Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs $16.5\pm5.8\times10^4/\mu l$) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001; −3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

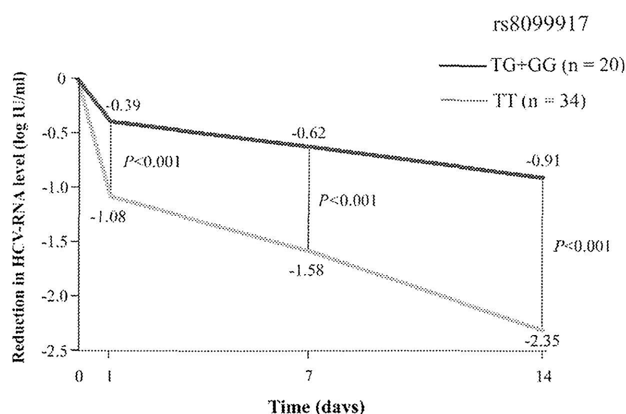

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels

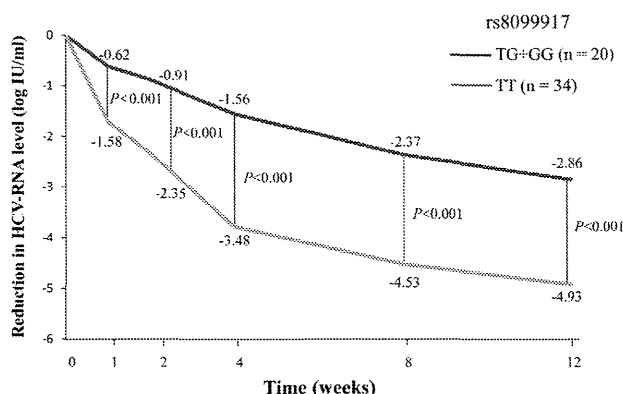

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable

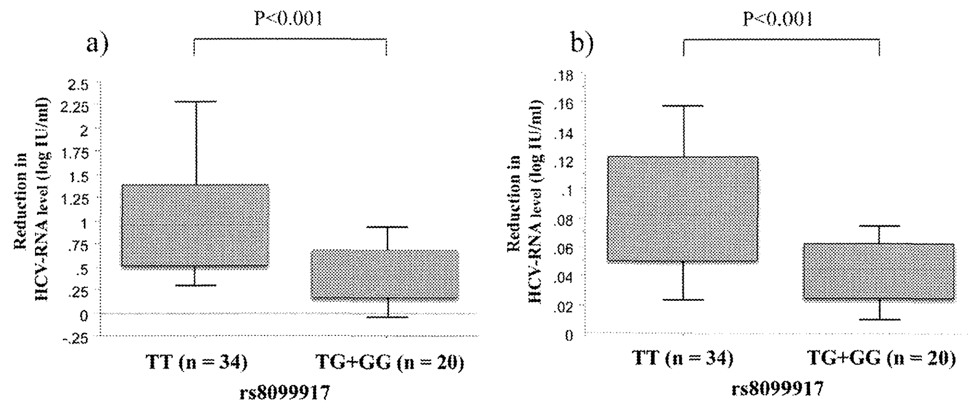

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

IL28B genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

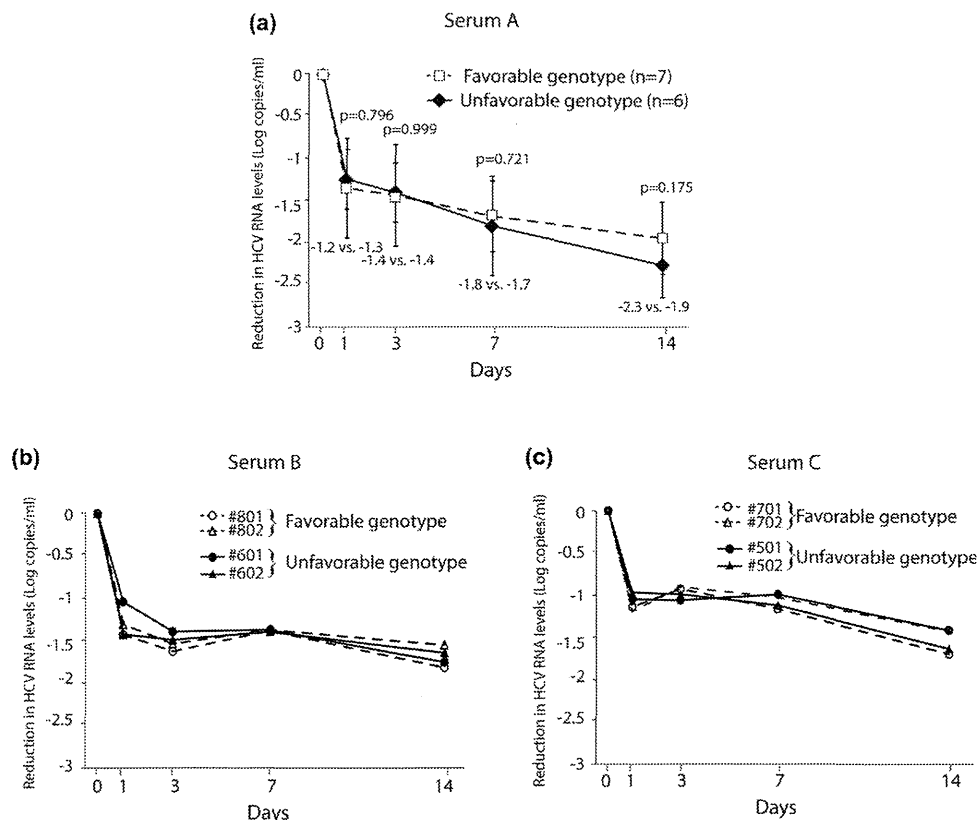

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

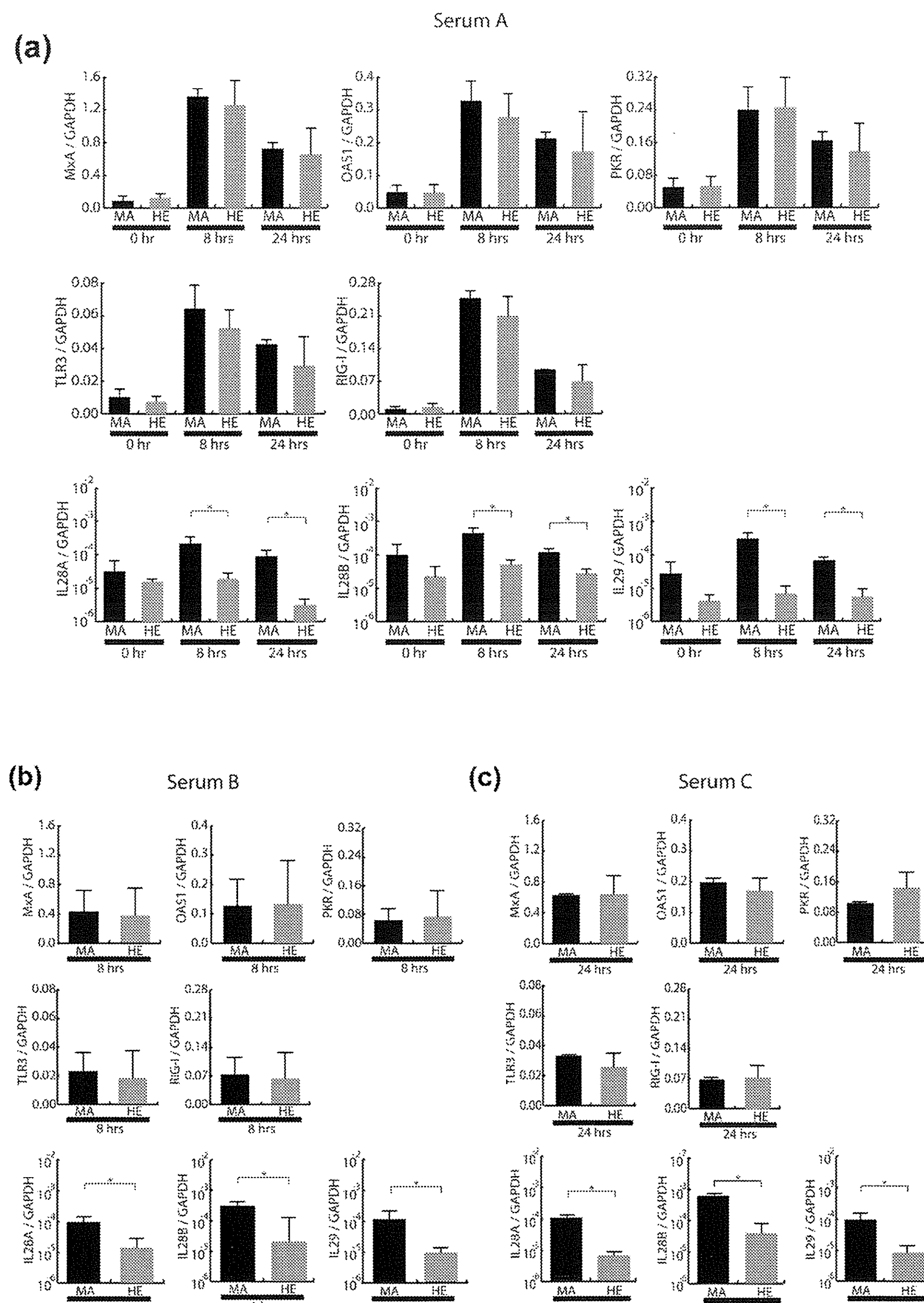

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean +SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *$p<0.05$. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

