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APPENDIX I

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Original Article

Diagnostic accuracy of α-fetoprotein and des-γ-carboxy prothrombin in screening for hepatocellular carcinoma in liver transplant candidates

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Aim: Although hepatocellular carcinoma (HCC)-specific serum tumor markers, α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), are used in the screening for HCC, their utility in pre-transplantation evaluation is not well established. This study aimed to evaluate the accuracy of AFP and DCP measurement for the diagnosis of HCC in liver transplant candidates.

Methods: A total of 315 consecutive adult patients (174 men and 141 women, mean age 52 years), who were to receive liver transplantation for end-stage liver diseases, were enrolled. The pre-transplant levels of AFP and DCP were compared with the histopathology of explanted liver.

Results: Hepatocellular carcinoma was present in the explanted liver of 106 recipients (median number of nodules 2, mean diameter 2.5 cm). The area under receiver operating characteristic curve for the diagnosis of HCC was 0.83 (95% confidence interval, 0.78–0.88) for AFP and 0.47 (0.41–0.54) for DCP. With the cut-off value of 100 mAU/mL, 20/106 (18.9%) patients with HCC and 54/194 (27.8%) patients without HCC were positive for DCP. DCP positivity was associated with vascular invasion, tumor differentiation and size among patients with HCC, which was associated with albumin level among patients without HCC. Vitamin K was administered prior to transplantation to 20 patients who were positive for DCP (two with and 18 without HCC), resulting in a decrease in DCP levels in 19 of them.

Conclusions: Serum DCP levels may be raised in end-stage liver disease patients without HCC, and cannot be used as a reliable marker for HCC among liver transplant candidates.

Key words: hepatocellular carcinoma, sensitivity and specificity, tumor marker, vitamin K deficiency

INTRODUCTION

CIRRHOSIS WITH EARLY-STAGE hepatocellular carcinoma (HCC) is currently one of the leading indications for liver transplantation. The introduction of the Milan criteria, i.e., a single tumor up to 5 cm in size or as many as three tumors up to 3 cm in size, and no vascular invasion has led to low incidence of recurrence post-transplantation in various transplant centers around the world.[1] The Milan criteria have now been adopted as the selection criteria for liver transplantation in the United States, Europe, and Japan.[2–4] Expanded criteria, such as the UCSF criteria or Tokyo rule, are proposed by several groups,[5,6] although the superiority of expanded criteria over Milan criteria is controversial.[7] Nevertheless, survival is reported to be poorer after liver transplantation for more advanced HCC outside the criteria, it is important to detect early-stage HCC that meets the eligibility requirements for liver transplantation.

In the screening for HCC, α-fetoprotein (AFP) measurement is widely used in conjunction with imaging studies, although the use of this marker alone has relatively low sensitivity (39–71%) and specificity (49–91%).[8,9] Other serum markers, such as des-γ-carboxy prothrombin (DCP) and the lens culinaris

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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

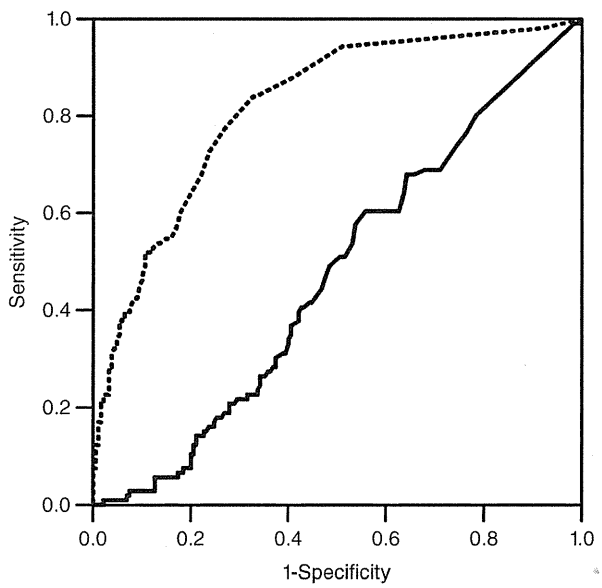

Figure 1 The receiver operating characteristic curves for des-γ-carboxy prothrombin (DCP) (solid line) and α-fetoprotein (AFP) (dashed line) levels in the diagnosis of hepatocellular carcinoma (HCC) are shown.

