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
Age[2]	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
ALT[3]	84.1 ± 154.5	94.6 ± 175	89.5 ± 93.3	NS
AST[3]	101.5 ± 233.3	53.7 ± 144	198 ± 313	0.0001
Albumin[3]	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.
[1]p: Mann-Whitney U test for continuous data, and χ^2 or Fisher's exact test for categorical data.
[2]Median (range).
[3]Mean ± 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

Int. J. Cancer: 00, 00–00 (2013) © 2013 UICC

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

[1]p: 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.
[1]p: 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

[1]*p*: 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.

References

1. Liaw YF, Chu CM. Hepatitis B virus infection. *Lancet* 2009;373:582–92.
2. Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005;42:1208–36.
3. Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001;34:590–4.
4. Tatematsu K, Tanaka Y, Kurbanov F, et al. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol* 2009;83:10538–47.
5. Yu H, Yuan Q, Ge SX, et al. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". *PLoS One* 2010;5:e9297.
6. Chan HL, Hui AY, Wong ML, et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 2004;53:1494–8.
7. Ito K, Tanaka Y, Orito E, et al. T1653 mutation in the Box-α increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. *Clin Infect Dis* 2006;42:1–7.
8. Sakamoto T, Tanaka Y, Simonetti J, et al. Classification of hepatitis B virus genotype B into 2 major types based on characterization of a novel subgenotype in Arctic indigenous populations. *J Infect Dis* 2007;196:1487–92.
9. Lau JY, Wright TL. Molecular virology and pathogenesis of hepatitis B. *Lancet* 1993;342:1335–40.
10. Tanaka Y, Mukaide M, Orito E, et al. Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. *J Hepatol* 2006;45:646–53.
11. Chou YC, Yu MW, Wu CF, et al. Temporal relationship between hepatitis B virus enhancer II/basal core promoter sequence variation and risk of hepatocellular carcinoma. *Gut* 2008;57:91–7.
12. Chen CH, Changchien CS, Lee CM, et al. A study on sequence variations in pre-S/surface, X and enhancer II/core promoter/precore regions of occult hepatitis B virus in non-B, non-C hepatocellular carcinoma patients in Taiwan. *Int J Cancer* 2009;125:621–9.
13. Kitab B, Essaid El Feydi A, Afifi R, et al. Variability in the precore and core promoter regions of HBV strains in morocco: characterization and impact on liver disease progression. *PLoS One* 2012;7:e42891.
14. Jang JW, Chun JY, Park YM, et al. Mutational complex genotype of the hepatitis B virus X/precore regions as a novel predictive marker for hepatocellular carcinoma. *Cancer Sci* 2012;103:296–304.
15. Chandra PK, Biswas A, Datta S, et al. Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. *J Viral Hepat* 2009;16:749–56.
16. Yousif M, Kramvis A. Genotype D of hepatitis B virus and its subgenotypes: an update. *Hepatol Res* 2013;43:355–64.
17. Abdo AA, Al-Jarallah BM, Sanai FM, et al. Hepatitis B genotypes: relation to clinical outcome in patients with chronic hepatitis B in Saudi Arabia. *World J Gastroenterol* 2006;12:7019–24.
18. Sanai FM, Helmy A, Bzeizi KI, et al. Discriminant value of serum HBV DNA levels as predictors of liver fibrosis in chronic hepatitis B. *J Viral Hepatitis* 2011;18:e217–e225.
19. Veazjalali M, Norder H, Magnius L, et al. A new core promoter mutation and premature stop codon in the S gene in HBV strains from Iranian patients with cirrhosis. *J Viral Hepat* 2009;16:259–64.
20. Sendi H, Mehrab-Mohseni M, Zali MR, et al. T1764G1766 core promoter double mutants are restricted to Hepatitis B virus strains with an A1757 and are common in genotype D. *J Gen Virol* 2005;86:2451–8.
21. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011;53:1020–2.
22. Abdo AA, Hassanain M, Al Jumah A, et al. Saudi guidelines for the diagnosis and management of hepatocellular carcinoma: technical review and practice guidelines. *Ann Saudi Med* 2012;32:174–99.

23. Tanaka Y, Hasegawa I, Kato T, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004;40:747–55.
24. Sugauchi F, Mizokami M, Orito E, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001;82:883–92.
25. Shin IT, Tanaka Y, Tateno Y, et al. Development and public release of a comprehensive hepatitis virus database. *Hepatol Res* 2008;38:234–43.
26. Hannachi N, Fredj NB, Bahri O, et al. Molecular analysis of HBV genotypes and subgenotypes in the Central-East region of Tunisia. *Virol J* 2010;7:302.
27. Sunbul M, Sugiyama M, Kurbanov F, et al. Specific mutations of basal core promoter are associated with chronic liver disease in hepatitis B virus subgenotype D1 prevalent in Turkey. *Microbiol Immunol* 2013;57:122–9.
28. Elkady A, Tanaka Y, Kurbanov F, et al. Virological and clinical implication of core promoter C1752/V1753 and T1764/G1766 mutations in hepatitis B virus genotype D infection in Mongolia. *J Gastroenterol Hepatol* 2008;23:474–81.
29. Sugiyama M, Tanaka Y, Sugauchi F, et al. Hepatitis virus genotype D1 specific mutations affecting viral replication and clinical course of hepatitis B patients. HBV Now in Asia: Single Topic Conference. 2009. 85.
30. Yu MW, Yeh SH, Chen PJ, et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J Natl Cancer Inst* 2005;97:265–72.
31. Guo X, Jin Y, Qian G, et al. Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qidong, China. *J Hepatol* 2008;49:718–25.
32. Tacke F, Gehrke C, Luedde T, et al. Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of Lamivudine-resistant mutants. *J Virol* 2004;78:8524–35.
33. Liang TJ, Hasegawa K, Rimon N, et al. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991;324:1705–9.
34. Hasegawa K, Huang JK, Wands JR, et al. Association of hepatitis B viral precore mutations with fulminant hepatitis B in Japan. *Virology* 1991;185:460–3.
35. Kao JH, Chen PJ, Lai MY, et al. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003;124:327–34.
36. Fang ZL, Yang J, Ge X, et al. Core promoter mutations (A(1762)T and G(1764)A) and viral genotype in chronic hepatitis B and hepatocellular carcinoma in Guangxi, China. *J Med Virol* 2002;68:33–40.
37. Chu CJ, Keeffe EB, Han SH, et al. Prevalence of HBV precore/core promoter variants in the United States. *Hepatology* 2003;38:619–28.
38. Fang ZL, Sabin CA, Dong BQ, et al. The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: a longitudinal analysis. *J Hepatol* 2009;50:273–80.
39. Livingston SE, Simonetti JP, Bulkow LR, et al. Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. *Gastroenterology* 2007;133:1452–7.

