Materials and methods

Patient selection

The study protocol was approved by the Institutional Ethics Committee of Ogaki Municipal Hospital in January 2011, and was in compliance with the Declaration of Helsinki. Written informed consent for the use of stored serum samples for the study was obtained from all patients.

Between 1998 and 2008, 1220 consecutive HBsAg-positive patients, who visited the Department of Gastroenterology and Hepatology at Ogaki Municipal Hospital, were prospectively enrolled in our HCC surveillance program. Of these, 785 patients met the following inclusion criteria: HBsAg positive for more than 6 months, no evidence of HCV co-infection, exclusion of other causes of chronic liver disease (alcohol consumption >80 g/day, hepatotoxic drugs, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease), follow-up duration of greater than 3 years, no evidence of HCC for at least 1 year from the start of the follow-up period, receiving no interferon treatment, and receiving NA therapy for more than 1 year before the detection of HCC (Fig. 1). In patients on NA therapy, the date of NA therapy initiation was considered the starting point of the follow-up period.

Of these 785 patients, 148 received NA therapy (NA group) and 637 patients did not receive NA therapy (non-NA group) during the follow-up period. To reduce the confounding effects of covariates, we used propensity scores to match NA patients to unique non-NA patients. Six covariates including age, sex, HBV DNA concentration, hepatitis B e antigen (HBeAg), platelet count, and alanine aminotransferase (ALT) activity were taken into account at the start of follow-up. We computed the propensity score by using logistic regression with the independent variable including age (≤40 years or >40 years), sex (female or male), HBV DNA concentration (≤5.0 log copies/ml or >5.0 log copies/ml), HBeAg (negative or positive), platelet count (>150 × 10^3/m^3 or ≤150 × 10^3/m^3), and ALT activity (≤40 IU/ml or >40 IU/ml), as shown in previous reported cut-off values according to the indication for NA therapy [16–19]. This model yielded a *c* statistic of 0.85 (95% confidence interval [CI], 0.82–0.88), indicating very good ability of the propensity score model to predict treatment status. We sought to match each patient who received NA therapy to a patient who did not receive NA therapy, having a propensity by using greedy 5–1 digit matching [20]. Once this threshold was exceeded, a patient with NA therapy was excluded. This score ranged from 0.09198 to 0.93867 and, in effect, represented the probability that a patient would be receiving NA. We were able to match 117 patients with NA therapy to 117 unique patients without NA therapy. The follow-up period ended on 31 December, 2011 or the date when HCC occurrence was identified.

Surveillance and diagnosis

All patients were followed up at our hospital at least every 6 months. During each follow-up examination, platelet count, ALT, gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, alkaline phosphatase (ALP), albumin, and alpha-fetoprotein (AFP) levels were measured. We used commercially available kits to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd., Tokyo, Japan). Before November 2007, the serum HBV DNA concentration was monitored by a polymerase chain reaction assay (COBAS Amplicor HBV monitor test, Roche Diagnostics K. K., Tokyo, Japan) with a lower detection limit of approximately 2.6 log copies/ml, and after December 2007, it was monitored with another polymerase chain reaction assay (COBAS AmpliPrep-COBAS TaqMan HBV Test, Roche Diagnostics K. K.), with a lower detection limit of approximately 2.1 log copies/ml. HBV genotyping was performed as described previously [21]. Serum levels of HBV core-related antigen (HBcrAg) were measured using a chemiluminescence enzyme immunoassay (CLEIA) as described previously [22,23]. Precore nucleotide 1896 and basal core promoter (BCP) dinucleotide 1762/1764 were determined using the line probe assay (INNO-LiPA HBV PreCore assay; Innogenetics NV) [24,25]. The probes were designed to determine the nucleotides at position 1896 (G vs. A) in the precore region and positions 1762 (A vs. T) and 1764 (G vs. A and G vs. T) in the BCP region. A line probe assay was used to identify any emergence of YMDD mutations (INNO-LiPA HBV DR assay; Innogenetics NV).

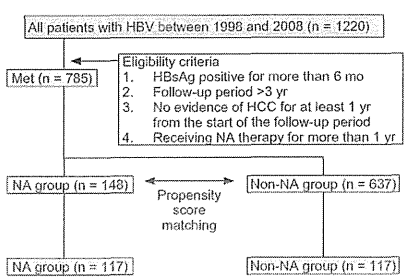

Fig. 1. Flowchart of the patient selection process.

Platelet count, ALT, gamma-GTP, total bilirubin, ALP, albumin, AFP, and HBV DNA values were expressed as average integration values [26,27] after the start of follow-up.

According to the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan [28], we performed ultrasound (US) and monitoring of 3 biomarkers (AFP, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein [AFP-L3], and des-gamma-carboxy prothrombin [DCP]) every 3–4 months, and dynamic magnetic resonance imaging (MRI) every 12 months, for patients with cirrhosis under surveillance. For patients with chronic hepatitis, we performed US and monitoring of the 3 biomarkers every 6 months. Histological examinations were performed in 91 out of 234 patients. Among them, cirrhosis was diagnosed in 32 patients. In the remaining 143 patients, the diagnosis of cirrhosis was made according to typical US findings, e.g., superficial nodularity, a coarse parenchymal echo pattern, and signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [29–31]. Patients who did not satisfy these criteria were classified as having chronic hepatitis. One hundred and forty-two patients were diagnosed with chronic hepatitis and 92 patients with cirrhosis. For diagnostic confirmation of HCC, patients underwent dynamic MRI. A histological diagnosis of HCC was made in 28 patients (surgical specimen, 23 patients; US-guided needle biopsy specimen, 5 patients). The remaining 29 patients were diagnosed with HCC based on typical dynamic MRI findings, including hypervascularity in the arterial phase with washout in the portal venous or delayed phase [32].

Treatments

In the NA group, 117 patients received NA therapy including 18 patients with lamivudine, 28 patients with lamivudine and adefovir dipivoxil, and 71 patients with entecavir. The indications for NA therapy followed the guidelines of the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), or the Asian Pacific Association for the Study of the Liver (APASL) [33–35]. In contrast, of the 117 patients not on NA therapy, 104 did not receive treatment before NA was not yet approved in Japan and the remaining 13 patients declined NA therapy.

Statistical analysis

Continuous variables are expressed as medians (range). The Mann–Whitney U test was used for continuous variables, and the Chi-square test with Yates' correction or Fisher's exact test was used for categorical variables. Actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed using the Kaplan–Meier method, and differences were tested with the log-rank test. The Cox proportional hazards model and the forward selection method were used to estimate the relative risk of HCC associated with age (≤40 years or >40 years), sex (female or male), treatment (NA or no NA), HBsAg (≤3.0 log IU/ml or >3.0 log IU/ml), HBV DNA level (≤5.0 log copies/ml or >5.0 log copies/ml), HBeAg (negative or positive), precore region (wild type or mutant), BCP (wild type or mutant type), HBcrAg (≤3.0 logU/ml or >3.0 logU/ml), platelet count (>150 × 10^3/m^3 or ≤150 × 10^3/m^3), ALT (≤40 IU/ml or >40 IU/ml), total bilirubin, gamma-GTP, ALP, albumin, and AFP (≤10 ng/ml or >10 ng/ml) for univariate and multivariate analyses. We used the minimum or maximum of the reference values at our institution as cut-off values for total bilirubin, gamma-GTP, ALP, and albumin. We conducted a sensitivity analysis to determine the magnitude of an unmeasured confounder [36].

We considered *p* values of 0.05 or less to be significant. Statistical analysis was performed with SPSS, version 18.0 for Windows (International Business Machines Corporation, Tokyo, Japan).

Table 1. Baseline characteristics of all patients.

	NA group (n = 148)	Non-NA group (n = 637)	*p* value	Standardized difference in %
Age (yr)	53 (26-81)	48 (4-85)	<0.0001	40.6
Sex (female/male)	60/88	285/352	0.5378	6.1
Genotype (A/B/C/D/F/n.d.)	2/5/137/0/1/2	24/60/389/2/0/162	<0.0001	37.6
HBsAg ($\log_{10}$ IU/ml)	3.5 (-0.1-5.5)	3.3 (-1.3-7.9)	<0.0001	53.8
HBV DNA ($\log_{10}$ copies/ml)	7.0 (2.6-9.6)	3.8 (2.3-9.9)	<0.0001	99.9
HBeAg (±)	76/72	151/486	<0.0001	62.8
Precore region (W/M/n.d.)	30/109/9	88/381/168	0.4652	0.0
BCP (W/M/n.d.)	33/123/10	135/279/205	0.0074	27.3
HBcrAg ($\log_{10}$ U/ml)	5.9 (2.9-7.0)	3.0 (2.9-7.0)	<0.0001	96.7
Platelet count (x10^3/m^3)	150 (32-388)	188 (37-503)	<0.0001	-59.7
ALT (IU/ml)	65 (7-1088)	26 (5-3410)	<0.0001	44.1
AFP (ng/ml)	3.9 (0.8-3363)	2.9 (0.8-3686)	0.0062	-6.2
Cirrhosis (presence/absence)	62/86	91/546	<0.0001	59.1
Child-Pugh classification (A/B)	132/16	618/19	0.0002	32.7
Follow-up duration (yr)	12.8 (3.1-19.6)	13.7 (3.1-20.0)	0.1565	-16.9
Administration period (yr)	6.5 (1.5-11.0)	-	-	-
Propensity score	0.58093 (0.09198-0.98686)	0.95253 (0.12913-0.98967)	<0.0001	-132.3

NA, nucleos(t)ide analogue; n.d., not done; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; W, wild type; M, mutant type; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; Child-Pugh classification, reference no [50]. Standardized difference in%: $100(\bar{x}_{NA} - \bar{x}_{non\text{-}NA})/([s^2_{NA} + s^2_{non\text{-}NA}]/2)^{1/2}$, where for each covariate $\bar{x}_{NA}$ and $\bar{x}_{non\text{-}NA}$ are the sample means in NA and non-NA groups, respectively, and s^2_{NA} and $s^2_{non\text{-}NA}$ are the corresponding sample variances.

Results

Patient characteristics

Table 1 shows baseline characteristics of all 785 patients before propensity matching. There were significant differences in age, HBV genotype, HBsAg, HBV DNA concentration, presence of HBeAg, BCP mutations, HBcrAg, platelet counts, ALT level, AFP level, presence of cirrhosis, and Child-Pugh classification. The baseline characteristics of the 234 study patients after propensity matching are summarized in Table 2. There are no significant differences in age, sex, HBV genotype, HBsAg, HBV DNA concentration, presence of HBeAg, precore region mutations, BCP mutations, platelet counts, ALT concentration, Child-Pugh classification, and follow-up duration. HBcrAg concentration was significantly higher in the NA group than in the non-NA group. NA was administered a median of 6.1 years (range: 1.5–10.7 years).

Factors associated with the incidence of hepatocarcinogenesis

Factors associated with the incidence of HCC as determined by the Cox proportional hazard models and the forward selection method were analyzed in all 785 patients. High age (hazard ratio, 6.43 [95% CI, 2.71–15.26], *p* <0.001), male sex (3.43 [1.67–7.02], *p* = 0.002), NA treatment (0.28 [0.21–0.85], *p* = 0.017), BCP mutation (19.96 [2.27–141.90], *p* = 0.03), high HBcrAg levels (8.21 [3.40–19.85], *p* <0.001), and high AFP levels (2.49 [1.43–4.34], *p* = 0.001) were significantly associated with the incidence of HCC.

HCC developed in 57 of 234 patients (24.4%) during follow-up after propensity matching. The 5-year, 7-year, and 10-year cumulative incidences of HCC were 9.6%, 20.4%, and 33.4%, respectively. The 5-year, 7-year, and 10-year cumulative incidences of HCC were 2.7%, 3.3%, and 3.3%, respectively, in patients on NA therapy (n = 117) and 11.3%, 26.0%, and 40.0% in patients not on NA therapy (n = 117). Hepatocarcinogenesis occurred at significantly higher rates in the non-NA group (*p* = 0.0094, Fig. 2). The 5-year, 7-year, and 10-year cumulative incidences of HCC were 0.0%, 0.0%, and 0.0%, respectively, in patients with wild type BCP (n = 38) and 11.0%, 25.2%, and 41.9% in patients with mutant BCP (n = 112; *p* = 0.0006, Fig. 3). Factors associated with the incidence of HCC as determined by the Cox proportional hazard models and the forward selection method are listed in Table 3. Higher age (hazard ratio, 4.36 [95% CI, 1.33–14.29], *p* = 0.015), NA treatment (0.28 [0.13–0.62], *p* = 0.002), BCP mutation (12.74 [1.74–93.11], *p* = 0.012), high HBcrAg levels (2.77 [1.07–7.17], *p* = 0.036), and high gamma-GTP levels (2.76 [1.49–5.12], *p* = 0.001) were significantly associated with the incidence of HCC. In addition, 2 patients died due to hepatic failure during the follow-up period in the non-NA group.

The sensitivity analysis found that the observed relationship between NA treatment and HCC incidence could be diminished by the unmeasured confounder that the high prevalence of the unmeasured confounder is greater in the non-NA group than in the NA group. For example, suppose a binary unmeasured confounder that increased the hazard of HCC incidence (hazard ratio, 1.50) was present in 40% of those who were treated with NA and 80% of those who were not treated with NA. Then, the study's result would become less extreme and would no longer be statistically significant (hazard ratio under sensitivity analysis, 0.48 [95% CI, 0.22–1.05]).

Follow-up data of various parameters in patients on or not on NA therapy

For this analysis, we used the average integration value during the follow-up period (Table 4). ALT, gamma-GTP, ALP, AFP, and

Table 2. Baseline characteristics of patients on NA therapy and propensity-matched controls.

