Table 3. Genotype frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

					HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
SNP	**Genotype**	**HBV carriers[a] (n = 449)**	**Resolved (n = 113)**	**Uninfected (n = 123)**	**OR (95% CI)**	***P* values**	**OR (95% CI)**	***P* values**
rs3077	CC	259 (57.7%)	43 (38.1%)	57 (46.3%)	1.00	-	1.00	-
HLA-DPA1	CT	153 (34.1%)	56 (49.6%)	46 (37.4%)	0.45 (0.29–0.71)	<0.001	0.73 (0.47–1.13)	0.161
	TT	37 (8.2%)	14 (12.4%)	20 (16.3%)	0.44 (0.22–0.88)	0.018	0.41 (0.22–0.75)	0.003
	Dominant[b]				**0.45 (0.30–0.69)**	**<0.001**	**0.63 (0.42–0.95)**	**0.025**
	HWEp	0.038	0.516	0.049				
rs9277378	GG	242 (53.9%)	40 (35.4%)	48 (39.0%)	1.00	-	1.00	-
HLA-DPB1	AG	177 (39.4%)	61 (54.0%)	54 (43.9%)	0.48 (0.31–0.75)	0.001	0.65 (0.42–1.00)	0.051
	AA	30 (6.7%)	12 (10.6%)	21 (17.1%)	0.41 (0.20–0.87)	0.018	0.28 (0.15–0.54)	<0.001
	Dominant				**0.47 (0.31–0.72)**	**<0.001**	**0.55 (0.36–0.82)**	**0.003**
	HWEp	0.757	0.110	0.390				
rs3128917	TT	99 (22.0%)	29 (25.7%)	38 (30.9%)	1.00	-	1.00	-
HLA-DPB1	TG	241 (53.7%)	60 (53.1%)	48 (39.0%)	1.18 (0.71–1.94)	0.525	1.93 (1.19–3.13)	0.008
	GG	109 (24.3%)	24 (21.2%)	37 (30.1%)	1.33 (0.73–2.44)	0.355	1.13 (0.67–1.92)	0.648
	Dominant				**1.22 (0.76–1.97)**	**0.413**	**1.58 (1.02–2.46)**	**0.042**
	HWEp	0.117	0.496	0.015				
rs1419881	TT	162 (36.1%)	31 (27.4%)	30 (24.4%)	1.00	-	1.00	-
TCF19	TC	213 (47.4%)	61 (54.0%)	60 (48.8%)	0.67 (0.41–1.08)	0.097	0.66 (0.41–1.07)	0.088
	CC	74 (16.5%)	21 (18.6%)	33 (26.8%)	0.67 (0.36–1.25)	0.210	0.42 (0.24–0.73)	0.002
	Dominant				**0.67 (0.42–1.06)**	**0.084**	**0.57 (0.36–0.90)**	**0.015**
	HWEp	0.778	0.349	0.792				
rs652888	TT	169 (37.6%)	50 (44.2%)	57 (46.3%)	1.00	-	1.00	-
EHMT2	TC	231 (51.4%)	50 (44.2%)	48 (39.0%)	1.37 (0.88–2.12)	0.162	1.62 (1.05–2.50)	0.027
	CC	49 (10.9%)	13 (11.5%)	18 (14.6%)	1.12 (0.56–2.22)	0.756	0.92 (0.49–1.70)	<0.001
	Dominant				**1.31 (0.87–2.00)**	**0.198**	**1.09 (0.65–1.82)**	**0.080**
	HWEp	0.022	0.926	0.142				

Abbreviation: CI, confidence interval; OR, odds ratio ; HWEp, Hardy-Weinberg equilibrium analysis.

[a]Defined as the combination between HCC and CHB.

[b]Defined as a minor dominant according to the comparison between heterozygous+minor homozygous genotype and major homozygous genotype (*eg.* rs3077; CT+TT vs. CC).

doi:10.1371/journal.pone.0086007.t003

DP region of MHC class II. The function of HLA-DP is to present bound peptide antigens, e.g. from HBV, at the surface of antigen-presenting cells. CD4+ T cells recognize these antigens and initiate the adaptive immune response. They assist the MHC class I-restricted CD8+ T cells which are the primary cellular effectors mediating HBV clearance from the liver during acute viral infection [22]. HBV infection will either be cleared by these means, or establish itself as a chronic infection. The reason for the latter is unclear but may be related to variation of *HLA-DP* alleles. Thus, the position of *HLA-DP* SNPs might be associated with possibility of clearance or chronicity. The rs3077 and rs9277535 SNPs are located within the 3′ untranslated region (UTR) of *HLA-DPA1* and *HLA-DPB1*, respectively while rs3128917 is located downstream of *HLA-DPB1*.

Recent investigations have identified 11 risk alleles for CHB related to mRNA expression of *HLA-DPA1* and *HLA-DPB1* [23]. The results showed that only these two alleles, rs3077 and rs9277535 were strongly associated with the risk of CHB and decreased expression of *HLA-DPA1* and *HLA-DPB1*, respectively. In contrast, while rs3128917 was associated with CHB, it was not associated with the level of HLA-DPB1 expression [23]. Variation at 5′ and 3′ UTRs can alter the binding sites of regulatory proteins which protect and stabilize newly synthesized RNA, either increasing or decreasing binding [24,25]. Nevertheless, the present study showed that rs3128917 was not associated with HBV carrier status in Thailand. Because rs3128917 is located downstream of the direction of transcription of the gene, this suggests that it does not affect regulation or coding of the gene and would have no effect on HLA protein expression.

The results from the present study not only establish the importance of variation at the *HLA-DP* gene but also explore two new SNPs, rs1419881 located in *TCF19* and rs652888 in the *EHMT2* gene [16]. *TCF19* (or transcription factor SC1) is a late growth regulatory gene like histone, thymidine kinase etc, maximally expressed at the onset of DNA synthesis at the G1-S boundary and S phase of cell cycle. This protein is also involved in regulations of growth and transcription factors controlling the number and development of peripheral-blood monocytes and erythrocytes [26]. The *EHMT2* gene is a histone methyltransferase [18] mainly responsible for mono- and di-methylation of H3K9 in euchromatin. This changes the conformation of chromatin from euchromatin to heterochromatin and then affects gene repression

[19]. Histone methylation has a critical role in gene transcription and epigenetic events [27–30].

According to recently published GWAS data [11], two SNPs associated with the risk for CHB in the Korea population were identified. These were the top signals in the genome-wide significance level analysis and were independently associated with *HLA-DP* and *HLA-DQ*, respectively. The authors then confirmed the results in a replication sample, showing that the frequency of their two SNPs strongly associated with CHB; OR = 0.76, 95% CI = 0.68–0.86, $p = 4.51E\text{-}11$ for rs1419881 and OR = 1.26, 95% CI = 1.07–1.47, $p = 2.78E\text{-}06$ for rs652888 [16]. Furthermore, another GWAS study focused on HLA, of hepatitis B vaccinated people in Indonesia, showed that rs652888 was also associated with risk of CHB ($p \leq 0.0001$) in that population [31].

In the present study, however, we found that rs1419881 tended to be associated with chronic HBV infection, based on the results of a comparison between HBV carriers and uninfected subjects. Nonetheless, it did not reach the significance by the Bonferroni corrections, as well as when HBV carriers were compared with patients who had their HBV infection resolved, no association with rs1419881 was observed. The second SNP, rs652888, was not associated with chronic HBV infection in the Thai population. Although our study had sampling error due to small samples, it might be another effect that the result between rs652888 in *EHMT2* gene and chronic hepatitis B in Thai population was not associated. The reason for these negative findings for the two SNPs might be due to the affected gene functions that were not involved with the immune system or processes of persistent infection. Data supporting this notion are to be found in the GWAS data for the Korean population, where pathway analysis of genes involved in the regulation of immune function showed that *TCF19* and *EHMT2* genes are not significantly involved in human immunity [16].

Mapping the position of the two new SNPs showed that rs1419881 located at the 3′ UTR of exon 4, with a tendency towards association with CHB and rs652888 which is not associated with CHB located on an intron. The position of each SNP might affect the phenotype of gene expression and susceptibility to disease, explaining why some are associated with chronic HBV infection, and others not. According to previous publications, the 3′ UTR of the *HLA-DP* region is strongly involved with regulating HLA-DP expression and influences the outcome of HBV infection [32]. In addition, another study showed that variation of the 3′ UTR of HLA-C was strongly associated with HLA-C expression levels and with control of human immunodeficiency virus [33]. This illustrated the general principle that the position of SNPs affects association with diseases.

The prevalence of HBV in Eastern countries, i.e. Asia, sub-Saharan Africa and the Pacific is much higher than in Western Europe and America. Most people in Eastern countries are infected with HBV during childhood and 8–10% of these develop CHB. In contract, the frequency of chronic carriers in Western Europe and North America is ≤1%. Furthermore, previous GWAS and meta-analysis reported that A alleles at rs3077 and rs9277353 have protective effects against CHB. Asian and African populations, especially Chinese, have lower frequencies of A alleles than European and American populations [10,34,35]. Moreover, the previous study showed no associations of rs3077 and rs9277535 with progressive CHB infection; however rs3077 was highly significant associated with HBV infection but not associated with rs9277353 in Caucasian populations [36].

