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

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The authors declare no conflicts of interest.

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.

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Short Communication

In vitro replication competence of a hepatitis B genotype D/A recombinant virus: dissimilar biological behaviour regarding its parental genotypes

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Hepatitis B virus (HBV) DNA recombinants contribute to ~30 % of the overall full-length sequences already deposited in GenBank. However, their biological behaviour has not been analysed so far. In this study, the *in vitro* replication kinetics of the first D/A recombinant from the American continent differed from its parental genotypes, exhibiting higher extracellular levels of HBV DNA and hepatitis B e antigen. Southern blots of intracellular core-associated HBV DNA were in agreement with such results. Because this recombinant was obtained from an Argentinian injecting drug user belonging to a vulnerable community, these results are of singular relevance for regional public health. Further *in vivo* studies are urgently needed to determine the pathogenicity of these replicative competent clones.

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Hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections among human beings. It often leads to cirrhosis and/or hepatocellular carcinoma, which is annually responsible for 1 million deaths worldwide. As a result, it is considered one of the major world health concerns.

Eight HBV genotypes (HBV/A–HBV/H) have been reported based on a sequence divergence greater than 8 % over the entire genome. Another two genotypes referred to as HBV/I and HBV/J have also been proposed. Genotypes are further subdivided into subgenotypes, which have been recognized in HBV/A–D and F, if the divergence in the whole genome reaches between 4 and 8 % (Lin & Kao, 2011). The global impact of HBV recombinants has also been described recently (Shi *et al.*, 2012).

Evidence for the influence of HBV genotypes and/or subgenotypes on the progression of liver diseases in acute, fulminant and chronic infection, the clinical outcome and the response to antiviral treatment have been reported by several researchers (Kramvis & Kew, 2005; Lin & Kao, 2011; Liu *et al.*, 2005). However, information about the effects of recombinant genomes on the clinical, prognostic and therapeutic aspects of the HBV infection is still lacking. Therefore, the aim of this study was to preliminarily analyse the very early replication dynamics of the infection of a HBV D/A recombinant and compare them with those

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Two supplementary figures are available with the online version of this paper.

of its parental genotypes (HBV/D and HBV/A) and of a highly replicative genotype (HBV/C) in an *in vitro* experimental system.

Serum samples were obtained from two previously recruited subjects (Trinks *et al.*, 2008): (i) H-IDU6 who was chronically infected with an HBV/D3 genome, as determined by partial S and pre-C/C phylogenetic analysis (PHYLIP package version 3.5c: Joseph Felsenstein, University of Washington, Seattle, WA, USA); and (ii) H-IDU7 who showed a HBV 'false' occult infection (Raimondo *et al.*, 2008) by a D3/A2 recombinant genome (breakpoints at nt 147 and 636, according to *Eco*RI restriction site numbering), as characterized by full-length phylogenetic and Simplot version 3.5.1 (Stuart Ray, John Hopkins University, Baltimore, MD, USA) analysis. Accordingly, this strain exhibited a recombinant HBV/A2 DNA region which corresponded to nt 147–636 of the S gene inserted in a backbone corresponding to HBV/D3.

HBV DNA was extracted from serum using QIAamp DNA blood kits (Qiagen). First, in order to fully characterize H-IDU6 HBV DNA, the complete genome was amplified and analysed by a reported method (Trinks *et al.*, 2008). Then, pUC19 plasmids deprived of promoters (Invitrogen) carrying a 1.24-fold HBV genome of each sample were constructed as described previously (Sugiyama *et al.*, 2006). Plasmids for HBV/A2 and HBV/C (Sugiyama *et al.*, 2006) were also included in this study.

After 24 h of culture, Huh7 cells were transfected with plasmids equivalent to 24 μg HBV DNA constructs using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection efficiency was monitored by GFP expression using flow cytometry (BD FACSCanto; BD Biosciences) after cell transfection with a pTARGET (Promega)–GFP expression vector. Except for Southern blotting, all experiments were conducted twice for each clone.

At 24 and 72 h post-transfection (p.t.), hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined from the supernatant by ARCHITECT (Abbott). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also measured to determine cell viability. Supernatants from cells treated solely with Lipofectamine were included as negative controls.

At 72 h p.t., in order to measure HBV DNA viral load in supernatants (ruling out extracellular free HBV DNA or HBV RNA), such fluids were ultracentrifuged at 22 000 ***g*** for 5 min to enrich HBV and HBV core particles; pellets were resuspended and jointly treated with DNase I and RNase A at 37 °C for 3 h (Sugiyama *et al.*, 2006). To confirm the validity of the DNA extraction method (only from virions and capsids, but not from free recombinant plasmid DNA) in the supernatants, Huh7 cells mock transfected with HBV/C plasmid in the absence of Lipofectamine were considered as a further (expected) negative control due to DNase and RNase treatment. HBV load was measured subsequently (COBAS TaqMan HBV Test; Roche).

At 72 h p.t., in order to confirm the HBV replication among all the studied clones, cells were lysed and the density of core-associated HBV DNA was compared by Southern blot hybridization with a mix of full-length probes of each genotype involved in the experiment (A2, C, D3 and D3/A2; Sugiyama *et al.*, 2006).

Student's *t*-test was used to compare the means and SD between any pair of samples: $P<0.05$ was considered statistically significant.

Biochemical and virological features of both patients from whom sera were obtained are shown in Table 1. The full-length genome was amplified from sample H-IDU6 and subjected to phylogenetic analysis. This sample was ascribed to HBV/D3 and the presence of recombination was ruled out by Simplot (Figs S1 and S2b, available in JGV Online). None of the isolates possessed the mutation G1896A, A1762T or G1764A, which could have interfered with the expression of HBeAg and the efficiency of pre-genome encapsidation for replication. As expected for HBV/D, T1858 was observed in both isolates.

For construction of HBV D3/A2 recombinant and HBV/D3 vectors (Sugiyama *et al.*, 2006), at least 25 clones for each PCR-amplified HBV hemigenome [fragments A (nt 17–1799) and B (nt 1595–239); Sugiyama *et al.*, 2006] from each sample were sequenced and phylogenetically analysed. All clones from the HBV/D3 sample were ascribed to the D3 subgenotype. With regard to the D3/A2 recombinant sample, all 25 clones derived from fragment B were ascribed to the D3 subgenotype; in contrast, 40 % of the analysed clones from fragment A were D3/A2 recombinants with breakpoints at nt 147 and 636, 32 % belonged to the A2 subgenotype, 16 % to recombinant clones with breakpoints at nt 505 and 630, 8 % to recombinant clones

Table 1. Biochemical and virological features of patients from whom HBV isolates were recovered

Feature	H-IDU6	H-IDU7
Gender	Male	Male
Age	35	26
HBsAg	+	–*
HBeAg	+	+
Anti-HBc Ab	+	+
Anti-HCV Ab	+	+
Anti-HIV Ab	+	+
HBV viral load	$>110\times10^6$ IU ml^{-1}	$>110\times10^6$ IU ml^{-1}
HBV genotype	D3	D3/A2 recombinant

*T113S and T131N mutants were detected within the major hydrophilic region of the deduced S amino acid sequence. A negative result for HBsAg had been originally obtained with the serum collected in 1995 and then studied with AxSYM (Abbott) (Trinks *et al.*, 2008). These mutants became detectable when supernatants collected from Huh7 transfected cells were tested by means of the ARCHITECT assay (Abbott) in this study.

with breakpoints at nt 519 and 630, and 4 % to D3 subgenotype (Fig. 1a). Because the most abundant recombinant clones were those exhibiting breakpoints at nt 147 and 636 (Fig. 1a and Fig. S2a), they were considered representative of the whole viral population and thus selected for D/A replicon construction.

Transfection efficiency ranged from 24.1 to 24.4 % and cell viability was similar in all groups ($P>0.05$; data not shown, available upon request).

At 24 h p.t., the D3/A2 clone produced the highest levels of both antigens ($P<0.0001$; Fig. 1b). Moreover, at 72 h p.t., the HBsAg levels from HBV/A2 and the recombinant clone were the highest ($P>0.05$), followed by HBV/C and HBV/D3 ($P<0.0001$; Fig. 1b). At this time point, the recombinant clone produced the highest levels of HBeAg compared with its parental genotypes and also the HBV/C clone ($P<0.0001$; Fig. 1b).

At 72 h p.t., the HBV/C clone showed the highest viral load in the supernatant, closely followed by the recombinant clone, whose extracellular HBV DNA level was, in turn, higher than those from its parental genotypes ($P>0.05$; Fig. 1c).

Southern blotting undoubtedly confirmed previously published results regarding the HBV/C clone, which exhibited the highest intracellular replication level (Sugiyama *et al.*, 2006). Interestingly, the level of the recombinant was higher than those from its parental genotypes (Fig. 1d).Negative controls processed in parallel confirmed the specificity of the above-mentioned results.

