Table 1 Etiology of background liver diseases

Etiology of background liver diseases	All patients ($n = 347$)	Histologically-confirmed HCC patients ($n = 106$)
HCV-related cirrhosis	105 (30.3%)	60 (56.6%)
HBV-related cirrhosis	56 (16.1%)	34 (32.0%)
HBV and HCV-related cirrhosis	2 (0.6%)	2 (1.8%)
Alcoholic liver cirrhosis	4 (1.2%)	4 (3.7%)
Cryptogenic cirrhosis†	21 (6.1%)	4 (3.7%)
Primary biliary cirrhosis	69 (19.9%)	2 (1.8%)
Primary sclerosing cholangitis	10 (2.9%)	0
Autoimmune hepatitis	12 (3.5%)	0
Biliary atresia	18 (5.2%)	0
Metabolic disease	11 (3.2%)	0
Fulminant hepatic failure	32 (9.2%)	0
Others	7 (2.0%)	0

†History of alcohol intake and persistent HBV or HCV infection were denied in patients in this category.
HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

agglutinin-reactive fraction of AFP (AFP-L3), are also used in combination with AFP.[10–13] DCP is an abnormal prothrombin that is frequently increased in the serum of patients with HCC.[12,14] DCP was reported to be a more specific marker than AFP in early studies and meta-analysis, although a few recent studies showed contradicting results.[9,15–18] The combined use of AFP with DCP or AFP-L3 improves sensitivity and thus may contribute to early detection.[16,18–20]

Screening for HCC is usually not recommended among patients who are at advanced stage of cirrhosis unless curative treatment such as liver transplantation is readily available. In the United States, development of early HCC will give the patient a priority MELD score while patients are waiting for liver transplantation. Currently surveillance for HCC using ultrasonography with or without AFP measurement is recommended by the guideline of American Association for the Study of Liver Diseases.[2] However, no data to date suggest the use of DCP during pre-transplantation screening or diagnosis. Level of DCP in patients with severe liver impairment may be increased due to altered vitamin K metabolism such as obstructive jaundice or wide-spectrum antibiotics use.[19–23] If DCP levels often increase false-positively due to causes other than HCC, diagnostic accuracy of DCP will be reduced. In this study, we therefore aimed to evaluate the accuracy of AFP and DCP measurement for the diagnosis of HCC in liver transplant candidates, and to identify factors that contribute to elevated DCP levels.

METHODS

Patients

FROM JANUARY 1996 to December 2008, 347 adult-to-adult primary liver transplantations were performed at the University of Tokyo hospital. A retrospective review of records of all liver transplant recipients at the University of Tokyo was approved by the University of Tokyo Institutional Review Board (No. 2140). Of the 347 transplantations, 191 were performed in men and 156 in women. Median (range) age was 52 (18–67) years. Etiologies of the liver diseases are summarized in Table 1. Thirty-two patients with fulminant hepatic failure were excluded. The remaining 315 patients were included in the analysis. None of these patients were on warfarinization.

Measurement of serum tumor markers

Serum tumor markers were measured routinely using a commercially available kit as a part of the pre-transplant evaluation; markers included were AFP, DCP, and carcinoembryonic antigen (CEA). Results obtained within one month prior to liver transplantation were considered to be valid data. If the test was repeated during this period, the highest value was adopted. Serum AFP and CEA levels were measured using an enzyme-linked immunoassay method until June 2001 and a fluorescence-enzyme immunoassay method thereafter. Commercially determined reference values were up to 9 ng/mL for AFP and up to 5.0 ng/mL for CEA. Of the

315 cases, 307 had valid AFP measurements and 299 cases valid CEA measurements within one month prior to liver transplantation. The median (range) levels of AFP and CEA were 8 (1–11 999) ng/mL and 4.1 (0.3–17.5) ng/nL, respectively. Serum DCP levels were measured using an enzyme-linked immunoassay (Eitest mono-P-II, or Eitest PIVKA-II kit, Sankyo Junyaku Co., Ltd, Tokyo, Japan) from 1996 to 2000. In 2000, we began using a Chemiluminescence assay (Picolumi PIVKA-II and Lumipluse, Sankyo Junyaku Co., Ltd, Tokyo). There were good correlations between the two measurement methods for DCP, with an r between 0.98–0.99 (data provided by Sankyo Junyaku Co., Ltd. and Eisai Co., Ltd). The commercially determined reference value was <40 mAU/mL. There were 300 cases with valid data available with a median of 25 (3–36 613) mAU/mL. AFP-L3 was measured using lectin-affinity electrophoresis followed by antibody-affinity blotting,[24] and levels are shown as a percentage of total AFP. This test was performed in only a limited number of patients (99 patients in the HCC group and 17 patients in the non-HCC group) and thus the results were not included in the analysis.

Imaging study prior to liver transplantation

Pre-transplant evaluation included multi-phase dynamic helical computed tomography (CT) with contrast enhancement taken within one month prior to liver transplantation. Images were reviewed by two independent radiologists; one of the radiologists was assigned to be a pre-transplant judge who was independent of the transplant surgical team (MA and KO). Protocol imaging examination was not performed in 25 of 315 recipients; films from the referral hospital were used in those cases. Nine patients underwent CT without contrast enhancement due to renal insufficiency; magnetic resonance imaging (MRI) and ultrasonography were used as adjunctive studies in such cases.

Evaluation of the liver explants

Histopathologic findings of explanted livers were regarded as the gold standard in this study. Official histologic reports issued by pathologists were reviewed. Removed livers were sliced in approximately 1-cm thick sections along the axial plane to check for tumors on the cut surface. Pathologic features, including histologic differentiation, vascular invasion, and intrahepatic metastasis, were examined. If the histologic grade differed between nodules in the same liver, the worst grade was adopted in this study. The diameter of the largest tumor nodule was adopted as the "tumor size"; lesion by lesion analysis was not performed in this study. Explanted livers with known HCC were examined prospectively by *ex situ* ultrasound study.[25] Because the pathologists were not blinded, livers known to contain tumors might have been examined more carefully.

Vitamin K administration

Our review of the patient charts indicated that vitamin K was administered in some cases. Physicians diagnosed patients with elevated DCP levels and a hemorrhagic tendency with a "coagulation disturbance due to vitamin K deficiency" and administered either vitamin K1 (phylloquinone 15 mg daily) or vitamin K2 (menatetrenone 20 mg daily). There was no uniform treatment protocol for the diagnosis of vitamin K deficiency; the durations of vitamin K treatment and repeat DCP measurements were determined by the treating physician.

Statistical analysis

Data are presented as median and range or mean ± standard deviation for quantitative variables, unless otherwise specified. Differences between groups were analyzed by the Mann–Whitney U-test for continuous variables and the χ^2 test for categorical variables. Log-normally distributed data were entered into analysis after log10 transformation. Two-tailed tests for significance were performed using a P-value of less than 0.05. Variables with a P-value of less than 0.05 were considered for entry into the multivariate logistic stepwise regression model. For the diagnostic performance of AFP and DCP, a receiver operating characteristics (ROC) curve was constructed and the area under the ROC curve (AUROC) was calculated. Data analysis was performed with SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

HEPATOCELLULAR CARCINOMAS MEETING Milan criteria were diagnosed prior to transplantation in 86 patients, exceeded the criteria in 15 patients, and was incidentally detected in the explanted liver in another five patients. In total, 106 patients were diagnosed with histologically confirmed HCC. Sixty-five patients had at least one history of treatment for HCC; percutaneous ethanol injection in 21, transcatheter arterial embolization in 49, radiofrequency ablation therapy in 13, and partial hepatic resection in 11. Of

Table 2 Patient characteristics

Factors	Valid data in analysis	Recipients with HCC ($n = 106$)	Recipients without HCC ($n = 209$)	*P*-value
Male gender	315	87 (82%)	87 (42%)	<0.001
Age	315	56 (40–67)	50 (18–66)	<0.001
Child class C	297†	35 (33%)	156 (82%)	<0.001
MELD score	315	12 (6–34)	14 (6–40)	0.007
Albumin (g/dl)	315	2.8 (1.8–4.4)	2.9 (1.5–4.4)	0.92
Total bilirubin (mg/dl)	315	2.6 (0.3–36.3)	7.0 (0.4–40.0)	<0.001
AST (IU/ml)	315	59 (18–281)	78 (17–481)	<0.001
Creatinine (mg/dl)	315	0.7 (0.4–2.8)	0.6 (0.2–4.6)	0.001
Prothrombin time (INR)	315	1.59 (0.97–3.24)	1.54 (0.87–7.48)	0.71
AFP (ng/ml)	307	20 (1–11 999)	3 (1–480)	<0.001
DCP (mAU/ml)	300	23 (7–10 592)	25 (5–36 613)	0.42
CEA (ng/ml)	299	5.4 (0.8–14.7)	3.7 (0.3–17.5)	0.001
AFP-L3 (% of total AFP)	116	0.5 (0.5–77.8)	0.5 (0.5–35.7)	0.092

†Patients with metabolic liver diseases, and other etiologies were excluded.
Data are presented as median and range or mean ± standard deviation.
AFP, α-fetoprotein; AFP-L3, L3 fraction of α-fetoprotein; AST, aspartate amino transferase; CEA, carcinoembryonic antigen; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; INR, international normalized ratio; MELD, model for end stage liver disease.

those 106 patients, 65 (61%) met the Milan criteria by histologic evaluation. The number of tumors in the explanted livers was one in 35 (33.0%), two in 26 (24.5%), three in 13 (12.2%), and four or more in 32 (30.1%). The diameter of the largest tumor was 2.5 ± 1.5 cm. Histologic grade was well differentiated in 26 (24.5%), moderately differentiated in 63 (59.4%), poorly differentiated in six (5.6%), combined HCC and cholangiocellular carcinoma in one (0.9%), and necrotic tissue of HCC in 10 (9.4%). Vascular invasion was diagnosed in 21 (19.8%).