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.

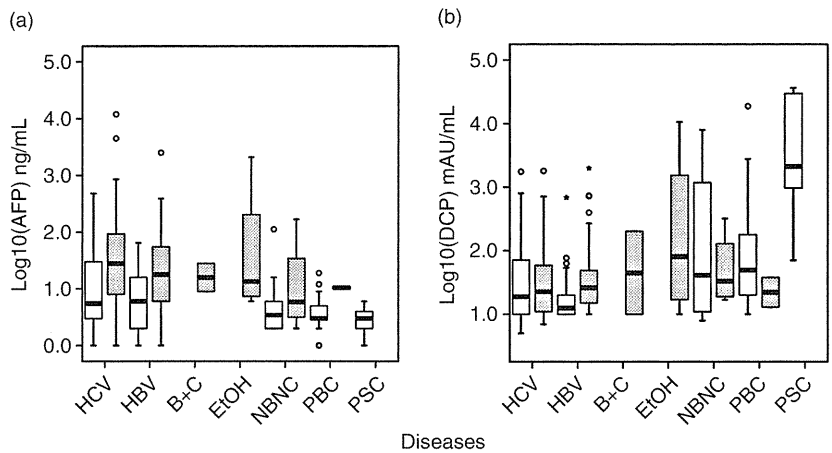

Figure 2 α-fetoprotein (AFP) (a) and des-γ-carboxy prothrombin (DCP) (b) levels according to etiology of liver diseases is shown as box-plot. White bar indicates non-hepatocellular carcinoma (HCC) group, and grey bar indicates HCC group. Serum level of AFP was higher in HCC group regardless of etiology. 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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ORIGINAL ARTICLES—LIVER, PANCREAS, AND BILIARY TRACT

Characteristics of Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma

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This article has an accompanying continuing medical education activity on page e50. Learning Objectives—At the end of this activity, the learner should identify the clinical features of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma and the role of hepatic fibrosis in the development of hepatocellular carcinoma.

See related article, Villanueva A et al, on page 1501 in *Gastroenterology*.

BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to characterize the clinical features of NASH patients with HCC. **METHODS:** In a cross-sectional multicenter study in Japan, we examined 87 patients (median age, 72 years; 62% male) with histologically proven NASH who developed HCC. The clinical data were collected at the time HCC was diagnosed. **RESULTS:** Obesity (body mass index ≥25 kg/m^2), diabetes, dyslipidemia, and hypertension were present in 54 (62%), 51 (59%), 24 (28%), and 47 (55%) patients, respectively. In nontumor liver tissues, the degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%). The prevalence of cirrhosis was significantly lower among male patients (21 of 54, 39%) compared with female patients (23 of 33, 70%) (P = .008). **CONCLUSIONS: Most patients with NASH who develop HCC are men; the patients have high rates of obesity, diabetes, and hypertension. Male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients.**

Keywords: Liver Cancer; Incidence; Sex; Retrospective Study.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality.[1] HCC mostly occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B and C viruses as well as alcohol consumption, are well-defined, 5%–30% of patients with HCC lack a readily identifiable risk factor for their cancer. It has been suggested that a more severe form of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH), might account for a substantial portion of cryptogenic cirrhosis and HCC cases.[2]

NAFLD is one of the most common causes of chronic liver disease in the world.[3,4] NAFLD is associated with obesity, diabetes, dyslipidemia, and insulin resistance and is recognized as a hepatic manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive NASH associated with fibrosis, necrosis, and inflammation. Despite its common occurrence and potentially serious nature, relatively little is known about the natural history or prognostic significance of NAFLD. Although prospective studies on the natural history of NAFLD and NASH with a larger cohort are awaited, these

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CT, computed tomography; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

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studies might be limited by the long and asymptomatic clinical course of these diseases, by their high prevalence in the general population, and by the lack of serologic markers for NASH. The evidence suggesting that NASH can progress to HCC comes from (1) case reports and case series,[5-8] (2) retrospective studies,[9-12] and (3) prospective studies.[13-17] These studies generally examined limited numbers of cases and follow-ups; therefore, the incidence of HCC and risk factors for HCC in NASH patients remain unclear.