Journal of Medical Virology 85:1900–1906 (2013)

Reactivation of Hepatitis B Virus in Patients With Undetectable HBsAg Undergoing Chemotherapy for Malignant Lymphoma or Multiple Myeloma

Takeshi Matsui,[1,2]* Jong-Hon Kang,[2] Masanori Nojima,[3] Akiko Tomonari,[2] Hironori Aoki,[2] Hajime Yamazaki,[2] Kei Yane,[2] Kunihiko Tsuji,[2] Seisho Andoh,[4] Sachiko Andoh,[4] Hajime Sakai,[4] Masayo Maemori,[4] Hiroyuki Maguchi,[2] and Yasuhito Tanaka[1]

[1]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[2]*Center for Gastroenterology, Teine-Keijinkai Hospital, Sapporo, Japan*
[3]*Department of Public Health, Sapporo Medical University School of Medicine, Sapporo, Japan*
[4]*Department of Hematology, Teine-Keijinkai Hospital, Sapporo, Japan*

Despite increasing reports of hepatitis B virus (HBV) reactivation in hematological malignancies, its incidence, and risk factors are still obscure. The aim of this study was to clarify the frequency and risk factors of HBV reactivation in hepatitis B surface antigen (HBsAg) undetectable patients with malignant lymphoma or multiple myeloma, during or after chemotherapy. A total of 109 patients with undetectable HBsAg undergoing chemotherapy for malignant lymphoma or multiple myeloma were enrolled in this study. Anti-hepatitis B surface (anti-HBs) and anti-hepatitis B core (anti-HBc) were checked before treatment, and HBV DNA in sera was quantified monthly during and after chemotherapy. Out of 109 patients, 42 (38.5%) had anti-HBs and 59 (54.1%) had anti-HBc. Among the 59 anti-HBc positive patients, four patients (4/59, 6.8%) showed HBV reactivation during 20.5 median follow-up months. In all four patients with HBV reactivation, peripheral lymphocyte counts before chemotherapy were lower than those without HBV reactivation ($P = 0.033$). HBV reactivation occurred during and after chemotherapy containing rituximab for non-Hodgkin lymphoma. Four patients, who had HBV reactivation, did not develop de novo hepatitis due to HBV reactivation and were able to undergo chemotherapy against malignant lymphoma as scheduled. Monitoring of HBV DNA in sera is useful for the early diagnosis of HBV reactivation, and preemptive therapy is an useful alternative to prevent hepatitis due to HBV reactivation. Patients must be monitored periodically for HBV-DNA levels during and after chemotherapy. ***J. Med. Virol. 85:1900–1906, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: reactivation; hepatitis B virus; chemotherapy; lymphocyte

INTRODUCTION

Reactivation of the hepatitis B virus (HBV) is a well-recognized complication following systemic chemotherapy for hematological malignancies [Francisci et al., 2010; Yagci et al., 2010; Sugauchi et al., 2011]. HBV infection has a wide clinical spectrum. Therefore different serologic markers or combinations of markers are used to identify different phases of HBV infection and to determine whether a patient has acute or chronic HBV infection, is immune to HBV as a result of prior infection or vaccination, or is susceptible. During acute or chronic hepatitis B infection, hepatitis B surface antigen (HBsAg) can be detected in high levels in serum. The presence of hepatitis B surface antibody (anti-HBs) is generally interpreted as an indication of recovery and immunity from HBV infection. Anti-HBs also develops in a person who has been successfully vaccinated against hepatitis B. Total hepatitis B core antibody (anti-HBc) appears at the onset of symptoms in acute hepatitis B and persists for life. The presence of anti-HBc indicates previous or ongoing infection with HBV in an undefined time frame. Therefore, in the past, anti-HBc and/or anti-HBs positive patients

*Correspondence to: Takeshi Matsui, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. E-mail: mtake0402@gmail.com

Accepted 6 June 2013

DOI 10.1002/jmv.23694
Published online 07 August 2013 in Wiley Online Library (wileyonlinelibrary.com).

without HBsAg was thought to be eradicated of HBV in the host after resolution of HBV infection. However, recently it has become known that a low level of virus replication continues in hepatocytes even after the end of the acute HBV infection [Yotsuyanagi et al., 1998]. The covalently closed circular DNA (cccDNA) persist for many years in the liver of patients, HBsAg and HBV DNA in the blood has fallen to an undetectable level. Immunosuppressive drugs interfere with the cellular and humoral arm of the immune system controlling hepatocellular HBV infection. In healthy patients, host immunity is able to control HBV in most of the cases. This changes in an immune impaired patient, whether it is due to immunosuppressive drugs or monoclonal antibodies like rituximab. The humoral arm is profoundly affected by rituximab which depletes the body's antibody producing B cells. Other immunosuppressive drugs also impair the innate immune response. Therefore, reactivation of HBV following systemic chemotherapy can develop not only in HBsAg positive patients with a sustained HBV infection, but also in HBsAg undetectable patients with a past history of HBV infection [Wu et al., 2009; Cheung et al., 2010; Matsue et al., 2010; Wursthorn et al., 2010]. Rituximab and corticosteroids are especially well known to cause reactivation of HBV in patients without HBsAg in sera [Kusumoto et al., 2011]. Rituximab is a human monoclonal antibody derived from chimeric mice that inhibits an immune response by attacking CD20 positive B cells [Hiddemann et al., 2005]. Corticosteroids block cytokine synthesis and act as immune-suppressing drugs [Auphan et al., 1995] and, in addition, stimulate HBV DNA, mRNA, and protein production in a stable expression system [Tur-Kaspa and Laub, 1990]. These agents are used for the treatment of malignant lymphoma or other hematological malignancies. Multiple myeloma is recognized as a disease with a risk of HBV reactivation, due to its treatment regime with a high dose of corticosteroids [Yoshida et al., 2010]. To date, there has been increasing reports of HBV reactivation in patients treated with chemo/immunosuppressive therapy including the agents noted above [Hui et al., 2006; Matsubara et al., 2009; Shinkai et al., 2010]. However, the incidence or risk factors of HBV reactivation remain unclear because only a few prospective cohorts have presented for this new clinical entity. This study aimed to assess the incidence and risk factors of HBV reactivation, and analyzing the clinical course of HBV reactivation that occurred in the patients with malignant lymphoma or multiple myeloma during and after treatment.