According to the histologic diagnosis of HCC, patients were divided into the HCC group ($n = 106$) or the non-HCC group ($n = 209$). The characteristics of the 315 recipients are summarized in Table 2. The HCC group was male dominant ($P < 0.001$), older in age ($P < 0.001$), and had lower model for end stage liver disease (MELD) scores ($P = 0.007$). AFP and CEA levels were significantly higher in the HCC group than in the non-HCC group, whereas DCP levels were similar between groups.

Performance of AFP and DCP

A ROC curve for predicting histological presence of HCC was created for all patients (Fig. 1). The AUROC for AFP (0.83, 95% confidence interval (CI): 0.78–0.88) was larger than that for DCP (0.47, 95% CI: 0.41–0.54).

The AUROC did not change after removing 10 cases with necrotic HCC cells; the AUROC was 0.83 (95%CI: 0.79–0.88) for AFP and 0.48 (95% CI: 0.41–0.54) for DCP. Sensitivity and specificity of tumor markers with a commercially defined reference value of AFP ≥ 9 ng/mL and DCP ≥ 40 mAU/mL are shown in Table 3. We also

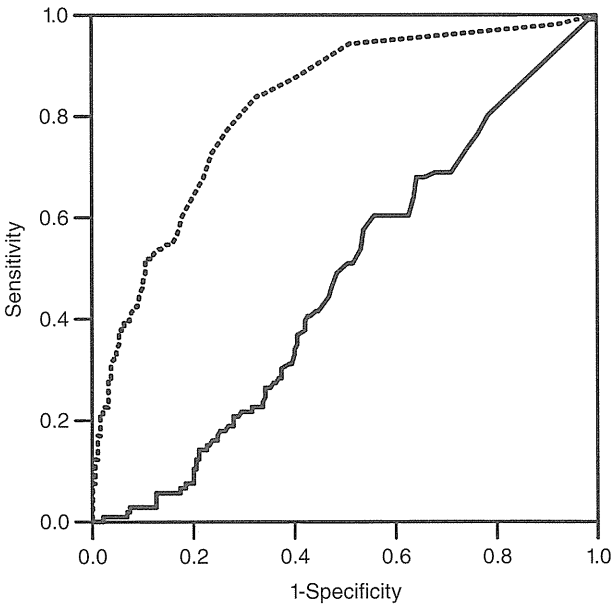

Figure 1 The receiver operating characteristic curves for des-γ-carboxy prothrombin (DCP) (solid line) and α-fetoprotein (AFP) (dashed line) levels in the diagnosis of hepatocellular carcinoma (HCC) are shown.

Table 3 Performance of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP)

	Number	Sensitivity	Number	Specificity
All patients				
AFP ≥ 9 ng/mL	68/106	64.1%	163/201	81.0%
AFP ≥ 20 ng/mL	55/106	51.8%	181/201	90.0%
DCP ≥ 40 mAU/mL	38/106	35.8%	115/194	58.3%
DCP ≥ 100 mAU/mL	20/106	18.8%	140/194	71.6%
Excluding PSC and PBC patients				
AFP ≥ 9 ng/mL	66/104	63.4%	92/127	72.4%
AFP ≥ 20 ng/mL	55/104	52.8%	107/127	84.2%
DCP ≥ 40 mAU/mL	38/104	36.5%	88/122	72.1%
DCP ≥ 100 mAU/mL	20/104	19.2%	98/122	80.3%

PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

adopted the cut-off values of AFP ≥ 20 ng/mL according to the ROC curve. For DCP, we tentatively set the cut-off level at 100 mAU/mL, since the majority of non-HCC patients showed DCP levels ≥ 40 mAU/mL. Performance of DCP was inferior to that of AFP. When patients with HCC were sub-stratified according to previous history of HCC treatment, the sensitivities for AFP and DCP with each cut-off value did not differ.

After removing patients with primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) from the analysis, AUROC for AFP and DCP were 0.77 (95% CI: 0.71–0.83) and 0.56 (95% CI: 0.49–0.64), respectively. Although sensitivity and specificity of DCP was improved (Table 3), the performance of DCP was still inferior to that of AFP.

Factors associated with elevated tumor marker level in non-HCC group

Associations between elevated serum AFP (≥20 ng/mL), and other factors were analyzed. By univariate analysis, hepatitis C virus (HCV; $P < 0.001$) and male gender ($P = 0.002$) were significantly associated with AFP ≥ 20 ng/mL among non-HCC patients. Multivariate analysis revealed both HCV (odds ratio [OR] 8.39, $P < 0.001$) and male gender (OR 3.32, $P = 0.035$) remained significant.

Associations between elevated serum DCP (≥100 mAU/mL), and other factors were then analyzed. Primary sclerosing cholangitis (PSC, $P = 0.003$), hepatitis B disease ($P = 0.031$), total bilirubin level ($P = 0.015$), and albumin level ($P = 0.012$) were significant factors. By multivariate analysis, PSC (OR 22.9, $P = 0.004$) and lower albumin level (OR: 0.44 per 1.0 g/dL, $P = 0.02$) remained significant factors associated with DCP levels ≥ 100 mAU/mL among non-HCC patients.

Since the association between DCP false positivity and the etiology of liver diseases was suggested, the differences in AFP and DCP levels between HCC and non-HCC groups were compared graphically as a box plot according to the etiology of liver disease (Fig. 2). AFP levels, shown as the log10 transformation, were higher in patients with HCC than in those without in each etiology group (Fig. 2a). On the other hand, DCP levels, also shown as the log10 transformation, were rather similar regardless of HCC status; instead, DCP levels were higher in patients with PSC, alcoholic liver disease, and cryptogenic cirrhosis (Fig. 2b) even without HCC.

Factors associated with elevated tumor marker level in HCC group

Among HCC group, the association between pathologic features and the level of tumor markers were analyzed. There was a positive association between AFP ≥ 20 ng/mL and the number of tumors ($P = 0.021$), and moderately to poorly differentiated HCC ($P = 0.036$) by univariate analysis, but the number of tumor (OR 1.22, $P = 0.021$) remained significant by multivariate analysis. DCP ≥ 100 mAU/mL was associated with vascular invasion ($P = 0.003$), size of HCC ($P = 0.008$), number of tumors ($P = 0.029$) and moderately to poorly differentiated HCC ($P = 0.019$) by univariate analysis. Multivariate analysis showed a positive association between DCP ≥ 100 mAU/mL and vascular invasion (OR 4.95, $P = 0.008$), size of HCC (OR 1.53, $P = 0.022$), and moderately to poorly differentiated HCC (OR 5.76, $P = 0.031$). These associations remained the same after removing 10 cases with necrotic HCC tissue (data not shown). None of these factors were associated with the level of CEA.

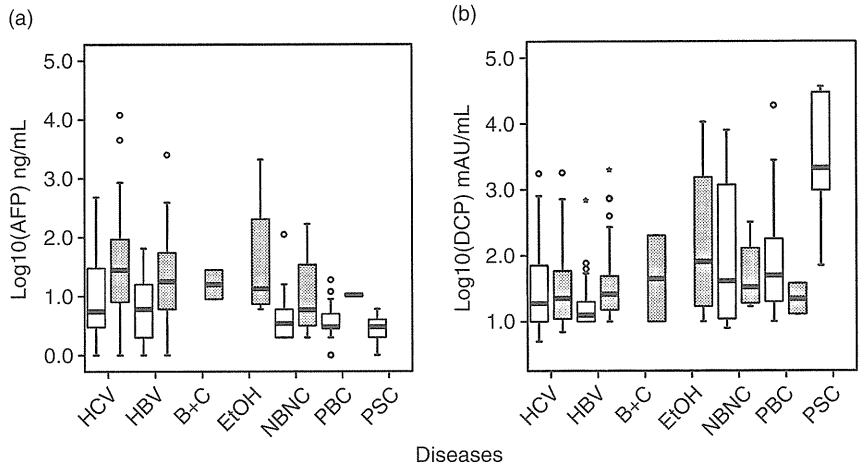

Figure 2 α-fetoprotein (AFP) (a) and des-γ-carboxy prothrombin (DCP) (b) levels according to etiology of liver diseases is shown as box-plot. White bar indicates non-hepatocellular carcinoma (HCC) group, and grey bar indicates HCC group. Serum level of AFP was higher in HCC group regardless of etiology. In contrast, DCP level in HCC group was similar to that in non-HCC group for each etiology groups. Longitudinal axis represents tumor marker levels after $\log_{10}$ transformation; upper horizontal line of box, 75th percentile; lower horizontal line, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, outliers are indicated by the circle (o), and extreme values are indicated by the asterisk. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNB, non B non C cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

Sequential changes in DCP levels by vitamin K administration

There were 74 cases whose DCP level was ≥ 100 mAU/mL, and 39 of these cases had more than two DCP measurements within one month before the transplantation. In 20 of these cases, either oral or parenteral vitamin K was administered after the first measurement of DCP until the last measurement (Table 4); DCP decreased in 19, and was unchanged in one. In the 19 patients without vitamin K administration, DCP levels increased in eight (two in the HCC group) and decreased in 11 (seven in the HCC group). Among those 19 cases, we could not find any specific markers that changed in parallel with or in inverse to the level of DCP. In cases 38 and 39, decrease of DCP levels might be related to transcatheter arterial chemoembolization (TACE), which was performed between the first and the last measurements of DCP.

