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Legends

Table 1. Selected baseline characteristics of cases and controls

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs) of primary liver cancer based on plasma adiponectin levels

Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs) of primary liver cancer associated with plasma adiponectin levels by subgroups

Figure 1. Box and whisker plot for plasma adiponectin levels of cases and controls

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Figure 1

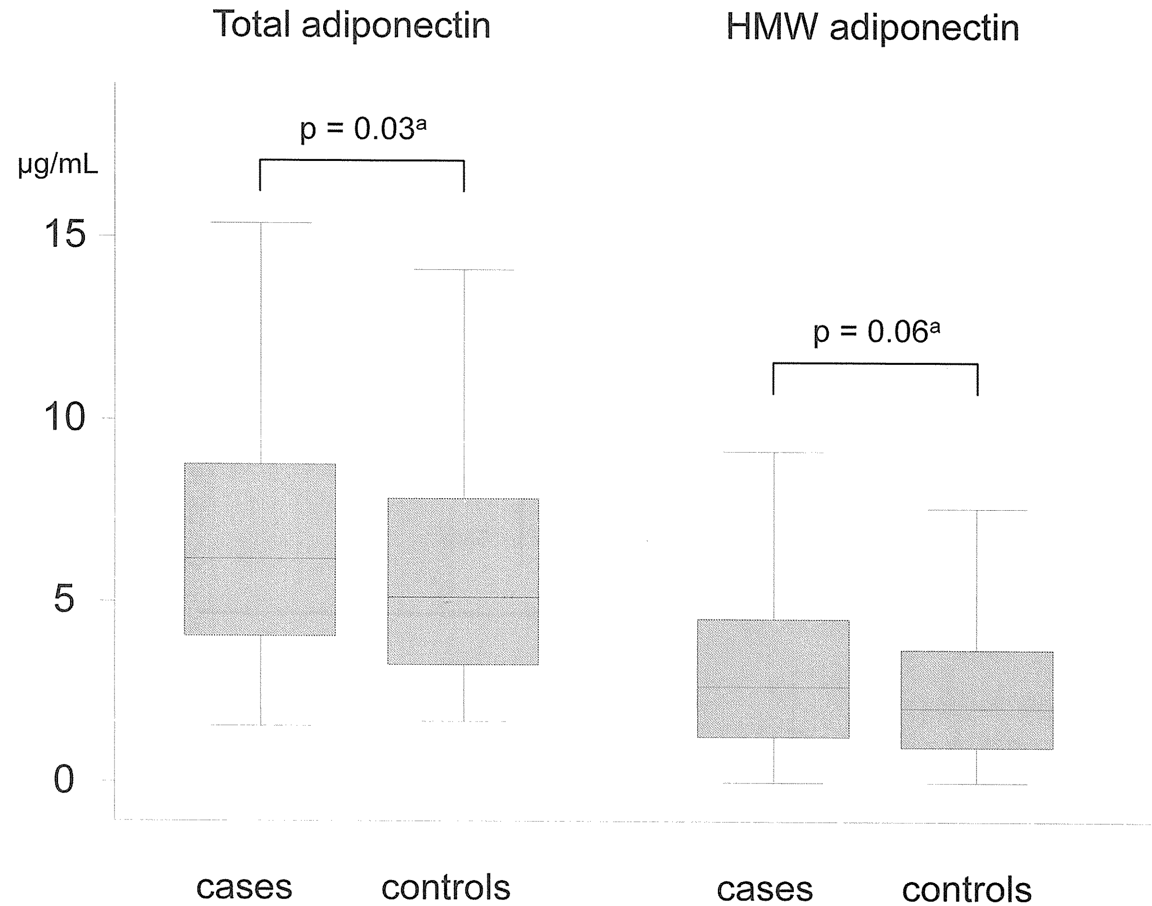

Figure 1. Box and whisker plot for plasma adiponectin levels of cases and controls

NOTE: HMW=high-molecular-weight
[a] p value for the difference between cases and controls were determined by the Mann-Whitney test.

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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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The GenBank/EMBL/DDBJ accession numbers for the HBV sequences determined in this study are KC012652 and KC012653.

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.

