

Title Page**Hepatitis B Virus RNA is Measurable in Serum and can be a New Marker for
Monitoring Lamivudine Therapy****Short title:** HBV RNA measurement in serum

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Summary

Purpose. Changes in serum hepatitis B virus (HBV) RNA level during lamivudine therapy were compared to those in serum HBV DNA and HBV core-related antigen (HBVcrAg) levels in 24 patients with chronic hepatitis B. **Methods.** For measurement of HBV RNA, total nucleic acid was extracted from serum samples and treated with RNase-Free DNase I. After cDNA synthesis from extracted RNA, HBV RNA was measured by real time detection polymerase chain reaction. **Results.** The peak fraction of HBV RNA in serum samples was consistent with peak fractions of HBV DNA and HBV core protein in sucrose gradient analysis, indicating that HBV RNA was incorporated in virus particles. All levels of HBV DNA, HBV RNA, and HBVcrAg decreased gradually during lamivudine therapy ($P < 0.001$ in all). The degree of decrease from the start of lamivudine therapy was significantly higher in HBV DNA level than in HBV RNA and HBVcrAg levels during 6 months of lamivudine therapy ($P < 0.001$ in all). However, such a difference was not seen between HBV RNA and HBVcrAg levels during that period. HBV RNA level was significantly ($P < 0.001$ in all) correlated both with levels of HBV DNA and HBVcrAg at the beginning and 2 months after the start of lamivudine therapy. **Conclusions.** HBV RNA is detectable in serum in a form incorporated in virus particles, and its serum level **is possible to be** a new viral marker with different significance than HBV DNA in lamivudine therapy.

Key words:

chronic hepatitis B, viral load, viral replication, cccDNA, sucrose gradient analysis

Introduction

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide (1). In some countries, hepatocellular carcinoma and cirrhosis accounts for more than 50% of all deaths among HBV carriers (2). Treatment of patients with hepatitis B has improved remarkably with the advent of oral nucleoside analogues such as lamivudine (3, 4). Lamivudine administration has been shown to cause rapid decrease in serum HBV DNA level followed by decrease of alanine aminotransferase level and improvement of liver histology (5-8). Therefore, measurement of serum HBV DNA is used widely in the clinical setting to monitor the effect of lamivudine.

It has been postulated that measurement of HBV covalently closed circular (ccc) DNA level in hepatocytes is valuable in a different way from serum HBV DNA for monitoring the effects of antiviral therapy since cccDNA is a key molecule in HBV replication (9-12). Practically, intrahepatic HBV cccDNA level has been reported to be superior to serum HBV DNA to predict sustained virologic response of antiviral therapy including lamivudine (13). However, the measurement of cccDNA seems ill-suited for clinical use because it requires a liver biopsy. Thus, serum markers that reflect the cccDNA level in the liver are desired.

Recently, an HBV core-related antigen (HBVcrAg) assay developed by our laboratory revealed a possible correlation with cccDNA level, especially during lamivudine therapy (14-16). This possibility was based on the fact that transcription of messenger RNA from cccDNA and subsequent translation of viral proteins are not inhibited by nucleoside analogues such as lamivudine. The same has been said for synthesis of pregenomic RNA (7). Therefore, in the present study, we measured serum HBV RNA and analyzed its virologic characteristics. Furthermore, changes in serum HBV RNA level during lamivudine therapy were compared to those of serum HBV DNA

and HBVcrAg levels to clarify whether there exists any clinical significance of HBV RNA measurement in serum.

Patients and Methods

Patients

A total of 24 patients with chronic hepatitis B who consented to participate in the present study. They were selected from a pool of 32 consecutive patients who underwent lamivudine therapy at Shinshu University Hospital between July, 2002 and June, 2003. The patients consisted of 18 men and 6 women and were a median age of 55 years (age range = 39-79 years). Chronic hepatitis B was defined as positive HBV surface antigen for more than 6 months with liver histological findings consistent with chronic hepatitis. All patients had elevations in serum alanine aminotransferase levels as well as HBV DNA for at least 6 months. Immediately prior to lamivudine administration, 16 patients were positive for HBV e antigen and 8 were positive for HBV e antibody but negative for HBV e antigen. The HBV genotype was C in all patients. Patients received 100 mg doses of lamivudine daily for at least 6 months. No patient had been treated with other antiviral agents, such as interferon, before or during the present study, and all patients were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by Ethics Committee of our institution. Written informed consent was obtained from each patient.