The Japan NASH Study Group (representative, Takeshi Okanoue)[18] was established in 2008 by the Ministry of Health, Labour and Welfare of Japan to address unmet research needs in the area of liver diseases. As a part of this mandate, the study group conducted a cross-sectional multicenter study to characterize the clinical features of histologically proven NASH patients who developed HCC.

Methods

Patients

We retrospectively identified and reviewed 87 Japanese patients with NASH, who developed HCC between 1993 and 2010, at 15 hepatology centers that belong to the Japan NASH Study Group[18] and their affiliated hospitals in Japan. The diagnosis of NASH was based on (1) the histologic features of steatohepatitis, (2) negligible alcohol consumption, and (3) exclusion of liver diseases of other etiology. To determine alcohol consumption as accurately as possible, we reviewed medical records in our institutions, and when patients had been transferred from other institutions, we also reviewed a summary of medical records from those institutions. According to the medical records, alcohol consumption was assessed on the basis of a detailed history that was obtained by physicians and by interviewing family members. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, positivity for anti-hepatitis C virus antibody, the presence of other types of liver diseases (eg, primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. The sections of nontumor liver tissues were reanalyzed by experienced hepatopathologists (T.O., E.H.) who were blinded to the laboratory parameters and clinical data. We excluded patients whose histologic diagnosis of NASH was not confirmed by central review and patients with insufficient or inconclusive information concerning alcohol consumption, body mass index (BMI), and laboratory data including fasting glucose and lipid.

Of the 87 patients, 14 patients had been previously diagnosed as NAFLD or NASH and had been followed at our institutions; 73 patients had been transferred from other institutions to our institutions for investigation and treatment of HCC. Most patients had been identified as having HCC during screening, which included ultrasound and/or computed tomography (CT) of the liver and alpha-fetoprotein (AFP) testing.

The diagnosis of HCC was based on liver histology and, in the absence of histology, on typical features of HCC as assessed by dynamic CT or magnetic resonance imaging (MRI) (ie, hypervascular with washout in the portal/venous phase).[19] Of the 87 patients, 49 patients were diagnosed as HCC after hepatic resection, 21 patients were diagnosed after ultrasound-guided tumor biopsy, and 17 patients were diagnosed by dynamic CT or MRI.

The Ethics Committees of each participating center approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Clinical Assessment and Laboratory Tests

The clinical and laboratory data were collected at the time HCC was diagnosed. BMI was calculated by using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as BMI $\geq$25 kg/m^2 according to the criteria of the Japan Society for the Study of Obesity.[20] Diabetes was defined as fasting plasma glucose concentration of $\geq$126 mg/dL or 2-hour plasma glucose concentration of $\geq$200 mg/dL during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose.[21] Hypertension was defined as systolic blood pressure $\geq$130 mm Hg or diastolic blood pressure $\geq$85 mm Hg or by the use of antihypertensive agents.[22] Dyslipidemia was defined as serum concentrations of triglycerides $\geq$150 mg/dL or high-density lipoprotein (HDL) cholesterol $<$40 mg/dL and $<$50 mg/dL for men and women, respectively, or by the use of specific medication.[22]

Venous blood samples were taken in the morning after 12-hour overnight fast. The laboratory evaluations included blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), fasting plasma glucose, HbA1c, total cholesterol, HDL cholesterol, triglyceride, ferritin, hyaluronic acid, AFP, and des-γ-carboxy prothrombin (DCP). These parameters were measured by using standard clinical chemistry techniques.

Histopathologic Examination

Nontumor liver tissues were obtained from all 87 patients to diagnose the background liver tissue at the time HCC was diagnosed. In 49 patients who underwent hepatic resection for HCC, we examined nontumor liver tissues that were surgically resected. In 21 patients who underwent ultrasound-guided tumor biopsy, nontumor liver tissues far from HCC tumors were biopsied separately. In 17 patients who were diagnosed as HCC by dynamic CT or MRI and did not undergo either hepatic resection or tumor biopsy, only nontumor liver tissues far from HCC tumors were obtained by ultrasound-guided biopsy.