	NA group (n = 117)	Non-NA group (n = 117)	*p* value	Standardized difference in %
Age (yr)	52 (27-77)	52 (21-77)	0.9223	1.7
Sex (female/male)	44/73	45/72	0.8929	6.1
Genotype (A/B/C/n.d.)	1/4/109/3	4/7/85/21	0.1232	26.8
HBsAg (log_{10} IU/ml)	3.6 (0.9-5.5)	3.6 (0.9-7.9)	0.1440	29.9
HBV DNA (log_{10} copies/ml)	6.7 (2.6-9.6)	6.5 (2.3-9.6)	0.1273	20.5
HBeAg (±)	57/60	58/59	0.8960	2.0
Precore region (W/M/n.d.)	22/87/8	16/75/26	0.6399	5.1
BCP (W/M/n.d.)	22/88/7	17/70/30	0.9359	0.0
HBcrAg (log_{10} U/ml)	5.9 (2.9-7.0)	4.9 (2.9-7.0)	0.0022	41.2
Platelet count (x10^3/m^2)	143 (32-262)	146 (37-396)	0.6340	-12.1
ALT (IU/ml)	68 (7-1088)	55 (9-3410)	0.0977	1.9
AFP (ng/ml)	2.8 (0.8-402)	3.9 (0.8-1010)	0.3118	-13.5
Cirrhosis (presence/absence)	48/69	44/73	0.6882	6.1
Child-Pugh classification (A/B)	108/9	104/13	0.5024	3.1
Follow-up duration (yr)	12.3 (3.1-19.4)	11.6 (3.1-18.3)	0.7346	-4.5
Administration period (yr)	6.1 (1.5-10.7)	-	-	-
Propensity score	0.65895 (0.11449-0.96977)	0.65895 (0.12913-0.96989)	0.9931	0.0

NA, nucleos(t)ide analogue; n.d., not done; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; W, wild type; M, mutant type; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; Child-Pugh classification, reference no [50]. Standardized difference in%: $100(\bar{X}_{NA} - \bar{X}_{non\text{-}NA})/([S^2_{NA} + S^2_{non\text{-}NA}]/2)^{1/2}$, where for each covariate X_{NA} and $X_{non\text{-}NA}$ are the sample means in NA and non-NA groups, respectively, and S^2_{NA} and $S^2_{non\text{-}NA}$ are the corresponding sample variances.

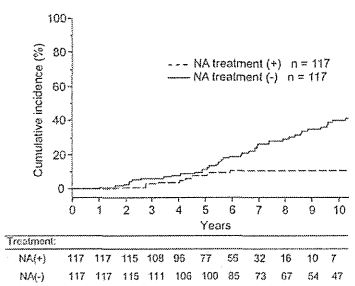

Fig. 2. Incidence of hepatocellular carcinoma (HCC) according to nucleos(t)ide analogue (NA) treatment status. The NA group had a significantly higher rate of progression to HCC than the non-NA group ($p = 0.0094$).

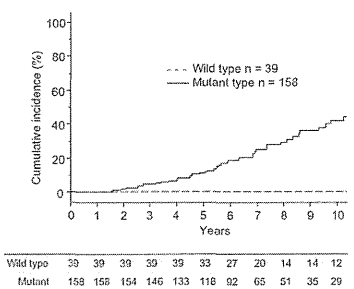

Fig. 3. Incidence of hepatocellular carcinoma (HCC) according to basal core promoter (BCP) mutations. Patients with mutant-type BCP had a significantly higher rate of progression to HCC than those with wild type BCP ($p = 0.0006$).

HBV DNA levels were significantly lower in patients on NA therapy than in patients not on NA therapy. In contrast, platelet counts and albumin levels were significantly higher in patients on NA therapy than in patients not on NA therapy.

Discussion

Our study shows that long-term NA maintenance therapy is associated with the suppression of progression to HCC. Liaw *et al.* reported that lamivudine decreased the risk of HCC in cirrhotic patients [7]. However, it is unclear whether the observed decreased risk of HCC with NA therapy was due to the short observation period in their study. It is very difficult to prove the preventive effect of NA on the development of HCC, because randomized control studies are not ethically possible. In this study, patients on NA therapy were compared to propensity score-matched untreated controls. In these control patients, NA therapy had not yet been approved or was not routinely used for chronic hepatitis B at the time, or was declined by the patient. As opposed to the entire population, these propensity-matched patients were well matched to patients on NA; significant differences included higher HBcrAg levels in the NA group.

Large community-based studies have confirmed that advanced age, male sex, HBeAg positivity, low platelet count, higher ALT levels, elevated AFP levels, and presence of cirrhosis are factors associated with the development of cirrhosis and HCC [17,18]. Platelet count is a useful surrogate marker for the diagnosis of cirrhosis [37]. All subjects were not histologically diagnosed in this study. Therefore, we selected platelet count as a marker of hepatic fibrosis instead of cirrhosis. An elevated ALT level indicates the presence of active disease, and persistently elevated AFP levels are a reflection of an enhanced regenerative state in the liver [16]. In the REVEAL study, a high HBV DNA load was associated with an increased rate of HCC development [17]. A direct correlation was observed between baseline HBV DNA levels and the incidence of HCC, independent of serum ALT concentration. In a model that integrated baseline and follow-up HBV DNA levels, the cumulative incidence of HCC ranged from 1.3% in patients with undetectable levels of HBV DNA to 14.9% in patients with HBV DNA levels greater than or equal to 10^6 copies/ml. Therefore, we have selected factors, such as age, sex, HBeAg serostatus, HBV DNA concentration, platelet count, and ALT for propensity matching.

Although the exact mechanisms of hepatocarcinogenesis by HBV remain unclear, two mechanisms have been proposed [38,39]. One mechanism involves chronic necroinflammation of hepatocytes, cellular injury, and hepatocyte regeneration [40]. The other mechanism involves the direct carcinogenicity of HBV through chromosomal integration [41]. Complete and sustained viral suppression by NA might block both pathways and prevent the development of HCC. It is well known that the rate of HCC is significantly higher in patients with virological breakthrough or no response. In our study, when virological or biochemical breakthrough was observed and the YMDD mutation was detected in patients on lamivudine, adefovir dipivoxil was immediately added. In patients with cirrhosis, especially in the decompensated stage, sustained viral response on NA therapy was not necessarily associated with a preventative effect against the development of HCC, even though the incidence was lower than in a group not on NA [14]. It is not surprising that viral suppression decreased but did not eliminate the risk of HCC, because HBV DNA may have already integrated into the host genome before the initiation of therapy and may have resulted in genomic alternations, chromosomal instability, or both [42,43].

It is reported that patients with HBV genotype C infection have higher HBV DNA levels, higher frequency of pre-S deletions, higher prevalence of BCP T1762/A1764 mutations, and significantly higher chances of developing HCC [16,44–46]. In our study, T1762/A1764 mutations were observed in 158 (80.2%) out of 197 patients and were associated with a higher risk of developing HCC (adjusted hazard ratio, 12.740 [95% CI 1.743–93.108]), independent of NA therapy. However, the BCP T1762/A1764 mutations were detected in HCC patients from Asia and Africa, where HBV genotype C infection is predominant [16].

HBcrAg is a new HBV marker that reflects HBV load and corresponds to HBV DNA levels [21]. HBcrAg is comprised of HBV core antigen (HBcAg) and HBeAg; both are products of the precore/core gene and share the first 149 amino acids of HBcAg. The HBcrAg assay measures HBcAg and HBeAg simultaneously by using monoclonal antibodies that recognize both denatured HBcAg and HBeAg [47]. Serum HBcrAg concentration is well correlated with intrahepatic levels of covalently closed circular DNA (cccDNA) [48]. It is reported that HBcrAg is a useful marker for guiding cessation of NA therapy and evaluation of disease activity [21,49]. In our study, elevated serum HBcrAg concentration was associated with a higher risk of developing HCC (adjusted hazard ratio, 2.767 [95% CI 1.067–7.172]). This is the first report demonstrating a relationship between HBcrAg and HCC.

The present study has several limitations. The retrospective design might have introduced an unintended bias. The propensity matching method was adopted to reduce the confounding effects of covariates. Characteristics of patients who did or did not receive NA therapy were similar except for HBcrAg concentration.

Table 3. Factors associated with progression to hepatocellular carcinoma among propensity-matched patients (Cox proportional hazard model).

		Adjusted hazard ratio (95% CI)	*p* value
Age (yr)	≤40	1	0.015
	>40	4.36 (1.33-14.29)	
Treatment	no NA	1	0.002
	NA	0.28 (0.13-0.62)	
BCP	wild-type	1	0.012
	mutant-type	12.74 (1.74-93.11)	
HBcrAg (log_{10} U/ml)	≤3.0	1	0.036
	>3.0	2.77 (1.07-7.17)	
γ-GTP (IU/L)	≤56	1	0.001
	>56	2.76 (1.49-5.12)	

NA, nucleos(t)ide analogue; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; γ-GTP, gamma glutamyl transpeptidase.

Table 4. Average integration values of various parameters in patients who did or did not receive NA therapy.

	NA group (n = 117)	Non-NA group (n = 117)	*p* value
Platelet count (x10^3/m^2)	17.0 (3.3-37.2)	14.8 (3.3-296)	0.0060
ALT (IU/ml)	28.2 (8.5-88.9)	39.1 (12.2-737.5)	<0.0001
γ-GTP (IU/L)	27.0 (10.9-267.6)	36.2 (9.5-269.7)	0.0427
Total bilirubin (mg/dl)	0.7 (0.3-2.0)	0.7 (0.3-2.6)	0.1554
ALP (IU/L)	242.7 (113.5-1028.8)	265.2 (140.5-1247.6)	0.0127
Albumin (g/dl)	4.4 (3.0-5.0)	4.0 (2.4-4.8)	<0.0001
Alpha-fetoprotein (ng/ml)	2.2 (0.8-106.0)	4.5 (0.9-723.8)	<0.0001
HBV DNA (log_{10} copies/ml)	2.5 (2.1-8.9)	4.6 (2.1-9.3)	<0.0001

NA, nucleos(t)ide analogue; ALT, alanine aminotransferase; γ-GTP, gamma glutamyl transpeptidase; ALP, alkaline phosphatase; HBV, hepatitis B virus.

However, the non-NA group included many historical cases when NA therapy was not yet available. In addition, the HBV DNA assay used between 1998 and 2007 was not the most sensitive one.

In conclusion, NA therapy reduces the risk of HCC compared with untreated controls. Higher serum HBcrAg levels and BCP mutations are associated with development of HCC, independent of NA therapy.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinicopathological features of liver injury in patients with type 2 diabetes mellitus and comparative study of histologically proven nonalcoholic fatty liver diseases with or without type 2 diabetes mellitus

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Abstract
Background The Japan Society of Diabetes Mellitus reported that the leading cause of death in patients with diabetes mellitus (DM) was chronic liver disease; however, there are limited studies investigating the cause of liver injury in these patients. Our study aimed to clarify the clinicopathological features of liver injury and the characteristics of nonalcoholic fatty liver disease (NAFLD) in DM patients.
Methods In total, 5,642 DM patients and 365 histologically proven NAFLD patients were enrolled. Clinical and laboratory parameters and liver biopsy results were, respectively, recorded and analyzed for the two sets of patients.
Results Positivity rates for Hepatitis B surface antigens (HBsAg) and anti-hepatitis C virus antibodies (anti-HCV Ab) were 1.7 and 5.1 %, respectively. The proportion of drinkers consuming 20–59 g and ≥60 g alcohol daily was 14.9 and 4.3 %, respectively. The percentage of DM patients with elevated serum alanine aminotransferase (ALT) levels (≥31 IU/L) was 28.6 %. Alcohol consumption had no significant effect on serum ALT levels. Seventy-two percent of HBsAg-positive patients were serum hepatitis B virus (HBV)-DNA negative, whereas 10 % exhibited high levels of the same (>4.0 log copies/ml). Thirty-eight percent of anti-HCV Ab-positive patients were serum HCV-RNA negative. Among the NAFLD patients, the frequencies of NASH and advanced stage NASH were significantly higher in male DM patients than in male patients without DM.
Conclusions Although HBsAg- and anti-HCV Ab-positivity rates were high in our Japanese DM patients, a majority of liver injuries could be associated with NAFLD/nonalcoholic steatohepatitis.

Keywords Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Diabetes mellitus · Hepatitis virus carrier · Alcoholic liver disease · Nationwide study

Abbreviations

HCC	Hepatocellular carcinoma
NAFLD	Nonalcoholic fatty liver disease
DM	Diabetes mellitus
NASH	Nonalcoholic steatohepatitis
HBV	Hepatitis B virus
HCV	Hepatitis C virus
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
FPG	Fasting plasma glucose
HOMA-IR	The homeostasis model assessment of insulin resistance index
HBsAg	Hepatitis B surface antigen
anti-HBc Ab	Anti-hepatitis B core antibody
anti-HCV Ab	Anti-hepatitis C virus antibody
HBV-DNA	Hepatitis B virus-deoxyribonucleic acid
HCV-RNA	Hepatitis C virus-ribonucleic acid
OR	Odds ratio
CI	Confidence interval

Introduction

As per the International Diabetes Federation, the number of diabetes mellitus (DM) sufferers rose to 366 million in 2011, representing 8.3 % of the global adult population, which is increasing in every country [1]. Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer mortality [2]. HCC largely occurs in patients with chronic liver disease. Persistent hepatitis C virus (HCV) or hepatitis B virus (HBV) infections are the main causes of HCC; however, non-HCV- and non-HBV-associated HCC cases are increasing in Japan [3].

In 2007, the Japan Society of DM reported that the most frequent cause of death among 18,385 DM patients who died in hospitals during 1991–2000 was malignancy (34.1 %), followed by ischemic heart disease (10.2 %) and cerebrovascular disease (9.8 %) [4]. Among the malignancies, HCC showed the highest frequency (8.6 %), followed by lung (5.3 %), pancreatic (4.8 %), and gastric cancer (3.5 %). Furthermore, the frequency of deaths caused by liver cirrhosis was 4.7 %, and in total, 13.3 % DM patients died of liver diseases. The cancer death rate in that study was quite different from that reported in the general Japanese population, in which lung (5.7 %), gastric (4.7 %), and colon (2.5 %) cancer occur with high frequencies [5]. Moreover, the death rate from liver diseases (13.3 %) was three times higher than that in the general Japanese population (HCC 3.2 %, liver cirrhosis 1.5 %, total 4.7 %) [6]. However, the incidences of HBV and HCV infection and the details of alcohol intake were not analyzed in that report.

