $n = 94$), −2 years (from 18 to 30 months before, $n = 97$), −1 year (from 6 to 18 months before, $n = 103$), and 0 year ($n = 104$) at the time of the HCC diagnosis were measured. In the non-HCC patients, similarly, stored serum samples at −3 years ($n = 99$), −2 years ($n = 104$), and −1 year ($n = 102$), and 0 year ($n = 104$) from the end of follow-up were measured.

Statistical analysis

To evaluate the diagnostic accuracy and predictive values of AFP, hs-AFP-L3, and DCP, sensitivity and specificity were calculated with cutoff values in the guidelines [7]. Furthermore, cutoff values of 5, 7, and 10 % for hs-AFP-L3 were used for this retrospective study according to previous reports [13, 16]. Serial changes of three biomarkers before the diagnosis of HCC were analyzed by Wilcoxon matched pair signed rank test. For the evaluation of prognosis, the long-term survival of patients with HCC was determined by the Kaplan–Meier method and the log-rank test was used to compare the survival rates. The values were considered significant when P value was <0.05. The analyses were performed using JMP10 statistical software (SAS Institute Japan, Japan).

The propensity score matching was performed with SPSS, version 18.0 for Windows (International Business Machines Corporation, Tokyo, Japan).

Results

Dynamic changes of biomarkers

The dynamic changes of hs-AFP-L3, AFP, and DCP in HCC patients at −3, −2, −1, and 0 year before diagnosis are shown in Fig. 1a, b, and c. The levels of hs-AFP-L3 at −1 year were significantly elevated from the levels at −2 years ($P = 0.0001$). The levels of hs-AFP-L3 at −0 year were significantly elevated from the levels at −1 year ($P = 0.0003$, Table 2). AFP and DCP were significantly elevated between −1 and 0 year ($P = 0.0315$

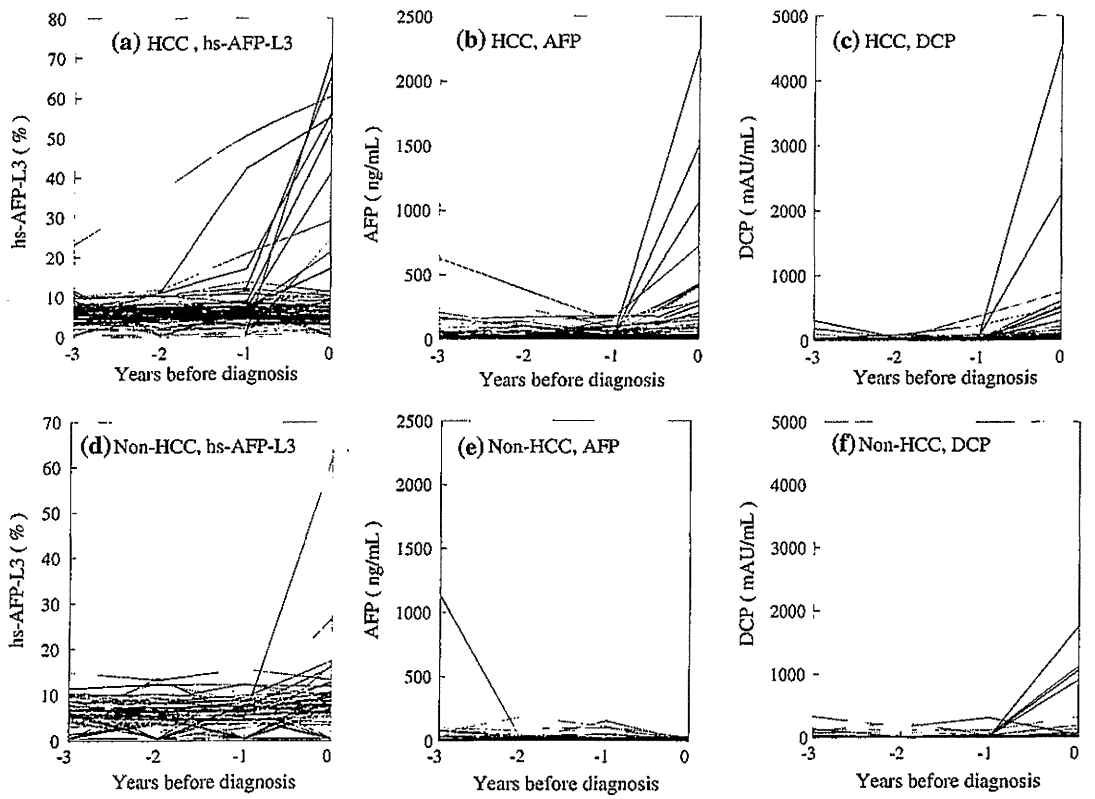

Fig. 1 Dynamic changes of biomarkers: **a** hs-AFP-L3, **b** AFP, and **c** DCP in each HCC patient ($n = 104$), and **d** hs-AFP-L3, **e** AFP, and **f** DCP in each non-HCC patient ($n = 104$)

Table 2 Serial changes of three biomarkers in HCC patients (Wilcoxon matched pair signed rank test)

Analyte	*P* value		
	At −3 year and −2 year	At −2 year and −1 year	At −1 year and 0 year
hs-AFP-L3	0.2935	0.0001	0.0003
AFP	0.4278	0.5359	0.0315
DCP	0.0926	0.6302	<0.0001

and $P < 0.0001$, respectively, Table 2). In non-HCC patients, no significant differences were observed for any markers (Fig. 1d–f). Only hs-AFP-L3 in HCC patients were significantly elevated 1 year prior to HCC diagnosis.

Sensitivity and specificity at diagnosis

Diagnostic sensitivity and specificity were evaluated for the hs-AFP-L3, AFP, DCP, and the combination of biomarkers (Table 3). The sensitivity was calculated by using HCC patient samples at diagnosis ($n = 104$) and the specificity was calculated by using non-HCC patient samples at −3 years ($n = 100$) to ensure that none had developed HCC for the following 3 years. Of the 104 HCC patients, 43 patients (41.3 %) had AFP < 10 ng/mL at which the conventional assay was not able to calculate AFP-L3 %. The sensitivity and specificity for hs-AFP-L3 were 11.5 and 100.0 %, respectively at a cutoff value of 15 %. A cutoff value of 7 % improved the sensitivity to 39.4 %. A combination assay with hs-AFP-L3, AFP, and DCP resulted in sensitivity of 60.6 % at diagnosis.

Sensitivity and specificity for 3 years before diagnosis

We calculated sensitivities using HCC samples at 3, 2, and 1 years prior to diagnosis. Similarly, specificities were calculated by using non-HCC samples (Table 4). The sensitivity and specificity for hs-AFP-L3 at −1 year were 34.3 and 74.7 %, respectively. The sensitivities at −1 year for AFP and DCP were 35.0 and 12.1 %, respectively. In HCC patients, hs-AFP-L3 turned positive at 34 patients (33.3 %) and stayed in positive at 27 patients (26.2 %) for two years till the diagnosis of HCC. In contrast, hs-AFP-L3 turned positive at 25 patients (24.3 %) and stayed in positive at 22 patients (21.4 %) for 2 years till the end of follow-up in non-HCC patients.

