Factors influencing SVR have been evaluated in many studies that reported IL-28B (a host factor) and Core 70 mutation (a viral factor) as factors predicting the treatment outcome [23, 24, 36–38]. Our present study also demonstrate that the SVR rate was lower in patients with IL-28B minor genotype and those with mutant Core 70, suggesting that IL-28B polymorphism and Core 70 mutation represent factors largely influencing the negative conversion of HCV RNA. Regarding the correlation between treatment response and SVR, Thompson et al. [38] reported that RVR and cEVR rates were lower in patients with the IL-28B minor genotype than in those with the major genotype but the SVR rate was not affected by the IL-28B genotype in patients with RVR or cEVR. In recent studies published after recognition of IL-28B polymorphism, virological response at week 4 and 12 was highly associated with SVR [39, 40]. In our present results, if RVR or EVR is achieved, a high SVR rate can be obtained regardless of the IL-28B polymorphism or Core 70 mutation status.

If RVR is achieved, PEG-IFNα-2a monotherapy exhibits a treatment effect equivalent to that of PEG-IFNα-2a/RBV combination therapy. Conversely, one patient receiving PEG-IFNα-2a/RBV combination therapy developed anemia caused by RBV, resulting in treatment discontinuation and non-SVR. In a phase III clinical trial in Japanese patients, the SVR rate in patients with RVR was 100 % (14/14) in control patients receiving PEG-IFNα-2a monotherapy but was 78 % (18/23) in those receiving PEG-IFNα-2a/RBV combination therapy [41]. Therefore, in terms of preventing treatment discontinuation due to adverse events of RBV, PEG-IFNα-2a monotherapy is recommended in cases with RVR.

In cases with cEVR, the SVR rate in patients who received biweekly PEG-IFNα-2/RBV combination therapy was comparable or even higher as compared to those who received weekly PEG-IFNα-2/RBV combination therapy. This means that biweekly PEG-IFNα-2a in a later treatment period did not reduce the antiviral effects in a subset of cases achieving a good antiviral effect (cEVR). This is partly because the half-life of PEG-IFNα2a is longer than that of PEG-IFNα2b [42–44], thus enabling the maintenance of antiviral effects. Therefore, this biweekly regimen appears possible only with PEG-IFNα2a. Regarding treatment discontinuation, the rate of treatment discontinuation was 3 % (1/31) in patients receiving biweekly PEG-IFNα-2 and 15 % (6/39) in those receiving weekly PEG-IFNα-2, suggesting that the reduced rate of adverse events and subsequent treatment discontinuation by biweekly administration may lead to the increased SVR rate.

Ikeda et al. [19] reported that one of the HMG-CoA reductase inhibitors, FLV, exhibits inhibitory effects on HCV RNA replication in a system of HCV RNA replication clone. In the clinical setting, Sezaki et al. and Rao and Pandya [20–22] reported that combined use of FLV from the treatment initiation period improved the SVR rate [21]. The HCV RNA is replicated using the lipid droplet in hepatocytes [45, 46], and HMG-CoA reductase inhibitors are reported to inhibit the proliferation of HCV RNA by suppressing the synthesis of mevalonic acid through geranylgeranylation [47].

We investigated whether the SVR rate is improved by the addition of FLV only in cases with LVR, because a high SVR rate is expected in patients showing rapid negative conversion of HCV RNA (such as RVR and cEVR cases) without the combined use of FLV. Our results showed that combined use of FLV yielded a higher SVR rate (62 %) as compared to the rate (29 %) obtained without the use of FLV, suggesting that the difference in the recurrence rate may reflect the difference in the SVR rate in patients negative for HCV RNA. Thus, because we used FLV in patients with LVR at high risk of recurrence, but not in those with RVR or cEVR at low risk of recurrence, the difference in anti-HCV activities by FLV was more pronounced. It has been reported that treatment with HMG-CoA reductase inhibitors does not increase the risk of severe hepatotoxicity in patients with chronic hepatitis C [48], which is consistent with our present results showing no adverse events associated with the addition of FLV.

In summary, the SVR rate was 52 % (32/61) in the group receiving PEG-IFNα-2a/RBV combination therapy and 70 % (38/54) in the group receiving modified treatment regimens according to response-guided therapy, showing a significant increase in the latter group. This result may be attributed to the difference in the rate of treatment discontinuation, which was significantly lower in the response-guided therapy group [2 % (1/54)] than in the PEG-IFNα-2a/RBV combination group [11 % (7/61)]. In addition, anti-HCV effects of FLV in patients with LVR at high risk of recurrence may contribute to the improved SVR in the response-guided therapy group. Our results demonstrated the safety and efficacy of PEG-IFNα-2a monotherapy in patients with RVR, biweekly PEG-IFNα-2a/RBV combination therapy in those with cEVR, and PEG-IFNα-2a/RBV/FLV combination therapy in those with LVR.

In conclusion, for the treatment of genotype 1b high virus titer chronic hepatitis C, the selection of an optimal response-guided therapy option, taking into consideration the viral response to initial treatment, the IL-28B polymorphism and Core 70 mutation status, and the safety of individual patients, can improve the SVR rate.

Acknowledgments We thank Ms. R. Nakatani for her assistance with data collection. We also thank Ms. N. Kanazawa for her excellent lab work on IL28B SNPs and Core 70 substitution in this study.

Conflict of interest Shuhei Nishiguchi received financial support from Chugai pharmaceutical, MSD, Dainippon Sumitomo Pharma,

Ajinomoto Pharma, and Otsuka pharmaceutical. The remaining authors declare no conflict of interest.

References

1. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet. 2001;358:958–65.
2. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
3. Kuboki M, Iino S, Okuno T, Omata M, et al. Peginterferon a-2a (40 KD) plus ribavirin for the treatment of chronic hepatitis C in Japanese patients. J Gastroenterol Hepatol. 2007;22:645–52.
4. Yamada G, Iino S, Okuno T, et al. Virological Response in patients with hepatitis C virus genotype 1b and a high viral load impact of peginterferon-α-2a plus ribavirin dose reductions and host-related factors. Clin Drug Invest. 2008;28(1):9–16.
5. Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol. 2012;56:78–84.
6. Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. J Viral Hepatol. 2012;19:e134–42.
7. Imai Y, Tamura S, Tanaka H, et al. Reduced risk of hepatocellular carcinoma after interferon therapy in aged patients with chronic hepatitis C is limited to sustained virological responders. J Viral Hepatol. 2010;17:185–91.
8. Tanaka T, Shakado S, Morihara D et al. The prognostic factors of sustained virologic response among patients of chronic hepatitis C treated with peg-interferon alpha 2a monotherapy. Kanzo 2008;49:417–25.
9. Berg T, von Wagner M, Nasser S, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
10. Sanchez-Tapias JM, Diago M, Escartin P, et al. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. Gastroenterology. 2006;131:451–60.
11. Ferenci P, Laferl H, Scherzer TM, et al. Peginterferon alfa-2a/ribavirin for 48 or 72 weeks in hepatitis C genotypes 1 and 4 patients with slow virologic response. Gastroenterology. 2010; 138:503–12.
12. Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. Hepatology. 2007;46:1688–94.
13. Nabci C Teoh et al. Individualisation of antiviral therapy for chronic hepatitis C. J Gastroenterol Hepatol. 2010; 25:1206–16.
14. Reddy KR, Lin F, Zoulim F. Response-guided and -unguided treatment of chronic hepatitis C. Liver Int. 2012;32:64–73.
15. Di Martino V, et al. Response-guided peg-interferon plus ribavirin treatment duration in chronic hepatitis C: meta-analyses of randomized, controlled trials and implications for the future. Hepatology. 2011;54:789–800.
16. Zeuzem S, et al. Pegylated-interferon plus ribavirin therapy in the treatment of CHC: individualization of treatment duration according to on-treatment virologic response. Curr Med Res Opin. 2010;26:1733–43.
17. Franik H, et al. Meta-analysis shows extended therapy improves response of patients with chronic hepatitis C virus genotype 1 infection. Clin Gastroenterol Hepatol. 2010;8:884–90.
18. Yu ML, Dai CY, Huang JF, et al. Rapid virological response and treatment duration for chronic hepatitis C genotype 1 patients: a randomized trial. Hepatology. 2008;47:1884–93.
19. Ikeda M, Abe K, Yamada M, et al. Different anti HCV profiles of statins and their potential for combination therapy with interferon. Hepatology. 2006;44:117–25.
20. Sezaki H,Suzuki F,Akuta N et al. Influence of HMG-CoA reductase inhibitor to virological response of peginterferon/ribavirin combination therapy in chronic hepatitis C. Kanzo 2008: 49;22–4.
21. Sezaki H, Suzuki F, Akuta N, et al. An open pilot study exploring the efficacy of fluvastatin, pegylated interferon and ribavirin in patients with hepatitis C virus genotype 1b in high viral loads. Intervirology. 2009;52:43–8.
22. Rao GA, Pandya PK. Statin therapy improves sustained virologic response among diabetic patients with chronic hepatitis C. Gastroenterology. 2011;140:144–52.
23. Akuta N, Suzuki F, Kawamura Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;46:403–10.
24. Akuta N, Suzuki F, Sezaki H, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology. 2005;48:372–80.
25. Enomoto N, Sakuma I, Asahina Y, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest. 1995;96:224–30.
26. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
27. Shirakawa H, Matsumoto A, Joshita S, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008;48:1753–60.
28. Oze T, Hiramatsu N, Yakushijin T, et al. Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy. J Hepatol. 2011;54:604–11.
29. Kogure T, Ueno Y, Fukushima K, et al. Pegylated interferon plus ribavirin for genotype Ib chronic hepatitis C in Japan. World J Gastroenterol. 2008;14:7225–30.
30. Sezaki H, Suzuki F, Kawamura Y, et al. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. Dig Dis Sci. 2009;54:1317–24.
31. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
32. Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon alpha and ribavirin therapy. Nat Genet. 2009;41:1100–4.
33. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41: 1105–9.

34. Miyase S, Haraoka K, Ouchida Y, et al. Randomized trial of peginterferon α-2a plus ribavirin versus peginterferon α-2b plus ribavirin for chronic hepatitis C in Japanese patients. J Gastroenterol. 2012;47:1014–21.
35. Minami T, Kishikawa T, Sato M et al. Meta-analysis: mortality and serious adverse events of peginterferon plus ribavirin therapy for chronic hepatitis C. J Gastroenterol. 2012. [Epub ahead of print].
36. Kobayashi M, Suzuki F, Akuta N et al. Relationship between SNPs in the *IL28B* region and amino acid substitutions in HCV core region in Japanese patients with chronic hepatitis C. Kanzo 2010;51:322–3.
37. Kurosaki M, Tanaka Y, Nishida N, et al. Pre-treatment prediction of response to pegylated -interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol. 2011;54:439–48.
38. Thompson AJ, Muir AJ, Sulkowski MS, et al. Interleukin-28b polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology. 2010;139:120–9.
39. Toyoda H, Kumada T, Tada T, et al. Predictive value of early viral dynamics during peginterferon and ribavirin combination therapy based on genetic polymorphisms near the IL28B gene in patients infected with HCV genotype 1b. J Med Virol. 2012; 84:61–70.
40. Marcellin P, Reau N, Ferenci P, et al. Refined prediction of week 12 response and SVR based on week 4 response in HCV genotype 1 patients treated with peginterferon alfa-2a (40KD) and ribavirin. J Hepatol. 2012;56:1276–82.
41. Sakai T.[PEG-interferonα-2a/ribavirin therapy for chronic hepatitis type 1b.] Kan Tan Sui 2006; 52:75–84. (in Japanese).
42. Perry CM, Jarvis B. Peginterferon-alpha-2a (40 kD): a review of its use in the management of chronic hepatitis C. Drugs. 2001; 61(15):2263–88.
43. Glue P, Fang JW, Rouzier-Panis R, Raffanel C, Sabo R, Gupta SK, et al. Pegylated interferon-alpha2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group. Clin Pharmacol Ther. 2000;68(5): 556–67.
44. Formann E, Jessner W, Bennett L, et al. Twice-weekly administration of peginterferon-α-2b improves viral kinetics in patients with chronic hepatitis C genotype 1. J Viral Hepat. 2003;10: 271–6.
45. Aizaki H, Lee KJ, Sung VM, et al. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology. 2004;324:450–61.
46. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol. 2007;9:1089–97.
47. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990;343:425–30.
48. Khorashadi S, Hasson NK, Cheung RC. Incidence of statin hepatotoxicity in patients with hepatitis C. Clin Gastroenterol Hepatol. 2006;4:902–7.