PATIENTS AND METHODS

Study Patients

Consecutive patients with undetectable HBsAg who received chemotherapy for malignant lymphoma or multiple myeloma from January 2007 to October 2010 were included in this study. After admission, all patients underwent a physical examination and blood chemistry analysis. The study patients consisted of 109 patients (60 male, 55%; 49 female, 45%). The median age was 68-years-old, with a range of 22–91 years. Ninety-six patients (88.1%) had malignant lymphoma and 13 (11.9%) had multiple myeloma. Diagnosis of subtypes in malignant lymphoma included Diffuse large B-cell lymphoma (n = 54, 56.3%), Follicular lymphoma (n = 22, 22.9%), Marginal zone B-cell lymphoma (n = 7, 7.3%), Mantle cell lymphoma (n = 2, 2.1%), Burkitt lymphoma (n = 2, 2.1%), Intravascular large B-cell lymphoma (n = 1, 1.0%), Lymphoplasmacytoid lymphoma (n = 1, 1.0%), Peripheral T-Cell lymphoma (n = 1, 1.0%), Angioimmunoblastic T-Cell Lymphoma (n = 1,1.0%), and Hodgkin lymphoma (n = 5, 5.2%).

Determination of HBV Serological Markers and HBV DNA Quantification

On admission, all patients were screened for HBsAg in sera using a commercially available kit (Architect, Abbott Japan, Tokyo, Japan). Patients with undetectable HBsAg were enrolled in this study. Before treatment of hematological malignancies, patients were tested for anti-HBc, anti-HBs and blood parameters, and were then followed up by monthly monitoring of HBV DNA loads in sera or plasma and blood parameters. All serial sera were stored at −40° C. HBV DNA levels were quantified using Amplicor (range from below 2.6 to 7.6 log copies/ml; Roche Diagnostics, Tokyo, Japan) up to December 2007 and real-time TaqMan PCR (range from below 1.8 to 8.8 log copies/ml; Roche Diagnostics) since then. HBsAg with a highly sensitive chemiluminescent enzyme immunoassay (CLEIA) [Shinkai et al., 2010] was checked in stored sera sampled from patients with HBV reactivation retrospectively.

Definition of HBV Related Hepatitis and HBV Reactivation

Hepatitis was defined as a serum level of alanine aminotransferase (ALT) threefold higher than the normal upper limit of two consecutive determinations, 5 days apart, in the absence of the clinical and laboratory features of acute hepatitis A, hepatitis C, hepatitis E, or other systematic infections [Matsue et al., 2010]. The definition of HBV reactivation was the detection of HBV DNA in sera, including when the DNA load was not quantifiable but a PCR signal was detectable.

Statistical Analysis

In order to assess the risk factors of HBV reactivation, Fisher's exact test was applied for categorical variables, and Mann–Whitney's *U*-test was used for numerical variables. Receiver operating characteristic (ROC) curve was constructed to evaluate the

TABLE I. Clinical Characteristics of HBsAg Undetectable Patients Undergoing Chemotherapy for ML or Multiple Myeloma

Sex (M/F)	60/49
Age of years, median (range)	67.9 (22–91)
Follow-up period, median (range)	20.5 months (1.0–58.5)
Anti-HBc positive	59
Anti-HBs positive	42
Diagnosis	
Multiple myeloma	13
Diffuse large B cell lymphoma	54
Follicular lymphoma	22
Marginal zone B cell lymphoma	7
Burkitt lymphoma	2
T-cell lymphoma	2
Hodgkin lymphoma	5
Others	4
No. of rituximab administration	81
No. of glucocorticoids administration	108

diagnostic ability of HBV reactivation using a measured variable. A *P*-value less than 0.05 was considered significant. The best cutoff was defined as the point on the ROC curve closest to the upper left corner. All statistical analyses were performed using SPSS18 (IBM).

RESULTS

Patient Characteristics

The background characteristics of patients are shown in Table I. Out of 109 patients, 59 (54.1%) had anti-HBc, 42 (38.5%) had anti-HBs, and 47 (43.1%) had neither. Thirty-nine (35.7%) had both anti-HBc and anti-HBs. The number of patients with multiple myeloma were 13, and 96 patients had malignant lymphoma. Of all patients with malignant lymphoma, Hodgkin lymphoma was diagnosed in 5 patients, and non-Hodgkin lymphoma was confirmed in 91 patients. Diffuse large B-cell lymphoma was the dominant subtype of lymphoma. Rituximab was administered in 81 patients and glucocorticoids were used in 108 patients. None were treated by autologous peripheral blood stem cell transplantation or allogenic stem cell transplantation.

Consequences of HBV Serology

Among the 109 patients with undetectable HBsAg at the follow-up period of 20.5 median months (1.0–58.5), 4 (3.7%) showed the emergence of HBV DNA in sera, and were therefore diagnosed as HBV reactivation. They had never received a blood transfusion. The background characteristics and clinical features in patients with HBV reactivation are shown in Table II. None of the 50 patients without anti-HBc revealed HBV reactivation. In contrast, out of the 59 anti-HBc positive patients, 4 (6.8%) became positive for HBV DNA in sera. Among 20 anti-HBc positive and anti-HBs negative patients, 3 (15.0%) patients developed HBV reactivation, and only 1 of the 39 (2.6%) positive for both had an emergence of HBV DNA in sera. Sufficient anti-HBs antibodies in sera among HBV-resolved patients might reduce the incidence of HBV reactivation.

