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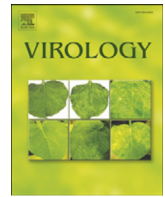

HBV mutations in untreated HIV-HBV co-infection using genomic length sequencing

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ABSTRACT

HIV infection has a significant impact on the natural progression of hepatitis B virus (HBV) related liver disease. In HIV-HBV co-infected patients, little is known about mutations in the HBV genome, which can influence severity of liver disease. The aim of this study was to characterize and to determine the frequency of known clinically significant mutations in the HBV genomes from HIV-HBV co-infected patients and from HBV mono-infected patients. To accomplish this, genomic length HBV sequencing was performed in highly-active anti-retroviral therapy (HAART)-naïve HIV-HBV co-infected patients ($n=74$) and in anti-HBV therapy-naïve HBV mono-infected patients ($n=55$).

The frequency of HBV mutations differed between the co-infected and mono-infected patients when comparing patients with the same genotype. BCP mutations A1762T and G1764A were significantly more frequent in HBV genotype C mono-infection and the -1G frameshift was significantly more frequent in co-infection and was only observed in HBV genotype A co-infection. PreS2 deletions were observed more frequently in the setting of co-infection. Further work is needed to determine if these mutational patterns influence the differences in liver disease progression in HIV-HBV co-infected and HBV mono-infected patients.

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Introduction

Co-infection with human immunodeficiency virus-1 (HIV) and hepatitis B virus (HBV) is common due to shared routes of transmission, with reported figures indicating approximately 10% of 33 million HIV-infected patients worldwide are chronically infected with HBV (Kellerman et al., 2003; Konopnicki et al., 2005; Lincoln et al., 2003; Ockenga et al., 1997; Thomas et al., 1994). HIV infection has a significant impact on the natural progression of HBV infection, with increased levels of HBV DNA and an elevated risk of liver-associated mortality (Colin et al., 1999; Gilson et al., 1997; Thio et al., 2002). However, an association of HIV co-infection with the emergence of specific mutations in the HBV genome is not known.

A number of clinically significant HBV genome mutations have been reported in HBV mono-infection. A Precore stop codon substitution at amino acid (aa) 28 (W28 stop, nucleotide (nt) G1896A) is associated with failure to synthesize hepatitis B e antigen (HBeAg) (Carman et al., 1989; Okamoto et al., 1994). In the basal core promoter (BCP) region, two associated nt changes, A1762T and G1764A have been associated with reduced Precore mRNA transcription and reduced HBeAg production (Buckwold et al., 1996; Gunther et al., 1998). These mutations also affect the X gene, due to overlapping reading frames, resulting in substitutions in the X protein at aa 130 and 131 (Funk et al., 2002; Moriyama et al., 1996; Okamoto et al., 1994). Both the BCP A1762T/G1764A mutations and the Precore stop codon mutation have been associated with advanced liver

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disease including hepatocellular carcinoma (HCC) and cirrhosis in mono-infected patients (Bläckberg and Kidd-Ljunggren, 2003; Chen et al., 2007b; Lindh et al., 1999; Tong et al., 2007). Combinations of these mutations, rather than the presence of a single mutation, have had the strongest association with advanced liver disease and HCC (Preikschat et al., 2002; Tanaka et al., 2006). Deletions in the PreS1 and PreS2 regions of the envelope gene and truncation of the S proteins due to premature stop codons have been reported in mono-infection (Bock et al., 1997; Chen et al., 2007a; Fang et al., 2008; Liu et al., 2002; Mun et al., 2008; Song et al., 2005; Trautwein et al., 1996). In addition, S proteins with truncations between aa positions 22 and 172 possess transactivational activity associated with an increased risk of HCC (Lauer et al., 1992). S proteins with truncations after aa position 175 (within the 3rd hydrophobic domain) do not confer transactivational activity, but are retained intracellularly. Intracellular retention of an additional envelope protein, the L protein, is associated with severe liver disease (Bruss and Ganem, 1991). Finally, in HIV-HBV co-infected patients with genotype A, we have recently described an HBV mutant that encodes for truncated core and Precore proteins (-1G mutant) (Revill et al., 2007). This mutant was associated with high HBV DNA levels (Revill et al., 2007).