Original Paper

Elevation of the AST to ALT Ratio in Association with the Severity of Esophageal Varices in Patients with HCV-Related Compensated Liver Cirrhosis

Yoshinori Iwata, Hirayuki Enomoto*, Yoshiyuki Sakai, Nobuhiro Aizawa, Hironori Tanaka, Naoto Ikeda, Tomoyuki Takashima, Akio Ishii, Kunihiro Hasegawa, Yukihisa Yuri, Kazunari Iwata, Masaki Saito, Hiroyasu Imanishi, Hiroko Iijima and Shuhei Nishiguchi*

**These authors contributed equally*

Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan

Corresponding author: Hirayuki Enomoto MD, PhD, Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo, 663-8501, Japan; Tel.: +81-798-45-6472, Fax: +81-798-45-6474; E-mail: enomoto@hyo-med.ac.jp

ABSTRACT

Background/Aims: The development of esophageal varices depends on the progression of liver fibrosis. However, it has not yet been sufficiently clarified whether biomarkers of liver fibrosis can be used to predict the incidence of varices in cirrhotic patients with a well-maintained liver function (Child-Pugh class A). **Methodology:** Three established markers of liver fibrosis, including AST-to-ALT ratios (AAR), FIB-4 and AST-to-platelet ratio indices (APRI), were analyzed in HCV-positive cirrhotic patients with Child-Pugh class A status, and the relationships between these markers and the risk of variceal bleeding were investigated. **Results:** The values of AAR and FIB-4 in the patients with varices with a high risk of hemorrhage were significantly higher than those in the patients without high-risk varices, whereas the value of APRI was not found to be related to the risk of variceal bleeding. Of all the parameters examined, the values of AAR were the most significantly different between the two (with or without high-risk varices) groups. In addition, the values of AAR increased in line with variceal severity. **Conclusions:** The value of AAR is related to the severity and risk of variceal bleeding in patients with HCV-related compensated cirrhosis.

Key Words: Liver cirrhosis; Portal hypertension; Esophageal varices; Biomarker; Fibrosis.

Abbreviations: EGD: Esophagogastroduodenoscopy; AAR: AST-to-ALT ratio; APRI: AST-to-Platelet Ratio Index; GA: Glycated Albumin; CLD: Chronic Liver Disease.

INTRODUCTION

The development of esophageal varices due to portal hypertension is a major complication of liver cirrhosis and variceal hemorrhaging is a life-threatening event that carries a significant risk of mortality. The risk of bleeding is related to variceal size, the presence of red signs on varices and advanced liver disease (Child-Pugh class B or C) (1). Although several biochemical parameters, such as low platelet counts, advanced Child-Pugh class, hypoalbuminemia and low prothrombin activity, have been reported to be associated with the presence of varices (2-4), esophagogastroduodenoscopy (EGD) is the most reliable method of evaluating variceal size and the presence of red signs.

Based on the concept that the development of portal hypertension is caused by the progression of liver fibrosis, non-invasive biomarkers of liver fibrosis have been used to predict the incidence of varices in cirrhotic patients (5). However, it has not yet been sufficiently clarified whether biomarkers of liver fibrosis can be used to predict the incidence of varices in cirrhotic patients with a well-maintained liver function (Child-Pugh class A). We previously analyzed the glycated albumin (GA)-to-glycated hemoglobin (HbA1c) ratios in HCV-related compensated cirrhotic patients and showed that the GA/HbA1c ratio in patients with high-risk varices is significantly higher than that in patients without high-risk varices (6).

In the present study, we examined the values of three established markers of fibrosis, including AST-to-ALT ratios (AAR) (7), FIB-4 indices (8) and AST-to-platelet ratio indices (APRI) (9), in HCV-positive compensated cirrhotic patients with a Child-Pugh class A status.

METHODOLOGY

EGD was routinely performed in outpatients with chronic liver disease (CLD) at our institution according to standard procedures. With regard to patients with esophageal varices, variceal size was graded from I-IV based on the Paquet grading system (10) and the presence of red signs on the varices was evaluated. Patients with large varices (grade III-IV) or small varices with red signs were categorized to be patients with high-risk varices. All HCV-associated compensated (Child-Pugh class A) cirrhotic patients admitted to our department for treatment of esophageal varices from June 2008 to July 2011 were included in the present study as "patients with high-risk varices." Liver cirrhosis as the cause of portal hypertension was diagnosed using histological criteria and/or clinical (laboratory, endoscopic and/or ultrasonographic) findings.

In order to obtain the values of several biomarkers in Child-Pugh class A cirrhotic patients without high-risk varices, we included consecutive HCV-positive CLD patients without high-risk varices who were pathologically diagnosed with cirrhosis using liver biopsies at our institution. Evaluations with EGD were performed

Hepato-Gastroenterology 2013; 60:149-152 doi 10.5754/hge12578

within two months of the liver biopsies. Liver biopsy examinations were carried out according to standard procedures and all liver specimens were evaluated by well-trained pathologists at our institute. The evaluation of the fibrosis stage and activity grade was made according to the METAVIR scoring system (11) and patients with F4 stage disease were diagnosed as having liver cirrhosis.

In the present study, the values of 3 useful biomarkers (AAR, FIB-4 and APRI) of the progression of liver fibrosis were compared between 2 groups (patients with or without treatment-requiring high-risk varices). The value of AAR was simply calculated as the AST/ALT ratio (7). The FIB-4 and APRI values were calculated based on formulas proposed by Vallet-Pichard et al. (8) and Wai et al. (9), respectively: FIB-4 = Age [years] × AST [U/L] / (platelets [109/ L] × (ALT [U/L])1/2), in which the age of the patient is the age at the time of endoscopic treatment or liver biopsy; APRI = 100× (AST level/upper limit of normal)/platelets [109/ L]. In addition, we further investigated whether the values of AAR differed between three groups: "patients without varices", "patients with low-risk varices (small varices without red signs)" and "patients with high-risk varices (large varices or small varices with red signs)."

HCV infection was diagnosed by detecting HCV antibodies and HCV-RNA in sera, and routine laboratory data, including platelet counts, prothrombin times and the results of liver functional tests (ALT, AST, γ-GTP, alkaline phosphatase and total bilirubin), were measured in all HCV-positive compensated cirrhotic patients with Child-Pugh class A status. All blood samples were obtained on the day of liver biopsy or endoscopic treatment for esophageal varices. Patients with the following conditions were excluded from the study: the presence of other liver diseases, the use of immunosuppressive therapy, hepatitis B virus co-infection and insufficient liver tissue available for evaluation of liver fibrotic staging. The study conformed to the ethical guidelines of the Helsinki declaration and written informed consent regarding liver biopsy and the use of all clinical data was obtained from all patients on admission.

Statistical analysis

The data were compared between the two groups and were analyzed using either Student's t-test or the Mann-Whitney U test, as appropriate. The data compared among three groups were analyzed using a non-repeated measurements ANOVA and statistical significance was further evaluated with the Bonferroni correction.

RESULTS

Characteristics of patients and clinical data

Of the CLD outpatients in our department, 75 were found to have a high risk of variceal bleeding due to the presence of large varices (grade III-IV) or small varices with red signs and 38 (38/75, 51%) were HCV-positive. Among the HCV-positive cirrhotic patients, the Child-Pugh classification was grade A in 13 patients, grade B in 23 patients and grade C in two patients. The 13 patients with Child-Pugh class A were enrolled as "patients with high-risk varices" (HCV-related compensated cirrhotic patients with a high risk of variceal hemorrhage). In order to obtain the characteristics of compensated cirrhotic patients without a high risk of variceal bleeding, we examined the patients who underwent liver biopsy as described in the "Methodology" section.

Out of a total of 226 HCV-positive patients who underwent liver biopsy, 38 were categorized as compensated (Child-Pugh class A) cirrhotic patients without a high risk of variceal bleeding, 25 were diagnosed with liver cirrhosis (METAVIR score F4) without detectable varices, and the remaining 13 were found to have small varices without red signs (low-risk varices). The characteristics of all 51 patients evaluated in the present study are shown in Table 1. The population consisted of 31 (61%) males and 20 (39%) females with ages ranging from 23 to 82 years (median 64 years). No patients showed any clinical symptoms of hepatic encephalopathy. In the patients with high-risk varices, two (2/13, 15%) had mild ascites, whereas none of the 38 patients without high-risk varices had ascites.

Liver fibrosis markers and other parameters in patients with HCV-related cirrhosis with a well-maintained liver function

Several biomarkers, such as AAR, FIB-4 and APRI, have been reported to be associated with the degree of liver

Figure 1

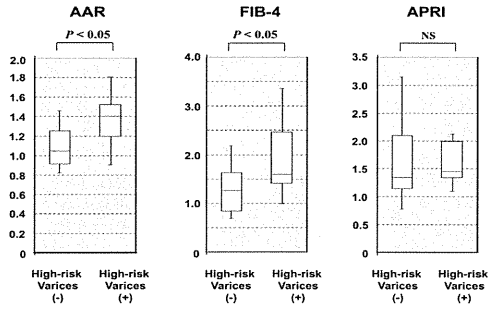

FIGURE 1. The values of three biomarkers of liver fibrosis in patients with HCV-related compensated (Child-Pugh class A) cirrhosis. The values of AST-to-ALT ratios (AAR), FIB-4 and AST-to-platelet ratio indices (APRI) were measured in HCV-related compensated cirrhotic patients with Child-Pugh class A status. The values of AAR and FIB-4 were elevated in patients with a high risk of variceal hemorrhage, while the values of APRI did not show any significant differences between the patients with a high risk of bleeding and the patients without a high risk of bleeding. (Box plots: 10th percentile, 25th percentile, median, 75th percentile and 90th percentile).

Figure 2

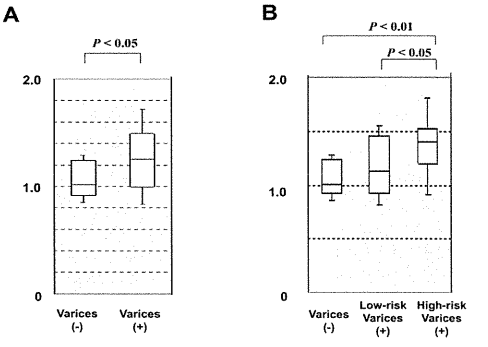

FIGURE 2. (A) The values of AST-to-ALT ratios (AAR) in patients with HCV-related compensated (Child-Pugh class A) cirrhosis with or without varices. The values of AAR in the 26 patients with varices (13 patients with high-risk varices and 13 patients with low-risk varices) were compared with those in the 25 patients without varices. The values of AAR in the patients with varices were significantly higher than those in the patients without varices. (Box plots: 10th percentile, 25th percentile, median, 75th percentile and 90th percentile). **(B)** The values of AAR increased as the severity of the esophageal varices increased. The values of AAR increased with the progression of variceal severity and significant differences between the patients "with high-risk varices" and the patients "with low-risk varices" or the patients "without varices" were observed. (Box plots: 10th percentile, 25th percentile, median, 75th percentile and 90th percentile).

fibrosis (7-9). Since portal hypertension is considered to be a major cause of variceal formation, we examined the relationships between these markers and the presence of high-risk varices in cirrhotic patients with a well-maintained liver function (Child-Pugh class A).