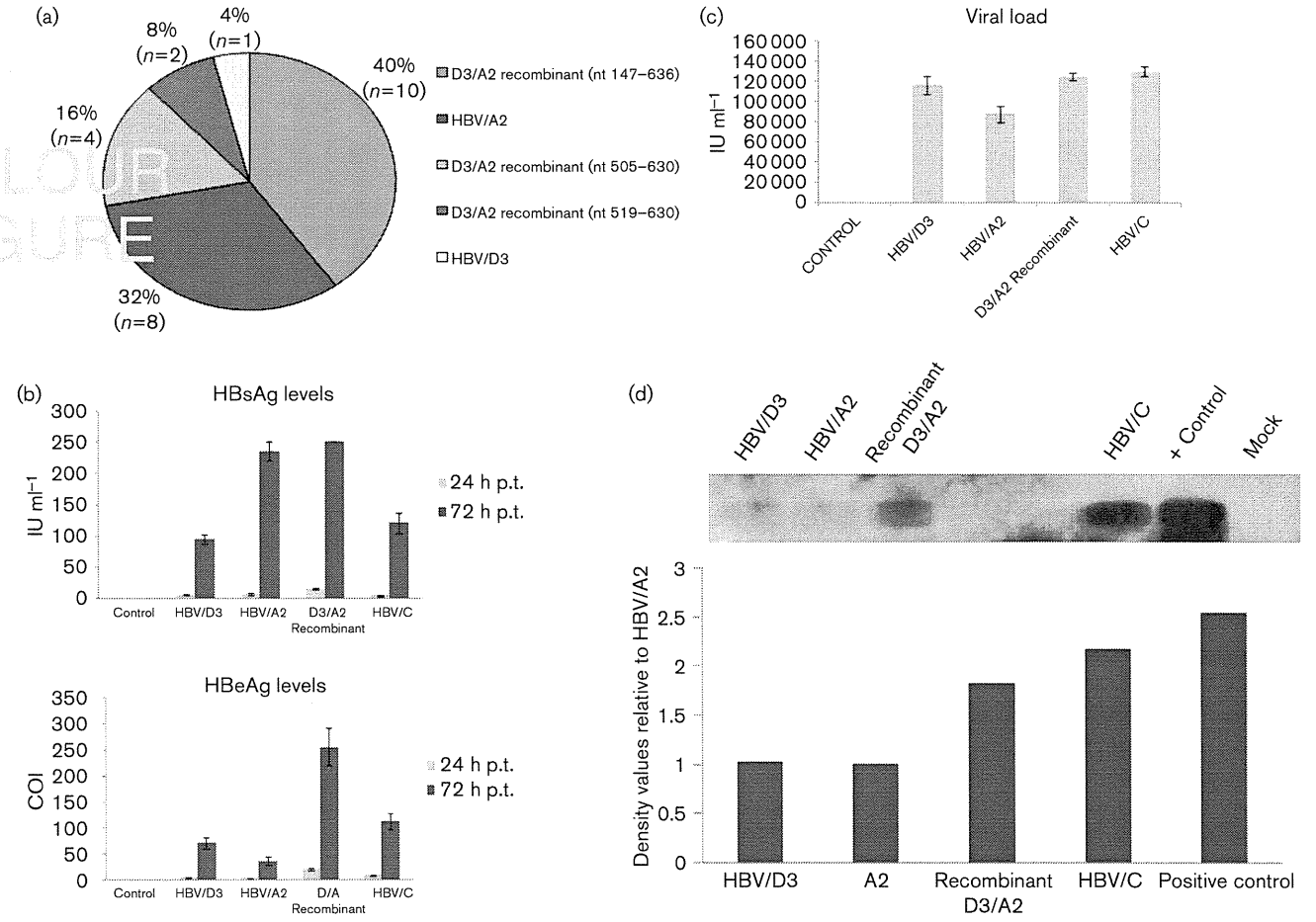

Fig. 1. (a) Analysis of clones derived from fragment A obtained from the recombinant strain H-IDU7. (b) HBsAg and HBeAg extracellular levels. COI, cut-off index. (c) HBV viral load in supernatant. (d) Core-associated HBV DNA in Southern blot analysis of Huh7 cell lysates transfected with plasmid constructs of genotype HBV/D3, HBV/A2, D3/A2 recombinant and HBV/C. An aliquot of non-transfected unlabelled full-length HBV/A2 probe (3.2 kb; positive control) and Huh7 cell lysates treated solely with transfection reagent (mock) were also included. The density of the bands corresponding to a hybridization signal was normalized to that obtained with the A2 clone, which exhibited the lowest density value (density=1). An asterisk represents a statistical difference of $P<0.0001$ when compared with all the remaining genotypes. Double asterisks indicate a statistical difference of $P<0.0001$ in comparison with genotypes HBV/D3 and HBV/C. The absence of asterisks represents no statistical difference.

The influence of genotypes and/or subgenotypes on disease progression and clinical outcome of HBV infection is well documented. However, information regarding HBV recombinant behaviour is unknown.

In a previous study (Trinks *et al.*, 2008), our group isolated a novel intergenotypic D/A recombinant strain from a patient (H-IDU7) co-infected with HIV/HCV. This strain, which was the first full-length D/A recombinant genome characterized from the American continent, exhibits a HBV/D3 backbone genome with an inserted segment of HBV/A2 within the Pol gene. After cloning this sample, we documented the co-circulation of HBV/D3, HBV/A2 genomes together with three different types of D/A recombinants of which one was dominant. In future studies, it will be interesting to compare the biological behaviour of these three dissimilar recombinants in an attempt to elucidate the reason(s) for the observed dominance of the D3/A2 recombinant clones with nt 147–636 breakpoints.

The observation of pure HBV/D3 and HBV/A2 clones confirms that co-infection with different HBV genotype strains is a prerequisite for recombination (Zhou *et al.*, 2012). However, the mechanism of selection of a given strain in mixed infections, i.e. DNA exchange or (less likely throughout the lifespan of a given individual) DNA mutation evolution, still remains unknown.

In this study, the replication kinetics of this recombinant differed from those of its parental genotypes, exhibiting higher extracellular levels of HBV DNA, similar (to A2) or higher (than D3) HBsAg, and higher (than both) HBeAg values. The significance of these findings should be explored by using the primary hepatocyte infection and also *in vivo* uPA-SCID mice models.

Taking into account that one of the HBV DNA-binding sites for CREB transcription factor is placed at nt 143–154 and that it enhances HBsAg expression levels, as previously shown for an A2 replicon (Tacke *et al.*, 2005), it seems plausible that those genomes showing the CCTGTG-ACGAAC binding site would exhibit similarly high HBsAg expression. This sequence was observed in the recombinant clone, as the 5′ breakpoint for the A2 insert is placed at nt 147. Interestingly, such a binding site is mutated in the HBV/D3 replicon (CCTGCGCTGAAC, mutations underlined), which could account for a lower level of pre-S/S transcription efficiency for such a genotype (in contrast to HBV/A2) and consequently for a lower level of HBsAg expression, as reported previously (Sugiyama *et al.*, 2006), the latter result also being observed in our study. Although HBsAg levels frequently reflect intrahepatic HBV replication in WT genomes (Chan *et al.*, 2011), they do not necessarily mirror HBV DNA levels in some mutated pre-S/S genomes (Pollicino *et al.*, 2012). Moreover, it has been shown that HBV/A2 is associated with higher HBsAg secretion and lower DNA replication compared with other genotypes (Sugiyama *et al.*, 2006). Interestingly, our recombinant clone produced high levels of HBsAg, HBeAg and DNA viral load, whose highest titres are usually associated with HBeAg secretion. These results might be explained by the presence of an A2 insert in the PreS2/S region and a D3 backbone in the pre-C/C region.

However, the recombinant exhibited even higher HBeAg values and intracellular HBV DNA levels than the parental D3 clone, which could be explained by the presence of mutations T1766 and A1770 in the D3 backbone of the recombinant D3/A2 clone, which form putative hepatocyte nuclear factors 1 (HNF1)- and HNF3-binding sites related to enhanced viral replication (Baumert *et al.*, 1996; Günther *et al.*, 1996; Fig. 2). Moreover, the single mutation T1664C observed within the core upstream regulatory sequence (CURS; nt 1636–1742) in the recombinant, but which was absent in the D3 parental genotype, might also account for such a difference in HBeAg secretion. As the CURS region exerts a strong stimulating effect on the basal core promoter (Yuh *et al.*, 1992), it is tempting to speculate that such a mutation might produce the higher HBeAg levels observed with the recombinant compared with the D3 parental clone.

Because this recombinant strain was obtained from an intravenous drug user belonging to a highly vulnerable

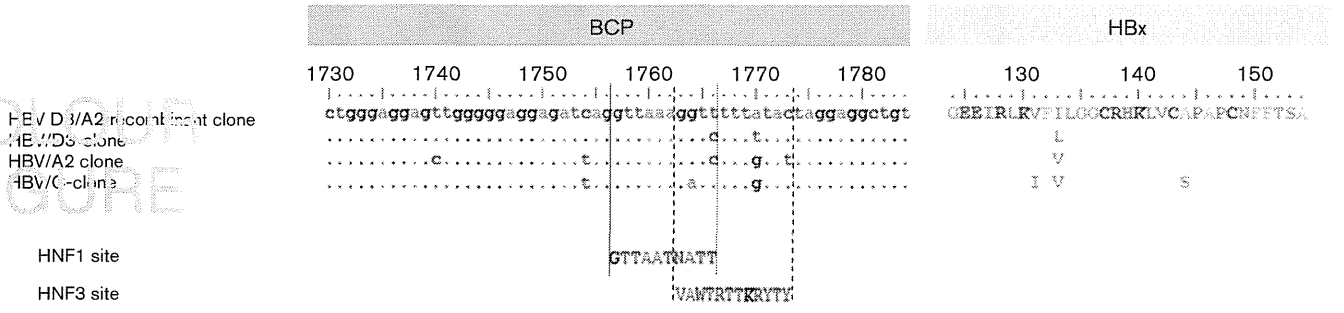

Fig. 2. Sequences of the basal core promoter (BCP) nt 1730–1785 and the overlapping region of the X protein of the HBV clones. The binding sites for HNF1 and HNF3 are aligned with the corresponding region of the HBV genome. The symbols for nucleotide ambiguities are as follows: V, A/C/G; W, A/T; R, A/G; K, G/T; Y, C/T.

group in Argentina, these results are of singular relevance for regional public health. Further *in vivo* studies are needed to determine the pathogenicity of these replicative competent clones.