While the frequency of alleles at rs3128917 and rs1419881 in Asian and African populations are quite similar, Northern and Western European populations have high frequencies of the protective T allele at rs3128917 but have low T allele frequencies (a risk allele for CHB) at rs1419881. The allele frequencies of populations in the worldwide for conspicuous details came from dbSNP Short Genetic Variations available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi. Lastly, both ethnic Eastern and Western populations have similar allele frequencies at rs652888, carrying a risk for CHB, with T allele frequencies very much higher than C allele frequencies, which has a protective effect. In addition, evolution of genomic characteristics, the migratory history of different populations, as well as HBV genotypes [37], HBV carrier rate [38] and pathological procession of liver disease [39] in each country may affect the distribution of *HLA* alleles. This was illustrated by a recent report in two Han Chinese populations (southern and northern) having different distributions of *HLA-DP* genes [39]. Thus, the genetics of the host is one of the factors influencing and predicting disease outcome [40].

According to less number of samples, it might influence statistical power in this study. Thus, we made another statistic meta-analysis of data obtained from previous reports and this study in Table S3. We compared HBV carriers with HBV uninfected subjects, because most previous studies also compared CHB with HBV clearance and/or healthy (negative for any HBV serological markers). Interestingly, all SNPs analyzed by the meta-analysis were significantly associated with HBV carriers. These results could support our data in Thailand. Additionally, no heterogeneity was observed between HBV carriers and HBV-resolved subjects ($P_{het} = 0.10$ for rs3077, 0.79 for rs9277378, and 0.07 for rs3128917), as well as between HBV carriers and HBV uninfected subjects ($P_{het} = 0.10$ for rs3077, 0.02 for rs9277378, 0.91 for rs1419881, and 0.04 for rs652888) except for rs9277378 ($P_{het} = 0.000$), for the minor allele frequency (MAF) of only rs9277378 was different between HapMap-CHB (MAF = 46.3% of G allele) and HapMap-JPT (MAF = 44.8% of T allele).

In the present study, we determined associations of variations at the *HLA-DP* gene with outcome in HBV infected Thai patients and the major homozygous genotypes of rs3077 and rs9277378, but not rs3128917, were significantly associated with HBV carrier status. Although genetic variation of two new SNPs, rs1419881 in the *TCF19* gene and rs652888 in the *EHMT2* gene, were not associated with the outcome of HBV infection in the Thai population, a large-scale study should be required.

Supporting Information

Figure S1 Association of 5 SNPs with HBV carriers, resolved HBV and uninfected subjects in Thailand. The results were compared between percentages of combination of heterozygous genotypes and minor homozygous genotypes (White square) with percentages of major homozygous genotypes (Grey square). Five SNPs applied in this study were rs3077, rs9277378 and rs3128917 in *HLA-DP* gene, rs1419881 in *TCF19* gene and rs652888 in *EHMT2* gene. OR, odds ratio; (lower-upper), 95% confidence interval.
(PPTX)

Table S1 Minor allele frequencies in HCC, CHB, resolved HBV and uninfected subjects in Thailand.
(DOC)

Table S2 The meta-analysis of minor allele frequencies in HBV carriers and resolved HBV.
(DOC)

Table S3 The meta-analysis of minor allele frequencies in HBV carriers and uninfected subject.
(DOC)

Author Contributions

Conceived and designed the experiments: SP TW YP YT. Performed the experiments: NP. Analyzed the data: NP SP SI KM NS. Contributed reagents/materials/analysis tools: PT SO SM. Wrote the paper: NP.

References

1. Kao JH, Chen DS (2002) Global control of hepatitis B virus infection. Lancet Infect Dis 2: 395–403.
2. Zanetti AR, Van Damme P, Shouval D (2008) The global impact of vaccination against hepatitis B: a historical overview. Vaccine 26: 6266–6273.
3. Dandri M, Locarnini S (2012) New insight in the pathobiology of hepatitis B virus infection. Gut 61 Suppl 1: i6–17.
4. Pan CQ, Zhang JX (2005) Natural History and Clinical Consequences of Hepatitis B Virus Infection. Int J Med Sci 2: 36–40.
5. Tran TT, Martin P (2004) Hepatitis B: epidemiology and natural history. Clin Liver Dis 8: 255–266.
6. Pumpens P, Grens E, Nassal M (2002) Molecular epidemiology and immunology of hepatitis B virus infection - an update. Intervirology 45: 218–232.
7. Elgouhari HM, Abu-Rajab Tamimi TI, Carey WD (2008) Hepatitis B virus infection: understanding its epidemiology, course, and diagnosis. Cleve Clin J Med 75: 881–889.
8. Singh R, Kaul R, Kaul A, Khan K (2007) A comparative review of HLA associations with hepatitis B and C viral infections across global populations. World J Gastroenterol 13: 1770–1787.
9. Thio CL, Thomas DL, Karacki P, Gao X, Marti D, et al. (2003) Comprehensive analysis of class I and class II HLA antigens and chronic hepatitis B virus infection. J Virol 77: 12083–12087.
10. Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, et al. (2009) A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 41: 591–595.
11. Mbarek H, Ochi H, Urabe Y, Kumar V, Kubo M, et al. (2011) A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. Hum Mol Genet 20: 3884–3892.
12. Wang L, Wu XP, Zhang W, Zhu DH, Wang Y, et al. (2011) Evaluation of genetic susceptibility loci for chronic hepatitis B in Chinese: two independent case-control studies. PLoS One 6: e17608.
13. An P, Winkler C, Guan L, O'Brien SJ, Zeng Z, Consortium HBVS (2011) A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. J Infect Dis 203: 943–947.
14. Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn SH, et al. (2012) Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. PLoS One 7: e39175.
15. Hu L, Zhai X, Liu J, Chu M, Pan S, et al. (2012) Genetic variants in human leukocyte antigen/DP-DQ influence both hepatitis B virus clearance and hepatocellular carcinoma development. Hepatology 55: 1426–1431.
16. Kim YJ, Young Kim H, Lee JH, Jong Yu S, Yoon JH, et al. (2013) A genome-wide association study identified new variants associated with the risk of chronic hepatitis B. Hum Mol Genet : In press.
17. Ku DH, Chang CD, Koniecki J, Cannizzaro LA, Boghosian-Sell L, et al. (1991) A new growth-regulated complementary DNA with the sequence of a putative trans-activating factor. Cell Growth Differ 2: 179–186.
18. Shinkai Y, Tachibana M (2011) H3K9 methyltransferase G9a and the related molecule GLP. Genes Dev 25: 781–788.
19. Tachibana M, Sugimoto K, Fukushima T, Shinkai Y (2001) Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. J Biol Chem 276: 25309–25317.
20. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.
21. Prokunina L, Alarcon-Riquelme ME (2004) Regulatory SNPs in complex diseases: their identification and functional validation. Expert Rev Mol Med 6: 1–15.
22. Yang PL, Althage A, Chung J, Maier H, Wieland S, et al. (2010) Immune effectors required for hepatitis B virus clearance. Proc Natl Acad Sci U S A 107: 798–802.
23. O'Brien TR, Kohaar I, Pfeiffer RM, Maeder D, Yeager M, et al. (2011) Risk alleles for chronic hepatitis B are associated with decreased mRNA expression of HLA-DPA1 and HLA-DPB1 in normal human liver. Genes Immun 12: 428–433.
24. Miller GM, Madras BK (2002) Polymorphisms in the 3′-untranslated region of human and monkey dopamine transporter genes affect reporter gene expression. Mol Psychiatry 7: 44–55.
25. Di Paola R, Frittitta L, Miscio G, Bozzali M, Baratta R, et al. (2002) A variation in 3′ UTR of hPTP1B increases specific gene expression and associates with insulin resistance. Am J Hum Genet 70: 806–812.
26. Ferreira MA, Hottenga JJ, Warrington NM, Medland SE, Willemsen G, et al. (2009) Sequence variants in three loci influence monocyte counts and erythrocyte volume. Am J Hum Genet 85: 745–749.
27. Cho HS, Kelly JD, Hayami S, Toyokawa G, Takawa M, et al. (2011) Enhanced expression of EHMT2 is involved in the proliferation of cancer cells through negative regulation of SIAH1. Neoplasia 13: 676–684.
28. Albert M, Helin K (2010) Histone methyltransferases in cancer. Semin Cell Dev Biol 21: 209–220.
29. Krivtsov AV, Armstrong SA (2007) MLL translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer 7: 823–833.
30. Lu Z, Tian Y, Salwen HR, Chlenski A, Godley LA, et al. (2013) Histone-lysine methyltransferase EHMT2 is involved in proliferation, apoptosis, cell invasion, and DNA methylation of human neuroblastoma cells. Anticancer Drugs 24: 484–493.
31. Png E, Thalamuthu A, Ong RT, Snippe H, Boland GJ, et al. (2011) A genome-wide association study of hepatitis B vaccine response in an Indonesian population reveals multiple independent risk variants in the HLA region. Hum Mol Genet 20: 3893–3898.
32. Thomas R, Thio CL, Apps R, Qi Y, Gao X, et al. (2012) A novel variant marking HLA-DP expression levels predicts recovery from hepatitis B virus infection. J Virol 86: 6979–6985.
33. Kulkarni S, Savan R, Qi Y, Gao X, Yuki Y, et al. (2011) Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature 472: 495–498.
34. Guo X, Zhang Y, Li J, Ma J, Wei Z, et al. (2011) Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. Hepatology 53: 422–428.
35. Yan Z, Tan S, Dan Y, Sun X, Deng G, et al. (2012) Relationship between HLA-DP gene polymorphisms and clearance of chronic hepatitis B virus infections: case-control study and meta-analysis. Infect Genet Evol 12: 1222–1228.
36. Vermehren J, Lotsch J, Susser S, Wicker S, Berger A, et al. (2012) A common HLA-DPA1 variant is associated with hepatitis B virus infection but fails to distinguish active from inactive Caucasian carriers. PLoS One 7: e32605.
37. Zeng G, Wang Z, Wen S, Jiang J, Wang L, et al. (2005) Geographic distribution, virologic and clinical characteristics of hepatitis B virus genotypes in China. J Viral Hepat 12: 609–617.
38. Hyams KC (1995) Risks of chronicity following acute hepatitis B virus infection: a review. Clin Infect Dis 20: 992–1000.
39. Li J, Yang D, He Y, Wang M, Wen Z, et al. (2011) Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. PLoS One 6: e24221.
40. Wong DK, Watanabe T, Tanaka Y, Seto WK, Lee CK, et al. (2013) Role of HLA-DP polymorphisms on chronicity and disease activity of hepatitis B infection in Southern Chinese. PLoS One 8: e66920.