DISCUSSION

IN THE PRESENT study, we evaluated pretransplant serum AFP and DCP levels as indicators of HCC presence in liver transplant candidates using histopathologic examination of explanted liver as the gold standard. Because the study subjects consisted entirely of liver transplantation candidates and none had advanced HCC, we had not expected high sensitivity for those tumor markers. On the other hand, specificity of DCP was revealed to be unexpectedly low, resulting in very low AUROC of 0.47, which indicates no diagnostic relevancy.

Des-γ-carboxy prothrombin positivity in HCC patients is reportedly associated with advanced features of HCC, such as vascular invasion and poorer differentiation, as shown indeed also in the present study.[26–28] In contrast, the tumor marker is reported to be fairly specific, at least in surveillance for HCC among chronic viral hepatitis patients, except in the cases of warfarinization or antibiotics-induced vitamin K deficiency.[19] The subjects of the present study, especially those without HCC, had poorer liver function than average chronic hepatitis patients under HCC surveillance. The difference in liver function may explain the very low specificity of DCP in the present study, and, indeed, lower albumin concentration was associated with false positivity in patients without HCC.

In advanced stages of cholestatic liver diseases, such as PBC and PSC, many patients show deficiency of fat-soluble vitamins including vitamin K.[29,30] In the present study, 22 of 67 (32.8%) patients with PBC and 8 of 10 (80%) patients with PSC, who had no HCC in liver

Table 4 Change of des-γ-carboxy prothrombin (DCP) level in 39 patients with pre-transplant DCP ≥ 100 mAU/mL

Case	Age/Sex	Etiology	DCP level (mAU/mL)		Interval (days)	Vitamin K administration	HCC	TACE for HCC†
			First DCP	Last DCP				
1	25/M	BA	118	35	10	K2, parenteral	No	
2	59/F	NBNC	709	17	12	K2, parenteral	No	
3	40/F	NBNC	8 041	15	28	K2, parenteral	No	
4	48/F	NBNC	1 198	47	13	K2, parenteral	No	
5	48/M	PBC	18 890	4 604	6	K2, parenteral	No	
6	50/F	PBC	1 257	387	8	K2, parenteral	No	
7	65/F	PBC	1 144	27	13	K2, parenteral	No	
8	56/F	PBC	524	59	24	K2, parenteral	No	
9	44/F	PSC	36 613	13 645	5	K2, parenteral	No	
10	57/M	PSC	2 853	1 571	3	K2, parenteral	No	
11	53/F	HCV	135	65	6	K2, parenteral	Yes	Yes
12	22/F	BA	7 490	26	27	K2, oral	No	
13	28/M	HCV	805	109	14	K2, oral	No	
14	55/F	PBC	397	389	14	K2, oral	No	
15	51/F	PBC	2 159	23	30	K2, oral	No	
16	38/F	PBC	603	21	26	K2, oral	No	
17	24/M	PSC	2 958	10	29	K1, oral	No	
18	33/F	PSC	972	699	14	K1, oral	No	
19	53/F	PSC	1 088	65	28	K1, oral	No	
20	42/M	HCV	1 089	105	19	K1, oral	Yes	No
21	24/M	BA	365	21	18	–	No	
22	48/M	HBV	692	369	30	–	No	
23	46/M	HCV	104	106	28	–	No	
24	59/F	HCV	33	287	21	–	No	
25	47/M	HCV	146	12	7	–	No	
26	54/F	HCV	340	378	26	–	No	
27	40/F	NBNC	128	310	30	–	No	
28	47/F	PBC	355	1 915	8	–	No	
29	29/F	PSC	593	919	30	–	No	
30	19/M	PSC	2 031	1 006	7	–	No	
31	57/M	Alcoholic	13 248	577	29	–	Yes	No
32	55/M	HBV	250	269	30	–	Yes	No
33	60/M	HBV	399	210	6	–	Yes	No
34	56/M	HBV	1 111	726	14	–	Yes	No
35	66/M	HCV	227	219	7	–	Yes	No
36	54/M	HCV	42	109	28	–	Yes	No
37	55/F	NBNC	323	168	14	–	Yes	No
38	54/M	HBV	1 994	682	6	–	Yes	Yes
39	44/M	HBV	302	40	20	–	Yes	Yes

†TACE performed only between the 1st and the last measurement of DCP was included.
BA, biliary atresia; DCP, des-γ carboxy prothrombin; F, female; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; K1, vitamin K1 (phylloquinone 15 mg q day); K2, vitamin K2 (menatetrenone 20 mg q day); M, male; NBNB, non B non C cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; TACE, transcatheter arterial chemoembolization.

explant, showed elevated levels of DCP (>100 mAU/mL). Thus, DCP is practically useless in the pre-transplant screening of HCC in patients with advanced cholestatic liver diseases. Furthermore, specificity of DCP was still low, 98/122 (80.3%), among pre-transplant patients after excluding those with PBC or PSC. Although elevated DCP levels were statistically associated with tumor factors suggesting poorer prognosis in patients with HCC, we cannot deny the possibility that elevated DCP levels were not related to HCC but

spurious and related to poor liver function. Although Liebman *et al.*[12] reported that vitamin K administration did not alter serum DCP levels in HCC patients, two patients with HCC in the present study showed decrease in DCP levels after vitamin K administration. The utility of DCP in pre-transplant screening seems to be very limited.

Vitamin K was administered to 20 patients with elevated DCP and the DCP levels decreased in 19 of them, suggesting that the increase in DCP levels was due to vitamin K deficiency. Except for patients with cholestatic liver diseases, who are likely to have vitamin K malabsorption, the mechanism of vitamin K deficiency is not clear. Possibilities include altered vitamin K metabolism, malnutrition, and prophylactic antibiotic use.[19] In addition, increase in serum DCP levels with normal serum concentration of vitamin has been reported in patients with alcoholic liver diseases,[31] although all patients with alcoholic cirrhosis in the present study had HCC and we do not have data concerning this issue. Several patients with HCC showed a decrease in DCP levels after vitamin K administration. Previous reports *in vitro* and *in vivo* have indicated suppression of HCC by the vitamin.[32–34] However, it is not clear whether tumor suppression by vitamin K contributed to the decrease in DCP levels in these patients.

Diagnosis of HCC should be based primarily on imaging studies. However, some HCC nodules fail to show typical enhancement patterns, requiring tumor biopsy. The guideline of American Association for the Study of Liver Diseases and that of European Association of the Study of Liver Diseases both state that biopsy of liver nodule is not required if serum AFP is greater than 200 ng/mL.[2,3] For DCP, however, data are insufficient regarding whether elevation of DCP could complement inconclusive imaging findings. Previous reports on the use of DCP as a prognostic factor showed that DCP levels over 300 mAU/mL predict histologic vascular invasion and tumor recurrence after transplantation.[26,27,35] However, among 23 patients in the present study with serum DCP levels over 1000 mAU/mL, only three (13%) had HCC in their explanted livers (data not shown).

In conclusion, DCP levels are associated with vascular invasion, poorer histologic grade, and a larger size of hepatocellular carcinoma among liver transplant recipients with HCC. Elevated DCP in pre-transplant patients with severe hepatic impairment levels, however, did not correlate with the presence of HCC in the explant. To screen for HCC in patients awaiting liver transplantation, repeated imaging studies would be desirable. Elevated DCP levels may lead to further imaging studies to detect HCC, but the cost-effectiveness of using DCP as a screening mode requires further investigation.

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Research Article

Cancer Epidemiology, Biomarkers & Prevention

Prediction of Hepatocellular Carcinoma Development by Plasma ADAMTS13 in Chronic Hepatitis B and C

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Abstract

Background: Chronic liver injury evokes a wound healing response, promoting fibrosis and finally hepatocellular carcinoma (HCC), in which hepatic stellate cells play an important role. Although a blood marker of hepatic stellate cells is not known, those cells importantly contribute to the regulation of plasma a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) activity, a defect of which causes thrombotic thrombocytopenic purpura.

Methods: Plasma ADAMTS13 was evaluated in chronic hepatitis B or C patients with or without HCC.