Comparing the 13 patients with high-risk varices and the 38 patients without high-risk varices (patients without varices or having only low-risk varices), the values of AAR and FIB-4 in the patients with a high risk of bleeding were significantly elevated compared to those in the patients without high-risk varices. Interestingly, the values of APRI were not significantly different between the two groups, indicating that APRI, an excellent marker of liver fibrosis, is not related to the presence of high-risk varices in compensated cirrhotic patients with Child-Pugh class A status (**Figure 1**). We found significant differences between the two (patients with or without high-risk varices) groups, with higher total bilirubin values and lower hemoglobin values found in association with the presence of high-risk varices. However, the values of AAR were the most significantly different (p=0.020) among all of the parameters examined in the present study (**Table 2**).

Since AAR was the most significantly different parameter between the two groups (patients with or without high-risk varices) of HCV-related Child-Pugh A cirrhotic patients, we next examined whether the AAR value was related to the presence of varices in the enrolled patients. The values of AAR in patients with varices were significantly higher than those in patients without varices (**Figure 2A**). Furthermore, the values of AAR increased with the progression of variceal severity (**Figure 2B**).

DISCUSSION

EGD is the gold standard method of determining whether a cirrhotic patient should receive treatment for varices (4,12-15). According to the Baveno IV Consensus Conference on portal hypertension, EGD should be performed at 2-3 year intervals in patients without varices and at 1-2 year intervals in patients with small varices (12). However, repeating EGD is invasive, expensive and sometimes poorly accepted by patients. Therefore, many non-invasive or minimally invasive methods to assess the presence/size of varices have been researched. For example, low platelet counts have been reported to be associated with the presence of varices or large varices in several reports (2-4,16,17). In addition, many other non-invasive approaches, including the Fibro-Test score, transient elastography, multi-detector CT and capsule endoscopy, have been used to predict the presence or size of varices (18). However, none of the available tools completely fulfill the criteria of an ideal (accurate, simple, inexpensive and easily reproducible) diagnostic tool (4,18).

Despite the fact that the risk of variceal hemorrhage is associated with variceal size, the presence of red signs on varices and advanced liver disease (Child-Pugh class B or C), it has not been sufficiently clarified which biomarker reflects the presence of high-risk varices in patients with a well-maintained liver function (Child-Pugh class A status). Recently, we reported that GA/HbA1c ratios become elevated with the progression of histological stage in patients with both HCV-related CLD and non-alcoholic steatohepatitis (19,20). We also showed the GA/HbA1c ratios to increase with the severity of esophageal varices in HCV-positive cirrhotic patients with a Child-Pugh Class A status (6).

Our findings suggest that the GA/HbA1c ratio is a simple and unique tool determined by the levels of two glycated proteins and is associated with the progression of liver fibrosis and the endoscopic severity of varices. However, the values of glycated proteins, especially GA, are not routinely measured in all countries, indicating that it is difficult to use GA/HbA1c ratios as daily clinical laboratory data.

In the present study, we examined various biomarkers of liver fibrosis in HCV-positive cirrhotic patients with a Child-Pugh class A status based on the concept that portal hypertension depends on the progression of liver fibrosis. We found AAR to be the most significantly different parameter, while another fibrosis marker, FIB-4, was also found to be related to the presence of high-risk varices. In contrast, although APRI is known to be an excellent marker of liver fibrosis and can be used to distinguish cirrhotic patients from non-cirrhotic patients (9), APRI was not found to be associated with the presence of high-risk varices in the patients with HCV-related compensated cirrhosis (**Figure 1**).

These findings are consistent with those of previous reports that investigated all types of cirrhotic patients (including all etiologies and all Child-Pugh classes) regarding the prediction of the presence of varices and the risk of variceal bleeding (5), thus suggesting that some fibrosis markers, such as AAR and FIB-4, should be useful for predicting the presence of varices and the risk of variceal bleeding, even in HCV-related cirrhotic patients with a well-maintained liver function (Child-Pugh class A) who do not present with any clinical symptoms.

In summary, we have herein shown that some fibrosis markers, especially AAR, are associated with the progression of variceal severity and the risk of variceal bleeding in HCV-related compensated cirrhotic patients. However, it will be necessary to confirm our findings in both larger and different populations.

TABLE 1. Characteristics of the 51 patients enrolled in the present study

Age (years)	63.0±11.8 (64, 23-82)
Gender (male/female)	33/18
AST (IU/L)	63.5±55.3 (47, 20-328)
ALT (IU/L)	59.3±54.2 (38, 10-310)
γ-GTP	66.8±64.8 (48, 12-259)
ALP (IU/L)	306±103 (269, 133-556)
Total bilirubin (mg/dL)	0.92±0.43 (0.8, 0.3-2.1)
Albumin (g/dL)	3.66±0.37 (3.7, 2.3-4.5)
Prothrombin time (%)	85.0±10.3 (85.0, 65.7-115.6)
White blood cell (/mm^3)	4350±1720 (4200, 1650-9980)
Hemoglobin (g/dL)	12.4±1.7 (12.1, 9.2-17.5)
Platelet (x10^4/mm^3)	10.8±3.4 (10.7, 4.6-19.6)

Quantitative variables were expressed as the mean values ±SD (median, range).

TABLE 2. Characteristics of the patients with or without high-risk varices.

	Varices with high risk of bleeding		
	Absent (n=38)	**Present (n=13)**	**p value**
Age (years)	63.5 (23-78)	72 (31-82)	NS
Gender (male/female)	22/16	11/2	NS
AST (IU/L)	44 (20-328)	48 (27-68)	NS
ALT (IU/L)	44 (10-310)	37 (18-86)	NS
γ-GTP (IU/L)	53.5 (12-259)	31 (12-159)	NS
ALP (IU/L)	268 (133-556)	266 (191-462)	NS
Total bilirubin (mg/dL)	0.7 (0.3-2.1)	1.1 (0.6-1.4)	0.049
Albumin (g/dL)	3.71±0.40	3.53±0.27	NS
Prothrombin time (%)	85.9±10.6	82.3±9.5	NS
White blood cell (mm^3)	4370 (1650-9980)	3180 (1850-5680)	NS
Hemoglobin (g/dL)	12.7±1.7	11.5±1.4	0.032
Platelet ($x10^4/mm^3$)	11.3±3.3	9.3±3.1	NS
AAR	1.05 (0.74-2.01)	1.41 (0.79-2.47)	0.020
FIB-4	1.26 (0.38-3.45)	1.60 (0.92-3.71)	0.032
APRI	1.34 (0.68-8.42)	1.46 (0.92-2.68)	NS

Quantitative variables were expressed as the mean values ±SD and those with an abnormal distribution were expressed as the median values (range). AAR: AST-to-ALT ratio, APRI: Ast-to-platelet count ratio index.

ACKNOWLEDGMENTS

Supported by a Grant-in-aid for Researchers, Hyogo College of Medicine.

REFERENCES

1. **North-Italian Endoscopic Club for the study and treatment of esophageal varices: P**rediction of the first variceal hemorrhage in patients with cirrhosis of the liver and esophageal varices. N Engl J Med 1988; 319:983-989.
2. **Pilette C, Oberti F, Aubé C, et al.:** Non-invasive diagnosis of esophageal varices in chronic liver diseases. J Hepatol 1999; 31:867-873.
3. **Burton JR, Liangpunsakul S, Lapidus J, et al.:** Validation of a multivariate model predicting presence and size of varices. J Clin Gastreoenterol 2007; 41:609-615.
4. **De Franchis R:** Non-invasive (and minimally invasive) diagnosis of esophageal varices. J Hepatology 2008; 49:520-527.
5. **Sebastiani G, Tempesta D, Fattovich G, et al.:** Prediction of esophageal varices in hepatic cirrhosis by simple serum non-invasive markers: Results of a multicenter, large-scale study. J Hepatology 2010; 53:630-638.
6. **Sakai Y, Enomoto H, Aizawa N, et al.:** Relationship between elevation of glycated albumin to glycated hemoglobin ratio in patients with a high bleeding risk of esophageal varices. Hepato-gastroenterol 2012; 59(119):2280-2284.
7. **Giannini E, Botta F, Fasoli A, et al.:** Progressive liver functional impairment is associated with an increase in AST/ALT ratio. Dig Dis Sci 1999; 44:1249-1253.
8. **Vallet-Pichard A, Mallet V, Nalpas B, et al.:** FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. Comparison with liver biopsy and fibrotest. Hepatology 2007; 46:32-36.
9. **Wai CT, Greenson JK, Fontana RJ, et al.:** A simple noninvasive index can predict both significant fibrosis and cirrhosis inpatients with chronic hepatitis C. Hepatology 2003; 38:518-526.
10. **Paquet KJ:** Prophylactic endoscopic sclerosing treatment of esophageal wall in varices: a prospective controlled trial. Endoscopy 1982; 14:4-5.
11. **The French METAVIR cooperative study group:** Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology 1994; 20:15-20.
12. **De Franchis R:** Evolving consensus in portal hypertension report of the Baveno IV consensus workshop on methodology of diagnosis and therapy in portal hypertension. J Hepatol 2005; 43:167-176.
13. **Garcia-Tsao G, Joseph L:** Management and treatment of patients with cirrhosis and portal hypertension: Recommendations from the Department of Veterans Affairs Hepatitis C Resource Center Program and the National Hepatitis C Program. Am J Gastroenterol 2009; 104:1802-1829.
14. **Runyon BA:** Management of adult patients with ascites due to cirrhosis: an update. Hepatology 2009; 49:2087-2107.
15. **Moore KP, Aithal GP:** Guidelines on the management of ascites in cirrhosis. Gut 2006; 55(6):vi1-12.
16. **Madhotra R, Mulcahy HE, Willner I, Reuben A:** Prediction of esophageal varices in patients with cirrhosis. J Clin Gastroenterol 2002; 34:81-85.
17. **Zein CO, Lindor KD, Angulo P:** Prevalence and predictors of esophageal varices in patients with primary sclerosing cholangitis. Hepatology 2004; 39:204-210.
18. **Zhang C, Thabut D, Kamath PS, Shah VH:** Esophageal var ices in cirrhotic patients: from variceal screening to primary prophylaxis of the first esophageal variceal bleeding. Liver Int 2010; 31:108-119.
19. **Aizawa N, Enomoto H, Imanishi H, et al.:** Elevation of the glycated albumin to glycated hemoglobin ratio during the progression of HCV-related liver fibrosis. World J Hepatol 2012; 27:4:11-17.
20. **Bando Y, Kanehara H, Aoki K, et al.:** The glycated albumin to HbA1c ratio is increasing along with fibrosis stage in non-alcoholic steatohepatitis. Ann Clin Biochem 2012 (in press).

ANNALS of Hepatology

ORIGINAL ARTICLE

May-June, Vol. 12 No.3, 2013: 471-478

Association of amino acid imbalance with the severity of liver fibrosis and esophageal varices

Hirayuki Enomoto, Yoshiyuki Sakai, Nobuhiro Aizawa, Yoshinori Iwata, Hironori Tanaka, Naoto Ikeda, Kunihiro Hasegawa, Kazunori Yoh, Akio Ishii, Tomoyuki Takashima, Kazunari Iwata, Masaki Saito, Hiroyasu Imanishi, Hiroko Iijima, Shuhei Nishiguchi

Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo, 663-8501, Japan.