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Application of a Newly Developed High-Sensitivity HBsAg Chemiluminescent Enzyme Immunoassay for Hepatitis B Patients with HBsAg Seroclearance

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We modified and automated a highly sensitive chemiluminescent enzyme immunoassay (CLEIA) for surface antigen (HBsAg) detection using a combination of monoclonal antibodies, each for a specific epitope of HBsAg, and by improving an earlier conjugation technique. Of 471 hepatitis B virus (HBV) carriers seen in our hospital between 2009 and 2012, 26 were HBsAg seronegative as determined by the Abbott Architect assay. The Lumipulse HBsAg-HQ assay was used to recheck those 26 patients who demonstrated seroclearance by the Abbott Architect assay. The performance of the Lumipulse HBsAg-HQ assay was compared with that of a quantitative HBsAg detection system (Abbott Architect) and the Roche Cobas TaqMan HBV DNA assay (CTM) (lower limit of detection, 2.1 log copies/ml) using blood serum samples from patients who were determined to be HBsAg seronegative by the Abbott Architect assay. Ten patients had spontaneous HBsAg loss. Of 8 patients treated with nucleotide analogues (NAs), two were HBsAg seronegative after stopping lamivudine therapy and 6 were HBsAg seronegative during entecavir therapy. Eight acute hepatitis B (AH) patients became HBsAg seronegative. Of the 26 patients, 16 were HBsAg positive by the Lumipulse HBsAg-HQ assay but negative by the Abbott Architect assay. The differences between the two assays in terms of detectable HBsAg persisted over the long term in the spontaneous loss group (median, 10 months), the NA-treated group (2.5 months), and the AH group (0.5 months). In 9 patients, the Lumipulse HBsAg-HQ assay detected HBsAg when HBV DNA was negative by the CTM assay. HBsAg was also detected by the Lumipulse HBsAg-HQ assay in 4 patients with an anti-HBs concentration of >10 mIU/ml, 3 of whom had no HBsAg escape mutations. The automatic, highly sensitive HBsAg CLEIA Lumipulse HBsAg-HQ is a convenient and precise assay for HBV monitoring.

Today, >400 million people worldwide are hepatitis B virus (HBV) carriers (1). We have monitored HBV markers, such as HBV DNA, hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and HB core-related antigen (HBcrAg), in chronic hepatitis B patients. The measurement of HBV DNA levels by a PCR-based method is the state-of-the-art technique for monitoring HBV replication in clinical practice (2). However, it is suboptimal for chronic hepatitis B patients who are medicated with nucleotide analogues (NAs), as those, in many cases, can decrease HBV DNA to below the limit of detection.

HBsAg is a secreted envelope protein that is continuously shed into the blood as long as HBV infection persists, irrespective of viral replication. Recent advances in HBsAg quantification (qHBsAg) have opened up new perspectives in the study of HBV; qHBsAg levels are correlated with intrahepatic covalently closed circular (ccc) DNA, which is used as a template for viral transcription and maintains the chronic HBV infection state (3–5). Additionally, a correlation between qHBsAg and HBV DNA has been suggested, with the possibility of a role for qHBsAg as a surrogate marker for viral replication put forward, which might identify chronic hepatitis B patients who are likely to be cured with pegylated alpha interferon (6–9).

In Japan, two HBsAg quantification assays are available: the Architect HBsAg-QT (Abbott Japan) (detection range, 50 to 250,000 mIU/ml) and the HISCL HBsAg (Sysmex) (detection range, 30 to 2,500,000 mIU/ml). These two methods have a good correlation and are sensitive over a wide detection range. Recently, Matsubara et al. (10) reported a novel highly sensitive chemiluminescent enzyme immunoassay (CLEIA) that was developed for quantitative HBsAg detection by combining monoclonal antibodies, each specific for a different epitope of the antigen, and employing an improved conjugation technique. It is as sensitive as nucleic acid testing for detecting early HBV infection. We further modified and improved the high-sensitivity assay reagent described above for adaptation to both ferrite microparticles as the solid phase and the automated analyzer system by modification of the optimum combination of monoclonal antibodies. As was recently reported (11), this assay (Lumipulse HBsAg-HQ) had good accuracy, reproducibility, specificity, and sensitivity, and the results correlate well with those of the Abbott Architect. The coefficient of variation in the Lumipulse HBsAg-HQ is <5.9% for samples with a low concentration of HBsAg (11), and the assay was approved by the Japanese government in 2013.

The sensitivity of this assay (5 mIU/ml) was approximately 10-fold higher than that of the Abbott Architect assay (50 mIU/ml). Here, we adapted this assay to monitor chronic hepatitis B

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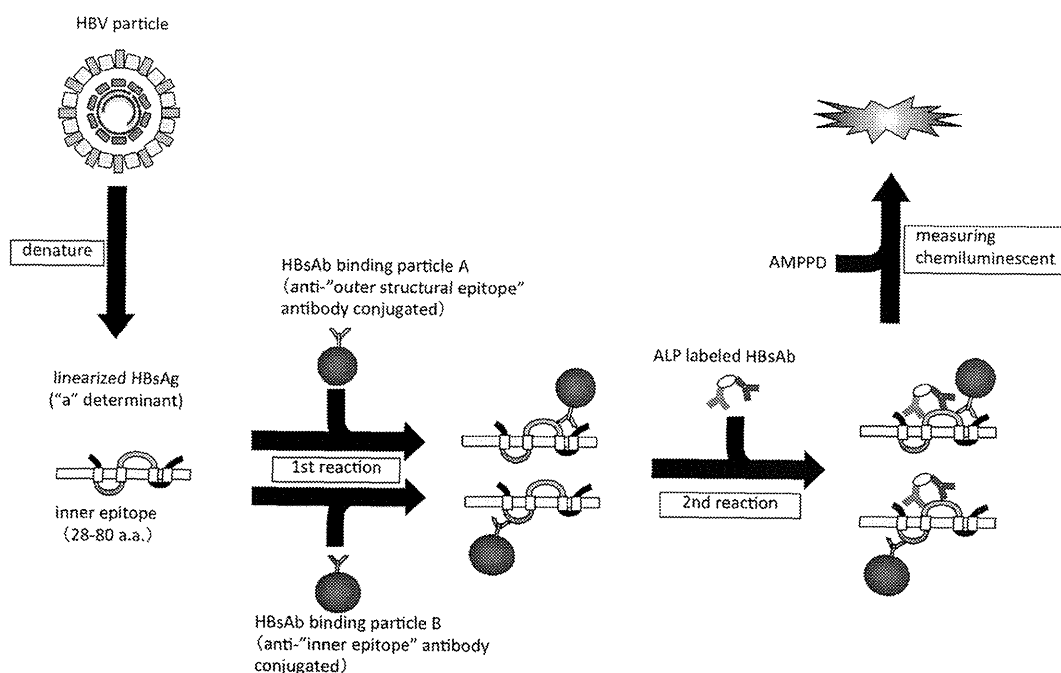

FIG 1 The principle of Lumipulse HBsAg-HQ.

patients with apparent HBsAg seroclearance as determined by the Abbott Architect assay.

MATERIALS AND METHODS

Samples. Four hundred seventy-one patients with chronic HBV infection visited our hospital from 2009 to 2012. One hundred eighty-one patients were asymptomatic carriers, 232 had chronic hepatitis B (CHB), and 58 had liver cirrhosis. Of these, 13 patients took lamivudine, one adefovir, 19 lamivudine plus adefovir, 140 entecavir, 8 entecavir plus adefovir, and 9 tenofovir. Thirty patients with acute HB (AH) infection (8 of whom developed chronic hepatitis) visited our hospital from January 2009 to 2012. We determined HBsAg seroclearance according to the Abbott Architect assay in 26 HBV-infected patients during the observation period. Of these, 10 were not treated with nucleotide analogues (spontaneous HBsAg loss group) and 8 were treated (NA-treated group). Of the 8 NA-treated patients, 2 on lamivudine therapy were HBsAg seronegative after stopping therapy, and the other 6 were HBsAg seronegative during entecavir therapy. Eight AH patients became HBsAg seronegative.

The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions, and informed consent was obtained from each carrier. We rechecked HBsAg status of the patients by the Lumipulse HBsAg-HQ assay in their serial blood serum samples and compared the results with those of the Architect HBsAg-QT assay.

Methods. (i) Measurement of HBsAg by Lumipulse HBsAg-HQ assay. HBsAg was measured on the two-step sandwich assay principle with a fully automated chemiluminescent enzyme immunoassay system (Lumipulse G1200; Fujirebio, Inc.). The assay principle for this new reagent was based on that previously reported by Matsubara et al. (10). Briefly, samples were pretreated with a solution, including surfactant to disrupt HBV particles, to dissociate HBsAg from HBsAg–anti-HBs complexes and to denature epitopes to a linear form. Linearized HBsAg were then detected using two monoclonal antibodies against external structural regions as determinant "a" and the internal epitope as a capture reagent, with two monoclonal antibodies coupled to alkaline phosphatase as the detector. For the assay procedures, 100 μl blood serum and/or plasma samples together with 20 μl pretreatment solution were incubated with the monoclonal antibodies binding ferrite microparticles at 37°C for 10 min. After automatic washing, 250 μl of the alkaline phosphatase-labeled antibodies were added and further incubated at 37°C for 10 min. After the washing step, 200 μl substrate solution (AMPPD [3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt]) (Applied Biosystems, Bedford, MA) was added and incubated at 37°C for 5 min. The relative intensity of chemiluminescence was measured and the HBsAg concentration was calculated by comparison with a standard curve. The range of HBsAg concentrations assayed was 5 to 150,000 mIU/ml, and retesting was accepted with a 200-fold dilution of samples that exceeded this range. In the present study, the cutoff value of HBsAg concentration was set at 5 mIU/ml. HBsAg in blood serum was also quantified at the same intervals using the Abbott Architect HBsAg-QT assay (cutoff value, 50 mIU/ml) (Fig. 1).