The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin, with Masson trichrome, and by silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory's hyaline (Mallory's body) or fibrosis.[23-25] The necroinflammatory grade and the degree of fibrosis were evaluated and scored according to the criteria proposed by Brunt et al.[26]

Statistical Analysis

Results are presented as numbers with percentages in parentheses for qualitative data or as the medians and ranges (25th-75th percentiles) for quantitative data. Comparisons were made by using a χ^2 test for qualitative factors or a Mann-

Table 1. Patient Characteristics

Characteristic	Total (n = 87)	Male (n = 54)	Female (n = 33)	*P* value[a]
Age (*y*)	72 (69–75)	72 (69–75)	72 (68–75)	.52
BMI (kg/m^2)	26.0 (23.8–28.3)	26.0 (23.8–28.8)	26.2 (23.9–27.7)	.54
Obesity	54 (62%)	35 (65%)	19 (58%)	.50
Diabetes	51 (59%)	31 (57%)	20 (61%)	.77
Dyslipidemia	24 (28%)	13 (24%)	11 (33%)	.35
Hypertension	47 (54%)	22 (41%)	25 (76%)	.001
Platelet count ($\times 10^4/\mu L$)	13.9 (10.1–18.0)	14.5 (11.7–18.0)	10.9 (7.8–18.0)	.05
AST (*IU/L*)	47 (30–59)	46 (27–60)	47 (35–58)	.45
ALT (*IU/L*)	36 (26–55)	43 (26–69)	34 (26–42)	.11
γ-GTP (*IU/L*)	75 (40–115)	68 (36–177)	75 (40–115)	.90
Fasting glucose (*mg/dL*)	114 (99–145)	112 (99–144)	120 (97–152)	.59
HbA1c (%)	6.1 (5.4–7.1)	5.9 (5.4–7.0)	6.3 (5.2–7.1)	.78
Total cholesterol (*mg/dL*)	169 (147–202)	169 (147–202)	169 (147–202)	.62
HDL cholesterol (*mg/dL*)	50 (41–60)	45 (41–58)	55 (50–73)	.03
Triglyceride (*mg/dL*)	100 (76–138)	118 (80–147)	96 (74–116)	.06
Ferritin (*ng/dL*)[b]	197 (74–401)	273 (154–703)	98 (23–172)	.005
Hyaluronic acid (*ng/mL*)[c]	166 (67–241)	151 (69–244)	174 (61–332)	.85
AFP (*ng/mL*)	7.1 (5.0–18.0)	6.0 (4.0–14.7)	10.8 (5.9–18.0)	.02
DCP (*mAU/mL*)	66 (22–298)	48 (22–243)	81 (21–942)	.42
HCC tumor size (*cm*)	3.0 (2.0–4.0)	3.1 (2.2–4.5)	2.6 (1.9–4.0)	.18
Number of HCC tumors				.78
1	65 (75%)	39 (72%)	26 (79%)	
2 or 3	16 (18%)	11 (20%)	5 (15%)	
≥4	6 (7%)	4 (8%)	2 (6%)	
Background liver tissue				
Steatosis grade				.64
0: <5%	1 (1%)	1 (2%)	0 (0%)	
1: 5%–33%	60 (69%)	36 (67%)	24 (73%)	
2: 34%–66%	19 (22%)	11 (20%)	8 (24%)	
3: >66%	7 (8%)	6 (11%)	1 (3%)	
Necroinflammatory grade[d]				.22
1: mild	31 (35%)	22 (41%)	9 (27%)	
2: moderate	45 (52%)	26 (48%)	19 (58%)	
3: severe	11 (13%)	6 (11%)	5 (15%)	
Fibrosis stage[d]				.003
1	10 (11%)	10 (18%)	0 (0%)	
2	15 (17%)	10 (18%)	5 (15%)	
3	18 (21%)	13 (25%)	5 (15%)	
4	44 (51%)	21 (39%)	23 (70%)	

NOTE. Values are medians (25th–75th percentiles) or numbers (%). Where no other unit is specified, values refer to number of patients.
[a]χ^2 test or Mann–Whitney *U* test.
[b]Missing data for 27 patients.
[c]Missing data for 29 patients.
[d]According to reference 26.

Whitney *U* test on ranks for quantitative factors with non-equal variance. *P* values less than .05 from two-sided tests were considered to be significant. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc, Chicago, IL).