The Japan Nonalcoholic Steatohepatitis (NASH) Study Group was founded in 2007 to investigate the cause of death in DM patients, the genetic factors in nonalcoholic fatty liver disease (NAFLD) patients, and the background of NASH-HCC patients [7]. This study focused on clarifying the cause of liver injury in Japanese DM patients and investigating the histological distribution of NAFLD in patients with and without DM.

Patients and methods

Patients

In total, 5,642 DM patients (3,238 males, 2,404 females) who visited nine DM clinics belonging to the Japan NASH Study Group (Saiseikai Suita Hospital; Kagoshima University Graduate School of Medical and Dental Sciences; Graduate School of Medicine, The University of Tokyo; Kanazawa University Graduate School of Medical Science; Department of Medicine, Asahikawa Medical College; Yamagata University Faculty of Medicine; Kyoto Prefectural University of Medicine; Okayama Saiseikai General Hospital; Fukui-ken Saiseikai Hospital) between January 2008 and December 2009 were enrolled in this observational study.

Three hundred and sixty-five NAFLD patients (182 males, 183 females) who visited Saiseikai Suita Hospital were enrolled in the histopathological study.

The study protocol was approved by the Human Ethics Committee of each participating hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinical and laboratory assessment

Demographic parameters, including age, sex, height, weight, and body mass index (BMI), and comorbidities, including alcohol consumption, hypertension, and dyslipidemia, were

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recorded for all subjects in addition to the treatment administered for DM and the frequency of HCC occurrence. Clinical laboratory tests were conducted to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), albumin, total cholesterol, triglyceride (TG), ferritin, uric acid, hemoglobin A1c, fasting plasma glucose (FPG), and insulin levels. The homeostasis model assessment of insulin resistance (HOMA-IR) index; platelet (PLT) count; and hyaluronic acid, type IV collagen 7S, hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibody (anti-HBc Ab), anti-HCV antibody (anti-HCV Ab), HBV-DNA, and HCV-RNA levels were also measured.

Blood samples were procured in the morning after overnight fasting. HOMA-IR was only calculated for subjects with FPG <140 mg/dL. HBV-DNA levels were measured by PCR (Amplicor HBV-DNA kit, Roche Diagnostics) or real-time PCR (TaqMan HBV-DNA kit, Roche Diagnostics) for HBsAg-positive, whereas HCV-RNA levels were measured by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) or real-time PCR (TaqMan HCV-RNA kit, Roche Diagnostics) for anti-HCV Ab-positive patients.

Histopathological examination

In total, 365 patients (177 non-DM and 188 DM) at Saiseikai Suita Hospital fulfilled the criteria for NAFLD, namely serum HBsAg and anti-HCV Ab negativity, no alcohol consumption, and the absence of autoimmune liver diseases or hereditary liver injury. These patients underwent an ultrasound-guided liver biopsy using a 16G needle.

Specimens were fixed in formalin, embedded in paraffin, and subjected to hematoxylin–eosin, Masson trichrome, and Perl's iron staining. Histological features of samples were interpreted according to a method described by Matteoni et al [8]. NASH stage was classified according to Brunt's classification [9].

Statistical analysis

All statistical analyses were performed using SPSS for Windows (SPSS Japan Inc.). Data were summarized by frequency for categorical variables and mean ± standard deviation (SD) for continuous variables. The Chi-square test was used to determine the differences between categorical variables. Student's t test was used to compare means of continuous valuables with equal variance, and the Mann–Whitney U test was used for non-normally distributed variables. The Cochran–Armitage test was used to study the trend of continuous variables. Forward stepwise logistic regression analysis was used to identify independent variables related to elevated serum ALT (≥31 IU/L) levels. A p value of <0.05, obtained by a two-tailed test, was considered statistically significant.

Since there is no official report on the HBV and HCV carrier rate in the general Japanese population, we utilized blood donor data for comparison with our patients [10].

Results

Baseline characteristics

The mean age and BMI of male and female DM patients was 62.2 and 64.8 years and 24.5 and 24.7 kg/m^2, respectively (Table 1). Hypertension and dyslipidemia occurred in 51.0 and 63.3 % of DM patients, respectively. Respective DM treatment types in DM patients were as follows: no medication, 20.5 %; oral drugs, 47.7 %; insulin, 28.9 %; and oral drugs and insulin, 2.8 % (Table 2).

Mean ALT level was significantly higher in males (30.6 IU/L) than in females (Table 1). Abnormal serum ALT levels (≥31 IU/L) were found in 28.6 % of DM patients (males 32.8 %, females 23.0 %). When the healthy upper limit of abnormal serum ALT level in females was defined as 20 IU/L according to Prati et al.'s [11] criteria, the frequency of abnormal ALT (≥21 IU/L) levels in females was 43 %. The mean PLT count was 20.8 × 10^4/μL in males and 21.9 × 10^4/μL in females. Mean values of other clinical laboratory tests are shown in Table 1.

Prevalence of HBV and HCV infection and drinking and their effects on liver function tests

HBsAg positivity was detected in 1.7 % of DM patients (M 1.8 %, F 1.6 %) (Table 2); this was significantly higher than that (total 0.9 %, M 1.0 %, F 0.7 %) detected in 1.7 million blood donors aged >40 years ($p < 0.001$). For both sexes, the HBsAg detection rate was significantly higher in DM patients than in blood donors in the 50- to 59- and 60- to 69-year age groups ($p < 0.05$) (Fig. 1). There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes.

Seventy-two percent of HBsAg-positive patients (M 69 %, F 79 %) demonstrated HBV-DNA negativity (<2.6 log copies/ml) (Table 3). Of the HBsAg-positive patients, only 10 % showed high serum HBV-DNA levels (≥4.0 log copies/ml); these could be HBV infection-induced liver injury cases. Mean values of age, serum ALT level, and PLT counts in HBV-DNA-negative HBV carriers were 63.6 years, 25.3 IU/L, and 20.5 × 10^4/μL, respectively. HBV-DNA-negative HBV carriers were older and exhibited lower ALT levels and higher PLT counts; however, the differences were not significant.

Table 1 Backgrounds of diabetes mellitus patients (1)

Characteristic	Total subjects		Males		Females		
	n	M ± SD	n	M ± SD	n	M ± SD	p
Age (years)	5,510	63.3 ± 12.7	3,164	62.2 ± 12.5	2,346	64.8 ± 12.9	<0.001
BMI (kg/m^2)	5,173	24.6 ± 4.7	2,987	24.5 ± 4.2	2,186	24.7 ± 5.2	0.629
Aspartate aminotransferase (IU/L)	5,568	26.4 ± 17.2	3,188	27.1 ± 18.0	2,380	25.5 ± 15.9	<0.001
Alanine aminotransferase (IU/L)	5,569	28.2 ± 24.5	3,190	30.6 ± 26.9	2,379	24.9 ± 20.5	<0.001
GGT (IU/L)	5,476	48.3 ± 72.5	3,131	59.6 ± 86.7	2,345	33.1 ± 42.9	<0.001
Albumin (g/dL)	5,031	4.2 ± 0.4	2,869	4.2 ± 0.5	2,162	4.1 ± 0.4	<0.001
Platelet (×10^4/μL)	5,419	21.3 ± 6.1	3,112	20.8 ± 6.0	2,307	21.9 ± 6.1	<0.001
Fasting plasma glucose (FPG; mg/dL)	5,123	152.7 ± 61.7	2,945	156.0 ± 63.9	2,178	148.3 ± 58.2	<0.001
HbA1c (%)	5,479	7.2 ± 1.7	3,143	7.2 ± 1.7	2,336	7.2 ± 1.6	0.744
HOMA-IR (FPG <140)	1,005	2.55 ± 2.60	570	2.51 ± 2.59	435	2.61 ± 2.60	0.209
Total cholesterol (mg/dL)	5,260	195.1 ± 39.5	3,016	191.6 ± 40.0	2,244	199.6 ± 38.5	<0.001
Triglycerides (mg/dL)	5,443	136.3 ± 102.7	3,119	145.1 ± 111.9	2,324	124.5 ± 87.5	<0.001
Hyaluronic acid (ng/mL)	559	74.5 ± 98.6	319	59.3 ± 73.0	240	94.6 ± 122.1	<0.001
Type 4 collagen 7S (ng/mL)	474	4.9 ± 2.0	269	4.8 ± 2.0	205	4.9 ± 1.9	0.544
Ferritin (ng/mL)	1,838	142.0 ± 157.0	1,084	171.9 ± 174.9	754	99.1 ± 114.1	<0.001
Uric acid (mg/dL)	3,645	5.4 ± 1.5	2,043	5.7 ± 1.4	1,602	4.9 ± 1.4	<0.001

Results are shown as mean ± SD

GGT gamma glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment of insulin resistance

Table 2 Backgrounds of diabetes mellitus patients (2)

Characteristic	Total subjects		Males		Females		
	n	Positive (%)	n	Positive (%)	n	Positive (%)	p
Hepatitis B surface antigen	4,867	83 (1.7 %)	2,796	50 (1.8 %)	2,071	33 (1.6 %)	0.604
Anti-hepatitis B core antibody	3,211	958 (29.8 %)	1,824	572 (31.4 %)	1,387	386 (27.8 %)	0.030
Anti-hepatitis C virus antibody	4,877	247 (5.1 %)	2,812	144 (5.1 %)	2,065	103 (5.0 %)	0.834
Alcohol	4,443		2,554		1,889		<0.001
<20 g/day		3589 (80.8 %)		1769 (69.3 %)		1820 (96.3 %)	
20–59 g/day		661 (14.9 %)		609 (23.8 %)		52 (2.8 %)	
≥60 g/day		193 (4.3 %)		176 (6.9 %)		17 (0.9 %)	
Hypertension	4,936	2515 (51.0 %)	2,841	1419 (49.9 %)	2,095	1096 (52.3 %)	0.100
Dyslipidemia	5,423	3434 (63.3 %)	3,091	1882 (60.9 %)	2,332	1552 (66.6 %)	<0.001
Diabetes mellitus (intervention)	5,227		3,013		2,214		0.002
None		1072 (20.5 %)		629 (20.9 %)		443 (20.0 %)	
Oral drugs		2495 (47.7 %)		1489 (49.4 %)		1006 (45.4 %)	
Insulin		1513 (28.9 %)		810 (26.9 %)		703 (31.8 %)	
Oral drugs + insulin		147 (2.8 %)		85 (2.8 %)		62 (2.8 %)	
Hepatocellular carcinoma	4,700	67 (1.4 %)	2,696	48 (1.8 %)	2,004	19 (0.9 %)	0.017

Anti-HCV Ab positivity was detected in 5.1 % (M 5.1 %, F 5.0 %) of DM patients; this rate was significantly higher than that (total 1.0 %, M 1.1 %, F 1.0 %) in blood donors of every age group of both sexes ($p < 0.001$), except for females aged 50–59 years (Table 2; Fig. 2). ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts ($p < 0.001$, $p < 0.05$) (Fig. 2). For both sexes,

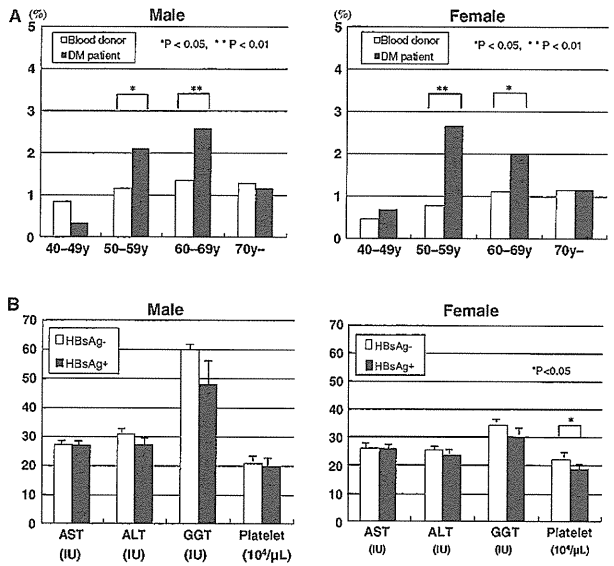


Fig. 1 Prevalence of HBV infection and the effect of HBV infection on laboratory tests in DM patients. a Prevalence of HBV infection in blood donors and DM patients. **b** The effect of HBV infection on laboratory tests in DM patients. There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes. *Error bars* SD

Table 3 Serum HBVDNA and HCVRNA levels, age, serum ALT level, and platelet (PLT) counts in HBsAg-positive patients and anti-HCV Ab-positive patients

	% (*n*)	Mean age (years)	Mean ALT levels (IU/L)	Mean PLT count($\times 10^4$/μL)
Serum HBV-DNA[a]				
Negative (<2.6 log copy/ml)	72 (29)	63.6	25.3	20.5
Positive (=2.6 log copy/ml)	28 (11)	55.6	28.0	18.5
=2.6<4.0	18 (7)	61.9	26.6	18.9
=4.0	10 (4)	50.0	30.5	17.8
Serum HCV-RNA[b]				
Negative (<2.7 log IU/ml)	38 (57)	67.2	28.2	17.7
Positive (=2.7 log IU/ml)	62 (91)	67.4	51.7	15.3
=2.7<5.0	3 (4)	65.1	28.0	16.4
=5.0	59 (87)	67.5	52.7	15.2

[a] Results are presented as either frequency or mean in 40 HBsAg-positive patients

[b] Results are presented as either frequency or mean in 148 anti-HCV Ab-positive patients

the PLT count was significantly lower in anti-HCV Ab-positive DM patients than in their negative counterparts ($p < 0.001$).

Thirty-eight percent of anti-HCV Ab-positive patients (M 36 %, F 42 %) demonstrated HCV-RNA negativity (Table 3), and 96 % of HCV-RNA-positive patients exhibited high serum HCV-RNA levels (≥5.0 log IU/ml). Serum ALT levels in anti-HCV Ab-positive patients with HCV-RNA positivity and those with HCV-RNA negativity were 51.7 ± 39.7 and 28.2 ± 18.1 IU/L, respectively, whereas those in anti-HCV Ab-negative patients were 27.7 ± 22.8 IU/L. Serum ALT levels were significantly higher in HCV-RNA-positive patients than in HCV-RNA-negative patients ($p < 0.001$).