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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

Received 18 March 2013 Returned for modification 29 April 2013
Accepted 6 August 2013
Published ahead of print 14 August 2013
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doi:10.1128/JCM.00726-13

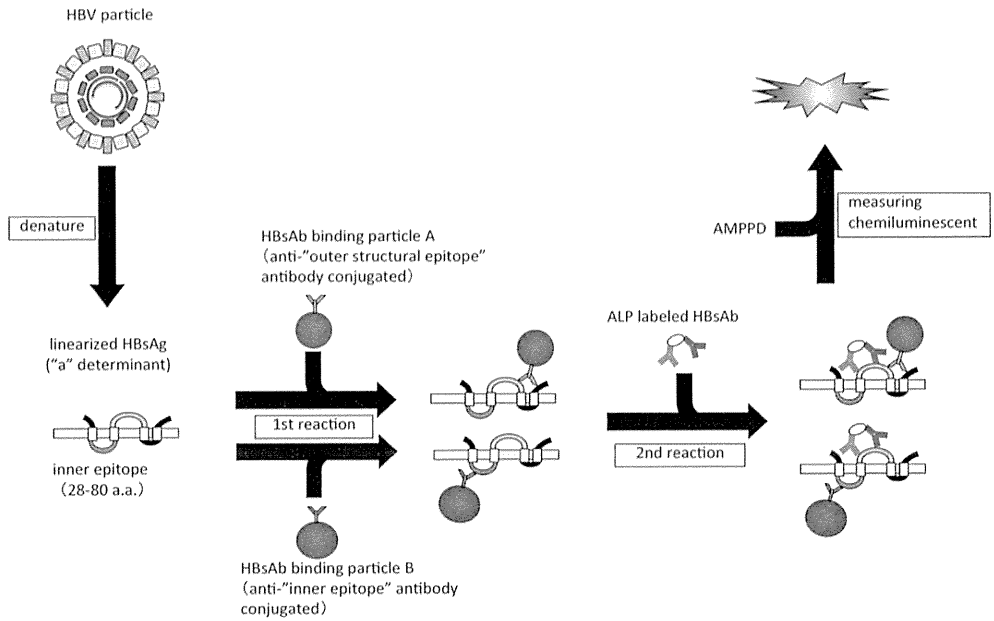

FIG 1 The principle of Lumipulse HBsAg-HQ.

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

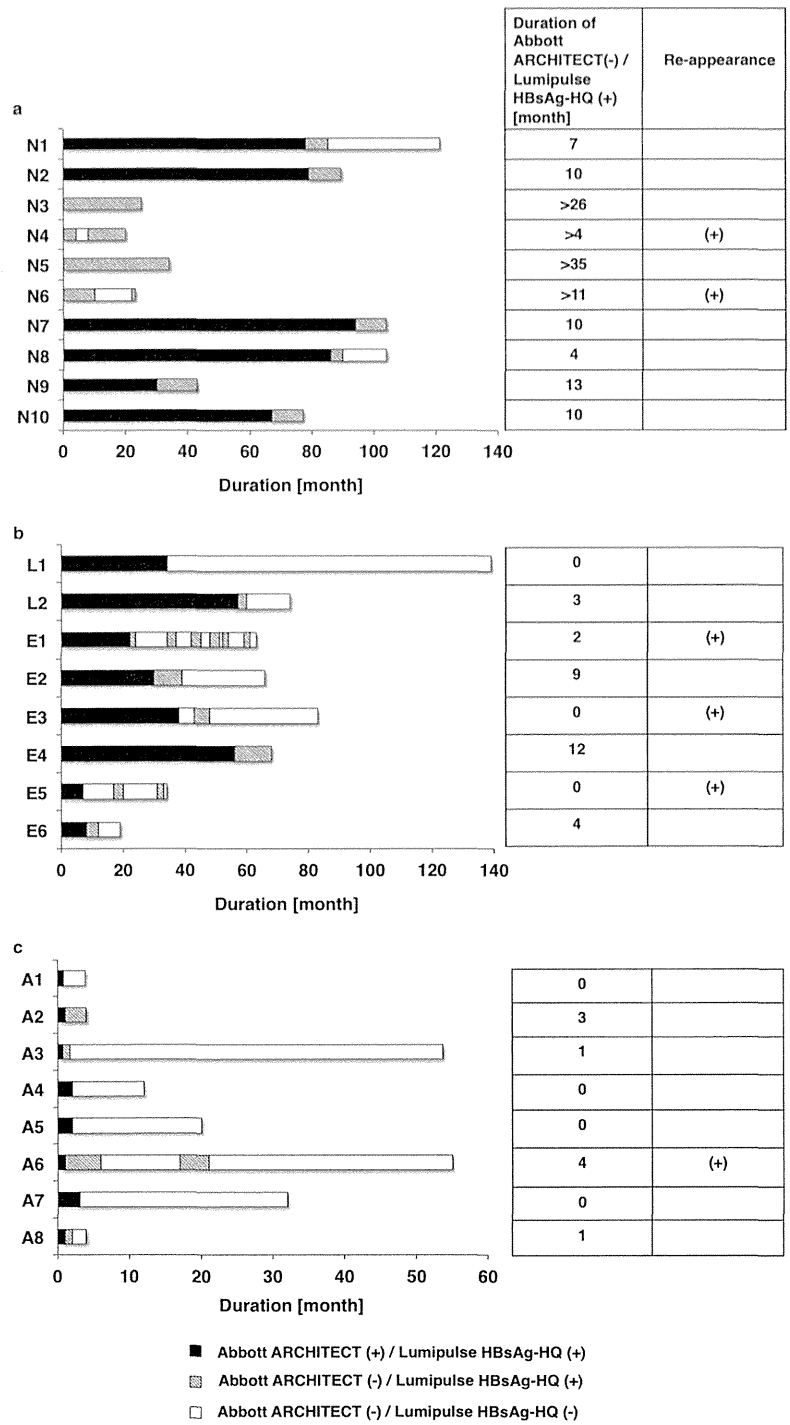

FIG 2 HBsAg dynamics by the Abbott Architect and Lumipulse HBsAg-HQ assays in the spontaneous HBsAg loss group (a), the NA-treated group (b), and the AH group (c).

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.