Results: Plasma ADAMTS13 activity significantly correlated with serum aspartate aminotransferase and alanine aminotransferase, liver stiffness value, and aspartate aminotransferase-to-platelet ratio index, irrespective of the presence of HCC, suggesting that it may reflect hepatocellular damage and subsequent wound healing and fibrosis as a result of hepatic stellate cell action. During the three-year follow-up period for patients without HCC, it developed in 10 among 81 patients. Plasma ADAMTS13 activity was significantly higher in patients with HCC development than in those without and was a significant risk for HCC development by univariate and multivariate analyses. Furthermore, during the one-year follow-up period for patients with HCC treated with radiofrequency ablation, HCC recurred in 55 among 107 patients. Plasma ADAMTS13 activity or antigen level was significantly higher in patients with HCC recurrence than in those without and was retained as a significant risk for HCC recurrence by multivariate analysis.

Conclusions: Higher plasma ADAMTS13 activity and antigen level was a risk of HCC development in chronic liver disease.

Impact: Plasma ADAMTS13 as a potential marker of hepatic stellate cells may be useful in the prediction of hepatocarcinogenesis. *Cancer Epidemiol Biomarkers Prev; 20(10); 2204–11. ©2011 AACR.*

Introduction

It is well known that chronic wound healing generally provides a microenvironment that gives rise to cancer (1). Indeed, chronic injury in the liver evokes a perpetuating wound healing response, promoting the development of fibrosis and finally hepatocellular carcinoma (HCC; ref. 2). Among the cells in the liver, hepatic stellate cells are known as a main effector of wound healing and fibrosis following liver injury of any etiology (3), however, a useful blood marker to reflect the activity of those cells has not been found yet in the clinical setting.

In this context, we have focused on a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13), a defect of which increases unusually large multimers of von Willebrand factor in the plasma, causes platelet thrombosis under high sheer stress, and results finally in thrombotic thrombocytopenic purpura (4–6). With regard to the site of production, *ADAMTS13* mRNA expression was shown exclusively in the liver (7–9) and then both *ADAMTS13* mRNA expression and ADAMTS13 activity were determined primarily in hepatic stellate cells among the liver cells in mice (10). ADAMTS13 expression was also detected in hepatic stellate cells in human and thereby ADAMTS13 is reportedly produced in those cells (11). To elucidate a regulatory mechanism of plasma ADAMTS13 activity, we previously determined that selective hepatic stellate cell damage caused by dimethylnitrosamine in rats leads to decreased plasma ADAMTS13 activity (12). On the other hand, plasma ADAMTS13 activity was upregulated during the process of liver fibrosis due to cholestasis caused by bile duct ligation and steatohepatitis induced by a choline-deficient L-amino acid–defined diet in rats, in which hepatic stellate cells actively proliferate (13).

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These results indicate that hepatic stellate cells play an important role in the regulation of plasma ADAMTS13 activity, although other sources of ADAMTS13 were reported (14–16).

On the basis of these previous findings, we wondered whether plasma ADAMTS13 could be a blood marker of hepatic stellate cells. To examine this, plasma ADAMTS13 was evaluated in patients with chronic hepatitis B or C, in whom chronic wound healing and fibrosis are observed with a high risk of HCC development (17), in which hepatic stellate cells play an important role (3). In this study, we have found that plasma ADAMTS13 was increased in relation with serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT), and the markers of liver fibrosis and that higher plasma ADAMTS13 was more frequently found in patients who later developed HCC.

Patients and Methods

Patients

Eighty-one patients with chronic hepatitis B and C, who visited the Department of Gastroenterology, the University of Tokyo Hospital, Tokyo, Japan, between April and August in 2007, were first enrolled. Chronic hepatitis B was defined as hepatitis B surface antigen (HBsAg) positivity, and chronic hepatitis C was defined as serum anti-hepatitis C virus antibody (HCVAb) positivity and a detectable HCV RNA level, having persistent liver damage for more than 6 months. Patients with HCC at the time of enrollment or with past history of HCC were excluded from this analysis.

Next, between July and September in 2009, 107 consecutive patients with chronic hepatitis B and C with HCC who were scheduled to undergo radiofrequency ablation (RFA) for HCC were enrolled.

All the studies were carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo. Informed consent from the patients was obtained for the use of the samples in this study.

Measurement of ADAMTS13 activity

ADAMTS13 enzymatic activity was measured manually using a chromogenic ELISA kit, ADAMTS13-act-ELISA (Kainos Inc./Technoclone GmbH), which captures products cleaved by ADAMTS13 using a sandwich method, and expressed as percentage of healthy control. The very high correlation of the values measured by classical VWF multimer assay and this novel chromogenic ADAMTS13-act-ELISA was reported previously (18).

Measurement of ADAMTS13 antigen level

ADAMTS13 antigen level was measured by a latex photometric immunoassay, in which suspended polystyrene latex particles coated with polyclonal antibody F(ab')2 fragment against ADAMTS13 were employed. Antisera against ADAMTS13 were obtained by immunization with pCAG-ADAMTS13 plasmid DNA (donated by Dr. Soejima from The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) using electroporation. Latex agglutination was analyzed using LPIA-A700 (Mitsubishi Chemical Medience Co.), a fully automated quantitative latex photometric immunoassay instrument. ADAMTS13 antigen level in sample of each patient was expressed as the percentage of that in pooled normal human plasma.

Measurement of liver stiffness

Liver stiffness was measured by transient elastography (FibroScan 502; EchoSens) as described previously (19–21). Briefly, the measurements were done in the right lobe of the liver through the intercostal spaces, with the patient lying in the dorsal decubitus position, and were considered valid only when at least 10 acquisitions were successful, with a success rate of at least 60% and the ratio of interquartile range to the median value was larger than 30%. Liver stiffness value was expressed in kilopascals (kPa).

Patient follow-up and diagnosis of HCC

Patients without HCC were followed up at the outpatient clinic with monthly blood tests, including tumor markers and ultrasonography every 4 to 6 months. Contrast-enhanced computed tomography (CT) was done when serum alpha-fetoprotein (AFP) levels and/or plasma des-gamma-carboxy prothrombin (DCP) levels showed an abnormal rise and/or tumors were detected as possible HCC on ultrasonography. The diagnosis of HCC was based on typical findings on CT, that is, hyperattenuation in the arterial phase and hypoattenuation in the equilibrium phase (22–24).

The end points consisted of the interval between the first measurement of plasma ADAMTS13 activity and the detection of HCC development, death without HCC development, or the last examination until 30 July 2010, whichever came first. Death without HCC development was treated as censored data.

Radiofrequency ablation, patient follow-up, and analysis of HCC recurrence

The detailed procedure of RFA was meticulously described elsewhere (25). The indication criteria for RFA consisted of total bilirubin concentration less than 3.0 mg/dL and platelet count more than $5 \times 10^4/\mu L$. Patients with portal vein tumor thrombosis, massive refractory ascites, or extrahepatic metastasis were excluded. In general, RFA was done on patients with 3 or fewer lesions, each less than 3.0 cm in diameter. However, RFA was also done on patients who did not meet these criteria when complete ablation could be anticipated in all tumors without deteriorating liver function. After RFA, dynamic CT was done to evaluate treatment efficacy. Complete ablation was defined as hypoattenuation of the whole lesion together with the surrounding liver parenchyma as a safety margin.

Patients received additional RFA until complete ablation was confirmed for each HCC nodule.

The follow-up consisted of monthly blood tests and monitoring of tumor markers at the outpatient clinic, with ultrasonography and dynamic CT scan done every 4 months. HCC recurrence was diagnosed on the basis of the criteria as described earlier.

The end points consisted of the interval between the first ablation and the detection of HCC recurrence, death without recurrence, or the last examination until 30 September 2010, whichever came first. Death without recurrence was treated as censored data.

Statistical analysis

Comparisons between groups were made using Student's t test or χ^2 test. The correlation between 2 groups, in which the data points were distribution free, was analyzed using Spearman's rank correlation coefficient (ρs). The cumulative incidence of HCC was estimated using the Kaplan–Meier method. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables obtained at the time of entry in univariate and multivariate Cox proportional hazard regression analyses: age, sex, positivity for HBsAg and HCVAb, albumin, total bilirubin, AST, ALT, prothrombin time, platelet counts, liver stiffness value, APRI, AFP, DCP, and either plasma ADAMTS13 activity or antigen level. Multichotomous categorical variables were represented by corresponding binary dummy variables. Factors that had a $P < 0.2$ in univariate analysis were subsequently included in a multivariate Cox proportional hazard regression model, with stepwise selection of variables based on the Akaike information criterion (AIC). Data processing and analysis were done by using the S-plus Ver. 7 (TIBCO Software Inc.).

Results

Characteristics of the patients without HCC and correlation between plasma ADAMTS13 activity and clinical variables

The characteristics of the patients, who were first enrolled for the measurement of plasma ADAMTS13 activity, are summarized in Table 1. There were 21 patients with chronic hepatitis B and 60 patients with chronic hepatitis C. All the patients were outpatients without HCC at the time of enrollment and past history of HCC.

Plasma ADAMTS13 activity in these patients was 114.0 ± 45.4% (mean ± SD) of control, ranged from 28.0% to 221.5%, as shown in Table 1. Relationships between plasma ADAMTS13 activity and clinical variables are shown in Table 2. The significant correlations were determined between plasma ADAMTS13 activity and serum AST and ALT levels ($P < 0.001$). On the other hand, the significant correlations were also determined between plasma ADAMTS13 activity and the variables predicting the stage of liver fibrosis, liver stiffness value ($P < 0.001$), and aspartate aminotransferase-to-platelet ratio index (APRI; $P = 0.027$). Of note is the finding that plasma ADAMTS13 activity significantly correlated with serum AFP level ($P < 0.001$).

