(33, 34). In addition, the utility of liver stiffness measurement is low in obese patients and in those with ascites and hepatic inflammation (35, 36). In the present study, serum miR-122 levels inversely correlated with liver fibrosis, and decreased miR-122 expression was associated with advanced fibrosis stage. Moreover, the ROC curves showed that the ability of the serum miR-122 to predict fibrosis was superior to that of hyaluronic acid and type IV collagen. Therefore, serum miR-122 may be a valuable tool to predict liver fibrosis.

In conclusion, hepatic and serum miR-122 levels are associated with hepatic steatosis and fibrosis, and the serum miR-122 level can serve as a useful predictive marker of liver fibrosis in patients with NAFLD.

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Frequency of elevated biomarkers in patients with cryptogenic hepatocellular carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AC 1 **Naota Taura**
C 1 **Tatsuki Ichikawa**
BC 1 **Hisamitsu Miyaaki**
B 2 **Eisuke Ozawa**
B 3 **Takuya Tsutsumi**
B 4 **Shotaro Tsuruta**
B 5 **Yuji Kato**
B 6 **Takashi Goto**
B 7 **Noboru Kinoshita**
B 8 **Masanori Fukushima**
B 9 **Hiroyuki Kato**
B 10 **Kazuyuki Ohata**
B 11 **Kazuo Ohba**
B 12 **Junichi Masuda**
B 13 **Keisuke Hamasaki**
BC 14 **Hiroshi Yatsuhashi**
ACD 1 **Kazuhiko Nakao**

1 Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences Nagasaki University, Nagasaki, Japan
2 Department of Gastroenterology and Hepatology, Sasebo City General Hospital, Sasebo, Japan
3 Department of Gastroenterology and Hepatology, Nagasaki Municipal Hospital, Nagasaki, Japan
4 Department of Gastroenterology and Hepatology, Japanese Red Cross Nagasaki Genbaku Hospital, Nagasaki, Japan
5 Department of Gastroenterology and Hepatology, Oita Prefectural Hospital, Oita, Japan
6 Director Digestive Organ Center, Japan Labour and Welfare Organization Nagasaki Labour Welfare Hospital, Nagasaki, Japan
7 Department of Gastroenterology and Hepatology, Sasebo Chuo Hospital, Sasebo, Japan
8 Department of Internal Medicine, Nagasaki Prefectural Goto Central Hospital, Nagasaki, Japan
9 Department of Internal Medicine, National Hospital Organization Saga National Hospital, Saga, Japan
10 Department of Internal Medicine, Kouseikai Hospital, Kouseikai, Japan
11 Department of Gastroenterology and Hepatology, Isahaya Health Insurance General Hospital, Isahaya, Japan
12 Department of Gastroenterology and Hepatology, Medical Inc. Kosei-kai Nijigaoka Hospital, Nijigaoka, Japan
13 Department of Internal Medicine, Caritas Clinic, Caritas, Japan
14 Clinical Research Center, National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan

Corresponding Author: Naota Taura, e-mail: ntaura-gi@umin.ac.jp
Source of support: Self financing

Background: The incidence of hepatocellular carcinoma (HCC) continues to increase in Japan, but the clinical characteristics of Japanese patients with HCC have not been well described. The aim of this study was to determine the frequencies and utilities of elevated α-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels as biomarkers in cryptogenic HCC.

Material/Methods: A total of 2638 patients with HCC diagnosed between 1999 and 2010 in the Nagasaki Association Study of Liver (NASLD) were recruited for this study. The cause of HCC was categorized into 4 groups; HCC-B, HCC-C, HCC-BC, and HCC-nonBC. The significance of factors was examined for HCC-nonBC using logistic regression analysis in all patients.

Results: Multivariate analysis identified age, sex, BMI, alcohol consumption, platelet count, AST, ALT, AFP, DCP, and TNM stage as independent and significant risk factors for HCC-nonBC. According to TNM stage, the median AFP levels in HCC-nonBC with TNM stages I, II, and III were significantly lower than in either HCC-B or HCC-C. In TNM stage IV, the median AFP level in HCC-nonBC was significantly lower than in either HCC-B or HCC-BC. The median DCP levels in HCC-nonBC with TNM stages I and II were significantly higher than those in either HCC-B or HCC-C. In TNM stage III, the median DCP level in HCC-nonBC was significantly higher than that in HCC-C.

Conclusions: DCP was more sensitive than AFP for the diagnosis of early stage cryptogenic HCC. DCP should be used as the main serum test for cryptogenic HCC detection.

Key words: HCC • DCP • AFP

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Background

Primary liver cancer accounts for approximately 6% of all human malignancies. It is estimated that half a million cases occur worldwide annually, making primary liver cancer the fifth most common malignancy in men and the ninth in women [1–6]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers [7] and the age-adjusted HCC mortality rate has increased in recent decades in Japan [8]. Similarly, a rising HCC trend has been reported in several developed countries in North America, Europe, and Asia [9,10]. HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses that cause HCC, HCV is predominant in Japan [11–14]. However, it has been reported that the absolute numbers and proportion of HBsAg and HCVab negative HCC (HCC-nonBC) have both been steadily increasing in Japan [15,16].

The prognosis for patients with HCC is still poor. Surgical resection and liver transplantation are the standard curative treatments available. Recently, radio-frequency ablation (RFA) and percutaneous ethanol injection (PEI) have also been recognized as effective methods of achieving complete tumor necrosis for small HCCs [17]. However, the chance of curative treatment is often limited by several features of HCC itself. HCCs are usually large before they produce symptoms. Bilobar or multifocal tumors are common. The incidence of associated cirrhosis is also high, exceeding 80% in most series [18–20]. Transcatheter intra-arterial chemoembolization (TACE), with which complete necrosis of HCCs is thought to be difficult to achieve, is also impacted by the above factors [21]. To increase opportunities for meaningful intervention and to improve survival, early detection of HCC by measuring alpha-fetoprotein (AFP) and/or imaging screening is implemented in many countries [15,22–25]. However, the poorer prognosis of patients with HCC-nonBC is reportedly attributable to its late detection in an advanced stage, owing to the lack of a surveillance system for early detection of HCC [26].

In this retrospective cohort study, our aim was to characterize consecutive patients who had been diagnosed with HCC-nonBC during an 11-year period (1999–2010) at the centers comprising the Nagasaki Association Study of Liver Disease (NASLD) group. We evaluated the clinical characteristics of patients with HCC-nonBC, their tumor stages, treatment, AFP and DPC as potential biomarkers, and survival.

Material and Methods

Patients

In total, 2638 patients with HCC diagnosed between 1999 and 2010 in the NASLD were recruited for this study. The diagnosis of HCC was based on AFP and/or DCP levels, as well as the results of imaging techniques such as ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), and hepatic angiography (HAG), and/or liver biopsy. The diagnostic criteria included characteristic liver biopsy findings, elevated AFP (≥20 ng/mL) and/or DCP (≥40 ng/mL), and neovascularization on HAG, CT, and/or MRI.

The diagnosis of chronic HCV infection was positive for both anti-HCV, by a third-generation enzyme-linked immunosorbent assay (ELISA), and for HCV RNA by polymerase chain reaction (PCR). The diagnosis of chronic HBV infection was based on the presence of HBsAg (enzyme-linked immunosorbent assay; Abbott Laboratories); serum AFP was measured by radioimmunoassay (Abbott Laboratories). The history of alcohol intake was obtained from medical records; habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol for a period of more than 10 years.

HCC etiologies were categorized into 4 groups: (1) HCC-B, HBsAg positive, and HCVAb negative; (2) HCC-C, HCVAb positive, and HBsAg negative; (3) HCC-BC, both HBsAg and HCVAb positive; and (4) HCC-nonBC, both HbsAg, and HCVAb negative. The significance of age, sex, body mass index (BMI), alcohol intake, diabetes mellitus, underlying liver disease Child-Pugh score, platelet count, prothrombin time (PT), albumin (ALB), total bilirubin (Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), AFP, DCP, and Tumor-Node-Metastasis (TNM) stage were examined to identify possible relationships with HCC-nonBC using logistic regression analysis.

Treatment modalities

Patients diagnosed with HCC were assessed for surgery based on the extent of lobar involvement and liver function status. The extent of lobar involvement was evaluated based on a combination of USG, CT, MRI, and HAG findings. Patients were considered to be poor candidates for resection if they met any of the following criteria: (1) bilobar involvement, (2) evidence of tumor infiltration into the main portal vein or thrombosis of the vein, (3) evidence of extrahepatic metastases, (4) Child's grade C cirrhosis, or (5) poor cardiac and/or respiratory status. If surgery was contraindicated or the patient refused to undergo an operation, RFA or PEI therapy was the second treatment choice, offered to those with HCCs less than 3 cm in diameter. The remaining patients without main portal vein thrombosis or extrahepatic metastasis were advised to undergo TACE, regardless of tumor size or number.