Table 3 Sensitivity and specificity at diagnosis

Analyte	Cutoff	Sensitivity (%)	Specificity (%)
hs-AFP-L3	5 %	50.9	51.0
	7 %	39.4	77.0
	10 %	16.3	96.0
	15 %	11.5	100.0
AFP	20 ng/mL	41.4	90.4
	200 ng/mL	12.5	99.0
DCP	40 mAU/mL	34.6	94.0
All biomarkers	7 % + 200 ng/mL + 40 mAU/mL	60.6	76.0

Comparison of tumor characteristics and survival rates

Comparing tumor characteristics at detection of HCC by a level of hs-AFP-L3 at −1 year, the tumor size, the number of tumors, and TNM stage between patients with hs-AFP-L3 ≥ 7 % and <7 % ($P = 0.064$, 0.821, and 0.504, respectively) were not statistically significant. The number of patients receiving curative treatments such as resection and LAT was significantly higher in patients with hs-AFP-L3 < 7 % ($P = 0.020$) (data not shown).

During the follow-up period after the diagnosis that was ranged from 4 to 110 months (median of 39 months), the survival rate of patients with hs-AFP-L3 ≥ 7 % was significantly lower than that of patients with hs-AFP-L3 < 7 % by using values at −1 year ($P = 0.039$) (Fig. 2). There was no statistical significance between patients with DCP ≥ 40 mAU/mL and patients with DCP < 40 mAU/mL ($P = 0.831$). No patients had AFP > 200 ng/mL at −1 year. The survival rate of patients with hs-AFP-L3 ≥ 7 % had a lower tendency than that of patients with hs-AFP-L3 < 7 % at HCC diagnosis ($P = 0.1501$).

Triggers to perform MRI for suspicious HCC and positivity rates for hs-AFP-L3

In this study population, US was performed median of 4 times between −1 year and diagnosis day. The 104 HCC

Table 4 Sensitivity and specificity for three years before diagnosis

Analyte	Year	Sensitivity (%)	Specificity (%)
hs-AFP-L3 ≥7 %	−1	34.3	74.7
	−2	25.3	80.6
	−3	24.5	77.0
AFP ≥20 ng/mL	−1	35.0	86.4
	−2	31.0	83.0
	−3	33.0	86.0
DCP ≥40 mAU/mL	−1	12.1	93.9
	−2	8.4	94.9
	−3	4.3	94.0

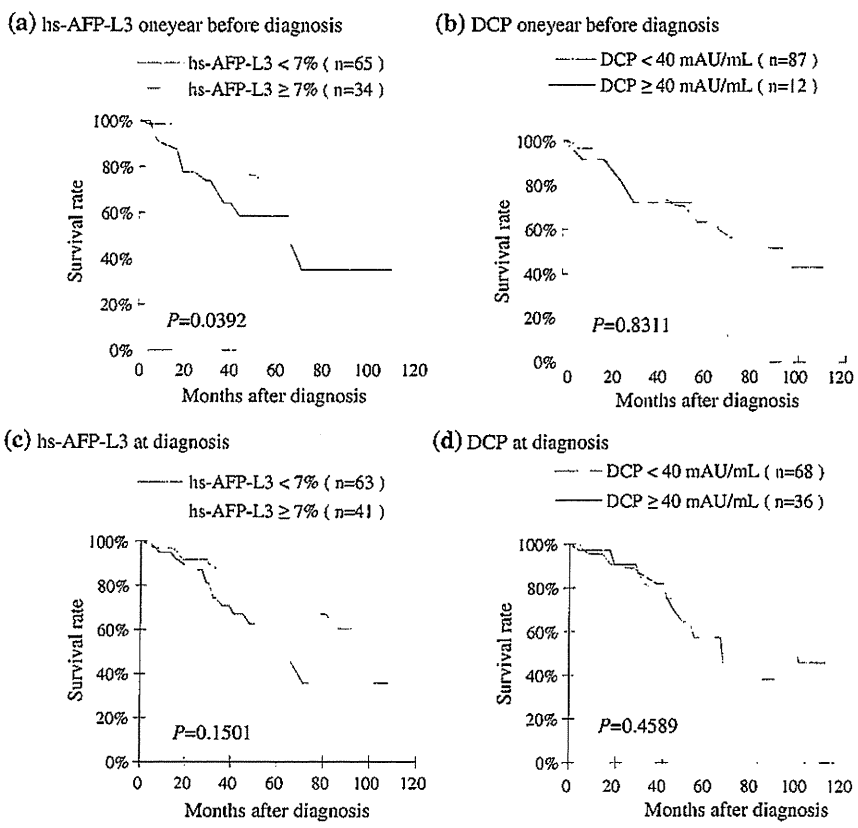

Fig. 2 Survival rates by levels of biomarkers: **a** hs-AFP-L3 and **b** DCP 1 year before, **c** hs-AFP-L3 and **d** DCP at diagnosis

patients were classified into three groups by a trigger to perform MRI for diagnostic confirmation (Table 5). US findings triggered MRI for 86 patients. The 86 patients were classified further by US findings: increase of the tumor number (51/86), increase of the tumor size (18/86), or change of the echo pattern in nodules (17/86). Five patients were monitored by MRI as results of elevated biomarkers. The remaining 13 patients were screened by MRI instead of US because interpretation of US was difficult in patients who were obese or had severe liver atrophy.

In the present retrospective study for hs-AFP-L3, 29.6 % of patients who were diagnosed with HCC by the trigger of US had hs-AFP-L3 $\geq$ 7 % 1 year prior to the diagnosis day. In the patients who had changes of the echo pattern in nodules, the positivity rate for hs-AFP-L3 at -1 year was 50.0 % and relatively higher compared to the other groups by US.

Table 5 Triggers to perform MRI for suspicious HCC and positivity rates for hs-AFP-L3

Triggers to perform MRI	*n*	hs-AFP-L3 >7 % At -1 year (%)	hs-AFP-L3 >7 % At diagnosis (%)
(a) Ultrasound	86	29.6	36.0
Increase of the tumor number	51	27.7	39.2
Increase of the tumor size	18	16.7	11.1
Change of the echo pattern in nodules	17	50.0	52.9
(b) Biomarkers	5	80.0	60.0
(c) Others	13	46.2	53.8

Discussion

Most studies on HCC biomarkers have focused on the accuracy at the time of diagnosis and the prediction of prognosis. So far there are a few studies which have evaluated early prediction of development of HCC in patients at high risk for HCC by biomarkers.

Taketa et al. [24] have reported that AFP-L3 values elevated above the cutoff value of 15 % with an average of 4.0 $\pm$ 4.9 months before the detection of HCC by imaging techniques. Sato et al. [25] also have demonstrated that lectin-reactive AFP elevated 3–18 months before the detection. However, only samples with AFP levels higher than 30 ng/mL were measured in their study. Recent data

indicated that the elevated AFP is not typical at HCC diagnosis for patients under in surveillance in Japan. Therefore, hs-AFP-L3 is expected to be more useful at low levels of AFP. Even though there were some differences in AFP concentration among the studies, they reported that elevation of AFP-L3 prior to diagnosis was associated with development of HCC.

Shiraki et al. [26] detected the small tumor <2 cm in maximum diameter in more than half of the patients. In the study population, they demonstrated clinical utility of lectin-reactive AFP as an early indicator while low AFP was reported limiting of the early recognition of HCC. Shimauchi et al. [27] demonstrated that AFP-L3 and DCP values showed elevated in about half of the patients at 6 months before the recognition of HCC by imaging techniques. These two markers were mutually complementary. In our study, DCP was not significantly elevated 1 year prior to diagnosis.