All four patients who developed HBV reactivation had lymphoma and were treated with rituxmab and glucocorticoids containing chemotherapy (Table II).

TABLE II. Clinical Characteristics of HBV Reactivation Patients

	Case 1	Case 2	Case 3	Case 4
Age/sex	75/F	70/M	66/M	83/F
Diagnosis	DLBCL	DLBCL	FL	BL
Stage[a]	IIIB	IIIB	IIA	IVB
Anti-HBc/HBs	+/−	+/−	+/+	+/−
Treatment	R-CHOP like	R-CHOP like	R-CHOP	R-MTX/CPM/VCR/ ADM/DEXA/ETP
Period from initiation of treatment (days)	42	46	398	148
Period from last rituximab (days)	3	26	159	23
Frequency of rituximab administration	5	1	12	8
During or after treatment	During treatment	During treatment	After treatment	During treatment
HBV DNA on the reactivation point (log copy/ml)	3.6	<1.8+[b]	3.6	<1.8+[b]
Peak HBV DNA (log copy/ml)	7.6	2.7	3.6	<1.8+[b]
HBV genotype	C	Ba	Not detected	Not detected
HBV pre-core	Wild	Wild	Not detected	Not detected
HBV core promoter	Wild	Mutant	Not detected	Not detected
Antiviral treatment	+	+	−	−
Outcome	Alive	Alive	Alive	Alive

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; R-CHOP, rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone; R-CHOP, rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone; R-MTX/CPM/VCR/ADM/DEXA/ETP, rituximab, methotrexate, cyclophosphamide, vincristine, adriamycin, dexamethasone, and etoposide; ALT, alanine aminotransferase.

[a]Ann Arbor staging.

[b]The DNA load was not quantifiable, but a PCR signal was detectable.

Reactivation of HBV occurred during the course of chemotherapy in three cases, and after maintenance therapy with rituximab in only one case (Fig. 1). Although there is no apparent correlation between HBV reactivation and complete blood count data prior to chemotherapy (data not shown), baseline counts of peripheral lymphocytes were associated with an incidence of HBV reactivation ($P = 0.033$). Nadir levels in peripheral lymphocytes for all subjects during treatment and baseline levels of immunoglobulin G were also assessed. However, none of the parameters were confirmed to be associated with the incidence of HBV reactivation, except for peripheral lymphocytes (Table III). The ROC analysis for the prediction of reactivation using lymphoid counts before treatment showed the area under the curve (AUC) to be 0.814, with the best cut-off to be 860/μl. In four cases with HBV reactivation, based on high sensitive HBsAg assay, HBsAg was examined from stored sera at the time of HBV reactivation, but none was detectable. The detailed clinical course are; (1) Case 1, initially negative for both HBsAg and anti-HBs, became positive for HBV DNA 42 days after the initiation of treatment. She had received chemotherapy using multiple agents such as rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone (R-CHOP like regimen). After an elevation in quantified HBV DNA in sera, the patient was treated with entecavir, at 1mg per day. HBV DNA was immediately undetectable without hepatitis. (2) Case 2 had detectable HBV-DNA 46 days after the initiation of an R-CHOP like regimen. One month after the transient emergence of HBV DNA in serum (signal positive, but not quantified), the HBV DNA became naturally undetectable. However, 6 weeks later, HBV DNA became detectable again, and after the confirmation of a sustained increase in the HBV DNA load, entecavir was continuously given. Thereafter, he showed a decrease in the HBV DNA load below the lower limit for detection without hepatitis, and ALT level became within normal range. (3) Case 3 had maintenance therapy with rituximab after the CHOP regimen. He showed an increase in the HBV DNA load at over 3 log copies/ml just once, 159 days after maintenance therapy with rituximab, but HBV DNA became undetectable again naturally. Although he had an anti-HBs titer of 601.2 mIU/ml before chemotherapy, the titer decreased to 500.8 mIU/ml at the showing of HBV reactivation. (4) Case 4 was not positive for quantified HBV DNA, but had a transient replication signal of HBV DNA in serum at day 148 by real time PCR. These two cases did not present with continuous viremia of HBV, and, as such, antiviral drugs were not administered. Although four cases showed HBV reactivation, they did not develop de novo hepatitis due to HBV reactivation and were able to undergo chemotherapy against malignant lymphoma as scheduled.

DISCUSSION

HBV reactivation is known as a significant complication of chemotherapy for hemodyscrasia [Francisci et al., 2010; Yagci et al., 2010; Sugauchi et al., 2011].