After initial treatment, the AFP levels and liver functions of the patients were assessed every 1 to 3 months, and USG imaging was performed every 3 to 6 months during the follow-up period. Patients suspected to have HCC recurrence were further evaluated by CT and/or MRI. The assessment of treatment for

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recurrent HCC was based on lobar involvement and liver function status as described for the initial treatment. RFA or liver transplantation to treat HCC was started at our institution in 2002. Furthermore, none of the subjects in our study received either of these treatments for recurrent HCC during the follow-up period.

Statistical analysis

The survival duration was the time from the diagnosis of HCC until the time of death or the time of preparation of this manuscript. The survival rate was analyzed using the Kaplan-Meier method, and differences between the survival probability curves were tested using the log-rank test. Descriptive summaries of study groups are reported as the median value (SD: standard deviation) and number (%). Data were analyzed using the Mann-Whitney *U* test for continuous ordinal data, and the chi-square test with Yates' correction and Fisher's exact test were used for intergroup comparisons to determine the association between 2 qualitative variables. *P* values <0.05 were considered to indicate a statistically significant difference. Variables achieving statistical significance according to univariate analysis were subsequently included in the multivariate analysis using a logistic regression model and were described as hazard ratios (HR) with 95% confidence intervals (CI). Coefficients were calculated from the linear discriminating function of the variables. Data analysis was performed using SPSS version 16.0 for Windows.

Results

Patient characteristics at enrollment

We diagnosed 2638 patients with HCC during the study period. Patient characteristics at the time of HCC diagnosis are presented in Table 1. The underlying causes of HCC were as follows: 474 (18%) patients were positive for HBsAg, 1533 (58%) were positive for HCVAb, 40 (2%) were positive for both HBsAg and HCVAb, and 591 (22%) were negative for HBsAg and anti-HCV.

Overall, the median survival of all 2638 patients was 1.8 years. The cumulative 5-year survival rates of the patients with HCC-B, HCC-C, HCC-BC, and HCC-nonBC were 43%, 52%, 49%, and 47%, respectively (Figure 1). Patients in the HCC-C group had a higher cumulative survival rate than those in the HCC-B and HCC-nonBC groups.

Univariate and multivariate analyses of the factors associated with HCC-nonBC

Univariate and multivariate analyses were performed to identify factors independently related to HCC-nonBC. In the univariate analysis, the following 13 factors significantly influenced HCC-nonBC: age, sex, BMI, alcohol consumption, diabetes mellitus, underlying liver disease, platelet count, Bil, AST, ALT, AFP, DCP, and TNM stage (Table 2). Multivariate analysis identified age (≥70 years, HR 1.63), sex (female, HR 1.73), BMI (≥25, HR 2.12), alcohol consumption (not excessive, HR 3.41; excessive, HR 14.73), diabetes mellitus (HR 2.42), underlying liver disease (chronic hepatitis, HR 0.46; cirrhosis, HR 0.52), platelet count (<116,000/μL, HR 1.88), AST (<56 IU/L, HR 1.47), ALT (<46 IU/L, HR 2.48), AFP (20–199 ng/mL, HR 0.60; ≥200 ng/mL, HR 0.63), DCP (20–199 mAU/mL, HR 1.64; ≥200 mAU/mL, HR 2.08), and TNM stage (II, HR1.67; III, HR1.88; IV, HR 2.40), as independent and significant factors associated with HCC-nonBC (Table 3).

Comparison of biomarkers according to liver disease cause

The positive rate of AFP (≥20 ng/ml) in HCC-B, HCC-C, HCC-BC, and HCC-nonBC were 62%, 55%, 61%, and 44%, respectively; whereas the positive rate of DCP (≥40 mAU/ml) were 67%, 55%, 75% and 77%, respectively (Figure 2). The positive rate of AFP in HCC-nonBC was significantly lower than those in the HCC-B and HCC-C groups, whereas the positive rate of DCP was significantly higher than that in the HCC-B and HCC-C groups.

The median AFP and DCP levels in HCC-B, HCC-C, HCC-BC, and HCC-nonBC according to TNM stage are presented in Table 4. The median AFP levels in HCC-B, HCC-C, HCC-BC, and HCC-nonBC were 60 ng/mL, 25 ng/mL, 29 ng/mL, and 13 ng/mL, respectively; whereas the DCP levels were 4990 mAU/mL, 418 mAU/mL, 612 mAU/mL, and 3077 mAU/mL, respectively. The median AFP level in HCC-nonBC was significantly lower than those in the other groups; whereas the median DCP level was significantly higher than that in the HCC-C group.

According to TNM stage, the median AFP levels in HCC-nonBC with TNM stages I, II, and III were significantly lower than that in either HCC-B or HCC-C. In TNM stage IV, the median AFP level in HCC-nonBC was significantly lower than that in either HCC-B or HCC-BC. The median DCP levels in HCC-nonBC with TNM stages I and II were significantly higher than that in either HCC-B or HCC-C. In TNM stage III, the median DCP level in HCC-nonBC was significantly higher than that in HCC-C. However, there were no significant differences in the median DCP level among TNM stage IV cases.

The survival rate of patients in the high DCP group (≥200 mAU/mL) was significantly lower than that of patients classified into the low DCP (40–199 mAU/mL) and DCP negative (<40 mAU/mL) groups among those with HCC-B, HCC-C, and HCC-nonBC ($p \leq 0.001$; log-rank test) (Figure 3).

Discussion

The age-adjusted mortality rate for HCC has increased over the past few decades in Japan [27]. However, most patients are

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Table 1. Characteristics of 2,638 HCC patients.

	(SD)	(%)		(SD)	(%)
All	2,638		Treatment		
Age (years)	70.0	10.3	Surgical resection	391	15
Sex			PEIT or RFA	740	28
Male	1,786	68	TACE or TAI	1,221	46
Female	852	32	Chemotherapy	51	2
Alcohol consumption (unknown 454)			Transplantation	10	1
Excessive	236	9	Only palliative care	225	9
Not excessive	511	13	Platelets (104/mL)	11.6	8.0
None	1,437	54	AST (IU/L)	56	101
DM (unknown 326)			ALT (IU/L)	45	402
(+)	651	25	Bil (mg/dL)	0.9	0.5
(–)	1,661	63	Alb (g/dL)	3.7	0.6
BMI (unknown 594)	22.7	0.5	PT (%)	83	18
Etiology of liver disease			AFP (ng/mL)	25	89,580
HBV	474	18	<20	1,209	46
HCV	1,533	58	20–199	761	29
HBV+HCV	40	2	≥200	668	25
NBNC	591	22	DCP (mAU/mL)	89	75,076
Underlying liver disease (unknown, 110)			<40	991	38
Normal	42	2	40–199	583	22
Chronic hepatitis	686	26	≥200	1,064	40
Cirrhosis	1,800	68	Observation period (years)	1.8	2.3
Child-Pugh Grade (unknown, 88)					
A	1796	68			
B	606	23			
C	148	6			
TNM stage					
I	682	26			
II	1,039	39			
III	579	22			
IV	338	12			

Alcohol consumption: excessive; average daily consumption of an amount equivalent to 80 g of pure ethanol for a period of more than 10 years. Not excessive; Alcohol consumption: excessive; average daily consumption of an amount equivalent to 1–79 g of pure ethanol for a period of more than 10 years. Data are presented as median value (SD: standard deviation) or frequency (%).