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References

Baumert, T. F., Rogers, S. A., Hasegawa, K. & Liang, T. J. (1996). Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J Clin Invest* **98**, 2268–2276.

Chan, H. L., Thompson, A., Martinot-Peignoux, M., Piratvisuth, T., Cornberg, M., Brunetto, M. R., Tillmann, H. L., Kao, J. H., Jia, J. D. & other authors (2011). Hepatitis B surface antigen quantification: why and how to use it in 2011: a core group report. *J Hepatol* **55**, 1121–1131.

Günther, S., Piwon, N., Iwanska, A., Schilling, R., Meisel, H. & Will, H. (1996). Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *J Virol* **70**, 8318–8331.

Kramvis, A. & Kew, M. C. (2005). Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. *J Viral Hepat* **12**, 456–464.

Lin, C. L. & Kao, J. H. (2011). The clinical implications of hepatitis B virus genotype: recent advances. *J Gastroenterol Hepatol* **26** (Suppl 1), 123–130.

Liu, C. J., Kao, J. H. & Chen, D. S. (2005). Therapeutic implications of hepatitis B virus genotypes. *Liver Int* **25**, 1097–1107.

Pollicino, T., Amaddeo, G., Restuccia, A., Raffa, G., Alibrandi, A., Cutroneo, G., Favaloro, A., Maimone, S., Squadrito, G. & Raimondo, G. (2012). Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. *Hepatology* **56**, 434–443.

Raimondo, G., Allain, J. P., Brunetto, M. R., Buendia, M. A., Chen, D. S., Colombo, M., Craxì, A., Donato, F., Ferrari, C. & other authors (2008). Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* **49**, 652–657.

Shi, W., Carr, M. J., Dunford, L., Zhu, C., Hall, W. W. & Higgins, D. G. (2012). Identification of novel inter-genotypic recombinants of human hepatitis B viruses by large-scale phylogenetic analysis. *Virology* **427**, 51–59.

Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S. K., Gish, R. G., Kramvis, A., Shimada, T. & other authors (2006). Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* **44**, 915–924.

Tacke, F., Liedtke, C., Bocklage, S., Manns, M. P. & Trautwein, C. (2005). CREB/PKA sensitive signalling pathways activate and maintain expression levels of the hepatitis B virus pre-S2/S promoter. *Gut* **54**, 1309–1317.

Trinks, J., Cuestas, M. L., Tanaka, Y., Mathet, V. L., Minassian, M. L., Rivero, C. W., Benetucci, J. A., Gímenez, E. D., Segura, M. & other authors (2008). Two simultaneous hepatitis B virus epidemics among injecting drug users and men who have sex with men in Buenos Aires, Argentina: characterization of the first D/A recombinant from the American continent. *J Viral Hepat* **15**, 827–838.

Yuh, C. H., Chang, Y. L. & Ting, L. P. (1992). Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J Virol* **66**, 4073–4084.

Zhou, B., Wang, Z., Yang, J., Sun, J., Li, H., Tanaka, Y., Mizokami, M. & Hou, J. (2012). Novel evidence of HBV recombination in family cluster infections in western China. *PLoS ONE* **7**, e38241.

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BRIEF ARTICLE

Incidence and characteristics of HBV reactivation in hematological malignant patients in south Egypt

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Abstract

AIM: To investigate characteristics of hepatitis B virus (HBV) implicated in HBV reactivation in patients with hematological malignancies receiving immunosuppressive therapy.

METHODS: Serum samples were collected from 53 patients with hematological malignancies negative for hepatitis B surface antigen (HBsAg) before the start of and throughout the chemotherapy course. HBV reactivation was diagnosed when the HBsAg status changed from negative to positive after the initiation of chemotherapy and/or when HBV DNA was detected by real-time detection polymerase chain reaction (RTD-PCR). For detecting the serological markers of HBV infection, HBsAg as well as antibodies to the core antigen (anti-HBc) and to the surface antigen (anti-HBs) were measured in the sera by CEIA. Nucleic acids were extracted from sera, and HBV DNA sequences spanning the S gene were amplified by RTD-PCR. The extracted DNA was further subjected to PCR to amplify the complete genome as well as the specific genomic sequences bearing the enhancer Ⅱ/core promoter/pre-core/core regions (nt 1628-2364). Amplicons were sequenced directly.

RESULTS: Thirty-five (66%) of the 53 HBsAg-negative patients were found to be negative serologically for anti-HBc, and the remaining 18 (34%) patients were positive for anti-HBc. Five of the 53 (9.4%) patients with hematologic malignancies experienced HBV reactivation. Genotype D1 was detected in all five patients. Four types of mutant strains were detected in the S gene of HBV strains and were isolated from 3 patients with HBV reactivation: T/S120, L143, and I126. HBV DNA was detected in the pretreatment HBsAg-negative samples in one of the five patients with HBV reactivation. In this patient, sequences encompassing the HBV full genome obtained from sera before the start of chemotherapy and at the time of *de novo* HBV hepatitis were detected and it showed 100% homology. Furthermore, in the phylogenetic tree, the sequences were clustered together, thereby indicating that this patient developed reactivation from an occult HBV infection.

CONCLUSION: Past infection with HBV is a risk fac-

tor for HBV reactivation in Egypt. Mandatory anti-HBc screening prior to chemotherapy in patients with hematological malignancies is recommended.

Key words: Hepatitis B virus; Occult infection; Reactivation; *De novo*; Hepatitis B surface antigen

Core tip: The study aimed to investigate characteristics of hepatitis B virus (HBV) implicated in HBV reactivation in patients with hematological malignancies receiving immunosuppressive therapy in Egypt. Fifty-three hepatitis B surface antigen (HBsAg)-negative patients treated with chemotherapy were included in the study. The incidence of HBV reactivation was 9.4% among the studied cohort, and all of the affected individuals were positive for HBsAg as well as antibodies to the core antigen. The present study provides further evidence via molecular evolutionary analysis of the development of HBV reactivation from an occult HBV infection. Past infection with HBV is a risk factor for HBV reactivation in Egypt. Mandatory anti-HBc screening prior to chemotherapy in patients with hematological malignancies is suggested.

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INTRODUCTION

Infection with hepatitis B remains one of the major causes of acute and chronic liver disease. An estimated 350-400 million people are chronically infected with hepatitis B virus (HBV) worldwide[1].

The reactivation of hepatitis B infection has been recorded in many clinical settings: chronic HBV infection after the cessation of HBV treatment, patients with malignant disease who receive immunosuppressant or chemotherapy, patients with end stage renal failure, and patients co-infected with HIV[2-6]. Patients with resolved HBV infection are diagnosed serologically by clearance of serum HBsAg and the appearance of the hepatitis B core antibody (anti-HBc), with or without antibodies to hepatitis B surface antigen (anti-HBs)[7]. These patients are at risk of hepatitis B reactivation due to any factor that can suppress the immune system[8,9]. *De novo* hepatitis B is of particular concern in this subset of patients because it commonly leads to severe liver dysfunction and fatal hepatitis[10,11].

Occult hepatitis B is defined by the presence of HBV DNA in the serum or the liver in the absence of HBsAg, with or without anti-HBc or antibodies to HBV surface antigen (anti-HBs). In these patients, a low level of HBV replication has been shown to persist in the liver and in peripheral blood mononuclear cells for decades[12]. Occult HBV infection is observed worldwide, and its prevalence is related closely to the endemicity of HBV infection[13,14].

Large scale geographic heterogeneity in the prevalence of HBV had been reported worldwide. Africa is one of the highly endemic regions of HBV, and an intermediate endemicity of HBV infection had been recorded in Egypt[15,16].

The aim of this study was to investigate the incidence of HBV reactivation and the underlying risk factors of hepatitis B reactivation in Egyptian patients who received cytotoxic chemotherapy for hematological malignancies.

MATERIALS AND METHODS

Patients

Fifty-nine consecutive patients with hematological malignancies were admitted to the oncology department of Sohag Faculty of Medicine and South Egypt Cancer Institution from November 2010 to October 2011. After admission, all patients underwent physical examination and blood and serum biochemistry analyses. All of patients received chest computed tomography and ultrasonography of the abdomen as an initial evaluation.

In clinical practice, patients are monitored during chemotherapy using liver function tests. HBsAg and HBV DNA are tested in patients with elevated liver enzymes. For the purpose of this study, serum samples were collected before and after the start of the chemotherapy course. The collected sera were stored at -80 ℃ for future examination of HBsAg, anti-HBs, and anti-HBc. HBV reactivation was diagnosed when the HBsAg status changed from negative to positive after the initiation of chemotherapy and/or when HBV DNA was detected as measured by real-time detection polymerase chain reaction (RTD-PCR) using stored samples from patients, as described latter.

Serological markers of HBV infection

HBsAg was measured by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of the IgG class was determined by radioimmunoassay (Abbott Japan). All serologic assays were performed according to the manufacturer's instructions.