HCC development and risk analysis

Next, a potential link between plasma ADAMTS13 activity and HCC was examined. During the mean follow-up period of 35.4 months, one patient had been lost to follow-up evaluation and one patient died before HCC was identified. By the end of the follow-up, HCC developed in 10 patients, among whom 2 patients died of HCC. The cumulative incidence rates of HCC at

Table 1. Characteristics of patients without HCC or with HCC

Variables	Patients without HCC	Patients with HCC
Age (y)	63 ± 12 (23–85)	68.9 ± 8.5 (43–86)
Man/Woman	49/32	68/39
HBV/HCV	21/60	15/92
Albumin (g/dL)	4.1 ± 0.4 (3.1–4.9)	3.7 ± 0.6 (2.0–5.1)
AST (U/L)	48 ± 35 (3–270)	61.4 ± 39.2 (16–289)
ALT (U/L)	53 ± 66 (11–542)	54.1 ± 38.6 (11–276)
Platelet count (× 10^4/μL)	15.2 ± 6.3 (3.4–30.8)	10.8 ± 4.7 (3.4–25.2)
Prothrombin time (%)	87.7 ± 11.6 (49.2–100.0)	98.3 ± 5.2 (73.0–100.0)
Plasma ADAMTS13 activity (%)	114.0 ± 45.4 (28.0–221.5)	125.0 ± 32.4 (62.0–223.0)
Plasma ADAMTS13 antigen level (%)	Not measured	128.6 ± 39.6 (48.9–258.3)
Liver stiffness (kPa)	11.4 ± 9.2 (3.1–48.0)	28.5 ± 17.9 (6.1–75.0)
APRI	1.07 ± 1.00 (0.08–5.92)	1.89 ± 1.44 (0.22–7.81)
AFP (ng/mL)	12.6 ± 38.2 (1–319)	99.4 ± 361.2 (1–3,399)
DCP (mAu/mL)	18.1 ± 17.6 (10–165)	70.7 ± 194.3 (8–1,462)
Maximum size of HCC (mm)	Not available	17.8 ± 6.0 (6.0–33.0)

NOTE: Values are expressed as the mean ± SD (range).

Table 2. Relation between plasma ADAMTS13 activity and clinical variables in patients without HCC or with HCC

Variables	Patients without HCC		Patients with HCC	
	ρs[a]	*P*	ρs[a]	*P*
Age	−0.067	0.554	−0.030	0.760
AST (U/L)	0.360	<0.001	0.531	<0.001
ALT (U/L)	0.426	<0.001	0.519	<0.001
Albumin (g/dL)	−0.114	0.309	−0.146	0.133
Platelet count (× 10^4/μL)	−0.091	0.418	−0.129	0.185
Prothrombin time (%)	−0.343	<0.005	−0.029	0.764
Liver stiffness (kPa)	0.379	<0.001	0.216	0.026
APRI	0.245	0.027	0.403	<0.001
AFP (ng/mL)	0.465	<0.001	0.554	<0.001
DCP (mAu/mL)	0.135	0.230	−0.281	0.003
Size of HCC (mm)[b]	Not available		−0.075	0.571

[a]Spearman's rank correlation coefficient.
[b]Analyzed in patients with single nodule of HCC.

1, 2, and 3 years estimated by the Kaplan–Meier method were 4.9%, 9.1%, and 11.1%, respectively, as shown in Figure 1A. In these patients who developed HCC, plasma ADAMTS13 activity was significantly higher than that in patients who did not develop HCC ($P < 0.001$), as depicted in Table 3; plasma ADAMTS13 activity was 161.9 ± 33.8% in patients who developed HCC and 108.8 ± 42.2% in patients who did not develop HCC. Liver stiffness value was also significantly higher in patients with HCC development, and serum albumin level and prothrombin time (%) were significantly lower in those patients. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$; Table 4). Other significant risk factors for HCC included lower albumin level, higher ALT level, lower prothrombin time (%), and higher liver stiffness value. Next, stepwise variable selection with AIC was used to find the best model in multivariate analysis (Table 4), which revealed that the higher plasma ADAMTS13 activity ($P = 0.03$) and the higher liver stiffness value ($P = 0.03$) were the significant risk factors for HCC. These results suggest that plasma ADAMTS13 activity may predict HCC development in patients with chronic hepatitis B or C.

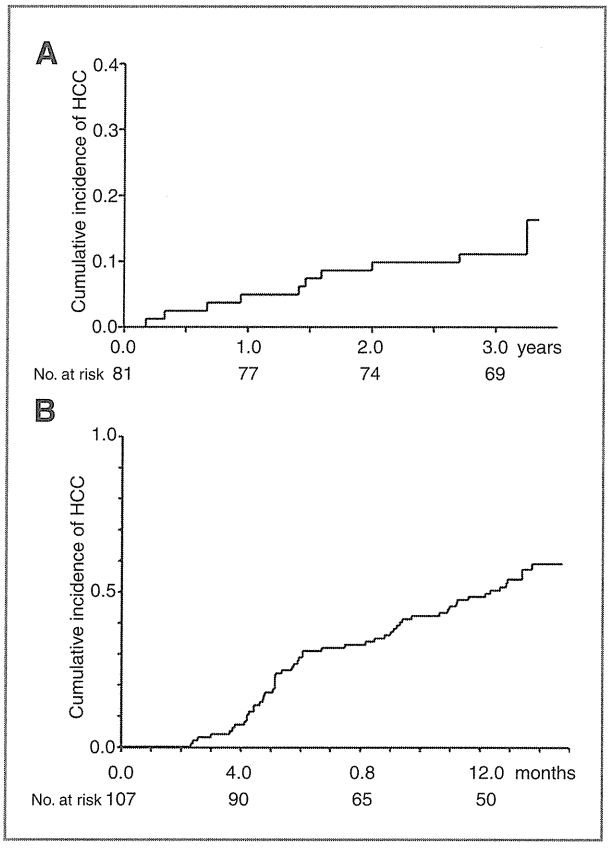

Figure 1. Cumulative incidence of HCC development (A) and recurrence (B).

Then, the relation between plasma ADAMTS13 activity and HCC development was analyzed separately in patients with chronic hepatitis B and with chronic hepatitis C. In patients with chronic hepatitis B ($n = 20$), plasma ADAMTS13 activity was significantly higher in patients who developed HCC than that in patients who did not develop HCC ($P < 0.005$); plasma ADAMTS13 activity was 158.9 ± 36.7% in patients who developed HCC and 95.3 ± 35.0% in patients who did not develop HCC. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$), and further multivariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC ($P = 0.03$) in these patients. In patients

Table 3. Characteristics of patients according to HCC development and recurrence

Variables	Development (−)	Development (+)	*P*	Recurrence (−)	Recurrence (+)	*P*
Age (y)	63.0 ± 12.4	61.4 ± 12.4	0.695	70.2 ± 7.5	68.3 ± 9.2	0.267
Man/Woman	40/29	6/4	0.82	23/19	36/19	0.39
HBV/HCV	16/53	4/6	0.45	7/35	7/48	0.80
Albumin (g/dL)	4.1 ± 0.3	3.8 ± 0.6	0.024	3.8 ± 0.5	3.6 ± 0.6	0.296
AST (IU/L)	46.9 ± 36.6	55.1 ± 23.0	0.494	61.0 ± 46.5	60.5 ± 33.3	0.951
ALT (IU/L)	52.0 ± 68.8	63.2 ± 46.9	0.620	56.9 ± 46.7	49.7 ± 29.5	0.359
Platelet count ($\times 10^4/\mu L$)	15.7 ± 6.5	12.2 ± 3.9	0.097	10.9 ± 5.5	10.5 ± 4.3	0.667
Prothrombin time (%)	89.5 ± 10.5	74.7 ± 11.6	<0.001	98.9 ± 3.5	97.5 ± 6.5	0.188
Plasma ADAMTS13 activity (%)	108.8 ± 42.2	161.9 ± 33.8	<0.001	116.8 ± 28.5	130.0 ± 30.8	0.039
Plasma ADAMTS13 antigen (%)	Not measured	Not measured		118.9 ± 35.4	134.3 ± 36.1	0.037
Liver stiffness (kPa)	9.2 ± 5.7	22.6 ± 13.7	<0.001	23.4 ± 15.0	30.6 ± 18.4	0.053
APRI	1.03 ± 1.03	1.35 ± 0.80	0.35	1.57 ± 0.81	1.47 ± 0.77	0.517
AFP (ng/mL)	10.7 ± 38.1	27.4 ± 41.4	0.203	131.5 ± 546.0	81.2 ± 158.1	0.521
DCP (mAu/mL)	17.9 ± 18.8	19.7 ± 8.2	0.766	114.0 ± 297.6	41.7 ± 64.3	0.082

NOTE: Values are expressed as the mean ± SD (range).

with chronic hepatitis C ($n = 59$), plasma ADAMTS13 activity was significantly higher in patients who develop HCC than that in patients who did not develop HCC ($P < 0.01$); plasma ADAMTS13 activity was 163.9 ± 35.2% in patients who developed HCC and 112.9 ± 43.6% in patients who did not develop HCC. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$), and multivariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC ($P = 0.02$) in these patients.