Lok et al. [28] have reported in a retrospective study of AFP and DCP values in patients in the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis Trial who had blood drawn every 3 months for 12 months prior to HCC diagnosis. They have concluded that the biomarkers are needed to complement ultrasound in the detection of early HCC but neither DCP nor AFP is optimal. For the study, early stage HCC was defined as a single tumor nodule <3 cm in diameter with no evidence of vascular invasion or metastasis, and only 61.5 % of patients presented with early stage HCC. In our study, median of tumor size was 1.9 cm and all patients with <3 cm. Tumor volume doubling time is reported to be 90–132 days [29] and it may take a half year or 1 year for a nodule to develop from <2 cm to >3 cm. Therefore, HCC patients in our study were diagnosed 1 year earlier than the patients in Lok's study. Clinically the tumor size between <2 cm and 3 cm is one of the factor for making decisions of treatments, and it has been reported that survival rate of patients with tumor size <2 cm is higher [20]. Therefore, HCC should be diagnosed at the earlier stage with tumor <2 cm in order to achieve better outcome.

It is well known that AFP-L3 concentration correlates well with AFP; however, AFP-L3 % is not correlated with AFP [24, 30]. AFP-L3 % is a marker that is independent of AFP. Therefore, we have used AFP-L3 % for analysis.

In the present study, hs-AFP-L3 was significantly elevated 1 year prior to HCC diagnosis in 34.3 % of patients at a cutoff value of 7 %. Tamura et al. [16] reported that a cutoff value of 7 % is most appropriate for discriminating HCC from benign liver disease using this assay. Therefore, patients with elevated hs-AFP-L3 value under surveillance should be followed up closely. The specificity of 80 % or less before diagnosis may actually mislead because the non-HCC patients selected by matching with the HCC patients were potentially higher risk group for HCC and would likely develop HCC later.

In previous studies, elevated AFP-L3 has been reported to be correlated to a shorter doubling time of tumor volume, increased hepatic arterial supply, and pathologic features such as infiltrative tumor growth pattern, capsule infiltration, vascular invasion, and intrahepatic metastasis [31, 32]. These findings are often difficult to diagnose by various imaging modalities in small HCCs. Such blood supply changes typically result in change of echo pattern in nodules. In this study, therefore, high positivity rates for hs-AFP-L3 at −1 year in the patients who had such changes of echo pattern may be associated with developing HCC. The survival rate of patients with hs-AFP-L3 > 7 % at −1 year was significantly poorer compared to patients with hs-AFP-L3 < 7 %. However, differences of the detected tumor size and number were not statistically significant between patients with hs-AFP-L3 ≥ 7 % and <7 %. AFP-L3-positive HCC nodules may be aggressive and have high malignancy potential even though the tumor size is small. Therefore, it may be useful in early detection of the aggressive tumor to perform enhanced imaging techniques such as MRI for patients with elevated hs-AFP-L3. Survival rate of patients with the hs-AFP-L3 elevation at HCC diagnosis showed a poorer tendency; however, there were no statistical differences. HCC treatments were done just after the HCC diagnosis. Therefore, HCC tumors in patients with the hs-AFP-L3 elevation 1 year before HCC diagnosis might have 1 year to grow. This 1 year may reflect the difference of survival of two groups. DCP is a good marker for poor prognosis of HCC. However, the difference of overall survival between patients with DCP ≥40 and <40 mAU/mL was not observed due to the early stage (small) HCC without obvious vascular invasion.

AFP is a good marker to distinguish high-risk group for HCC development in the future [22]; however, AFP was not elevated 1 year prior to HCC development. AFP-L3 was elevated 1 year prior to diagnosis of small HCC in 34.3 % of patients.

Interpretation of US can be challenging without comparison to previous imaging results and performance of US can be limited in patients who are obese or have severe background liver cirrhosis. In the present study, sensitivity of the combined three biomarkers was 60.6 % at diagnosis, and measurements of biomarkers are expected to complement to US in surveillance.

In conclusion, elevation of hs-AFP-L3 was early predictive of development of HCC even at low AFP levels and in absence of US findings of suspicious HCC. Prognosis of patients with elevated hs-AFP-L3 was significantly poorer. HCC may be diagnosed earlier to receive curative treatments by the elevated hs-AFP-L3 as a trigger of enhanced imaging techniques. Additional prospective studies are

expected to demonstrate whether routine measurements of hs-AFP-L3 in HCC surveillance can improve overall patient survival.

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Conflict of interest All authors declare that the authors report no conflicts of interest.

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Impact of Virus Clearance for the Development of Hemorrhagic Stroke in Chronic Hepatitis C

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The aim of this retrospective cohort study was to assess the cumulative incidence and predictive factors for intracerebral hemorrhagic stroke after the termination of interferon (IFN) therapy in Japanese patients with hepatitis C virus (HCV). A total of 4,649 HCV-positive patients treated with IFN were enrolled. The primary goal is the first onset of intracerebral hemorrhagic stroke. The mean observation period was 8.0 years. Evaluation was performed using the Kaplan–Meier method and the Cox proportional hazard model. A *P*-value of less than 0.05 was considered statistically significant. A total of 28 developed intracerebral hemorrhagic stroke. The cumulative incidence of intracerebral hemorrhagic stroke was 0.3% at 5 years, 0.8% at 10 years, and 1.7% at 15 years. Intracerebral hemorrhagic stroke occurred when patients had age increments of 10 years (hazard ratio: 2.77; 95% confidence interval (CI) 1.48–5.18; $P=0.001$), hypertension (hazard ratio: 2.30; 95% CI 1.09–4.83; $P=0.021$), liver cirrhosis (hazard ratio: 4.50; 95% CI 2.07–9.78; $P<0.001$), and HCV non-clearance (hazard ratio: 3.22; 95% CI 1.22–8.53; $P=0.018$). On the intracerebral hemorrhagic stroke based on the difference of liver fibrosis and efficacy of IFN therapy, HCV clearance reduced to 24.3% (1/4.11) compared to HCV non-clearance in cirrhotic patients ($P=0.040$). In conclusion, HCV clearance reduced the development of intracerebral hemorrhagic stroke. In particular, HCV clearance reduced intracerebral hemorrhagic stroke to about one-fourth in cirrhotic patients. ***J. Med. Virol. 86:169–175, 2014.***

KEY WORDS: hepatitis C virus; interferon therapy; hemorrhagic stroke

INTRODUCTION

There are 170 million people affected with chronic hepatitis C virus (HCV) infection worldwide, which may cause an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20–50% of cases over a period of 10–30 years [Kiyosawa and Furuta, 1991; Alter et al., 1992]. In addition, HCV is a major risk for hepatocellular carcinoma (HCC) [Hasan et al., 1990; Kew et al., 1990; Ikeda et al., 1993; Tsukuma et al., 1993; Arase et al., 2012]. In addition, several authors have reported that HCV clearance decreases the rate of fibrosis progression and the development of HCC in patients with chronic HCV infection [Kasahara et al., 1998; Yoshida et al., 2002; Arase et al., 2013].

On the other hand, hemorrhagic stroke is a medical emergency and can cause permanent neurological damage and death [Truelsen et al., 2003; Iso et al., 2007; Donnan et al., 2008]. It is becoming a great health burden in most countries. However, there is a little information on the incidence and risk factors on the incidence of hemorrhagic stroke in HCV patients treated with interferon (IFN). Furthermore, it is not clear whether the HCV clearance is useful for

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CT, computed tomography; GGT, gamma-glutamyltransferase; HbA_{1C}, hemoglobin A_{1C}; HCV, hepatitis C virus; HDL, high density lipoprotein; IFN, interferon; LDL, low density lipoprotein

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Biochemical tests were conducted at each examination together with regular check-up. Four hundred fifty patients were lost to follow-up. The final date of follow-up in 452 patients with loss of follow-up was regarded as last consulting day.