Results

The characteristics of the 87 NASH patients who developed HCC are summarized in Table 1. The median age was 72 years (25th percentile, 69; 75th percentile, 75); the mean age (standard deviation) was 71.2 (6.7) years. There were 54 male patients (62%) and 33 female patients (38%); the male:female ratio was 1.6:1. The median BMI was 26.0 kg/m^2, and 54 patients (62%) were obese (BMI ≥25 kg/m^2). Diabetes, dyslipidemia, and hypertension were present in 51 (59%), 24 (28%), and 47 (55%) patients, respectively.

The diagnosis of NASH was proved by histologic examination of nontumor liver tissues at the time HCC was diagnosed. The degree of steatosis was grade 1 (5%–33%) in 60 patients (69%), grade 2 (34%–66%) in 19 (22%), and grade 3 (>66%) in 7 (8%). One patient who showed less than 5% steatosis was diagnosed as "burn-out" NASH, because a previous liver biopsy that was performed before development of HCC had demonstrated typical histologic features of NASH. The necroinflammatory grade was mild (grade 1) in 31 patients (35%), moderate (grade 2) in 45 (52%), and severe (grade 3) in 11 (13%). The degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%).

The median diameter of HCC tumors was 3.0 cm (25th percentile, 2.0; 75th percentile, 4.0). A single HCC lesion was present in 65 of 87 patients (75%).

Data were stratified according to sex (Table 1). Compared with female patients, male patients had significantly less hypertension, lower HDL cholesterol and AFP, higher ferritin, and a less advanced stage of fibrosis. The prevalence of cirrhosis was significantly lower in male patients (21 of 54, 39%) than in female patients (23 of 33, 70%) ($P = .008$).

Discussion

In this cross-sectional multicenter study in Japan, we showed the clinical features of a relatively large number (n = 87) of NASH patients with HCC. The male:female ratio was 1.6:1. Men have higher HCC rates than women in almost all populations, with male:female ratios usually averaging between 2:1 and 4:1.[2] In the latest nationwide survey of HCC in Japan,[27] this ratio was 2.5:1. The reasons underlying higher rates of HCC in men might relate to sex-specific differences in exposure to risk factors. Men are more likely to be infected with hepatitis B and C viruses, consume alcohol, smoke cigarettes, and have increased iron stores.[2] Moreover, androgens are considered to influence the development of HCC. With regard to the male: female ratio of HCC associated with NASH, a male:female ratio of 1.3:1 was reported in a summary of 16 published cases of HCC associated with NASH.[28] Ratios of 2.8:1 and 0.67:1 were reported in 2 retrospective studies of HCC arising from cryptogenic cirrhosis in Italy (n = 44)[10] and the United States (n = 30),[9] respectively, and a ratio of 1.6:1 was reported for 36 cases of NASH-associated HCC from a single center in Japan.[15] Overall, NASH patients with HCC are more often men. However, these male:female ratios might be lower than the ratios for HCC of other etiologies, including viral hepatitis and alcohol consumption.

Although it is well-known that male gender is a risk factor for HCC in patients infected with hepatitis B and C viruses,[2] it remains unclear whether male gender is a factor associated with the development of HCC in NASH patients. It is now suspected that there is an even distribution of NASH among men and women.[29] In another study by our group,[30] the male:female ratio was 0.85:1 in 342 NASH patients without cirrhosis and HCC. The male:female ratio (1.6:1) of NASH patients with HCC in the present study is higher than this ratio. In agreement with our observations, a case-control study showed that the male: female ratio was 1.6:1 in 34 NASH patients with HCC, whereas the ratio was 0.69:1 in 348 NASH patients without HCC.[15] A recent prospective study indicated that older age and alcohol consumption were independent risk factors for the development of HCC in patients with NASH-cirrhosis and that male gender tended to be associated with the development of HCC, although this trend did not reach statistical significance.[17]

The median age of our patients was 72 years. There was no significant difference in age between men and women. Although the global age distribution of HCC varies by geographic region, sex, and etiology, in almost all areas the peak female age group in HCC patients is 5 years older than in male HCC patients.[2] In a nationwide survey of HCC in Japan,[27] the mean ages were 65.5 years for men and 69.4 years for women. The male patients in the present study are slightly older than the mean ages reported in these previous studies.