ABSTRACT

Background. The relationships between the metabolic parameters and the endoscopic findings of esophageal varices have been poorly investigated. We investigated the association of the branched-chain amino acids to tyrosine ratio (BTR) with the severity of liver fibrosis and esophageal varices. **Material and methods.** We studied hepatitis C virus (HCV)-positive chronic liver disease patients who had undergone liver biopsy (n = 149). The relationship between the BTR values and the liver fibrotic stage was investigated. We also studied whether the BTR value was associated with the presence and bleeding risk of varices in patients with HCV-related compensated cirrhosis. **Results.** The mean values of the BTR decreased with the progression of the fibrosis (METAVIR score: F0-1: 6.40 ± 1.19; F2: 5.85 ± 1.33; F3: 5.24 ± 0.97, F4: 4.78 ± 1.14). In the 58 patients with HCV-related compensated cirrhosis, the mean values of the BTR decreased with the severity of varices (patients without varices: 5.01 ± 1.15, patients with a low-risk varices: 4.42 ± 1.06, patients with a high-risk varices: 3.86 ± 1.02). The BTR value was significantly lower in the patients with varices than in those without varices (4.17 ± 1.07 *vs.* 5.01 ± 1.15, P < 0.01). The BTR value was also significantly lower in the patients with a high risk of hemorrhage than in those with a low risk (3.86 ± 1.02 *vs.* 4.78 ± 1.14, P < 0.01). Furthermore, the BTR value was the most significantly different parameter, with the smallest P-value among all the factors examined, including the platelet count and albumin level. **Conclusion.** A decreased BTR value was found to be associated with the progression of liver fibrosis and severity of varices.

Key words. Portal hypertension. Biomarker. Branched-chain amino acids to Tyr ratio. Metabolism.

INTRODUCTION

In patients with liver cirrhosis, variceal hemorrhage due to portal hypertension is a severe complication that can occasionally cause an unfavorable prognosis. Three factors have been identified as conditions that are associated with a high risk of variceal bleeding: a large variceal size, red signs on the varices, and advanced liver disease (Child-Pugh class B or C status).[1] Since esophagogastroduodenoscopy (EGD) is the gold standard method of evaluating variceal size and the presence of red signs, EGD should be performed in all cirrhotic patients.[2,3]

Despite the importance of EGD examination, this uncomfortable and expensive method is sometimes difficult for patients to accept. Therefore, several noninvasive biomarkers of liver fibrosis have so far been proposed to predict the presence and bleeding risk of varices in cirrhotic patients,[4-6] because the progression of liver fibrosis is thought to be a major cause of portal hypertension. The major target population for a screening test should be the compensated cirrhotic patients without any clinical symptoms, rather than patients with obvious cirrhosis-related symptoms.[6] However, in the case of cirrhotic patients with well-maintained liver function (Child-Pugh class A), there are few clinical parameters that have been reported to be related to the presence of treatment-requiring high-risk varices.

Correspondence and reprint request: Hirayuki Enomoto, M.D. & Ph.D.
Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo, 663-8501, Japan.
Tel.: +81-798-45-6472. Fax: +81-798-45-6474
E-mail: enomoto@hyo-med.ac.jp

Manuscript received: October 17, 2012.
Manuscript accepted: December 03, 2012.

In particular, the relationships between the metabolic parameters and the endoscopic findings of esophageal varices have up to now been poorly investigated, although the liver plays a central role in various functions associated with the metabolism.

It is well known that cirrhotic patients have low serum levels of branched-chain amino acids (BCAA), such as valine, leucine and isoleucine, and high serum levels of aromatic amino acids (AAA) such as tyrosine (Tyr) and phenylalanine. Therefore, the decreased serum ratio of BCAA and AAA (the Fisher's ratio) in cirrhotic patients has been used as an indicator of an amino acid imbalance.[7-9] The ratio of the BCAA and Tyr level (BCAA to Tyr ratio: BTR) has also been reported to decline in patients with liver cirrhosis, and the BTR can therefore be used as an easily measurable and inexpensive parameter to assess the amino acid imbalance, and is currently used in Japan.[10,11]

In the present study, we investigated the relationship between the histological grading of liver fibrosis and the value of BTR in patients with HCV-positive CLD. We further analyzed the values of BTR in HCV-related compensated cirrhotic patients with Child-Pugh class A status.

MATERIAL AND METHODS

In order to examine the relationship between the BTR value and the progression of liver fibrosis in CLD patients, we studied 149 patients with HCV-related disease who had undergone a percutaneous liver biopsy from January 2008 to July 2011. The HCV infection was diagnosed by the detection of HCV antibodies and HCV-RNA in serum, and liver biopsy examinations were performed using the standard techniques. All liver samples were evaluated by well-trained pathologists at this institute, with an evaluation of the fibrosis stage and activity grade. Fibrosis was staged on a scale of F0-F4 (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis with rare septa; F3: numerous septa without cirrhosis. F4: liver cirrhosis) according to the METAVIR scoring system.[12] The histological evaluation of the biopsy samples was also routinely performed in our department. All authors participated in the conferences about the histological evaluation, and the final results were confirmed by two authors (HE and HI) who received training for histological studies.

EGD was routinely performed in CLD outpatients at our institution according to the standard techniques. Esophageal varices detected by EGD was graded as I-IV according to the Paquet grading system,[13] and the presence of red signs on the varices was also evaluated. Patients with large varices (grade III-IV) or small varices with red signs were categorized to be treatment-requiring (high-risk) varices. All HCV-related compensated (Child-Pugh class A) cirrhotic patients admitted to our department for the treatment of esophageal varices from January 2008 to July 2011 were included in the present study as the high-risk varices group. Liver cirrhosis as the cause of portal hypertension was diagnosed by histological criteria and/or by the clinical (laboratory, endoscopic and/or ultrasonographic) findings.

In order to collect the BTR values in the compensated (Child-Pugh class A) cirrhotic patients without high-risk varices, we enrolled consecutive HCV-positive CLD patients who had been diagnosed with cirrhosis (F4 stage) by liver biopsy, but who did not have treatment-requiring risky varices. The evaluation by EGD was performed within two months of the liver biopsy. The cirrhotic patients who did not have high-risk varices were divided into two groups; patients without detectable varices were classified as the no varices group and patients with small varices without red signs were classified as the low-risk varices group.

All blood samples were obtained on the day of liver biopsy or endoscopic treatment for esophageal varices, and patients without a complete data set on the day of the liver biopsy or the endoscopic treatment were excluded from the study. Patients with the following conditions were also excluded from the study: the presence of other liver diseases, using immunosuppressive therapy, hepatitis B virus co-infection and cases with insufficient liver tissue available for the evaluation of liver fibrotic staging. The study conformed to the ethical guidelines of the Helsinki declaration, and was approved by the ethics committees of the institutional review board. Written informed consent was obtained from all patients on admission.

Statistical analysis

In the present study, we attempted to clarify whether the BTR value was associated with the progression of liver fibrosis in HCV-related CLD. The data for the comparisons among the groups F0-1 *vs.* F2 *vs.* F3 *vs.* F4 were analyzed by a non-repeated measurements ANOVA, and statistical significance was further examined with the Bonferroni correction. We also investigated whether the BTR values diffe-

red among the three groups (no varices group, low-risk varices group and high-risk varices group). The data for the comparisons among the groups were analyzed by a non-repeated measurements ANOVA with a subsequent Bonferroni correction. In addition, we investigated whether the BTR differed between the groups with or without varices, and the differences in the baseline characteristics of the two groups were also investigated. We also evaluated whether the BTR values were different between the groups with and without high-risk (treatment-requiring) varices. Numerical variables with a normal distribution were expressed as the mean values ± SD, and the statistical significance of differences between two groups was evaluated using Student's t-test. Numerical variables with an abnormal distribution were expressed as the median values (range) and the statistical analysis between two groups was done using the Mann-Whitney U test.

RESULTS

The BTR values decreased with the progression of liver fibrosis in patients with HCV-related CLD

A total of 149 patients with HCV-related CLD were included in this study to investigate the association between the BTR value and the histological progression of liver fibrosis, based on the criteria described in the Material and Methods section. The characteristics of the enrolled patients are summarized in table 1. The population included 63 (42%) males and 86 (58%) females, and the age of patients ranged from 23 to 83 years of age (median 61). The METAVIR liver fibrosis staging 12 showed that 92 (62%) patients had significant fibrosis (F2-F4), 75 (50%) patients had severe fibrosis (F3-F4) and 44 (30%) had cirrhosis (F4). Five out of a total of 149 patients were administered BCAA, and all five patients were histologically diagnosed to have cirrhosis by liver biopsy.

Table 1. Characteristics of the 149 HCV-positive patients who received liver biopsy.

Age (years)	61 (23-83)
Gender (male/female)	63/86
AST (IU/L)	38 (13-328)
ALT (IU/L)	35 (8-315)
γ-GTP (IU/L)	32 (8-306)
ALP (IU/L)	222 (97-556)
Total bilirubin (mg/dL)	0.8 (0.1-2.1)
Albumin (g/dL)	3.94 ± 0.36
Prothrombin time (%)	91.6 ± 9.8
White blood cell (mm^3)	4,200 (1,440-9,980)
Hemoglobin (g/dL)	13.3 ± 1.8
Platelet (x $10^4/mm^3$)	15.6 ± 5.7
BCAA treatment (present/absent)	5/144
Fibrosis stage: F0-1/F2/F3/F4	57/17/31/44

BCAA: branched-chain amino acids. Quantitative variables were expressed as the mean values ± SD or median (range).

The values of the BTR were decreased with the progression of the fibrosis (Figure 1), indicating that the value of the BTR is associated with the degree of liver fibrosis in HCV-associated CLD patients.

Characteristics of compensated cirrhotic patients and clinical data

Since several biomarkers of liver fibrosis were reported to be associated with the presence and/or bleeding risk of esophageal varices,[4-6] we examined whether the values of BTR were related to the severity of esophageal varices in patients with HCV-related compensated cirrhosis (Figure 2). In order to

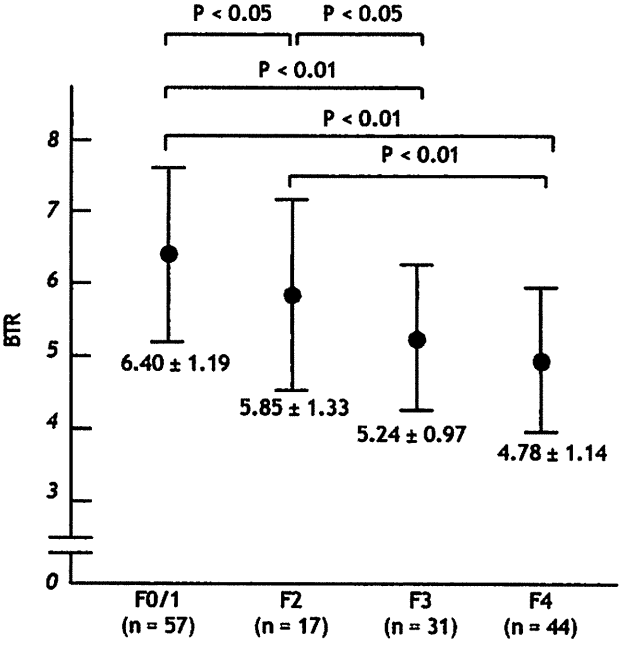

Figure 1. The values of BTR in relation to the METAVIR histological grading for liver fibrosis in patients with HCV-positive disease. The BTR value decreased as the fibrosis progressed. There was a significant difference between the F0/1 vs. F4, F3 and F2 groups. There was also a significant difference between the F2 vs. F4 and F2 vs. F3 groups.