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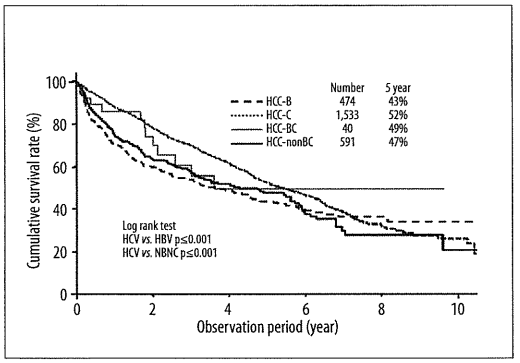

Figure 1. Cumulative survival rate of HCC patients according to chronic viral hepatitis infection.

still diagnosed at an advanced stage and their survival time is therefore short. Patients with chronic HBV and/or HCV infection in addition to cirrhosis should be monitored with USG and/or CT and/or MRI of the liver to detect tumors at an early stage. HCC surveillance using imaging modalities is usually performed at 6-month intervals [28,29]. There is a large population of individuals infected with HCV, HBV, or both in whom cancer is in the latency period. For those who harbor chronic HCV and/or HBV infections, attention must be focused on the detection of HCC at an early stage. In this study, more than 75% of patients with HCC were positive for HBV and/or HCV. Additionally, our data showed patients with HCC-nonBC to generally be diagnosed at an advanced stage. Thus, the target population for HCC surveillance must be easily identifiable. However, it will not be easy to select appropriate subjects for screening of HCC among those negative for both HBsAg and HCVAb.

AFP has long been considered the ideal serological marker for detecting HCC. Persistently elevated AFP is well known to be related to the presence of HCC and its determination can facilitate better identification of patients at risk. However, in our present dataset, the median serum AFP level in HCC-nonBC was not abnormal, whereas those in HCC-B and HCC-C cases were abnormal. Few early-stage HCC-nonBC cases present with abnormal AFP serum levels. Several reports have shown elevated AFP to be a risk factor for HCC development in HCV and/or HBV patients [24,30–36]. However, our results suggest AFP alone to be insufficient for HCC-nonBC surveillance.

Since Liebman et al. demonstrated DCP to be a useful marker for HCC diagnosis, many studies have compared DCP and AFP. Several investigations have made comparisons of the usefulness of DCP and AFP for HCC diagnosis [37–40]. However, whether AFP is superior to DCP in all cases is still controversial. Even the sensitivities and specificities reported by these studies were quite different. One reason for these differences involves the use of different cut-off

Table 2. Univariate analysis of factors associated with HCC-nonBC.

Parameters	Hazard ratio	P value
Age (years) ≥70	1.59	<0.001
Sex Female	0.67	<0.001
BMI (kg/m²) ≥25	1.85	<0.001
Alcohol consumption		
None	1	–
Not excessive	2.57	<0.001
Excessive	12.41	<0.001
Diabetes mellitus (%)		
+	2.96	<0.001
Underlying liver disease		
Normal	1	–
Chronic hepatitis	0.20	<0.001
Cirrhosis	0.20	<0.001
Child-Pugh grade		
A	1	–
B	0.92	0.446
C	1.34	0.131
Platelets (10³/μL) ≥116	2.22	<0.001
AST (IU/L) <56	2.15	<0.001
ALT (IU/L) <46	2.64	<0.001
PT (%) ≥83	1.26	0.016
Bil (mg/dL) <0.9	0.89	0.229
Alb (mg/dL) ≥3.7	1.06	0.547
AFP (ng/mL)		
<20	1	–
20–199	0.48	<0.001
≥200	0.82	0.079
DCP (mAU/mL)		
<40	1	–
40–199	1.66	<0.001
≥200	2.73	<0.001
TNM stage		
I	1	–
II	1.66	<0.001
III	2.73	<0.001
IV	2.22	<0.001

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Table 3. Multivariate analysis of factors associated with HCC-nonBC.

Parameters	Hazard ratio	95% CI	P value
Age (years) ≥70	1.63	1.21–2.20	0.001
Sex Female	1.73	1.33–2.85	<0.001
BMI (kg/m^2) ≥25	2.12	1.58–2.83	<0.001
Alcohol consumption			
None	1	–	–
Not excessive	3.41	2.43–4.79	<0.001
Excessive	14.73	9.48–22.9	<0.001
Diabetes mellitus (%)			
(+)	2.42	1.82–3.22	<0.001
Underlying liver disease			
Normal	1	–	–
Chronic hepatitis	0.46	0.01–0.16	<0.001
Cirrhosis	0.52	0.02–0.19	<0.001
Platelets (10^3/μL) ≥116	1.88	1.35–2.60	<0.001
AST (IU/L) <56	1.47	1.01–2.10	0.411
ALT (IU/L) <46	2.08	1.47–2.94	<0.001
PT (%) ≥83	0.97	0.71–1.32	0.826
AFP (ng/mL)			
<20	1	–	–
20–199	0.60	0.42–0.85	0.005
≥200	0.63	0.43–0.92	0.079
DCP (mAU/mL)			
<40	1	–	–
40–199	1.64	1.13–2.39	0.010
≥200	1.88	1.35–2.60	0.018
TNM stage			
I	1	–	–
II	1.67	1.13–2.48	0.011
III	1.88	1.19–2.96	0.007
IV	2.40	1.32–4.35	<0.001

CI – confidence interval.

values in the various studies (e.g., 40, 60, and 100 mAU/mL for DCP; and 20, 100, and 200 ng/mL for AFP). Other possible reasons include differences in the causes of the underlying liver diseases, and patients with cirrhosis tending to have higher AFP levels than those with chronic hepatitis [36,41]. Another possible reason for these differences might be etiological differences in liver diseases among the patients examined in prior studies. In this study, the median AFP level in HCC-nonBC was significantly lower than that in either HCC-B or HCC-C, whereas the median DCP level was significantly higher. Our data suggest that DCP levels differ among liver diseases with different etiologies. The high value identified in our study may be related to the higher DCP values in patients without hepatitis virus infection.

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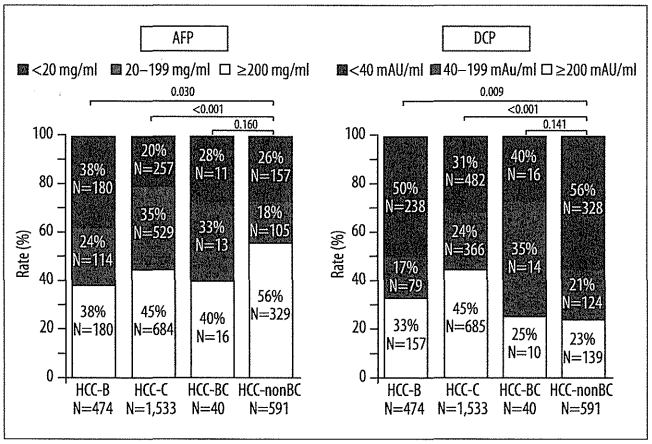

Figure 2. The positive rate of AFP (≥20 ng/ml) and DCP (≥40 mAU/ml) in HCC-B, HCC-C, HCC-BC and HCC-nonBC.

Table 4. Median AFP and DCP levels in HCC-B, HCC-C, HCC-BC and HCC-nonBC according to TNM stage.

	HCC-B	HCC-C	HCC-BC	HCC-nonBC
All patients				
Number	474	1,533	40	591
AFP (ng/mL) (range)	60 (1–2,920,000)*	25 (1–1,438,472)*	29 (3–189,850)***	13 (1–8,145,000)
DCP (mAU/mL) (range)	4,990 (4–1,497,560)	418 (1–871,700)*	612 (10–266,260)	3,077 (5–265,000,000)
TNM stage I				
Number	103	480	13	77
AFP (ng/mL) (range)	15 (1–6,300)**	16 (1–3,188)*	28 (3–2,120)*	6 (1–9,820)
DCP (mAU/mL) (range)	26 (3–1,038)**	25 (1–20,448)*	39 (14–353)	44 (12–12,224)
TNM stage II				
Number	150	624	11	254
AFP (ng/mL) (range)	14 (1–181,150)**	24 (1–200,000)*	17 (5–952)	8 (1–114,907)
DCP (mAU/mL) (range)	72 (4–233,780)*	53 (2–74,493)*	173 (10–831)	255 (5–369,000)
TNM stage III				
Number	92	319	12	157
AFP (ng/mL) (range)	77 (2–453,000)***	48 (1–196,000)**	47 (4–72,727)	25 (1–246,940)
DCP (mAU/mL) (range)	535 (10–172,000)	305 (4–14,410) ***	206 (16–4,039)	460 (10–109,350)
TNM stage IV				
Number	129	102	4	103
AFP (ng/mL) (range)	4,450 (1.5–2,920,000)**	2,379 (2–1,438,472)	64,838 (2882–189,850)***	914 (1–8,145,000)
DCP (mAU/mL) (range)	9,573 (10–1,497,560)	8,954 (19–871,700)	104,254 (755–266,260)	3,340 (12–265,000,000)

* p≤0.001 *vs.* HCC-nonBC; ** p≤0.01 *vs.* HCC-nonBC; *** p≤0.05 *vs.* HCC-nonBC.