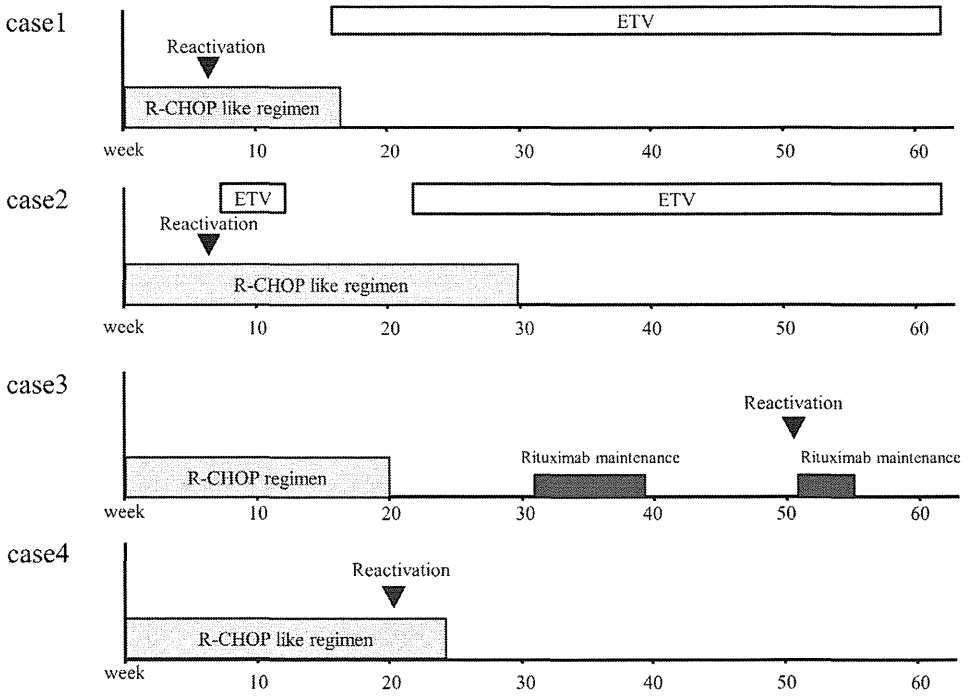

Fig. 1. Reactivation of HBV occurred during the course of chemotherapy in three cases, and after maintenance therapy with rituximab in only one case.

TABLE III. Comparison of Background Between Patients With or Without HBV Reactivation

			Reactivation proportion		
	Reactivation	Non-reactivation	Estimates(%)	95%CI(%)	*P*-value[a]
Number	4	105	3.7	(1.0–9.1)	
Sex(M/F)	2/2	58/47	3.3/4.1	(0.4–11.5)/(0.5–14.0)	1.000
Age[b], mean	73.5	67.7			0.420
Anti-HBc positive	4	55	6.8	(1.9–16.5)	0.123
Anti-HBs positive	1	41	2.4	(0.0–12.6)	1.000
Diagnosis					
Multiple myeloma	0	13	0.0	(0.0–24.7)	1.000
Diffuse large B cell lymphoma	2	52	3.8	(0.5–12.8)	1.000
Follicular lymphoma	1	21	4.8	(0.1–22.8)	1.000
Marginal zone B cell lymphoma	0	7	0.0	(0.0–41.0)	1.000
Burkitt lymphoma	1	1	50.0	(1.3–98.7)	0.072
T-cell lymphoma	0	2	0.0	(0.0–84.2)	1.000
Hodgkin lymphoma	0	5	0.0	(0.0–52.2)	1.000
Others	0	4	0.0	(0.0–60.2)	1.000
No. of rituximab administration	4	84	4.5	(1.3–11.2)	1.000
No. of glucocorticoid administration	4	104	3.7	(1.0–9.2)	1.000
Lymphocyte before chemotherapy median[b] (/μl) (range)	776 (460–1368)	1363 (274–10156)			0.033
Nadir lymphocyte during and after chemotherapy median[b] (/μl) (range)	133 (8–217)	247 (0–1729)			0.130
Immunoglobulin G before chemotherapy median[b] (mg/dl) (range)	1237 (1103–1479)	1421 (82–9085)			0.733

CI, confidence interval.
[a]Fisher's exact test.
[b]Medians and ranges are presented, compared by Mann–Whitney test.

Recently, the risk for development of HBV reactivation after chemotherapy in HBsAg undetectable patients has been reported [Wu et al., 2009; Cheung et al., 2010; Matsue et al., 2010]. Hui et al. [2006] described that 6 of 49 patients with undetectable HBsAg with malignant lymphoma receiving rituxmab plus corticosteroid chemotherapy developed new onset hepatitis B, and the risk factor was rituximab plus corticosteroid administration. Yeo et al. [2009] noted that 5 of 21 HBsAg undetectable, anti-HBc positive patients with diffuse large B-cell lymphoma who were treated with rituximab combination chemotherapy had reactivated HBV, and the risk factors were male, anti-HBs negative, and rituximab combination chemotherapy. It was recently reported that HBV reactivation had occurred in HBsAg undetectable multiple myeloma patients who underwent chemotherapy; 1 of 61 HBsAg undetectable multiple myeloma patients had reactivated HBV following chemotherapy [Yoshida et al., 2010]. However, additional prospective study would be required to know the precise frequency and risk factors for HBV reactivation among patients treated for malignant lymphoma or multiple myeloma. In addition, HBV reactivation was reported to be associated with the presence of anti-HBc and anti-HBs in baseline sera [Hui et al., 2006; Yeo et al., 2009], but the other factors associated with HBV reactivation have not yet been described.

In this study, all four cases with HBV reactivation were positive for anti-HBc before chemotherapy, and three of them were negative for anti-HBs (Table III). However, because of the limitation of size of samples in this study, this study could not evaluate the significance of anti-HBc for HBV reactivation ($P = 0.06$). This might be one of the key results of this study, but it could also be a chance finding. Therefore, serological markers including the titrations of anti-HBc and anti-HBs should be analyzed for the purpose of determining their relationship with HBV reactivation in larger scaled studies.

Out of four patients with HBV reactivation, two patients were treated with entecavir because they showed a persistent increase in the HBV DNA load. In contrast, in the remaining two patients, one patient showed a temporary replication signal of HBV DNA by real-time PCR, and another patient revealed a slight increase in the DNA load during a close follow-up. As HBV DNA of the latter two cases immediately became undetectable by real-time PCR, they were not given antiviral drugs. None of the four patients with HBV reactivation presented de novo hepatitis due to HBV reactivation. All cases were able to receive chemotherapy for underlying diseases as scheduled initially. Although there has been no proposal for the optimal time point for initiation of an anti-HBV treatment for this disease setting, preemptive therapy should be started immediately in patients with sustained viral replication quantified by real-time PCR.

In the present study, periodical quantitation of HBV DNA was useful for monitoring active

replication of HBV in patients receiving chemotherapy. HBsAg was also measured in serial sera in all cases of HBV reactivation, using novel CLEIA which was reported to be highly sensitive. However, HBsAg was not detected in any serum obtained from patients with HBV reactivation, indicating insufficient sensitivity of the assay for detecting HBV reactivation. As shown in the Japanese guidelines [Hirohito Tsubouchi et al., 2009], at present, routine measurement of HBV DNA levels would be preferable to an assay for HBsAg for the purpose of the early diagnosis of HBV reactivation.