Characteristics of the patients with HCC and correlation between plasma ADAMTS13 activity or antigen level and clinical variables

To further examine a potential link between plasma ADAMTS13 and HCC, plasma ADAMTS13 activity and antigen level were measured in 107 patients with HCC. Their characteristics are summarized in Table 1. There were 15 patients with chronic hepatitis B and 92 patients with chronic hepatitis C.

Plasma ADAMTS13 activity in these patients was 124.9% ± 32.3% (mean ± SD) of control, ranged from 62.0% to 223.0%, and plasma ADAMTS13 antigen level, 128.3% ± 39.3% (mean ± SD) of control, ranged from 48.9% to 258.3%, respectively (Table 1). Of note, the strong correlation between plasma ADAMTS13 activity and plasma ADAMTS13 antigen level was observed (Spearman's rank; $\rho s = 0.803$, $P < 0.00001$, $n = 107$). Relationships between plasma ADAMTS13 activity and clinical variables are shown in Table 2. Same as in patients without HCC, the significant correlations were determined between plasma ADAMTS13 activity and serum AST and ALT levels ($P < 0.001$), liver stiffness value ($P = 0.026$), APRI ($P < 0.001$), and serum AFP level ($P < 0.001$). Of note, there was no significant correlation between plasma ADAMTS13 activity and maximum tumor size in patients with single nodule, suggesting that plasma ADAMTS13 activity is not a tumor marker of HCC.

Table 4. Risk factors for HCC development–univariate and multivariate analyses

Variable	HR (95% CI)	*P*
Univariate analysis		
ADAMTS13 (per 10% increase)	1.29 (1.11–1.50)	<0.001
Age (per 1 year increase)	0.990 (0.943–1.04)	0.68
Sex (male vs. female)	1.07 (0.563–2.02)	0.84
Hepatitis virus (HCV vs. HBV)	0.718 (0.380–1.36)	0.31
Albumin (per 1 g/dL increase)	0.208 (0.0477–0.905)	0.04
AST >40 U/L	1.73 (0.881–3.41)	0.11
ALT >40 U/L	2.06 (1.05–4.07)	0.04
PLT <15 $\times 10^4/\mu L$	1.97 (0.908–4.29)	0.09
Prothrombin time (%; per 10% increase)	0.490 (0.324–0.743)	<0.001
Liver stiffness (per 10% increase)	1.16 (1.07–1.26)	<0.001
APRI (per 10% increase)	1.05 (0.983–1.13)	0.14
AFP >20 ng/mL	1.71 (0.785–3.71)	0.18
DCP >40 mAU/mL[a]	NA	
Multivariate analysis		
ADAMTS13 (per 10% increase)	1.20 (1.02–1.40)	0.03
Liver stiffness (per 10% increase)	1.12 (1.01–1.23)	0.03

[a]Not accessed as only DCP was more than 40 mAU/mL in only 1 patient.

HCC recurrence and risk analysis

During the follow-up period of 12 months, 1 patient died without HCC. Two patients who developed extrahepatic recurrence and 3 patients who developed recurrence at a site adjacent to the treated site were excluded from the analysis. Four patients who were treated with IFN were not analyzed because IFN is known to reduce the risk of HCC development in chronic hepatitis B and C (26, 27). By the end of the follow-up, HCC recurrence was determined in 55 patients. The cumulative recurrence rates of HCC by the Kaplan–Meier method are shown in Figure 1B. The characteristics of patients with or without HCC recurrence are shown in Table 3. Among the various parameters, plasma ADAMTS13 activity ($P = 0.039$) and antigen level ($P = 0.037$) were significantly higher in patients with HCC recurrence than those in patients without HCC recurrence (Table 3). No significant differences were determined in other parameters between patients with and without HCC recurrence. Although there was no significant risk factor for HCC recurrence in univariate analyses (Table 5), plasma ADAMTS13 activity was retained as a significant risk factor of HCC recurrence ($P = 0.028$) in the multivariate Cox proportional hazard model, as shown in Table 5. When plasma ADAMTS13 antigen level was analyzed instead of plasma ADAMTS13 activity level, plasma ADAMTS13 antigen level was also a significant risk factor of HCC recurrence ($P = 0.007$) in multivariate analysis. These results suggest that plasma ADAMTS13 activity may predict HCC recurrence in patients with chronic hepatitis B or C.

Table 5. Risk factors for HCC recurrence–univariate and multivariate analyses

Variable	HR (95%CI)	P
Univariate analysis		
ADAMTS13 activity (per 10% increase)	1.106 (0.997–1.228)	0.052
Age (per 1 year increase)	0.982 (0.953–1.013)	0.25
Sex (male vs. female)	1.22 (0.70–2.13)	0.49
Hepatitis virus (HCV vs. HBV)	1.10 (0.50–2.44)	0.81
Albumin (per 1g/dL increase)	0.723 (0.432–1.210)	0.22
AST > 40 IU/L	1.35 (0.75–2.42)	0.31
ALT > 40 IU/L	1.00 (0.58–1.71)	0.99
PLT < 15 × 10^4/μL	1.25 (0.65–2.43)	0.51
Prothrombin Activity (per 10% increase)	0.88 (0.42–1.07)	0.20
Liver stiffness (per 10% increase)	1.12 (0.98–1.28)	0.11
APRI (per 10% increase)	1.00 (0.973–1.04)	0.81
AFP > 20 ng/mL	1.24 (0.73–2.11)	0.42
DCP > 40 mAU/mL	1.17 (0.64–2.14)	0.62
Multivariate analysis		
ADAMTS13 activity (per 10% increase)	1.14 (1.01–1.29)	0.028

The relation between plasma ADAMTS13 activity and HCC recurrence was also analyzed separately in patients with chronic hepatitis B and with chronic hepatitis C. In patients with chronic hepatitis B ($n = 14$), plasma ADAMTS13 activity or antigen level was not different between patients with ($105.0 \pm 34.0\%$ or $97.3 \pm 24.7\%$) and without HCC recurrence ($104.0 \pm 16.3\%$ or $98.9 \pm 14.4\%$), possibly because the number of patients analyzed was small. On the other hand, in patients with chronic hepatitis C ($n = 83$), plasma ADAMTS13 activity or antigen level was significantly higher in patients with HCC recurrence ($133.2 \pm 28.9\%$ or $139.7 \pm 34.4\%$) than that in patients without HCC recurrence ($119.4 \pm 29.8\%$ or 122.9 ± 37.1; $P = 0.037$ or $P = 0.036$). Multivariate analysis revealed that the higher plasma ADAMTS13 activity or antigen level was a significant risk factor for HCC ($P = 0.024$ or $P = 0.005$) in these patients.

Discussion

In the current study, plasma ADAMTS13 activity or antigen level significantly correlated with serum AST and ALT levels and also the variables predicting the stage of liver fibrosis, liver stiffness value, and APRI in patients with chronic hepatitis B or C, irrespective of the presence of HCC. Serum levels of AST and ALT reflect hepatocellular damage, and higher hepatocellular damage generally induces a higher wound healing response. Thus, our current findings may be in line with our speculation that plasma ADAMTS13 activity or antigen level reflects the activity of hepatic stellate cells as a main effector of wound healing and fibrosis in the liver.

Major finding of this study is that the higher plasma ADAMTS13 activity or antigen level was a significant risk factor for HCC development. With regard to HCC development among patients with chronic hepatitis B or C without the past history of HCC, plasma ADAMTS13 activity was higher in the patients who developed HCC than in those who did not develop HCC. Among the various clinical parameters, univariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC development. Then, multivariate analysis showed that the higher plasma ADAMTS13 activity was a significantly predicting factor for hepatocarcinogenesis, independent of other significant risk factors for HCC development, including the variables predicting the stage of liver fibrosis. This potential link between plasma ADAMTS13 activity and HCC development was further observed in the analysis of HCC recurrence: the patients who had HCC recurrence during the 1-year follow-up period had also significantly higher plasma ADAMTS13 activity or antigen level than those who did not have HCC recurrence. Then, only plasma ADAMTS13 activity or antigen level was retained in the multivariate Cox proportional hazard model as a significant risk factor of recurrence.

Then, we wondered how HCC development might be predictable by the activity or antigen level of plasma ADAMTS13, whose source is mainly hepatic stellate cells, as a key player of liver fibrosis. To explain this, the notion that advanced liver fibrosis is the strong risk factor for HCC development (17) may be important. Furthermore, the recent evidence suggests a potential direct link between hepatic stellate cells and HCC (3), as follows.