The biological function of AFP is still not well identified. Since AFP is similar to albumin, it is possible that AFP function as a carrier for several ligands such as bilirubin, fatty acids, steroids, heavy metals, flavonoids, phytoestrogens, dioxin, and various drugs [42]. The increase of AFP levels to 500 ng/ml is correlated with the tumor size; 80% of small HCC show no increase of AFP

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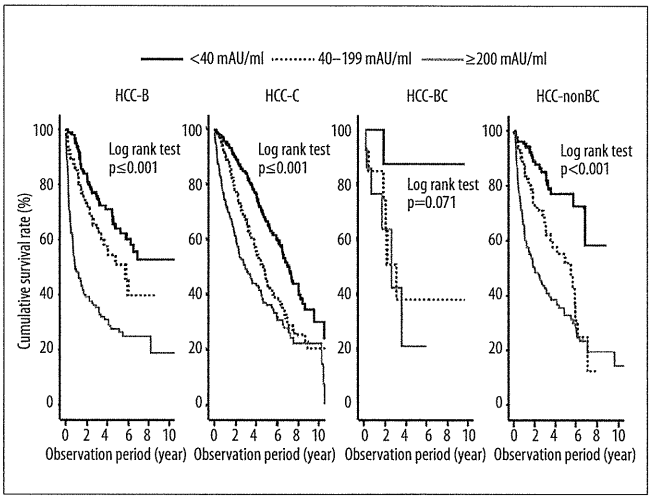

Figure 3. Cumulative survival rate of HCC patients according to DCP levels stratified by chronic viral hepatitis infection.

concentration. Furthermore, sensitivity of AFP decreases from 52% to 25% when tumor diameter is >3 and <3 cm, respectively [42].

There are various differences between DCP and total AFP. Firstly, DCP is a more specific HCC marker than AFP because other liver diseases do not cause an increase of DCP serum levels. DCP measurement for HCC has a sensitivity of 48–62% and a specificity of 81–98% [43]. However, we often encounter patients with liver disease who have slightly elevated DCP levels, but undetectable HCC as assessed by imaging studies. It has been reported that aberrant elevation of DCP is occasionally observed in patients with alcoholic cirrhosis, obstructive jaundice, or vitamin K deficiency [44]. Recently, Toyoda et al. measured a novel DCP (NX-DCP) in serum using a newly developed sandwich ECLIA with new anti-DCP monoclonal antibodies p11 and p16, and reported preliminary data from only 20 HCC patients. They showed that the DCP/NX-DCP ratio may be useful for the diagnosis of HCC among warfarin users [45].

Neither DCP alone nor AFP alone was optimal for the detection of HCC, but the combination of both markers enhanced sensitivity, indicating that these 2 markers are complementary. Several other studies have shown DCP and AFP to be complementary, which is consistent with the production of DCP and AFP in HCC occurring through different pathways, possibly explaining why sex, race, underlying liver disease, and hepatic disease etiologies had opposite effects on these 2 markers [46–48].

Conclusions

In conclusion, this retrospective cohort study demonstrated DCP to be more sensitive than AFP for the diagnosis of early-stage cryptogenic HCC. We advocate that DCP be used as the main serum test for detecting cryptogenic HCC.

Conflict of interest

The following people have nothing to disclose: Naota Taura, Tatsuki Ichikawa, Hisamitsu Miyaaki, Eisuke Ozawa, Takuya Tsutsumi, Shotaro Tsuruta, Yuji Kato, Takashi Goto, Noboru Kinoshita, Masanori Fukushima, Hiroyuki Kato, Kazuyuki Ohata, Kazuo Ohba, Junichi Masuda, Keisuke Hamasaki, Hiroshi Yatsuhashi, and Kazuhiko Nakao.

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HEPATOLOGY

Significance of hepatitis B virus core-related antigen and covalently closed circular DNA levels as markers of hepatitis B virus re-infection after liver transplantation

Toshihisa Matsuzaki,* Ichikawa Tatsuki,* Masashi Otani,* Motohisa Akiyama,* Eisuke Ozawa,* Satoshi Miuma,* Hisamitsu Miyaaki,* Naota Taura,* Tomayoshi Hayashi,‡ Sadayuki Okudaira,† Mitsuhisa Takatsuki,† Hajime Isomoto,* Fuminao Takeshima,* Susumu Eguchi† and Kazuhiko Nakao*

Departments of *Gastroenterology and Hepatology and †Surgery, Nagasaki University Graduate School of Biomedical Sciences, and ‡Department of Pathology, Nagasaki University Hospital, Nagasaki, Japan

Key words
cccDNA, HBcrAg, HBV, liver transplantation.

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Correspondence
Dr Toshihisa Matsuzaki, Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: tmatsuzaki6@gmail.com

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Abstract

Background and Aim: Currently, hepatitis B virus (HBV) re-infection after liver transplantation (LT) can be almost completely suppressed by the administration of HBV reverse transcriptase inhibitors and hepatitis B immunoglobulins. However, after transplantation, there is no indicator of HBV replication because tests for the serum hepatitis B surface antigen and HBV-DNA are both negative. Therefore, the criteria for reducing and discontinuing these precautions are unclear. In this study, we examined the serum HBV core-related antigen (HBcrAg) and intrahepatic covalently closed circular DNA (cccDNA) in order to determine if these could be useful markers for HBV re-infection.

Methods: Thirty-one patients underwent LT for HBV-related liver disease at Nagasaki University Hospital from 2001 to 2010. Of these, 20 cases were followed up for more than 1 year (median follow-up period, 903 days). We measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue. In addition, in nine cases, we assessed the serial changes of HBcrAg and intrahepatic cccDNA levels from preoperative periods to stable periods.

Results: We examined serum HBcrAg and intrahepatic cccDNA levels in 20 patients (35 samples). HBcrAg and cccDNA levels were significantly correlated with each other ($r = 0.616$, $P < 0.001$). From a clinical aspect, the fibrosis stage was significantly lower in both HBcrAg- and cccDNA-negative patients than in HBcrAg- or cccDNA-positive patients.

Conclusions: HBcrAg and cccDNA were useful as HBV re-infection markers after LT. Keeping patients' HBcrAg and cccDNA negative after LT might contribute to long-term graft survival.

Authors' Contributions:
Toshihisa Matsuzaki: acquisition of data, study concept and design, statistical analysis, writing of manuscript.
Tatsuki Ichikawa: study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content.
Masashi Otani: critical revision of the manuscript for important intellectual content.
Motohisa Akiyama: critical revision of the manuscript for important intellectual content.
Eisuke Ozawa: critical revision of the manuscript for important intellectual content.
Satoshi Miuma: critical revision of the manuscript for important intellectual content.
Sadayuki Okudaira: acquisition of data, critical revision of the manuscript for important intellectual content.
Tomayoshi Hayashi: acquisition of data, critical revision of the manuscript for important intellectual content.
Naota Taura: critical revision of the manuscript for important intellectual content.
Hisamitsu Miyaaki: critical revision of the manuscript for important intellectual content.
Susumu Eguchi: critical revision of the manuscript for important intellectual content.
Takashi Kanematsu: critical revision of the manuscript for important intellectual content.
Hajime Isomoto: critical revision of the manuscript for important intellectual content.
Fuminao Takeshima: critical revision of the manuscript for important intellectual content.
Kazuhiko Nakao: study supervision, critical revision of the manuscript for important intellectual content.

Introduction

Liver transplantation (LT) is an established procedure for the treatment of end-stage liver disease. However, the recurrence of hepatitis B virus (HBV) is implicated in life-threatening graft failure.[1] Therefore, the prevention of HBV recurrence following LT is a serious concern. The advent of hepatitis B immunoglobulins (HBIg) and the HBV reverse transcriptase inhibitor (RTI) was a major breakthrough in the management of HBV recurrence. Currently, an ideal recurrence rate for HBV has been observed in patients who received HBIg and RTI combination therapy.[2] However, several studies have reported that HBV can be detected in the transplanted liver and peripheral blood mononuclear cells of recipients even when they have a hepatitis B surface antigen (HBsAg)-negative status.[3] Therefore, prophylaxis currently must be continued for the patient's lifetime. However, there are concerns with the long-term administration of HBIg and RTI with respect to safety, medical costs, and resistant mutations of HBV.[4] In order to discontinue the prophylaxis, several groups have attempted to vaccinate LT recipients against HBV, but most of these studies involve relatively low seroconversion rates because of the immunosuppressive environment.[5]

Recently, new agents against HBV, such as adefovir and entecavir, which hardly develop resistant mutations, have become available. Some have reported that HBIg can be discontinued after LT by using the new anti-HBV agents even if the vaccination does not succeed.[6] Angus *et al.* reported that when adefovir dipivoxil was substituted for low-dose HBIg, all patients were alive at the study completion without recurrence.[7] In addition, low-risk cases, such as those with fulminant hepatitis, and hepatitis B core antibody (HBcAb)-positive donors are not necessary for the administration of high-dose HBIg.[8] However, after transplantation, RTI and HBIg may mask the appearance of HBV-DNA, regardless of the presence of intrahepatic HBV covalently closed circular DNA (cccDNA). These factors make it difficult to detect HBV dynamics following LT, and we are therefore unable to determine the feasibility of the discontinuation of prophylaxis.

