

The Significance of Enzyme Immunoassay for the Assessment of Hepatitis B Virus Core-Related Antigen following Liver Transplantation

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Abstract

Purpose Recently, a new enzyme immunoassay for the detection of hepatitis B virus (HBV) core-related antigen (HBcrAg) has been reported. In this study, we proposed to account for feasibility of HBcrAg assay, and discuss the dynamics of HBV seen in patients following HBV-related living donor liver transplantation (LDLT).

Methods and results This study involved 12 patients; 11 patients had positive serum HBcrAg, and 6 patients had negative HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg in sera under prophylaxis therapy. At post-operation, 5 of the 12 had positive serum HBcrAg, and at stable state, 6 had positive serum HBcrAg postoperatively. The mean levels of HBcrAg following LDLT were significantly lower than those seen in the preoperative-operation stage.

Conclusion This enzyme immunoassay is a readily utilizable marker of HBV replication in the post transplantation stage. Furthermore, the evaluation of HBV activity by HBcrAg assay must be studied to determine the appropriate prophylaxis for controlling replication of HBV following LDLT.

Key words: hepatitis B virus, liver transplantation, hepatitis B virus core-related antigen

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Introduction

Liver transplantation (LT) is a long established procedure for the treatment of end-stage liver disease. Patients with chronic or fulminant hepatitis B virus (HBV) infection are major candidates for LT. However, the recurrence of HBV following LT is implicated in severe and life-threatening graft failure (1). Therefore, the prevention of HBV recurrence following LT has been a serious concern. The advent of anti-HBsAg immune globulin (HBIG, Hebsulin-IH, Mitsubishi Pharma Corporation, Tokyo, Japan), and HBV reverse transcriptase inhibitor, namely lamivudine (Lam,

Zeffix, GlaxoSmithKline K.K., Tokyo, Japan) and adefovir dipivoxil (Adv, Hepsera, GlaxoSmithKline K.K., Tokyo, Japan), was a major breakthrough in controlling HBV recurrence in patients who received transplants for HBV-related liver disease. The ideal recurrence rate for HBV (<10%), has been observed in patients receiving HBIG and Lam combination prophylaxis versus just HBIG monotherapy (2, 3) or Lam monotherapy (4, 5). Lam monotherapy has been shown to be ineffective in controlling recurring HBV, and the long term administration of HBIG was necessary (6, 7). Therefore, presently, continuous combination therapy is the standard prophylaxis in the control of HBV recurrence following HBV-related LT.

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Table 1. Clinical Characteristics

Case	Age at LDLT	Gender	Indication disease	HCC	HBV-DNA at LDLT (logcopy/mL)	Adefovir before LDLT	HBsAg (COI)	HBsAb (IU/mL)	HBeAg (IU/mL)	HbeAb (COI)	HBcrAg (logU/mL)
1	56	male	LC	+	<2.6	-	>2000	2.3	0.6	82.4	4.2
2	60	male	LC	+	<2.6	-	1789	0.1	0.2	97.6	5.8
3	59	male	LC	+	<2.6	-	>2000	0.3	0.1	>100	3.2
4	55	female	LC	-	<2.6	-	>2000	0.2	36.0	0	6.0
5	56	male	LC	+	<2.6	-	>2000	0.1	1.4	75.4	5.6
6	68	male	LC	+	<2.6	-	47.7	0.1	0.1	>100	<3.0
7	37	female	LC	+	2.6	+	>2000	0.1	0.2	81.5	5.5
8	57	male	LC	+	<2.6	+	188.5	0.5	0.8	54.0	5.1
9	48	male	LC	-	<2.6	+	562.5	0.1	1.1	57.7	5.0
10	53	male	LC	-	4.4	+	>2000	0.1	49.2	96.1	7.5
11	34	female	FHF	-	4.9	-	374	7.9	0.8	93.9	5.7
12	28	female	FHF	-	4.6	-	19.5	133.8	4.6	54.4	7.4