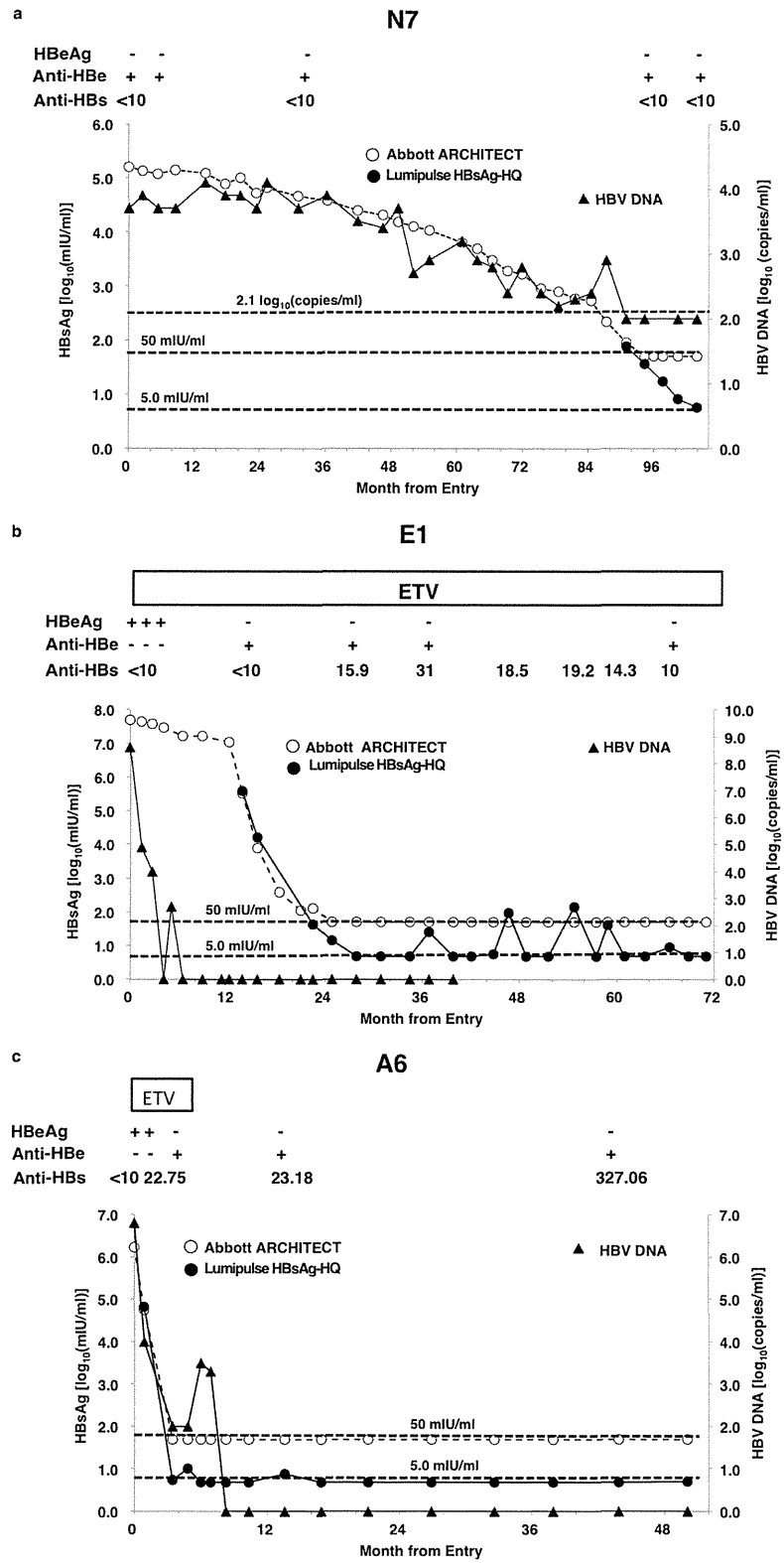

FIG 3 (a) HBsAg and HBV DNA dynamics of case no. N7. The Lumipulse HBsAg-HQ was still positive even 10 months after Abbott Architect results became negative. (b) HBsAg and HBV DNA dynamics of case no. E1. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was detectable for 3 months after HBsAg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

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.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan.

The authors declare no conflicts of interest.

REFERENCES

1. Pan CQ, Zhang JX. 2005. Natural history and clinical consequences of hepatitis B virus infection. Int. J. Med. Sci. 2:36–40.
2. Kohmoto M, Enomoto M, Tamori A, Habu D, Takeda T, Kawada N, Sakaguchi H, Seki S, Shiomi S, Nishiguchi S. 2005. Quantitative detection of hepatitis B surface antigen by chemiluminescent microparticle immunoassay during lamivudine treatment of chronic hepatitis B virus carriers. J. Med. Virol. 75:235–239.
3. Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, Sung JJ. 2007. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. Clin. Gastroenterol. Hepatol. 5:1462–1468.
4. Werle B, Cinquin K, Marcellin P, Pol S, Maynard M, Trépo C, Zoulim F. 2004. Evolution of hepatitis B viral load and viral genome sequence during adefovir dipivoxil therapy. J. Viral Hepat. 11:74–83.
5. Wursthorn K, Lutgehetmann M, Dandri M, Volz T, Buggisch P, Zollner B, Longerich T, Schirmacher P, Metzler F, Zankel M, Fischer C, Currie G, Brosgart C, Petersen J. 2006. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology 44:675–684.
6. Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Luo K, Wang Y, Hadziyannis S, Wolf E, McCloud P, Batrla R, Marcellin P. 2009. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology 49:1141–1150.
7. Chen CH, Lee CM, Wang JH, Tung HD, Hung CH, Lu SN. 2004. Correlation of quantitative assay of hepatitis B surface antigen and HBV DNA levels in asymptomatic hepatitis B virus carriers. Eur. J. Gastroenterol. Hepatol. 16:1213–1218.
8. Deguchi M, Yamashita N, Kagita M, Asari S, Iwatani Y, Tsuchida T, Iinuma K, Mushahwar IK. 2004. Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay. J. Virol. Methods 115:217–222.
9. Martinot-Peignoux M, Maylin S, Moucari R, Ripault MP, Boyer N, Cardoso AC, Giuily N, Castelnau C, Pouteau M, Stern C, Aupérin A, Bedossa P, Asselah T, Marcellin P. 2009. Virological response at 4 weeks to predict outcome of hepatitis C treatment with pegylated interferon and ribavirin. Antivir. Ther. 14:501–511.
10. 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.
11. 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. (Article in Japanese.)
12. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J. Clin. Microbiol. 40:439–445.
13. Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. 2007. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J. Clin. Microbiol. 45:3942–3947.
14. Moucari R, Korevaar A, Lada O, Martinot-Peignoux M, Boyer N, Mackiewicz V, Dauvergne A, Cardoso AC, Asselah T, Nicolas-Chanoine MH, Vidaud M, Valla D, Bedossa P, Marcellin P. 2009. High rates of HBsAg seroconversion in HBeAg-positive chronic hepatitis B patients responding to interferon: a long-term follow-up study. J. Hepatol. 50:1084–1092.
15. van Zonneveld M, Honkoop P, Hansen BE, Niesters HG, Darwish Murad S, de Man RA, Schalm SW, Janssen HL. 2004. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 39:804–810.
16. Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, Williams J, Livingston SE. 2010. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. Hepatology 51:1531–1537.
17. Yuen MF, Wong DK, Fung J, Ip P, But D, Hung I, Lau K, Yuen JC, Lai CL. 2008. HBsAg seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. Gastroenterology 135:1192–1199.
18. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. 2009. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J. Med. Virol. 81:27–33.
19. Shinkai N, Tanaka Y, Orito E, Ito K, Ohno T, Hirashima N, Hasegawa I, Sugauchi F, Ueda R, Mizokami M. 2006. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. Hepatol Res. 36:272–276.
20. Hollinger FB. 2008. Hepatitis B virus infection and transfusion medicine: science and the occult. Transfusion 48:1001–1026.
21. Raimondo G, Pollicino T, Cacciola I, Squadrito G. 2007. Occult hepatitis B virus infection. J. Hepatol. 46:160–170.
22. van Hemert FJ, Zaaijer HL, Berkhout B, Lukashov VV. 2008. Occult hepatitis B infection: an evolutionary scenario. Virol. J. 5:146. doi:10.1186/1743-422X-5-146.
23. Dettori S, Candido A, Kondili LA, Chionne P, Taffon S, Genovese D, Iudicone P, Miceli M, Rapicetta M. 2009. Identification of low HBV-DNA levels by nucleic acid amplification test (NAT) in blood donors. J. Infect. 59:128–133.
24. Satake M, Taira R, Yugi H, Hino S, Kanemitsu K, Ikeda H, Tadokoro K. 2007. Infectivity of blood components with low hepatitis B virus DNA levels identified in a lookback program. Transfusion 47:1197–1205.
25. Fukushima N, Mizuta T, Tanaka M, Yokoo M, Ide M, Hisatomi T, Kuwahara N, Tomimasu R, Tsuneyoshi N, Funai N, Sueoka E. 2009. Retrospective and prospective studies of hepatitis B virus reactivation in malignant lymphoma with occult HBV carrier. Ann. Oncol. 20:2013–2017.
26. Law JK, Ho JK, Hoskins PJ, Erb SR, Steinbrecher UP, Yoshida EM. 2005. Fatal reactivation of hepatitis B post-chemotherapy for lymphoma in a hepatitis B surface antigen-negative, hepatitis B core antibody-positive patient: potential implications for future prophylaxis recommendations. Leuk. Lymphoma 46:1085–1089.
27. Pei SN, Chen CH, Lee CM, Wang MC, Ma MC, Hu TH, Kuo CY. 2010. Reactivation of hepatitis B virus following rituximab-based regimens: a serious complication in both HBsAg-positive and HBsAg-negative patients. Ann. Hematol. 89:255–262.
28. 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.
29. 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.
30. Chen WN, Oon CJ. 2000. Hepatitis B virus surface antigen (HBsAg) mutants in Singapore adults and vaccinated children with high anti-hepatitis B virus antibody levels but negative for HBsAg. J. Clin. Microbiol. 38:2793–2794.
31. Wu C, Deng W, Deng L, Cao L, Qin B, Li S, Wang Y, Pei R, Yang D, Lu M, Chen X. 2012. Amino acid substitutions at positions 122 and 145 of hepatitis B virus surface antigen (HBsAg) determine the antigenicity and immunogenicity of HBsAg and influence *in vivo* HBsAg clearance. J. Virol. 86:4658–4669.
32. Oon CJ, Chen WN, Goh KT, Mesenas S, Ng HS, Chiang G, Tan C, Koh S, Teng SW, Toh I, Moh MC, Goo KS, Tan K, Leong AL, Tan GS. 2002. Molecular characterization of hepatitis B virus surface antigen mutants in Singapore patients with hepatocellular carcinoma and hepatitis B virus carriers negative for HBsAg but positive for anti-HBs and anti-HBc. J. Gastroenterol. Hepatol. 17(Suppl):S491–S496.
33. Coleman PF. 2006. Detecting hepatitis B surface antigen mutants. Emerg. Infect. Dis. 12:198–203.