Recently, a new enzyme immunoassay that detects hepatitis B core-related antigen (HBcrAg) has been reported.[9] HBcrAg changes in parallel with HBV-DNA in the serum and has a wide detection range.[10] Moreover, its levels are correlated with the intrahepatic cccDNA levels of patients with chronic hepatitis B.[11] In addition, we previously reported on the usefulness of HBcrAg in patients receiving anti-HBV prophylaxis following LT.[12]

Therefore, in this study, we simultaneously measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue and studied the HBV dynamics in patients following HBV-related LTs.

Methods

Patients and samples. From 2001 to 2010, a total of 31 patients with HBV-related end-stage liver disease underwent LTs at Nagasaki University Hospital, Nagasaki, Japan. Of these, we enrolled 20 patients who could be followed up for more than approximately 1 year (median 902 days; range 323–2456 days). There were 17 men and 3 women, with a median age of 56.5 years (range 33–68 years). All 20 patients were diagnosed with liver cirrhosis, and 12 were diagnosed with hepatocellular carcinoma. In addition, two patients were coinfected with the hepatitis C virus (Table 1).

Table 1 Baseline clinical features of the enrolled patients

Case	Age	Gender	Indication disease	HBV-DNA	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb	Donor HBcAb	HBcrAg
1	55	F	LC-B	< 2.6	> 2000	0.2	36.0	0.0	> 100.0	5.0	6.0
2	56	M	LC-B	< 2.6	> 2000	2.3	0.6	82.4	99.9	5.0	4.2
3	48	M	LC-B, HCC	< 2.6	562.5	0.1	1.1	57.7	> 100.0	31.3	5.0
4	60	M	LC-B	< 2.6	1789	0.1	0.2	97.6	> 100.0	70.1	5.8
5	59	M	LC-B, HCC	< 2.6	> 2000	0.1	0.1	> 100.0	> 100.0	5.0	3.2
6	57	M	LC-B, HCC	3.9	188.5	0.5	0.8	54.0	> 100.0	10.3	5.1
7	56	M	LC-B, HCC	< 2.6	> 2000	0.1	1.4	75.4	> 100.0	91.9	5.6
8	68	M	LC-B, HCC	< 2.6	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	3.0
9	33	F	LC-B	3.0	> 2000	0.2	0.2	81.5	99.9	99.6	5.5
10	58	M	LC-B, HCC	3.0	> 2000	0.1	0.1	93.6	> 100.0	93.4	5.1
11	59	M	LC-B	< 2.6	378.3	0.3	0.1	61.6	> 100.0	93.0	3.8
12	57	M	LC-B + C, HCC	< 2.6	519.9	0.1	0.1	> 100.0	99.9	5.0	2.0
13	49	M	LC-B	< 2.6	> 2000	0.1	0.9	52.9	> 100.0	34.1	5.2
14	65	F	LC-B	6.9	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	6.8
15	55	M	LC-B, HCC	< 2.1	> 2000	0.2	0.1	99.3	> 100.0	31.6	4.5
16	46	M	LC-B + C	4.3	1100.4	0.2	0.1	> 100.0	> 100.0	81.9	3.7
17	59	M	LC-B, HCC	< 2.1	> 2000	0.1	0.1	99.2	> 100.0	38.6	3.7
18	51	M	LC-B, HCC	2.1	> 2000	0.2	0.4	62.8	99.4	50.0	4.7
19	67	M	LC-B, HCC	3.9	> 2000	0.1	34.3	60.2	> 100.0	91.1	6.3
20	54	M	LC-B, HCC	2.1	> 2000	0.1	104.8	37.4	> 100.0	9.7	4.3

HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAb, hepatitis B envelope antibody; HBeAg, hepatitis B envelope antigen; HBsAb, antibody against hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; LC-B, LC due to HBV; LC-B + C, LC due to HBV-HCV coinfection.

All patients had been receiving RTI since preoperative periods. The HBsAg was negative in all donors, but eight donors were HBcAb-positive (cut-off, 50%), which was suggested to be due to prior exposures to HBV.

The prophylactic infusion of HBIg was administered to all patients according to a fixed-dose schedule; 10 000 units were given intravenously at the anhepatic period during the operation and the next day after the living donor LT (LDLT). Afterwards, 2000 units of HBIg were given routinely in order to keep the serum hepatitis B surface antibody (HBsAb) titers above 100 units/L. After the LDLT, serum HBsAg, hepatitis B envelope antigen (HBeAg), and HBV-DNA were not detected in any of the patients in this study.

Serum samples and biopsy specimens were obtained from 20 patients who received protocol biopsies 1 year after the LDLT at Nagasaki University Hospital after providing informed consent. Nine patients were followed up from the preoperative period to the stable period. Serum samples were obtained at the following three specified intervals: (i) in the preoperative period, samples were obtained just before the operation; (ii) in the postoperative period, samples were obtained during the operation of LT; and (iii) in the stable period, samples were obtained during admission for protocol biopsy. Liver tissue samples were obtained during the following three specified procedures: (i) biopsy from explanted liver during the operation; (ii) time-zero biopsy from the implanted liver during the operation; and (iii) protocol biopsy 1 year after the LDLT.

Serological markers for HBV. HBsAg, HBsAb, HBeAg, hepatitis B envelope antibodies (HBeAb), and HBcAb levels were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Inc., Tokyo, Japan). Serum concentrations of HBV-DNA were determined using a polymerase chain reaction (PCR) HBV monitoring kit (Roche Diagnostics K.K., Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copies/mL.

HBcrAg test. Serum HBcrAg levels were measured by a CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, FujiRebio, Inc.). HBcrAg concentrations were expressed as units/mL (U/mL). In this study, HBcrAg values were expressed as log U/mL, and the cut-off value was set at 3.0 log U/mL.[9,13]

Measurement of cccDNA. Liver tissues were stored at −80°C before DNA extraction. HBV-DNA was extracted using a high pure PCR template preparation kit (Roche Diagnostics K.K.). The concentration of purified DNA was measured at an absorbance of 260 nm.

cccDNA levels were measured with the real-time PCR method. With reference to a previous study,[11] we designed two oligonucleotide primers, cccF2 (5′-CGTCTGTGCCTTCTCATCTGA-3′, nucleotides: 1424-1444) and cccR4 (5′-GCACAGCTTGGAGGC TTGAA-3′, nucleotides: 1755-1737), and a cccP2 probe (5′-FAM-ACCAATTTATGCCTACAG-MGB-3′, nucleotides: 1672-1655). Reaction volume (20.0 μL) containing 500 ng of extracted DNA, 0.5 μmol/L of each primer, 0.2 μmol/L of the probes, and LightCycler TaqMan Master (Roche Diagnostics K.K.) was administered. The initial activation step was heated at 95°C for 10 min. The subsequent PCR conditions consisted of 60 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s per cycle. Real-time PCR was performed in a LightCycler (Roche Diagnostics K.K.). Serial dilutions of a plasmid containing an HBV monomer were used as quantitation standards.

Liver histology. Liver histology was evaluated by the same two pathologists. The degrees of necroinflammation and fibrosis were assessed based on the New Inuyama classification.[14] The degrees of rejection were assessed with the Rejection Activity Index according to the Banff working classification of hepatic allograft pathology.[15]

Liver function test. Blood biochemical tests were performed in all patients, and liver function was evaluated. Liver function was assessed using Pugh's modification of Child's scoring system.[16]

Statistical analyses. Student's *t*-tests and Fisher's exact tests were used for comparisons between groups of parametric quantitative data, and Mann–Whitney *U*-tests were used for comparisons between independent groups of non-parametric data. Categorical variables were compared with chi-square tests. The correlations between continuous variables were analyzed by the Pearson's correlation test. Two-tailed *P* values less than 0.05 were considered statistically significant.