The aim of this study was to characterize HBV genome mutations observed in HIV-HBV co-infected and HBV mono-infected patients. To accomplish this, full genomic length HBV sequencing was performed in an international cohort of highly-active anti-retroviral therapy (HAART)-naïve HIV-HBV co-infected patients and anti-HBV therapy-naïve HBV mono-infected patients to examine the type and frequency of known clinically-significant mutations.

Results

Study participants and clinical features

This study included 88 HIV-HBV co-infected and 64 HBV mono-infected patients (Table 1). Statistically significant differences between HIV-HBV co-infected and HBV mono-infected patients were observed for gender (more females in the mono-infected patients, $p<0.001$), ethnicity (more Caucasians in the co-infected patients, $p<0.001$) and HBeAg status (more HBeAg positive patients in the co-infected patients, $p=0.02$). Median age of the co-infected patients at the time of the preHAART sample was 37.2 years, age was not available for the mono-infected patients. There was no difference in the plasma HBV DNA ($\log_{10}$ IU/ml) between the groups ($p=0.54$). Mixtures of more than one HBV genotype were identified in seven co-infected samples but none of the samples from HBV mono-infected patients. In the mixed genotype cases, the genome sequence generated by direct sequencing was based on the predominant HBV genotype. There was a significant difference in the genotype distribution between the co- and mono-infected patients when all genotypes were included in a Chi-squared analysis ($p\leq0.001$), however in a series of pairwise analyses of genotypes A, B and C there were no significant differences in the prevalence. For the co-infected patients, the median duration of HIV was 4.4 years, the major transmission risk factor was sexual and the median CD4 nadir was 87 cells/μl. As the inclusion criteria for co-infected patients was pre-HAART rather than anti-HBV therapy naïve, a small number of patients had either past or current lamivudine (LMV) experience ($n=7$) and past experience with pegylated interferon ($n=2$).

Phylogenetic analysis

Phylogenetic analysis was restricted to genomic length HBV sequences only (co-infected $n=74$, mono-infected $n=55$). HBV has been classified into 10 genotypes, A to J, based on nt sequence diversity of at least 8% in the genome-length sequence (Tatematsu et al., 2009). Within these genotypes, subgenotypes have been identified that differ by between 4 and 7.5% at the nt level. The majority of patients belonged predominantly to subgenotypes A2, B2, and C1 (Fig. 1). HBV sequences from eight mono-infected patients were identified as subgenotype C2 and one as C5. In the co-infected patients, one A1 and one A/D recombinant were identified. The majority of C1 sequences isolated from co-infected patients in Thailand formed a separate clade from the C1 sequences of mono-infected patients from Hong Kong. However, the Hong Kong and Thai HBV sequences only diverged by an average of 2.7% (not shown), indicating they were the same subgenotype.

Clinically significant mutations

We examined the frequency of the following mutations in the HBV genomes of the mono-infected and co-infected patients: BCP A1762T and G1764A (reduced Precore mRNA transcription), Precore G1896A (W28 stop, failure to synthesize HBeAg), Pre S1 and PreS2 deletions (associated with accelerated disease progression in HBV mono-infection), and the -1G frameshift (associated with HIV co-infection). As some co-infected participants ($n=7$) were exposed to LMV, we also examined the frequency of polymerase rt L180M and rt M204V (signature LMV resistance mutations (Allen et al., 1998).