(ii) Quantification of HBV DNA. Serum HBV DNA was measured using the TaqMan PCR assay (Cobas TaqMan; Roche Molecular Systems [lower limit of detection, 2.1 log copies/ml]).

(iii) Quantification of HBcrAg. Serum HBcrAg was measured using CLEIA, as described previously (12, 13). Briefly, sodium dodecyl sulfate pretreated serum was incubated with monoclonal antibodies against denatured HBcAg and HBeAg. After washing and incubation with alkaline phosphatase-labeled secondary antibodies, the relative chemiluminescence intensity was measured, and the HBcAg concentration was calculated by comparison with a standard curve generated using a known concentration of recombinant HBeAg-containing peptide. The cutoff value of HBcrAg was 3 log U/ml.

(iv) Quantification of anti-HBs. Serum anti-HBs was measured using the Architect system's anti-HBs. A specimen was considered positive for anti-HBs when the concentration was ≥10.0 mIU/ml.

RESULTS

Table 1 shows clinical data at baseline for the three groups with HBsAg seroclearance according to data from the Abbott Architect assay. In four of 10 spontaneous HBsAg loss cases, HBsAg had already been <50 mIU/ml as measured by the Abbott Architect assay at the first visit. Table 1 shows the characteristics of all 26 patients in these 3 groups. The HBV DNA and HBcrAg levels at

TABLE 1 Clinical data at baseline of 3 groups with HBsAg seroclearance as determined by the Abbott Architect assay

Patient characteristic	Data for group (*n*):		
	Spontaneous HBsAg loss (10)	NA treated (8)[a]	Acute hepatitis (8)
Age at first visit or medication (yr)	60.6 ± 12.6	46.8 ± 12.2	50.5 ± 10.8
Sex (no. of males/no. of females)	10/0	7/1	8/0
Route of infection (no. of vertical/no. of horizontal)	10/0	4/4	0/8
No. with genotype Aa/Ae/Ba/Bj/C	0/0/0/2/8	1/1/1/1/4	1/4/1/0/2
Clinical data			
ALT (median [range]) (IU/liter)	23.5 (8–51)	76 (11–220)	1,682 (455–3,622)
HBeAg (no. positive/no. negative)	0/10	5/3	8/0
HBV DNA (median [range]) (log copies/ml)	2.3 (<2.1 to 3.4)	7.4 (4.1 to >9.1)	6.5 (3.8–8.5)
HBcrAg (median [range]) (log IU/ml)	<3 (<3 to 3.3)	6.8 (4.2–8.6)	7.1 (6.6–8)
Abbott Architect HBsAg-QT detection (median [range]) (mIU/ml)	1,300 (<50 to 10,880)	2,676,800 (9,680–89,679,600)	362,500 (91,200–40,000,000)
NA therapy (no. with none/no. with LVD/no. with ETV)[b]	10/0/0	0/2/6	5/0/3

[a] NA, nucleotide analogue.
[b] LVD, lamivudine; ETV, entecavir.

baseline were significantly higher in the NA-treated and AH groups than in the spontaneous HBsAg loss group. The HBsAg levels at baseline were also significantly higher in the AH group and the NA-treated group than in the spontaneous HBsAg loss group. However, HBsAg became undetectable by the Abbott Architect assay immediately in the AH group (median, 1 month), compared with the NA-treated group (32 months) and the spontaneous HBsAg loss group (78.5 months [excluding 4 patients with HBsAg of ≤50 mIU/ml by the Abbott Architect assay at the first visit]). In 19 of the 26 cases, the HBsAg levels were still detectable by the Lumipulse HBsAg-HQ assay at the time point when they were undetectable by the Abbott Architect assay. At the last time point with detectable HBsAg by Lumipulse HBsAg-HQ assay, the Abbott Architect assay could not detect HBsAg in all 10 spontaneous HBsAg loss patients, but the Abbott Architect assay was also able to detect at the last time point in three (case no. L1, E3, and E5) of eight NA-treated group patients and four (case no. A1, A4, A5, and A7) of eight AH patients. In the spontaneous HBsAg loss group, the decline in HBsAg was slower than in the NA-treated and AH groups (Fig. 2a to 2c). Differences in the median duration between the Abbott Architect and Lumipulse HBsAg-HQ assays were seen at 10 months (excluding 4 patients with HBsAg of <50 mIU/ml by the Abbott Architect assay at the first visit), 2.5 months, and 0.5 months in the spontaneous HBsAg loss group, NA-treated group, and AH group, respectively. We observed the reappearance of HBsAg measured by Lumipulse HBsAg-HQ assay in 2 patients (case no. N4 and N6) in the spontaneous HBsAg loss group, 3 (case no. E1, E3, and E5) in the NA-treated group, and one (case no. A6) in the AH group (Fig. 2a to 2c). At the last time point with detectable HBsAg by the Lumipulse HBsAg-HQ assay, HBV DNA was undetectable by the Cobas TaqMan assay in 4 of 10 spontaneous HBsAg loss patients (40%), 4 of 8 NA-treated patients (50%), and one of 8 AH patients (12.5%). At the last time of detection by the Lumipulse HBsAg-HQ assay, HBcrAg was <3 log U/ml in 8 of 10 spontaneous HBsAg loss patients (80%), 2 of 8 NA-treated patients (25%), and none of the 10 AH patients (0%). At the last time point of detection by the Lumipulse HBsAg-HQ assay, anti-HBs was positive in one of 10 spontaneous HBsAg loss patients (10%), none of the 8 NA-treated patients (0%), and 2 of 10 AH patients (20%) (Tables 2 to 4). In case no. A1 and A7, HBsAg was relatively high at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay (Table 4). In case no. A1, however, HBsAg was undetectable by the Abbott Architect and Lumipulse HBsAg-HQ assays after 1 month. In case no. A7, HBsAg was undetectable by the Abbott Architect and Lumipulse HBsAg-HQ assays after 3 months.

To elucidate possible HBs escape mutants, we examined the S gene sequences of all 26 patients at the first visit. Patient N2 had an amino acid G145S mutation, L1 had an amino acid S143T mutation, and L2 had amino acid I126N and F134Y mutations. None had an amino acid G145R mutation. At the last time point that HBsAg was detected by the Abbott Architect assay, anti-HBs was positive in patient N2 (from the spontaneous HBsAg loss group) with an amino acid G145S mutation. We performed an inhibition assay for samples N1 and N2 at the time of Abbott Architect undetectability but Lumipulse HBsAg-HQ detectability to confirm whether the identification of HBsAg by the Lumipulse HBsAg-HQ assay was specific. HBsAg detection of these samples was inhibited, indicating that the Lumipulse HBsAg-HQ assay was indeed specific. The following are three representative cases.

(i) Case no. N7 was a 71-year-old male. His alanine transaminase (ALT) was 19 IU/liter, HBV DNA was 3.7 log copies/ml at his first visit, the HBV genotype was C, HBeAg was negative, and anti-HBe was positive. The HBsAg level as measured by the Abbott Architect assay was 162,000 mIU/ml. The patient was followed as an inactive HB carrier. The last time at which HBsAg was detectable by the Abbott Architect assay was 87 months after the first visit, and it became undetectable in 3 months. However, it was still detectable by the Lumipulse HBsAg-HQ assay (78 mIU/ml). HBV DNA by Cobas TaqMan assay decreased to <2.1 log copies/ml. The Lumipulse HBsAg-HQ assay was still positive even 10 months after the Abbott Architect assay became negative. The HBsAg level measured by the Lumipulse HBsAg-HQ assay was 5.8 mIU/ml at this time (Fig. 3a).