Results

Correlation between HBcrAg and cccDNA. The correlation between HBcrAg and cccDNA levels in all 35 samples is summarized in Figure 1. A statistically significant positive

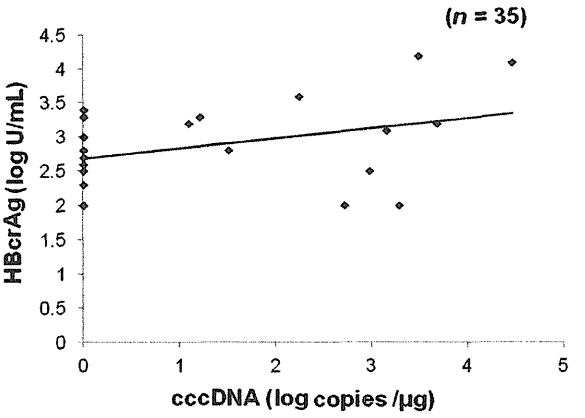

Figure 1 Correlation between serum hepatitis B core-related antigen (HBcrAg) and intrahepatic hepatitis B virus covalently closed circular DNA (cccDNA). $r = 0.616$, $P < 0.001$ ($y = 0.40x + 2.62$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

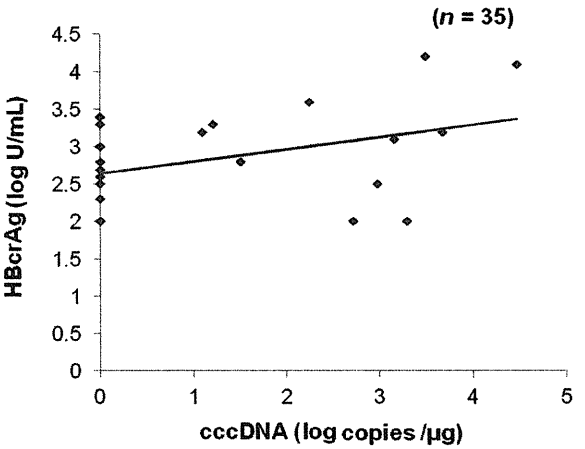

Figure 2 Correlation between hepatitis B core-related antigen (HBcrAg) and covalently closed circular DNA (cccDNA) levels after transplantation. $r = 0.402$, $P = 0.046$ ($y = 0.16 \times + 2.64$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

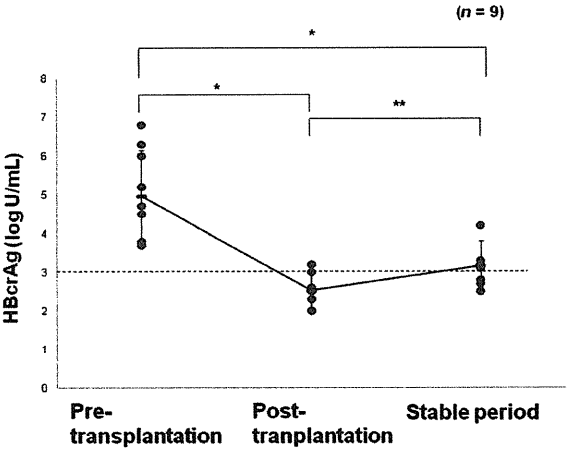

Figure 3 Serial changes of the hepatitis B core-related antigen (HBcrAg) levels. HBcrAg levels are represented as mean values; the closed circles show the values of the HBcrAg levels in all phases. The error bars indicate standard deviations. The detection range is above 3.0 log U/mL. In order to obtain the mean value, the values of 3.0 log U/mL or less, and 2.0 log U/mL or more were added to the calculation. The mean values of HBcrAg levels dropped during the postoperative period but then gradually increased again during the stable period (*$P < 0.001$ and **$P = 0.035$ indicate the significant differences between each period).

correlation was observed ($r = 0.616$, $P < 0.001$). Similarly, in the 23 samples that were obtained after LT only (that is, preoperative state samples were excluded), HBcrAg levels were significantly correlated with cccDNA levels (Fig. 2, $r = 0.402$, $P = 0.046$). These results supported the hypothesis that HBcrAg can be useful as an HBV marker instead of cccDNA after LT.

Serial changes in HBcrAg and cccDNA levels. HBcrAg and cccDNA levels showed similar dynamics during each period (Figs 3,4). All nine cases had positive levels of HBcrAg. However, seven of them were negative for HBV-DNA. During the post-transplantation period, HBcrAg levels of seven cases and cccDNA levels of eight cases became negative. Subsequently, HBcrAg and cccDNA levels of five cases became positive again during the stable period. These dynamics implicated the re-infection of HBV in the graft liver.

Comparisons of the clinical features of HBcrAg and cccDNA levels. We divided patients into two groups according to their status of HBcrAg and cccDNA, and investigated their clinical features (Table 2). Positive group includes the patients with positive cccDNA or HBcrAg, negative group includes the patients with both negative.

In comparisons between the positive group and negative group, the number of patients being treated with entecavir was significantly lower in negative group ($P = 0.022$). Additionally, the stage of the graft liver was significantly lower ($P = 0.012$) in negative group. The grafts of the HBcrAg- and cccDNA-negative patients were in good condition in the lower fibrosis stages (median 0; range 0–1).

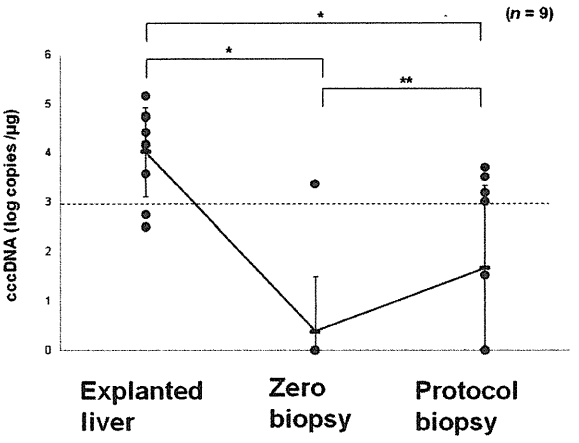

Figure 4 Serial changes of the covalently closed circular DNA (cccDNA) levels. cccDNA levels are represented as mean values; the closed circles show the values of the cccDNA levels in all phases. The error bars indicate standard deviations. The mean values of the cccDNA levels dropped during the time-zero biopsy but then gradually increased during the protocol biopsy (*$P < 0.001$ and **$P = 0.078$ indicate the significant differences between each period).

Discussion

In the present study, we demonstrated the usefulness of HBcrAg and cccDNA as markers of HBV after transplantation. As in our previous report,[12] we suggest that HBcrAg, which is a newly developed enzyme immunoassay,[9] is a possible method for detecting the dynamics of HBV after LT. However, HBcrAg consists of HBcAg, HBeAg, and p22cr, which is generated from cccDNA,

Table 2 Comparisons of the clinical features of HBcrAg and cccDNA levels

HBcrAg/cccDNA status	Positive group	Negative group	Positive versus negative
Patient M/F	10/2	7/1	NS
Day after transplantation†	854 (323–2163)	1674.5 (353–2456)	NS
Age†	55.5 (33–68)	56.5 (48–65)	NS
Serum HBV-DNA positive at LT (p/n)	7/5 (58.3%)	2/6 (33.3%)	NS
Serum HBeAg positive at LT (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
HBcAb-positive donor (p/n)	7/5 (58.3%)	1/7 (14.3%)	NS
Blood incompatibly (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
Presence of HCC at LT (p/n)	9/3 (75%)	7/1 (87.5%)	NS
RTI for prophylactic therapy after LT			
Use of LAM	3/12 (25%)	4/8 (50%)	NS
Use of ETV	9/12 (75%)	1/8 (12.5%)	$P = 0.022$
Use of ADV	0 (0%)	2/8 (25%)	NS
Use of LAM + ADV	0 (0%)	1/8 (12.5%)	NS
Immunosuppression after LT			
Use of TAC	10/12 (83.3%)	5/8 (62.5%)	NS
Use of CYA	0 (0%)	2/8 (25%)	NS
Use of MMF	2/12 (16.6%)	0 (0%)	NS
Use of TAC + MMF	0 (0%)	1/8 (12.5%)	NS
Liver function test			
Serum albumin (g/L)‡	39.2 (4.7)	40.0 (4.8)	NS
Child–Pugh score†	5.0 (5.0–9.0)	5.0 (5.0–6.0)	NS
Histology of LB			
Grade†	1.0 (0.0–3.0)	0.5 (0.0–1.0)	NS
Stage†	1.0 (0.0–3.0)	0.0 (0.0–1.0)	$P = 0.0027$
RAI score†	2.5 (0.0–5.0)	1.5 (0–4)	NS

Fisher's exact test for categorical variables.