Detection and quantitation of serum HBV DNA

HBV-DNA sequences spanning the S gene were amplified by RTD-PCR according to the previously described protocol with a slight modification and a detection limit of 100 copies/mL (equivalent to 20 IU/mL)[17].

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100

Table 1 Characteristics of 53 patients with malignant hematologic disease who were negative for hepatitis B surface antigen

	Total (n = 53)	Anti-HBc positive (n = 18)	Anti-HBc negative (n = 35)	P-value
Age[1] (yr)	27.8 ± 26.2	34.4 ± 27.9	27.7 ± 25.4	0.42
Gender (Male)	26 (49.1)	10 (55.6)	16 (45.7)	0.56
Diagnosis				
Malignant lymphoma	26 (40.1)	9 (50)	17 (48.6)	1.00
Acute leukemia	25 (47.2)	9 (50)	15 (42.9)	0.77
Chronic leukemia	1 (1.9)	0	1 (2.9)	1.00
Multiple myeloma	1 (1.9)	0	1 (2.9)	1.00

[1]mean ± SD. anti-HBc: Hepatitis B surface antigen as well as antibodies to the core antigen.

mL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany)

Extracted DNA was subjected to PCR for amplifying the complete genome and the specific genomic sequences bearing enhancer II /core promoter/pre-core/core regions [nt 1628-2364], as described previously[18]

Amplicons were sequenced directly using the ABI Prism Big Dye ver. 3.1 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, United States).

All sequences were analyzed in both the forward and reverse directions. HBV genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTALX, and genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/)[19]. Based on the obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times for analysis by the ODEN program of the National Institute of Genetics.

Statistical analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent t-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL, United States). P-values (two-tailed) less than 0.05 were considered statistically significant.

Ethical consideration

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from all patients.

RESULTS

Patient characteristics

Six of the 59 patients with hematologic malignancies were found to be HBsAg positive and were excluded from the analysis. Therefore, a total of 53 HBsAg-negative patients were checked for the serological markers of infection with hepatitis B. The background general characteristics of the 53 HBsAg-negative patients are presented in Table 1. The mean age of the analyzed cohort was 27.8 ± 26.2 years-old. Thirty-five (66%) of 53 HBsAg-negative patients were found to be anti-HBc-negative, and 18 (34%) patients were serologically positive for anti-HBc. The predominance of male patients was observed in both the anti-HBc-positive and -negative patient groups. Twenty-six patients (40.1%) were diagnosed with malignant lymphoma, whereas 25 patients (47.2%) were diagnosed with acute leukemia. Solitary cases of chronic leukemia and multiple myeloma were also included in the studied cohort. An insignificantly higher incidence of acute leukemia cases was observed in the anti-HBc-positive patients (9/18; 50%) compared with the anti-HBc-negative patients (15/35; 42.9%).

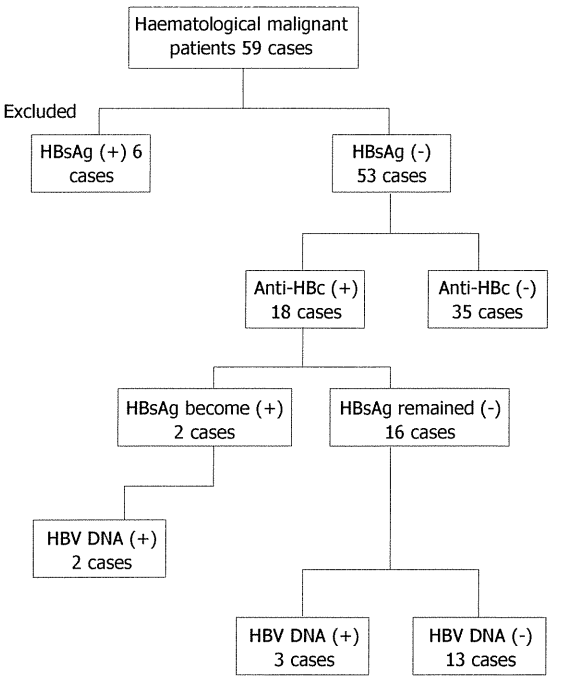

Figure 1 Longitudinal representation of hepatitis B reactivation after chemotherapy in patients with hematological malignancies. HBsAg: Hepatitis B surface antigen; anti-HBc: Antibody to hepatitis B core antigen.

Consequences of HBV serology after receiving anticancer treatment

After the initiation of systemic chemotherapy, examination of the HBV serology revealed that two (3.8%) of the HBsAg-negative patients became serologically positive for HBsAg. In addition, 3 more patients (5.8%) exhibited detectable HBV DNA in their sera after the start of the anticancer therapy (Figure 1). Interestingly, none of the serologically negative patients for anti-HBc became serologically positive for HBsAg or molecularly detectable for HBV DNA. In contrast, 2 of the 18 anti-HBc-positive patients (11.1%) became serologically positive for the

Table 2 Clinical and virological characteristics of patients who experienced hepatitis B reactivation

	Case 1	Case 2	Case 3	Case 4	Case 5
Age/gender	79/F	8/M	11/F	5/M	20/M
Diagnosis	NHL (stage III)	AML	ALL	ALL	ALL
Treatment*	CVP	St Jude protocol	St Jude protocol	St Jude protocol	St Jude protocol
HBV serology and DNA prior to chemotherapy					
HBsAg/anti-HBs/HBV DNA	(-)/(+)/1.8 log copy/ml	(-)/(+)/Negative	(-)/(-)/Negative	(-)/(+)/Negative	(-)/(nt)/Negative
HBV reactivation months after anti-cancer therapy	12	4	5	6	4
HBV serology and DNA after chemotherapy					
HBsAg/anti-HBs/HBV DNA	(+)/(nt)/7.6 log copy/mL	(+)/(+)/5.8 log copy/mL	(-)/(-)/3.1 log copy/mL	(-)/(+)/2.9 log copy/mL	(-)/(nt)/2.0 log copy/mL
ALT (IU/mL)	35	195	27	86	17
Total bilirubin (mg/dL)	1	1.1	1.3	1.1	0.2
Outcome	Died	Died	Died	Alive	Alive
HBV genotype	D1	D1	D1	D1	D1
Core promoter mutation	Wild	T1764/G1766	A1764	Wild	-
Pre-core A1896	Mutant	Wild	Wild	Wild	-
Amino acid mutation in S gene product	P120S S143L	P120T	-	T126I	-

M: Male; F: Female; NHL: Non-Hodgkin lymphoma; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; HBsAg: Hepatitis B surface antigen; anti-HBs: Antibody to hepatitis B surface antigen; CVP: Cyclophosphamide, vincristine, prednisone; ALT: Alanine amino transferase enzyme. St Jude protocol: (1) prephase: vincristine + steroid; (2) induction: vincristine + farmarabin + aracytine + etoposide, intrathecal; (3) consolidation: high dose methotrexate + mercaptopurine; (4) continuation: methotexate+mercaptopurine.

HBsAg, and 3 (16.7%) became molecularly detectable for the HBV DNA. In brief, 5 of the 53 HBsAg negative patients (9.4%), representing 27.8% (5/18) of the anti-HBc-positive patients in the studied cohort, manifested the criteria of HBV reactivation (Figure 1).

Clinical and virological criteria of the patients who manifested HBV reactivation

Five of the 53 patients (9.4%) treated for hematologic malignancies manifested HBV reactivation throughout the anti-cancer therapy regimen. The demographic, clinical and virological criteria of the HBV infection of the five patients who experienced HBV reactivation are summarized in Table 2 (cases 1-5). The mean age of the five patients was 24.6 ± 30.9 years old. Three of the patients were males (cases 2, 4 and 5), and two were females. Four patients were diagnosed with acute leukemia (cases 2, 3, 4, and 5), and only one patient (case 1) was diagnosed with malignant lymphoma. All of the 5 patients received a steroid regimen as a part of their anticancer therapy. All 5 patients were positive for anti-HBc. Three patients (cases 1, 2 and 4) were positive for anti-HBs (cases 1, 2, and 4), and only one patient was serologically negative for the anti-HBs (case 3). Because of small volume of serum sample obtained from case 5, anti-HBs could not be tested. After HB reactivation, two cases (cases 2 and 4) exhibited abnormal ALT levels, and one patient (case 2) experienced a more than 3-fold increase in the ALT level, indicating the emergence of hepatitis in this patient. None of the 5 cases who experienced had the HBV reactivation after cancer chemotherapy received an antiviral treatment for HBV.

The virological and molecular criteria are summarized in Table 2. The infecting genotype of the HBV strains was HBV genotype D, subtype D1 in all five cases. Two core promoter HBV variants were detected in 2 patients. The two variants were T1764/G1766 and A1764 in cases 2 and 3, respectively. The stop codon pre-core HBV mutant (A1896) was detected in one patient (case 1).

Infection with HBV mutant strains in the S gene product was detected in 3 patients. The amino acid escape mutant strains are as follows: S120 and L143 (case 1), T120 (case 2) and I126 (case 4). Four types of mutant strains (T/S120, L143, and I126) were detected in the S gene strains of 3 patients (cases 1, 2 and 4, respectively).