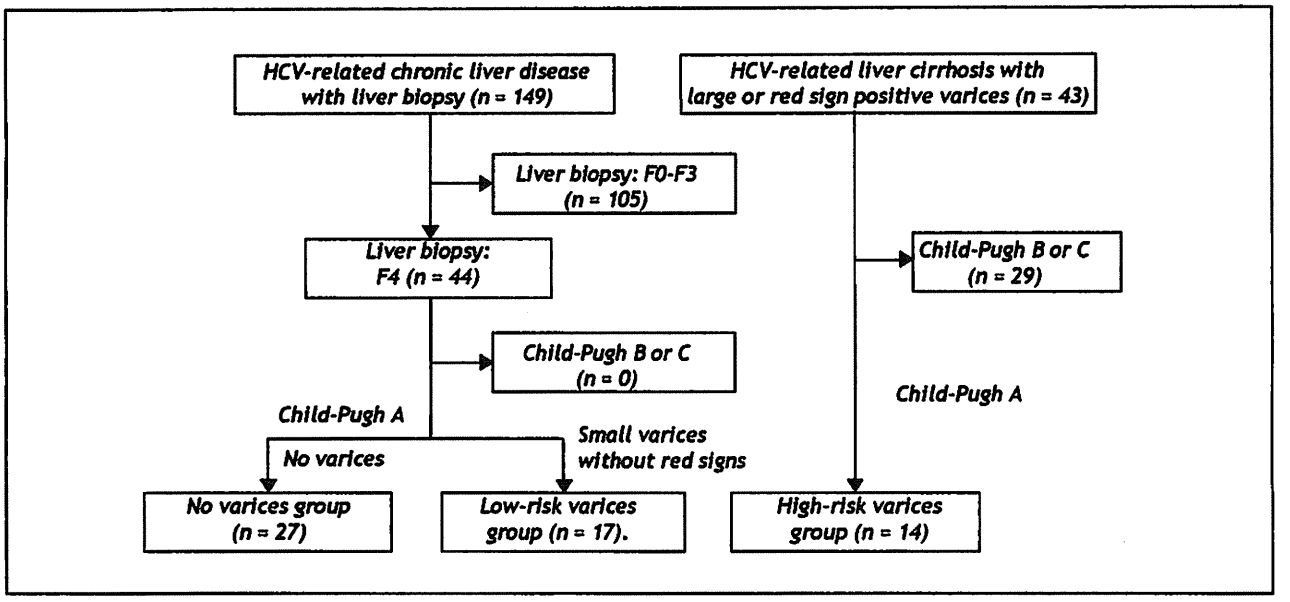

Figure 2. Algorithm for the classification of the patients with HCV-related compensated (Child-Pugh class A) cirrhosis. The 44 patients with a METAVIR score of F4 (shown in figure 1) were categorized as compensated cirrhotic patients with a low risk of variceal bleeding; 27 patients were diagnosed with liver cirrhosis without detectable varices (no varices group) and the remaining 17 patients had small varices without red signs (low-risk varices group). Of the CLD patients in our department, 43 HCV-positive patients were found to have a high risk of variceal bleeding because they had large varices (grade III-IV) or small varices with red signs. In these HCV-related cirrhotic patients, the 14 patients with Child-Pugh class A were categorized as the HCV-related compensated cirrhotic patients with a high risk of variceal hemorrhage (high-risk varices group).

ascertain the characteristics of compensated cirrhotic patients with a low risk of variceal bleeding, we examined the 44 patients who were categorized as compensated cirrhotic patients (44 patients with a METAVIR score of F4, as shown in figure 1) with a low risk of variceal bleeding; 27 patients were diagnosed with liver cirrhosis without detectable varices (no varices group) and the remaining 17 patients had small varices without red signs (low-risk varices group). Of the CLD patients in our department, 43 HCV-positive patients were found to have a high risk of variceal bleeding because they had large varices (grade III-IV) or small varices with red signs. In these HCV-related cirrhotic patients, the Child-Pugh classification was grade A in 14 patients, grade B in 27 patients and grade C in two patients. The 14 patients with Child-Pugh class A were categorized as the HCV-related compensated cirrhotic patients with a high risk of variceal hemorrhage (high-risk varices group).

The characteristics of all 58 compensated cirrhotic patients (27 patients in the no varices group, 17 patents in the low-risk varices group, and 14 patients in the high-risk varices group) are shown in table 2. The population consisted of 30 males (52%) and 28 females (48%), and the age of patients ranged from 23 to 82 years old (median 64.5). BCAA were administrated to none of the 27 patients (0%) in the no varices group, 5 of 17 patients (29%) in the low-risk varices group, and 5 of 14 (36%) patients in the high-risk varices group.

Table2. Characteristics of the total 58 patients with HCV-positive compensated cirrhosis.

Age (years)	64.5 (23-82)
Gender (male/female)	30/28
AST (IU/L)	45.5 (25-328)
ALT (IU/L)	38.5 (12-315)
γ-GTP (IU/L)	44.5 (12-259)
ALP (IU/L)	267.5 (133-556)
Total bilirubin (mg/dL)	0.8 (0.3-2.1)
Albumin (g/dL)	3.67 ± 0.39
Hemoglobin (g/dL)	12.3 ± 2.0
Platelet ($x10^4/mm^3$)	12.7 ± 3.3
Prothrombin time (%)	84.6 ± 9.3
BCAA treatment (present/absent)	10/48
Severity of varices (no/low-risk/high-risk)	27/17/14

BCAA: branched-chain amino acids. BTR: BCAA to tyrosine ratio. Quantitative variables were expressed as the mean values ± SD or median (range).

The BTR value is associated with the severity of varices in patients with HCV-related cirrhosis

Irrespective of the higher ratio of BCAA-treated patients, the values of the BTR were decreased as the bleeding risk increased, suggesting that the low BTR value was correlated with the severity of portal hypertension (Figure 3).

We also examined the differences in the BTR values between the patients with varices and patients without varices. Comparing the 31 patients with varices (the high-risk varices group and the low-risk varices group) and 27 patients without varices (the no varices group), the BTR value was significantly lower in patients with varices than that in patients without varices, although the percentage of BCAA-treated patients was significantly higher in the group with varices than in the patients without varices, suggesting that the decreased BTR values was associated with the presence of varices (Figure 4A). The low BTR value was found to be significantly different in the presence of varices, as well as a higher ALT value and lower albumin and hemoglobin values (Table 3).

When we compared the 14 patients in the high-risk varices group with the 44 patients in the no varices group or the low-risk varices group, the BTR value was significantly lower in the patients in the high-risk varices group, although the percentage of BCAA-treated patients was higher in the patients with high-risk varices than in patients without high-risk varices (Figure 4B), thus suggesting that a low BTR value is related to an increased risk of variceal hemorrhage. We also found a significantly higher ALT and lower hemoglobin level in the high-risk varices group. Interestingly, the BTR value showed the smallest p-value ($P = 0.0093 < 0.01$) among all of the parameters examined (Table 4).

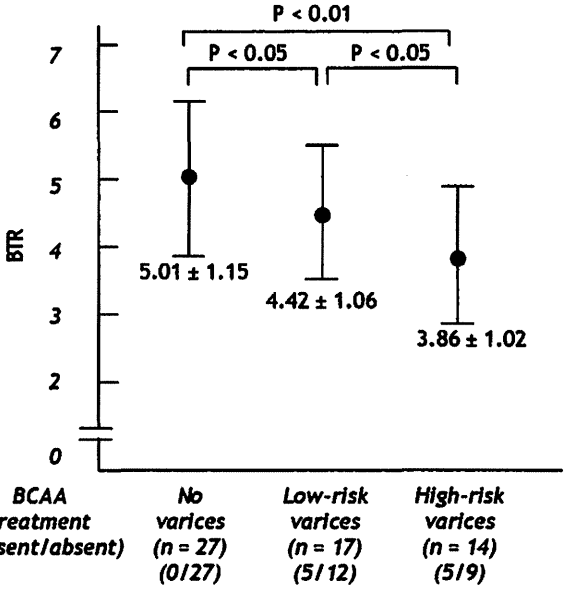

Figure 3. The values of the branched-chain amino acids to Tyr ratio (BTR) in patients with HCV-related compensated (Child-Pugh class A) cirrhosis. The BTR values were reciprocally decreased as the bleeding risk of esophageal varices increased. There was a significant difference between the no varices group vs. the low-risk varices group, the no varices group vs. the high-risk varices group and the low-risk varices group vs. the high-risk varices group.

Table 3. Characteristics of the compensated cirrhotic patients with or without varices.

	Varices detected by EGD		
	Absent (n = 27)	Present (n = 31)	P-value
Age (years)	65 (23-77)	64 (31-82)	NS
Gender (male/female)	10/17	20/11	NS
AST (IU/L)	47 (25-328)	43 (27-251)	NS
ALT (IU/L)	49 (12-315)	35 (18-160)	< 0.05
γ-GTP (IU/L)	52 (14-259)	44 (12-242)	NS
ALP (IU/L)	269 (133-494)	266 (177-556)	NS
Total bilirubin (mg/dL)	0.7 (0.3-1.7)	1.0 (0.3-2.1)	NS
Albumin (g/dL)	3.85 ± 0.31	3.52 ± 0.39	< 0.01
Hemoglobin (g/dL)	13.0 ± 2.0	11.6 ± 1.8	< 0.05
Platelet (x10^4/ mm^3)	12.6 ± 4.0	12.0 ± 7.4	NS
Prothrombin time (%)	86.9 ± 9.2	82.6 ± 9.0	NS
BCAA treatment (present /absent)	0/27	10/21	< 0.01
BTR	5.01 ± 1.15	4.17 ± 1.07	< 0.01

BCAA: branched-chain amino acids. BTR: BCAA to tyrosine ratio. Quantitative variables were expressed as the mean values ± SD or median (range).

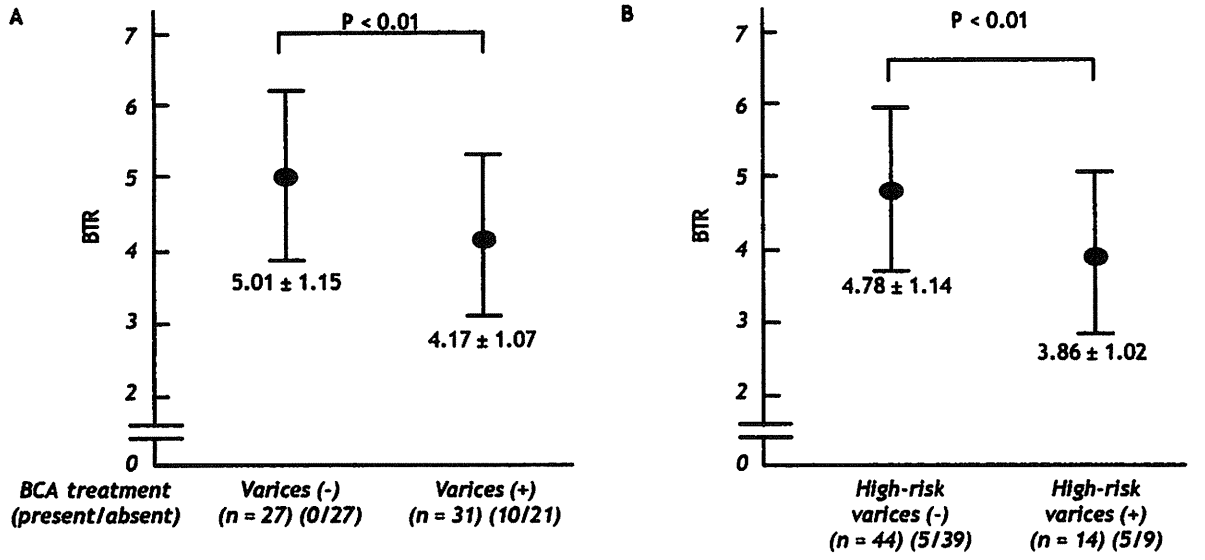

Figure 4. The decreased values of the branched-chain amino acids to Tyr ratio (BTR) in relation to the severity of varices in patients with HCV-related compensated (Child-Pugh class A) cirrhosis. A. The branched-chain amino acids to Tyr ratio (BTR) in patients with HCV-related compensated (Child-Pugh class A) cirrhosis with or without varices. A comparison between the 31 patients with varices (in the high-risk varices group or the low-risk varices group) and the 27 patients in the no varices group is shown. The BTR value was significantly lower in the patients with varices than that in the patients without varices. B. The branched-chain amino acids to Tyr ratio (BTR) in patients with HCV-related compensated (Child-Pugh class A) cirrhosis with or without high-risk varices. A comparison between the 14 patients in the high-risk varices group and the 35 patients without high-risk varices (in either the no varices group or the low-risk varices group) is shown. The BTR ratio was significantly lower in patients with high-risk varices than that in patients without high-risk varices.