In both the co-infected and mono-infected patients, the BCP A1762T and G1764A mutations were the most common mutations (Table 2a) but were significantly more frequent in the mono-infected patients ($p=0.02$ and 0.01 respectively). The PreS2 deletions and -1G frameshift were present in co-infected patients with a frequency of 14.6% and 10.8%, respectively; however these mutations were uncommon in the mono-infected patients (3.3% and 0%, respectively). PreS2 deletions were usually observed in combination with BCP 1762/4 mutations (Fig. 2). The proportions of G1896A, pol rt M204V, pol rt L180M or PreS1 deletions were similar in both mono and co-infected patients. None of the core or envelope deletions that have been previously associated with HBV mono-infected patients on immunosuppressive therapy were found in the co-infected or mono-infected patients (Günther et al., 2000; Märschenz et al., 2006).

In order to determine whether the differences in frequencies of the BCP A1762T, G1764A, PreS2 deletions and -1G frameshift between the co-infected and mono-infected patients were influenced by genotype, we compared the co-infected and mono-infected patients within each HBV genotype (Table 2b). The frequency of BCP A1762T and G1764A mutations was significantly higher in HBV mono-infected patients compared to co-infected patients with genotype C, but the frequency was similar in patients with HBV genotype A. The -1G frameshift was only present in genotype A co-infected patients, ($p<0.001$ for genotype A compared to genotype B and C combined). The frequency of PreS2 deletions was higher in HBV co-infected patients with both genotypes A and C, but did not reach statistical significance.

Association of clinical and virological variables with the frequency of HBV mutations

Multivariate Poisson regression analysis was performed combining the co-infected and mono-infected patients to determine if the frequency of BCP mutations and amino acid substitutions in functional domains (including the *a* determinant of the S protein) were associated with any clinical and/or HBV parameters. The *a* determinant contains the major antigenic region located within the S protein (aa positions 110–160), with upstream and downstream regions located at aa1–109 and aa161–226 respectively. Associations with mutation frequency were identified for HBeAg positive status and genotype. Positive HBeAg status was associated with a 0.3-fold decrease in the frequency of mutations in the Precore gene, as well as decreases in the BCP (0.7-fold), PreS1 (0.8-fold), and PreS2 (0.6-fold) regions (Table 3a). In addition, lower mutation frequency in association with HBeAg positive status was observed in the regions

upstream (0.6-fold) and downstream (0.7-fold) of the S protein *a* determinant.

Genotype B was associated with a 0.6-fold decrease in the number of mutations in the region upstream of the *a* determinant and a 0.6-fold decrease in PreS2. However, genotype C was associated with a 1.5-fold increase in mutation frequency in the BCP, a 1.7- and 2.3-fold increase in mutations upstream or downstream of the *a* determinant respectively, a three-fold increase in PreS1 mutations and a four-fold increase in the number of mutations in the catalytic domain D region of the HBV reverse transcriptase in Pol (pol rt D). HBV mono-infection was associated with a 2-fold increase in the number of Precore mutations and a 2.5-fold increase in pol rt D mutations.

We repeated this analysis, restricting it to the HIV-HBV co-infected patients, to identify possible clinical factors associated with mutation frequency in the setting of HIV-HBV co-infection. Only HBeAg status and genotype were significantly associated with the frequency of mutations in the final multivariate model (Table 3b), which included the following additional HIV-related variables: HIV duration, pre-HAART AIDS defining illness, nadir CD4 and age at the preHAART sample. Positive HBeAg status was associated with a decrease in the frequency of mutations both upstream, within and downstream of the S protein *a* determinant, (0.6-, 0.5- and 0.7-fold reductions, respectively). HBV genotype A was significantly associated with a 2-fold increase in the number of PreS1mutations and HBV genotype C with an increase in the number of mutations upstream, within, and downstream of the S protein *a* determinant (1.7-, 2.4-, and 1.9-fold increases respectively), and PreS1 region (3.5-fold).