Previous reports showed only trace amounts of HBV replication in extra-hepatic sites following LT (8). If HBV was present in hepatocytes, Lam would have masked the appearance of HBV-DNA regardless of the presence of intrahepatic HBV covalently closed circular (ccc)DNA (9, 10). These factors make it difficult to understand HBV dynamics following LT. Recently, new enzyme immunoassays for detecting HBV core antigen (HBcAg) (11) and HBV core-related antigen (HBcrAg) (12, 13) have been reported. These antigens move parallel with HBV-DNA in the serum and have a wide detection range (14). In particular, the assay for HBcrAg is able to detect both HBcAg and HBeAg even in anti-HBc antibody and anti-HBe antibody-positive specimens. Additionally, it has shown a higher sensitivity than HBV-DNA transcription mediated amplification (TMA), and equivalent sensitivity to in-house real time detection PCR (15). Different from the assay for HBV genome, the HBcrAg assay detects translational products of HBV and is presumed to be a reflection of cccDNA (16, 17). The HBcrAg assay has never been used to assess transplant patients undergoing HBV prophylaxis, and the status of HBV replication markers has also never been discussed in the case of post-transplanted patients, negative for HBsAg and HBV-DNA, who were undergoing anti-HBV prophylaxis. Therefore, in this study, we proposed to account for availability of HBcrAg assay, and discuss HBV dynamics in patients following HBV-related LT.

Abbreviation: HBsAg: hepatitis B virus s antigen, HBeAg: hepatitis B virus e antigen, HBcAg: hepatitis B virus core antigen, HBcrAg: Hepatitis B virus core-related antigen, cccDNA: covalently closed circular DNA, Lam: Lamivudin, HBIG: anti-HBs antigen immune globulin

Materials and Methods

Patients and clinical samples

From 2001 to 2006, a total of 12 patients with HBV-related severe liver disease, were admitted to Nagasaki University Hospital, Nagasaki, Japan, and enrolled in this study (Table 1). There were 8 men and 4 women with a median age of 52.0 years (range 28-68 years). All 12 patients had received LDLT at this hospital. The graft survival rate was

100%, and not one showed evidence of graft hepatitis. Of the 12 patients, 10 had been diagnosed with liver cirrhosis (LC) (with 7 of those having hepatocellular carcinoma), and 2 patients had been diagnosed with fulminant hepatic failure (FHF). All patients had been receiving a daily dose of 100 mg Lam since the pre-operation period in order to prevent the recurrence of HBV infection [range 0.1-22 months, mean (standard deviation: SD); 7.81 (8.17) months] and following LT, 4 patients began receiving Adv therapy [range; 19-250 days, mean (SD) 102.3 (128.2) days] in addition to Lam due to Lam resistant HBV mutations present before and after LT. Donor status of HBV serological makers such as HBsAg, HBsAb and HBcAb were negative. Prophylactic infusion of HBIG was administered to all patients using a fixed dosing schedule: 10,000 units intravenously at the anhepatic period and on the day following LDLT. Afterwards, a dose of 2,000 units of HBIG was given routinely over the long term with the aim of keeping the serum titer greater than 100 units/L. After LDLT, serum HBsAg, HBeAg or HBV-DNA were not detected in any patient. Serum samples for HBV were collected from each patient at the following three specified intervals: 1) at the pre-operation period during Lam or Lam/Adv treatment (ranges; 7.9±8.7 days prior to LT). 2) at the immediate post-operation period during which patients received combined prophylaxis, and immunosuppression with steroid and calcineurin inhibitor (within 37.7±20.3 days after LT), and 3) at the stable state period when patients received combined prophylaxis, and immunosuppression without steroid (18.1±16.7 months after LT). Serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), prothrombin time (PT), and albumin (Alb) were obtained from patient's medical records. All patients underwent needle liver biopsy every year after transplantation.