(ii) Case no. E1 was a 51-year-old male who had been infected

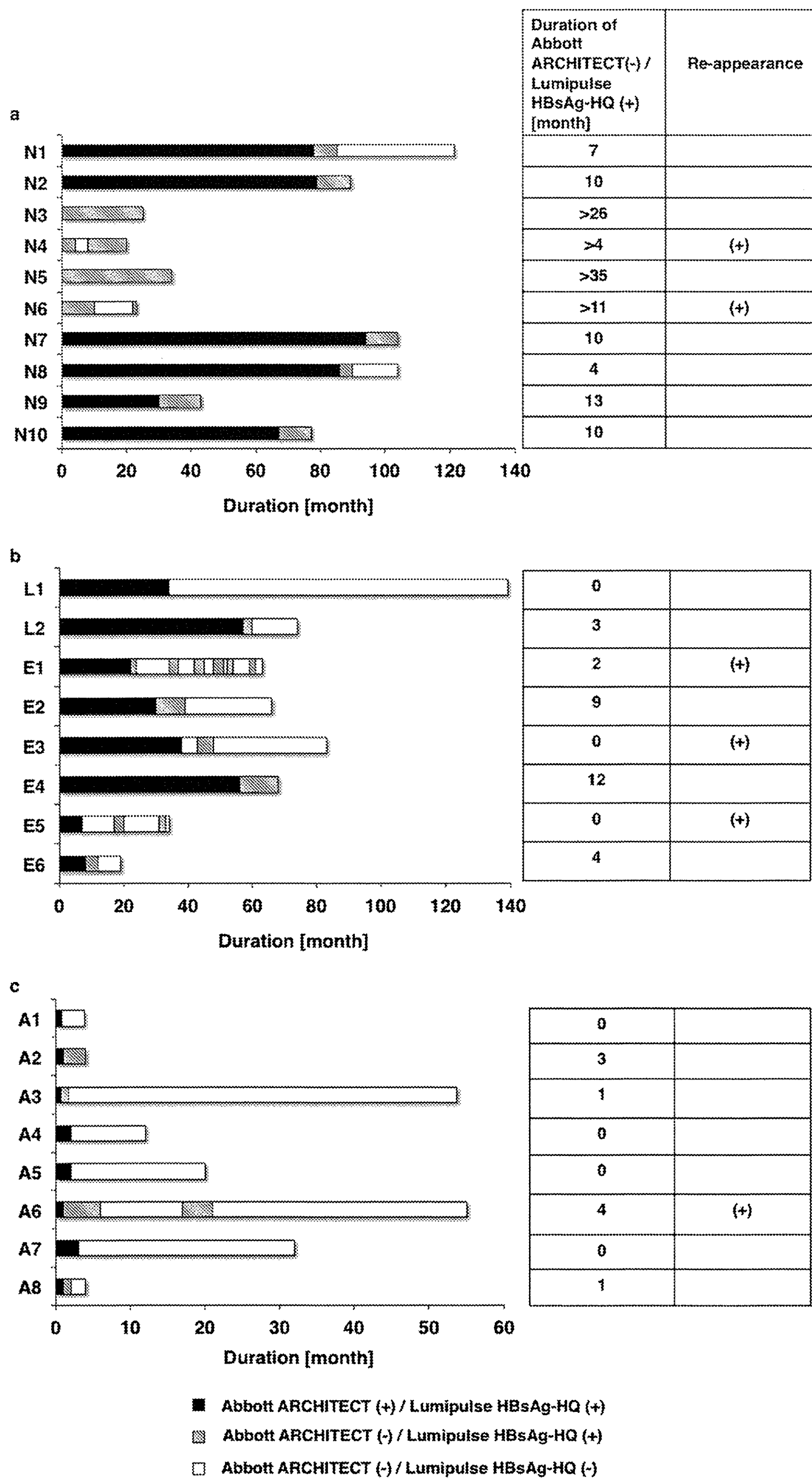

FIG 2 HBsAg dynamics by the Abbott Architect and Lumipulse HBsAg-HQ assays in the spontaneous HBsAg loss group (a), the NA-treated group (b), and the AH group (c).

with HBV by transfusion in adulthood and had developed chronic hepatitis B. His ALT was 57 IU/liter, HBV DNA was 8.6 copies/ml by the Cobas TaqMan assay, the HBV DNA genotype was Ba, HBeAg was positive, and anti-HBe was negative. The HBsAg level as measured by the Abbott Architect assay was 4,983,730 mIU/ml. The patient was treated with entecavir. After 24 months, HBsAg became undetectable by the Abbott Architect assay, and from this point to the last observation point, the Abbott Architect assay was

TABLE 2 Clinical data of spontaneous HBsAg loss patients at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay

Clinical data	Values for patient no.: N1	N2[b]	N3[a,b]	N4[a,b]	N5[a,b]	N6[a,b]	N7[b]	N8	N9[b]	N10[b]
Nucleotide analogue therapy	None	None	None	None	None	None	None	None	None	None
Age (yr)	61	54	91	50	76	63	71	62	62	65
HBeAg (+/−)	−	−	−	−	−	−	−	−	−	−
Abbott Architect HBsAg-QT detection (mIU/ml)	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	8.0	51.0	12.0	8.9	10.4	5	5.8	20.4	11.7	30.3
HBV DNA (log copies/ml)	Not detected	Not detected	<2.1	<2.1	2.9	2.6	<2.1	Not detected	2.7	Not detected
HBcrAg (log IU/ml)	<3	3	<3	<3	3.2	<3	<3	<3	<3	<3
Anti-HBs (mIU/ml)	<10	973.8	<10	<10	<10	<10	<10	<10	<10	<10

[a] Abbott Architect HBsAg-QT assay (IU/ml) was already negative at first visit.
[b] Lumipulse HBsAg-HQ assay was still able to detect HBsAg at the last observation time.

continuously unable to detect HBsAg. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was 14.7 mIU/ml at the first point that was undetectable by the Abbott Architect assay, and it had been detectable for 3 months. After 3 months, HBsAg became undetectable by the Lumipulse HBsAg-HQ assay and anti-HBs reached >10 mIU/ml. From this point, anti-HBs was continually >10 mIU/ml. Interestingly, after 1 year, HBsAg measured by Lumipulse HBsAg-HQ assay became detectable again (25.2 mIU/ml), although HBV DNA by the Cobas TaqMan and HBsAg by the Abbott Architect assays remained undetectable. At some time points, HBsAg as determined by the Lumipulse HBsAg-HQ assay was detectable, and at the same time, anti-HBs was >10 mIU/ml (Fig. 3b).

(iii) Case no. A6 was a 38-year-old male diagnosed as having acute hepatitis B. After 1 month, HBeAg became seronegative and anti-HBe became seropositive. Three months after the first visit, HBV DNA was <2.1 log copies/ml, HBsAg became undetectable by the Abbott Architect assay, anti-HBs was 22.75 IU/ml, and the Lumipulse HBsAg-HQ assay detected HBsAg. After this time, anti-HBs was continually >10 mIU/ml. Thirteen months after the first visit, the Lumipulse HBsAg-HQ assay detected the reappearance of HBsAg (7.6 mIU/ml), although anti-HBs was still positive at 23.18 IU/ml (Fig. 3c).

DISCUSSION

The Lumipulse HBsAg-HQ assay showed improved sensitivity after disrupting HBV particles, dissociating HBsAg from HBsAg/anti-HBs complexes, and denaturing epitopes into linear forms. A major difference between the Abbott Architect and the Lumipulse HBsAg-HQ assays is that the latter detects HBsAg-anti-HBs complexes as well as small S proteins, which are present 10,000 to 1,000,000 times more frequently than Dane particles. The detection limit of the Lumipulse HBsAg-HQ assay (5 mIU/ml) was 10 times lower than that of the Abbott Architect assay, but there was otherwise a good correlation between the two. In clinical practice, more precise and broader HBsAg dynamics might therefore be followed by using the Lumipulse HBsAg-HQ assay. Differences between the two assays in detectable HBsAg persisted for a long time in the spontaneous HBsAg loss group (median, 10 months), followed by the NA-treated group (2.5 months) and the AH group (0.5 months).

In addition to the significant decrease or loss of all HBV replication in the blood serum, the long-term outcome after HBsAg seroclearance is good if there is no preexisting cirrhosis or viral superinfection. This view is supported by studies showing increased survival, a lower rate of hepatic decompensation, and a reduced frequency of hepatocellular carcinoma (HCC) in patients who have cleared HBsAg (14, 15). In carriers without cirrhosis and with no evidence of viral superinfection (hepatitis C virus [HCV] and/or hepatitis D virus [HDV]) at HBsAg seroclearance, liver function can improve or remain stable and hepatic decompensation rarely occurs; however, the incidence of HCC varies significantly, as was previously reported (16, 17). These discrepancies might depend on concurrent hepatitis, the severity of liver disease, age, and other factors. Yuen et al. (17) reported that HBsAg seroclearance of patients aged ≥50 years was associated with a higher risk of developing HCC than in patients of age <50 years, suggest-

TABLE 3 Clinical data of NA-treated patients at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay

Clinical data	Values for patient no.: L1	L2	E1	E2	E3	E4[a]	E5	E6
Nucleotide analogue therapy	LVD	LVD	ETV	ETV	ETV	ETV	ETV	ETV
Age (yr)	62	49	53	40	44	44	67	39
HBeAg (+/−)	−	−	−	−	−	−	−	−
Abbott Architect HBsAg-QT detection (mIU/ml)	80[b]	<50	<50	<50	90[b]	<50	90[b]	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	77.3	5	14.7	8	44.6	6.5	42.5	89
HBV DNA (log copies/ml)	<2.1	Not detected	Not detected	Not detected	3.3	2.2	<2.1	Not detected
HBcrAg (log IU/ml)	<3	3.3	4.3	4.1	3.2	<3	3.8	4.3
Anti-HBs (mIU/ml)	<10	<10	<10	<10	<10	<10	<10	<10

[a] The Lumipulse HBsAg-HQ assay was still able to detect HBsAg at the last observation time.
[b] HBsAg was detectable by both assays at this point, but HBsAg became undetectable at the next point.

TABLE 4 Clinical data of AH patients at the last time point at which HBsAg was detectable by Lumipulse HBsAg-HQassay

Clinical data	Values for patient no.:							
	A1	A2	A3	A4	A5	A6	A7	A8
Nucleotide analogue therapy	None	None	None	None	None	ETV	ETV	ETV
Age (yr)	62	34	53	50	39	39	53	54
HBeAg (+/−)	−	−	−	−	−	−	+	+
Abbott Architect HBsAg-QT detection (mIU/ml)	91,200[a]	<50	<50	240[a]	680[a]	<50	11,500[a]	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	112,289.3	5.6	13.6	180.4	771.9	7.6	12,358.4	34.3
HBV DNA (copies/ml)	3.8	Not detected	2.3	2.2	3	<2.1	<2.1	<2.1
HBcrAg (log IU/ml)	6.8	4.0	5.4	4.9	3.2	3.1	3.7	4.3
Anti-HBs (mIU/ml)	<10	24.41	<10	<10	<10	23.18	<10	<10

[a] HBsAg was detectable by both assays at this point, but HBsAg became undetectable at the next point.

ing that we have to consider the age at which HBsAg becomes undetectable.