DISCUSSION

EGD is the most reliable method to determine whether a cirrhotic patient should receive a prophylactic treatment for variceal hemorrhage.[2,3] However, patients with a well-maintained liver function (Child-Pugh class A) usually do not have any clinical symptoms, sometimes leading to a negative attitude toward undergoing the EGD examination. Therefore, it should be attractive to find a biomarker which is related to the presence and the bleeding risk of varices even in compensated (asymptomatic) cirrhotic patients.

Although several biomarkers of liver fibrosis have been reported to predict the presence of varices,[4-6] there have been few studies that have investigated the relationships between the endoscopic variceal findings and the fibrosis-related markers in compensated cirrhotic patients with well-maintained liver function (Child-Pugh class A status). In particular, there have been few studies that have investigated the relationship between the metabolic parameters and the severity of varices in compensated cirrhotic patients.

We previously examined several biomarkers, including the AST-to-ALT ratio (AAR),[14] the FIB-4 indices[15] and the AST-to-platelet count ratio index (APRI)[16] in HCV-related cirrhotic patients with Child-Pugh class A status, and reported that the AAR was associated with both with presence and the bleeding risk of varices.[17] In addition, we examined the two glycated proteins (glycated albumin: GA and glycated hemoglobin: HbA1c) proteins in patients with CLD, and reported that the GA/HbA1c ratio was correlated with the severity of the esophageal varices in HCV-related cirrhotic patients.[18] We also reported that this ratio (the GA/HbA1c ratio) was increased with the progression of liver fibrosis in patients with HCV-related CLD and non-alcoholic steatohepatitis.[19,20]

In the present study, we focused on the amino acid imbalance in CLD and found the BTR to be decreased with the severity of varices. Amino acid imbalances are well-known metabolic disorders in patients with CLD, and a previous report examined the relationship between the progression of liver fibrosis and the BTR value in CLD patients with various etiologies, including HCV infection, HBV

Table 4. Characteristics of the compensated cirrhotic patients with or without high-risk varices.

	High risk varices detected by EGD		
	Absent (n = 44)	Present (n = 14)	P-value
Age (years)	64 (23-78)	71.5 (31-82)	NS
Gender (male/female)	18/ 26	12/2	NS
AST (IU/L)	44 (25-328)	47.5 (27-68)	NS
ALT (IU/L)	45.5 (12-315)	35.5 (18-86)	< 0.05
γ-GTP (IU/L)	51 (12-259)	29.5 (12-159)	NS
ALP (IU/L)	262.5 (133-556)	281 (191-462)	NS
Total bilirubin (mg/dL)	0.8 (0.3-2.1)	1.0 (0.6-1.4)	NS
Albumin (g/dL)	3.71 ± 0.41	3.56 ± 0.27	NS
Hemoglobin(g/dL)	12.6 ± 2.0	11.2 ± 1.7	< 0.05
Platelet ($x10^4/mm^3$)	12.6 ± 5.4	11.2 ± 7.6	NS
Prothrombin time (%)	85.2 ± 9.3	82.7 ± 9.2	NS
BCAA treatment (present/absent)	5/39	5/9	NS
BTR	4.78 ± 1.14	3.86 ± 1.02	< 0.01

BCAA: branched-chain amino acids. BTR: BCAA to tyrosine ratio. Quantitative variables were expressed as the mean values ± SD or median (range).

infection, autoimmune hepatitis and cryptogenic hepatitis.[21] Furthermore, Habu, *et al.*[22] showed that there was an association between a low BTR value and the presence of a porto-systemic shunt in patients with compensated liver cirrhosis. We herein showed that the value of BTR decreased with the progression of liver fibrosis and with the severity of varices in HCV-related CLD patients, and these results were in agreement with previous reports regarding CLD patients with various etiologies.[21,22] Interestingly, despite the fact that the ratio of BCAA-treated patients was increased with the severity of varices, the BTR value was inversely decreased with the severity. Therefore, if none of the patients had received the BCAA treatment, the decrease of the BTR value with the progression of liver fibrosis and the severity of varices would have been even more remarkable. Our findings suggest that the BTR value is a unique parameter that is strongly associated with various clinical conditions in patients with HCV-positive CLD, including the degree of liver fibrosis and the severity of varices, as well as the amino acid imbalance. It is interesting to note that recent reports.[23,24] suggest that the values of BTR are associated with the prognosis of HCC. Although the main focus of the disorders of amino acid metabolism has been on patients with cirrhosis, an evaluation of the amino acid imbalance by measuring the BTR should provide new information with regard to the various clinical statuses in patients with CLD, as well as in cirrhotic patients.

It is of interest to clinicians to determine cut-off values of the BTR that can discriminate between compensated cirrhotic patients with or without varices and with or without high-risk varices. We performed the receiver operating characteristic (ROC) analyses and obtained possible cut-off values (BTR values of 4.39 and 3.93, respectively). However, it was not easy to identify the cut-off values of the BTR that had a sufficient diagnostic performance, perhaps because of the relatively small number of patients in the present study. Therefore, further investigations in a larger number of patients are needed.

In summary, we showed that the BTR value is associated with the progression of liver fibrosis and the severity of esophageal varices. The liver plays an important role in many aspects of metabolism, and various metabolic disorders involving glucose, amino acids and lipids are observed in patients with CLD.[25,26] Therefore, it would be interesting and important to investigate the metabolic parameters, including the GA/HbA1c ratio and the BTR value, for their correlations with the presence of varices or the bleeding risk of varices in a larger number of patients.

ABBREVIATIONS

- **AAA:** aromatic amino acids.
- **BCAA:** branched-chain amino acids.
- **BTR:** BCAA to Tyr ratio.
- **CLD:** chronic liver disease.
- **EGD:** esophagogastroduodenoscopy.

FINANCIAL SUPPORT

Supported by a Grant-in-Aid for Health and Labor Sciences Research from the Ministry of Health, Labour, and Welfare of Japan.

REFERENCES

1. North-Italian Endoscopic Club for the study and treatment of oesophageal varices. Prediction of the first variceal hemorrhage in patients with cirrhosis of the liver and oesophageal varices. *N Engl J Med* 1988; 319: 983-9.
2. Garcia-Tsao G, Joseph L. Management and treatment of patients with cirrhosis and portal hypertension: Recommendations from the Department of Veterans Affairs Hepatitis C Resource Center Program and the National Hepatitis C Program. *Am J Gastroenterol* 2009; 104: 1802-29.
3. de Franchis R; Baveno V Faculty. Revising consensus in portal hypertension: report of the Baveno V consensus workshop on methodology of diagnosis and therapy in portal hypertension. *J Hepatol* 2010; 53: 762-8.
4. Sebastiani G, Tempesta D, Fattovich G, Castera L, Halfon P, Bourliere M, Noventa F, et al. Prediction of oesophageal varices in hepatic cirrhosis by simple serum non-invasive markers: Results of a multicenter, large-scale study. *J Hepatology* 2010; 53: 630-8.
5. de Franchis R. Non-invasive (and minimally invasive) diagnosis of oesophageal varices. *J Hepatology* 2008; 49: 520-7.
6. Thabut D, Moreau R, Lebrec D. Noninvasive assessment of portal hypertension in patients with cirrhosis. *Hepatology* 2011; 53: 683-94.
7. Fischer JE, Rosen HM, Ebeid AM, James JH, Keane JM, Soeters PB. The effect of normalization of plasma aminoacids on hepatic encephalopathy in man. *Surgery* 1976; 80: 77-91.
8. James JH, Ziparo V, Jeppsson B, Fischer JE. Hyperammonaemia, plasma aminoacid imbalance, and blood-brain aminoacid transport: a unified theory of portal-systemic encephalopathy. *Lancet* 1979; 2: 772-5.
9. Fischer JE. Branched-chain-enriched amino acid solutions in patients with liver failure: an early example of nutritional pharmacology. *J Parenter Enteral Nutr* 1990; 14: 249S-256S.
10. Azuma Y, Maekawa M, Kuwabara Y, Nakajima T, Taniguchi K, Kanno T. Determination of branched-chain amino acids and tyrosine in serum of patients with various hepaticdiseases, and its clinical usefulness. *Clin Chem* 1989; 35: 1399-403.
11. Suzuki K, Suzuki K, Koizumi K, Ichimura H, Oka S, Takada H, Kuwayama H. Measurement of serum branched-chain amino acids to tyrosine ratio level is useful in a prediction of a change of serum albumin level in chronic liver disease. *Hepatol Res* 2008; 38: 267-72.
12. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology* 1994; 20: 15-20.
13. Paquet KJ. Prophylactic endoscopic sclerosing treatment of esophageal wall in varices: a prospective controlled trial. *Endoscopy* 1982; 14: 4-5.
14. Giannini E, Botta F, Fasoli A, Ceppa P, Risso D, Lantieri PB, Celle G, et al. Progressive liver functional impairment is associated with an increase in AST/ALT ratio. *Dig Dis Sci* 1999; 44: 1249-53.
15. Vallet-Pichard A, Mallet V, Nalpas B, Verkarre V, Nalpas A, Dhalluin-Venier V, Fontaine H, et al. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. Comparison with liver biopsy and fibrotest. *Hepatology* 2007; 46: 32-6.
16. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis inpatients with chronic hepatitis C. *Hepatology* 2003; 38: 518-26.
17. Iwata Y, Enomoto H, Sakai Y, Aizawa N, Tanaka H, Ikeda N, Takashima T, et al. Elevation of the AST to ALT ratio in association with the severity of esophageal varices in patients with HCV-related compensated liver cirrhosis. *Hepatogastroenterology* [In press].
18. Sakai Y, Enomoto H, Aizawa N, Iwata Y, Tanaka H, Ikeda N, Takashima T, et al. Relationship between Elevation of Glycated Albumin to Glycated Hemoglobin Ratio in Patients with a High Bleeding Risk of Esophageal Varices. *Hepatogastroenterology* [In press].
19. Aizawa N, Enomoto H, Imanishi H, Saito M, Iwata Y, Tanaka H, Ikeda N, et al. Elevation of the glycated albumin to glycated hemoglobin ratio during the progression of HCV-related liver fibrosis. *World J Hepatol* 2012; 4: 11-7.
20. Bando Y, Kanehara H, Aoki K, Toya D, Notsumata K, Tanaka N, Enomoto H, et al. The glycated albumin to HbA1c ratio is increasing along with fibrosis stage in non-alcoholic steatohepatitis. *Ann Clin Biochem* 2012; 49(Pt. 4): 387-90.
21. Michitaka K, Hiraoka A, Kume M, Uehara T, Hidaka S, Ninomiya T, Hasebe A, et al. Amino acid imbalance in patients with chronic liver diseases. *Hepatol Res* 2010; 40: 393-8.
22. Habu D, Nishiguchi S, Nakatani S, Kawamura E, Ishizu H, Lee C, Ohfuji S, et al. Relationship between branched-chain amino acid to tyrosine ratio (BTR) and porto-systemic shunt in the Child-Pugh grade A cirrhosis determined by per-rectal portal scintigraphy. *Hepatol Res* 2003; 27: 57-61.
23. Nakamura Y, Mizuguchi T, Kawamoto M, Meguro M, Harada K, Ota S, Hirata K. Cluster analysis of indicators of liver functional and preoperative low branched-chain amino acid tyrosine ration indicate a high risk of early recurrence in analysis of 165 hepatocellular carcinoma patients after initial hepatectomy. *Surgery* 2011; 150: 250-62.
24. Ishikawa T. Branched-chain amino acids to tyrosine ratio value as a potential prognostic factor for hepatocellular carcinoma. *World J Gastroenterol* 2012; 18: 2005-8.
25. Soeters PB, Fischer JE. Insulin, glucagon, aminoacid imbalance, and hepatic encephalopathy. *Lancet* 1976; 2: 880-2.
26. Oster JR, Perez GO. Acid-base disturbances in liver disease. *J Hepatol* 1986; 2: 299-306.