J Gastroenterol
DOI 10.1007/s00535-014-1010-7

The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impact of alpha-fetoprotein on hepatocellular carcinoma development during entecavir treatment of chronic hepatitis B virus infection

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Received: 22 August 2014 / Accepted: 21 October 2014

Abstract
Background Entecavir (ETV) is one of the first-line nucleoside analogs for treating patients with chronic hepatitis B virus (HBV) infection. However, the hepatocellular carcinoma (HCC) risk for ETV-treated patients remains unclear.
Methods A total of 496 Japanese patients with chronic HBV infection undergoing ETV treatment were enrolled in this study. The baseline characteristics were as follows: age 52.6 ± 12.0 years, males 58 %, positive for hepatitis B e antigen 45 %, cirrhosis 19 %, and median HBV DNA level 6.9 log copies (LC) per milliliter. The mean treatment duration was 49.9 ± 17.5 months.
Results The proportions of HBV DNA negativity (below 2.6 LC/mL) were 68 % at 24 weeks and 86 % at 1 year, and the rates of alanine aminotransferase (ALT) level normalization were 62 and 72 %, respectively. The mean serum alpha-fetoprotein (AFP) levels decreased significantly at 24 weeks after ETV treatment initiation (from 29.0 ± 137.1 to 5.7 ± 27.9 ng/mL, $p < 0.001$). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively, among all enrolled patients. In a multivariate analysis, advanced age [55 years or older, hazard ratio (HR) 2.84; $p = 0.018$], cirrhosis (HR 5.59, $p < 0.001$), and a higher AFP level (10 ng/mL or greater) at 24 weeks (HR 2.38, $p = 0.034$) were independent risk

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Electronic supplementary material The online version of this article (doi:10.1007/s00535-014-1010-7) contains supplementary material, which is available to authorized users.

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Published online: 11 November 2014

factors for HCC incidence. HCC incidence was not affected by HBV DNA negativity or by ALT level normalization at 24 weeks.

Conclusions The AFP level at 24 weeks after ETV treatment initiation can be the on-treatment predictive factor for HCC incidence among patients with chronic HBV infection.

Keywords Hepatitis B virus · Entecavir · Risk factors for hepatocellular carcinoma incidence · Alpha-fetoprotein

Abbreviations

AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
cccDNA	Covalently closed circular DNA
ETV	Entecavir
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
NA	Nucleos(t)ide analog
ROC	Receiver operating characteristic

Introduction

More than 350 million people worldwide have hepatitis B virus (HBV) infection, and persistent hepatic damage following HBV infection is associated with liver disease progression [1–3]. Chronic HBV infection accounts for approximately 52.3 % of hepatocellular carcinoma (HCC) cases worldwide [4], and antiviral treatment such as interferon (IFN) or nucleos(t)ide analogs (NAs) that aims to improve the prognosis of patients with chronic HBV infection has been developed [5]. Entecavir (ETV), one of the first-choice NAs, is a more potent antiviral agent with a higher genetic barrier to resistance than lamivudine; ETV administration over the long term has been reported to enable most patients to maintain a state of viral suppression [6–9]. With regard to the suppressive effect of NAs on HCC, in a randomized controlled trial of patients who were treated with lamivudine or placebo, the lamivudine-treatment group showed a significantly lower HCC rate than the placebo group during the observation period of 32.4 months (3.9 % vs 7.4 %, $p = 0.047$) [10]. In other cohort studies of patients who were treated with lamivudine, HCC incidence has been reported to be significantly lower in those who maintained low HBV DNA levels [less than 4 or 5 log copies (LC) per milliliter], especially in those with cirrhosis [11–13]. In contrast, the suppressive effect of ETV on HCC incidence remains unclear because a randomized controlled study of patients treated with ETV or placebo has not been performed.

To date, many studies have assessed the relationship between clinical factors and HCC incidence, such as male gender, advanced age, presence of cirrhosis, and high HBV DNA levels, during the natural course of chronic HBV infection [14, 15]. Among patients who were treated with IFN, it has been reported that hepatitis B e antigen seroconversion achieved with IFN treatment was associated with lower HCC incidence rates compared with nonseroconversion [16]. However, neither the pretreatment factors nor the on-treatment factors that are associated with HCC incidence among patients receiving ETV have been fully examined. ETV treatment for patients with chronic HBV infection reduces serum HBV DNA levels and may also have anti-inflammatory and antineoplastic effects. That is, among patients receiving ETV, various factors, such as HBV DNA, alanine aminotransferase (ALT), total bilirubin, albumin, and alpha-fetoprotein (AFP) levels, have the possibility to change and be associated with HCC suppression.

In this study, we evaluated the risk factors for HCC, especially the on-treatment factors in patients with chronic HBV infection who were undergoing ETV treatment.

Patients and methods

Study population

This study was a retrospective, multicenter study conducted by Osaka University Hospital and other institutions

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that participate in the Osaka Liver Forum. A total of 840 NA-naïve patients chronically infected with HBV started treatment with 0.5 mg of ETV per day between July 2004 and July 2012. Of these patients, we excluded 51 patients with HBV DNA levels under 3 LC/mL at the baseline, 13 patients who were co-infected with hepatitis C virus (HCV) or with human immunodeficiency virus, one patient who had undergone liver transplantation, and 140 patients with a history of HCC at the baseline. In addition, we excluded 51 patients who had been treated with ETV for less than 1 year and 88 patients who developed HCC within 1 year after the initiation of ETV treatment. As a result, 496 patients were enrolled in this cohort study. This study was conducted according to the ethical guidelines of the Declaration of Helsinki, amended in 2002, and was approved by the Institutional Review Board of Osaka University Hospital (approval number 12380-2).

HCC surveillance and data collection

The patients were followed up once every 3–6 months, and clinical symptoms, HBV DNA and other virological markers, complete blood count, liver biochemistry, and AFP levels were assessed. AFP levels measured between 20 and 28 weeks from the initiation of ETV treatment were regarded as valid AFP levels at 24 weeks. Ultrasonography of the abdomen, computed tomography, and/or magnetic resonance imaging was performed every 3–6 months for HCC surveillance. HCC was diagnosed by the presence of typical hypervascular characteristics evident on the computed tomography and/or magnetic resonance imaging scans. If no typical signs of HCC were observed, either hepatic angiography or fine-needle aspiration biopsy was performed with the patient's consent, or the patient was carefully followed until a diagnosis was possible on the basis of a definite observation. Liver cirrhosis was defined by a shrunken, small liver with a nodular surface as noted on liver imaging and by clinical features of portal hypertension.

Definition of treatment response

The surveillance start date was defined as the time of ETV treatment initiation. HBV DNA was measured by the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan) with a linear range of detection from 2.6 to 7.6 LC/mL or by the COBAS Taqman HBV Test v2.0 (Roche Diagnostics) with a linear range of detection from 2.1 to 9.0 LC/mL. The achievement of a virological response by ETV treatment was defined by serum HBV DNA levels that were continuously under 2.6 LC/mL. ALT level normalization was defined by serum ALT levels that were 30 IU/L or less.

Statistical analyses

Statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA) and SAS for Windows version 9.3 (SAS Institute, Cary, NC, USA). The continuous variables were expressed as the mean ± standard deviation or standard error of the mean or as the median (range), as appropriate, whereas the categorical variables were expressed as frequencies. The Wilcoxon signed-rank sum test was used to analyze differences between continuous variables before and after treatment. The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was assessed by the time-dependent receiver operating characteristic (ROC) curve, and the 95 % confidence interval for the area under the ROC curve was constructed using the bootstrap method. The Kaplan–Meier method was used to assess the cumulative HCC incidence, and the groups were compared using the log-rank test. The Cox proportional-hazards model was used to identify the independent factors associated with HCC incidence. The factors that were selected as significant by simple Cox regression analysis were evaluated by multiple Cox regression analysis. The risks were expressed as hazard ratios and 95 % confidence intervals. We considered $p < 0.05$ as significant.

Results

The characteristics of the 496 patients at the baseline and at 24 weeks after ETV treatment initiation are summarized in Table 1. The average age of the patients was 52.6 ± 12.0 years at the baseline, and there were 288 males (58 %) and 92 patients with cirrhosis (19 %). The patients were followed up for an average of 49.9 ± 17.5 months.