In most patients in our study (9 of 10 in the spontaneous HBsAg loss group and 7 of 8 in each of the NA-treated and AH groups), HBV DNA or HBcrAg was still detectable by the Abbott Architect assay at the time of HBsAg seroclearance (data not shown). Suzuki et al. (18) reported that HBcrAg correlates with intrahepatic covalently closed circular DNA in chronic hepatitis B patients. Hence, as the current CLEIA HBsAg quantification methods are inadequate for following some cases of HBV infection, the use of the Lumipulse HBsAg-HQ assay together with HBcrAg and HBV DNA testing might be valuable for evaluating patient response to treatment with interferon and NAs. Additionally, we reported that the measurement of HBcrAg is useful for predicting relapse after the cessation of lamivudine therapy for chronic hepatitis B; an HBcrAg level of <3.4 log U/ml at this time was the only independent predictive factor for the absence of post-treatment relapse (19). Thus, the combination of highly sensitive HBsAg detection by the Lumipulse HBsAg-HQ assay and HBcrAg might improve the accuracy of predicting response to treatment and relapse. Highly sensitive HBsAg detection by the Lumipulse HBsAg-HQ assay might be useful for several clinical applications. First, the Lumipulse HBsAg-HQ assay might replace HBV DNA monitoring by a PCR-based method for blood screening. As shown in Tables 2 to 4, at the last time point that HBsAg was detectable by the Lumipulse HBsAg-HQ assay, HBV DNA was undetectable in 9 of 26 patients (34%) by the Cobas TaqMan assay. This suggests that the sensitivity of the Lumipulse HBsAg-HQ assay for HBV detection was at least as high as that for the Cobas TaqMan assay at some time points. The Lumipulse HBsAg-HQ assay is simpler, more convenient, and less expensive than HBV DNA quantification by real-time PCR. At present in Japan, nucleic acid testing is used for detecting HBV in blood donors, but the Lumipulse HBsAg-HQ assay might substitute for nucleic acid testing for screening HBV if the sensitivity could be improved.

Second, the Lumipulse HBsAg-HQ assay may be useful for detecting occult HBV infection as well as HBV reactivation. Occult HBV infection is defined as infection with detectable HBV DNA but undetectable HBsAg with or without antibodies to HBV core antigen (anti-HBc) and/or anti-HBs (20–22). Recent interest in occult HBV infection has focused on the potential of donors with such infections to transmit the virus to susceptible recipients (23, 24). In this study, we detected HBsAg by the Lumipulse HBsAg-HQ assay in occult hepatitis B virus infection (OBI) patients, including those with HBsAg clearance as determined by the Architect assay (case no. N1, N3, N4, N5, N6, N7, N10, E3, E4, E5, E6, A3, A6, A8, and A9). In case no. N5, even >35 months after HBsAg became undetectable by the Abbott Architect assay, HBsAg was still detectable by the Lumipulse HBsAg-HQ assay. The Lumipulse HBsAg-HQ assay may change the diagnosis of patients defined as having current occult HBV infection. In case no. E1, HBsAg was detectable by the Lumipulse HBsAg-HQ assay at some time points, although HBV DNA by the Cobas TaqMan assay and HBsAg by Abbott Architect assay remained undetectable. In many cases (cases N1, N2, N4, N6, N8, N10, L2, E1, E2, E3, E5, E6, A2, A4, and A6), the HBV DNA and Lumipulse HBsAg-HQ results did not correlate. Interestingly, the original highly sensitive HBsAg assay reported by Matsubara et al. (10) had a similar sensitivity with HBV DNA detection during the acute phase of HBV infection. If the sensitivity of the Lumipulse HBsAg-HQ assay is improved, it would be sensitive enough to monitor HBV reactivation instead of needing to rely on HBV DNA monitoring. More importantly, there have been cases of HBV reactivation in patients with resolved infection (HBsAg-negative, anti-HBc, and/or anti-HBs positive) during the course of chemotherapy and/or immunotherapy (especially therapy with rituximab plus steroids), sometimes proving fatal (25–29). The Lumipulse HBsAg-HQ assay might be more convenient for such screening than TaqMan PCR.

Third, previous CLEIA HBsAg quantification methods, including the Abbott Architect assay, apply monoclonal/polyclonal antibodies against external structural regions within the determinant "a" loop. HBsAg escape mutations, such as G130D, T131N, M133T, and G145R, were found in patients who were positive for anti-HBs but negative for HBsAg (9, 30). Oon et al. (32) reported that HBV carriers, including HCC patients who were negative for HBsAg but positive for anti-HBc and anti-HBs, had the T126S, Q129D, M133L, T140I, and G145R mutations within the S region. Wu et al. (31) reported that amino acid residues at positions 122 and 145 of HBsAg had a major effect on antigenicity and immunogenicity. HBsAg mutants can escape current detection and persist in HBV-infected individuals after the loss of HBsAg (32). In the present study, we therefore determined the HBs amino acid sequences of all cases (with detectable HBV DNA), some of which had amino acid I126N, F134Y, S143T, and G145S (not G145R) mutations. It is possible that these HBsAg mutants escape detection by current HBsAg assays and the sensitivity becomes low (33). Based on the pretreatment, however, the Lumipulse HBsAg-HQ assay was able to detect HBsAg mutants because it uses two monoclonal antibodies against the external structural region as determinant "a" and the internal epitope as the capture target. Additionally, the Lumipulse HBsAg-HQ assay can detect HBsAg from samples with anti-HBs.

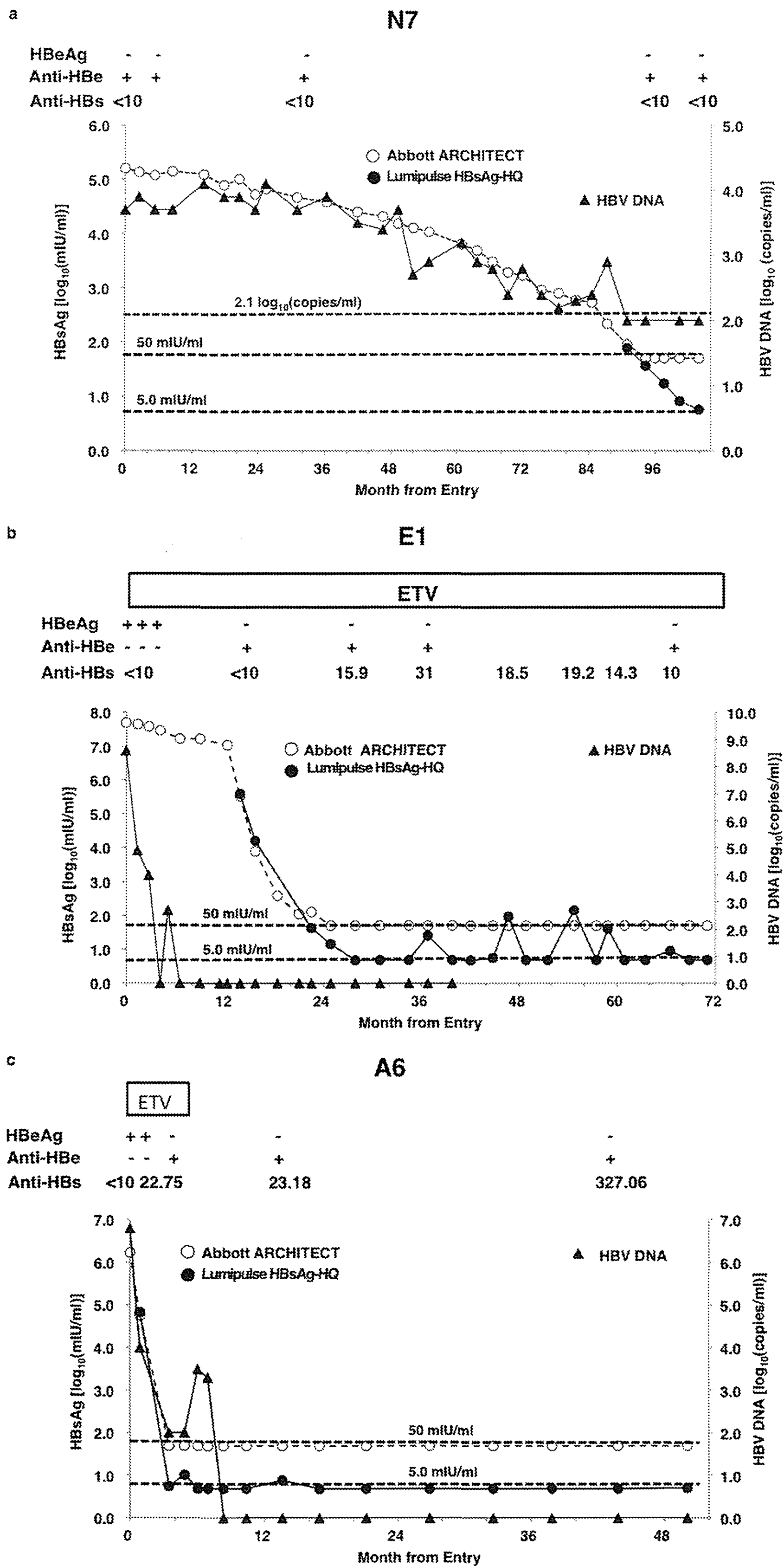

FIG 3 (a) HBsAg and HBV DNA dynamics of case no. N7. The Lumipulse HBsAg-HQ was still positive even 10 months after Abbott Architect results became negative. (b) HBsAg and HBV DNA dynamics of case no. E1. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was detectable for 3 months after HBsAg became negative by the Abbott Architect assay. After 1 year, HBsAg became detectable by the Lumipulse HBsAg-HQ assay, although HBV DNA was undetectable by the Cobas TaqMan and HBsAg was undetectable by the Abbott Architect assay. At 5 points, HBsAg was detectable by the Lumipulse HBsAg-HQ assay, and the anti-HBs concentration was >10 mIU/ml. (c) HBsAg and HBV DNA dynamics of case no. A6. HBsAg was detectable by the Lumipulse HBsAg-HQ assay for 3 months after HBsAg became negative by the Abbott Architect assay.