Serum samples were collected at the start of lamivudine therapy, and at 2 and 6 months after commencement. Samples were stored frozen at -20 °C or below until assayed.

Routine laboratory tests

HBV surface antigen, HBV e antigen, HBV e antibody, hepatitis C virus

antibody, and human immunodeficiency virus antibody were measured by commercially available enzyme-linked immunosorbent assay kits (ABBOTT JAPAN, Tokyo, Japan). HBV genotypes were determined by the method as reported by Mizokami et al. and classified into six major genotypes, A to F (17).

Serum level of HBV DNA was determined using Amplicor HBV Monitor kit (Roche Diagnostics, Tokyo, Japan) which has a quantitative range from 2.6 to 7.6 log copy/mL. Sera containing over 7.0 log copy/mL HBV DNA were diluted 10 or 100 fold with normal human serum and re-tested to obtain the end titer.

HBV core and core-related antigen assay

HBV core antigen (HBVcAg) and HBVcrAg in serum was measured using a chemiluminescence enzyme immunoassay as reported previously (14, 15, 18). In brief, 100 μ L serum was mixed with pretreatment solution containing 15% sodium dodecylsulfate. After incubation at 70°C for 30 min, 50 μ L pretreated serum was added to wells coated with monoclonal antibodies against denatured HBV core and e antigens (HB44, HB61, and HB114) and filled with 100 μ L assay buffer. The mixture was then incubated for 2 hrs at room temperature. After washing with buffer, either alkaline phosphatase-labeled HB50 monoclonal antibody (specific for denatured HBV core antigen) or a mixture of HB91 and HB110 monoclonal antibodies (against denatured HBV core and e antigens) were added to wells and incubated for 1 hr at room temperature. After washing again, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and plates were incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and HBVcAg and HBVcrAg concentrations were read by comparison to a standard curve generated using recombinant pro-hepatitis B e antigen (amino acids -10 to 183 of the precore/core gene

product). The concentrations of HBVcAg and HBVcrAg were expressed as unit/mL and the immunoreactivity of recombinant pro-hepatitis B e antigen at 10 fg/mL was defined as 1 unit/mL. The cut-off value of both assays was set at 3 log units/mL (11). Sera containing over 7 log unit/mL of antigen were diluted 10 or 100 fold in normal human serum and measured again to obtain the end titer.

HBV RNA assay

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) was used for isolation of HBV RNA from serum. Briefly, 200 μ L of serum was added to 250 μ L of freshly prepared working solution (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl [pH 4.4] and 20% [vol/vol] Triton X-100) supplemented with 20 μ g of poly(A) carrier RNA and 900 μ g Proteinase K. After incubation for 10 min at 72°C, 100 μ L of isopropanol was added and the mixture was transferred into a High Pure filter tube combined with a collection tube. The filter tube was centrifuged for 1 min at 8,000 rpm in a standard tabletop centrifuge at room temperature and combined with a new collection tube. The inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl [pH 6.6] in ethanol) was added to the upper reservoir and centrifuged for 1 min at 8,000rpm. After being washed with 250 μ L of wash buffer (20 mM NaCl, 2 mM Tris-HCl [pH 7.5] in ethanol), 80 μ L of RNase-Free DNase I solution (QIAGEN, Hilden, Germany) was added and incubated to digest HBV DNA for 15 min at room temperature. A volume of 200 μ L of wash buffer was added to the filter tube and centrifuged for 15 sec at 10,000 rpm. After being washed with 450 μ L of buffer, the filter was placed in a new collection tube and 50 μ L of RNase- and DNase-free water was added to elute the RNA. After centrifugation for 1 min at 8,000 rpm, the eluted RNA was stored at -80°C.