DNA sequencing and phylogenetic analysis

HBV DNA was quantified retrospectively by RTD-PCR in the stored samples of the five patients with HBV reactivation. Evidence of occult HBV infection at the time of the HBsAg-negative status (before the start of anticancer therapy) was detected by RTD-PCR in one patient (case 1). To determine the source of HBV infection, sera from case 1 before (case 1-A) and at the time of HBV reactivation (case 1-B) were subjected to HBV full genome amplification and sequencing. Sequences encompassing the HBV full genome obtained from sera before the start of chemotherapy and at the time of de novo HBV hepatitis revealed 100% homology, and the two sequences clustered together in the phylogenetic tree (Figure 2). These results demonstrate that case 1 developed reactivation from an occult HBV infection.

DISCUSSION

This study is considered the first step in documenting

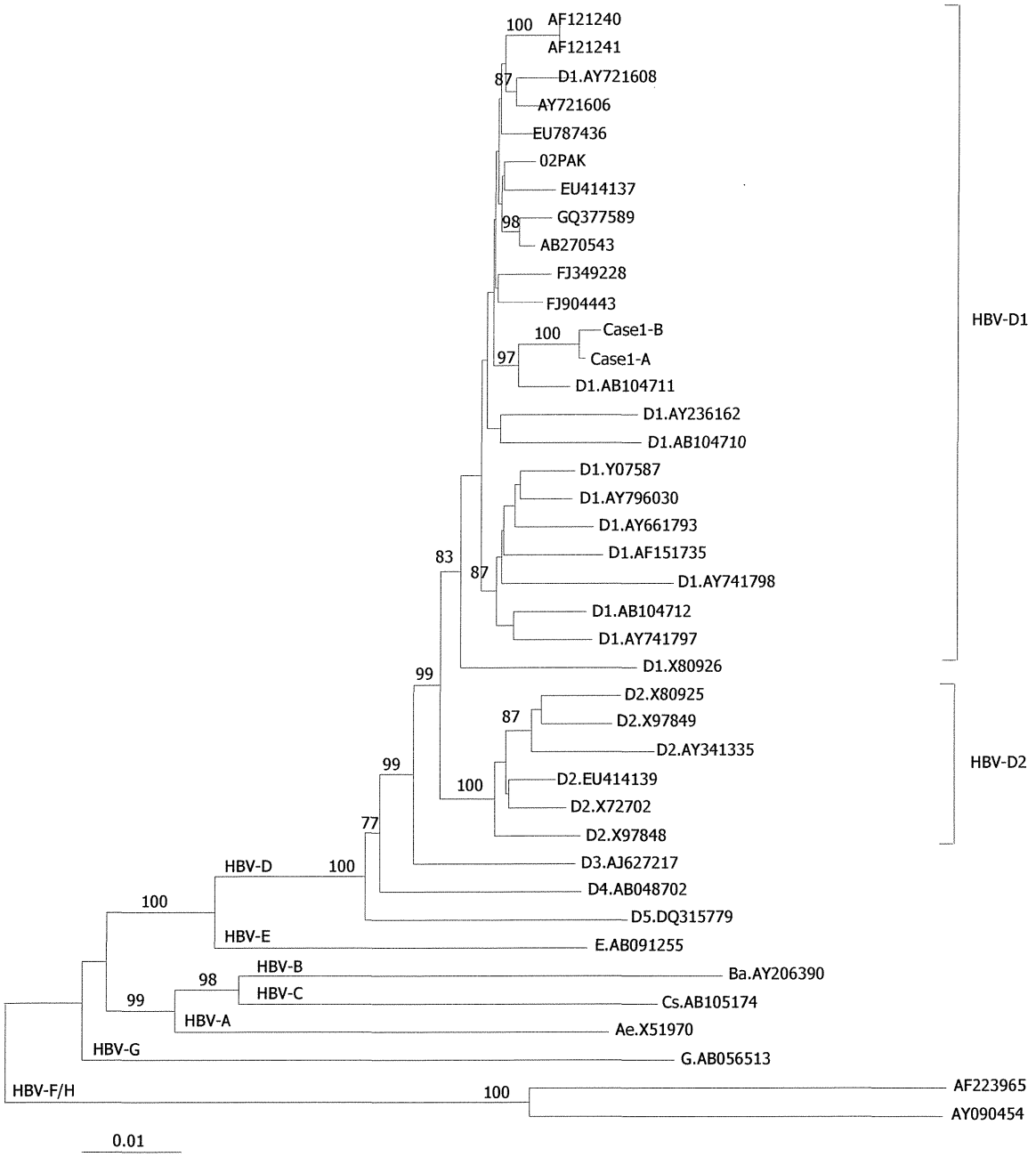

Figure 2 The complete genome of the hepatitis B virus was isolated and sequenced (Case 1) prior to the start of chemotherapy (Case 1-A) and after the emergence of hepatitis B virus reactivation (Case 1-B). The phylogenetic analysis demonstrated that the patient (Case 1) developed an hepatitis B virus (HBV) reactivation of an occult HBV infection.

and characterizing the reactivation of hepatitis B in Egypt among patients negative for the HBsAg who received immunosuppressive therapy. The current study presented further evidence that resolved hepatitis B infection and occult HBV infection may represent a hidden risk factor for the development of *de novo* hepatitis B.

The incidence of hepatitis B reactivation in the HBsAg-negative group was 9.4%, and all cases of reactivation occurred in patients with resolved or past infection with hepatitis B, as evidenced by the absence of HBsAg and the serological detection of anti-HBc. The patients who had HBV reactivation represent 27% of the HBsAg-negative/anti-HBc-positive patients. This incidence was comparable to the incidence that was described by Hui et al. In their study, Hui and his group described an HBV reactivation incidence of 3.3% (8/244) in their studied cohort, which included HBsAg-negative lymphoma patients receiving systemic chemotherapy[20]. Of note, all 8 patients were seropositive for either anti-HBc or anti-HBs antibody. Recently, Matsue et al. conducted a retrospective study on consecutive patients with CD20-positive B cell lymphoma before and after rituximab-containing treatment. In the latter study, 5 out of 230 patients negative for HBsAg (2.2%) experienced HBV reactivation, representing an incidence of 8.9% of the anti-HBc-positive patients[21]. In a prospective observational study

of patients with hematological malignancies (a study cohort similar to the current study), Francisci et al. reported the incidence of HBV reactivation was (18%), which is close to that detected in the present study[22]. The reasons for the difference in the incidence in HBV reactivation among different studies remain to be elucidated. However, the intensity of treatment, patient characteristics, and geographic differences in HBV prevalence and its genotypes may account for these differences[23]. Furthermore, the lack of a clear definition of HBV reactivation should not be ignored as a possible explanation for this variation in the incidence[21]. In this study, the inclusion of patients who had detectable HBV DNA after cancer chemotherapy plus patients who exhibited HBsAg seroconversion after receiving the anticancer therapy dramatically increased the incidence of HBV reactivation among the studied cohort. This criterion of including cases with detectable HBV DNA after cancer chemotherapy as a sign of HBV reactivation was not used to define cases with HBV reactivation in the related studies[20,21]. The variations in the cohort size among the different studies cannot be ignored as a possible factor that may be implicated in such discrepancy.

Occult HBV infection is defined by the detection of HBV DNA in the sera or in the livers of serologically HBsAg-negative patients[14]. Until recently, the clinical effects of occult HBV infection were unclear regarding the influence on the progression of liver disease, the development of hepatocellular carcinoma, the risk for HBV reactivation, and the transmission of HBV infection[24]. The underlying mechanisms for the pathogenesis of occult HBV infection may be due to either viral or host factors[25]. One of the important viral factors is the presence of mutations in the HBV DNA sequence, which may interfere with the detection of HBsAg by the commercial assays, *i.e.*, "escape mutations"[26]. In the present study, 4 types of possible escape mutants were detected in 3 of the 5 patients who experienced HBV reactivation[27]. Previous *in vitro* studies have reported that escape mutations are associated with an increased immune evasive capacity and are capable of causing symptomatic flare up and high viral loads[28]. Furthermore, studying the viral genome isolated from case 1 revealed a complete match of the sequences obtained before the start of chemotherapy and at the time of reactivation. The present study provides further evidence of the emergence of HBV reactivation of occult hepatitis B as confirmed by the molecular evolutionary analysis[29]. Furthermore, two amino acid escape mutations in the S gene product, P120S and S143L, were detected in the HBV viral genome isolated from case 1[27].

Patients with malignancies in Egypt are monitored only by testing ALT levels throughout the chemotherapy course. Therefore, the present study, which is the first to explore HBV reactivation in Egypt, suggests mandatory serological screening for anti-HBc and anti-HBs in patients planning to receive immunosuppressant therapy. Patients found to be positive for anti-HBc, particularly patients who are negative for anti-HBs, should be closely monitored with HBsAg, HBV DNA and serum biochemistry during chemotherapy and for at least 6 mo after the completion of therapy. Further prospective multicenter studies are needed to explore the incidence and risk factors of HBV reactivation in Egypt. Further studies are recommended to determine whether specific genomic mutations are implicated in de novo hepatitis in this subset of patients infected with HBV genotype D1.

COMMENTS

Background

The reactivation of hepatitis B is a syndrome characterized by an abrupt appearance or rise of the hepatitis B virus (HBV) DNA in the sera of patients with resolved or inactive hepatitis B infection. Reactivation can be spontaneous but is typically triggered by cancer chemotherapy, immune suppression or alterations in immune system function. Hepatitis B reactivation is of special clinical concern in immunocompromised patients because it leads to severe liver dysfunction and hepatic failure. However, hepatitis B reactivation is easy to prevent by introducing a prophylactic oral antiviral therapy. Occult hepatitis B is defined by the presence of HBV DNA in the serum or the liver in the absence of HBsAg with or without anti-HBc or antibodies to HBV surface antigen (anti-HBs). These patients are at risk of developing hepatitis B reactivation due to any factor suppressing the immune system. In Egypt, patients receiving cancer chemotherapy are typically monitored by liver function tests, with no screening for HBsAg or HBV DNA except in cases with elevated liver enzymes. This study aimed to investigate the incidence of HBV reactivation and the underlying risk factors of reactivation in Egyptian patients with hematological malignancies who were receiving cancer chemotherapy.