In conclusion, the automatic, highly sensitive HBsAg CLEIA Lumipulse HBsAg-HQ assay is a very convenient and precise assay for HBV monitoring in clinical practice.

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The authors declare no conflicts of interest.

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Novel point mutations and mutational complexes in the enhancer II, core promoter and precore regions of hepatitis B virus genotype D1 associated with hepatocellular carcinoma in Saudi Arabia

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In this study, a cohort of 182 patients [55 hepatocellular carcinoma (HCC) and 127 non-HCC] infected with hepatitis B virus (HBV) in Saudi Arabia was investigated to study the relationship between sequence variation in the enhancer II (EnhII), basal core promoter (BCP) and precore regions of HBV genotype D (HBV/D) and the risk of HCC. HBV genotypes were determined by sequencing analysis and/or enzyme-linked immunosorbent assay. Variations in the EnhII, BCP and precore regions were compared between 107 non-HCC and 45 HCC patients infected with HBV/D, followed by age-matched analysis of 40 cases *versus* equal number of controls. Age and male gender were significantly associated with HCC ($p = 0.0001$ and $p = 0.03$, respectively). Serological markers such as aspartate aminotransferase, albumin and anti-HBe were significantly associated with HCC ($p = 0.0001$ for all), whereas HBeAg positivity was associated with non-HCC ($p = 0.0001$). The most prevalent HBV genotype was HBV/D (94%), followed by HBV/E (4%), HBV/A (1.6%) and HBV/C (0.5%). For HBV/D1, genomic mutations associated with HCC were T1673/G1679, G1727, C1741, C1761, A1757/T1764/G1766, T1773, T1773/G1775 and C1909. Age- and gender-adjusted stepwise logistic regression analysis indicated that mutations G1727 [odds ratio (OR) = 18.3; 95% confidence interval (CI) = 2.8–118.4; $p = 0.002$], A1757/T1764/G1766 (OR = 4.7; 95% CI = 1.3–17.2; $p = 0.01$) and T1773 (OR = 14.06; 95% CI = 2.3–84.8; $p = 0.004$) are independent predictors of HCC development. These results implicate novel individual and combination patterns of mutations in the *X*/precore region of HBV/D1 as predictors of HCC. Risk stratification based on these mutation complexes would be useful in determining high-risk patients and improving diagnostic and treatment strategies for HBV/D1.

Key words: hepatitis B virus, sequence analysis, genotype D, *X*-gene, point mutations, mutational complexes, hepatocellular carcinoma
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Approximately three billion people in the world are exposed to hepatitis B virus (HBV), of whom 350–400 million are persistently infected with it.[1] The two primary clinical manifestations associated with chronicity of the disease are cirrhosis and hepatocellular carcinoma (HCC), either of which may lead to liver-related death. It is estimated that chronic HBV carriers have a 100-fold increased risk for developing HCC compared to noncarriers[2]; however, the incidence rates of developing HCC vary widely around the world.

HBV has been characterized into eight genotypes (A–H) based on a divergence over the entire genome of greater than 8%.[3] Two new genotypes, I and J, have recently been reported and await international recognition.[4,5] These HBV genotypes are known to have a distinct geographical distribution. Most studies on clinical outcome in relation to HBV

What's new?

The accumulation of mutations in hepatitis B virus (HBV) over the course of long-term infection may increase its carcinogenicity, leading to the development of hepatocellular carcinoma (HCC). The combined effect of multiple mutations, however, has not been explored in detail. Here, novel mutations in the BCP and precore regions of HBV subgenotype D1 were associated with HCC in a study population in Saudi Arabia. The associations held for individual mutations and for combination patterns involving multiple mutations. The mutation complexes may help identify patients at high risk for HCC and could influence treatment strategies for HBV/D1.

genotypes have their origin in East Asia, having been conducted on patients infected with genotypes B and C. Genotype C is generally considered to be more virulent than genotype B.[6,7] Reports are emerging that subgenotypes within a genotype may also differ in the capacity to induce HCC based on viral sequence variations and recombination.[8]

In addition to the HBV genotype, variations in the *X*/basal core promoter (BCP)/precore regions have been shown to affect viral functions *in vitro*. Many functional sequences of HBV genome, such as enhancer II (EnhII), BCP, X-terminal signal, start points of two pregenomic messenger RNA, poly A signal and Epsilon, lie within this region.[9] Nucleotide changes in this region are therefore presumed to have a high carcinogenic capability.[10–12] Most of the previous studies have focused on the evaluation of individual mutations or only the combined effect of BCP double mutation T1762/1764 with respect to the development of HCC rather than evaluating the combined effect of multiple mutations or pattern of combinations.[13,14] As mutations accumulate gradually during long-term HBV infection, it is better to evaluate the combined effect of multiple mutations, which is expected to impart greater stress on the liver leading to HCC.

HBV genotype D (HBV/D) is the most prevalent genotype in South and Central Asia and the Middle East. To date, Six subgenotypes of HBV/D (D1–D6) have been identified and are distributed throughout the world.[15,16] The predominance of each subgenotype differs geographically and as such its role in the natural history of HBV infection may differ. It has been reported that genotype D is the most prevalent genotype in Saudi Arabia; however, there is no information about the prevalence of subgenotypes of HBV/D and their relation to advanced liver disease.[17,18] Very few studies related HBV/D1 with severity of disease; however, these studies were hampered by a small sample size and unavailability of balanced clinical groups in comparison.[17,19,20]

Our study was conducted on a cohort of HBV-infected patients in Saudi Arabia recruited from different hospitals in the Kingdom of Saudi Arabia (KSA). We examined HBV genotypes in these patients and analyzed the sequence variations in the EnhII/BCP/precore regions of HBV/D1 associated with the clinical course of the disease. Data on other viral factors, including viral load, HBeAg and antibodies against HBeAg (anti-HBe), were also included to analyze their associations with sequence variations in HBV disease sequelae.

Material and Methods

Patients

A total of 182 serum/plasma samples were obtained from chronic carriers of HBV [presence of HBsAg for >6 months and detection of antibody to hepatitis B core antigen (anti-HBc)] enrolled in different hospitals of KSA. The diagnosis of HCC was based on published guidelines for the diagnosis and management of HCC.[21,22] In brief, enhancement of a liver lesion during the arterial phase and contrast washout during the portal phase in patients with background cirrhosis was considered diagnostic of HCC. Computed tomography and magnetic resonance imaging were the imaging modalities used for diagnosis. Trucut biopsy or fine-needle aspiration was obtained only where considerable doubt existed after imaging studies. Chronic carriers of HBV infection, who had been regularly screened with imaging studies (showing no concerning lesion), performed 6 months apart for 12 months, along with normal α-fetoprotein (AFP) levels were selected as controls. The exclusion criteria for all patients were as follows: (*i*) coinfection with hepatitis C, human immunodeficiency virus or delta virus; (*ii*) coexistent autoimmune or metabolic liver disease; (*iii*) hepatotoxic medications in the preceding 3 months; (*iv*) another hepatobiliary malignancy; (*v*) alcohol consumption >20 g/day and (*vi*) organ transplantation.

Serological markers of infection

Serum samples collected at each hospital were tested for alanine aminotransferase and aspartate aminotransferase (AST), albumin levels and serology for HBeAg and anti-HBe using commercial kits (Abbott laboratories, Diagnostics Division, Abbott Park, IL 60064, USA). HBV genotypes were determined by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies directed to distinct epitopes on the preS2-region (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan).