Patients with either of the following criteria during follow-up were regarded as censored data in statistical analysis [Fleming et al., 1984]: (1) they were retreated with IFN (N = 949); (2) they had new onset of carcinogenesis (N = 645); and (3) they had been given anticoagulant and antiplatelet drugs (N = 28). The final date of follow-up in these patients with censored data was regarded as the time of the initiation of criteria described above. The mean follow-up period was 6.7 [standard deviation (SD) 4.3] years in 452 patients with loss of follow-up and 7.4 (SD 4.7) years in 1,722 patients who had censored data. Patients with loss of follow-up and censored data were counted in the analysis.

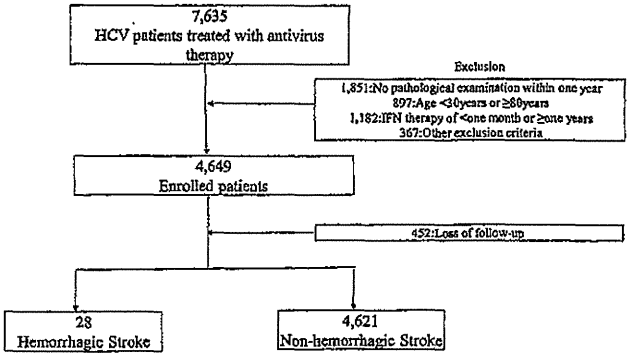


Fig. 1. An algorithm of the study population.

Statistical Analysis

Clinical differences between patients with hemorrhagic stroke and those without events were evaluated using Mann–Whitney test. The cumulative incidence of hemorrhagic stroke were calculated by using the Kaplan–Meier technique, and differences in the curves were tested using the log-rank test [Kaplan and Meier, 1958; Harrington and Fleming, 1983]. Independent risk factors associated with hemorrhagic stroke were studied using the stepwise Cox regression analysis [Cox, 1972]. The following

TABLE I. Clinical Backgrounds at the Initiation of Follow-Up in Enrolled Patients

	Total	Hemorrhagic stroke group	Without events group	*P*-value
N	4,649	28	4,621	
Age (years)	51.9 ± 11.8	60.4 ± 6.7	51.8 ± 11.9	<0.001
Gender (M/F)	2,966/1,883	16/12	2,950/1,871	0.781
Height (cm)	163.1 ± 9.2	159.5 ± 9.4	163.2 ± 9.2	0.171
Weight (kg)	61.4 ± 12.8	57.9 ± 8.0	61.4 ± 12.7	0.113
BMI	22.7 ± 3.1	23.4 ± 2.8	22.7 ± 3.1	0.582
BP (systolic, mmHg)	128 ± 18	140 ± 20	127 ± 18	0.007
BP (diastolic, mmHg)	77 ± 13	86 ± 15	77 ± 13	0.001
Total alcohol intake (kg)[a]	95 ± 92	148 ± 105	94 ± 92	0.002
Smoking index[a]	6.5 ± 9.5	11.8 ± 12.4	6.4 ± 9.4	<0.001
AST (IU/L)	41 ± 43	48 ± 28	41 ± 43	<0.001
ALT (IU/L)	44 ± 53	53 ± 38	43 ± 52	0.004
GGT (IU/L)	53 ± 60	59 ± 47	52 ± 61	0.078
Albumin (g/dl)	4.0 ± 0.3	3.5 ± 0.4	4.0 ± 0.3	0.110
Triglyceride (mg/dl)	101 ± 52	108 ± 46	100 ± 52	0.097
Cholesterol (mg/dl)	170 ± 31	171 ± 27	170 ± 31	0.893
HDL-C (mg/dl)	48 ± 14	45 ± 12	48 ± 14	0.002
LDL-C (mg/dl)	104 ± 29	108 ± 37	103 ± 29	0.049
Fasting plasma glucose (mg/dl)	99 ± 22	103 ± 23	100 ± 22	0.093
HbA_{1C} (%)	5.7 ± 1.1	5.9 ± 1.2	5.7 ± 1.1	0.024
Platelet ($\times 10^4/mm^3$)	17.2 ± 5.2	14.1 ± 6.2	17.3 ± 5.4	0.001
Staging (cirrhosis/non-cirrhosis)[b]	485/4,164	12/16	473/4,148	<0.001
HCV genotype (1b/2a/2b/other)[b]	2,859/1,109/497/184	22/5/1/0	2,837/1,104/496/184	0.104
HCV RNA (log IU/ml)[b]	6.07 ± 1.05	6.03 ± 1.03	6.08 ± 1.05	0.387
IFN monotherapy/combination therapy[c]	3,000/1,649	24/4	2,976/1,645	<0.001
Efficacy (HCV; clearance/non-clearance)	2,103/2,546	5/23	2,098/2,523	0.006

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; GGT, gamma-glutamyltransferase; HbA_{1C}; hemoglobin A_{1C}; HCV, hepatitis C virus; HDL, high density lipoprotein; IFN, interferon.
Data are number of patients or mean ± standard deviation.
[a]Smoking index is defined as package per day × year; total alcohol intake and smoking index indicate the sum before and after first consultation.
[b]Value before IFN treatment.
[c]Outbreak of IFN monotherapy: recombinant IFN alpha 2a, 238 cases; recombinant IFN alpha 2b, 183 cases; natural IFN alpha, 1,750 cases; natural IFN beta, 750 cases; total dose of IFN = 554 ± 164 MU. Outbreak of peg IFN monotherapy: peg IFN alpha 2a, 93 cases, total dose of peg IFN = 7.54 ± 2.20 mg.
Outbreak of combination therapy: recombinant IFN alpha 2b + ribavirin, 335 cases, total dose of IFN = 508 ± 184 MU, total dose of ribavirin = 160 ± 68 g; natural IFN beta + ribavirin, 127 cases, total dose of IFN = 502 ± 177 MU, total dose of ribavirin = 155 ± 67 g; peg IFN alpha 2b + ribavirin, 1,173 cases, total dose of peg IFN = 4.12 ± 1.10 mg, total dose of ribavirin = 205 ± 58 g.

TABLE II. Predictive Factors for the Development of Intracerebral Hemorrhagic Stroke