Synthesis of cDNA was performed at 42°C for 30 min in a 20 μ L reaction

mixture containing 10 μ L of the extracted RNA, 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM dNTP (1 mM each dATP, dGTP, dCTP and dTTP), 1 mM DTT, 100 nM reverse primer for the HBV surface gene (5'-GGTTGGTGAGTGATTGGAGGTT-3'; nt. 345 to 324), 40 units of RNasin (TaKaRa, Kyoto, Japan), and 200 units of SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA). The reaction mixture was inactivated by heating to 70°C for 15 min and then cooled to -80°C until real-time detection polymerase chain reaction (RTD-PCR) assay. A 4 μ L aliquot of cDNA solution was used for RTD-PCR which was performed with the Light Cycler System (Roche Diagnostics) as reported previously (14). The two primers and TaqMan probe used were designed from a region of the HBV surface gene: forward primer; 5'-ACAACATCAGGATTCCTAGGAC-3' (nt. 166 to 187), reverse primer as stated above (nt. 345 to 324), and TaqMan probe; 5'-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-TAMRA-3' (nt. 244 to 269). An HBV genome (nt. 20 to 1805) which had been subcloned into a pUC vector was used as an internal standard. The lower detection limit for the HBV RNA assay was set at 2.6 log copy/ml. HBV DNA was tested on extracted HBV RNA samples not having undergone the preceding process by RTD-PCR and was confirmed to be negative in all samples.

Sucrose density gradient ultracentrifugation

Serum (0.1 mL) was layered on a linear 10 to 60% (wt/wt) sucrose gradient, then underwent centrifugation at 200,000 x g (45,000 rpm) for 15 hrs at 4°C with a Beckman SW50.1 rotor (Beckman Coulter, Inc., CA). In total, 24 fractions of 200 μ L were collected by micropipette. Each fraction was diluted 5-fold and tested for HBV DNA, HBV RNA, and HBVcAg.

Statistical analyses

Statistical analyses including the Mann-Whitney's U test, Friedman's test, and Spearman rank correlation test were performed using the SPSS 10.0J statistical software package (SPSS Inc., Chicago, IL). A P value of less than 0.05 was considered statistically significant.

Results

Serum levels of HBV DNA ($P < 0.001$), HBV RNA ($P < 0.001$), and HBVcrAg ($P < 0.001$) all decreased significantly throughout the course of lamivudine therapy (Fig. 1). The degree of decrease at 2 months following commencement of lamivudine therapy was significantly higher for HBV DNA level than for HBV RNA and HBVcrAg levels (median = 2.45, 25% - 75% range = 1.90 - 3.00 log copy/mL vs. median = 0.40, 25% - 75% range = 0.00 - 0.85 log copy/mL, $P < 0.001$ and median = 0.30, 25% - 75% range = 0.10 - 0.65 log unit/mL, $P < 0.001$, respectively). Similarly, the degree of decrease at 6 months of treatment was significantly higher in HBV DNA level than in HBV RNA and HBVcrAg levels (median = 3.20, 25% - 75% range = 2.00 - 4.55 log copy/mL vs. median = 0.90, 25% - 75% range = 0.45 - 1.90 log copy/mL, $P < 0.001$ and median = 0.90, 25% - 75% range = 0.20 - 1.55 log unit/mL, $P < 0.001$, respectively). The degree of decrease did not differ between HBV RNA and HBVcrAg levels at either 2 ($P > 0.2$) or 6 ($P > 0.2$) months after commencement.

As shown in Fig. 2, the serum level of HBV RNA was significantly correlated with HBV DNA both at the start of lamivudine therapy ($r = 0.801$, $P < 0.001$) and 2 months afterwards ($r = 0.837$, $P < 0.001$). Serum HBV RNA was also significantly correlated with that of HBVcrAg both at the start of treatment ($r = 0.841$, $P < 0.001$) and at 2 months later ($r = 0.777$, $P < 0.001$). Ratios of HBV DNA and HBVcrAg levels to HBV RNA level were calculated in log scale in patients who were positive for the above two

parameters. The HBV DNA per HBV RNA ratio at the start of lamivudine therapy (n = 21, median = 1.36, 25% - 75% range = 1.20 – 1.71) decreased significantly ($P < 0.001$) at 2 months after treatment commencement (n = 15, median = 0.98, 25% - 75% range = 0.86 – 1.11). On the other hand, the HBVcrAg per HBV RNA ratio at the start of treatment (n = 21, median = 1.32, 25% - 75% range = 1.21 – 1.52) did not change ($P > 0.2$) after 2 months (n = 17, median = 1.36, 25% - 75% range = 1.15 – 1.54).

Serial serum samples obtained at the start of lamivudine therapy and at 1 and 2 months afterwards were subjected to sucrose density gradient fractionation and tested for HBV DNA, HBV RNA, and HBVcrAg. Each of the three viral markers showed a single peak at the same fraction (Fig. 3). This suggested that HBV RNA was incorporated in virus particles, similarly to HBV DNA. Viral particles containing HBV DNA were dominant at the start of treatment, while those containing HBV RNA became more prevalent 1 and 2 months afterwards.