Extraction and quantification of HBV DNA

Total DNA was extracted from 200 µl of serum using a QIAmpDNA mini kit (Qiagen). Quantitative HBV DNA levels were measured by Abbott Real-Time HBV assay (Abbott

Molecular, Des Plaines, IL), allowing detection up to 100 viral DNA copies per milliliter used for DNA quantification.[23]

Amplification of HBV DNA, sequencing and molecular evolutionary analysis

HBV DNA sequences bearing the partial S- and *X*-gene were obtained according to the method proposed by Sugauchi *et al.*[24] with slight modifications. The amplification in the BCP and precore region was carried out with a forward primer HB7F: 5′-GAGACCACCGTGAACGCCCA-3′ (nt. 1611–1630) and an antisense primer HB7R: 5′-CCTGAGTG CAGTATGGTGAGG-3′ (nt. 2072–2052). HBV DNA sequences spanning the S-gene were amplified by two PCR reactions with heminested primers. The first round of PCR was performed with a sense primer HB1F: 5′-AAACTCTGCAAGAT CCCAGAGT-3′ (nt. 18–39) and an antisense primer HB2R: 5′-CAGACTTTCCAATCAATAGG-3′ (nt. 989–970). In the second round, PCR products were obtained in two overlapping fragments. For fragment 1, PCR was performed with the sense primer HB1F and an antisense primer HB1R: 5′-GATACATAGAGGTTCCTTGAGCAG-3′ (nt. 557–534), and for fragment 2, PCR was performed with the sense primer HB2F: 5′-TGCTGCTATGCCTCATCTTC-3′ (nt. 414–433) and the antisense primer HB2R. The amplicons obtained were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. HBV genotypes were determined by phylogenetic analysis in the partial S and core regions of HBV genome. Reference sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GenBank. Alignments were performed using CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-e.html), and neighbor-joining trees were constructed with six-parametric method and bootstrapped 1,000 times to confirm the reliability of the phylogenetic tree.[25]

Analysis in the EnhII/BCP and precore regions

Analysis of the whole set of HBV/D1-infected cohort was performed in the partial BCP, precore and core regions using BioEdit program version 7.0. The nucleotide mutation was defined by comparing the sequences with differences in the reference HBV/D1 sequence (GenBank accession number AY721612), whereas dual type or deletion was considered as a mutant type. This reference sequence was chosen to be very close to represent the consensus sequence for HBV/D1 based on previously published sequences.

A case–control analysis

Age-matched control patients ($n = 40$) were selected from within the non-HCC cohort with a cutoff age of 60 years. They were matched to within 5 years of the age of case HCC patient. HBeAg was a nonsignificant factor between both groups (p = NS; Table 4). Absence of HCC in the control patient was ascertained by a lack of any definite markers of HCC (normal AFP levels and imaging results showing absence of any concerning lesion) at the point of diagnosis of HCC in the case patient.

Statistical analysis

The nonparametric Mann-Whitney *U* test, Fisher's exact test or χ^2 test with Yate's correction were used to compare data, as appropriate. Stepwise logistic regression analysis was conducted to identify factors independently associated with the development of HCC. All tests were two-sided, and a *p*-value of less than 0.05 was considered significant. SPSS (version 19) was used to perform the analysis.

Results

Baseline characteristics of patients

The demographic and clinical characteristics of the 182 patients with chronic liver disease (non-HCC = 127 and HCC = 55) are shown in Table 1. Overall, the mean age of the patients was 52.6 ± 20.1 years, and HCC patients were significantly older ($p = 0.0001$) than non-HCC patients. Male gender was significantly abundant among HCC patients ($p = 0.03$). AST, albumin and anti-HBe were significantly higher in HCC patients compared to the non-HCC patients ($p = 0.0001$); inversely, HBeAg positivity was significantly more frequent among non-HCC patients ($p = 0.0001$).

HBV/D was the most prevalent genotype found in 94% (171/182) of patients followed by genotype E in 3.8% (7/182), genotype A2 in 1.6% (3/182) and C2 in 0.5% (1/182) with no statistical significance between both groups. Phylogenetic analysis was used as a major tool to determine the HBV genotypes and subgenotypes. HBV genotyping by ELISA was performed in a total of 18 cases who were either difficult to amplify by PCR or had a short S or core region sequence. These 18 cases along with one subgenotype D2 case were excluded from further analysis. Table 2 presents the baseline characteristics of 152 patients with HBV/D1. Consistent with the findings in the overall cohort (Table 1), age, male gender, anti-HBe, albumin and AST were significantly higher in HCC patients compared to non-HCC patients with HBV/D1 (Table 2).

Patterns of EnhII/BCP and precore mutations

The patterns of the BCP and precore mutations in patients infected with HBV/D1, with or without HCC, are presented in Table 2. A novel double mutation T1673/G1679 located in between Box ά and Box β[10] was found to be significantly higher in the HCC group ($p = 0.007$) compared to the non-HCC group. The frequency of G1727 and C1741 was also significantly higher in the HCC group than in those without HCC ($p = 0.005$ and $p = 0.0006$, respectively). The presence of C1761 was also more frequent in the HCC group ($p = 0.0005$). Apart from this, different kinds of single, double and triple mutation patterns were observed in the region encompassing nucleotides 1757–1768. The polymorphism at position A or G1757 shaped these patterns as double mutation T1762/A1764 and was found in both patterns but did

Table 1. Baseline and clinical characteristics of 182 patients with chronic liver disease infected with HBV in Saudi Arabia

Features	Total ($n = 182$)	Non-HCC ($n = 127$)	HCC ($n = 55$)	p^1
Age²	59 (6–93)	56 (6–85)	68 (40–93)	0.0001
Gender (M/F)	131/51	85/42	46/9	0.03
HBeAg+	94 (51.6)	85 (66.9)	9 (16.3)	0.0001
Anti-HBe+	99 (54.3)	50 (39.4)	49 (89)	0.0001
ALT³	84.1 ± 154.5	94.6 ± 175	89.5 ± 93.3	NS
AST³	101.5 ± 233.3	53.7 ± 144	198 ± 313	0.0001
Albumin³	82.1 ± 109.5	42 ± 8.2	183.2 ± 154.9	0.0001
Genotypes				
D	171 (93.9)	119 (93.7)	52 (94.5)	NS
E	7 (3.8)	4 (3.1)	3 (5.4)	NS
A	3 (1.6)	3 (2.3)	0	NS
C	1 (0.5)	1 (0.7)	0	NS

Numbers in parenthesis represent % age.
¹p: Mann-Whitney U test for continuous data, and χ^2 or Fisher's exact test for categorical data.
²Median (range).
³Mean ± SD. Abbreviation: ALT: alanine aminotransferase; AST: aspartate aminotransferase; NS: nonsignificant.

not show statistical significance between both clinical groups. However, it was observed that a combination of A1757/C or T1764/G1766 (triple mutation) was significantly higher in HCC patients compared to non-HCC patients ($p = 0.0004$). The point mutation at nucleotide position 1773 (C1773T), alone or in combination with A1775G was significantly higher in HCC patients compared to non-HCC patients ($p = 0.005$ and $p = 0.003$, respectively). The mutations A1896 and A1899 in the precore region were frequent in both groups showing no statistical significance, whereas a novel mutation in the core gene C1909 appeared significantly higher in HCC patients compared to the non-HCC group ($p = 0.014$). Stepwise logistic regression analysis in HBV/D1-infected patients showed older age (>58 years), male gender and viral mutations G1727, T1773, A1757/T1764/G1766 as independent predictive markers of HCC (Table 3).

Age-matched case–control analysis

Age- and gender-matched case–control analysis of 40 patients in each non-HCC and HCC group is shown in Table 4. Cases and controls did not differ significantly for eAg or eAb status in the matched set of samples. There were five controls and two HCC cases that were dually positive for HBeAg and anti-HBe, whereas four controls did not seroconvert. HBV viral load was significantly higher in HCC compared to non-HCC patients. The double mutation T1673/G1679 and the point mutations G1727 and C1741 remained significantly higher in HCC patients ($p = 0.01$, $p = 0.0007$ and $p = 0.006$, respectively). G1757 alone was significantly higher in the non-HCC group ($p = 0.03$), whereas in combination with T1762/A1764, it turned up as a protective mutation pattern, relatively higher in non-HCC compared to HCC patients ($p = 0.08$). The presence of triple mutation A1757/T1764/G1766 in association with HCC was consistent with the overall findings, as shown in Table 1 ($p = 0.01$). As expected, T1773 was more frequent in HCC ($p = 0.0001$), whereas in combination with G1775, it appeared only in the HCC group ($p = 0.01$). Stop-codon mutation A1896 did not reach statistical significance; however, A1899 was associated with non-HCC ($p = 0.04$). The stepwise logistic regression analysis confirmed A1727, A1757/T1764/G1766 and T1773 as independent predictive markers for HCC in this case–control analysis (Table 5).

Discussion

It is believed that HBV genotypes and even subgenotypes may differ in the clinical presentation of the disease and its treatment outcome. In our study, we found that the majority of patients were infected with HBV/D, subgenotype D1 (HBV/D1), followed by genotype E. These results are consistent with previous findings from the region.[13,17] HBV/D1 association with a benign course of disease has been suggested from studies elsewhere; however, most of these studies were hampered by their small sample size. Considering the data arising only from the Mediterranean region, HBV/D7 strains have been reported from Morocco and Tunisia but were not associated with advanced liver diseases.[13,26]

To our knowledge, this is the first case–control study nested within a cohort study of HBV carriers infected with HBV/D1. A significant positive correlation of HBV variants in the *X*/precore region with HCC was found individually and in combination. In the cohort analysis between 107 non-HCC and 45 HCC patients, individual mutations G1727, C1741, C1761 and T1773 were significantly associated with HCC. These mutations, except for C1761, were also associated with HCC in the case–control analysis. The magnitude

Table 2. Comparison of demographic characteristics and BCP and PC mutation factors among 152 patients with chronic liver disease infected with HBV/D1