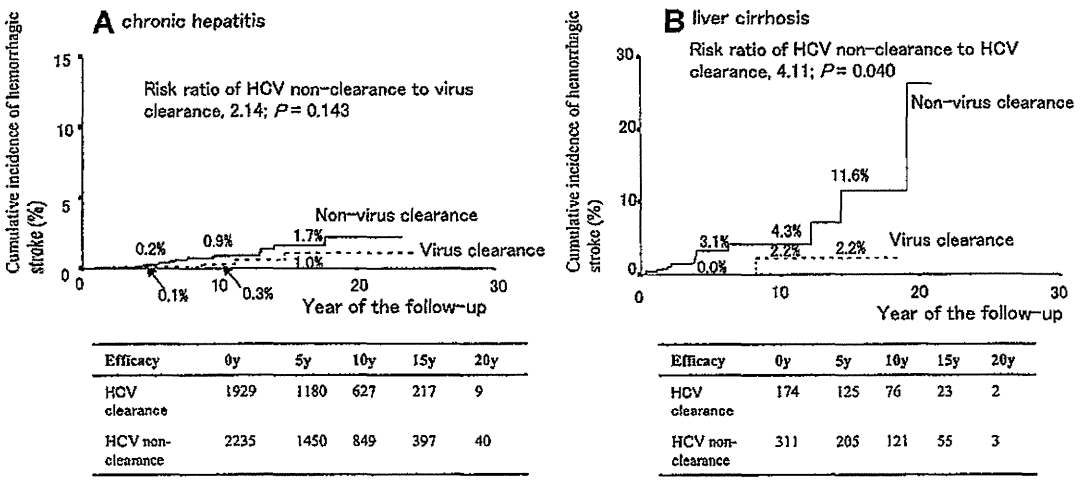
	Univariate analysis		Cox regression	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years, per 10)	3.55 (1.96–6.43)	<0.001	2.77 (1.48–5.18)	0.001
Gender (M/F)	1.26 (0.65–2.44)	0.334		
BMI (≥22/<22)	0.97 (0.75–1.24)	0.767		
Diabetes (+/−)	3.40 (1.26–9.15)	0.015		
Hypertension (+/−)	4.07 (1.94–8.54)	<0.001	2.30 (1.09–4.83)	0.021
Smoking index (≥20/<20)[a]	2.12 (0.95–4.76)	0.068		
Total alcohol intake (kg, ≥200/<200)[a]	1.10 (0.53–4.37)	0.138		
AST (IU/L, ≥34/<34)	2.79 (1.17–6.66)	0.020		
ALT (IU/L, ≥36/<36)	2.68 (1.14–6.29)	0.023		
GGT (IU/L, ≥109/<109)	1.28 (0.610–1.89)	0.655		
Albumin (g/dl, <3.9/≥3.9)	2.96 (1.24–7.09)	0.015		
Triglyceride (mg/dl, ≥100/<100)	1.19 (0.83–1.49)	0.283		
Total cholesterol (mg/dl, <150/≥150)	1.06 (0.48–1.91)	0.936		
HDL-C (mg/dl, ≥40/<40)	0.96 (0.38–2.50)	0.960		
LDL-C (mg/dl, ≥120/<120)	0.81 (0.50–2.51)	0.572		
Platelet ($\times 10^4$/mm^3, <15/≥15)	3.22 (1.41–7.35)	0.005		
Histological diagnosis (cirrhosis/non-cirrhosis)	7.40 (3.30–16.77)	<0.001	4.50 (2.07–9.78)	<0.001
Combination of ribavirin (+/−)	0.80 (0.25–2.54)	0.701		
Type of IFN (α/β)	1.29 (0.65–2.33)	0.116		
Total dose of IFN (MU, ≥500/<500)	0.87 (0.39–1.99)	0.744		
HCV genotype (1/2)	1.53 (0.62–3.80)	0.360		
HCV RNA (log IU/ml, ≥5/<5)	1.35 (1.02–1.79)	0.035		
Efficacy (HCV: non-clearance/clearance)	2.98 (1.13–6.59)	0.020	3.22 (1.22–8.53)	0.018

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; GGT, gamma-glutamyltransferase; HCV, hepatitis C virus; IFN, interferon.
[a]Smoking index is defined as package per day × year; total alcohol intake and smoking index indicate the sum before and after first consultation.

(hazard ratio: 2.30; 95% CI 1.09–4.83; $P=0.021$), liver cirrhosis (hazard ratio: 4.50; 95% 2.07–9.78; $P<0.001$), and HCV non-clearance (hazard ratio: 3.22; 95% CI 1.22–8.53; $P=0.018$). Figure 2B–E shows the cumulative incidence of hemorrhagic stroke based on difference of age, blood pressure, liver fibrosis, and efficacy of IFN therapy.

Hemorrhagic Stroke Based on the Difference of Liver Fibrosis and Efficacy

Figure 3A,B shows the cumulative incidence of intracerebral hemorrhagic stroke based on the difference of liver fibrosis and efficacy of IFN therapy. As shown in Figure 3B, HCV clearance reduced



Efficacy	0y	5y	10y	15y	20y
HCV clearance	1929	1180	627	217	9
HCV non-clearance	2235	1450	849	397	40

Efficacy	0y	5y	10y	15y	20y
HCV clearance	174	125	76	23	2
HCV non-clearance	311	205	121	55	3

Fig. 3. **Panel A**: Cumulative development rate of intracerebral hemorrhagic stroke based on difference of efficacy after interferon treatment in HCV patients with chronic hepatitis. **Panel B**: Cumulative development rate of intracerebral hemorrhagic stroke based on the difference of efficacy after interferon treatment in HCV patients with liver cirrhosis.

patients with hypertension, liver cirrhosis, and HCV non-clearance should be noted the development of hemorrhagic stroke.

The present study was limited by a retrospective cohort trial. Another limitation of the study was that patients were treated with different types of antivirus therapy for different duration. In addition, these patients were treated with different types of drugs for diabetes, hypertension, and dyslipidemia during follow-up. Finally, our cohort contains Japanese subjects only. On the other hand, the strengths of the present study are a long-term follow-up in the large numbers of patients included.

In conclusion, HCV clearance reduced hemorrhagic stroke to about one-fourth in cirrhotic patients.

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Original Article

Oral supplementation with branched-chain amino acid granules prevents hepatocarcinogenesis in patients with hepatitis C-related cirrhosis: A propensity score analysis

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Aim: It has been reported that branched-chain amino acids (BCAA) supplementation can improve nutritional status and reduce liver-related complications in patients with decompensated cirrhosis. BCAA supplementation reportedly reduces the incidence of hepatocellular carcinoma (HCC) in obese cirrhotic patients infected with hepatitis C virus (HCV). We investigated the effects of oral supplementation with BCAA granules on hepatocarcinogenesis in patients with HCV-related cirrhosis using propensity score matching.

Methods: A total of 60 patients with HCV-related cirrhosis and without history of HCC who were selected by one-to-one matching of propensity scores: 30 patients receiving 12 g/day of BCAA granules for 3 months or more (BCAA group) and 30 being observed without BCAA supplementation (control group). The impact of BCAA supplementation was analyzed on the incidence of HCC.

Results: The 3- and 5-year rates of HCC development were 13.7% and 13.7% in the BCAA group and 35.1% and 44.5% in the control group, respectively. The BCAA group had a significantly lower rate of HCC than the control group ($P = 0.032$). Multivariate analysis for factors that were associated with hepatocarcinogenesis indicated that BCAA supplementation was independently associated with a reduced incidence of HCC (hazard ratio 0.131; 95% confidence interval, 0.032–0.530; $P = 0.004$) along with sex and serum α-fetoprotein. Obesity (body mass index, ≥25 kg/m^2) was not significantly associated with an increased incidence of HCC.

Conclusion: Oral supplementation with BCAA granules is associated with a reduced incidence of HCC in patients with HCV-related cirrhosis regardless of the presence of obesity based on the propensity score analysis.