The cumulative incidence of virological response (HBV DNA level less than 2.6 LC/mL) at 24 weeks, 1 year, and 3 years after the initiation of ETV treatment was 68, 86, and 95 %, respectively. The median levels of HBV DNA were significantly decreased among noncirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) and cirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation (Table 1). ALT level normalization (30 IU/L or lower) was achieved in 62 % of patients at 24 weeks and in 72 % of patients at 1 year. The median ALT levels were significantly decreased among noncirrhotic (72.0–25.0 IU/L, $p < 0.001$) and cirrhotic (51.0–29.0 IU/L, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation. The following parameters were also significantly increased from the baseline to 24 weeks after ETV treatment initiation: platelet counts and serum albumin levels

Table 1 Characteristics of patients at the baseline and 24 weeks after initiation of entecavir (ETV) treatment

	All patients, $n = 496$		Noncirrhotic patients, $n = 404$		Cirrhotic patients, $n = 92$	
	Baseline	24 weeks	Baseline	24 weeks	Baseline	24 weeks
Age (years)	52.6 ± 12.0 (15–82)		51.3 ± 12.1 (15–82)		58.2 ± 9.8 (32–81)	
Gender: male/female	288/208 (58 %)		233/171 (58 %)		55/37 (60 %)	
HBeAg[a]: positive/negative	220/270 (45 %)		181/219 (45 %)		39/51 (43 %)	
Histology[b], activity: A0/1/2/3	3/82/74/14		3/75/63/12		0/7/11/2	
Histology[b], fibrosis: F0/1/2/3/4	8/63/51/32/20		8/63/52/32/0		0/0/0/0/20	
History of IFN therapy: presence	50 (11 %)		44 (11 %)		6 (7 %)	
Platelet count ($\times 10^4/\mu L$)	16.0 ± 5.8	16.5 ± 6.4*	17.3 ± 5.2	17.7 ± 5.3*	10.3 ± 5.8	11.5 ± 7.9
Total bilirubin (mg/dL)	1.01 ± 1.48	0.83 ± 0.45*	0.91 ± 0.95	0.78 ± 0.42*	1.45 ± 2.78	1.09 ± 0.48
Albumin (g/dL)	3.94 ± 0.52	4.11 ± 0.44*	4.03 ± 0.44	4.18 ± 0.39*	3.56 ± 0.64	3.79 ± 0.50*
PT (%)	83.8 ± 16.3		86.7 ± 15.7		72.4±16.3	
ALT (IU/L)	143.7 ± 199.3 (9–1,885)	29.6 ± 16.5* (6–166)	156.1 ± 210.8 (9–1,885)	29.2 ± 16.9* (6–166)	89.2 ± 124.7 (12–763)	31.5 ± 14.0* (10–84)
ALT ≤ 30 (IU/L)	11 %	62 %	10 %	64 %	13 %	53 %
30 < ALT ≤ 60 (IU/L)	31 %	33 %	28 %	31 %	48 %	43 %
60 < ALT (IU/L)	58 %	5 %	62 %	5 %	39 %	4 %
HBV DNA (LC/mL) (median)	6.9	<2.6*	6.9	<2.6*	6.9	<2.6*
HBV DNA < 2.6 (LC/mL)	–	68 %	–	68 %	–	70 %
2.6 ≤ HBV DNA < 4.0 (LC/mL)	4 %	24 %	4 %	21 %	3 %	30 %
4.0 ≤ HBV DNA (LC/mL)	96 %	8 %	96 %	11 %	97 %	0 %
AFP (ng/mL)[c]	29.0 ± 137.1 (1–2,225)	5.7 ± 7.9* (1–126)	29.5 ± 152.7 (1–2,225)	4.9 ± 4.6* (1–126)	27.4 ± 48.0 (1–318)	9.3 ± 14.6* (1–52)
Observation periods (months)	49.9 ± 17.5 (14–109)		49.2 ± 17.6 (14–109)		52.8 ± 16.6 (18–82)	

Data are expressed as the mean ± standard deviation except for hepatitis B virus (*HBV*) DNA (median)

AFP alpha-fetoprotein, *ALT* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *IFN* interferon, *LC* log copies, *PT* prothrombin time

* $p < 0.05$ (Wilcoxon signed-rank sum test)

[a] HBeAg measurement at the baseline was missing in six patients

[b] Liver biopsy was performed in 174 patients

[c] AFP data were missing in 78 noncirrhotic patients and five cirrhotic patients with cirrhosis

among noncirrhotic patients ($p = 0.008$ and $p < 0.001$, respectively) and serum albumin levels in cirrhotic patients ($p < 0.001$).

Mean serum AFP levels decreased significantly from 29.0 ± 137.1 ng/mL at the baseline to 5.7 ± 7.9 ng/mL at 24 weeks after the initiation of ETV treatment ($p < 0.001$). Mean AFP levels were assessed according to the severity of liver disease and decreased significantly from the baseline to 24 weeks in both the noncirrhotic group and the cirrhotic group (noncirrhotic group 29.5 ± 152.7 to 4.9 ± 4.6 ng/mL, $p < 0.001$; cirrhotic group 27.4 ± 48.0 to 9.3 ± 14.6 ng/mL, $p < 0.001$; Table 1). The proportion of patients with AFP levels below 10 ng/mL increased from 73 % at the baseline to 95 % at 24 weeks among noncirrhotic patients and from 48 % at the baseline to 76 % at 24 weeks among cirrhotic patients (Fig. 1).

A total of 42 patients developed HCC during the observation period (16 noncirrhotic patients, 26 cirrhotic patients). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively. The mean time point of HCC development was 34.0 ± 18.4 months from the initiation of ETV treatment. AFP levels among patients who developed HCC decreased from 24 weeks (13.1 ± 3.9 ng/mL) (mean ± standard error of the mean) to 48 weeks (10.2 ± 3.0 ng/mL) after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence (7.6 ± 1.6 ng/mL) to the time of HCC incidence (35.4 ± 12.8 ng/mL) (Fig. S1). The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was set as 10 ng/mL on the basis of the calculated cutoff value (12.1 ng/mL) assessed using the time-dependent ROC curve (Table S1).

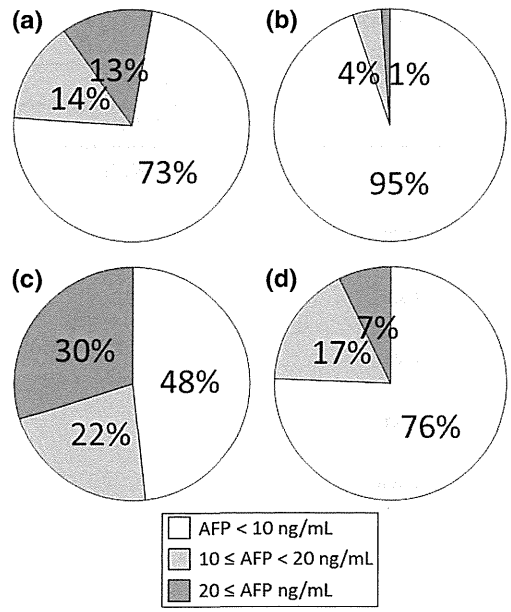

Fig. 1 Distribution of alpha-fetoprotein (*AFP*) levels at the baseline and at 24 weeks after the initiation of entecavir (ETV) treatment according to the severity of liver disease: **a** patients without cirrhosis at the baseline ($n = 326$); **b** patients without cirrhosis at 24 weeks after ETV treatment initiation ($n = 326$); **c** patients with cirrhosis at the baseline ($n = 87$); **d** patients with cirrhosis at 24 weeks after ETV treatment initiation ($n = 87$)

Factors associated with HCC incidence at the baseline

In a univariate analysis, factors at the baseline such as advanced age, cirrhosis, lower platelet counts, and higher total bilirubin, lower albumin, and higher AFP levels were significant, and a multivariate analysis demonstrated that advanced age (55 years or older) and cirrhosis were significant independent risk factors for HCC incidence (Table 2). After a stratified analysis of HCC incidence according to those risk factors at the baseline, the cumulative incidence of HCC at 5 years was 2.5 % in younger patients (younger than 55 years) and 18.6 % in older patients (55 years or older, $p < 0.001$; Fig. 2a). The cumulative incidence of HCC at 5 years was 5.3 % in noncirrhotic patients and was 30.0 % in cirrhotic patients ($p < 0.001$; Fig. 2b).

Factors associated with HCC incidence at 24 weeks after the initiation of ETV treatment

The association between HCC incidence and posttreatment factors at 24 weeks after the initiation of ETV treatment was estimated. In a univariate analysis, advanced age, cirrhosis, lower platelet counts,and lower albumin, higher total bilirubin, and higher AFP levels at 24 weeks were significant, and a multivariate analysis showed that a higher AFP level (10 ng/mL or greater) at 24 weeks was the only additional factor independently associated with HCC incidence other than advanced age and cirrhosis, which were found to be significant risk factors at the baseline (Table 3). The cumulative incidence of HCC at 5 years was 8.2 % among patients with an AFP level below 10 ng/mL at 24 weeks and was 34.2 % among patients with an AFP level of 10 ng/mL or higher at 24 weeks (Fig. 3a). Although the American Association for the Study of Liver Disease practical guidelines for chronic hepatitis B indicate that the aims of treatment for patients infected with HBV are to achieve a reduction in the serum HBV DNA levels and a normalization of serum ALT levels [17], in this study, neither virological response nor biochemical response (ALT level of 30 IU/L or lower) at 24 weeks by ETV treatment affected HCC incidence (Table 3). The cumulative incidence of HCC was almost equivalent between patients with and without virological response at 24 weeks in the analysis among all enrolled patients ($p = 0.685$; Fig. 3b). Additionally, there was no significant difference in the cumulative incidence of HCC between patients with or without normalization of ALT levels at 24 weeks ($p = 0.076$; Fig. 3c). The cumulative incidence of HCC significantly increased with higher AFP levels (10 ng/mL or greater) at 24 weeks even among patients who achieved virological response ($p = 0.023$) or normalization of ALT levels at 24 weeks ($p = 0.002$). The AFP levels at 24 weeks were closely related to HCC incidence irrespective of the virological response or biochemical response at 24 weeks in patients with HBV infection who were undergoing treatment with ETV.