Consistent with the literature,[9-12] more than half of our patients displayed obesity, diabetes, and hypertension. Obesity constitutes a significant risk factor for cancer mortality in general and is an increasingly recognized risk factor for HCC in particular.[31,32] In the present study, body weight was measured at the time HCC was diagnosed. Because advanced HCC might cause weight loss, it is likely that our patients were obese before the development of HCC. Diabetes has also been proposed as a risk factor for HCC.[2] Thus, HCC shares 2 major risk factors, obesity and diabetes, with NASH.

Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. As NASH progresses to cirrhosis, steatosis tends to disappear, so-called burn-out NASH.[5] As expected, the grade of steatosis was mild in most of our cases. It was possible to diagnose 1 case without steatosis as burn-out NASH, because a previous liver biopsy specimen (liver biopsy was performed 25 years prior) was preserved and available. It is likely that many cases of NASH-associated HCC might have been missed because of loss of the telltale sign of steatosis.

Most HCC arises on a background of cirrhosis. It is less clear whether cirrhosis is a necessary predisposition for the development of HCC in patients with NASH. Case reports of HCC arising from NAFLD and NASH patients without fibrosis or cirrhosis have been accumulating.[33-36] Cirrhosis (fibrosis stage 4) was present in 51% of cases, and advanced stages of fibrosis (stage 3 or 4) were found in 72% of cases in the present study. Indeed, cirrhosis or advanced fibrosis appeared to be the predominant risk factors for HCC development. However, in the remaining 28% of cases, HCC developed in patients with less fibrosis (stage 1 or 2). Interestingly, male patients developed HCC at a less advanced stage of fibrosis than female patients, and the prevalence of cirrhosis was significantly lower in men (39%) than in women (70%). Although the reason for the sex differences is unclear, these findings indicate that screening for HCC is needed not only in NASH patients with advanced fibrosis but also in those with less fibrosis, particularly if they are men. Further studies are needed to confirm this potentially important observation. Paradis et al[37] reported that in patients whose only risk factors for chronic liver disease are features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis. In addition, they found that some of these HCCs developed on preexisting liver cell adenomas. However, no preexisting adenomas were observed in the present cases.

Compared with female patients, male patients had significantly higher serum ferritin value. The normal value for ferritin varies according to the age and gender of the individual. Adult men have serum ferritin values averaging approximately 100 ng/mL (range, 75–250), whereas adult women have levels averaging approximately 30 ng/mL (range, 20–75).[38] Thus, normal men have higher ferritin levels than women. Elevation of ferritin levels is associated with NASH.[39] Because we excluded patients with alcohol consumption as rigorously as possible, we believe that alcohol consumption did not contribute to the elevation of ferritin levels in our patients.

The median diameter of the HCCs in the present study was 3.0 cm, which is equal to or smaller than the size of previously reported HCCs.[9,10,12,28,37] This is probably because most of our patients had been identified as having HCC during screening. A single HCC lesion was present in 75% of patients. For early detection of NASH-associated HCC, vigilant screening is important,[9] and the development of serologic markers for NASH is necessary.

The mechanisms of carcinogenesis in NASH remain to be elucidated. Possible mechanisms include hyperinsulinemia

caused by insulin resistance in NASH, increased levels of insulin-like growth factor that promotes tumor growth, increased susceptibility of the steatotic liver to lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, disordered energy and hormonal regulation in obesity, and aberrations in regenerative processes occurring in cirrhosis.[25]

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding the causality of NASH in HCC. Second, the study did not include a control group of HCC patients with other liver diseases. Third, there might be a bias in patient selection, because patients were retrospectively identified as having NASH-associated HCC. Finally, although our patients were negative for hepatitis B virus surface antigen, it is still possible that occult hepatitis B virus infection might be associated with the development of HCC in some of our cases.

In summary, we showed the clinical features of NASH patients with HCC. NASH patients with HCC were more often men and frequently displayed obesity, diabetes, and hypertension. Our results suggest that male patients might develop HCC at a less advanced stage of fibrosis than female patients. Further prospective studies with a longer follow-up time and larger cohorts are needed to determine the causal association of NASH with HCC and to identify risk factors for the development of HCC in NASH patients.

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Conflicts of interest

The authors disclose no conflicts.