Discussion

Replication of the HBV DNA genome proceeds via pregenomic RNA transcribed from the cccDNA present in the nuclei of infected hepatocytes (9, 11, 12). The pregenomic RNA is then packaged into nucleocapsids and reverse transcribed to form minus-strand DNA. Plus-strand DNA synthesis is initiated following degradation of the pregenome. It has been reported that nucleocapsids containing completely minus strand DNA can be enveloped and then secreted from hepatocytes (19). Thus, HBV virions in circulation have been considered to contain only mature viral genomes. In spite of this, HBV RNA could be detected in serum in the present study. HBV RNA was considered to be incorporated in virus particles because HBV RNA made a single peak at the same fraction where both HBV DNA and HBVcrAg made single peaks in sucrose gradient analyses conducted at three different time points during lamivudine therapy.

Detection of HBV particles with RNA genome does not necessarily contradict the previous report (19), since HBV RNA particles seemed to exist in only a small portion (0.1 – 1 %) of HBV virions in patients without lamivudine administration. The possibility that our HBV RNA assay detected HBV DNA which was left undigested by DNAase was considered to be negligible because HBV DNA was not detected by RTD-PCR in extracted HBV RNA samples, and that movement of HBV RNA level during lamivudine therapy differed significantly from HBV DNA.

Detection of HBV RNA in serum has rarely been reported thus far. Su et al. reported the presence of full length and truncated RNAs in serum (20), though their results seem to be quite different in nature because the HBV RNA they observed was not incorporated in core particles. Zhang et al. reported an existence of RNA genome with partially reverse transcribed minus-strand DNA in serum and speculated that the genome was in a virus particle (21). Such particles with HBV RNA accounted for about 1% of all HBV virions under untreated conditions, but became a major component under lamivudine administration. **Although their analysis was done in a single case,** those observations are consistent with our observations that the rate of decrease of HBV DNA in serum was much faster than HBV RNA during lamivudine administration.

The HBVcrAg assay is a unique assay which measures the total amount of antigen coded by pre-core core genes such as HBV core and e antigens (14, 15). During the HBVcrAg assay, core antigen was released from the pretreated HBV virion and denatured, along with free e antigen and e antigen/antibody-complex. The HBVcrAg assay employs monoclonal antibodies that are reactive with the common epitopes of denatured core and e antigens. Under these conditions, the HBVcrAg assay simultaneously measures the core and e antigens within a given sample, irrespective of their source of origin. Serum HBVcrAg levels reflect viral load in the untreated state because these levels correlate linearly with those of HBV DNA. On the

other hand, it has been reported that HBVcrAg level shows different characteristics than HBV DNA level under lamivudine administration (15, 16). It is noteworthy that HBVcrAg and HBV RNA levels both decreased significantly more slowly than HBV DNA after starting lamivudine administration, but at a similar rate. This phenomenon is quite possible because synthesis of mRNA from cccDNA episomes and subsequent production of viral proteins is not inhibited by lamivudine like the synthesis of pregenomic RNA. Furthermore, it has been reported that the level of cccDNA decreases quite slowly proceeding administration of nucleoside analogues (22), indicating that serum levels of HBV RNA and HBVcrAg can be independent markers from serum level of HBV DNA.

Measurement of serum HBV DNA is widely used for predicting and monitoring the effect of lamivudine therapy. However, a negative result of HBV DNA in serum does not necessarily indicate a good subsequent clinical course since the appearance of lamivudine resistant strains during drug administration and reactivation of HBV replication after discontinuation of treatment often occurs, even in patients who test negatively for serum HBV DNA during therapy (23-25). Sung et al. reported that intrahepatic HBV cccDNA level at the end of lamivudine monotherapy or peginterferon and lamivudine combination therapy is a better predictor of sustained virologic response than serum HBV DNA level (13). Their conclusion seems quite reasonable because cccDNA in infected hepatocytes, not HBV in circulation, serves as a template for HBV pregenomic and messenger RNA in HBV replication. Although measurement of cccDNA in the liver is a good marker for monitoring the effect of antiviral therapy, the measurement is not easy to apply for clinical use since it requires liver biopsy. Thus, serum markers that reflect the cccDNA level in hepatocytes are more desired for clinical use. We previously reported that serum HBVcrAg level was an independent marker from serum HBV DNA level in predicting the appearance of lamivudine

resistance, and suggested that HBVcrAg level reflects the level of cccDNA in hepatocytes (16). In the present study, serum levels of HBVcrAg and HBV RNA correlated significantly, and the two levels decreased in a similar manner during lamivudine therapy (Fig. 1 and 2). These results further indicate that both serum levels of HBVcrAg and HBV RNA reflect cccDNA level in hepatocytes since neither the synthesis of only messenger RNA, nor pregenomic RNA synthesized directly from cccDNA which may be incorporated in viral particles, is inhibited by lamivudine.