Factors	Non-HCC ($n = 107$)	HCC ($n = 45$)	p^1
Age[2]	56 (26–65)	68 (65.5–71.5)	0.0001
Gender (M/F)	71/36	39/6	0.01
HBeAg+	66 (61.6)	5 (11.1)	0.0001
Anti-HBe+	47 (43.9)	41 (91.1)	0.0001
ALT[3]	86.5 ± 187.8	93.5 ± 94.5	NS
AST[3]	69.7 ± 197.9	206.9 ± 333.7	0.002
AFP[3]	3 (2.6–7.3)	36.5 (6.8–1,000)	0.0001
Albumin[3]	40.9 ± 8.02	186.6 ± 165.4	0.0001
Viral load (log IU/ml)[3]	6.03 ± 3.3	6.65 ± 3.7	NS
C1653 T or Y	14 (13)	8 (17.7)	NS
T1678 C	10 (9.3)	4 (8.8)	NS
A1679 G (alone)	10	3	0.08
C1673T/A1679G (double)	3 (2.8)	7 (15.5)	0.007
A1727 G	13 (12.1)	15 (33.3)	0.005
T1741 C	6 (5.6)	12 (26.6)	0.0006
T1753 C or A	43 (40.1)	16 (35.5)	NS
A1757G	38 (35.5)	12 (26.6)	NS
T1761C	2 (1.8)	8 (17.7)	0.0005
A1762T (alone)	5 (4.6)	1 (2.2)	NS
G1764A or T (alone)	4 (3.7)	0	ND
G1757/T1762/A1764 (triple)	26 (24.2)	9 (20)	NS
A1757/T1762/A1764 (triple)	13 (12.1)	4 (8.8)	NS
A1757/T or C1764/G1766 (triple)	12 (11.2)	16 (35.6)	0.0004
C1766T (alone)	7 (6.5)	5 (11.1)	NS
C1766T/T1768A (double)	9 (8.4)	5 (11.1)	NS
C1773T (alone)	60 (56.0)	36 (80)	0.005
A1775G (alone)	4 (3.7)	0	ND
C1773 T/A1775G (double)	2 (1.8)	7 (15.5)	0.003
G1896A	47 (43.9)	25 (55.5)	NS
G1899A	37 (34.5)	19 (42.2)	NS
T1909C	8 (7.4)	10 (22.2)	0.014
T1912C	10 (9.3)	2 (4.4)	NS

Numbers in parenthesis represent % age.
[1]p: Mann-Whitney U test for continuous data, and χ^2 or Fisher's exact test for categorical data.
[2]Median (interquartile range).
[3]Mean ± SD. Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; NS: nonsignificant; ND: not determined.

of OR was highest for T1773 mutation (silent mutation) in the cohort and the case–control analysis, that is, 11.8 and 14, respectively. The presence of this mutation in severe liver disease has also recently been reported among Turkish patients infected with HBV/D1.[27] The clinical impact of the point mutation A1727G (silent mutation) is not clear in HBV/D1 infection; however, the reverse mutation, G1727A, has been reported as a marker of HCC in occult HBV infection from Taiwan where infections by genotypes B and C are common.[12] The missense mutation C1761, causing amino acid change K130Q, has previously been reported from Iran in connection with severe liver disease.[19] The missense point mutation C1741 causing amino acid change L123S is novel; however, the mechanism whereby its interaction exists with other BCP mutations is yet unclear.

Our observation of an increased risk of HCC associated with infection by HBV strains in combination of mutations in the *X*-gene is far more novel and interesting. In the case–control analysis, the missense point mutation C or A1753 (causing amino acid change I1127N/T) appears to be a

protective one ($p = 0.03$), in contrast to the previous finding where this mutation has been found associated with the development of HCC in HBV/C-infected patients. Polymorphism at nucleotide position 1757 (sense mutation) has been evidenced in relation with the BCP double mutations T1762/A1764 (CP1) or T1764/G1766 (CP2).[19,28,29] In our study, the CP1 mutation appeared in combination with G at position 1757, showing a protective trend from HCC ($p = 0.08$); however, this needs to be further studied and confirmed by larger studies. CP1 affects amino acid changes K130M and V131I and, contrary to our findings, is a characteristic HCC-related double mutation in HBV/C and/or HBV/Ba infections.[6,10,30] The CP2 mutation that results in amino acid change C131L was observed in combination with A1757 and was significantly associated with HCC ($p = 0.01$). Our results are in agreement with previous reports where this double mutation has been reported in association with severe liver disease in HBV/D infections.[19,28] A recent *in vitro* study[29] showed that the CP2 mutation induced high levels of viral replication and transcription efficiency in HuH7 and HepG2 cells, which were comparable to those induced by the CP1 mutation. The effect of the CP2 mutation was significantly increased by the addition of the 1757A mutation by creating a binding site for the transcription factor HNF3, thereby increasing its

Table 3. Stepwise logistic regression analysis for factors independently associated with the development of HCC in patients infected with HBV/D1

Factors	Odds ratio (95% CI)	p^1
Age (>58 years)	6.78 (2.5–18.3)	0.0001
Male	2.96 (0.93–9.4)	0.06
G1727	3.97 (1.34–11.7)	0.01
T1764/G1766	2.8 (1.01–7.8)	0.04
T1773	11.8 (2.5–55.7)	0.002

^1p: Wald test.

Table 4. Age- and gender-matched case–control analysis in the BCP and PC regions of HBV/D1 in patients with chronic liver disease

Factors	Non-HCC ($n = 40$)	HCC ($n = 40$)	p^1
HBeAg	4 (10)	0	NS
HBeAg + anti-HBe	5 (12.5)	2 (5)	NS
Viral load ($\log_{IU}$ ml^{-1})^2	4.33 ± 3.0	6.48 ± 3.9	0.008
C1653T	6 (15)	7 (17.5)	NS
A1679G (alone)	3 (7.5)	3 (7.5)	0.06
C1673T/A1679G (double)	0	7 (17.5)	0.01
T1678C	8 (20)	4 (10)	NS
A1727G	2 (5)	14 (35)	0.0007
T1741C	3 (7.5)	12 (30)	0.006
T1753C or A	21 (52.5)	12 (30)	0.07
A1757G	21 (52.5)	11 (27.5)	0.03
A1761C	1 (2.5)	6 (15)	NS
A1762T (alone)	3 (7.5)	1 (2.5)	NS
G1764A or T (alone)	1 (2.5)	1 (2.5)	NS
G1757G/T1762T/A1764 (triple)	16 (40)	8 (20)	0.08
A1757/T1762/A1764 (triple)	4 (10)	3 (7.5)	NS
A1757/T or C1764/G1766 (triple)	5 (12.5)	15 (37.5)	0.01
C1766T (alone)	2 (5)	3 (7.5)	NS
C1766T/T1768A (double)	3 (7.5)	3 (7.5)	NS
C1773T	21 (52.5)	38 (95)	0.0001
C1773T/A1775G (double)	0	7 (17.5)	0.01
G1896A	29 (72.5)	22 (55)	NS
G1899A	24 (60)	16 (40)	0.04
T1909C	5 (12.5)	9(20)	NS
T1912C	4 (12.5)	2 (5)	NS

Numbers in parenthesis represent % age.
^1p: Mann-Whitney U test for continuous data, and χ^2 or Fisher's exact test for categorical data.
^{2}Mean ± SD. Abbreviation: NS: nonsignificant.

Table 5. Stepwise logistic regression analysis for factors independently associated with HCC development for age- and gender-matched case–control subjects

Factors	Odds ratio (95% CI)	p^1
G1727	18.3 (2.8–118.4)	0.002
T1764/G1766	4.7 (1.31–17.2)	0.01
T1773	14.06 (2.3–84.8)	0.004

^1p: Wald test.

transcriptional activity. In contrast, introduction of the 1757A mutation reduced the transcriptional activity of CP1, abolishing the viral replication *via* a reduction in HNF1 binding affinity. The double mutation T1766/A1768 was found in a small population of HCC patients and controls. The exact significance of this double mutation is not entirely clear; however, a few studies have described it as a predictive marker for cirrhosis.[31] Apart from these double mutations, a quadruple mutation T1673/G1679/T1773/G1775 was observed in a group of seven HCC patients. Being silent mutations, the exact biological significance of this combination of mutations is not entirely clear. Interestingly, these cases were also carrying point mutations G1727 and C1741. It is possible that G1727 and C1741 interact with the quadruple mutation in a similar way as CP1 and CP2 mutations interact with G or A1757, affecting the transcription factor binding site and inducing high levels of viral replication. It is possible that silent and missense mutations may synergistically act for a significantly altered function of X-protein, promoting hepatocarcinogenesis by interfering with cell growth control and DNA repair. According to a previous study, there may be a dose-risk relationship of mutation number with HCC and suggested using the mutation count as a diagnostic indicator for HCC.[14]

The G to A change at position 1896 is a hot-spot mutation in the precore region, which creates a premature stop-codon and has been associated with HBeAg levels.[32] Inconsistent results have been reported about the relationship of this mutation with liver disease. It has been associated with fulminant hepatitis in some studies[33,34] or less hepatic inflammation,[33] whereas some other studies did not find any notable association with liver disease.[35–38] Our study findings showed its association with HBeAg seroconversion, but could not relate it to the development of HCC. Furthermore, the accumulating evidence suggests that HBV/D exists more as HBeAg-negative phenotype. Various patients do seroconvert in the initial stages of infection, although not clearing the virus itself but remaining a carrier for life, suggesting an immune selection phenomenon as opposed to a replication advantage.[38,39]

In conclusion, we have shown several novel mutations in the EnhII/BCP regions of the HBV genome associated with the development of HCC. Each specific mutation may be sufficiently associated with HCC; however, the synergistic effect of combination patterns of mutations may be much more critical in escalating the development of HCC. These mutation complexes are novel risk factors that may facilitate early prediction of HCC in the chronic carriers of HBV/D1 infection.

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