Abbreviation: HBsAb: hepatitis B virus s antibody, HBcAb: hepatitis B virus core antibody

Serological markers for HBV

HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method, using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Tokyo, Japan). Serum concentrations of HBV DNA were determined using a

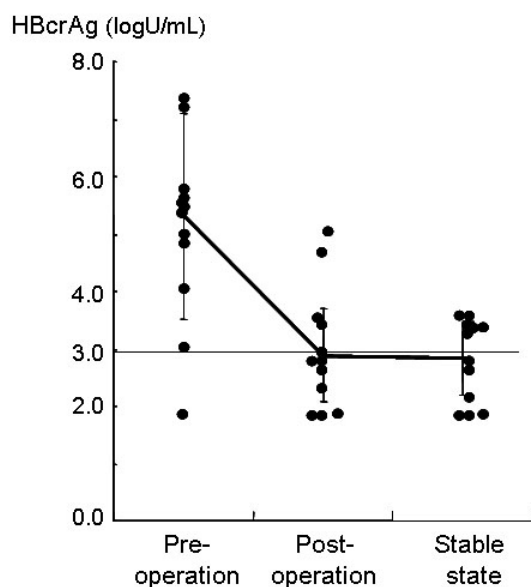


Figure 1. Serial changes of the HBcAg levels. The HBcAg levels are represented as mean values; the closed circles show the value of HBcAg levels in all phases. Error bar is standard deviation. The mean value of HBcAg levels in the post-operation period and in the stable state period were significantly lower than that in the pre-operation period (*t*-test, $p < 0.05$). The detection range is above 3.0 logU/mL. To obtain the mean value, the value of 3.0 logU/mL or less and 2.0 logU/mL or more was added to the calculation.

polymerase chain reaction HBV monitoring kit (Roche, Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copy/mL.

Serum concentrations of HBcAg were measured using the CLEIA method reported previously (12, 18). In brief, 100 mL serum was mixed with 50mL of a pretreatment solution containing 15% sodium dodecylsulfate, and 2% Tween 60. After incubation at 70°C for 30 minutes, 50mL of pretreated serum was added to test wells coated with monoclonal antibodies specific for denatured HBcAg and HBeAg (HB44, HB61, and HB114), and then filled with 100 mL assay buffer. The plate was incubated for 2 hours at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies specific for denatured HBcAg and HBeAg (HB91 and HB 110), were added to the wells, and the plate was again incubated at room temperature, this time for 1 hour. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated at room temperature one more time for 20 minutes. The relative chemiluminescent intensity was measured, and the HBcAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBeAg (amino acids, 10-183 of the precore/core gene product). The HBcAg concentration was expressed as units/mL (U/mL) and the immunoreactivity of recombinant pro-HBeAg at 10 fg/ml corresponded to 1 U/mL. In this study, the cutoff value was tentatively set at 3.0 logU/mL (12).

Table 2. Comparison of the HBcAg Levels between Lam Group and Combination Lam/Adv Group at Each Period

Group	Number	pre-operation	post-operation	stable
L: Lam	6	4.47 (1.62)	2.92 (1.19)	5.14 (0.72)
A: Lam+Adv	4	5.78 (1.17)	3.58 (0.78)	3.45 (0.17)

Abbreviation: HBeAb: hepatitis B virus e antibody

Statistical analyses

Statistical analyses were performed using the SPSS 11.0.1 J statistical software package (SPSS, Inc., Chicago, IL). The *p*-values of less than 0.05 were considered statistically significant.