Key words: branched-chain amino acids, hepatitis C, hepatocarcinogenesis, hepatocellular carcinoma, propensity score

INTRODUCTION

THE DECREASE IN the ratio of serum branched-chain amino acids (BCAA) to aromatic amino acids (AAA) is a feature of liver cirrhosis. This decrease is due to factors including reduced nutritional intake, ammonia detoxification in skeletal muscle and hypermetabolism.[1] A low serum BCAA/AAA ratio reduces the biosynthesis and secretion of albumin in hepatocytes,[2] and is associated with poor prognosis in patients with chronic liver disease.[3,4]

Branched-chain amino acid granules, which consist of leucine, isoleucine and valine, have been used for patients with cirrhosis to improve protein malnutrition.[5] Recent clinical studies have reported that long-term oral supplementation with BCAA improves complication-free survival and prevents progression of liver failure in patients with chronic liver disease.[6–8] Additionally, several clinical studies have also reported that long-term oral supplementation with BCAA is associated with decreased incidence of hepatocellular carcinoma (HCC) and decreased recurrence after treatment with radiofrequency ablation in patients with cirrhosis.[9–11]

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HCC is the sixth most common cancer worldwide and the third most common cause of cancer-related death.[12,13] In Japan, HCC currently represents the third and fifth most common cause of death from cancer in men and women, respectively,[14] and hepatitis C virus (HCV)-related HCC accounts for 70% of all HCC cases.[14] Hepatocarcinogenesis can be prevented in patients infected with HCV when HCV is eradicated and transaminases are normalized by interferon (IFN)-based antiviral therapy.[15–18] In contrast, it has been controversial whether maintenance IFN treatment can reduce the incidence of HCC in patients who failed previous IFN therapy.[19–21] The investigation for measures that can potentially prevent HCC development is an important issue.

Recently, several *in vivo* studies have reported the effects of BCAA on suppressing carcinogenesis.[22–26] The object of the present study was to further confirm the impact of oral supplementation with BCAA granules on hepatocarcinogenesis in patients with HCV-related cirrhosis, using propensity score analysis to reduce biases associated with the selection of study patients.[27–30]

METHODS

Patients

HEPATITIS C VIRUS infection was confirmed in a total of 2752 patients at the Ogaki Municipal Hospital, between July 2005 and December 2011, by the detection of serum HCV RNA by a polymerase chain reaction (PCR) assay. Of these 2752 patients, 307 met the following eligibility criteria: (i) observation or oral supplementation with BCAA granules during the follow-up period; (ii) platelet count of less than $130 \times 10^3/\mu L$; (iii) no history of IFN therapy; (iv) absence of uncontrollable ascites, edema and encephalopathy; (v) absence of other causes of liver disease (excessive alcohol consumption, hepatitis B, autoimmune liver disease, Wilson's disease, hemochromatosis, α1-antitrypsin deficiency); (vi) no previous history of HCC; (vii) regular surveillance for HCC during follow-up period; (viii) duration of follow-up period of more than 6 months; (ix) oral BCAA supplementation for more than 3 months (patients in BCAA group); and (x) diagnosis of HCC more than 1 year after the start of the follow-up period and the start of oral supplementation with BCAA granules (patients in the BCAA group), if HCC developed. In the BCAA group, the start of oral supplementation with BCAA granules was defined as the start of follow-up period. In control group, the date of the first visit was defined as the start of follow up.

Table 1 Baseline characteristics of patients ($n = 307$)

Age (years)†	72.0 (33.0–91.0)
Sex (female/male)	166/141
BMI (kg/m^2)†	21.8 (13.9–31.6)
Obese patients (BMI, ≥25 kg/m^2) (%)	69 (22.5)
Albumin (g/dL)†	3.8 (1.8–4.8)
Total bilirubin (mg/dL)†	0.8 (0.2–3.8)
Alanine aminotransferase (IU/L)†	40 (8–321)
Total cholesterol (mg/dL)†	149 (62–256)
Platelet count ($\times 10^3/\mu L$)†	97 (30–129)
AFP (ng/mL)†	4.8 (0.8–1622.0)
Duration of follow up (years)†	4.6 (0.5–6.4)

†Data expressed as medians (range).
AFP, α-fetoprotein; BMI, body mass index.

Regarding the end of follow up, the final visit was defined as the end of follow up in patients without HCC, and the date of the detection of HCC was defined as the end of follow up in patients in whom HCC developed during follow up. Table 1 shows the characteristics of these 307 patients.

Of these 307 patients, 39 patients received oral supplementation with BCAA granules, and the other 268 patients did not receive BCAA supplementation. We then constructed one-to-one matched pairs using propensity score analysis. In total, 30 patients who received oral supplementation with BCAA granules (BCAA group) and 30 patients who did not receive BCAA treatment (control group) were selected by propensity score matching and were analyzed (Fig. 1).

The study protocol was in compliance with the Declaration of Helsinki and was approved by the institutional review board. Written informed consent was obtained from all patients prior to the study for use of their laboratory data.

Oral supplementation with BCAA granules

Branched-chain amino acid granules (LIVACT; Ajinomoto Pharmaceuticals; Tokyo, Japan) contain 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine per sachet. Patients in the BCAA group took one sachet three times daily after meals.

Surveillance for and diagnosis of HCC

Patients underwent ultrasonography, serum liver function tests, and tumor marker examination including α-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP and des-γ-carboxy-prothrombin every 3 months. Dynamic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) was per-

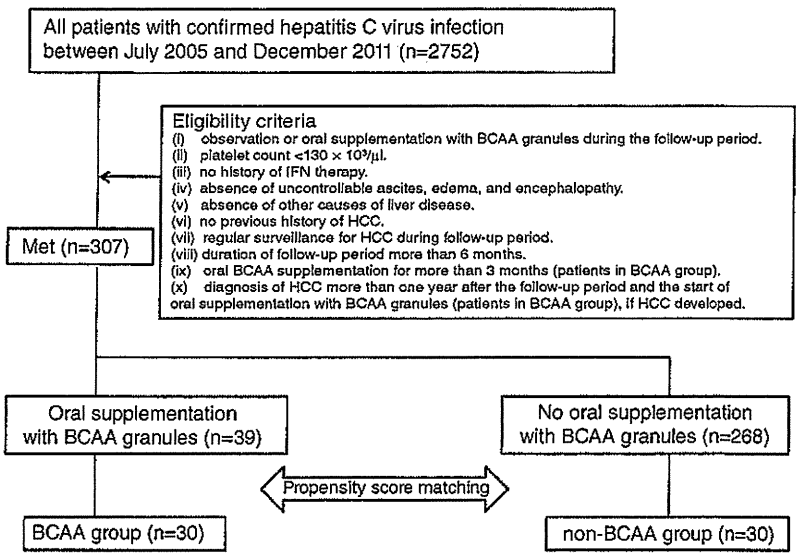

Figure 1 Patient selection criteria. BCAA, branched-chain amino acids; HCC, hepatocellular carcinoma; IFN, interferon.

formed annually. HCC was diagnosed principally based on the results from ultrasonography and dynamic CT (hyperattenuation during the arterial phase in the entire or part of the tumor, and hypoattenuation in the portal venous phase) and/or MRI mainly as recommended by the diagnosis algorithm of Japan Society of Hepatology.[31]

Statistical analysis

Statistical analyses were performed using SPSS software ver. 18.0 for Windows (SPSS, Chicago, IL, USA). Quantitative values are expressed as medians (range). Between-group differences were analyzed using the χ^2-test and Fisher's exact test. Differences between two groups in quantitative values were analyzed using the Mann–Whitney *U*-test. Curves for the HCC incidence were constructed using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazards modeling with the variable increasing method was used for multivariate analyses of factors related to hepatocarcinogenesis. A two-sided *P*-value of less than 0.05 was accepted as statistically significant.