Additionally, baseline lymphocyte counts in patients who had HBV reactivation were significantly less than those in patients who did not, although there was no difference in the nadir level of peripheral lymphocytes between patients who developed HBV reactivation and those who did not during or after chemotherapy. Based on these results, it is possible that lower levels of baseline peripheral lymphocytes might have a correlation with the occurrence of reactivated HBV in patients with malignant lymphoma or multiple myeloma. Although there is no similar data, further clinical studies are needed to evaluate the association between HBV reactivation and differential count of lymphocytes. In hepatocytes of chronic hepatitis B patients, cellular and humoral immunity could be associated with viral clearance, especially cytotoxic T lymphocytes (CTL) and natural killer (NK) cells which have roles to suppress proliferation of HBV. Gu et al. [2009] showed that serum HBV DNA levels in chronic hepatitis B patients were correlated to the frequency of HBV-specific CTL. Li et al. [2011] also reported that patients with acute hepatitis B possess a higher frequency of HBV-specific CTL than chronic hepatitis B patients. These reports may indicate that the HBV-specific CTL would be associated with suppression of HBV proliferation. This study could not evaluate differential counts of lymphocytes, and functional analyses of CTL. Further studies with CTL would provide a better understanding of the mechanism of this condition.

In conclusion, among the 59 anti-HBc positive patients with malignant lymphoma or multiple myeloma, four patients (6.8%) showed HBV reactivation during and after chemotherapy. HBV reactivation did not occur among patients without anti-HBc in this study. Monitoring of HBV DNA in sera is useful for the early diagnosis of HBV reactivation, and preemptive therapy is a useful alternative to prevent hepatitis due to HBV reactivation. Patients must be monitored periodically for HBV-DNA levels during and after chemotherapy.

REFERENCES

Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. 1995. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 270:286–290.

Cheung WI, Chan HL, Leung VK, Tse CH, Fung K, Lin SY, Wong A, Wong VW, Chau TN. 2010. Reactivation of hepatitis B virus infection with persistently negative HBsAg on three HBsAg assays in a lymphoma patient undergoing chemotherapy. J Clin Virol 47:193–195.

Francisci D, Falcinelli F, Schiaroli E, Capponi M, Belfiori B, Flenghi L, Baldelli F. 2010. Management of hepatitis B virus reactivation in patients with hematological malignancies treated with chemotherapy. Infection 38:58–61.

Gu XB, Yang XJ, Wang D, Hua Z, Xu YQ, Lu ZH. 2009. Relationship between serum HBV DNA level and HBV-specific, nonspecific cytotoxic T lymphocytes and natural killer cells in patients with chronic hepatitis B. Chin Med J 122:2129–2132.

Hiddemann W, Kneba M, Dreyling M, Schmitz N, Lengfelder E, Schmits R, Reiser M, Metzner B, Harder H, Hegewisch-Becker S, Fischer T, Kropff M, Reis HE, Freund M, Wormann B, Fuchs R, Planker M, Schimke J, Eimermacher H, Trumper L, Aldaoud A, Parwaresch R, Unterhalt M. 2005. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood 106:3725–3732.

Hirohito Tsubouchi HK, Kendo K, Satoshi M, Isao S, Eiji T, Takafumi I, Masashi M, Kazuyuki S, Shinsyo Y, Hisataka M, Toshifumi H, Norio H, Norihiro K, Tomoo F, Hiromi I, Yasuhiko S, Hiroshi Y, Akio I, Yasuhiro T, Kazuaki I, Makoto O, Hirofumi U, Nobuaki N, Takafumi N, Shinichiro T, Shinichi K, Koji Y, Ryujin E, Yasuhito T, Takeji U, Kotaro K. 2009. Prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection—Joint report of the intractable liver diseases study group of Japan and the Japanese study group of the standard antiviral therapy for viral hepatitis. Kanzo 50:38–42.

Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. 2006. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology 131:59–68.

Kusumoto S, Tanaka Y, Ueda R, Mizokami M. 2011. Reactivation of hepatitis B virus following rituximab-plus-steroid combination chemotherapy. J Gastroenterol 46:9–16.

Li J, Han Y, Jin K, Wan Y, Wang S, Liu B, Liu Y, Lu S, Huang Z. 2011. Dynamic changes of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and natural killer T (NKT) cells in patients with acute hepatitis B infection. Virol J 8:199.

Matsubara N, Kusano O, Sugamata Y, Itoh T, Mizuii M, Tanaka J, Yoshizawa H. 2009. A novel hepatitis B virus surface antigen immunoassay as sensitive as hepatitis B virus nucleic acid testing in detecting early infection. Transfusion 49:585–595.

Matsue K, Kimura S, Takanashi Y, Iwama K, Fujiwara H, Yamakura M, Takeuchi M. 2010. Reactivation of hepatitis B virus after rituximab-containing treatment in patients with CD20-positive B-cell lymphoma. Cancer 116:4769–4776.

Shinkai N, Tanaka Y, Matsuura K, Kani S, Naganuma H, Mizokami M. 2010. Evaluation and application of a newly developed highly sensitive HBsAg chemiluminescent enzyme immunoassay for chronic hepatitis B patients. Rinsho Byori 58:1078–1084.

Sugauchi F, Tanaka Y, Kusumoto S, Matsuura K, Sugiyama M, Kurbanov F, Ueda R, Mizokami M. 2011. Virological and clinical characteristics on reactivation of occult hepatitis B in patients with hematological malignancy. J Med Virol 83:412–418.

Tur-Kaspa R, Laub O. 1990. Corticosteroids stimulate hepatitis B virus DNA, mRNA and protein production in a stable expression system. J Hepatol 11:34–36.

Wu JM, Huang YH, Lee PC, Lin HC, Lee SD. 2009. Fatal reactivation of hepatitis B virus in a patient who was hepatitis B surface antigen negative and core antibody positive before receiving chemotherapy for non-Hodgkin lymphoma. J Clin Gastroenterol 43:496–498.

Wursthorn K, Wedemeyer H, Manns MP. 2010. Managing HBV in patients with impaired immunity. Gut 59:1430–1445.