Positive and negative selection in patients with HBV genotype C

While the total number of sites undergoing positive or negative selection did not significantly differ between the co-infected and mono-infected patients with HBV genotype C (not shown), three mutations were identified as under positive selection in the HIV-HBV co-infected patients. One was located in the polymerase gene at aa rt 223 and two were located in the X gene (aa 30 and 47), one of which overlapped with pol (RNaseH117=> HB X 47). None of these mutations were located within known T-cell epitopes (Desmond et al., 2008). No mutations undergoing positive selection were identified in the HBV mono-infected patients. However, a total of 53 nucleotide changes undergoing negative selection were identified in the co-infected patients and 50 were identified in the HBV mono-infected patients. Four codons undergoing negative selection in HIV-HBV co-infected patients were located in known T-cell epitopes (Desmond et al., 2008). These were at Core 23 (epitope at 18–27), RNaseH 66 in polymerase (epitope at 63–71), PreS2 4 (epitope at 1–15), and surface 212 (epitope at 207–216). No mutations were identified in T-cell epitopes of HBV mono-infected patients, although one mutation was identified in the *a* determinant of the S protein (surface126).

Discussion

To our knowledge, this is the first study to describe full HBV genome sequences in a large cohort of HIV-HBV co-infected patients prior to HAART and HBV mono-infected patients naïve to HBV therapy to determine the frequency of clinically significant mutations in both settings. We compared the frequency of HBV mutations in HIV-HBV co-infected and HBV mono-infected patients adjusted for HBV genotype. Two BCP mutations were significantly more common in HBV from mono-infected patients with HBV genotype C. In addition, in HIV-HBV co-infected patients, the -1G frameshift mutation was significantly more common, and was only observed in HBV genotype A. HBV PreS2 deletions were more frequent in the setting of co-infection, and this was not explained by differences in HBV genotypes between the two groups.

Table 1
Demographics and clinical characteristics.

Characteristic	Cohort group		*p* value
	Co-infection	Mono-infection	
Participants, *n*	88	63	
Gender[a], m/f	76/11	36/27	<0.001
Ethnicity[b], *n*			
Caucasian	46	0	<0.001
Asian	39	42	
African	2	0	
Not stated	1	21	
Median age at sample date, years (IQR)[c]	37.2 (32.1–43.6)	–	–
HBV genotype, *n* (%)			
A	42 (47.7%)	13 (20.6%)	0.08
B	3 (3.4%)	17 (26.9%)	0.37
C	35 (39.7%)	33 (52.4%)	0.31
D	3 (3.4%)	0	–
G	5	0	–
A/G[d]	5	–	–
C/D	1	–	–
C/G	1	–	–
HBeAg status, positive/negative	44/39	15/41	0.02
Median (IQR) nadir CD4 count, cells/μL	87 (33–222)	–	–
Median (IQR) HBV viral load, $\log_{10}$ IU/mL	7.25 (5.61–7.79)	7.20 (6.49–8.15)	0.54
Median (IQR) HIV viral load at CD4 nadir, $\log_{10}$ copies/mL	4.87 (4.34–5.25)	–	–
Median duration of HIV at preHAART sample date, years (IQR)	4.4 (0.4–9.5)	–	–
Risk factor for HIV/HBV		–	–
Sexual contact	79		
Sexual contact and IDU	7		
Unknown	2		

IDU: intravenous drug use; IQR: inter-quartile range.
[a] Co-infection group also included one male-to-female transgendered individual.
[b] Includes Hispanic and non-Hispanic Caucasians.
[c] Age data were not available for the mono-infection group.
[d] For the analysis, mixed genotypes were reported according to the predominant genotype present. Three of the A/G samples and the one C/G sample were predominantly genotype G and the C/D sample predominantly genotype C. Two A/G samples had equivalent amounts of the two genotypes, but the polymerase sequence was predominantly genotype A, so were included as polymerase only genotype A sequences.

BCP A1762T and G1764A mutations were more common in patients with HBV genotype C mono-infection, as previously described in patients from Taiwan and China (Chen et al., 2005, 2007a,b; Du et al., 2007). These BCP mutations have been associated with reduced Precore mRNA transcription and reduced HBeAg production (Buckwold et al., 1996; Gunther et al., 1998). High frequency of mutations in genotype C may be linked to the longer duration of infection, however when comparing genotype B and C Asian cohorts where all infections were likely acquired in the perinatal period, genotype C was still associated with these mutations suggesting that duration of infection was not the only explanation for such findings in genotype C. BCP A1762T and G1764A have a reported association with HCC in particular, and more severe clinical outcomes in general (Bläckberg and Kidd-Ljunggren, 2003; Chen et al., 2005, 2007a,b; Du et al., 2007; Lindh et al., 1999; Tong et al., 2007).