†Mann–Whitney *U*-test for non-normally distributed variables, expressed as median (range).

‡Student's *t*-test for normally distributed variables, expressed as mean (SD).

ADV, adefovir; cccDNA, covalently closed circular DNA; CYA, cyclosporin A; ETV, entecavir; HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B envelope antigen; HCC, hepatocellular carcinoma; LAM, lamivudine; LB, liver biopsy; LT, liver transplantation; MMF, mycophenolate mofetil; n, negative; NS, not significant; p, positive; RAI, Rejection Activity Index; RTI, reverse transcriptase inhibitor; SD, standard deviation; TAC, taclolimus.

and thus, it is questionable if HBcrAg truly reflects the viral pattern of HBV. Therefore, we designed this study to examine the usefulness of further analysis of cccDNA, which truly functions as a reservoir of HBV replication.

In the results of this study, a positive correlation between HBcrAg and cccDNA was shown, and this was consistent with a previous report on chronic hepatitis B.[11] These findings suggest the usefulness of monitoring HBV dynamics of patients after LTs because examinations of serum HBcrAg are less invasive methods compared with examinations of cccDNA levels in liver tissue. HBcrAg enables us to frequently check the HBV dynamics of patients, and it contributes to a reduction in the risk of HBV reactivation.[13]

However, as shown in Table 2, the results of the HBcrAg and cccDNA levels were not matched in 35% (7 of 20) of the patients. This may be due to a problem with the sensitivity of these two markers. We should use these markers cautiously because HBV might exist even if these were negative. Suzuki *et al.* reported that among the 13 patients with negative results for HBsAg, HBeAg, and HBV-DNA, all had positive results with cccDNA, while HBcrAg was positive in only seven patients.[11] In addition, cccDNA was also examined in a limited way because it was extracted from tissue from only a small part of the liver. Moreover, some reports have suggested that cccDNA can be detected in extrahepatic sites,[17] and thus, it is impossible to determine whether HBV exists with only one method. Therefore, we preferred to assess HBV dynamics with these two methods in order to overcome problems with sensitivity.

Interestingly, in the group with negative results for both of the two markers, the fibrosis stage was significantly lower compared with the other. This might reflect HBV activity after the LT. In addition, it was considered that keeping the two markers negative after LT may suggest the possibility of an extension of graft survival. But we observed only a limited period, further study of long-term outcome will be required.

The goal of this study was to determine the criteria for the appropriate prophylaxis of HBV related to LT with these two markers. Lenci *et al.* reported that 80.1% of the patients with undetectable intrahepatic cccDNA levels did not exhibit signs of HBV recurrence, even after withdrawal of the prophylaxis.[18] We thought that it might be possible to select patients more efficiently and correctly by using a method that combines examinations of HBcrAg and cccDNA. We observed one patient with both HBcrAg- and cccDNA-negative discontinued antiviral therapy.

Although the patient stopped antiviral therapy, he has not relapsed for 29 months (data not shown).

In conclusion, HBcrAg and cccDNA were helpful for the monitoring of HBV dynamics after LT and keeping a negative status of these markers might contribute to graft survival. In addition, using these methods, the criteria for the discontinuation of HBV prophylaxis could be clarified in the future.

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Review Article

Recent topics on α-fetoprotein

Kazuhiko Nakao and Tatsuki Ichikawa

Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Zinc-fingers and homeoboxes 2 (ZHX2) and zinc-finger and BTB domain containing 20 (ZBTB20) repress the postnatal expression of α-fetoprotein (AFP) by interacting with the AFP gene promoter regions. ZHX2 inhibits the expression of AFP and cyclins A and E. ZBTB20 is negatively regulated by CUX1, which promotes cell-cycle progression, suggesting that AFP reactivation is closely linked to hepatocyte proliferation. A slight elevation in the serum AFP level often occurs in patients with chronic hepatitis C in the absence of hepatocellular carcinoma (HCC) and is an independent risk factor for HCC development to complement the fibrosis stage. In addition, the sustained elevation of AFP after interferon therapy is a risk factor of HCC development. AFP levels are clinically useful in predicting the outcomes of liver transplantation and sorafenib therapy for HCC patients. A low preoperative AFP level is a predictor of long-term survival and is associated with a low recurrence rate of HCC after liver transplantation. AFP response (≥20% decrease in AFP during 6–8 weeks of treatment) rather than radiological outcomes is a significant prognostic factor for survival in sorafenib-treated HCC patients. Highly sensitive *Lens culinaris* agglutinin-reactive AFP (AFP-L3) is 5–10 times more sensitive than conventional AFP-L3, and useful for early detection of HCC in patients with total AFP below 20 ng/mL.

Key words: α-fetoprotein, chronic hepatitis C, hepatocellular carcinoma, highly sensitive *Lens culinaris* agglutinin-reactive α-fetoprotein, liver transplantation, sorafenib

INTRODUCTION

THE A-FETOPROTEIN (AFP) and albumin genes are similar in structure and tandemly arranged on the q arm of chromosome 4. Both genes are expressed at high levels in fetal liver. After birth, AFP expression decreases rapidly to an almost undetectable level, whereas albumin expression remains high.[1,2] The AFP gene is reactivated in pathological conditions such as hepatocellular carcinoma (HCC). The release of AFP gene repression in hepatocytes may be linked to hepatocarcinogenesis. The serum level of AFP is elevated in benign liver diseases, such as chronic viral hepatitis and liver cirrhosis without HCC.[3,4] Elevated AFP levels are linked to alanine aminotransferase elevation, hepatocyte regeneration and hepatic fibrosis.[3,4] A rising level of AFP over the first few hospital days indicates a better prognosis of acute liver failure.[4] In the present study, we reviewed the relationship between clinical features and serum AFP levels in patients with chronic hepatitis C (CHC) without HCC.

Assessment of AFP response after locoregional therapy for HCC, including surgical resection, radiofrequency ablation and transcatheter arterial chemoembolization, is simple and sensitive for detecting radiological tumor response, as well as an early objective screening tool for progression by imaging.[5,6] We reviewed the clinical usefulness of monitoring the serum AFP level during two newly established therapies for HCC, liver transplantation and administration of sorafenib. Finally, clinical usefulness of highly sensitive *Lens culinaris* agglutinin-reactive AFP (hs-AFP-L3) for detection and management of HCC is discussed.

AFP GENE REGULATION

THE AFP GENE is positively regulated by transcription factors including HNF-1, HNF-3, HNF-4 and C/EBP that bind to specific elements in the promoter and enhancer regions.[7–9] These factors also bind to regulatory regions of the albumin gene,[10,11] which is consti-

Correspondence: Dr Kazuhiko Nakao, Department of Gastroenterology and Hepatology Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: kazuhiko@nagasaki-u.ac.jp

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tutively expressed in adult liver. Therefore, the existence of factors that specifically silence the AFP gene in adult liver has been supposed.[1,2,7–11] Indeed, two novel factors involved in postnatal AFP silencing have been identified, zinc-fingers and homeoboxes 2 (ZHX2)[12] and zinc-finger and BTB domain containing 20 (ZBTB20).[13]

In a study of the hereditary persistence of AFP in the liver of BALB/cJ mice, ZHX2 was identified as a postnatal repressor of AFP expression.[12] Shen *et al.* showed that ZHX2 overexpression decreases AFP secretion in human hepatoma cells expressing high AFP levels and that ZHX2 repression is governed by the human AFP promoter and requires intact HNF1 binding sites.[14] Hypermethylation of CpG islands in the ZHX2 promoter and the resultant loss of ZHX2 expression were detected in human HCC tissues, but not in surrounding non-tumor tissues.[15] These data suggest that ZHX2 contributes to human AFP repression in adult liver and may be involved in AFP reactivation in HCC. ZHX2 also represses glypican 3, an oncofetal gene.[16] Yue *et al.* reported that ZHX2 inhibits HCC cell proliferation by preventing the expression of cyclins A and E (Fig. 1a) and reduces the growth of xenograft tumors in mice.[17] Thus, they proposed that the loss of nuclear ZHX2 may be an early step in the development of HCC.