It is well known that HCC usually develops in the liver already suffering from chronic liver disease (2). In particular, HCV-related cirrhosis is associated with an extremely high risk of HCC development, with a reported annual incidence ranging between 3% and 8% (28–30). Thus, advanced liver fibrosis is one of the strongest risk factors for HCC development. In fact, the higher liver stiffness value is reportedly a strong risk for HCC development (21). In this study, a significant correlation was observed between plasma ADAMTS13 activity or antigen level and the variables predicting the stage of liver fibrosis such as liver stiffness value. Thus, we have first speculated that plasma ADAMTS13 activity is retained as a risk factor for HCC development by univariate analysis because plasma ADAMTS13 activity may reflect liver fibrosis. However, the higher plasma ADAMTS13 activity was a significant risk factor for HCC development, independent of liver stiffness value by multivariate analysis. Furthermore, in the analysis of HCC recurrence, plasma ADAMTS13 activity or antigen level was retained as a significant risk for HCC development, but not liver stiffness value, by multivariate analysis. The current finding that plasma ADAMTS13 activity or antigen level significantly correlated with serum AST and ALT levels may explain this. Of note, it was previously shown that the higher serum ALT is associated with the higher rate of incidence of HCC development (31) and HCC recurrence after the surgical treatment (32) in HCV-related cirrhosis, suggesting that more hepatocellular damage increases a risk for HCC development in the liver of the same stage of fibrosis. Because plasma ADAMTS13 activity or antigen level reflect hepatocellular damage and subsequent wound healing as well as liver fibrosis stage, plasma ADAMTS13 activity, or antigen level may act distinctly from liver stiffness value in the risk analysis of HCC development.

Alternatively, the prediction of HCC development by plasma ADAMTS13 activity or antigen level may be explained by a potential direct link between hepatic stellate cells and HCC, which has been recently reported (3). This concept is suggested based on the findings that hepatic stellate cells express the stem cell marker of CD133 (33) and both hedgehog (34, 35) and Wnt signaling (36) are found in hepatic stellate cells, two pathways implicated in stem cell differentiation and cancer (37). Furthermore, the direct promotion of tumorigenicity of HCC by hepatic stellate cells has been reported (38).

In human studies, the alteration of plasma ADAMTS13 activity in chronic liver disease has already been reported (39–44). In patients with liver cirrhosis, plasma ADAMTS13 activity was shown to be decreased (39) in relation to the severity of cirrhosis (44), although the wide range of values were detected compared with normal controls (43). In contrast, Lisman and colleagues showed that plasma ADAMTS13 activity in patients with liver cirrhosis was highly variable and not significantly different from that in normal controls (42). In line with the latter report, plasma ADAMTS13 activity in chronic hepatitis B and C was variable in the current study. We speculate that these distinct results of plasma ADAMTS13 activity in chronic liver disease may be caused by the characteristics of the patients enrolled in the analysis. The patients with reduced plasma ADAMTS13 activity in the previous reports (39, 43, 44) might have minimal hepatitis activity, that is, minimal wound healing response. Highly variable activity of plasma ADAMTS13 in liver cirrhosis (42) might also be explained by the variable hepatitis activity in those patients. This issue should be further clarified.

In conclusion, the higher plasma ADAMTS13 activity or antigen level was a significantly independent risk factor for HCC development in chronic hepatitis B or C, suggesting that plasma ADAMTS13 activity and antigen level may be useful in the prediction of hepatocarcinogenesis in chronic liver disease. It should be further evaluated whether plasma ADAMTS13 activity and antigen level could be useful as a predictor of HCC development with a larger sample size and also with other etiology of underlying chronic liver disease such as NASH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Original Article

Cancer preventive effect of pegylated interferon α-2b plus ribavirin in a real-life clinical setting in Japan: PERFECT interim analysis

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Aim: This study was conducted to clarify the incidence of hepatocellular carcinoma (HCC) and the factors contributing to its occurrence by following chronic hepatitis C patients who received pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Methods: Patients who received PEG-IFN α-2b and RBV combination therapy with no history of HCC or HCC within 3 months after the start of treatment were observed for the onset of HCC at 67 centers.

Results: Sustained virological response (SVR) was observed in 999 (53.5%) of 1865 patients eligible for analysis. During the observation period (median duration: 4 years and 3 months), HCC developed in 59 patients (3.1%). A significant difference was observed in the 5-year cumulative incidence of HCC between SVR and non-SVR patients (1.1% vs. 7.1%). Factors contributing to HCC selected in multivariate analysis were therapeutic efficacy, sex, age, alanine aminotransferase (ALT) level at 24 weeks after the end of treatment, and platelet count. Non-SVR patients with ALT improvement after the end of treatment had a significantly lower 5-year cumulative incidence of HCC than those without (3.4% vs. 11.0%). HCC developed in 10 patients who achieved SVR, and multivariate analysis indicated that ALT level at 24 weeks after the end of treatment was the only significant factor contributing to HCC.

Conclusion: Several known risk factors for HCC contributed to HCC in patients who received PEG-IFN α-2b and RBV combination therapy, and ALT abnormality after the end of treatment contributes to the onset of HCC in both non-SVR and SVR patients.

Key words: alanine aminotransferase, chronic hepatitis C virus, hepatocellullar carcinoma, pegylated inteferon, ribavirin

Abbreviations: AFP, alpha fetoprotein; ALT, alanine aminotransferase; BR, biochemical response; CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; IFN, interferon; LVR, late virological response; NR, no response; NVR, non-virological response; PEG-IFN, pegylated interferon; ; RBV, ribavirin; SVR, sustained virological response; TR, transient response.

INTRODUCTION

THE INCREASE IN the incidence of hepatocellular carcinoma (HCC) in Japan peaked in 2004 and is now in a declining trend.[1] The HCC mortality rate, however, is still particularly high among developed countries,[2] and even now nearly 35 000 people die

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annually from HCC. In Japan, about 70% of patients diagnosed with HCC are positive for hepatitis C virus antibody.[3] The hepatitis C virus infection rate[2] and incidence of HCC both increase with the age of the patient,[4] and curing chronic hepatitis C (CHC) to reduce HCC and deaths due to HCC is a pressing issue.

With the discovery of interferon (IFN), CHC became a curable disease, and with the addition of ribavirin (RBV), therapeutic outcomes have improved dramatically. Currently, about 50%[5–8] of patients with HCV genotype 1b and high virus load and more than 80%[9] of genotype 2 patients achieve sustained virologic response (SVR), and the SVR rate is reported to improve further with long-term treatment[10,11] and with combination therapy plus a statin.[12]

The efficacy achieved with these IFN therapies is also reported to lead to the inhibition of the onset of HCC and deaths due to HCC[13-19], but only a few reports are available of long-term observation of patients receiving PEG-IFN α plus RBV combination therapy.

We therefore examined the HCC preventive effect of combination therapy in 1865 patients who received PEG-IFN α-2b and RBV.

METHODS

Patients and treatment

PERFECT (THE PEG-IFN and Ribavirin, Find Evidence of Chronic Hepatitis C Therapy in Tokyo) Study Group, consisting of 67 centers in Tokyo and Yamanashi Prefecture, conducted a retrospective study to investigate the efficacy and safety of PEG-IFN α-2b plus RBV in CHC patients in a real-life clinical setting. The participating centers, targeted patients, and the treatment method have already been reported[10] and are summarized below.

Patients seen from December 2004 who completed PEG-IFN α-2b plus RBV combination therapy by September 2007 were registered regardless of genotype, history of IFN treatment, or alanine aminotransferase (ALT) levels. Excluded from this study were pregnant or possibly pregnant and lactating women, and patients with severe heart disease, chronic kidney failure or creatinine clearance of ≤50 mL/min, current or history of severe psychiatric disorder, and autoimmune hepatitis. Doses of PEG-IFN α-2b and RBV and dose adjustment followed the Japanese package insert. The duration of treatment was 48 weeks, the standard of care for patients with genotype 1 and high virus load. In patients with late viral response (LVR) who did not achieve viral negativity by week 12, treatment could be extended up to 72 weeks. Patients other than those with genotype 1 and high virus load were treated for 24 weeks.

Included in this analysis were the patients registered in the PERFECT Study who had no history of HCC and for whom SVR/non-SVR status could be confirmed. The patients who developed HCC within 3 months of the start of treatment were excluded from analysis to rule out the possibility of inclusion of patients with HCC already present at the start of treatment.

The start of the follow-up period was defined as the first day of PEG-IFN α-2b and RBV treatment. The patients were monitored for the onset of HCC by routine follow-up methods practiced by each center. The diagnosis of HCC was based on the presence of typical hypervascular characteristics on angiography in addition to the findings on computed tomography and ultrasonography. Microscopic examination of fine-needle biopsy specimens was performed in patients whose angiograms did not demonstrate a typical image of HCC.

This multicenter study was approved by the institutional review board of each participating center. The study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent was obtained from each patient.

Statistical analysis

All statistical analyses were performed using SAS, version 9.13 (SAS Institute, Cary, NC, USA). Intergroup comparison of background variables was performed by Fisher's exact test and Mann–Whitney *U*-test.

The cumulative incidence of HCC was calculated by the Kaplan–Meier method, and intergroup comparison was conducted using the log-rank test. The determination of the factors contributing to HCC was conducted by Cox proportional hazards regression model using a stepwise procedure, incorporating the factors exhibiting $P < 0.2$ by the log-rank test and excluding factors with more than 30% of values missing. The determination of factors associated with biochemical response (BR) was conducted by a stepwise procedure using the results of logistic univariate analysis ($P < 0.2$) in logistic multivariate analysis.

All tests were two-sided, with a significance level set at $P < 0.05$.