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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B

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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B▽†

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We focused on determining the most accurate and convenient genotyping methods and most appropriate single nucleotide polymorphism (SNP) among four such polymorphisms associated with interleukin-28B (IL-28B) in order to design tailor-made therapy for patients with chronic hepatitis C virus (HCV) patients. First, five different methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe [HP], the InvaderPlus assay [Invader], and the TaqMan SNP genotyping assay [TaqMan]) were developed for genotyping four SNPs (rs11881222, rs8103142, rs8099917, and rs12979860) associated with IL-28B, and their accuracies were compared for 292 Japanese patients. Next, the four SNPs associated with IL-28B were genotyped by Invader for 416 additional Japanese patients, and the response to pegylated interferon/ribavirin (PEG-IFN/RBV) treatment was evaluated when the four SNPs were not in linkage disequilibrium (LD). HRM failed to genotype one of the four SNPs in five patients. In 2 of 287 patients, the results of genotyping rs8099917 by direct sequencing differed from the results of the other three methods. The HP, TaqMan, and Invader methods were accurate for determination of the SNPs associated with IL-28B. In 10 of the 708 (1.4%) patients, the four SNPs were not in LD. Eight of nine (88.9%) patients whose rs8099917 was homozygous for the major allele were virological responders, even though one or more of the other SNPs were heterozygous. The HP, TaqMan, and Invader methods were suitable to determine the SNPs associated with IL-28B. The rs8099917 polymorphism should be the best predictor for the response to the PEG-IFN/RBV treatment among Japanese chronic hepatitis C patients.

Hepatitis C virus (HCV) infection is a global health problem, with worldwide estimates of 120 to 130 million carriers (7). Chronic HCV infection can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma (25). The current standard of care treatment for suitable patients with chronic HCV infection consists of pegylated alpha 2a or 2b interferon (PEG-IFN) given by injection in combination with oral ribavirin (RBV), for 24 or 48 weeks, dependent on HCV genotype. Large-scale treatment programs in the United States and Europe showed that 42 to 52% of patients with HCV genotype 1 achieved a sustained virological response (SVR) (3, 8, 13), and similar results were found in Japan. This treatment is associated with well-described side effects (such as a flu-like syndrome, hematologic abnormalities, and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment (2). It is valuable to predict an individual's response before treatment with PEG-IFN/RBV to avoid these side effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with HCV genotype 2 or 3 have a relatively high rate of SVR (70 to 80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR despite 48 weeks of treatment (8).

Recently, we reported from genome-wide association stud-

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TABLE 1. Characteristics of the patients examined

Parameter	Result for:	
	1st stage (n = 292)	2nd stage (n = 416)
Age (yr)	57.2 ± 10.2	56.6 ± 10.9
No. of patients male/female	145/147	194/222
No. (%) of patients in institution[a]:		
1	18 (6.2)	0 (0)
2	178 (61.0)	0 (0)
3	57 (19.5)	0 (0)
4	39 (13.3)	0 (0)
5	0 (0)	249 (59.9)
6	0 (0)	94 (22.6)
7	0 (0)	52 (12.5)
8	0 (0)	21 (5.0)

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ies (GWAS) that several highly correlated common single nucleotide polymorphisms (SNPs), located in the vicinity of the lambda 3 interferon (IFN-λ3), coded for by the interleukin-28B (IL-28B) gene on chromosome 19, are implicated in nonvirological response (NVR) to PEG-IFN/RBV among patients with HCV genotype 1 (21). At almost exactly the same time as our report, the association between response to PEG-IFN/RBV and SNPs associated with IL-28B was reported from the results of GWAS by two other groups (6, 19). Determination of these SNPs associated with IL-28B before PEG-IFN/RBV treatment will provide extremely valuable information, because the patients predicted as showing NVR to PEG-IFN/RBV treatment could avoid the treatment. There are two questions to be asked before using these SNPs in clinical practice: (i) which methods for genotyping these SNPs are efficient, and (ii) which SNP is most informative in cases where the SNPs are not in linkage disequilibrium (LD)? We have developed five different methods for detecting the SNPs associated with IL-28B and compared their accuracies to establish the most efficient genotyping method. The response to PEG-IFN/RBV treatment was evaluated, when the SNPs associated with IL-28B were not in LD, to determine the best SNP to predict the response to PEG-IFN/RBV treatment.