In conclusion, HBV RNA which is incorporated in viral particles is detectable in the serum of chronic HBV carriers. HBV RNA in serum can be a new marker which may reflect cccDNA level in hepatocytes and may be useful for monitoring lamivudine therapy. We could not clarify relationship between HBV RNA level and clinical outcome in the present study. Thus, further studies are required to elucidate the clinical significance of HBV RNA in serum and its relationship to serum HBVcrAg.

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Figure Legends

Figure 1.

Changes in serum levels of HBV DNA, HBV RNA, and HBVcrAg during lamivudine therapy in 24 patients with chronic hepatitis B. Open squares indicate HBV DNA, open circles indicate HBVcrAg, and closed triangles indicate HBV RNA. Data are expressed as median and 25th and 75th percentiles. HBV (hepatitis B virus), HBVcrAg (HBV core-related antigen).

Figure 2.

Correlation between serum levels of HBV DNA and HBVcrAg and HBV RNA at the start of lamivudine therapy and 2 months afterwards. HBV (hepatitis B virus), HBVcrAg (HBV core-related antigen).

Figure 3.

Distribution of HBV DNA, HBV RNA, and HBVcAg in sucrose density gradient fractions. Serum samples obtained at the start of lamivudine therapy and 1 and 2 months after were subjected to ultracentrifugation on a 10 to 60% (wt/wt) sucrose density gradient. Open squares indicate HBV DNA, open circles indicate HBVcAg, and closed triangles indicate HBV RNA. HBV (hepatitis B virus), HBVcAg (HBV core antigen).