RESULTS

Study population

A TOTAL OF 1865 subjects, consisting of 999 SVR patients (SVR rate 53.5%) and 866 non-SVR patients, were eligible for analysis. Of the non-SVR patients, 441 had transient response (TR) defined as viral negativity achieved during treatment (relapse: 408, virus breakthrough: 33), 400 patients had non-virological response (NVR) defined as viral negativity not being achieved, and the change in viral load during treatment was not known for 25 patients.

The duration of observation ranged from 3 months to 5 years and 8 months, with a median of 4 years and 3 months.

During the observation period, HCC developed in 59 patients (3.1%). Between patients who developed HCC and those who did not, significant differences in background factors were detected in age ($P < 0.0001$), hepatic fibrosis ($P = 0.0002$), virological efficacy ($P < 0.0001$), ALT levels ($P = 0.0089$), ALT level at 24 weeks after the end of treatment (≤40 vs. >40 IU/L) ($P < 0.0001$), platelet count ($P = 0.0001$), serum albumin ($P = 0.0062$), and alpha fetoprotein (AFP) ($P < 0.0001$) (Table 1).

Virological efficacy and incidence of HCC

The 5-year cumulative incidence of HCC by the Kaplan–Meier method was 1.1% in SVR patients and 7.1% in non-SVR patients, a difference that was significant ($P < 0.001$) (Fig. 1). No significant difference was observed in the incidence of HCC between TR and NVR patients among non-SVR patients, but the difference between TR and SVR patients was significant ($P < 0.0001$) (Fig. 2). This trend was also observed regardless of gender, with no significant difference in the incidence of HCC observed between TR and NVR in either male or female patients and a significant difference observed between TR and SVR in both male patients ($P = 0.0007$) and female ($P = 0.0065$) patients.

Factors contributing to HCC

The factors contributing to HCC selected in the multivariate analysis were therapeutic efficacy (SVR vs. NVR), sex, age (<60 vs. ≥60 years), ALT level at 24 weeks after the end of treatment (≤40 vs. >40 IU/L), and platelet count (<10 vs. ≥10 × 10 000/mm^3) (Table 2).

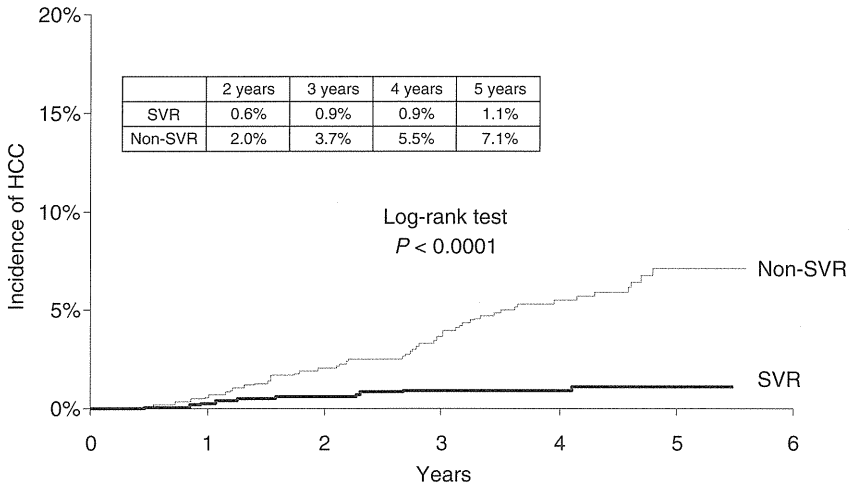
Biochemical response and incidence of HCC in non-SVR patients

Since ALT levels at 24 weeks after the end of treatment was selected as one factor contributing to HCC, the changes in ALT levels and onset of HCC were examined in 514 non-SVR patients with a pretreatment ALT level of more than 40 IU/L whose ALT level at 24 weeks after the end of treatment was obtained. Of these 514

Table 1 Patient background by onset of hepatocellular carcinoma (HCC) (1865 patients)

Factor	With onset of HCC ($n = 59$)	Without onset of HCC ($n = 1806$)	*P*-value
Gender (male/female)	40/19	1014/792	0.0832
Age	62 (44–74)	56 (17–77)	<0.0001
Diabetes (yes/no/unknown)	6/33/20	100/1040/666	0.1539
Hypertension (yes/no/unknown)	4/6/49	116/569/1121	0.0763
Alcohol abuse (yes/no/unknown)	11/16/32	195/493/1118	0.1930
Fibrosis (0/1/2/3/4/unknown)	0/12/13/15/4/15	57/573/355/205/56/560	0.0002
Genotype (1/2/3/unknown)	52/5/0/2	1421/365/2/18	0.0876
Effect of IFN (SVR/non-SVR)	10/49	989/817	<0.0001
Body mass index (kg/m^2)	22.6 (14.2–34.0)	22.9 (14.9–41.2)	0.8546
ALT (IU/L)	79 (24–343)	60 (8–984)	0.0089
ALT at 24 weeks after end of treatment (IU/L) (≤40/>40/unknown)	16/30/13	1105/352/349	<0.0001
Platelet count (×10 000/mm^3)	13.3 (4.3–22.2)	16.3 (3.6–213.3)	0.0001
Serum albumin (g/dL)	3.9 (2.9–4.7)	4.1 (2.8–5.9)	0.0062
AFP (ng/mL)	13 (2.2–327.9)	5 (0–875)	<0.0001

Median (minimum – maximum).
AFP, alpha fetoprotein; ALT, alanine aminotransferase; IFN, interferon; SVR, sustained virological response.



	2 years	3 years	4 years	5 years
SVR	0.6%	0.9%	0.9%	1.1%
Non-SVR	2.0%	3.7%	5.5%	7.1%

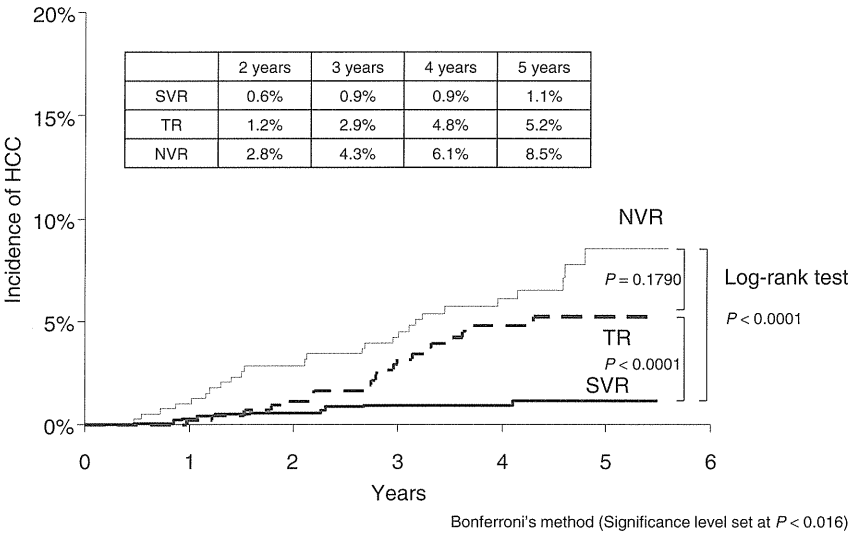
Figure 1 Onset of hepatocellular carcinoma (HCC) by therapeutic efficacy (1865 patients) (sustained virological response [SVR] vs. non-virological response [NVR]). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. The difference between SVR and non-SVR was examined using the log-rank test.

patients, ALT level at 24 weeks after the end of treatment was reduced to less or equal to 40 IU/L (biochemical response: BR) in 234 patients, and the remaining 280 patients had values of more than 40 IU/L (non-BR). There were significant differences between BR and non-BR patients in the background factors of pretreatment ALT level, age, hepatic fibrosis, platelet count, AFP, and treatment duration. Selected as the factors contributing to BR in non-SVR patients in the multivariate analysis were TR, long treatment duration, and high platelet count before the start of treatment (Table 3).

The 5-year cumulative incidence of HCC was 3.4% in BR patients and 11.0% in non-BR patients, and the difference in incidence was significant ($P = 0.0012$) (Fig. 3). The 5-year cumulative incidence of HCC in male patients was 3.6% in BR patients and 13.9% in non-BR patients, and the difference was significant ($P = 0.0012$). In female patients, however, it was 3.5% in BR patients and 7.6% in non-BR patients, and although the incidence of HCC was lower in BR patients, the difference was not significant ($P = 0.0706$).

Incidence of HCC in patients with normal pretreatment ALT levels

When the incidence of HCC was compared between SVR (288) and non-SVR (214) patients among 502 patients with pretreatment ALT levels less or equal to 40 IU/L, the 5-year cumulative incidence of HCC was 0% in SVR patients and 4.8% in non-SVR patients, indicating a significant difference ($P = 0.0005$) between the groups



	2 years	3 years	4 years	5 years
SVR	0.6%	0.9%	0.9%	1.1%
TR	1.2%	2.9%	4.8%	5.2%
NVR	2.8%	4.3%	6.1%	8.5%

Figure 2 Onset of hepatocellular carcinoma (HCC) by therapeutic efficacy (sustained virological response [SVR] vs. transient response [TR] vs. non-virological response [NVR]). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. The difference between each group was examined using the log-rank test (Bonferroni's Method, significance level set at $P < 0.016$).