Results

Serial changes in HBcAg levels at indicated periods

Results of the HBcAg assay showed differences in titers during the specific periods (Table 1 and Fig. 1); 11 cases had positive levels of HBcAg, however 8 of them were negative for HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg, however 5 of them (cases 4, 5, 7, 9 and 10) had positive levels of HBcAg. In the stable state period, 6 of the cases (cases 2, 5, 7, 8, 9 and 10) had positive levels of HBcAg. The 2 cases with FHF (cases 11 and 12) had negative levels of HBcAg in both post operation and the stable state periods. Of the 4 patients who received combined Lam/Adv treatment, 3 patients (cases 7, 9, and 10) also had positive HBcAg levels in both post operation and the stable state periods. Two cases (cases 2 and 8) had negative levels of HBcAg in the post operation period, but positive levels in the stable state period. The overall mean level of HBcAg following LT [post-operation 3.05 (1.026) logU/mL and stable state periods 2.875 [(0.66) logU/mL] was significantly lower than that at pre-operation period [mean (SD); 5.25 (2.445) logU/mL] (Fig. 1). After LT, the levels of serum HBcAg were decreased and steroid administration on early post-operation period did not seem to influence HBcAg levels.

Comparison of the HBcAg levels between combination Lam/Adv group and Lam group

A comparison of the mean values of HBcAg levels between the group receiving only Lam treatment (6 patients with LC: Group L) and the group receiving combination Lam/Adv treatment (4 patients with LC: Group A) was made (Table 2). The mean value of HBcAg in Group A was higher than that in Group L through all periods of the study [mean (SD) value (logU/mL) is as follows: pre-operation, Group A; 5.78 (1.17), Group L; 4.47 (1.62), post-operation, Group A; 3.58 (0.78), Group L; 2.92 (1.19), stable state, Group A 3.45 (0.17), Group L: 5.14 (0.72)]. No

prior to LT. His serum HBV-DNA became negative 1 month prior to LT. Liver function became worse and LT was performed for hepatic failure. His level of serum HBcrAg prior to LT was 5.8 logU/mL, and HBsAg was positive. Following LT, HBsAg became negative. His HBsAb titer was high due to HBIG. At post operation, his serum HBcrAg level fell below the cut-off level (2.8 logU/mL). However, levels of HBcrAg then rose to 3.5 logU/mL despite normal levels of ALT and total bilirubin. Negative levels of both HBsAg and HBV-DNA have continued to present. In case 8, whose HBcrAg level was negative in the post operation period but became positive in the stable state period, the levels of ALT and total bilirubin remained at normal levels in the observation period.

Case number 10 (Fig. 3B), a 53-year-old man with LC, began receiving Lam therapy 19 months prior to LT, however his ALT and T-Bil were in relapse due to Lam resistant HBV mutation. Therefore, the addition of Adv therapy was started 3 weeks prior to LT. Hepatic failure could not be prevented despite the addition of Adv, and LT was performed. At the time of LT, serum HBV-DNA and HBcrAg were 4.4 logU/mL and 7.5 logU/mL, respectively. Following LT, Lam and Adv therapy was continued and his levels of HBV-DNA have remained below the cut-off level (<2.6 logU/mL), but levels of HBcrAg have been positive, throughout both the post-operation period; 4.7 logU/mL, and the stable state period; 3.6 logU/mL. His liver function became stable following after LT.

Case number 12 (Fig. 3C), a 28-year-old woman with FHF, suffered from acute HBV infection. Since several courses of plasma aphaeresis, along with Lam therapy did not improve her condition, she underwent LT despite positive levels both of HBV-DNA (4.6 logU/mL) and HBcrAg (7.4 logU/mL). Following LT, serum HBV-DNA became negative, and serum HBcrAg levels have remained below the cut-off level (post-operating period; 2.0 logU/mL, stable state period; 2.3logU/mL). Her liver function became stable following LT.

Every case entered in this study underwent an annual liver biopsy in our hospital. All of the biopsy specimens in the stable state did not show any pathological features of chronic viral hepatitis despite the titer of HBcrAg in serum.