Fig. 1

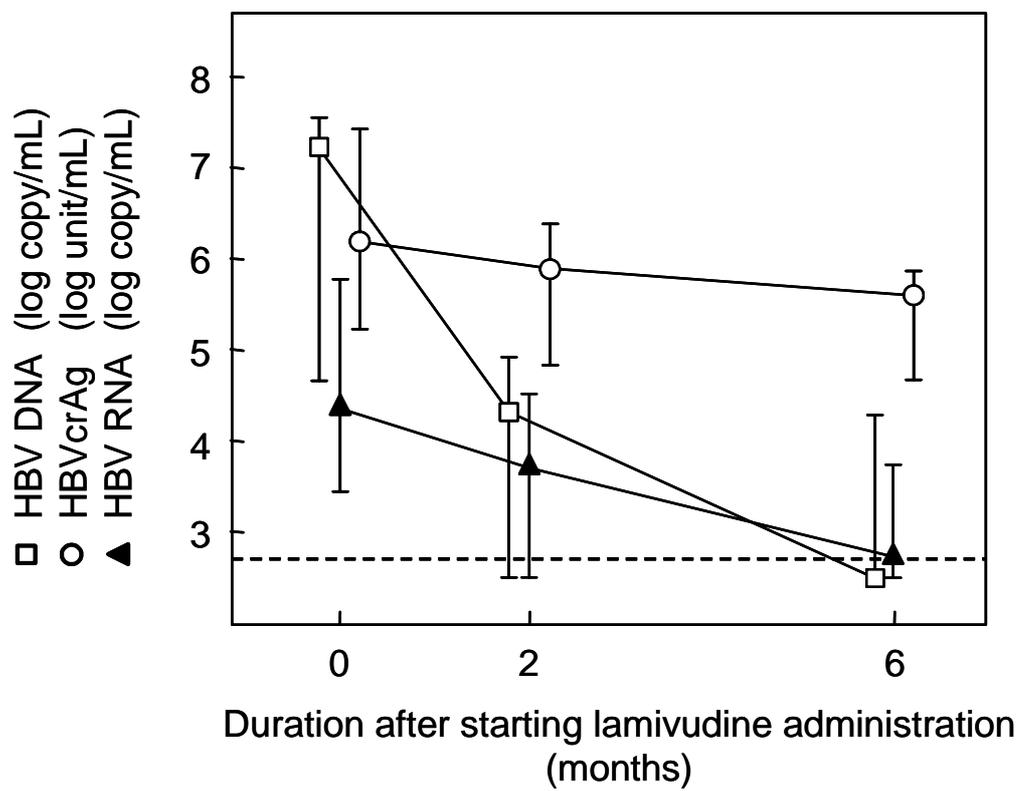


Fig. 2

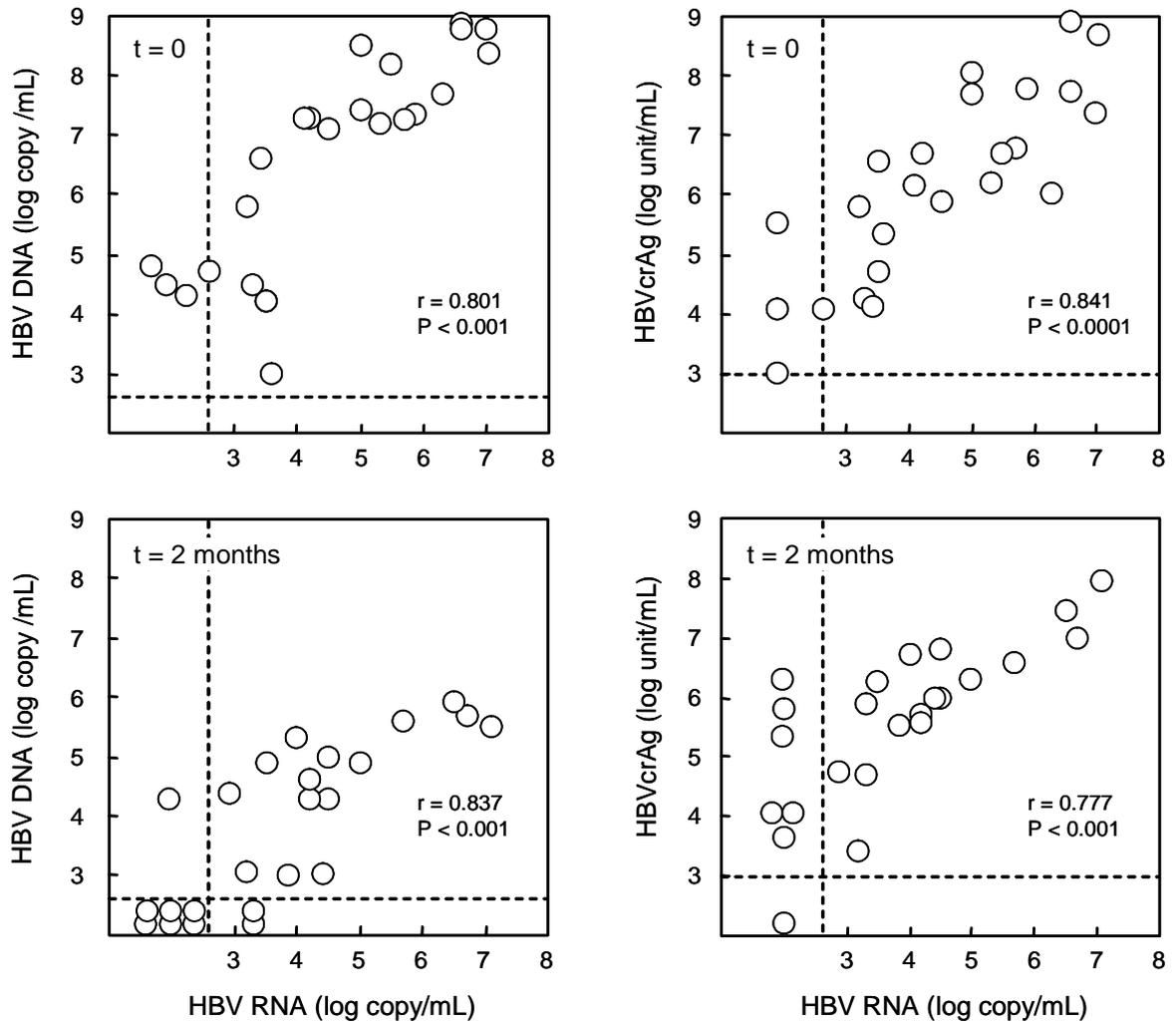


Fig. 3