Research frontiers

In a cohort of 53 patients with hematological malignancies receiving cancer chemotherapy who were negative for HBsAg, 18 patients (34%) were found to be positive for the hepatitis B core antibody (anti-HBc), and five of the 53 (9.4%) patients with hematologic malignancies experienced HBV reactivation. All five patients were positive for anti-HBc. HBV DNA was detected in pretreatment HBsAg-negative samples in one of the five patients with HBV reactivation. In this patient, sera were obtained before the start of chemotherapy and at the time of *de novo* HBV hepatitis; the molecular evolutionary analysis of the sequences encompassing the HBV full genome obtained from the sera revealed that this patient developed reactivation from an occult HBV infection.

Innovations and breakthroughs

This study is the first in Egypt to characterize HBV reactivation in Egypt. The study introduces more evidence through molecular evolutionary analysis that occult HBV infection is a risk factor for reactivation of hepatitis B in patients with hematological malignancies receiving cancer chemotherapy.

Applications

The study strongly recommends mandatory serological screening for anti-HBc and anti-HBs in this subset of patients before the commencement of chemotherapy. Patients found to be positive for anti-HBc, particularly patients who are negative for anti-HBs, should be closely observed for signs of HBV reactivation through the regular monitoring of HBsAg and HBV DNA.

Peer review

The manuscript is easy to read. In the study, performance of sequencing and molecular analysis of HBV genomes seems relevant in characterization of the strains associated with HBV reactivation. Their findings are significant and beneficial for the readers.

REFERENCES

1 **Lee WM**. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745 [PMID: 9392700 DOI: 10.1056/NEJM199712113372406]
2 **Freudiger H**, Sitavanc R. Reverse seroconversion of hepatitis B in a haemodialysis patient. *Nephrol Dial Transplant* 2004; **19**: 238-241 [PMID: 14671065 DOI: 10.1093/ndt/gfg448]
3 **Schnepf N**, Sellier P, Bendenoun M, Zini JM, Sanson-le

Pors MJ, Mazeron MC. Reactivation of lamivudine-resistant occult hepatitis B in an HIV-infected patient undergoing cytotoxic chemotherapy. *J Clin Virol* 2007; **39**: 48-50 [PMID: 17368969 DOI: 10.1016/j.jcv.2007.01.011]

4 **Chakvetadze C**, Bani-Sadr F, Le Pendeven C, Lamontagne F, Vincensini JP, Pialoux G. Reactivation of hepatitis B virus replication during peginterferon-ribavirin therapy in an HIV/hepatitis C virus-co-infected patient with isolated anti-hepatitis B core antibodies. *AIDS* 2007; **21**: 393-394 [PMID: 17255759 DOI: 10.1097/QAD.0b013e328012b5d3]

5 **Clark SJ**, Creighton S, Horner M, Smith HM, Portmann B, Taylor C, Cramp ME. Reactivation of latent hepatitis B virus infection with HIV-related immunosuppression. *Int J STD AIDS* 2006; **17**: 67-69 [PMID: 16409685 DOI: 10.1258/095646206775220612]

6 **Bortolotti F**, Crivellaro C, Brunetto MR, Cadrobbi P, Bertolini A, Alberti A. Selection of a precore mutant of hepatitis B virus and reactivation of chronic hepatitis B acquired in childhood. *J Pediatr* 1993; **123**: 583-585 [PMID: 8410512 DOI: 10.1016/S0022-3476(05)80956-4]

7 **Lowell JA**, Howard TK, White HM, Shenoy S, Huettner PC, Brennan DC, Peters MG. Serological evidence of past hepatitis B infection in liver donor and hepatitis B infection in liver allograft. *Lancet* 1995; **345**: 1084-1085 [PMID: 7715341 DOI: 10.1016/S0140-6736(95)90819-6]

8 **Nordbø SA**, Skaug K, Holter E, Waage A, Brinch L. Reactivation of hepatitis B virus infection in an anti-HBc and anti-HBs positive patient after allogeneic bone marrow transplantation. *Eur J Haematol* 2000; **65**: 86-87 [PMID: 10914949 DOI: 10.1034/j.1600-0609.2000.91228.x]

9 **Chamorro AJ**, Casado JL, Bellido D, Moreno S. Reactivation of hepatitis B in an HIV-infected patient with antibodies against hepatitis B core antigen as the only serological marker. *Eur J Clin Microbiol Infect Dis* 2005; **24**: 492-494 [PMID: 15990987 DOI: 10.1007/s10096-005-1355-1]

10 **Kitano K**, Kobayashi H, Hanamura M, Furuta K, Ueno M, Rokuhara A, Tanaka E, Umemura T, Kiyosawa K. Fulminant hepatitis after allogenic bone marrow transplantation caused by reactivation of hepatitis B virus with gene mutations in the core promotor region. *Eur J Haematol* 2006; **77**: 255-258 [PMID: 16923112 DOI: 10.1111/j.1600-0609.2006.00678.x]

11 **Lin PC**, Poh SB, Lee MY, Hsiao LT, Chen PM, Chiou TJ. Fatal fulminant hepatitis B after withdrawal of prophylactic lamivudine in hematopoietic stem cell transplantation patients. *Int J Hematol* 2005; **81**: 349-351 [PMID: 15914368 DOI: 10.1532/IJH97.A10411]

12 **Hu KQ**. Occult hepatitis B virus infection and its clinical implications. *J Viral Hepat* 2002; **9**: 243-257 [PMID: 12081601 DOI: 10.1046/j.1365-2893.2002.00344.x]

13 **Ozaslan E**, Purnak T. Controversies about occult hepatitis B virus infection. *World J Gastroenterol* 2009; **15**: 4986-4987 [PMID: 19842236 DOI: 10.3748/wjg.15.4986]

14 **Raimondo G**, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. *J Hepatol* 2007; **46**: 160-170 [PMID: 17112622 DOI: 10.1016/j.jhep.2006.10.007]

15 **Sugiyama M**, Tanaka Y, Kurbanov F, Maruyama I, Shimada T, Takahashi S, Shirai T, Hino K, Sakaida I, Mizokami M. Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. *Gastroenterology* 2009; **136**: 652-62.e3 [PMID: 19041311]

16 **Sugauchi F**, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol* 2002; **76**: 5985-5992 [PMID: 12021331 DOI: 10.1128/JVI.76.12.5985-5992.2002]

17 **Shin-I T**, Tanaka Y, Tateno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. *Hepatol Res* 2008; **38**: 234-243 [PMID: 17877727 DOI: 10.1111/j.1872-034X.2007.00262.x]

18 **Hui CK**, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology* 2006; **131**: 59-68 [PMID: 16831590 DOI: 10.1053/j.gastro.2006.04.015]

19 **Matsue K**, Kimura S, Takanashi Y, Iwama K, Fujiwara H, Yamakura M, Takeuchi M. Reactivation of hepatitis B virus after rituximab-containing treatment in patients with CD20-positive B-cell lymphoma. *Cancer* 2010; **116**: 4769-4776 [PMID: 20597091 DOI: 10.1002/cncr.25253]

20 **Francisci D**, Falcinelli F, Schiaroli E, Capponi M, Belfiori B, Cecchini E, Baldelli F. Reactivation of hepatitis B virus replication due to cytotoxic therapy: a five-year prospective study. *Tumori* 2012; **98**: 220-224 [PMID: 22677988]

21 **Chu CM**, Liaw YF. Genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B: a longitudinal study of hepatitis B e antigen-positive patients with normal aminotransferase levels at baseline. *J Hepatol* 2005; **43**: 411-417 [PMID: 16006001 DOI: 10.1016/j.jhep.2005.03.018]

22 **Romero M**, Madejón A, Fernández-Rodríguez C, García-Samaniego J. Clinical significance of occult hepatitis B virus infection. *World J Gastroenterol* 2011; **17**: 1549-1552 [PMID: 21472119 DOI: 10.3748/wjg.v17.i12.1549]

23 **Said ZN**. An overview of occult hepatitis B virus infection. *World J Gastroenterol* 2011; **17**: 1927-1938 [PMID: 21528070 DOI: 10.3748/wjg.v17.i15.1927]

24 **Samal J**, Kandpal M, Vivekanandan P. Molecular mechanisms underlying occult hepatitis B virus infection. *Clin Microbiol Rev* 2012; **25**: 142-163 [PMID: 22232374 DOI: 10.1128/CMR.00018-11]

25 **Zaaijer HL**, Torres P, Ontañón A, Ponte LG, Koppelman MH, Lelie PN, Hemert FJ, Boot HJ. Multiple surface antigen mutations in five blood donors with occult hepatitis B virus infection. *J Med Virol* 2008; **80**: 1344-1349 [PMID: 18551607 DOI: 10.1002/jmv.21233]

26 **Henke-Gendo C**, Amini-Bavil-Olyaee S, Challapalli D, Trautwein C, Deppe H, Schulz TF, Heim A, Tacke F. Symptomatic hepatitis B virus (HBV) reactivation despite reduced viral fitness is associated with HBV test and immune escape mutations in an HIV-coinfected patient. *J Infect Dis* 2008; **198**: 1620-1624 [PMID: 18847320 DOI: 10.1086/592987]

27 **Sugauchi F**, Tanaka Y, Kusumoto S, Matsuura K, Sugiyama M, Kurbanov F, Ueda R, Mizokami M. Virological and clinical characteristics on reactivation of occult hepatitis B in patients with hematological malignancy. *J Med Virol* 2011; **83**: 412-418 [PMID: 21264861 DOI: 10.1002/jmv.21995]

28 **Miyakawa Y**, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; **46**: 329-338 [PMID: 14688448 DOI: 10.1159/000074988]

29 **Sugauchi F**, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, Kuramitsu T, Ueda R, Miyakawa Y, Mizokami M. Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease. *J Med Virol* 2003; **70**: 537-544 [PMID: 12794715 DOI: 10.1002/jmv.10428]

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Is Antiviral Prophylaxis Necessary to Prevent Hepatitis B Virus (HBV) Reactivation in Patients With HBV-Resolved Infection Receiving Rituximab-Containing Chemotherapy?