The impact of AFP at 24 weeks on HCC incidence according to baseline factors

Because AFP levels at 24 weeks were found to be a significant factor related to HCC incidence among multiple factors that varied during treatment, the impact of AFP at 24 weeks on HCC incidence was assessed in the subgroups stratified by HCC-related factors at the baseline: age and the severity of liver disease. In the subgroup analysis stratified by age, AFP levels at 24 weeks were significantly related to HCC incidence, and the cumulative incidence of HCC at 5 years was significantly higher in patients with AFP levels of 10 ng/mL or higher at 24 weeks than those with AFP levels below 10 ng/mL, irrespective of age (younger than 55 years, 16.1 % vs 2.2 %, $p = 0.009$; 55 years or older, 45.4 % vs 14.9 %, $p < 0.001$; Fig. 4a, b). In the subgroup analysis that was stratified according to the severity of liver disease, the AFP level at 24 weeks was a significant factor in the cirrhotic group ($p = 0.029$) but not in the noncirrhotic group ($p = 0.377$); the cumulative incidence of HCC at 5 years in the cirrhotic group was

Table 2 Risk factors at the baseline for hepatocellular carcinoma (HCC) incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis		*p*	Multivariate analysis		*p*
		HR	95 % CI		HR	95 % CI	
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.592–8.560	0.002
	1:≥55	5.869			3.691		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.415–9.404	<0.001
	1:cirrhosis	7.553			4.765		
HBeAg	0:negative	1	0.412–1.436	0.410			
	1:positive	0.770					
Histology: activity	0:A0-1	1	0.352–3.800	0.810			
	1:A2-3	1.157					
Histology: fibrosis	0:F0-2	1	0.865–5.910	0.096			
	1:F3-4	2.262					
History of IFN therapy	0:none	1	0.032–1.718	0.154			
	1:presence	0.236					
Platelet count ($\times 10^4/\mu L$)	0:<15	1	0.103–0.449	<0.001			
	1:≥15	0.215					
Total bilirubin (mg/dL)	0:<1.0	1	1.235–4.141	0.008			
	1:≥1.0	2.261					
Albumin (g/dL)	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
PT (%)	0:<80	1	0.301–1.056	0.074			
	1:≥80	0.564					
ALT (IU/L)	0:<80	1	0.345–1.246	0.197			
	1:≥80	0.656					
HBV DNA(LC/mL)	0:<6.5	1	0.748–2.701	0.283			
	1:≥6.5	1.422					
AFP (ng/mL)	0:<10	1	1.040–3.721	0.038			
	1:≥10	1.967					

CI confidence interval, *HR* hazard ratio

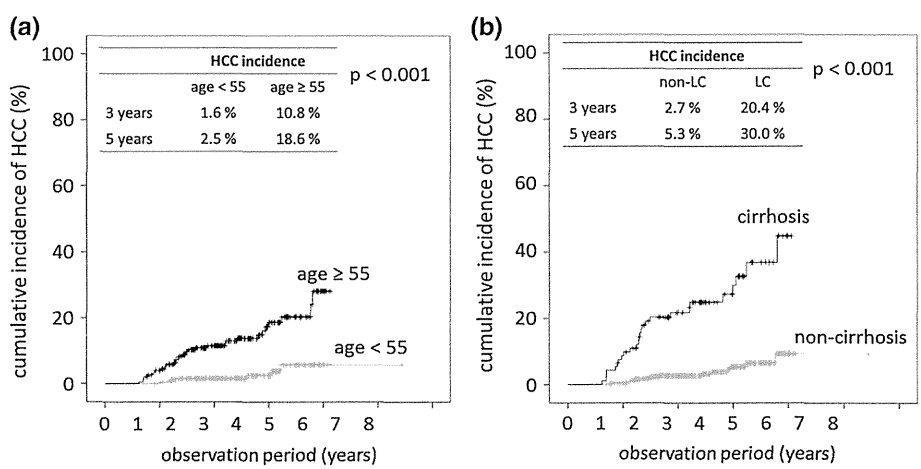

Fig. 2 Cumulative hepatocellular carcinoma (*HCC*) incidence among patients with hepatitis B virus (HBV) infection according to factors at the baseline (log-rank test). **a** Cumulative HCC incidence according to the age at the baseline (*black line* 55 years or older, *gray line* younger than 55 years). **b** Cumulative HCC incidence according to the severity of liver disease (*black line* cirrhosis, *gray line* no cirrhosis)

Table 3 Risk factors at 24 weeks after initiation of ETV treatment for HCC incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis			Multivariate analysis		
		HR	95 % CI	*p*	HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.198–6.748	0.018
	1:≥55	5.869			2.843		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.518–12.411	<0.001
	1:cirrhosis	7.553			5.590		
Platelet count ($\times 10^4/\mu L$) at 24 weeks	0:<15	1	0.114–0.473	<0.001			
	1:≥15	0.233					
Total bilirubin (mg/dL) at 24 weeks	0:<1.0	1	1.360–4.569	0.003			
	1:≥1.0	2.493					
Albumin (g/dL) at 24 weeks	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
ALT (IU/L) at 24 weeks	0:≤30	1	0.938–3.157	0.080			
	1:>30	1.720					
VR[a] at 24 weeks	0:none	1	0.461–1.664	0.685			
	1:presence	0.875					
AFP (ng/mL) at 24 weeks	0:<10	1	2.589–11.496	<0.001	1	1.066–5.316	0.034
	1:≥10	5.456			2.381		

VR virological response

[a] VR is defined as HBV DNA of less than 2.6 LC/mL

higher in patients with AFP levels of 10 ng/mL or greater at 24 weeks than in those with AFP levels below 10 ng/mL (50.0 % vs 24.7 %; Fig. 4c, d).

Risk analysis for HCC incidence among patients who achieved virological response by ETV treatment

Among patients with HBV infection who achieved virological response by ETV treatment, the risk analysis for HCC incidence was performed in a Cox proportional-hazards model according to the number of the following three risk factors: AFP levels at 24 weeks, age, and the presence of cirrhosis (Fig. S2). When the AFP level remained high (10 ng/mL or higher) at 24 weeks, the cumulative incidence of HCC at 5 years was 6.7 % with no other risk factors (Fig. S2a), 14.8 % with the factor of age of 55 years or older, 27.9 % with the factor of cirrhosis, and 57.7 % with the factors of age of 55 years or older and cirrhosis (Fig. S2b).

Discussion

ETV treatment has been reported to reduce serum HBV DNA levels and ALT levels in patients with chronic HBV infection and to improve hepatitis [18]. On the basis of a study that showed that a higher HBV DNA level at the baseline is associated with a higher HCC incidence in the natural history cohort (the REVEAL study) [15], a reduction of HBV DNA levels by ETV treatment has been considered to have the possibility to suppress HCC incidence among patients with chronic HBV infection. However, it was still unknown whether a lower or an undetectable level of serum HBV DNA, which was achieved by ETV treatment, has a suppressive effect on HCC incidence as shown in the natural course. In the present study, factors associated with HCC incidence during ETV treatment among patients with chronic HBV infection were investigated.

In a previous study that used a historical control group, a significant suppressive effect of ETV on HCC incidence was shown in cirrhotic but not noncirrhotic patients [19]. Furthermore, Wong et al. [20] reported that HCC incidence was significantly lower among patients with cirrhosis who had undetectable levels of HBV DNA compared with those with detectable levels of HBV DNA. In the present study, reduced serum HBV DNA levels were associated with a decrease in the cumulative incidence of HCC only in patients with cirrhosis, and not in those without cirrhosis (Fig. S3). Originally, HBV covalently closed circular DNA (cccDNA) levels in the hepatocyte nuclei were nearly parallel to the serum HBV DNA levels in the natural