Yagci M, Ozkurt ZN, Yegin ZA, Aki Z, Sucak GT, Haznedar R. 2010. Hepatitus B virus reactivation in HBV-DNA negative and positive patients with hematological malignancies. Hematology 15:240–244.

Yeo W, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, Chan HL, Hui EP, Lei KI, Mok TS, Chan PK. 2009. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. J Clin Oncol 27:605–611.

Yoshida T, Kusumoto S, Inagaki A, Mori F, Ito A, Ri M, Ishida T, Komatsu H, Iida S, Sugauchi F, Tanaka Y, Mizokami M, Ueda R. 2010. Reactivation of hepatitis B virus in HBsAg-negative patients with multiple myeloma: two case reports. Int J Hematol 91:844–849.

Yotsuyanagi H, Yasuda K, Iino S, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Kimura S, Koike K. 1998. Persistent viremia after recovery from self-limited acute hepatitis B. Hepatology 27: 1377–1382.

JCM
Journals.ASM.org

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

Noboru Shinkai,[a,b] Kentaro Matsuura,[a,b] Fuminaka Sugauchi,[a,b] Tsunamasa Watanabe,[a] Shuko Murakami,[a] Etsuko Iio,[a,b] Shintaro Ogawa,[a] Shunsuke Nojiri,[b] Takashi Joh,[b] Yasuhito Tanaka[a]

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan[a]; Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan[b]

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

Received 18 March 2013 Returned for modification 29 April 2013
Accepted 6 August 2013
Published ahead of print 14 August 2013
Address correspondence to Yasuhito Tanaka, ytanaka@med.nagoya-cu.ac.jp.

doi:10.1128/JCM.00726-13

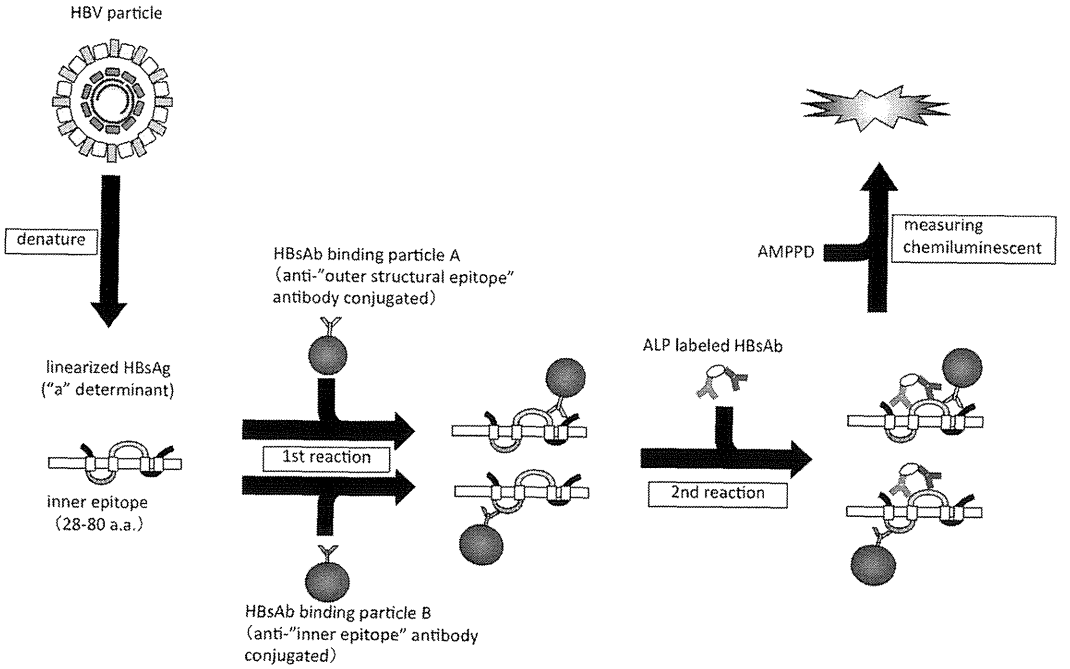

FIG 1 The principle of Lumipulse HBsAg-HQ.