MATERIALS AND METHODS

Study population. Samples were obtained from 708 Japanese chronic hepatitis C patients and divided into groups of 292 patients (145 males and 147 females; mean age, 57.2 years) and 416 patients (194 males and 222 females; mean age, 56.6 years) for the first and second stages (Table 1). In the first stage, we focused on analyzing the effective methods for determining the genotypes of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B (Fig. 1A). Figure 2 shows the locations of these four SNPs in chromosome 19; rs11881222 and rs8103142 are located in the IL-28B gene, and rs12979860 and rs8099917 are located downstream from the IL-28B gene. The results of genotyping the four SNPs by five different methods, described below, were compared and evaluated for consistency. For this first stage, the 292 chronic hepatitis C patients were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan (Table 1). From the results of the first stage, the InvaderPlus assay was chosen as one of the best methods to determine the genotypes of the four SNPs associated with IL-28B and was used for genotyping 416 patients (Fig.

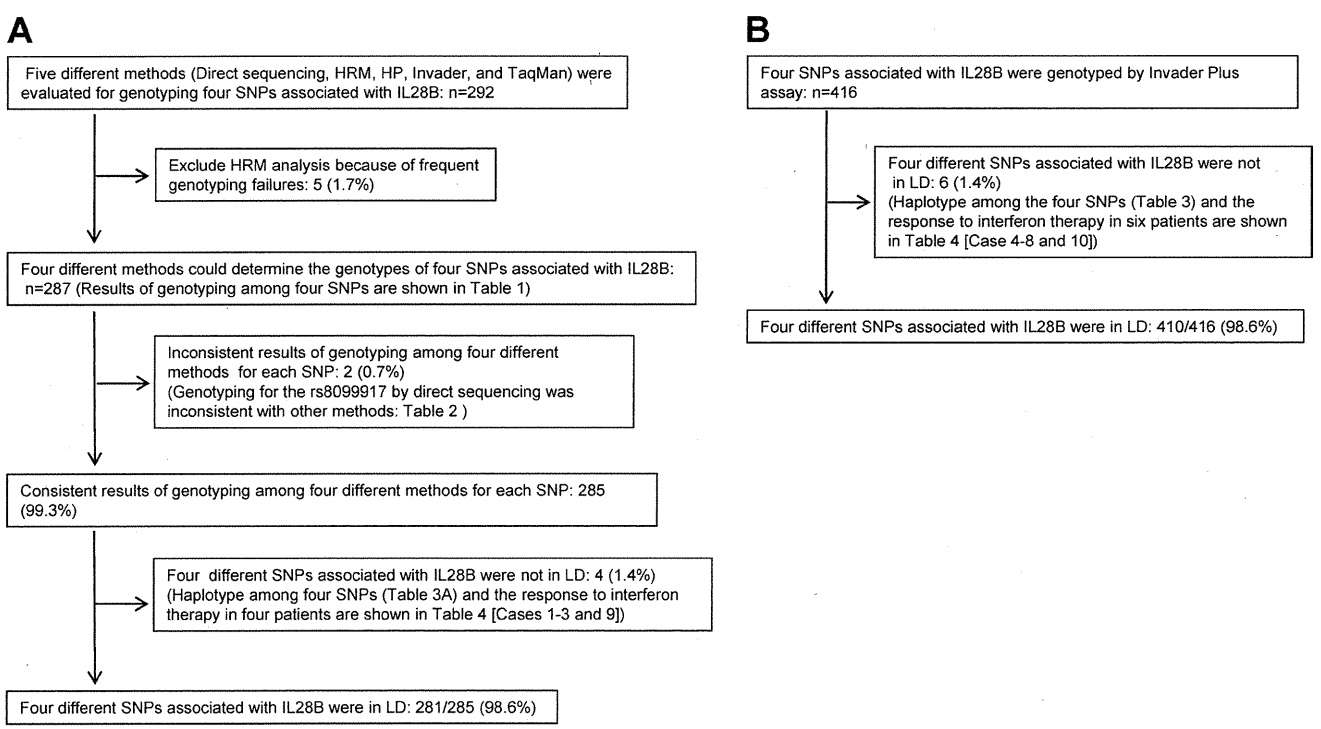

FIG. 1. Schema for the flowchart of the examinations.