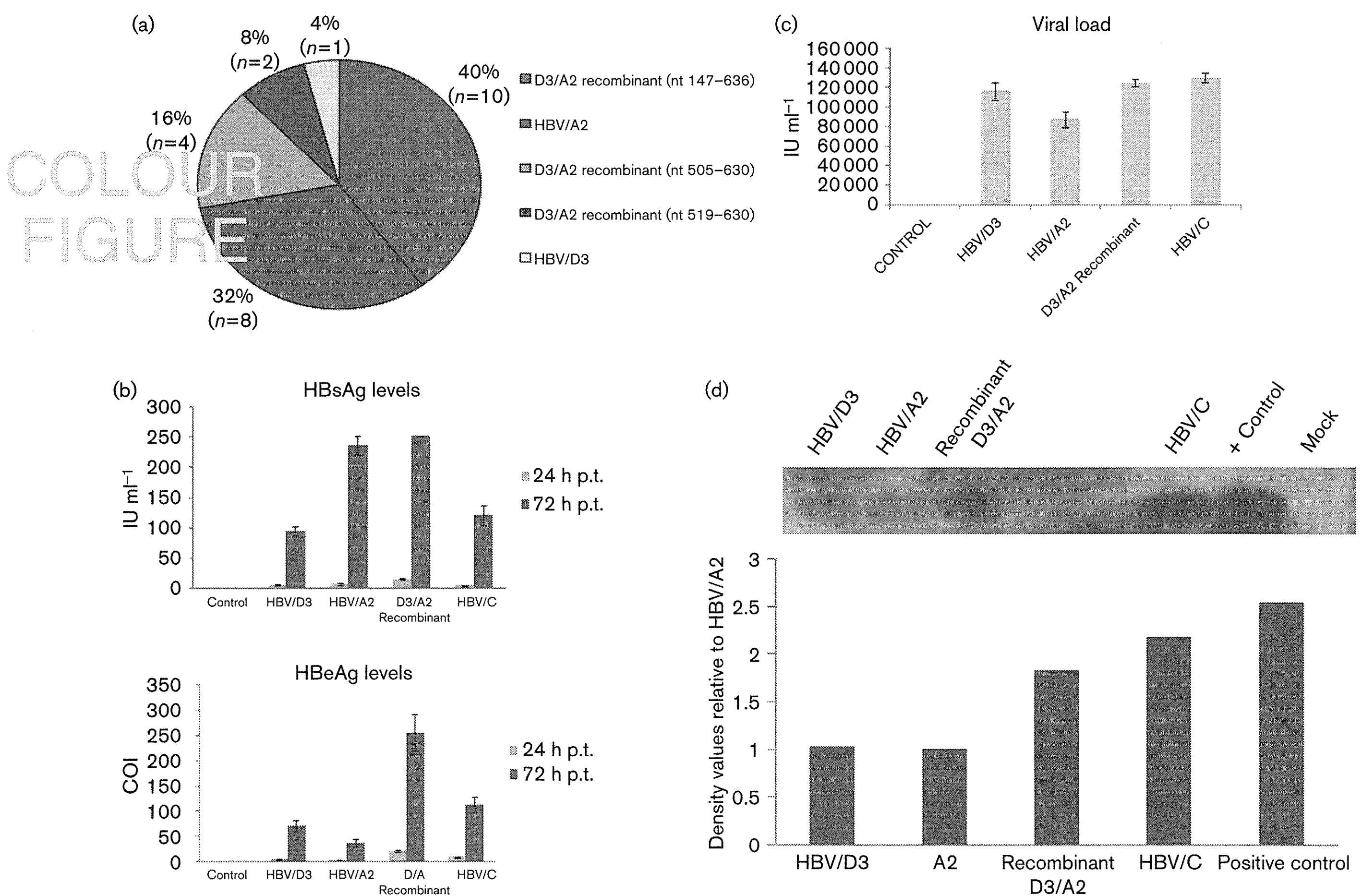
with breakpoints at nt 519 and 630, and 4 % to D3 subgenotype (Fig. 1a). Because the most abundant recombinant clones were those exhibiting breakpoints at nt 147 and 636 (Fig. 1a and Fig. S2a), they were considered representative of the whole viral population and thus selected for D/A replicon construction.

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.



COLOUR FIGURE

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.

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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

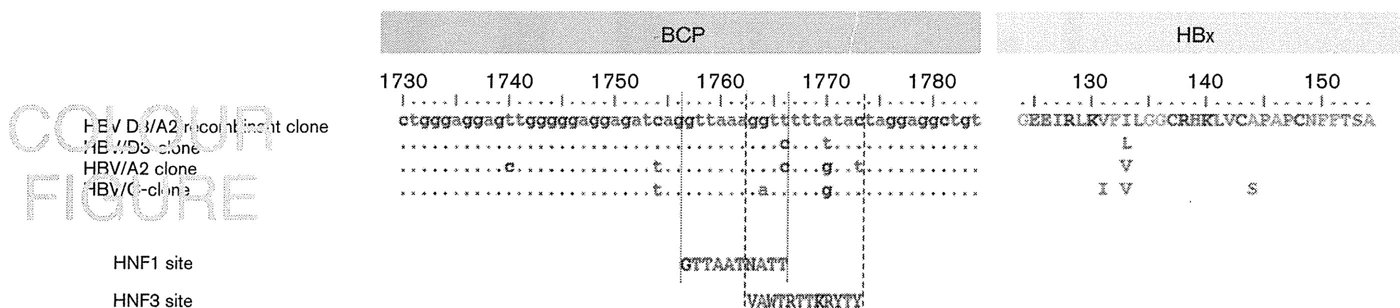

Fig. 2. Sequences of the basal core promoter (BCP) nt 1730–1785 and the overlapping region of the X protein of the HBV clones. The binding sites for HNF1 and HNF3 are aligned with the corresponding region of the HBV genome. The symbols for nucleotide ambiguities are as follows: V, A/C/G; W, A/T; R, A/G; 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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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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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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