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

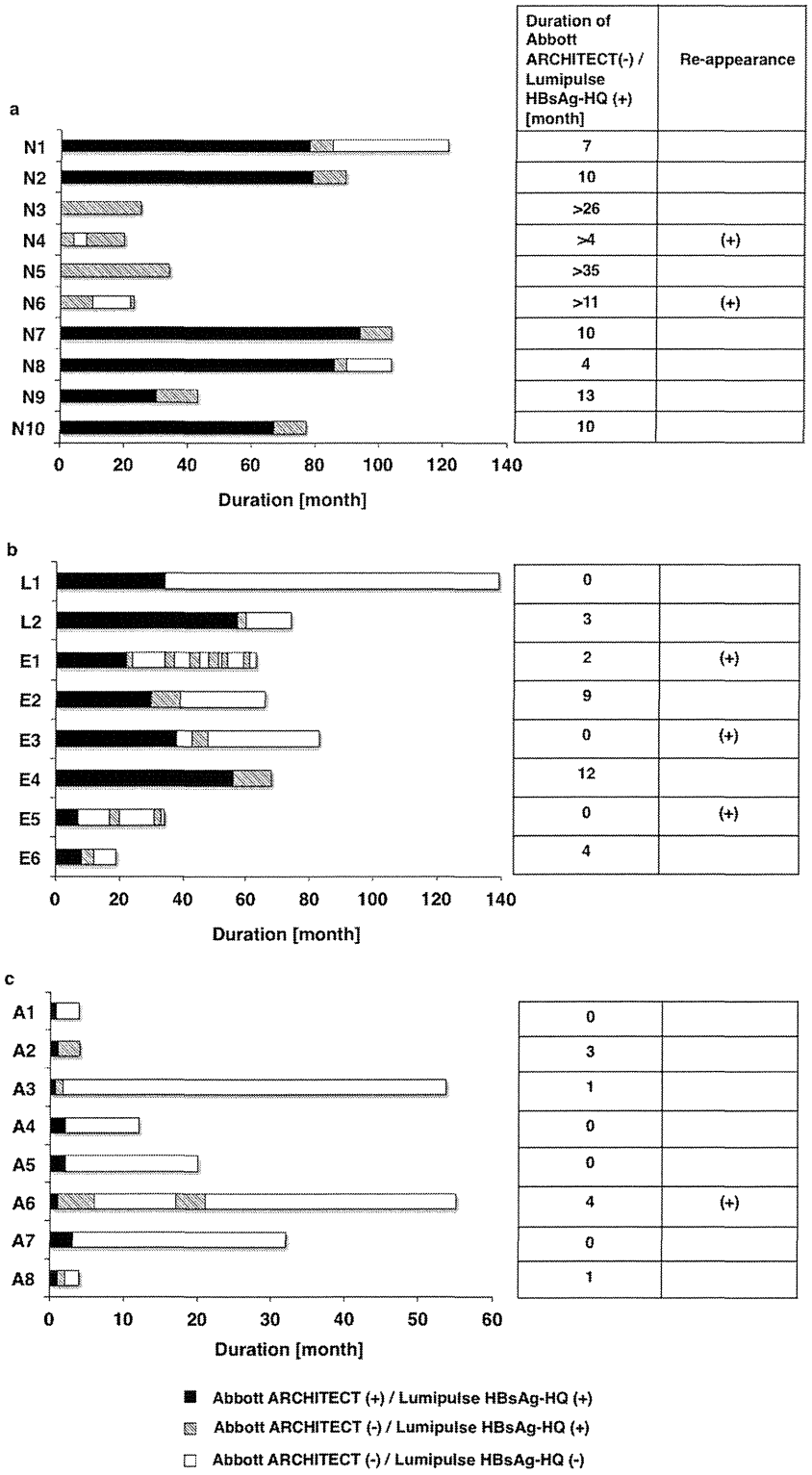

FIG 2 HBsAg dynamics by the Abbott Architect and Lumipulse HBsAg-HQ assays in the spontaneous HBsAg loss group (a), the NA-treated group (b), and the AH group (c).

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.

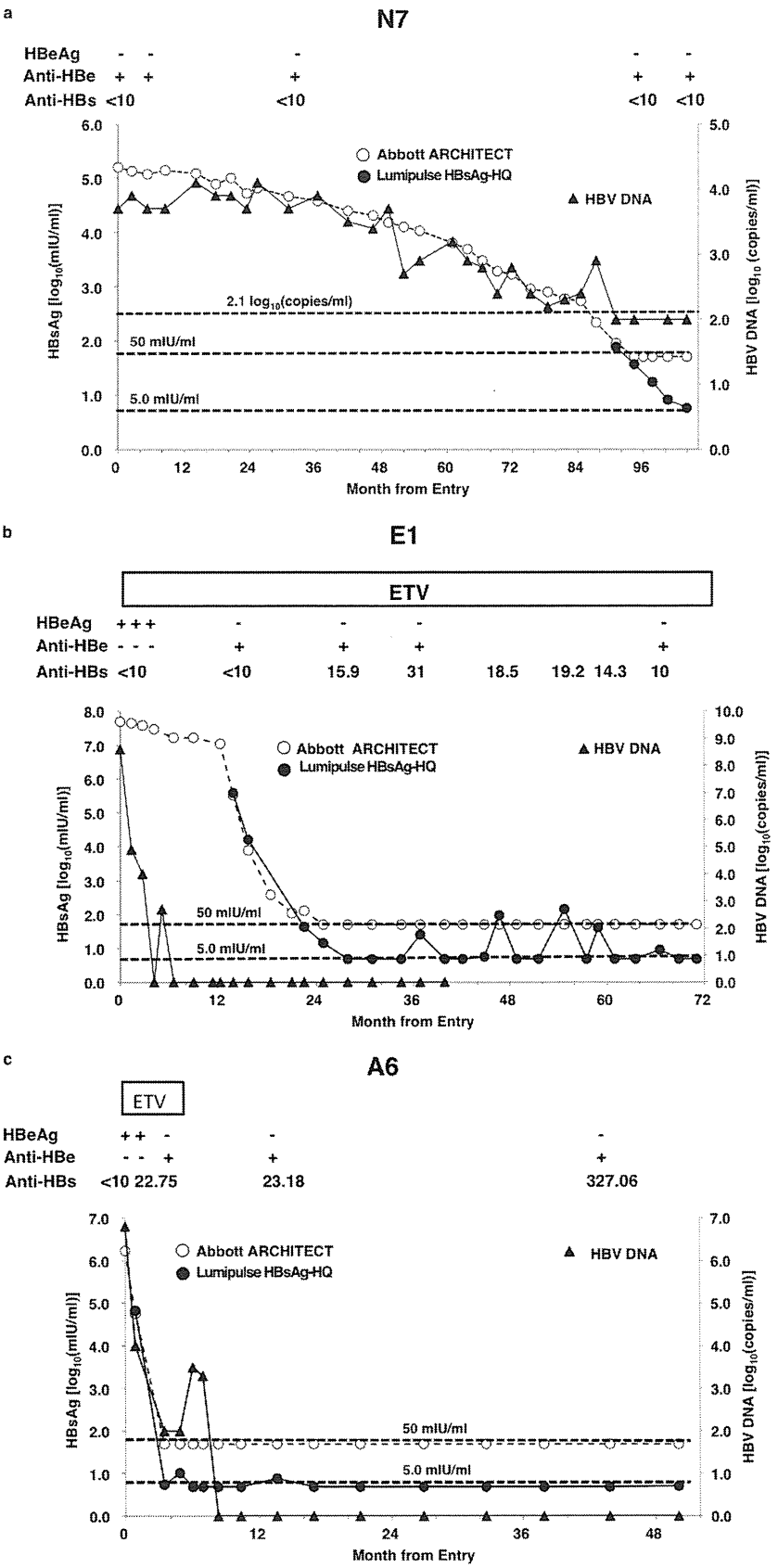

FIG 3 (a) HBsAg and HBV DNA dynamics of case no. N7. The Lumipulse HBsAg-HQ was still positive even 10 months after Abbott Architect results became negative. (b) HBsAg and HBV DNA dynamics of case no. E1. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was detectable for 3 months after HBsAg 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.