J Gastroenterol
DOI 10.1007/s00535-013-0830-1

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

New malignant grading system for hepatocellular carcinoma using the Sonazoid contrast agent for ultrasonography

Hironori Tanaka · Hiroko Iijima · Akiko Higashiura · Kazunori Yoh · Akio Ishii · Tomoyuki Takashima · Yoshiyuki Sakai · Nobuhiro Aizawa · Kazunari Iwata · Naoto Ikeda · Yoshinori Iwata · Hirayuki Enomoto · Masaki Saito · Hiroyasu Imanishi · Seiichi Hirota · Jiro Fujimoto · Shuhei Nishiguchi

Received: 24 July 2012 / Accepted: 1 May 2013

Abstract
Background The ultrasonography contrast agent Sonazoid provides parenchyma-specific contrast imaging (Kupffer imaging) based on its accumulation in Kupffer cells. This agent also facilitates imaging of the fine vascular architecture in tumors through maximum intensity projection (MIP). We examined the clinical utility of the malignancy grading system for hepatocellular carcinoma (HCC) using a combination of 2 different contrast-enhanced ultrasonography images.
Methods We studied 121 histologically confirmed cases of HCC (well-differentiated, 45; moderately differentiated, 70; poorly differentiated, 6). The results of Kupffer imaging were classified as (1) iso-echoic pattern or (2) hypo-echoic pattern. The MIP patterns produced were classified into one of the following categories: fine, tumor vessels were not clearly visualized and only fine vessels were visualized; vascular, tumor vessels were visualized clearly; irregular, tumor vessels were thick and irregular. Based on the combined assessment of Kupffer imaging and the MIP pattern, the samples were classified into 4 grades: Grade 1 (iso-fine/vascular), Grade 2 (hypo-fine), Grade 3 (hypo-vascular), and Grade 4 (hypo-irregular).
Results The distribution of moderately and poorly differentiated HCCs was as follows: Grade 1, 4 % (1/24); Grade 2, 52 % (15/29); Grade 3, 85 % (44/52); and Grade 4, 100 % (16/16). The grading system also predicted portal vein invasion in 72 resected HCCs: Grade 1, 0 % (0/4); Grade 2, 13 % (1/8); Grade 3, 23 % (11/48); and Grade 4, 67 % (8/12).
Conclusions This new malignant grading system is useful for estimation of histological differentiation and portal vein invasion of HCC.

Keywords Hepatocellular carcinoma · Contrast enhanced ultrasonography · Sonazoid · Malignant grade

H. Tanaka and H. Iijima contributed equally to this work.

H. Tanaka (✉) · H. Iijima · A. Higashiura · A. Ishii
Ultrasound Imaging Center, Hyogo College of Medicine,
1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
e-mail: hironori@hyo-med.ac.jp

H. Tanaka · H. Iijima · K. Yoh · T. Takashima · Y. Sakai · N. Aizawa · K. Iwata · N. Ikeda · Y. Iwata · H. Enomoto · M. Saito · H. Imanishi · S. Nishiguchi
Division of Hepatobiliary and Pancreas Disease, Department of Internal of Medicine, Hyogo College of Medicine,
1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

S. Hirota
Department of Surgical Pathology, Hyogo College of Medicine,
1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

J. Fujimoto
Division of Hepatobiliary and Pancreatic Disease,
Department of External Medicine, Hyogo College of Medicine,
1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

Introduction

Hepatocellular carcinoma (HCC) represents the most common liver cancer and the third most common cause of cancer-related deaths [1, 2]. Knowledge of the histological grade of differentiation of HCC is useful in establishing a therapeutic strategy and in predicting therapeutic outcome, prognosis [3], and recurrence (especially in the case of internal metastases) [4]. However, tumor biopsy is the only strategy available for obtaining tumor tissue prior to therapy. Performing a biopsy of HCC has traditionally been avoided, because several cases of tumor seeding after biopsy have been reported [5, 6]. The risk of seeding is in

Published online: 30 May 2013

addition to the risk of complications, such as bleeding. Therefore, in order to determine the ideal therapeutic strategy, alternatives to biopsy are required.

The principal methods of diagnosis for HCC are imaging studies such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI). CT and MRI are superior to US in terms of objectivity. However, prediction of histological differentiation of HCC only by contrast-enhanced CT and MRI has limited effectiveness, because most classical HCCs are hyper-vascular in the arterial phase and hypo-vascular in the portal phase. Thus, it is difficult to distinguish histological differentiation by factors such as the vascular structure of the tumor.

Currently, there are only 2 US contrast agents available, Sonazoid and Levovist, which can be used for Kupffer imaging in the post-vascular phase (i.e., 10 min after injecting these agents). Bubbles made from Levovist, the first-generation US contrast agent, are very fragile and are easily collapsed by US emissions. Therefore, Kupffer imaging in the post-vascular phase using Levovist should be performed using a single sweep scan of the liver, which is insufficient for surveillance. However, Sonazoid, a second-generation US contrast agent, is a lipid-stabilized suspension of perfluorobutane and is composed of a hard shell containing bubbles. Due to this structure, microbubbles made from Sonazoid are chemically stable in blood vessels [7–9] and produce stable, non-linear oscillations in the low-power acoustic field. This feature allows Sonazoid to provide detailed perfusion features during imaging in the vascular phase and Kupffer imaging in the post-vascular phase, without bubble collapse. Specifically, Sonazoid is stable for at least 3 h after injection and, since the Sonazoid microbubbles are phagocytosed by liver Kupffer cells, this agent allows for multiple real-time scans. Malignant hepatic tumors, including HCC, contain few or no Kupffer cells, leading to an area clear of contrast material or a perfusion defect in Kupffer imaging. Therefore, perfusion defects seen on Kupffer imaging and the degree of histological malignancy of HCC are correlated [10–12]. Moderately or poorly differentiated HCC requires prompt therapy and Kupffer imaging has been a key imaging modality for the estimation of these histologic grades.

Moreover, using maximum intensity projection (MIP) [13], this type of contrast-enhanced ultrasonography (CEUS) could enable visualization of the fine vascular architecture in tumors, which also has been correlated with the histological differentiation of HCC [14]. The MIP pattern is an image that takes advantage of Sonazoid CEUS characteristics, such as high time and spatial resolution.

Hence, in comparison with other imaging modalities, these 2 imaging techniques, Kupffer imaging and MIP, could provide more relevant information for estimating the malignant grade of HCC. In this study, we examined the clinical utility of the malignant grading system for HCC using a combination of 2 different CEUS images, namely, Kupffer imaging and the MIP pattern.

Methods

Patients

We studied 116 patients with histologically confirmed HCC who were admitted to our institution between January 2008 and October 2010. Eighty patients were male and 36 were female, with a mean age of 69.9 years (range 38–92 years). Most of the 116 patients had a history of chronic liver disease, including hepatitis C virus (HCV) infection in 79 (68.1 %) patients and hepatitis B virus (HBV) infection in 14 (12.1 %) patients. Of the 116 patients included, 23 (19.8 %) were negative for both HCV and HBV. For the 23 patients that were negative for both viral markers, 13 (11 %) patients had alcoholic liver disease, 2 (2 %) patients had autoimmune hepatitis (AIH), 1 (1 %) patient had non-alcoholic steatohepatitis (NASH), and 7 (6 %) patients had cryptogenic hepatitis.

Liver specimens of 72 tumors (obtained from 71 patients who underwent partial hepatectomy) were analyzed in this study. The liver specimens obtained from 71 patients showed 34 patients with cirrhosis and the remaining 37 patients with chronic hepatitis. Presence of portal vein tumor invasion in these resected tumors was diagnosed by histological examination. An additional 49 tumors, obtained by 21-gauge needle core biopsy (Majima needle, Top Surgical Manufacturing, Tokyo, Japan) from 45 patients, were also evaluated for validation of the system. HCCs with regions of varying histological grades were classified as belonging to the predominating histological characteristic. The degree of differentiation was determined according to the International Working Party classification [15]. The final histological diagnoses of the 121 HCCs were as follows: 45 (37 %) well-differentiated, 70 (58 %) moderately differentiated, and 6 (5 %) poorly differentiated. This study was approved by the institutional ethics review board of Hyogo College of Medicine, Hyogo, Japan and all patients provided informed consent.

Sonologists

Two sonologists from our institution, with 20 (HT) and 30 (HI) years of experience in liver US imaging, were involved in this retrospective study. Each sonologist had at least 10 years of experience in microbubble contrast-enhanced US of the liver. They were aware of the patients' clinical histories and were blinded to the biopsy results.

Contrast-enhanced US study

The intravenously injected sonographic contrast agent, Sonazoid (Daiichi Sankyo, Tokyo, Japan; GE Healthcare, Little Chalfont, UK), was used in all studies. The suspension was prepared by vigorously shaking the powder with 2 mL of sterile water for 5–10 s. After the suspension was allowed to stand for 2 min to achieve equilibrium and the dissolution of large bubbles, the suspension was injected into an antecubital vein through a 21-gauge cannula at a speed of 1 mL/s and immediately flushed with 5–10 mL of normal saline.

US equipment included SSA-770A, SSA-790A, and TUS-A500 (Aplio; Toshiba Medical Systems, Tokyo, Japan) with a 3.75-MHz convex transducer (PSK-375BT). The imaging mode was wideband harmonic imaging (commercially called pulse subtraction) with transmission and reception frequencies of 3.75 and 7.5 MHz, respectively. When a suspected lesion was identified, CEUS was performed with the focus depth beyond the lesion of interest using the following settings: frame rate, 15 fps and dynamic range, 35 dB. A low mechanical index (MI) (0.16–0.30) was selected to avoid the disruption of microbubbles.

The region of interest was observed continuously for approximately 3 min from the time of injection. The arterial phase was timed for 45 s after completion of the flash. Approximately 20 min after the injection via the peripheral venous line, the liver was scanned again to observe Kupffer imaging (Fig. 1). Arterial-phase findings and Kupffer imaging were classified as follows: (1) hyper-echoic pattern, (2) iso-echoic pattern, and (3) hypo-echoic pattern.

After the Kupffer imaging was acquired, an MIP pattern was evaluated by reinjection of Sonazoid using micro-flow imaging (MFI), as was introduced by Sugimoto et al. [14]. Briefly, the maximum-hold processing started just after the burst scan. The burst scan consisted of high-MI (1.3–1.6) scanning of 5 frames. Low-MI (0.16–0.30) scanning was started again, just after the MI burst scanning, to visualize fresh microbubble contrast agent flowing into the scanning volume. The maximum intensity holding sequence was started simultaneously with flash replenishment low-MI imaging, which maintained maximum brightness on each pixel and was displayed as a persistent vision. The accumulation time for each MFI sequence was 10–15 s, depending on the perfusion of the target tissue.

MIP classification

The MIP pattern was classified into 1 of the following 3 patterns: (1) fine pattern: where tumor vessels were not clearly visualized and only fine vessels were visualized; (2) vascular pattern: where tumor vessels were visualized clearly; and (3) irregular pattern: where tumor vessels were thick and irregular (Fig. 1). The "tumor vessels" were defined as the vascular pattern in which vessels were obvious to the surrounding fifth or sixth branches. In cases where the vascular pattern was similar to that of the surrounding vessels, the tumor was classified as fine pattern. For accurate diagnose of the MIP pattern, the entire tumor was observed in vascular phase and the most suitable cross-sectional direction was chosen to enable identification of important signs of vascular and irregular patterns.