The proportion of DM patients consuming >60 g and 20–59 g alcohol daily was 4.3 % (M 6.9 %, F 0.9 %) and

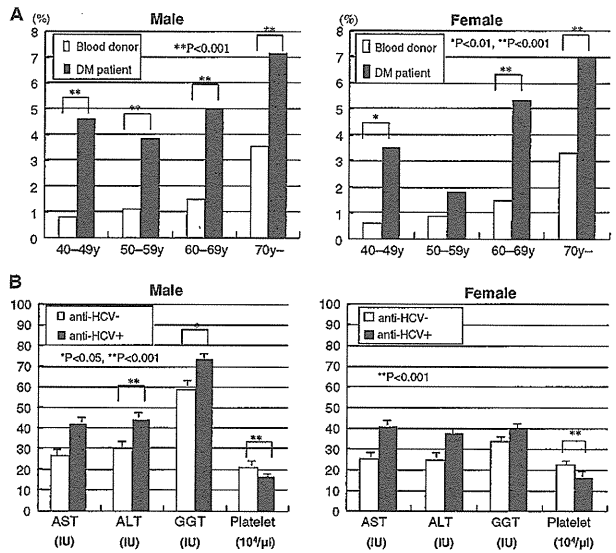


Fig. 2 Prevalence of HCV infection and the effect of HCV infection on laboratory tests in DM patients. a Prevalence of HCV infection in blood donors and DM patients. **b** The effect of HCV infection on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts. *Error bars* SD

14.9 % (M 23.8 %, F 2.8 %), respectively (Table 2). The highest percentage of drinkers were males in the 60- to 69-year age group and females in the <40-year age group. Male drinkers consuming >60 g alcohol daily had significantly higher serum AST and GGT levels compared with nondrinkers (patients consuming <20 g of daily alcohol intake) ($p < 0.001$). Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. Drinkers of both sexes consuming 20–59 g alcohol daily had significantly higher serum GGT levels ($p < 0.001$) (Fig. 3).

Factors related to serum ALT levels

With increasing age in both sexes, the number of DM patients with elevated serum ALT levels and high BMI decreased, whereas those with decreased PLT counts increased. The number of DM patients with elevated serum ALT levels increased with increasing BMI in both sexes (Fig. 4).

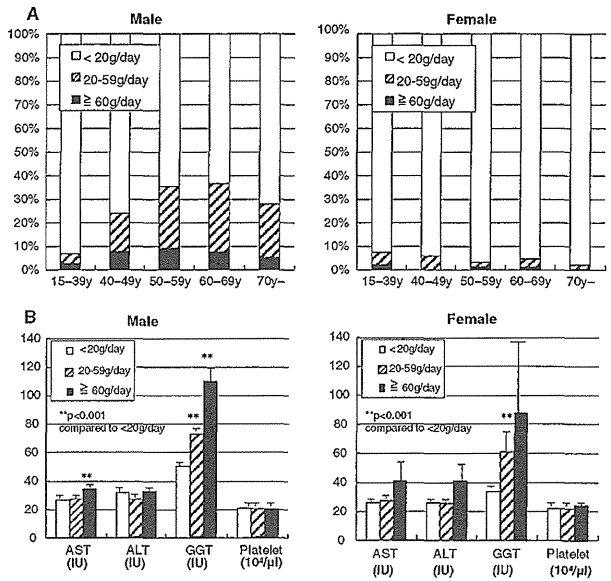
A forward stepwise logistic regression model yielding odds ratios (ORs) and 95 % confidence intervals (CIs) was used to analyze the factors related to elevated serum ALT levels. The model included BMI, age, drinking status, HBsAg status, anti-HCV Ab status, PLT count, hypertension status, and dyslipidemia status as independent variables. The odds ratio shown indicates the change in odds for one SD increase in each variable.

Multivariate analysis showed that age (M: OR 0.674, CI 0.613–0.741; $p < 0.001$; F: OR 0.767, CI 0.683–0.861; $p < 0.001$), PLT count (M: OR 0.806, CI 0.732–0.886; $p < 0.001$, F: OR 0.714, CI 0.632–0.808, $p < 0.001$), anti-HCV Ab status (M: OR 1.321, CI 1.218–1.433; $p < 0.001$; F: OR 1.232, CI 1.117–1.359; $p < 0.001$), and BMI (M: OR 1.509, CI 1.374–1.657; $p < 0.001$; F: OR 1.487, CI 1.330–1.663; $p < 0.001$) were significantly associated with elevated serum ALT levels (Table 4).

For both sexes, AST and ALT levels were similar in drinkers consuming 20–59 g alcohol daily and those consuming <20 g alcohol daily (Fig. 3). After eliminating HBV-positive patients and/or HCV carriers and heavy drinkers consuming >60 g alcohol daily, the number of male, female, and total DM patients with elevated serum ALT levels were 33.4, 23.3, and 28.3 %, respectively. These values were comparable with those in all DM patients, including those with hepatitis and/or those consuming alcohol (M 32.8 %, F 23.0 %, total 28.6 %).

Liver histology in DM patients

The median age of histologically proven, DM- ($n = 87$) and non-DM-associated ($n = 95$) male NAFLD patients



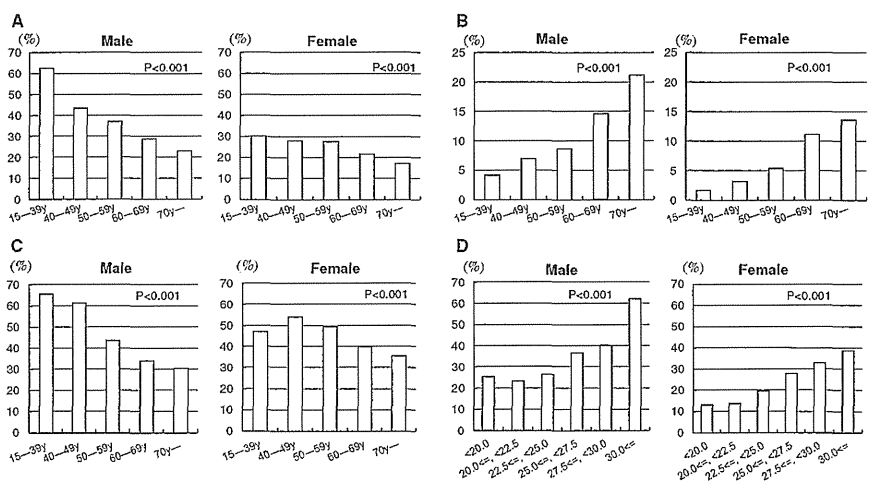


Fig. 3 *Drinking habits* and the effect of alcohol consumption on laboratory tests in DM patients. **a** Drinking habits in individual age. **b** The effect of alcohol consumption on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. *Error bars* SD

Fig. 4 Influence of age on the ratio of patients with elevated serum ALT level. decreased PLT count and abnormal BMI. and the relationship between BMI and the ratio of patients with elevated serum ALT level. **a** The ratio of patients with elevated serum ALT level (≥31 IU/L). **b** The ratio of patients with decreased PLT count (<15 × 10^4/μL). **c** The ratio of patients with abnormal BMI (≥25). **d** The relationship between BMI and the ratio of patients with elevated serum ALT level (≥31 IU/L)

Table 4 Multivariate analysis to identify independent variables related to elevated serum ALT level (≥31 IU/L)

	Regression coefficient	Standard error	Odds ratio	95 % confidence interval	*p*
Males					
Age	−0.394	0.048	0.674	0.613–0.741	<0.001
Platelet	−0.216	0.049	0.806	0.732–0.886	<0.001
Anti-hepatitis C virus	0.278	0.042	1.321	1.218–1.433	<0.001
Body mass index	0.411	0.048	1.509	1.374–1.657	<0.001
Females					
Age	−0.265	0.059	0.767	0.683–0.861	<0.001
Platelet	−0.336	0.063	0.714	0.632–0.808	<0.001
Anti-hepatitis C	0.208	0.050	1.232	1.117–1.359	<0.001
Body mass index	0.397	0.057	1.487	1.330–1.663	<0.001

was 60 and 45 years, respectively; in corresponding females, the median age was 66 (n = 101) and 61 years (n = 82), respectively. No significant difference was noted in BMI between DM and non-DM NAFLD patients (M 26.0 and 27.5 kg/m^2, respectively; F 26.0 and 27.0 kg/m^2, respectively). Male NAFLD patients without DM were significantly younger than those with DM ($p < 0.001$).

NAFLD patients were classified according to Matteoni's classification. Type 1, 2, 3, and 4 cases were 14 (16 %), 14 (16 %), 4 (5 %), and 55 (63 %), respectively, among male DM patients and 28 (29 %), 23 (24 %), 11 (12 %), and 33 (35 %), respectively, among male non-DM patients. Type 1, 2, 3, and 4 cases were 16 (16 %), 8 (8 %), 4 (4 %), and 73 (72 %), respectively, among female DM patients and 9 (11 %), 9 (11 %), 7 (9 %), and 57 (69 %), respectively, among female non-DM patients (Fig. 5). The frequency of Type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$).The rate of Type 4 NASH was high in both female DM and non-DM patients.

In total, 244 (M 103, F 141) NASH patients were classified according to Brunt's classification. The number of patients with stage 0 (Matteoni Type 3), 1, 2, 3, and 4 were 2 (3 %), 27 (46 %), 10 (17 %), 17 (29 %), and 3 (5 %), respectively, among male DM patients and 10 (23 %), 16 (36 %), 8 (18 %), 9 (21 %), and 1 (2 %), respectively, among male non-DM patients. Stage 0, 1, 2, 3, and 4 cases were 4 (5 %), 25 (33 %), 24 (31 %), 13 (17 %), and 11 (14 %), respectively, among female DM patients and 4 (6 %), 28 (44 %), 20 (31 %), 10 (16 %), and 2 (3 %), respectively, among female non-DM patients (Fig. 6). The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$).The rate of Stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$).

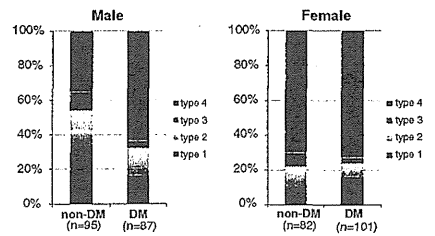


Fig. 5 Distribution of Matteoni's type classification in individual status of glucose metabolism among NAFLD patients. The frequency of type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$). The rate of type 4 NASH was high in both female DM and non-DM patients

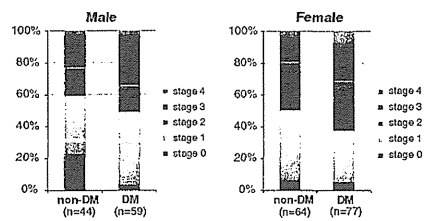


Fig. 6 Distribution of Brunt's stage in individual status of glucose metabolism among NASH patients. The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$). The rate of stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$)

HCC incidence in DM patients

In total, 67 (M 48, F 19) HCC cases (1.4 %) were reviewed (Table 2). HCC incidence was significantly higher in males

than in females. Five of 67 HCC patients consumed >60 g alcohol daily, and two of these five patients were anti-HCV Ab positive. HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence in the HCC patients was 8.6, 50.0, and 41.4 %, respectively. In a Japanese nationwide survey of 19,499 HCC patients [3], HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence was 15.0, 67.7, and 17.3 %, respectively. Non-B non-C prevalence was higher in our DM patients with HCC than in the nationwide HCC survey participants ($p < 0.001$). Mean PLT count in DM patients with HCC was as follows: HBsAg-positive patients, 12.4 ± 6.8; anti-HCV Ab-positive patients, 12.4 ± 5.6; and non-B non-C patients, 16.0 ± 7.0 ($\times 10^4/\mu L$); PLT count was significantly higher in the non-B non-C patients than in the anti-HCV-positive patients ($p < 0.05$). Mean BMI in these three patient groups was as follows: HBsAg-positive patients, 23.2 ± 5.1; anti-HCV Ab-positive patients, 22.8 ± 3.3; and non-B non-C patients, 27.2 ± 4.4 (kg/m^2); BMI was significantly higher in the non-B non-C patients than in the anti-HCV Ab-positive patients ($p < 0.001$).

Discussion

This is the first multicenter study, as per our knowledge, that clarifies the cause of liver injury in DM patients in Japan. Most Japanese HBV carriers are genotype C, acquired via perinatal vertical transmission or early childhood infection [12]. The HBV carrier rate in Japan is higher than that in western countries and significantly lower than that in other Asian countries [13]. In 1986, the Japanese government initiated a nationwide hepatitis B immunization program for infants born to HBV carrier mothers to prevent perinatal transmission. Consequently, the number of young serum HBsAg-positive individuals is extremely low. In our study, although the HBV carrier rate in DM patients was significantly higher than that in blood donors, 72 % of HBsAg-positive patients were serum HBV-DNA negative. Only 10 % of HBsAg-positive patients exhibited high serum HBV-DNA levels (≥4.0 log copies/ml), which is likely to induce hepatitis. These results indicate that a majority of DM patients who are HBV carriers may be asymptomatic.

Chronic hepatitis C may result in life-threatening complications, including cirrhosis and HCC. Worldwide, cirrhosis can be attributed to HBV (30 %) and HCV infection (27 %) [14]. The leading cause of cirrhosis among HBV and HCV sufferers and alcohol consumers varies with individual countries. A recent nationwide Japanese survey reported the etiology of cirrhosis in Japan as follows: HCV 60.9 %, HBV 13.9 %, alcoholism 13.6 %, primary biliary cirrhosis 2.4 %, NASH-related 2.1 %, and autoimmune hepatitis 1.9 % [15]. However, we must consider that hepatic triglycerides diminish with liver fibrosis progression in NASH patients (so-called "burned-out" NASH), resulting in difficulty in diagnosing NASH. Sixty-two percent of anti-HCV Ab-positive DM patients were HCV-RNA positive; these patients showed significantly higher serum ALT levels compared with HCV-RNA-negative patients. These results indicate that HCV infection is involved in the etiology of liver disease in DM patients.