Multiple logistic regression analysis was used to determine predictors of receiving BCAA supplementation. We used the minimum or maximum of the reference values at our instruction as cut-off values for total bilirubin, albumin and alanine aminotransferase (ALT). Propensity scores were calculated using binary logistic regression with covariates that determined whether BCAA supplementation was received. Propensity scores were rounded to two decimal places. We conducted one-to-one matching of patients by consistency of the propensity score to the second decimal place. Discrimination of the propensity score model was assessed using the area under the receiver operating characteristic (ROC) curve,[32] with higher values indicating better discrimination. Calibration was assessed using the Hosmer–Lemeshow goodness-of-fit test.[33] The Hosmer–Lemeshow test compares model performance (observed vs expected) across deciles of risk to test whether the model is biased (i.e. performs differently at the extremes of risk). A non-significant value for the Hosmer–Lemeshow test suggests an absence of such bias.

RESULTS

Analysis of propensity score and matching

SERUM LEVELS OF total bilirubin (<1.2 mg/dL) and albumin (<3.5 g/dL) were selected as covariates for propensity score analysis based on multiple logistic regression analysis of BCAA supplementation status. This multiple logistic regression model involved the covariates of sex, age (<65/≥65 years), body mass index (BMI, <25/≥25 kg/m^2), ALT (<35/≥35 IU/L), total bilirubin (<1.2/≥1.2 mg/dL), albumin (<3.5/≥3.5 g/dL) and platelet count (<100 × 10^3/≥100 × 10^3/μL). We then calculated the propensity score by binary logistic regression analysis using the covariates of sex, age, total bilirubin and albumin. The *P*-value of the calculated propensity score was 0.947 based on the Hosmer–

Lemeshow test. The area under the curve (AUC) calculated propensity score was 0.804 (95% confidence interval [CI], 0.723–0.886).

Characteristics of patients after propensity score matching

Table 2 shows the characteristics of the 60 patients selected by one-to-one matching based on consistency of the propensity score to the second decimal place. Between the BCAA and the control groups, sex and quantitative values such as age, BMI, albumin, total bilirubin, ALT, total cholesterol, white blood cell counts, total lymphocyte counts, platelet count, AFP and follow-up duration were not significantly different. There was no difference in the percentage of obese patients between groups. The percentage of patients with esophageal or gastric varices was higher in the BCAA group ($P = 0.010$) at the start of follow up. The median period of oral supplementation with BCAA granules was 2.0 years (range, 0.3–6.8).

Serum albumin level, white blood cell counts and lymphocyte counts were not significantly different between the BCAA and control groups at the end of the follow-up period. There was no significant difference between groups in the percentage of patients who experienced the rupture of varices during the follow-up period.

Incidence and characteristics of HCC

The overall 3- and 5-year rates for the development of HCC were 24.8% and 30.6%, respectively. The size of HCC tumors was 2.3 ± 0.8 cm (range, 1.1–3.6) and the number of tumors was 1.1 ± 0.3 (range, 1–2). There was no HCC with vascular invasion and extrahepatic metastases based on the imaging examinations.

Factors associated with hepatocarcinogenesis

Multivariate analysis with Cox proportional hazards modeling using the covariates of sex (34 female vs 26 male), age (years, <65, $n = 13$ vs ≥65, $n = 47$), BMI (kg/m^2, <25, $n = 45$ vs ≥25, $n = 15$), platelet count (×10^3/μL, <100, $n = 47$ vs ≥100, $n = 13$), AFP level (ng/mL, <10, $n = 30$ vs ≥10, $n = 28$) and BCAA supplementation

Table 2 Characteristics of patients after propensity score matching

	BCAA group ($n = 30$)	Control group ($n = 30$)	*P*-value
Age (years)†	71.5 (46.0–87.0)	72.0 (47.0–86.0)	0.998
Sex (female/male)	16/14	18/12	0.602
BMI (kg/m^2)†	22.5 (14.2–29.7)	22.5 (14.6–31.1)	0.938
Obese patients (BMI, ≥25 kg/m^2) (%)	7 (23.3)	8 (26.7)	0.765
Albumin (g/dL)†			
Start of follow up	3.1 (1.8–4.4)	3.3 (2.4–4.3)	0.509
End of follow up	3.1 (2.4–4.6)	3.4 (2.1–4.6)	0.111
Total bilirubin (mg/dL)†	1.2 (0.5–3.8)	1.2 (0.2–3.1)	0.830
Alanine aminotransferase (IU/L)†	48 (18–321)	40 (16–128)	0.107
Total cholesterol (mg/dL)†	142 (82–191)	138 (62–210)	0.888
White blood cell count (/μL)†			
Start of follow up	3760 (2070–7490)	3560 (2520–9080)	0.663
End of follow up	4020 (2740–9240)	4230 (2760–8550)	0.796
Total lymphocyte cell count (/μL)†			
Start of follow up	984 (468–3146)	1189 (610–2214)	0.256
End of follow up	1012 (373–4149)	1157 (357–2062)	0.296
Platelet count (×10^3/μL)†	70 (50–127)	80 (50–120)	0.549
AFP (ng/mL)†	10.3 (0.9–254.7)	7.7 (0.8–211.5)	0.876
Esophageal or gastric varices			
Present/absent at the start of follow up	19 (63%)/11 (37%)	9 (30%)/21 (70%)	0.010
Rupture of varices during follow up	5 (17%)	1 (3%)	0.097
Duration of follow up (years)†	3.7 (0.5–6.4)	4.1 (0.5–6.4)	0.894
Duration of follow-up oral supplementation of BCAA (years)†	2.0 (0.3–6.8)		

†Data expressed as medians (range).
AFP, α-fetoprotein; BCAA, branched-chain amino acids; BMI, body mass index.

Table 3 Multivariate analysis of factors related to hepatocarcinogenesis

Factor	Hazard ratio	95% CI	*P*-value
Oral supplementation with BCAA granules			
Control group ($n = 30$)	1	0.032–0.530	0.004
BCAA group ($n = 30$)	0.131		
Sex			
Female ($n = 34$)	1	1.862–25.169	0.004
Male ($n = 26$)	6.847		
AFP			
<10 ng/mL ($n = 30$)	1	2.056–30.972	0.003
≥10 ng/mL ($n = 28$)	7.980		

95% CI, 95% confidence interval; AFP, α-fetoprotein; BCAA, branched-chain amino acids.

status (BCAA group, $n = 30$ vs control group, $n = 30$) showed that oral supplementation with BCAA granules was inversely associated with hepatocarcinogenesis (hazard ratio [HR], 0.131; 95% CI, 0.032–0.530; $P = 0.004$), whereas male sex (HR, 6.847; 95% CI, 1.862–25.169; $P = 0.004$) and AFP 10 ng/mL or more (HR, 7.980; 95% CI, 2.056–30.972; $P = 0.003$) were also significantly associated with hepatocarcinogenesis (Table 3).