TO THE EDITOR: In a recent article in *Journal of Clinical Oncology*, Huang et al[1] reported a randomized controlled trial (NCT00926757) of entecavir prophylaxis to prevent hepatitis B virus (HBV) reactivation in 80 patients with HBV-resolved infection receiving rituximab-containing chemotherapy, in which interesting and important data were included. However, some concerns regarding study design and results in this report are worth considering.

First, Huang et al[1] reported that the incidence of HBV reactivation, the primary end point in this study, was defined as elevation of HBV viral load to 2,000 IU/mL with two consecutive determinations (> 2 weeks apart). However, the ClinicalTrials.gov archive[2] indicated that the primary end point had been changed, whereby HBV reactivation was defined as greater than 10-fold increase, compared with previous nadir levels of HBV DNA in the serum as of June 2009 at the beginning of the study. Previous secondary end points were defined as hepatitis and hepatic failure attributed to HBV reactivation. It is important for the reader to be aware of the reason why the authors changed the definition of the primary end point, and to be able to assess the incidence of HBV reactivation according to the previous original definition. Because some patients had HBV reactivation with high viral loads, readers need to know the kinetics of HBV viral load development as well as the clinical outcomes attributed to HBV reactivation during follow-up.

Second, Huang et al[1] reported that the HBV viral load was determined using a Cobas Amplicor HBV monitor (Roche Molecular Systems, Pleasanton, CA), with a detection limit of 12 IU/mL. However, it has been reported by others that the detection limit with this Cobas Amplicor HBV monitor is 60 IU/mL.[3] It may be necessary to amend the description regarding HBV viral load measurement for evaluation of the primary end point.

Third, Huang et al[1] reported that seven of 39 patients (17.9%) developed HBV reactivation (2,000 IU/mL), but only one (2.6%) had hepatitis attributed to HBV reactivation in the control group shown in Table 2. Furthermore, they also reported that no patients developed HBV-related liver decompensation or mortality in this study. These data might be unrepresentative, but if they are confirmed, the low incidence of hepatitis and no mortality associated with HBV reactivation is interesting in this prospective study, which would strongly suggest that antiviral prophylaxis is not cost effective for all patients with resolved hepatitis B receiving rituximab-containing chemotherapy. As Huang et al[1] suggested, regular monitoring of HBV viral load is more reasonable and cost effective, and some guidelines have already recommended this strategy to prevent HBV reactivation.[4,5] Recently, we presented data showing that monthly monitoring of HBV DNA could prevent hepatitis associated with HBV reactivation, even in HBV-resolved patients with highly replicative viral clones (interim analysis of a prospective study).[6]

The identification of risk factors associated with HBV reactivation is an important research question in patients with HBV-resolved infection following systemic chemotherapy, especially when the latter contains molecularly targeted drugs. If high-risk patients can be accurately predicted, we will be able to prevent HBV reactivation more effectively.

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REFERENCES

1. Huang YH, Hsiao LY, Hong YC, et al: Randomized controlled trial of entecavir prophylaxis for rituximab-associated hepatitis B virus reactivation in patients with lymphoma and resolved hepatitis B. J Clin Oncol 31:2765-2772, 2013
2. US National Institutes of Health: ClinicalTrials.gov archive. http://clinicaltrials.gov/archive/NCT00926757/2013_05_28/changes
3. Roche Diagnostics: Package insert, COBAS AMPLICOR HBV MONITOR Test. Revision 6.0, 2009
4. Oketani M, Ido A, Uto H, et al: Prevention of hepatitis B virus reactivation in patients receiving immunosuppressive therapy or chemotherapy. Hepatol Res 42:627-636, 2012
5. European Association for the Study of the Liver: EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 57:167-185, 2012
6. Kusumoto S, Tanaka Y, Suzuki R, et al: Prospective nationwide observational study of hepatitis B virus (HBV) DNA monitoring and preemptive antiviral therapy for HBV reactivation in patients with B-cell non-Hodgkin lymphoma following rituximab containing chemotherapy: Results of interim analysis. Blood 120, 2012 (abstr 2641)

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Genetic Association of Human Leukocyte Antigens with Chronicity or Resolution of Hepatitis B Infection in Thai Population

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Abstract

Background: Previous studies showed that single nucleotide polymorphisms (SNPs) in the *HLA-DP*, *TCF19* and *EHMT2* genes may affect the chronic hepatitis B (CHB). To predict the degree of risk for chronicity of HBV, this study determined associations with these SNPs.

Methods: The participants for this study were defined into 4 groups; HCC (n = 230), CHB (n = 219), resolved HBV infection (n = 113) and HBV uninfected subjects (n = 123). The *HLA-DP* SNPs (rs3077, rs9277378 and rs3128917), *TCF19* SNP (rs1419881) and *EHMT2* SNP (rs652888) were genotyped.

Results: Due to similar distribution of genotype frequencies in HCC and CHB, we combined these two groups (HBV carriers). The genotype distribution in HBV carriers relative to those who resolved HBV showed that rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, $p<0.001$ and OR = 0.47, $p<0.001$). The other SNPs rs3128917, rs1419881 and rs652888 were not associated with HBV carriers.

Conclusions: Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population.

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Introduction

The hepatitis B virus (HBV) is one of the most common causes of chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC). Globally more than 2 billion people have been infected with HBV and 378 million are suffering from chronic hepatitis. Over 600,000 people die each year because of HBV infection. In high prevalence areas such as the central Asian republics, Southeast Asia, Sub-Saharan Africa and the Amazon basin over 8% of the population may be HBV carriers [1]. The main route of HBV infection is vertical transmission from mother to infant and horizontal transmission between children, whereby 90% will develop chronic hepatitis as infants or in early childhood and never clear the virus [1–3]. In contrast, 15% of HBV infections in adulthood develop into chronic hepatitis with viral persistence.

The frequency of HBV infection which develops into chronic hepatitis depends on the age at which the person is infected [1,2]. However, the factors determining HBV persistence or clearance are not clearly understood [4–6]. Risk factors for viral persistence include the following: virological factors (viral load, genotype, viral gene mutations and co-infection with another virus), host factors (age at infection, gender, immune status and genetic variability) and extrinsic factors (e.g. alcohol consumption and chemotherapy) [7]. Whether viral infection results in acute or chronic infection also depends on cellular immune responses influenced by human leukocyte antigen (*HLA*) class I and II molecules which must present the viral antigens to CD8+ T cells and CD4+ T cells, respectively [8]. The genes encoding *HLA* are the most

polymorphic in the human genome, presumably in order to be able to respond to all potential foreign antigens [9].

Recently, many genome-wide association studies (GWAS) have been performed to seek associations between human genetic variation and the outcome of HBV infection [10–15]. Studies in the Japanese population showed that 11 single nucleotide polymorphisms (SNPs) located within or around the *HLA-DPA1* and *HLA-DPB1* loci are significantly associated with the occurrence of CHB. Of these 11 SNPs, the most strongly associated with the outcome of HBV infection were rs9277535 and rs3128917 in *HLA-DPB1* and rs3077 in *HLA-DPA1* [10].

Thereafter, GWAS studies in the Korean population confirmed the presence of these host factors related to HBV outcome and reported two new SNPs significantly associated with CHB within the *HLA* region, namely rs1419881 and rs652888 in transcription factor 19 (*TCF19*) and euchromatic histone-lysine methyltransferase 2 (*EHMT2*), respectively [16]. *TCF19* (or transcription factor SC1) is a *trans*-activating factor that mainly influences the transcription of genes required for late growth regulation at the G1-S checkpoint and during S phase [17]. *EHMT2* is a histone methyltransferase responsible for mono- and di-methylation of H3K9 (lysine at 9th residue of histone subunit 3) in euchromatin [18], which modifies the conformation of chromatin from its tightly packed form, heterochromatin, and thus influences gene repression or transcriptional silencing [19].

In the present study, we determined associations between the SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), *TCF19* (rs1419881) and *EHMT2* (rs652888) in HBV infected patients compared to those with resolved infections and those who had never been infected.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Faculty of Medicine, University (Bangkok, Thailand) code IRB.455/54. Written informed consent was obtained from each patient and all samples were anonymized.