There is no doubt that the positive rates of serum HBsAg and anti-HCV Ab in the general population are higher than in blood donors. Unfortunately, there were no data in the distribution of the rate of hepatitis virus carriers in each age group in Japan. In the present study, the positive rates of HBsAg and anti-HCV Ab in DM patients were significantly higher than that in blood donors. However, the present study demonstrated that most of HBsAg positive patients were negative for serum HBV DNA or had low serum HBV DNA levels and around one-third of anti-HCV Ab positive patients were negative for serum HCV RNA.

These results indicate the possibility that the frequency of hepatitis virus carriers in DM patients is higher than that in general population but no significant differences might be noted between DM patients and the general population.

Alcohol consumption is reportedly a significant factor associated with the risk of HCC development in patients with NASH-associated cirrhosis [16]. In our study, serum AST and ALT levels were comparable between drinkers consuming 20–59 g alcohol daily and nondrinkers. The ratio of heavy drinkers consuming >60 g alcohol daily was low (4.3 %) in our study. Moreover, drinking was not chosen as a variable related to elevated serum ALT levels. These results suggest that alcohol intake is not an important factor in the pathogenesis of liver disease in DM patients.

In our study, the frequency of anti-HCV Ab-positive DM patients was 5 %, whereas the serum HCV-RNA positivity rate in anti-HCV Ab-positive patients was 62 %. Therefore, the HCV carrier rate was calculated as 3 %. Since the proportion of HCV carriers and patients with elevated ALT levels were 3 % and up to 29 %, respectively, the influence of HCV infection is estimated to be no more than 10 % (3 % divided by 29 %) among DM patients with elevated ALT levels. There was no significant change in the number of DM patients with elevated ALT levels before and after elimination of HBV and/or HCV carriers and heavy drinkers. These results suggested that the major cause (up to 90 %) of liver injury in DM patients may be NAFLD.

In the present study, the frequency of advanced stage NASH was significantly higher in male DM patients than

in male non-DM patients. Neuschwander-Tetri et al. [17] reported that patients with advanced stage NASH were more likely to have DM. Mayaaki et al. [18] also examined the relationship between hepatic fibrosis stage and DM prevalence. In the mild fibrosis group, only 42 % were complicated with DM, whereas in the severe fibrosis group, the prevalence was as high as 71 % ($p = 0.020$). Lo et al. [19] reported that DM exacerbated diet-induced NASH fibrosis in mice. Therefore, DM may be an important factor in hepatic fibrosis development in NAFLD patients.

HCC frequency is significantly higher in obese and DM patients than in non-obese and non-DM patients [20, 21]. Recently, Tokushige et al. [22] reported on the backgrounds of Japanese HCC patients, and non-B non-C HCC accounted for 16 % of cases. A recent report has shown that NASH patients are likely to develop HCC in an earlier stage of fibrosis compared with chronic hepatitis C patients [23]. Our previous study analyzed 87 histologically proven NASH-HCC patients [24]; 37 % (20/54) of male HCC patients had a mild to moderate stage of liver fibrosis (F1 or F2); however, no female HCC patients were F1 stage, and only 15 % (5/33) were F2 stage. In the present study, DM patients with non-B non-C HCC exhibited a tendency to have higher PLT counts than those in DM patients with HCV-HCC, indicating that non-B non-C HCC is more likely to occur in DM patients with less advanced liver disease than in those with viral hepatitis.

In conclusion, HBsAg and anti-HCV Ab positivity rates were high; however, most of these patients were HBV-DNA negative or had low serum HBV-DNA levels. One-third of anti-HCV Ab-positive patients were HCV-RNA negative, and 4.3 % patients were drinkers whose ALT levels were comparable with those of nondrinkers. From these results, we conclude that up to 90 % of Japanese DM patients with liver injury may have NAFLD/NASH.

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Conflict of interest The authors declare that they have no conflicts of interest to disclose.

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学術助成論文

血液透析患者における肝炎ウイルス感染率と生命予後

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key words：生命予後，血液透析，肝疾患，C型肝炎ウイルス，B型肝炎ウイルス

要　旨

血液透析患者集団では，肝炎ウイルス陽性率が高いことが以前より知られているが，肝発がん予防まで考慮した積極的な抗ウイルス治療は現実には進んでいない．肝炎ウイルスキャリアへの抗ウイルス療法に対して公費助成金制度が2008年より開始され，また2011年には透析患者におけるC型肝炎治療ガイドラインが策定されている．我々は，血液透析患者における肝炎ウイルスの感染とその予後との関連についての検討を行った．

広島県内9透析施設で行った肝炎ウイルス感染状況調査（1999年から2004年）に登録された3,096例を対象として，2010年に転帰，合併症の有無，原疾患などを調査するとともに，採血によるB型肝炎ウイルス（HBV）検査，C型肝炎ウイルス（HCV）検査を行い，関連性について検討した．

解析対象とした3,087例中，HBs抗原単独陽性は2.2%（68例），HCV RNA単独陽性は13.8%（425例），重複感染は0.2%（7例），両方陰性は83.8%（2,587例）であった．観察期間中の累積死亡率はそれぞれのグループで45.6%，60.2%，57.1%，47.2%であり，HCV感染で有意に高くなっていた（p<0.001）．また肝疾患関連死はそれぞれ死亡の9.7%，8.6%，0%，1.3%であった．

転帰日の明らかな3,064例について，死亡をエンドポイントとした生存分析により，HBVとHCV共に感染の有無による生存率の差は認められなかった．一方，原疾患が「糖尿病性腎症」，あるいは「糖尿病を合併している」と有意に生存率が低い結果であった（p<0.0001）．

血液透析患者では一般住民集団よりも肝炎ウイルス陽性率が高いにもかかわらず，肝疾患関連死は少ないことが示された．肝炎ウイルス感染は生存率との関連性は認められなかった．

1　はじめに

血液透析患者では，頻回の観血的処置により肝炎ウイルス感染のハイリスク集団であることがよく知られている．現在では透析施設にて院内感染防止の措置がとられ，新規の感染予防対策が行われているが，いまだに肝炎ウイルス陽性率（有病率・キャリア率）は高いままの状態である[1]．

我が国では，ウイルス性肝炎患者に対する公費医療助成が2008年より開始され，肝炎ウイルス感染者の新規受療が促進されている．血液透析患者に対しても，慢性に感染が持続するいわゆるキャリアの状態にある者に，定期的検査や抗ウイルス療法介入の推奨がなされている．2011年には，透析患者におけるC型肝炎治療ガイドラインも発表された[2]．

我々は，1999年から行っている広島県下の血液透析患者集団のコホート調査にて，肝炎ウイルス陽性率，

A Study of prevalence of hepatitis virus among hemodialysis patients and its relevance to their prognosis
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HCV 罹患率，院内感染に対する予防策などについて報告してきた[3]．今回の研究は，肝炎ウイルス感染のある血液透析患者の生命予後について検討するため，1999 年からの血液透析患者集団コホートにて，肝炎ウイルス感染とその生命予後に関する調査を行った．

2　対象と方法

広島県内の九つの透析施設（表 1）において，1999 年 11 月から 2004 年 8 月まで行った，肝炎ウイルス感染状況調査のさいに，登録された 3,096 例を対象とした．男性 1,818 例，女性 1,278 例，1999 年時の平均年齢は 61.3±13.2 歳であった．

2-1　転帰調査

2010 年に各施設にて 3,096 例の転帰調査を行った．予後に関する項目として，臨床経過，転帰，転帰日，死亡の場合は死因，各施設での肝炎ウイルス検査の結果，肝疾患の有無と，透析開始日，透析に至った原疾患，糖尿病の有無などについて調査を行った．

2-2　肝炎ウイルス検査

今回の調査で，採血可能な症例は同意を得て新規採血を行い，行えなかった症例では保存血清にて肝炎ウイルス検査を行った．HBV 検査について，HBs 抗原検出のためマイセル II HBsAg 検査（凝集法）を行った．HCV 検査については，HCV RNA の 5'NC 領域の nested PCR 検査[3]にて検出を行った．観察期間中に一度でも HBs 抗原が陽性，あるいは HCV RNA が陽性であるものをそれぞれ「陽性」と判定した．

2-3　予後の解析

肝炎ウイルス感染と予後の関連を見るための解析として，HBs 抗原の有無，HCV RNA の有無の肝炎ウイルス感染別にみた，肝疾患関連死亡率を算出した．各群の比較は二元配置分散分析にて行った．またカプランマイヤー法による生存分析を，肝炎ウイルス感染別：HBs 抗原，HCV RNA の有無，原疾患別：糸球体腎炎・糖尿病性腎症・その他，糖尿病合併の有無別で行った．

なお，この調査は連結可能匿名化データの調査として，広島大学疫学研究倫理審査委員会の承認を得ている．

3　結　果

調査対象 3,096 例から臨時透析 2 例，転帰不明 3 例，透析導入日不明 3 例，肝炎ウイルス検査結果不明 1 例の 9 例を除き，3,087 例を解析対象とした．男性 1,815 例，女性 1,272 例，観察終了までの平均透析期間は 10.2±7.5 年であった．

全体の肝炎ウイルスの感染状況（図 1）は，HBs 抗原単独陽性 2.2%（68/3,087），HCV RNA 単独陽性 13.8%（425/3,087），重複感染 0.2%（7/3,087），両方陰性 83.8%（2,587/3,087）であった．

3-1　肝炎ウイルス感染の有無別転帰

観察開始の 1999 年 11 月から 2010 年 12 月までの転帰をまとめると，観察期間中の死亡は対象の約半数の 48.9%（1,511/3,087）であった（図 2）．肝炎ウイルス感染状況別にそれぞれ転帰をみると，HCV RNA 単独陽性グループの死亡率は 60.2%（256/425）であった．各 4 群の死亡率を比較すると，二元配置分散分析では HCV の感染により死亡率が高いことが明らかとなった（図 3，$p<0.001$）．

また，全体での肝疾患関連死は，肝不全・肝硬変が 1.9%，肝細胞がんが 0.9% であった（図 4）．肝炎ウイルス感染状況別での肝疾患関連死は，HBs 抗原単独

表 1　調査対象施設

特定医療法人あかね会　土谷総合病院
医療法人一陽会　原田病院，同 一陽会クリニック，同 イーストクリニック
医療法人社団仁友会　尾道クリニック
医療法人社団スマイル　博愛クリニック
医療法人辰川会　山陽病院
医療法人　中央内科クリニック
医療法人社団博寿会　山下医院
医療法人社団　博美医院
医療法人社団光仁会　フェニックスクリニック

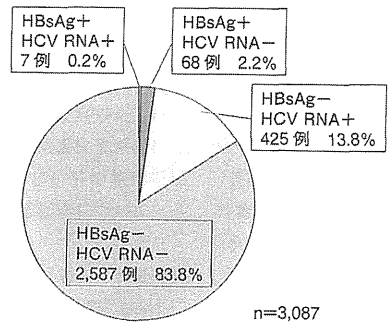

図 1　透析患者 3,087 例の肝炎ウイルス感染状況の内訳

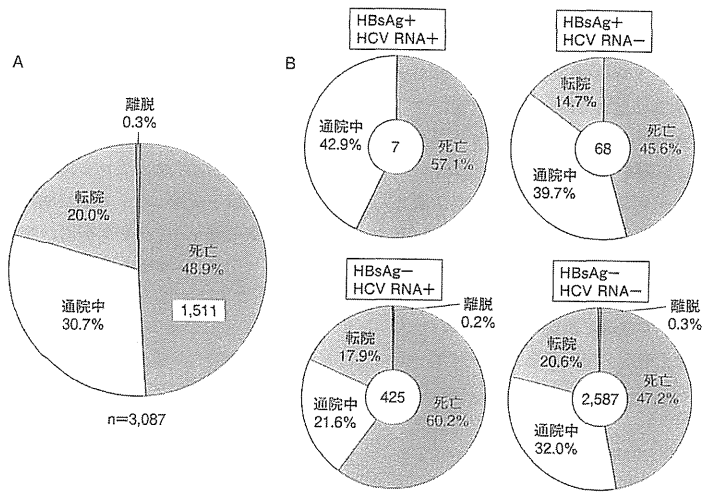

図 2　観察した 3,087 例の転帰の内訳
（A）全体では 1,511 例が死亡した．（B）肝炎ウイルスの感染状況別にみた転帰の内訳とその割合を示す．

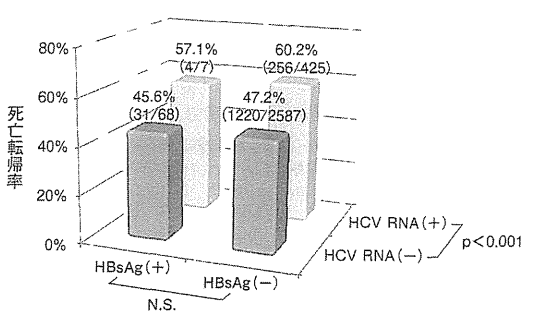

図 3　肝炎ウイルス感染状況別にみた死亡転帰の割合

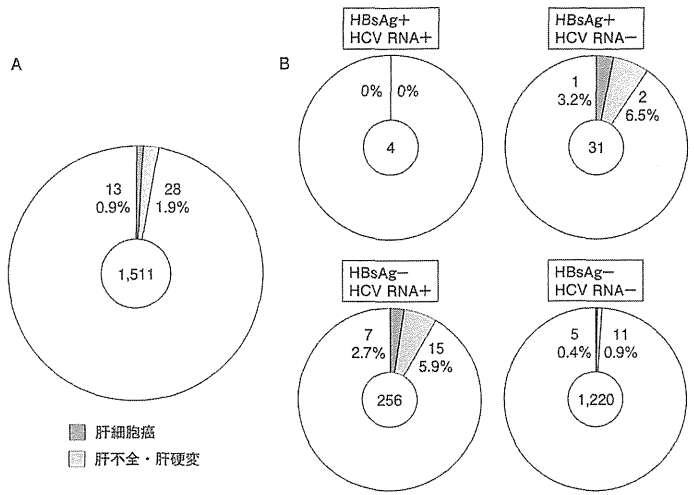

図4 観察期間中に死亡した1,511例の肝疾患関連死亡
(A) 全体と (B) 肝炎ウイルスの感染状況別を示す．死亡時の平均年齢は72.2±11.2歳 (24〜99歳)．