ZBTB20 is another repressor of AFP gene transcription in the liver. The main isoforms of ZBTB20 in humans and mice are 741 and 733 amino acids in length.[9] The liver-specific deletion of ZBTB20 resulted in the persistence of AFP expression in adult mouse liver.[13] ZBTB20 directly binds to a region of the AFP promoter between −108 and −53 and represses the AFP promoter activity.[13] Recently, it was shown that miR122, a liver-specific miRNA, indirectly modulates the expression of ZBTB20 and regulates AFP expression.[18] The miR122-silenced HCC cells exhibit a more invasive phenotype and produce more abundant AFP. In the miR122-silenced cells, the expression of CUX1, a transcription factor that regulates multiple processes including cell-cycle progression, is upregulated. CUX1 is a positive regulator of miR214. Because ZBTB20 is a target of miR214, the elevated expression of miR214 represses the ZBTB20 translation, followed by increased expression of AFP (Fig. 1b).[18]

Accordingly, ZHX2 inhibits the expression of both AFP and cyclins A and E.[17] ZBTB20 inhibits AFP expression and is regulated by CUX1, which promotes cell-cycle progression.[18] These findings suggest that AFP reactivation is closely linked to hepatocyte proliferation (Fig. 1).

AFP ELEVATION IN CHRONIC HEPATITIS C

MILDLY ELEVATED SERUM AFP levels are often seen in patients with CHC without HCC. The clinical significance of this mild elevation in serum AFP has been investigated. Hu *et al.* reported that in 357 patients with CHC without HCC, 82 (23.0%) patients had AFP levels of 10 ng/mL or more, and the AFP elevation was independently associated with stage III/IV hepatic fibrosis, the serum level of aspartate aminotransferase (AST) and prolonged prothrombin time.[3] The prevalence of elevated AFP (≥10 ng/mL) was 15.3% (28/183), 24.5% (25/102) and 42.0% (29/69) in stages 0–II, III and IV hepatic fibrosis, respectively.[3] In another report, elevated AFP levels (≥15 ng/mL) were observed in 23.9% (156/654) of CHC patients, and thrombocytopenia, AST elevation and AFP levels of 6 ng/mL or more were associated with advanced hepatic fibrosis.[4] Richardson *et al.* analyzed 258 275 AFP tests in a cohort

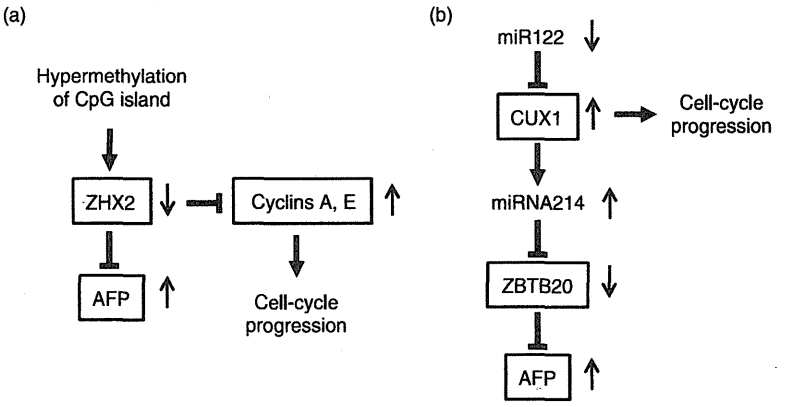

Figure 1 Schema of α-fetoprotein (AFP) gene regulation and cell-cycle control by zinc-fingers and homeoboxes 2 (ZHX2) (a) and CUX1/zinc-finger and BTB domain containing 20 (ZBTB20) (a).

of 76 347 hepatitis C virus (HCV)-infected patients.[19] Of these, 12 775 (16.6%) patients had cirrhosis, and 1488 (1.9%) patients developed HCC during the observation period. Among patients without HCC, significant determinants for increased levels of AFP included cirrhosis, high Model for End-Stage Liver Disease (MELD) score, and increased level of alanine aminotransferase.[19] Tateyama *et al.* reported that a slightly elevated AFP level is an independent risk factor for HCC to complement the fibrosis stage in a retrospective study of 707 CHC patients without HCC.[20] The 10-year cumulative incidence rates of HCC in patients with AFP levels of less than 6, 6–20 and 20 mg/mL or more at entry were 6.0%, 24.6% and 47.3%, respectively.[20]

In addition, the change in AFP level during interferon (IFN) therapy in CHC patients has been investigated. The serum level of AFP before pegylated (PEG) IFN/ribavirin (RBV) therapy predicts treatment outcome in CHC patients regardless of HCV genotype.[21] The serial AFP levels decreased after PEG IFN/RBV treatment, presenting in a time-dependent manner, specifically in patients who achieved a sustained virological response.[22] A decrease of serum AFP level after low-dose IFN therapy regardless of virological response has also been reported.[23–25] Moreover, Tamura *et al.* reported that increased serum AFP levels (≥10 ng/mL) at the end of IFN therapy was a significant variable affecting the development of HCC.[26] Osaki *et al.* also reported that among patients without a sustained virological response, a decrease in the AFP value (<10 mg/mL) by IFN therapy correlates with a reduced risk of HCC incidence after treatment.[27] Taken together, the results of these studies indicate that the sustained elevation of AFP (≥10 ng/mL) after IFN therapy is a risk factor of HCC development. In this regard, Akuta *et al.* reported that the substitution of amino acid 70 in the HCV core region of genotype 1b is an important predictor of elevated AFP in CHC patients without HCC.[28] The substitution of amino acid 70 in the HCV core region is related to non-sustained virological response by IFN therapy[29] and also to hepatocarcinogenesis.[30,31]

AFP LEVELS IN LIVER TRANSPLANTATION FOR HCC

THE SERUM LEVEL of AFP before liver transplantation for HCC is clinically significant. Several studies found that a low preoperative AFP level is a predictor of long-term survival and associated with a low recurrence rate of HCC after liver transplantation.[32–37] Mailey *et al.* analyzed 2253 patients who underwent orthotopic liver transplantation.[38] In this patient group, 1210 (53.7%), 805 (35.7%) and 238 (10.6%) patients had low (<20 ng/mL), medium (20–399 ng/mL) or high (≥400 ng/mL) AFP levels, respectively. The low AFP group had the greatest 4-year survival rate (76%) as compared to the medium (65%; $P < 0.001$) and high (57%; $P < 0.001$) AFP groups, and the improved survival in the low AFP group was still observed in patients with only stage II HCC.[38] Todo *et al.* analyzed a total of 653 patients with HCC who received living donor liver transplants in Japan.[39] In this study, the preoperative serum AFP levels were inversely correlated with patient survival: 83.8% at 1 year, 77.3% at 3 years and 72.2% at 5 years when AFP was less than 200 ng/mL ($n = 473$), and 64.9% at 1 year, 42.5% at 3 years and 34.0% at 5 years when AFP was 1000 ng/mL or more ($n = 48$).[39] Wang *et al.* reported 1-, 2- and 3-year recurrence-free survival rates of 83%, 63% and 53%, respectively, for patients with AFP levels of less than 20 ng/mL.[40] These survival rates were much greater than the corresponding rates for patients with AFP levels of 700 ng/mL or more (68%, 49% and 32% for the 1-, 2- and 3-year recurrence-free survival rates, respectively).[40] Fujiki *et al.* studied 144 HCC patients who received living donor liver transplants.[41] The 1-, 3- and 5-year recurrence-free survival rates for patients with AFP levels were less than 200 ng/mL in comparison to patients with AFP levels of 800 ng/mL or more were 97% versus 65%, 91% versus 40% and 90% versus 40%, respectively. However, the preoperative level of des-γ-carboxy prothrombin (DCP) (≥400 mAU/mL) was a stronger predictor of recurrence than the AFP level (≥800 ng/mL), and the DCP level (≥400 mAU/mL) was significantly related to microvascular invasion and poor differentiation of HCC cells.[41]

Pretransplant treatment of HCC patients with high AFP levels could be feasible and lead to similar intent-to-treat and post-transplant survival rates to those of patients with persistently low AFP levels.[42] Merani *et al.* analyzed 6871 HCC patients listed for liver transplantation and reported that patients with AFP levels decreased to 400 ng/mL or less by local pretransplant HCC treatment and patients with AFP levels persistently 400 ng/mL or less had similar dropout rates from the transplant list (10% for both groups) and similar post-transplant survival rates (89% vs 78% at 3 years, $P = 0.11$).[43] They concluded that only the last pretransplant AFP level independently predicted survival ($P < 0.001$), unlike the AFP level at the time of listing or AFP changes.[43] Toso *et al.* analyzed 5498 adult candidates for liver transplantation for HCC and 43 528 liver