Sample Collection

All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Subjects were defined into 4 groups: 230 hepatitis B surface antigen (HBsAg)-positive HCC, and 219 CHB who had been HBsAg-positive for at least 6 months were recruited at the King Chulalongkorn Memorial Hospital, whereas patients with resolved HBV and uninfected subjects were from the Thai Red Cross Society and from the north-eastern part of Thailand (age>40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to hepatitis B surface antigen (anti-HBs) and antibody to hepatitis B core protein (anti-HBc). Of these subjects, 113 were negative for HBsAg but positive for anti-HBc and/or positive for anti-HBs after resolution of infection, while 123 uninfected subjects were all negative for HBsAg, anti-HBc and anti-HBs. All samples in this study were collected from subjects who have lived at the same area in Thailand, suggesting that the genetic background would be balanced between a case and control.

Genotyping assays

DNA was extracted from peripheral blood mononuclear cell using phenol-chloroform DNA extraction. The concentration of DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). We determined SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), and the genes *TCF19* (rs1419881) and *EHMT2* (rs652888) by commercial TaqMan PCR assays (Applied Biosystems, USA). In this study we investigated *HLA-DPB1* (rs9277378) because this SNP had a high level of linkage disequilibrium with rs9277535 ($D' = 1.00$, $R^2 = 0.954$) [20] and was clearly detectable by the TaqMan assay rather than rs9277535.

Statistical analyses

In this study, Hardy-Weinberg equilibrium was performed on each SNP. The Chi-square test of independence and Odds Ratio (OR) from two-by-two tables for comparisons between case and control groups was performed using Microsoft Excel. Statistical significance was defined by $P<0.05$. The calculated of possibility level was established using Chi-square contingency table analysis.

Results

Subjects were defined into 4 groups: group 1) HCC (age = 58.2±12 years, 190/230 (82.6%) male); group 2) CHB (age = 46.6±10 years, 144/219 (65.7%) male); group 3) those with resolved HBV (age = 48.2±6 years, 83/113 (73.5%) male); and group 4) HBV uninfected subjects (age = 46.7±6 years, 73/123 (59.3%) male). The details are given in Table 1. To find the genetic factor associated with chronicity of HBV infection, however, the two groups (group 1 and 2) were combined (designated "HBV carriers"). Indeed, according to the frequencies of minor alleles of the SNPs in the *HLA-DP*, *TCF19* and *EHMT2* genes listed in Table 2, the frequencies of minor alleles of these 5 SNPs in HCC and CHB were similar (data shown in Table S1). The composite HBV carriers group had a minor allele frequency for rs3077 and rs9277378 lower than in groups 3 and 4 (OR = 0.57, 95% CI = 0.42–0.78, $p<0.001$ and OR = 0.63, 95% CI = 0.47–0.85, $p = 0.008$ for rs3077, OR = 0.59, 95% CI = 0.44–0.81, $p = 0.001$ and OR = 0.56, 95% CI = 0.42–0.75, $p<0.001$ for rs9277378, respectively). In contrast, the minor allele frequency for rs1419881 in HBV carriers was similar to group 3 (OR = 0.80, 95% CI = 0.60–1.08, $p = 0.142$) but lower than in group 4 (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$). Moreover, minor allele frequency for rs3128917 and rs652888 in HBV carriers was comparable to groups 3 and 4 (OR = 1.14, 95% CI = 0.85–1.53, $p = 0.371$ and OR = 1.06, 95% CI = 0.80–1.41, $p = 0.673$ for rs3128917; OR = 1.14, 95% CI = 0.84–1.55, $p = 0.400$ and OR = 1.12, 95% CI = 0.83–1.50, $p = 0.471$ for rs652888, respectively).

The results of Hardy-Weinberg equilibrium analysis of each SNPs were shown in Table 3. All data were over 0.01 ($p>0.01$), indicating that the frequencies did not deviate from Hardy-Weinberg equilibrium. The genotype distribution in HBV carriers compared to subjects with HBV resolution showed that both rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, 95% CI = 0.30–0.69, $p<0.001$ for rs3077 and OR = 0.47, 95% CI = 0.31–0.72, $p<0.001$ for rs9277378, are described in Table 3), suggesting that major homozygous genotypes were risk factors with the chronicity of HBV. The other SNPs rs3128917, rs1419881 and rs652888 were not associated against HBV carrier status (OR = 1.22, 95% CI = 0.76–1.97, $p = 0.413$ for rs3128917, OR = 0.67, 95% CI = 0.42–1.06, $p = 0.084$ for rs1419881 and OR = 1.31, 95% CI = 0.87–2.00, $p = 0.198$ for rs652888, respectively).

The genotype frequencies for 5 SNPs are shown in Table 3. Comparing HBV carriers with uninfected subjects showed that rs3077, rs9277378 and rs1419881 were all protectively associated with chronic HBV infection (OR = 0.63, 95% CI = 0.42–0.95,

Table 1. Characteristics of participants in HCC, CHB, resolved HBV and HBV uninfected subjects in Thailand.

	HCC (n = 230)	CHB[a] (n = 219)	Resolved[b] (n = 113)	Uninfected[c] (n = 123)
Age (years)	58.2±12	46.6±10	48.2±6	46.7±6
Male	190 (82.6%)	144 (65.7%)	83 (73.5%)	73 (59.3%)
HBsAg positive	230 (100%)	219 (100%)	0	0
ALT>40 (IU/L)	43 (18.7%)	61 (27.8%)	-	-
Alb (g/dl)	3.7 (2.5–5.6)	4.5 (3–5.2)	-	-
TB (mg/dl)	1.2 (0.17–14.8)	0.56 (0.2–2.67)	-	-

Abbreviation: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; ALT, Alanine transaminase; Alb, Albumin; TB, Total bilirubin.
[a]Defined as chronic hepatitis B includes chronic HBV infection but not cirrhosis and HCC.
[b]Defined as HBsAg negative but anti-HBc or/and anti-HBs positive.
[c]Defined as any HBV serological markers negative.
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$p = 0.025$ for rs3077 and OR = 0.55, 95% CI = 0.36–0.82, $p = 0.003$ for rs9277378 and OR = 0.57, 95% CI = 0.36–0.90, $p = 0.015$ for rs1419881, respectively). Comparing HBV carriers and uninfected subjects rather than those with resolved infection regarding rs1419881 was significantly protective association against CHB, but rs3128917 and rs652888 were not associated against CHB (OR = 1.58, 95% CI = 1.02–2.46, $p = 0.042$ for rs3128917 and OR = 1.09, 95% CI = 0.65–1.82, $p = 0.080$ for rs652888). When we consider the Bonferroni corrections (5 SNPs), however, the P value for rs1419881 did not reach the level of significant difference (0.015>0.05/5) between HBV carriers and HBV uninfected subjects. These data suggested that other SNPs, rs1419881, rs3128917 and rs652888 were not associated with HBV carriers in this study.

Results of meta-analysis for 3 SNPs (rs3077, rs9277378 and rs3128917) in the *HLA* gene were shown in Table S2 and S3; HBV carriers were compared to HBV resolved or HBV uninfected subjects, respectively. While the other 2 SNPs were published only from Korean population, thus the meta-analysis appeared only between HBV carriers and HBV uninfected subjects. All SNPs analyzed by the meta-analysis were significantly associated with HBV carriers.

The associations between these 5 SNPs and HBV status are depicted graphically in Figure S1. Each histogram compares HBV carriers with subjects that have resolved HBV infection or were never infected. The results showed that the minor dominant model of rs3077 and rs9277378 was highly protective associated against chronic HBV, while no significant associations were observed with rs3128917 and rs652888. Furthermore, comparing the frequency of rs1419881 between HBV carriers and uninfected subjects also revealed its association against chronic HBV infection but the association with resolved HBV did not achieve statistical significance.

Discussion

Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population. In the human genome, single nucleotide polymorphisms are found in every 300–570 nucleotides. Many SNPs have no effect on the function of the encoded proteins, but some variants do appear in regulatory or coding part of the gene and affect gene expression level or protein function which can give rise to disease [21] such as the 3 SNPs including rs3077, rs9277378 and rs3128917 in *HLA-*

Table 2. Minor allele frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

						HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
SNPs	Gene	Minor alleles[a]	HBV carriers[b] (2n = 898)	Resolved (2n = 226)	Uninfected (2n = 246)	OR (95% CI)	*P* values	OR (95% CI)	*P* values
rs3077	*HLA-DPA1*	T	227 (25.3%)	84 (37.2%)	86 (35.0%)	0.57 (0.42–0.78)	<0.001	0.63 (0.47–0.85)	0.008
rs9277378	*HLA-DPB1*	A	237 (26.4%)	85 (37.6%)	96 (39.0%)	0.59 (0.44–0.81)	0.001	0.56 (0.42–0.75)	<0.001
rs3128917	*HLA-DPB1*	G	459 (51.1%)	108 (47.8%)	122 (49.6%)	1.14 (0.85–1.53)	0.372	1.06 (0.80–1.41)	0.673
rs1419881	*TCF19*	C	361 (40.2%)	103 (45.6%)	126 (51.2%)	0.80 (0.60–1.08)	0.142	0.64 (0.48–0.85)	0.002
rs652888	*EHMT2*	C	329 (36.6%)	76 (33.6%)	84 (34.1%)	1.14 (0.84–1.55)	0.400	1.11 (0.83–1.50)	0.478

Abbreviation: CI, confidence interval; OR, odds ratio.
[a]Defined by using data from public database (NCBI).
[b]Defined as the combination between HCC and CHB.
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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).
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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, el at. (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.