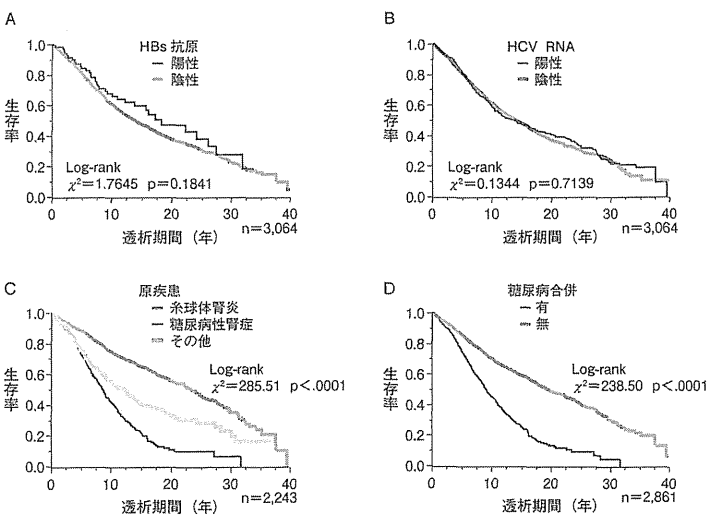

図5 カプランマイヤー法による各因子での生存時間分析
(A) HBV感染の有無，(B) HCV感染の有無では生存率に違いは無く (p=0.1841，p=0.7139)，(C)，(D) 糖尿病性腎症，糖尿病の合併有で，生存率が低くなった (p<0.0001)．

陽性グループで9.7% (3/31人，肝不全・肝硬変6.5%，肝細胞がん3.2%)，HCV RNA単独陽性グループで8.6% (22/256人，5.9%，2.7%)，重複感染のグループ0%，両方陰性のグループで1.3% (16/1,220人，0.9%，0.4%) であった．

3-2 生存分析

解析対象3,087例中，転帰日の明らかな3,064例について，死亡をエンドポイントとして，生存分析を行った (図5)．各因子に関する情報が得られなかった対象を除外すると，肝炎ウイルス感染の有無については3,064例，原疾患について2,243例，糖尿病について2,861例で解析が行えた．

HBV，HCVともに感染の有無での生命予後の差は認められず，一方「原疾患が糖尿病性腎症である」こと，「糖尿病を合併している」ことで有意に生存率が低くなっていた．

4 考 察

肝炎ウイルスの感染は一般に肝発がんの大きなリスクファクターであり，また発がんしなくても，肝硬変，肝不全への肝疾患の進展もあるため，大きく死亡リスクと関与している．そのため，感染に終止符を打つ事を目的とした抗ウイルス療法が現在の肝炎治療の大きな柱となっている．

血液透析患者にとっても同様に，抗ウイルス療法に言及した，「透析患者のC型ウイルス肝炎治療ガイドライン」が2011年，日本透析医学会より発表された[2]．それによると，C型慢性肝炎に対しては，

① 生命予後の期待できるHCV感染透析患者に対しては積極的に抗ウイルス療法を推奨
② 腎移植が予定されているHCV感染透析患者にも抗ウイルス療法が推奨

とされている．

ガイドラインにも示されているが，副作用の発現頻度がやや高いこと，血液透析患者では元々ALTが低いので，肝炎が起こっていてもALTが正常値に近く重篤感に欠け，透析病院以外への受診が患者に受け入れられにくく，肝臓専門医への紹介が困難なことなどより，実際には抗ウイルス療法はまだ血液透析患者に普及しているとはいえず，これからも急速に普及が進むとは考えにくい．

血液透析患者集団のHCV抗体陽性者について，その予後はHCV抗体陰性者に比べ悪いとの報告がある[4]．しかし，本研究では，二次元配置による解析ではHCV感染で死亡率が高いことが示されたが，生存分析を行ったところ，血液透析患者における肝炎ウイルス感染とその生存率に関連は認められず，血液透析患者の予後を大きく左右しているのではないことが示された．その報告との大きな違いは，我々はHCV抗体ではなく，HCV RNAの検出を用い，C型肝炎ウイルスの感染を確定して解析したことである．そのため，HCV抗体陽性かつHCV RNA陰性の，いわゆる感染既往者を解析から除外できているため，結果に相違が出た可能性がある．

我々も以前報告したように[3]，血液透析患者のHCV感染は，院内感染として高率に起こっており，いまだに肝炎ウイルス感染のハイリスクグループとしてとらえられている．近年，一般住民検診や，初回献血者での肝炎ウイルス陽性率 (キャリア率) は大変低く，HBVは0.71%，HCVは0.63%である[5]．一方，血液透析患者のHCV陽性率はこのコホートグループで2003年に報告したものでは12.9%[3]，安藤等の血液透析施設における2006年調査の報告でもHBV陽性率は2.39%，HCV陽性率は11.27%[1]と大変高くなっている．

血液透析患者の高い肝炎ウイルス陽性率 (キャリア率) より，それらの死因，生命予後には肝疾患が関連するのではという仮説を考えた．しかし，肝炎ウイルスの感染状況別での死因の内訳をみると，約10年間の累積肝疾患関連死はHBV陽性のグループで9.7%，HCV陽性のグループで8.6%であった．血液透析患者集団では一般集団よりも大変キャリア率が高いにもかかわらず，肝疾患関連死は予想に反して，少ないことが特徴であることが今回の研究で示された．

この集団では，約10年間の観察期間中の累積死亡率は約50%と高く，おそらく「肝疾患を長く患う」前に，全身状態が悪化し死亡する経過と推測される．血液透析患者の死因として，2010年死亡をみると，心血管疾患である心不全，脳血管疾患，心筋梗塞と感染症，悪性腫瘍が多かった[6]．糖尿病は，動脈硬化を進展させ，易感染性を増大させるため，これら死因の上位に占める疾患に直結したものであるので，今回明らかとなった，原疾患が「糖尿病性腎症」あるいは「糖尿病の合併がある」ということが，生存率の低下と関連していたという結果は矛盾しないと考えられる．

我々が観察した血液透析患者集団において，「肝炎ウイルス感染」はHBVにしても，HCVにしても，感染の存在自体に生存率との関連性は認められなかった．さらに詳しく死因や生命予後に関連する因子についての検討が必要と考えた．

実際には血液透析患者においても肝疾患関連死が存

在する．「基本的にすべての症例に」ではなく，それぞれの症例でガイドラインに示されたように，生命予後の改善が期待できるかどうかの判断のうえでの積極的な抗ウイルス療法が必要と考えられる．

謝　辞

この研究は，日本透析医会平成22年度学術研究助成事業，科学研究費助成事業（学術研究助成基金助成金），厚生労働科学研究費補助金肝炎等克服緊急対策研究事業によって行った．また，広島透析患者肝炎Study Groupの協力により調査研究が行われたことを深謝する．なお，広島透析患者肝炎Study Groupのメンバーは，表1の透析医療施設と吉澤浩司（広島大学名誉教授），頼岡德在（広島腎臓機構代表），田中純子（広島大学教授），広島大学疫学・疾病制御学である．

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＜短　報＞

岡山県における肝炎ウイルス検診陽性者の医療機関受診等に関する追跡調査

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山本　和秀[4)]　田中　純子[5)]　日野　啓輔[1)*]

緒言：平成14年から18年までの5年間に老人保健法に基づく保健事業として40歳から70歳までの受診対象者に対して5歳刻みで節目検診が行われ，また節目検診の対象者とならないが肝炎ウイルス感染の可能性が高い者（過去に肝機能異常を指摘されたことのある者，広範な外科的処置を受けたことのある者又は妊娠・分娩時に多量に出血したことのある者であって定期的に肝機能検査を受けていない者，基本健康診査の結果，ALT（GPT）値により要指導とされた者）に対して節目外検診が行われた．これらの検診により新たな肝炎ウイルス感染者が見いだされたが，その後の医療機関受診状況や肝炎に対する治療状況については，岡山県が平成17年度に市町村の協力の下で調査を行って以来，実態が把握されていないのが現状であった．田中らは「広島県における検診結果」として広島県12市町において聞き取り調査を行い，平成21年度報告書に報告している[5)]．それによると，HBVキャリアにおいては，回答率を考慮した医療機関受診率48%，把握されているHBVキャリア709名中440名から回答があった（回答率62.1%）．また，HCVキャリアにおいては，回答率を考慮した医療機関受診率65%，把握されているHCVキャリア630名中439名から回答があった（回答率69.7%）．また，医療機関受診率に関しては，HBVキャリアにおいては「現在受診中」が62%，「以前受診した」が15%，「受診していない」が23%であり，HCVキャリアにおいては「現在受診中」が80%，「以前受診した」が13%，「受診していない」が7%にすぎなかった．

そこで，厚生労働科学研究費補助金肝炎等克服緊急対策研究事業「肝炎ウイルス感染状況・長期経過と予後調査及び治療導入に関する研究」の一環として，岡山県における平成14年度から18年度までの節目・節目外検診で新たに見いだされた肝炎ウイルス感染者について，その後の医療機関受診状況や肝炎に対する治療状況を把握する目的でアンケートによる追跡調査を行ったので報告する．

対象と方法：対象は岡山県において平成14年度から18年度までの検診（節目・節目外）で肝炎ウイルス感染が判明した2,566人（B型974人，C型1,592人）のうち，調査可能であった24市町村において既に追跡調査等が行われていた肝炎ウイルス感染者を除いた1,352人（52.7%）（B型549人，C型803人）であった．調査用紙は各市町村より直接肝炎ウイルス陽性者へ郵送され，また一部の市町村（笠岡市58人，勝央町11人）においては保健師が直接聞き取り調査を行った．回答後の調査用紙は肝炎ウイルス陽性者から匿名の形で本研究の事務局である川崎医科大学肝胆膵内科学研究室へ直接郵送された．

アンケートは以下の項目について調査を行った．1. 在住市町村．2. 陽性と通知されたのはB型肝炎ウイルス（HBV）かC型肝炎ウイルス（HCV）か．3. 性別と年齢．4.「肝炎ウイルス感染の可能性が高い」と通知を受けて医療機関を受診したか否か．5. 受診していない場合その理由．6-1. 受診した場合は受診先がかかりつけ医か専門医療機関か．6-2. 受診先での診断名．7. 現在も通院を継続しているか否か．8. 通院を中止した場合はその理由．9. 治療を受けている場合は主な内容．

結果：調査を行った1,352人のうち716人（53%）より回答が得られた．このうち11人は既にウイルス性慢性肝炎として医療機関に通院していながら検診を受けており，8人は調査用紙の返送はあったものの無回答であり，1人は肝炎ウイルス陰性という回答であったため，

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2) 岡山県保健福祉部健康推進課
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4) 岡山大学大学院医歯薬学総合研究科消化器・肝臓内科学
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＜受付日2012年9月18日＞＜採択日2012年11月16日＞

るHCVキャリア630名中439名から回答があった（回答率69.7%）．それによると医療機関受診率に関しては，HBVキャリアにおいては「現在受診中」が62%，「以前受診した」が15%，「受診していない」が23%であった．一方HCVキャリアにおいては「現在受診中」が80%と高く，「以前受診した」が13%，「受診していない」は7%にすぎなかった．B型陽性者の通院中断の理由として，担当医から「通院しなくてもよい」と言われた割合が71.8%認めた点も今後の課題と考えられる．したがって，抗ウイルス療法が適切に行われていない可能性もあり，ウイルス肝炎治療のガイドラインに準じた治療の啓発をさらに推進する必要があると考えられた．現在，B型肝炎ウイルス感染者の肝発癌危険因子はウイルス量（HBV-DNA）であることが明らかにされているので[2]，トランスアミナーゼの値でフォローを中断するようなことは慎むべきであり，こうした点は肝臓専門医以外の医師にもっと広く啓発していく必要があると考えられる．

わが国の肝癌患者は高齢化が進んでおり，岡山県も例外ではない．今回の調査においてもアンケート回答者の平均年齢は高齢であった．肝炎ウイルス陽性の高齢者は肝癌の高危険群であり[3)~5)]，医療機関での経過観察あるいは治療が極めて重要である．これを実現するためには全県的な肝炎ウイルス陽性者の把握とともに地域に密接した保健活動を行政も含めてさらに推進していく必要がある．

結論：岡山県において平成14年度から18年度に実施された肝炎ウイルス検診（節目・節目外検診）で見出された陽性者について，その後の医療機関受診状況や受療状況を把握する目的で追跡調査を行った．肝炎ウイルス検診陽性者に対する医療機関への受診勧告や通院継続率の引き上げなどが今後の更なる課題と考えられた．

索引用語：ウイルス性肝炎，健康診断，追跡調査

文献：1）田中純子，他．「平成21年度　厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業　肝炎状況・長期予後の疫学に関する研究　研究報告書」2010，p11—14　2）Chen CJ, Yang HI, Su J, et al. JAMA 2006; 295: 65—67　3）Ikeda K, Saitoh S, Arase Y, et al. Hepatology 1999; 29: 1124—1130　4）Kasahara A, Hayashi N, Mochizuki K, et al. Hepatology 1998; 27: 1394—1402　5）Tseng TC, Liu CJ, Yang HC, et al. Gastroenterology 2012; 142: 1140—1149

英文要旨

A follow-up survey of hepatitis virus carriers after notification of their infection in Okayama prefecture

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In Okayama prefecture we investigated by questionnaires whether hepatitis B virus (HBV) (n = 549) or hepatitis C virus (HCV) carriers (n = 803) consulted a doctor after they had been informed of their infection at their initial checkup for viral hepatitis between 2002 and 2006. The ratio of patients who consulted a doctor after notification of infection was 38.4% (211/549) of HBV carriers and 49.4% (397/803) of HCV carriers, respectively. Among those patients, 53.1% of HBV carriers and 73.4% of HCV carriers were on follow care at the start of this investigation. These results indicated a need to establish a more effective follow up system for hepatitis virus carriers following notification at the initial medical checkup.