Discussion

This newly developed enzyme immunoassay for HBcrAg could be a useful measure of HBV activity in patients receiving anti-HBV prophylaxis following LT. Serum HBcrAg was detected prior to LT in all patients, and the levels varied in the early and late post operation period. Our use of HBcrAg assay shows that HBV replication is occurring in patients receiving combination prophylaxis following LT, and that LT itself decreased levels of HBcrAg. Since LT decreased the levels of serum HBcrAg, then the use of steroid did not have any influence on HBcrAg levels. The value of HBcrAg varies over time, but it has no relationship to he-

patic function. However, further observation is necessary to evaluate the relationship between the detection of HBcrAg and the long-term prognosis of these patients.

It has been reported that serum HBcrAg levels can be thought of as a non-relapse marker at the time of Lam cessation (15), and a risk marker for HBV resistance at the 6 month point in Lam treatment (16). Lam blocked the reversed transcription of HBV-RNA to HBV-DNA, but did not inhibit translation or transcription. Cessation of Lam at the absence of serum HBV-DNA causing a flare up of HBV replication, due to the existence of HBV cccDNA, which is a template for the HBV pregenome RNA, may be a source of Lam resistant HBV strains in hepatocytes (8, 19). The levels of cccDNA in hepatocytes, as well as HBcrAg in serum, but not HBV-DNA in serum, are also a prediction marker of sustained anti-viral response in Lam treatment (20, 21). Production of HBcrAg in hepatocytes as a reflection of HBV replication activity, indicates the existence of cccDNA in hepatocytes. Therefore, the concentration of HBcrAg in the serum of a patient receiving Lam treatment may indicate an altered HBV replication status within the hepatocytes (22). We feel the HBcrAg assay is a reliable means for identifying HBV replication following HBV-related LT, and think that HBV replication continues following LT despite combination HBIG and Lam prophylaxis. The sensitivity of HBcrAg is not very high in HBsAg seroclearance patients (17). Since HBsAg and HBV-DNA had not been detected in post LT patients receiving combination prophylaxis, HBcrAg assay can be a predictive maker of HBV replication at this stage. Recently, it was reported that HBV cccDNA in hepatocytes (23), HBV-RNA (22, 24) and serum HBsAg quantitative (25) are HBV replication markers. In addition to the HBcrAg assay, we should evaluate these markers to fully understand HBV dynamics after LT.

Previous reports have suggested that Lam resistant, HBV-infection related-LT was as safe as wild type HBV-infection-related LT (26). These reports concluded that a combination of Adv and Lam therapy provides effective prophylaxis in patients with pre-LT Lam resistant HBV mutants (26, 27). However, positive HBV-DNA was observed in all of the patients in the present study, and Adv and Lam resistant HBV has recently been observed (28). In our study, in the stable period, the titer of HBcrAg in Lam group was relatively higher than Adv add-on group. Further study is needed to evaluate Adv add-on Lam combined prophylaxis.

The production site of HBcrAg was unclear in the post-LT period. In cases of HCV-related LT, non-hepatic virus sources, at the most, account for 4% of the total viral production, and post-LT viral clearance, after rapid initial decline, slows, possibly due to the filling of absorption sites in newly grafted liver (29). HBV re-infection may be caused by the over-production of HBV in extrahepatic sites or HBV circulating particles following LT (30). Escaped mutants from HBIG and Lam may also cause re-infection (31). According to a recent report (32), highly sensitive real-time PCR of cccDNA found that cccDNA in PBMCs is detected

only to a small degree. As such, PBMCs are unlikely to function as a reserve of HBV. In HCV-related LT, it has been reported that the virus immediately re-infects liver grafts (33, 34), but re-infection of the graft is not apparent in HBV. We can not disregard production of HBcAg in hepatocytes following LT, but further studies are necessary to fully understand HBV replication sites following LT.

In addition to HBsAg and HBV-DNA, HBcAg assess-

ment could be a practical tool as a marker of HBV replication after LT. Because the levels of HBcAg are a reflection of cccDNA, we think that the HBcAg positive cases need continual prophylaxis following LT. In addition, the evaluation of HBV dynamics by HBcAg assay must be studied to determine the appropriate prophylaxis against replication of HBV following LT.

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