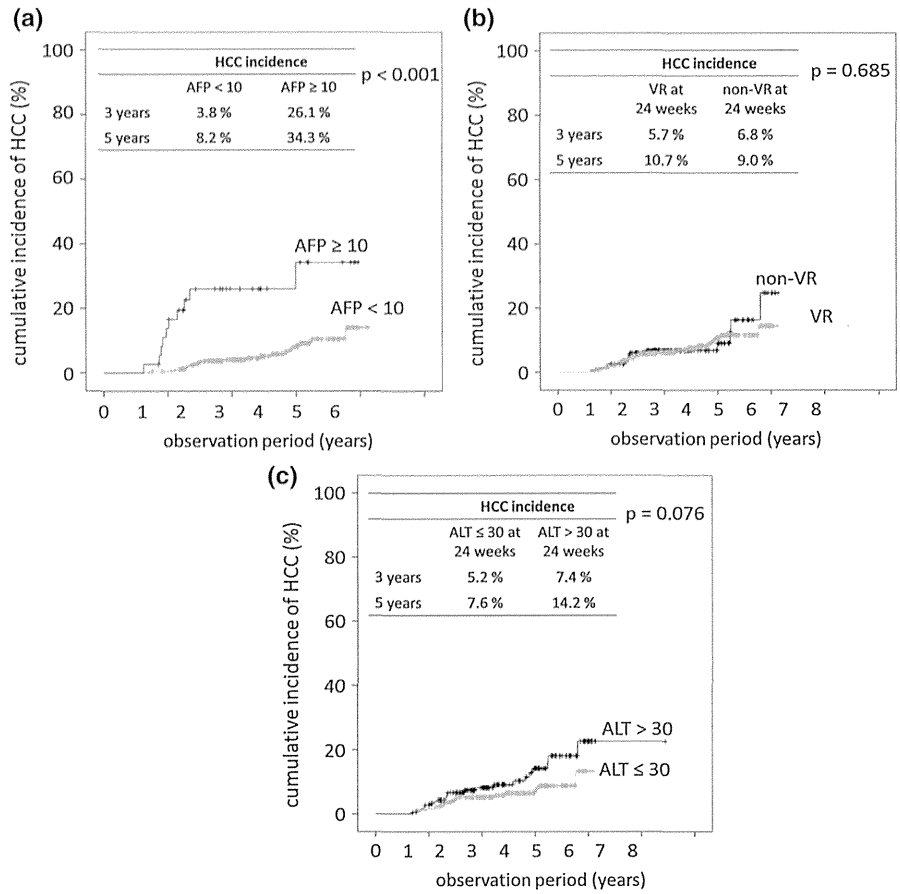

Fig. 3 Cumulative HCC incidence among patients with HBV infection according to factors at 24 weeks after ETV treatment initiation (log-rank test). Virological response (*VR*) is defined as HBV DNA of less than 2.6 log copies per milliliter. **a** Cumulative HCC incidence according to AFP levels at 24 weeks (*back line* AFP level of 10 ng/mL or greater at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks). **b** Cumulative HCC incidence according to virological response at 24 weeks (*black line* no VR at 24 weeks, *gray line* VR at 24 weeks). **c** Cumulative HCC incidence according to biochemical response at 24 weeks [*black line* alanine aminotransferase (*ALT*) level above 30 IU/L at 24 weeks, *gray line* ALT level of 30 IU/L or lower at 24 weeks]

course. However, low levels of serum HBV DNA achieved by ETV treatment do not always indicate low intracellular HBV cccDNA levels [21, 22]. Therefore, it is possible that an insufficient decrease of intracellular HBV DNA levels cannot bring the apparent HCC suppression in noncirrhotic liver with low malignant potential. A longer observation period is required to clarify the suppressive effect on HCC incidence among noncirrhotic patients. The relationship between HBV cccDNA levels in the liver and HCC incidence should also be examined.

In this study, in the analysis of the relationship between on-treatment factors and HCC incidence, only higher AFP levels (10 ng/mL or higher) at 24 weeks after the initiation of ETV treatment were found to be associated with HCC incidence. This is the first study to investigate the significance of AFP levels as a representative marker for the potential of HCC development among patients with chronic HBV infection undergoing ETV treatment. Originally, AFP was known as a tumor-associated antigen in HCC and as a target for immunotherapy. AFP has been used in the surveillance of HCC and in the evaluation of treatment response in HCC patients. The use of AFP as a marker to identify HCC among patients with HBV infection has previously been shown in patients with a natural course of the disease [23]. In recent reports that have focused on AFP levels for HCC diagnosis in patients undergoing ETV treatment, elevated AFP levels at 6 months before or at the time of HCC incidence were shown to be useful in detecting existing HCC [24, 25]; that is, elevated AFP levels implied the existence of cancer cells. However, the present study clarified that a high AFP level at 24 weeks did not suggest the existence of cancer cells, but indicates a potential for HCC incidence before the initiation of carcinogenesis. A possible reason is as follows. The AFP levels among patients who developed HCC decreased from 24 to 48 weeks after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence to the time of HCC incidence. Furthermore, it took a considerably long time before HCC incidence, on average 32.6 months of the observation period (Fig. S1). With regard to the relationship between serum AFP levels and HCC incidence among HCV-infected patients, AFP levels at 24 weeks after the end of IFN treatment have been associated with HCC [26, 27]. AFP levels after the

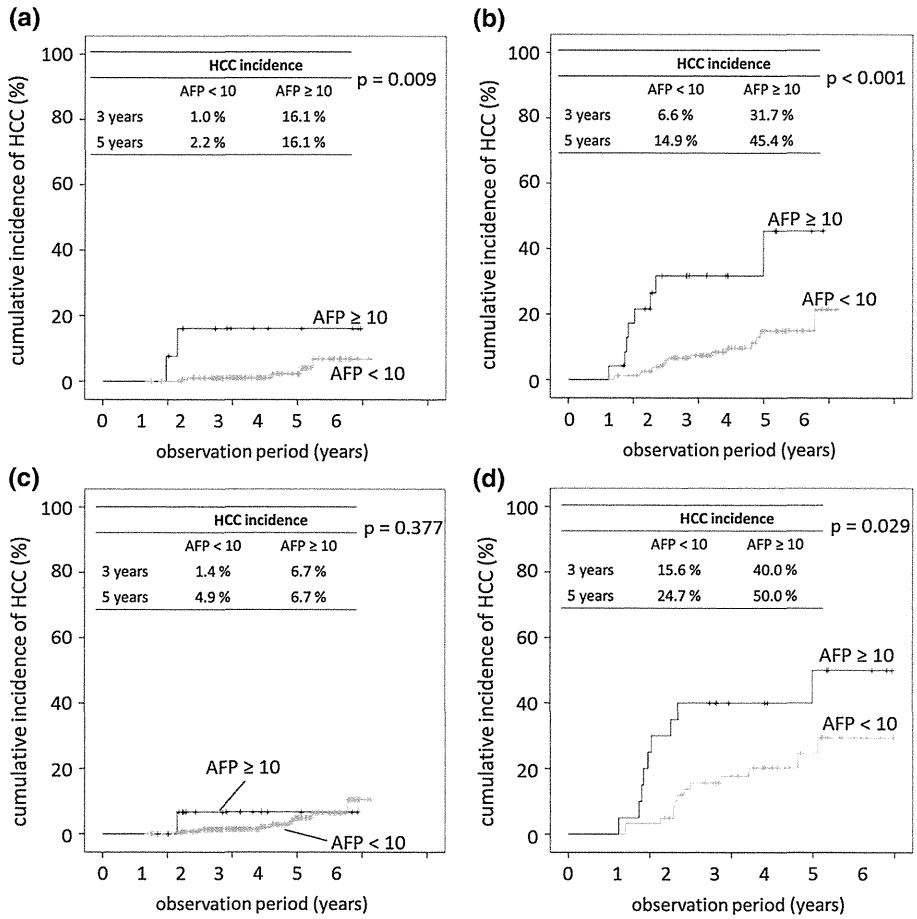

Fig. 4 Cumulative HCC incidence among patients with HBV infection according to AFP levels at 24 weeks after ETV treatment initiation, stratified with baseline factors (log-rank test). **a** Patients younger than 55 years. **b** Patients 55 years or older. **c** Patients without cirrhosis. **d** Patients with cirrhosis. *Black line* AFP level of 10 ng/mL or higher at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks

initiation of treatment of both HBV infection and HCV infection appear to have important implications for HCC incidence.

What the AFP levels at 24 weeks actually represent in patients undergoing ETV treatment is uncertain. The AFP level is a surrogate marker that appears to predict a disease condition from various pathological factors including inflammation, fibrosis, and liver regeneration, which involve carcinogenesis. Moreover, a previous study reported that the activation of natural killer cells by dendritic cells was inhibited when they were co-cultured with AFP; this result suggests an association between HCC development and the maintenance of high AFP levels [28]. Therefore, AFP is thought to be an important biomarker that can reflect various aspects of liver disease.

American Association for the Study of Liver Disease practice guidelines for the management of HBV have defined the goal of NA treatment as to decrease serum HBV DNA levels to undetectable levels to suppress HCC development. In this study, the HBV DNA levels and ALT levels were rapidly lowered in most patients. However, this study shows that the virological and biochemical treatment responses had no association with HCC development, whereas advanced age, liver cirrhosis, and a higher AFP level at 24 weeks after the initiation of ETV treatment were independent risk factors that were significantly associated with HCC development. It is considered that decreasing serum HBV DNA levels to undetectable levels is the necessary, but not sufficient condition to suppress HCC development. In fact, the HCC incidence rate even in patients undergoing ETV treatment who achieved virological response at 24 weeks with the three factors of age of 55 years or older, liver cirrhosis, and AFP level of 10 ng/mL or higher increased to as high as approximately 60 % at 5 years (Fig. S2). Accordingly, the undetectable HBV DNA level in patients with chronic HBV infection undergoing ETV treatment is in itself of little consequence and does not mean a riskless environment.

The limitation of this study is that analysis including other HCC-related factors, such as hepatitis B surface antigen levels, precore and core promotor mutations, and family history of HCC or alcohol consumption, was not