Key words: virus hepatitis, medical checkup, follow-up survey

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ORIGINAL ARTICLE

The Outcomes of Patients with Severe Hyperbilirubinemia Following Living Donor Liver Transplantation

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Abstract
Background Prolonged hyperbilirubinemia (HB) following living donor liver transplantation (LDLT) can be a risk factor for early graft loss and mortality. However, some recipients who present with postoperative hyperbilirubinemia do recover and maintain a good liver function.
Aim The purpose of this study was to investigate the risk factors for hyperbilirubinemia following LDLT and to identify predictors of the outcomes in patients with post-transplant hyperbilirubinemia.
Methods A total of 107 consecutive adults who underwent LDLT in Nagasaki University Hospital were investigated retrospectively. The patients were divided into two groups according to postoperative peak serum bilirubin level (HB group: ≥30 mg/dl; non-HB group: <30 mg/dl). These two groups of patients and the prognosis of patients in the HB group were analyzed using several parameters.
Results Seventeen patients (15.9 %) presented with hyperbilirubinemia, and their overall survival was significantly worse than patients in the non-HB group ($n = 90$). Donor age was significantly higher in the HB group ($P < 0.05$). Of the 17 patients in the HB group, nine survived. The postoperative serum prothrombin level at the time when the serum bilirubin level was >30 mg/dl was significantly higher in surviving patients ($P < 0.01$).
Conclusions The use of a partial liver graft from an aged donor is a significant risk factor for severe hyperbilirubinemia and a poorer outcome. However, those patients who maintain their liver synthetic function while suffering from hyperbilirubinemia may recover from hyperbilirubinemia and eventually achieve good liver function, thus resulting in a favorable survival.

Keywords Living donor liver transplantation · Hyperbilirubinemia · Partial graft · Small-for-size graft syndrome · Acute cellular rejection

Introduction

Hyperbilirubinemia following living donor liver transplantation (LDLT) can be caused by several mechanisms, such as initial poor function, acute cellular rejection, surgical complications, small-for-size syndrome, drug toxicity, among others. Hyperbilirubinemia has also been reported to be a risk factor for early graft loss and mortality [1]. However, some recipients can overcome hyperbilirubinemia, and these patients subsequently achieve and maintain a good liver function after their eventual recovery from hyperbilirubinemia. The aim of this study was to retrospectively clarify the risk factors for the development of postoperative severe hyperbilirubinemia and to identify any predictors for the outcomes in patients who present with hyperbilirubinemia following LDLT.

Patients and Methods

We retrospectively analyzed the data of 107 consecutive adult patients (67 males, 40 females, median age 55 years, age range 16–68 years) who underwent LDLT in the Department of Surgery of Nagasaki University Hospital between November 1997 and January 2010. The etiologies

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of the liver disease were hepatitis C virus infection (35 patients), hepatitis B virus infection (25 patients), non-viral causes (40 patients), and fulminant liver failure (7 patients) (Table 1). During this period, we occasionally treated patients with a postoperative bilirubin level of >20 mg/dl. Marubashi et al. [1] reported that a postoperative peak serum bilirubin level of >27 mg/dl could be a predictor of short-term graft outcome. Therefore, we defined those patients who had presented with a postoperative peak serum bilirubin level of >30 mg/dl as having hyperbilirubinemia (HB group); the remaining patients formed the non-HB group.

The two groups of patients were compared for preoperative serum bilirubin level; donor age; the postoperative peak alanine aminotransferase (ALT); model for end-stage liver disease (MELD) score; graft weight (GW)/standard liver volume ratio [SLV; SLV (ml) = 706.2 × body surface area (m^2) + 2.4] [2]; type of graft; development of acute cellular rejection [as proven by biopsy within postoperative day (POD) 60]; ABO compatibility; the development of biliary complications. We defined a biliary complication as anastomotic stenosis that needed interventions by means of balloon dilatation, stent placement, or re-operation. We divided the types of grafts into those for the right lobe and left lobe, respectively. The right lobe included the right lateral sector, and the left lobe included the left lateral segment.

In the HB group, we compared surviving and non-surviving patients for all of the above-mentioned parameters as well as for serum prothrombin [PT (%)] and creatinine levels at the time when the serum bilirubin level was >30 mg/dl. In the HB group, no patients received administration of fresh frozen plasma at the time of diagnosis. We used log-rank test for survival comparison. Group data were compared with the Mann–Whitney *U* test, and differences between proportions of categorical data were compared with the χ^2 test. Furthermore, several factors detected in the univariate analysis with *P* values of <0.15 were entered into a multivariate analysis. We used multivariate logistic regression analysis for the multivariate analysis. A *P* value <0.05 was considered to be statistically significant.

Table 1 Indication for liver transplantation

Cause of liver disease	Total (n = 107)	HB group (n = 17)	Non-HB group (n = 90)
Liver cirrhosis (hepatitis virus C)	35	6	29
Liver cirrhosis (hepatitis virus B)	25	4	21
Alcoholism	11	2	9
Primary biliary cirrhosis	8	3	5
Fulminant hepatitis	7	0	7
Liver cirrhosis (non-B non-C)	6	0	6
Primary sclerosing cholangitis	3	0	3
Budd–Chiari syndrome	1	0	1
Caroli's disease	1	0	1
Graft failure	4	2	2
Others	6	0	6

HB Hyperbilirubinemia

Results

Of the 107 consecutive adult patients who underwent LDLT at our hospital during the study period, 17 (15.9 %) met our criteria for HB and were included in the HB group; the remaining 90 patients (84.1 %) formed to the non-HB group. The overall survival rate was significantly different between the groups ($P < 0.01$) (Fig. 1). Time-zero biopsies showed no apparent differences between patients in the HB and non-HB group. Protocol biopsy was not performed postoperatively except in cases of cellular rejection or recurrence of hepatitis was suspected. The median donor age was significantly higher in the HB versus the non-HB group [50 (range 22–63) vs. 36 (19–67) years, respectively; $P < 0.05$], and ABO incompatibility was identified as a risk factor for posttransplant hyperbilirubinemia. The median preoperative serum bilirubin level tended to be higher in the HB group than in the non-HB group [5.4 (range 1.1–39.5) vs. 3.3 (0.6–42.7) mg/dl, respectively; $P = 0.06$]. The median postoperative peak ALT level was significantly higher in the HB group than in the non-HB group [569 (range 120–1,907) vs. 339 (79–3,359) IU/l, respectively; $P = 0.02$]. There were no significant differences in the GW/SLV, MELD score, type of graft, and incidence of biliary complication and acute cellular rejection between the groups (Table 2). The multivariate logistic analysis identified donor age ($P = 0.0125$) and preoperative serum bilirubin level ($P = 0.032$) as preoperative risk factors for postoperative hyperbilirubinemia (Table 3).

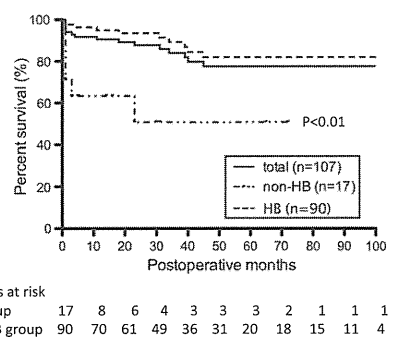

Fig. 1 Kaplan–Meier curves of the postoperative survival of patients with hyperbilirubinemia (*HB* group) and without hyperbilirubinemia (*non-HB* group)

Table 2 Analysis of predictive factors for hyperbilirubinemia (univariate analysis)

Predictive factors	HB group[a] (n = 17)		Non-HB group[a] (n = 90)		P value
GW/SLV (%)	39.9 (24.9–56.3)		44.1 (23.6–85.3)		0.139
Donor age (years)	50 (22–63)		36 (19–67)		0.035
MELD score	22 (9–32)		18 (7–40)		0.217
Preoperative serum total bilirubin (mg/dl)	5.4 (1.1–39.5)		3.3 (0.6–42.7)		0.061
postoperative peak ALT(IU/l)	569 (120–1,907)		339 (79–3,359)		0.02
	+ (n)	%	+ (n)	%	
Acute cellular rejection (<POD 60)	5/17	29	26/90	29	0.804
Biliary complication	0/17	0	18/90	20	0.07
Type of graft					
Right lobe	10/17	59	36/90	40	
Left lobe	7/17	41	54/90	60	0.241
ABO incompatibility	6/17	35	9/90	10	0.01

GW/SLV Graft weight/standard liver volume ratio, *MELD* model for end-stage liver disease, *POD* postoperative days, *ALT* alanine aminotransferase

[a] Values are presented as the median with the range in parenthesis

Table 3 Multivariate analysis of postoperative hyperbilirubinemia

Preoperative risk factors	Yes/no	P value
GW/SLV (%)	–	0.107
Donor age (years)	–	0.0125
Preoperative serum total bilirubin (mg/dl)	–	0.032
ABO incompatibility	Yes	0.163

Of the 17 patients in the HB group, nine were alive at the writing of this manuscript. The results of the comparison between surviving and non-surviving patients are shown in Table 4. The median postoperative PT (%) at the time when the serum bilirubin level was >30 mg/dl was significantly higher in surviving patients than in those that did not survive [52 (range 26–59) vs. 33.5 (20–60) %, respectively; $P < 0.01$]. The median postoperative serum creatinine level at the time when the serum bilirubin level exceeded 30 mg/dl tended to be lower in surviving patients than in those that had not survived [1.2 (range 0.5–2.9) vs. 1.86 (0.4–3.1) mg/dl, respectively; $P = 0.06$]. There were no significant differences between surviving patients and non-surviving patients for donor age, GW/SLV, preoperative serum bilirubin level, MELD score, postoperative duration when the serum bilirubin level was >30 mg/dl, ABO incompatibilty, and acute cellular rejection (Table 4). The multivariate logistic analysis was not performed because of the small number of patients. Table 5 summarizes the characteristics and the postoperative course of patients in the HB group. Eight patients did not survive—one patient due to severe acute cellular rejection and seven patients due to infection. The indications for liver transplantation for non-surviviors were liver cirrhosis (hepatitis C virus; 3 patients), primary biliary cirrhosis (3 patients), (hepatitis B virus; 1 patient), and graft failure (1 patients) (Table 5). None of these patients had suffered from short-term recurrence of viral hepatitis and hepatocellular carcinoma after transplantation. One patient (Table 5, case no. 10) was considered to be small-for-size syndrome with massive ascites and prolonged hyperbilirubinemia without arterial or portal occlusion and rejection. However, she had maintained PT (%) and survived. Although postoperative biopsies were performed for 11 patients in the HB group, no specific causes of hyperbilirubinemia were detected besides the findings of acute cellular rejection or recurrent hepatitis.

Discussion

In this study, we analyzed the risk factors for postoperative HB and the prognosis of patients who belonged to the HB group. Our results indicate that the donor age was most strongly correlated with the development of HB. A multivariate analysis also identified donor age and patient preoperative total bilirubin level as significant risk factors for post-transplant HB. The outcome of liver transplantation from aged donors is controversial. Some studies have shown that the outcomes of using grafts from donors older than 50 years without additional risk factors are similar to those of using grafts from donors younger than 50 years [3, 4]. However, the data from a registry of the Japanese Liver Transplantation Society show that patients who received a graft from an older donor had a significantly

Table 4 Comparison of risk factors for mortality in HB group (univariate analysis)

Risk factors	Surviving group[a] ($n = 9$)		Non-surviving group[a] ($n = 8$)		P value
GW/SLV(%)	40 (24.9–56.3)		39.2 (26.9–48.4)		0.847
Donor age	50 (22–61)		50.5 (22–63)		0.847
MELD score	22 (13–32)		22 (9–40)		1
Preoperative total bilirubin (mg/dl)	3.2 (1.9–39.5)		14.2 (1.1–28.7)		0.289
Timing of diagnosing HB	19 (5–28)		17 (6–32)		0.885
Prothrombin time (%) at HB diagnosis	52 (26–59)		33.5 (20–60)		0.004
Serum creatinine (mg/dl) at HB diagnosis	1.2 (0.5–2.9)		1.86 (0.4–3.1)		0.067
	+ (n)	%	+ (n)	%	
ABO incompatibility	3/9	33	3/8	38	1
Acute cellular rejection (<POD 60)	2/9	22	3/8	38	0.619

[a] Values are presented as the median with the range in parenthesis

Table 5 Characteristics and postoperative courses of patients in HB group

Case no.	Gender	Age	Indication for transplantation	ABO incompatibility	GW/SLV	Timing of diagnosing HB	Prothrombin (%) at HB diagnosis[a]	Outcomes	Cause of death
1	Male	63	B-LC, HCC	+	36.9	36	45	Dead	Infection
2	Female	61	PBC	+	26.9	26	25	Dead	Infection
3	Female	61	C-LC, HCC	–	43.6	12	29	Dead	Infection
4	Female	62	PBC	–	38.4	45	31	Dead	Infection
5	Male	57	C-LC, HCC	–	40	18	37	Dead	Infection
6	Male	57	C-LC, HCC	–	48.4	15	36	Dead	Infection
7	Male	41	PBC	–	44.6	16	31	Dead	ACR
8	Female	56	Graft failure	+	36.3	14	43	Dead	Infection
9	Female	54	C-LC, HCC	+	41.2	28	61	Alive	
10	Female	59	C-LC, HCC	–	24.9	26	45	Alive	
11	Male	58	B-LC, HCC	–	29.7	17	46	Alive	
12	Male	56	B-LC, HCC	–	44.2	37	76	Alive	
13	Female	53	C-LC	+	40	11	55	Alive	
14	Male	22	Graft failure	–	56.3	5	41	Alive	
15	Male	52	B-LC, HCC	+	36.1	34	52	Alive	
16	Male	62	Alcoholism	–	43.5	19	60	Alive	
17	Female	46	Alcoholism	–	37.8	17	34	Alive	

C-LC Liver cirrhosis type C, *B-LC* liver cirrhosis type B, *PBC* primry biliary cirrhosis, *ACR* acute cellular rejection

[a] At the time when the serum bilirubin level was >30 mg/dl

worse survival [5]. Notable findings of two studies which investigated non-transplanted aged livers were: 40 and 50 % decreases in vascular inflow and biliary flow, respectively, impairment of energy- and microtubule-dependent transport processes, with reduced endoplasmic reticulum mass, cumulative pigmented waste deposition, and a reduced ability to scavenge reactive oxygen intermediates [6, 7].