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

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

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

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

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

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

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

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

Material and Methods

Patients

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

Serological markers of infection

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

Extraction and quantification of HBV DNA

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

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

Amplification of HBV DNA, sequencing and molecular evolutionary analysis

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

Analysis in the EnhII/BCP and precore regions

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

A case–control analysis

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

Statistical analysis

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

Results

Baseline characteristics of patients

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

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

Patterns of EnhII/BCP and precore mutations

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

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

Features	Total ($n = 182$)	Non-HCC ($n = 127$)	HCC ($n = 55$)	p^1
Age[2]	59 (6–93)	56 (6–85)	68 (40–93)	0.0001
Gender (M/F)	131/51	85/42	46/9	0.03
HBeAg+	94 (51.6)	85 (66.9)	9 (16.3)	0.0001
Anti-HBe+	99 (54.3)	50 (39.4)	49 (89)	0.0001
ALT[3]	84.1 ± 154.5	94.6 ± 175	89.5 ± 93.3	NS
AST[3]	101.5 ± 233.3	53.7 ± 144	198 ± 313	0.0001
Albumin[3]	82.1 ± 109.5	42 ± 8.2	183.2 ± 154.9	0.0001
Genotypes				
D	171 (93.9)	119 (93.7)	52 (94.5)	NS
E	7 (3.8)	4 (3.1)	3 (5.4)	NS
A	3 (1.6)	3 (2.3)	0	NS
C	1 (0.5)	1 (0.7)	0	NS

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

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

Age-matched case–control analysis

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

Discussion

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

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

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

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

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

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

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

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

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

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

[1]*p*: Wald test.

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

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

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

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

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

^1p: Wald test.

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

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

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

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Title:

Strategy for preventing hepatitis B reactivation in patients with resolved HBV infection following rituximab-containing chemotherapy

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To the editor:

In a recent article in Hepatology, Hsu et al.[1] reported a prospective study (NCT00931299) to determine the incidence of hepatitis B virus (HBV) reactivation in 150 patients with resolved HBV infection receiving rituximab-CHOP chemotherapy. The authors indicated that HBV reactivation is not uncommon and can be managed with regular monitoring of HBV DNA in serum. However, there are some concerns regarding the management of HBV DNA monitoring as described in this report.

First, Hsu et al.[1] reported that no HBV-related death occurred during the study period, but HBV-related severe hepatitis and chemotherapy delay occurred in 7 (4.6%) and 2 (1.3%) patients, respectively. Furthermore, patients with HBV reactivation may have a poorer prognosis than those without reactivation, suggesting that HBV DNA monitoring could not enable the successful management of HBV reactivation in this setting. In fact, the authors have already described the usefulness of a more sensitive HBV DNA assay and they should show whether a second PCR assay (detection limit 300 copies/mL, assay #2) could prevent severe hepatitis flare due to HBV reactivation by estimating in their retrospective analysis the exact time between early HBV DNA detection and the onset of hepatitis.

Second, Hsu et al.[1] concluded that re-appearance of HBsAg was the most important

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predictor of HBV-related hepatitis flare, but there is no information regarding the sensitivity and specificity of the HBsAg assay, and these might influence clinical outcome. The authors should provide information regarding the HBsAg assay in the methods section and specify the time between the re-appearance of HBsAg and the onset of HBV-related hepatitis. In addition, they should specify the incidence of re-appearance of HBsAg with persistence for more than 6 months in patients with HBV reactivation, because the chronic HBV carrier state might negatively influence long-term outcomes, regardless of fulminant hepatitis and HBV-related death.

Third, Hsu et al.[1] discussed the importance of host factors associated with HBV reactivation, but several papers have reported that the development of fulminant hepatitis was associated with viral factors, which especially included high levels of replication associated with mutations in the precore region[2,3]. The authors should specify whether the kinetics of HBV DNA and severe hepatitis were associated with precore and/or basal core promoter mutations in the patients with HBV reactivation, because general readers need to be aware of such important viral factors to perform safe monitoring of HBV DNA.

Preemptive antiviral therapy guided by regular monitoring of HBV DNA is a reasonable strategy to prevent HBV reactivation in patients with resolved HBV