Malignant grading system

The combination of Kupffer imaging and MIP patterns classified HCCs into 4 grades: Grade 1 (iso-fine/vascular),

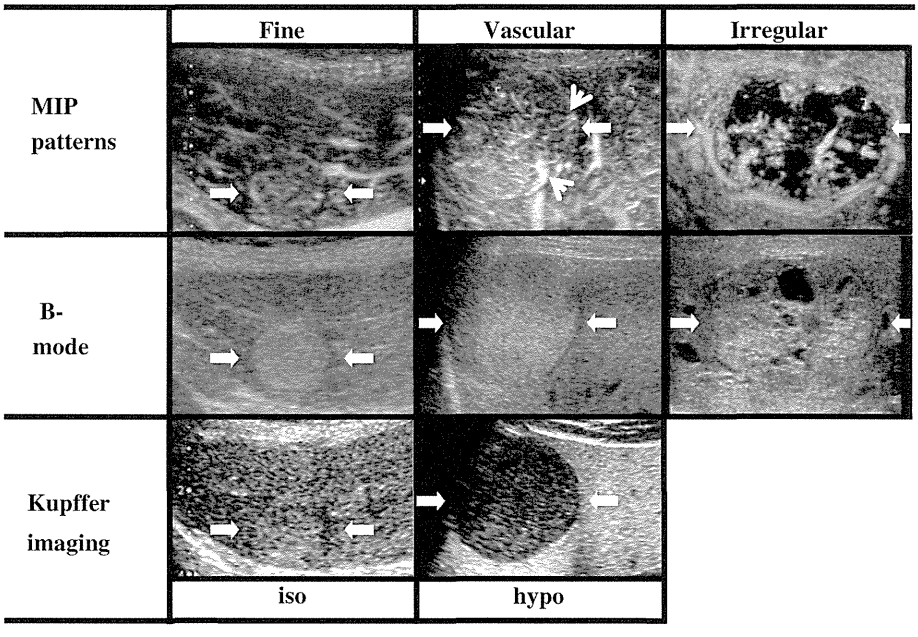

Fig. 1 Classification of MIP and Kupffer imaging. The MIP pattern is classified as 1 of the following 3 patterns: (1) fine pattern: where tumor vessels were not clearly visualized and only fine vessels were visualized; (2) vascular pattern: where tumor vessels were visualized clearly; and (3) irregular pattern: where tumor vessels were thick and irregular. *Small arrows* in "vascular" category of MIP patterns show tumor vessels of vascular pattern. Kupffer imaging is classified as 1 of following 2 patterns: (1) iso-echoic pattern, (2) hypo-echoic pattern

Grade 2 (hypo-fine), Grade 3 (hypo-vascular), and Grade 4 (hypo-irregular).

Statistical analysis

In the case of categorical variables, statistical analysis was performed using the Fisher's exact test. The Kruskal–Wallis H test was used for continuous variables. The Tukey–Kramer honestly significant difference was used for multiple comparisons. Relationships among the clinical parameters, such as malignant grade, tumor size, portal vein invasion, and histological differentiation were analyzed using Spearman's rank correlation coefficient. Unless otherwise noted, all data are presented as mean ± SD. $P < 0.05$ was considered statistically significant. The statistical analysis was performed with the JMP 8 (SAS Institute Inc., Cary, NC, USA).

Results

Arterial phase of HCCs and Kupffer imaging of HCCs

When compared with the adjacent liver tissue, 68 (94 %) lesions showed a hyper-echoic pattern and 4 (6 %) lesions showed an iso-echoic pattern during the arterial phase. As the degree of histological differentiation of HCC decreased, tumor hyper-vascularity increased: well-differentiated, 80 % (8/10); moderately differentiated, 96 % (54/56); and poorly differentiated, 100 % (6/6) (Table 1). Kupffer imaging of HCC according to histological differentiation showed the opposite tendency in that tumor hypo-intensity increased as the degree of histological differentiation of HCC decreased [well-differentiated, 60 % (6/10); moderately differentiated, 98 % (55/56); and poorly differentiated, 100 % (6/6)]. In Kupffer imaging, a hypo-echoic pattern was significantly larger in moderately or poorly differentiated HCCs, compared to well-differentiated HCCs ($P < 0.001$).

Table 1 Correlations between histological differentiated and patterns of contrast enhanced ultrasonography

	n	Arterial phase			Kupffer phase		
		Iso	Hyper	P^a	Iso	Hypo	P^b
Well	10	2	8	0.090	4	6	<0.001
Mod	56	2	54		1	55	
Poor	6	0	6		0	6	
Total	72	4	68		5	67	

Well Well differentiated HCC, *mod* moderately differentiated HCC, *poor* poorly differentiated HCC

[a] Proportion of hyper in arterial phase (well vs. mod/poor)

[b] Proportion of hyper in arterial phase (well vs. mod/poor)

Relationship between MIP patterns and histological differentiation or CEUS findings

In this study, intratumoral vessels of 72 tumors were clearly delineated using MIP. The vascular architecture of the tumors was as follows: fine, 12 (17 %); vascular, 48 (67 %); and irregular, 12 (17 %). Correlations between the MIP patterns and CEUS findings are presented in Fig. 2a, b. Most cases were hyper-echoic pattern in arterial phase (94 %) and hypo-echoic pattern in Kupffer imaging (93 %) and it was difficult to classify the MIP patterns using the CEUS patterns. Next, the correlations between the MIP patterns and histological differentiation were examined (Fig. 2c). We observed that 50 % (5/10) of well-differentiated HCCs showed a Fine pattern and 77 % (43/56) of moderately differentiated HCCs showed a vascular pattern, while the all poorly differentiated HCCs showed an irregular pattern. Furthermore, the irregular pattern was found only in moderately and poorly differentiated HCCs (Fig. 2c).

Histological differentiation and CEUS malignant grading system

As shown in Fig. 3a, Grade 1 included no moderately differentiated HCC tumors. In contrast, all poorly differentiated HCCs were Grade 4. This tendency became more clear when we considered the relationship among all 121 nodules, including biopsy-confirmed HCCs. There was a close relationship between malignant grade and histologic differentiation ($r = 0.712$, $P < 0.0001$). Thus, this grading system could predict moderately and poorly differentiated HCCs: Grade 1, 4 % (1/24); Grade 2, 52 % (15/29); Grade 3, 85 % (44/52); and Grade 4, 100 % (16/16).

Tumor size and malignant grading system

When evaluating tumor size according to the grading system, mean tumor size increased: Grade 1, 18.2 ± 4.7 mm; Grade 2, 16.6 ± 4.2 mm; Grade 3, 30.6 ± 14.7 mm; and Grade 4, 53.2 ± 21.3 mm ($r = 0.590$, $P < 0.001$). Tumor sizes were similar between Grades 1 and 2, but there was a greater increase in size between Grades 3 and 4 (Fig. 4a).

Portal vein invasion and clinical parameters

When portal vein invasion was compared with 72 resected HCCs, all HCCs with portal vein invasion were high-echoic pattern in arterial phase and hypo-echoic pattern in Kupffer imaging. There were only 4 (8 %) HCCs without portal vein invasion that showed an iso-echoic pattern

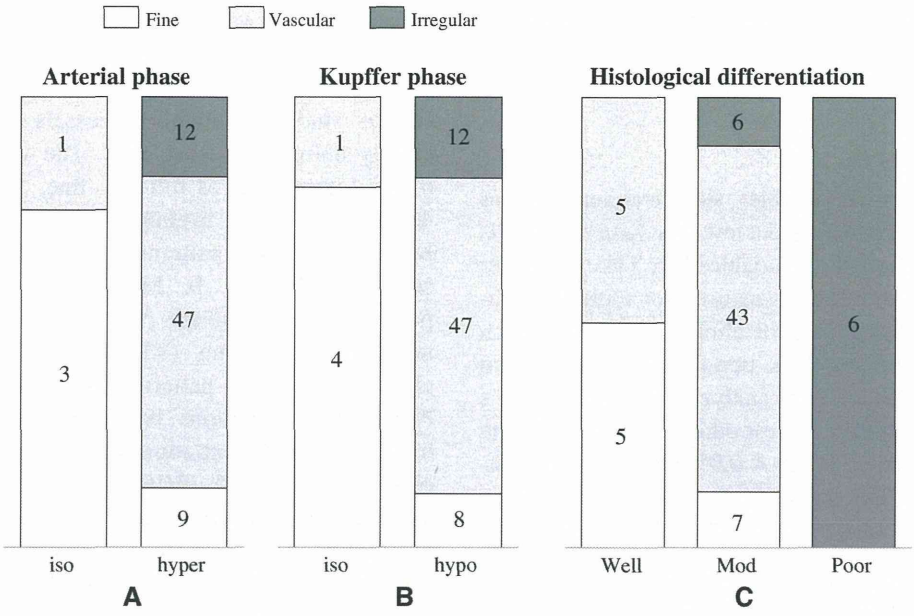

Fig. 2 Correlation between maximum intensity projection (MIP) patterns and contrast-enhanced ultrasonography (CEUS) findings or histological differentiation of hepatocellular carcinoma (HCC). **a** In the arterial phase, most HCCs depicted as an iso-echoic pattern showed a fine pattern and most hyper-echoic HCCs were vascular. All HCCs depicted as irregular showed a hyper-echoic pattern. **b** Kupffer imaging. In a similar fashion, most HCCs with iso-echoic patterns showed a fine pattern and most with hypo-echoic patterns were vascular. All HCCs depicted as irregular showed a hyper-echoic pattern. **c** In histological differentiation, 77 % (43/56) of moderately differentiated HCCs showed a vascular pattern and the irregular pattern was found only in moderately and poorly-differentiated HCCs

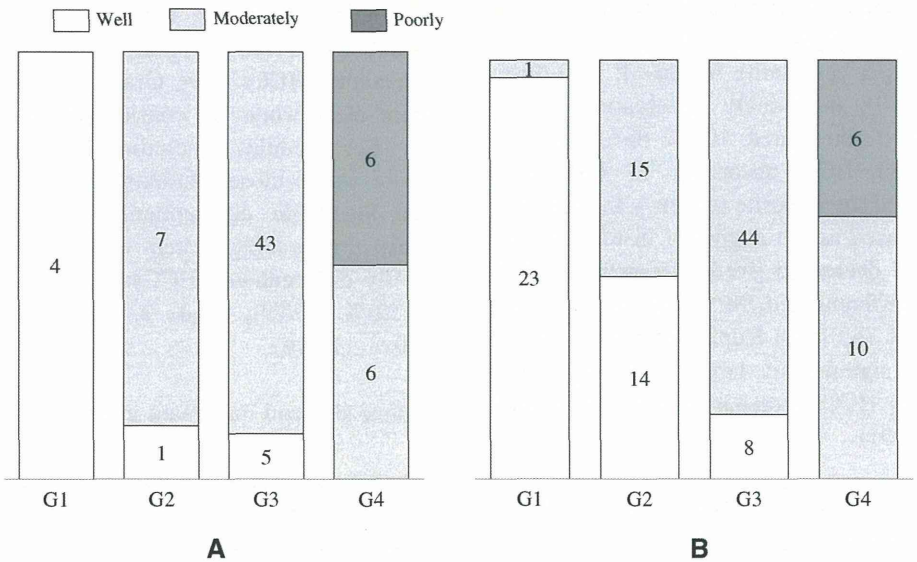

Fig. 3 Correlations between malignant grading system and HCC differentiation. The malignant grading system classified HCC into 4 grades. The combination of Kupffer imaging and MIP patterns classified HCC into 4 grades: Grade 1 (iso-fine/vascular), Grade 2 (hypo-fine), Grade 3 (hypo-vascular), and Grade 4 (hypo-irregular). **a** 72 resected HCCs. **b** All 121 HCCs, including the 72 resected HCCs and 49 HCCs diagnosed by biopsy specimen. Most malignant Grade 1 (G1) HCCs were well-differentiated and there were no well-differentiated HCCs in Grade 4 (G4). Poorly differentiated HCCs were identified only in G4. The proportions of moderately or poorly differentiated HCCs increased with increasing malignant grade. The close relationship between malignant grade and histological differentiation was confirmed, especially in all 121 HCCs ($r = 0.755$, $P < 0.0001$)

both in arterial phase and Kupffer imaging and almost all echo patterns of HCCs without portal vein invasion were hyper-echoic in both arterial phase and hypo-echoic in Kupffer imaging (Fig. 5a, b). Hence, using only CEUS patterns, it is difficult to distinguish the HCCs with portal vein invasion. However, the rates of positive portal vein