It has been reported that patients who receive a graft from an aged donor tend to have a greater incidence of delayed graft function [8, 9]. A multivariate analysis also revealed that the use of these grafts is associated with an increased incidence of recurrent hepatitis C [10]. A relative poorer regeneration of the liver graft from an aged donor has also been reported [11]. Taken together, these findings indicate that clinicians should be aware that the use of grafts from aged donors could lead to the development of severe hyperbilirubinemia by a multifactorial mechanism.

The HB group included significantly more patients who had undergone ABO blood type-incompatible LDLT. The outcomes of ABO blood type-incompatible LDLT have improved over the years, and many institutes have adopted ABO-incompatible LDLT owing to the various treatments that can be used to overcome antibody-mediated rejection (AMR). AMR is the result of a circulatory disturbance that is caused by injury to the endothelium due to an antibody–

antigen–complement reaction. The typical clinical manifestations of AMR are hepatic necrosis and intrahepatic biliary complications [12]. Although no patients in our study had developed hepatic necrosis or apparent intrahepatic biliary complications with the prophylaxis, including rituximab and plasma exchange, our results suggest that patients undergoing ABO-incompatible LDLT may have a greater chance of developing postoperative severe hyperbilirubinemia.

The prognosis of the HB group was significantly worse than that of the non-HB group. Marubashi et al. [1] reported devastating outcomes in patients with a postoperative peak serum bilirubin level of >27 mg/dl, with eight of their grafts resulting in early graft loss within 1 year. In contrast, we experienced a number of patients with severe hyperbilirubinemia post-LDLT who eventually recovered their liver function; in fact, nine of the 17 patients in the HB group survived. Therefore, we investigated the perioperative parameters to clarify the risk factors for decreased survival. Our analysis revealed that the postoperative PT (%) at the time when the serum bilirubin level exceeded 30 mg/dl for the first time was significantly correlated with the prognosis based on the univariate analyses. Based on these results, the patients who were able to maintain their liver synthesis function were able to recover their liver function despite a temporal deterioration in bilirubin excretion.

Cholestasis has been recognized as a clinical manifestation of small-for-size graft syndrome, and the improvement of temporal cholestasis in proportion to the liver regeneration can be expected in cases of partial liver graft transplantation. We tried to exclude small-for-size syndrome with massive ascites. Although there is no consensus on the definition of small-for-size syndrome, there was one patient in the HB group who was suspected to have small-for-size syndrome, and she recovered spontaneously [normal range PT (%)] [13, 14]. In fact, GW/SLV was not a significant risk factor for the development of hyperbilirubinemia in our present study.

In addition, the postoperative serum creatinine level at the time when the serum bilirubin level exceeded 30 mg/dl for the first time tended to be lower in surviving patients. Acute kidney injury following liver transplantation has been reported to be associated with a worse outcome [15]. It is not hard to comprehend that HB patients with multiple organ dysfunction would have be a worse prognosis.

In conclusion, the use of a partial liver graft from an aged donor is considered to be a significant risk factor for postoperative severe hyperbilirubinemia. Although the outcomes of the HB patients were worse than those for the non-HB group, we should recognize that recovery is possible even from severe hyperbilirubinemia in those patients who are able to maintain their liver synthetic function during the postoperative course.

Conflict of interest None.

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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

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Key words
cccDNA, HBcrAg, HBV, liver transplantation.

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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.
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Sadayuki Okudaira: acquisition of data, critical revision of the manuscript for important intellectual content.
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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.

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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.

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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

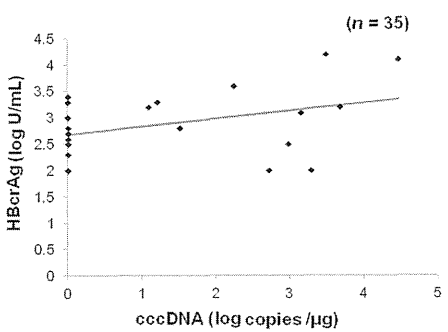

Figure 1 Correlation between serum hepatitis B core-related antigen (HBcrAg) and intrahepatic hepatitis B virus covalently closed circular DNA (cccDNA). $r = 0.616$, $P < 0.001$ ($y = 0.40x + 2.62$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

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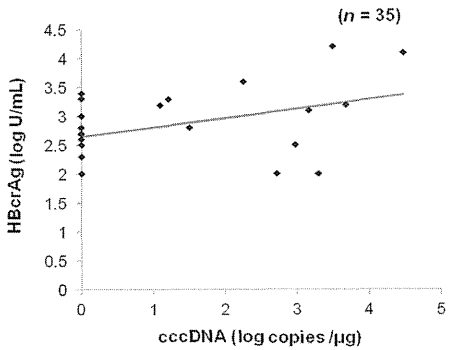

Figure 2 Correlation between hepatitis B core-related antigen (HBcrAg) and covalently closed circular DNA (cccDNA) levels after transplantation. $r = 0.402$, $P = 0.046$ ($y = 0.16 \times + 2.64$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

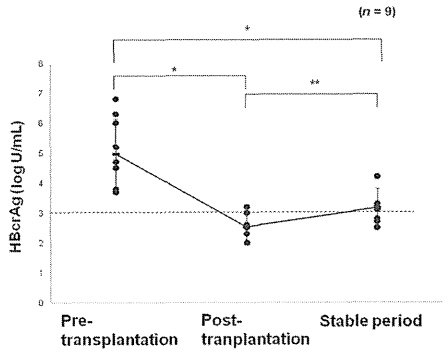

Figure 3 Serial changes of the hepatitis B core-related antigen (HBcrAg) levels. HBcrAg levels are represented as mean values; the closed circles show the values of the HBcrAg levels in all phases. The error bars indicate standard deviations. The detection range is above 3.0 log U/mL. In order to obtain 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).

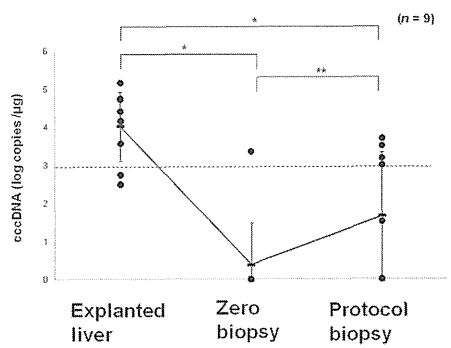

Figure 4 Serial changes of the covalently closed circular DNA (cccDNA) levels. cccDNA levels are represented as mean values; the closed circles show the values of the cccDNA levels in all phases. The error bars indicate standard deviations. The mean values of the cccDNA levels dropped during the time-zero 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,

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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.

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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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LIVER TRANSPLANTATION 19:666, 2013

LETTER TO THE EDITORS

False Positivity for the Human Immunodeficiency Virus Antibody After Influenza Vaccination in a Living Donor for Liver Transplantation

Received February 13, 2013; accepted February 25, 2013.

TO THE EDITORS:

Because of increased productivity and availability, more people have had the chance to undergo prophylactic influenza vaccination. It has been reported that influenza vaccination has cross-reactivity with human immunodeficiency virus (HIV) antibody assays, but this information is not well known in the field of transplantation.[1] Recently, we experienced a case of living donor liver transplantation in which a healthy donor candidate was frightened and was further screened for the HIV antibody.

The patient was a 43-year-old female who was a candidate for partial liver donation for her husband, who was suffering from hepatocellular carcinoma associated with hepatitis B liver cirrhosis. She had never undergone a blood transfusion or abused drugs before her screening for living partial liver donation. According to her laboratory results, she was positive for the HIV antibody (1.7 cut off index). Otherwise, all data, including hepatitis B antibody results, were within normal limits. It was found that she had undergone vaccination for influenza 1 week before the screening. She was referred to a specialist in HIV infection, and western blotting for all antibodies (GP160, GP110/120, P68/66, P55, P52/51, GP41, P40, P34/31, P24/25, and P18/17) was negative. HIV RNA was undetectable in her blood (<40 copies/mL). Thus, she was considered to be HIV-negative with a high level of confidence and subsequently donated the left lobe of her liver. The recipient remained negative for the HIV antibody even after living donor liver transplantation.

With the prevalence of influenza vaccination and organ donation, physicians should keep in mind that recent inoculation with any brand of influenza vaccine is associated with a false-positive screening assay for HIV antibodies.[2]

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The protocol for our living donor liver transplantation received a priori approval by the institutional review committee.

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EDITORIAL

Is low central venous pressure effective for postoperative care after liver transplantation?

Susumu Eguchi

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The central venous pressure (CVP) has been regarded as an important factor for reducing blood loss and the blood transfusion rate during major hepatectomy, and can be controlled by positive end-expiratory pressure (PEEP) or certain drugs and the optimal positioning of the patient [1–4].

In this issue of *Surgery Today*, Wang et al. [5] describe the beneficial effects of lowering the CVP for achieving a better postoperative outcome compared with conventional fluid management in deceased donor liver transplantation based on a prospective randomized controlled study. They report that the low CVP group showed (1) less intraoperative blood loss, (2) a decreased need for intraoperative blood transfusion, (3) fewer lung-related complications at 1 month postoperatively, (4) a shorter intubation period and (5) equal patient survival at 1 year after liver transplantation. A previous retrospective study showed intraoperative blood transfusion to be a risk factor for postoperative lung complications [6]. The present study was done in a prospective, randomized manner, which yielded the same results as those seen in the previous retrospective study. The methods used to reduce the CVP in the present study were the use of the Fowler position, fluid restriction and drugs (e.g., nitroglycerin, furosemide and somatostatin). These methods have also been used in previous studies to reduce the intraoperative CVP, and therefore they appear to be valid for this kind of study [2].

Although the results provided in the article were of high importance, lowering the CVP during liver transplantation might still be controversial and may have ambivalent aspects with regard to the lack of a relationship between the early complication rates, including renal, hepatic and pulmonary complications, and the CVP following liver transplantation [7–10]. For example, apart from the reduced pulmonary complication rate, and the lower blood loss and blood transfusion rate, what would be the influence of lowering the CVP on the postoperative care following liver transplantation? If blood product administration during the intensive care period is increased, then the policy to limit CVP during surgery would be in vain. Therefore, the readers will also want to know: How would the perfusion in the organ be affected? How would the lactate level in the blood after LT be affected, not only at the end of surgery but also during the postoperative period? How would the post-transplant blood product requirements be affected?

In fact, the period in which the CVP is lowered may be of importance. For example, Feng et al. [7] reported that a low CVP during the pre-anhepatic phase reduced the intraoperative blood loss, protected the liver function and it also had no detrimental effects on the renal function after LT. On the other hand, Cywinski et al. reported that a low CVP during the post-anhepatic phase was not associated with any benefit in terms of immediate postoperative allograft function, graft survival or patient survival [10]. In addition, the cut-off value for CVP monitoring in previous studies varied between 5 and 10 mmHg.

We therefore await further reports from other investigators before drawing any definitive conclusions about the above-mentioned issues, since liver transplant surgery, especially partial liver transplantation, is often affected by multiple factors [11].

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Review Article

Liver transplantation for HIV/hepatitis C virus co-infected patients

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Since the introduction of antiretroviral therapy (ART) in the mid-1990s, AIDS-related death has been dramatically reduced, and hepatitis-C-virus (HCV)-related liver failure or hepatocellular carcinoma has currently become the leading cause of death in HIV/HCV co-infected patients. Liver transplantation may be one of the treatments of choices in such cases, but the indications for transplantation, perioperative management including both HIV and HCV treatments, immunosuppression and the prevention/treatment of infectious complications are all still topics of debate. With the improved understanding of the viral behaviors of both HIV and HCV and the development of novel strategies, especially to avoid drug interactions between ART and immunosuppression, liver transplantation has become a realistic treatment for HIV/HCV co-infected patients.

Key words: hepatitis C virus, HIV, liver transplantation

INTRODUCTION

IN JAPAN, IN the late 1980s, contaminated blood production of coagulation factor for hemophilia caused co-infection of HIV and hepatitis C virus (HCV). Actually, greater than 90% of HIV-infected patients have HCV as well.[1]

After antiretroviral therapy (ART) was introduced in the late 1990s, successful control of HIV was achieved in most cases and death due to AIDS was dramatically reduced, but HCV-related death due to liver failure or hepatocellular carcinoma became a serious problem, not only in Japan, but all over the world.[2–6] In such cases, liver transplantation (LT) is the only treatment option to achieve long-term survival, but several modifications of perioperative management are required. In this review, the outcome and the points of management of LT for HIV/HCV co-infected patients were reviewed.

REPORTED OUTCOME OF LT FOR HIV/HCV PATIENTS

THE REPORTED OUTCOMES of LT for HIV and HIV/HCV co-infected patients from Western countries after the introduction of ART are summarized in Table 1.[7–11] In general, most reports concluded that the results were worse than in the cases with HCV mono-infection, with a 3-year survival of approximately 60–70%. In Japan, the Tokyo group reported six cases of living donor liver transplantation (LDLT) between 2001 and 2004, of whom four died.[12] These unfavorable outcomes are likely related to the difficulties of determining the indications for LT and of perioperative management, including HIV/HCV treatment and the prevention and treatment of infectious complications. Terrault *et al.* reported that older donor age, combined kidney–liver transplantation, an anti-HCV positive donor and a body mass index of less than 21 kg/m^2 were independent predictors of graft loss.[10] After transplantation, several studies showed that acute cellular rejection was more frequent and severer in HIV/HCV co-infected patients than that in HCV mono-infected patients, possibly due to the difficulties in achieving optimal immunosuppression because of interactions between antiretroviral agents and immunosuppression.[10,11]

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