Incidence of HCC based on variables that showed significant differences in the multivariate analysis

The rate of HCC development based on oral supplementation of BCAA is shown in Figure 2(a). The HCC incidence of patients in the BCAA group was significantly lower than that of patients in the control group ($P = 0.032$). The 3- and 5-year HCC incidence of patients in the BCAA group were 13.7% and 13.7%, respectively, and those of patients in the control group were 35.1% and 44.5%, respectively. The rate of HCC development based on patient sex is shown in Figure 2(b). The HCC incidence of females was significantly lower than that of males ($P = 0.042$). The 3- and 5-year HCC incidence in females were 14.9% and 19.1%, respectively, and those of males were 38.0% and 48.3%, respectively. The rate of HCC development based on AFP level is shown in Figure 2(c). Patients with low AFP (<10 ng/mL) had a significantly lower rate of HCC development than patients with high AFP

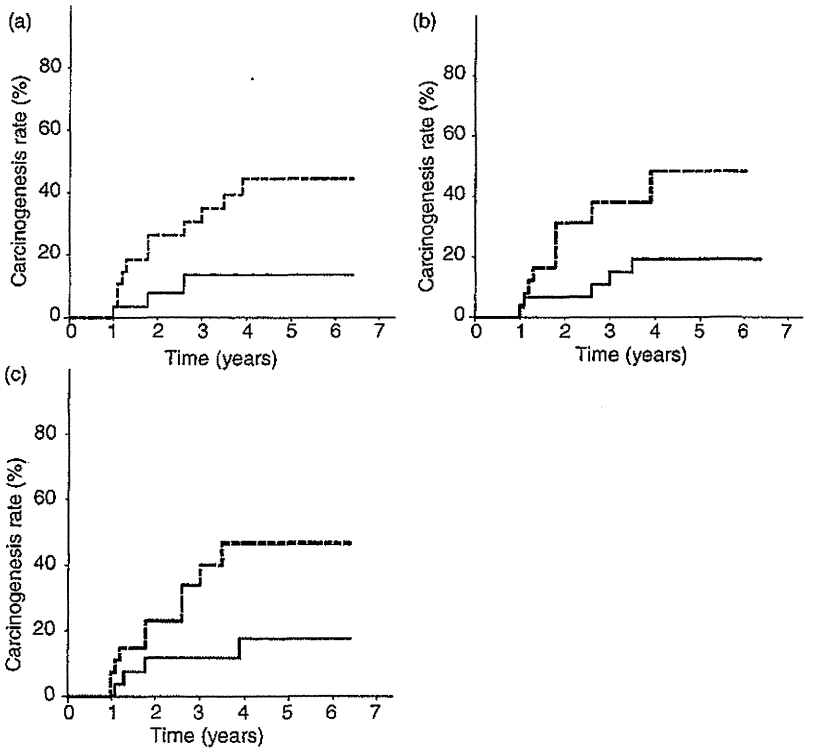

Figure 2 Incidence of hepatocellular carcinoma (HCC) based on factors that are associated with hepatocarcinogenesis by multivariate analysis. (a) Incidence of HCC according to oral branched-chain amino acids (BCAA) supplementation. HCC incidence of patients in the BCAA group was significantly lower than that of patients in the control group ($P = 0.032$). ----, non-BCAA group ($n = 30$); ——, BCAA group ($n = 30$). (b) Incidence of HCC according to sex. HCC incidence of females was significantly lower than that of males ($P = 0.042$). ----, male ($n = 26$); ——, female ($n = 34$). (c) Incidence of HCC according to α-fetoprotein (AFP) level. HCC incidence of patients with low AFP (<10 ng/mL) had a significantly lower than that of patients with high AFP (≥10 ng/mL) ($P = 0.043$). ----, AFP ≥10 ng/mL ($n = 28$); ——, AFP <10 ng/mL ($n = 30$).

(≥10 ng/mL) ($P = 0.043$). The 3- and 5-year HCC incidence of patients with low AFP were 11.6% and 17.5%, respectively, and those of patients with high AFP were 39.9% and 46.6%, respectively.

DISCUSSION

PROTEIN–ENERGY MALNUTRITION is frequently observed in patients with cirrhosis, resulting in decreased skeletal muscle volume and serum albumin levels, and a non-protein respiratory quotient.[34] Supplementation with BCAA has been tried as an intervention to improve protein malnutrition in patients with cirrhosis.[5] Two previous randomized studies demonstrated that oral supplementation with BCAA granules decreases the frequency of cirrhosis-related complications and improves complication-free survival in patients with decompensated cirrhosis.[7,8] Based on these findings, oral supplementation with BCAA granules is recommended in Japanese guidelines for the treatment of HCV-related cirrhosis.[26] Oral supplementation with BCAA granules has also been reported to be useful as an adjuvant nutritional therapy after hepatectomy, radiofrequency ablation and transarterial chemoembolization with a reduced risk of complications and maintenance of liver function.[10,35–37]

Recent studies have also suggested that BCAA have anti-hepatocarcinogenic activity,[38,39] in obese diabetic mice with insulin resistance. Because insulin resistance is closely linked to hepatocarcinogenesis,[40] it is possible that BCAA may inhibit hepatocarcinogenesis through amelioration of insulin resistance. A previous randomized trial[9] showed that oral supplementation with BCAA granules was associated with reduced incidence of HCC in cirrhotic patients who are obese (BMI, ≥25 kg/m^2) and have HCV infection (HR, 0.28; 95% CI, 0.10–0.78). Because patients who are obese and/or infected with HCV frequently have insulin resistance,[41,42] the findings of this study also support the hypothesis that BCAA suppresses hepatocarcinogenesis through amelioration of insulin resistance.

The results of our present study further confirmed the suppressive effect of BCAA on hepatocarcinogenesis and, in addition, the results showed that this suppressive effect of BCAA was regardless of the presence of obesity. The baseline clinical characteristics of the BCAA group and the control group were matched using propensity score analysis including the obesity, and the percentage of obese patients (BMI, ≥25 kg/m^2) were less than 30% in both groups. Oral supplementation with BCAA granules was significantly associated with lower incidence of HCC in the multivariate analyses (HR, 0.131; 95% CI, 0.032–0.530; $P = 0.004$), as well as male sex (HR, 0.131; 95% CI, 0.032–0.530; $P = 0.004$) and baseline AFP levels (HR, 7.980; 95% CI, 2.056–30.972; $P = 0.003$), both of which are reportedly associated with increased incidence of HCC.[43,44] The incidence of HCC was significantly lower in patients with BCAA supplementation than without ($P = 0.032$). In contrast, overweight (BMI ≥25 kg/m^2) was not significantly associated with the incidence of HCC in patients with HCV-related cirrhosis by multivariate analyses. These results suggested the presence of possible mechanisms unrelated to those associated with insulin resistance through which oral supplementation with BCAA granules prevents hepatocarcinogenesis in patients with HCV-related cirrhosis and the results enhanced the necessity for additional prospective trials to confirm these results. Several experimental and clinical studies reported the possible mechanisms of BCAA granules to suppress hepatocarcinogenesis, not via the improvement of insulin resistance, including the decreased iron-related oxidative stress,[45] suppressed anti-angiogenesis via inhibition of vascular endothelial growth factor[23] and improvement of immune function.[46] Because we could not find differences in immune competence such as white blood cell counts and total lymphocyte counts at the start and end of the follow-up period between groups, further studies will be necessary to elucidate the mechanisms of BCAA to suppress hepatocarcinogenesis that is unrelated with insulin resistance.

Because the present study is retrospective in nature, we used propensity score analysis to reduce selection bias associated with indications for BCAA supplementation. A *P*-value of 0.947 by the Hosmer–Lemeshow test that evaluates the goodness-of-fit for the calculated propensity score was reassuring.[33] Additionally, an AUC of 0.804 (95% CI, 0.723–0.886) yielded in the ROC analysis, suggesting excellent discrimination for the calculated propensity score.[32] Consequently, sex and quantitative values such as age, BMI, albumin, total bilirubin, ALT, total cholesterol, platelet count, AFP and follow-up duration were not statistically significantly different between the BCAA and control groups.

The present study has several limitations. First, findings from propensity score analyses may be potentially limited by biases related to unmeasured and hidden covariates. One-to-one matching based on propensity scores resulted in reducing the number of patients included. Second, the patients in both groups had not undergone dietary therapy containing defined daily energy, protein intake and a late-evening snack in both