The -1G frameshift mutation, which has been associated with HIV-HBV co-infection and high levels of HBV DNA (Revill et al., 2007), was only observed in samples from the co-infected patients with HBV genotype A. This mutation results in a frameshift in the Precore/core genes that truncates the deduced Precore and core proteins in a key T-cell epitope (Revill et al., 2007). Unpublished data show that the truncated proteins encoded by the -1G mutant accumulate in the hepatocyte ER (Revill, unpublished) and the effect of this

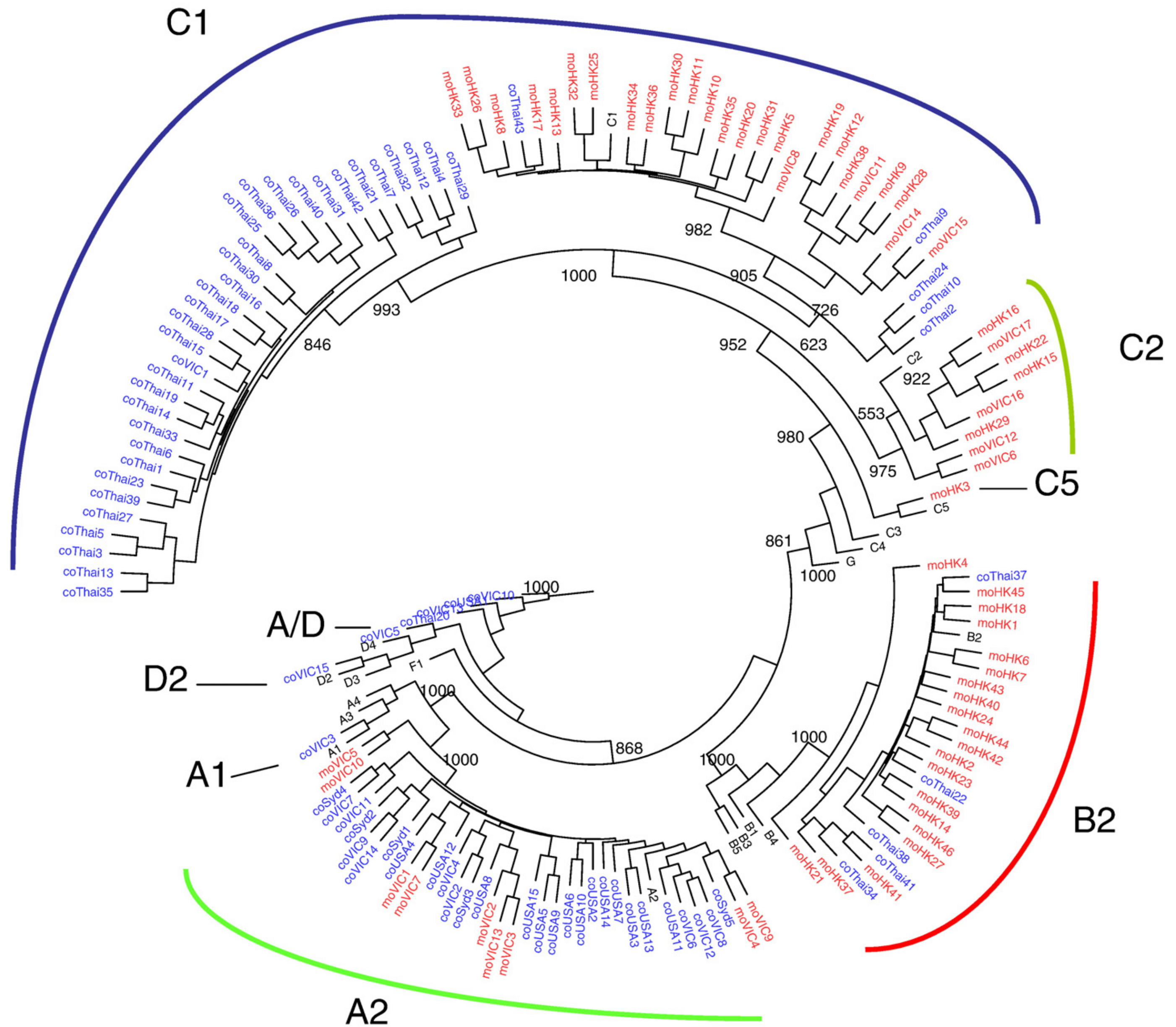

Fig. 1. Unrooted neighbour-joining tree of all genomic-length HBV sequences from the total cohort. Bootstrap values (1000 replicates) are shown at selected nodes. The subgenotype of major clades is indicated at the periphery of the tree. All sequences from HIV-HBV co-infected individuals are prefixed by "co" (shaded blue) and all sequences from HBV mono-infected individuals are prefixed by "mo" (shaded red). Other abbreviations are as follows: Thai = Thailand, Vic = Victoria, Syd = Sydney, HK = Hong Kong. Reference sequences are labelled as A1, A2 to G.

accumulation is currently under investigation. Previous studies of HBV mono-infected renal transplant recipients on immunosuppressive therapy observed deletion mutations in the Precore/Core region of HBV (Günther et al., 2000; Märschenz et al., 2006). We did not observe these deletions in the HBV sequences from co-infected patients in our study.

PreS2 deletions were more frequent in co-infection, however there was no significant association with genotype. The majority of PreS2 deletions were located close to the N-terminus of the PreS2 protein. HBV with mutations in PreS can accumulate in the endoplasmic reticulum (ER) of hepatocytes during late stages of infection, causing oxidative stress and the formation of ground glass hepatocytes (Fan et al., 2001; Hsieh et al., 2004). Furthermore, *in vitro* studies have shown that intracellular accumulation of the HBV large surface protein or PreS1/PreS2 mutant proteins can induce ER stress, increase activation of the HBV S promoter and alter HBV protein expression, increase nuclear factor-κB transcription and the over-expression of the COX-2 gene; which have been associated with increased cell cycle progression, oxidative stress and DNA damage (Hsieh et al., 2004; Wang et al., 2003; Xu and Yen, 1996). The majority of the PreS2 mutants identified in our study also encoded A1762T/G1764A basal core mutations. The presence of PreS2 deletions together with BCP A1762T/G1764A mutations have been reported in immunosuppressed genotype A HBV mono-infected patients with advanced liver disease (Märschenz et al., 2006). In that study the PreS2 deletion was located towards the C-terminus (aa 140/141), whereas in our study the PreS2 deletions were located towards the N-terminus. The PreS2 deletion mutants near the C-terminus were found to have increased replication phenotypes relative to wild-type virus (Märschenz et al., 2006). Further studies are needed to determine if PreS2 deletions near the N-terminus result in intracellular accumulation of aberrant envelope proteins or have increased replication phenotypes.

Table 2a
Number (percentage) of individuals with mutations at reported clinically significant locations for co-infected and mono-infected individuals.

Location	Co-infected	Mono-infected	*p*-value
Precore G1896A	9/75 (12.0)	9/57 (15.8)	0.53
BCP A1762T	33/83 (39.8)	35/59 (59.3)	0.02
BCP G1764A	33/83 (39.8)	36/59 (687.0)	0.01
pol rt L180M	1/87 (1.2)	1/63 (1.6)	0.82
pol rt M204V	2/87 (2.3)	0/63 (0.0)	0.23
PreS1 deletions	5/74 (6.8)	1/61 (1.6)	0.22
PreS2 deletions	12/82 (14.6)	2/60 (3.3)	0.04
-1G frameshift	8/74 (10.8)	0/55 (0.0)	0.02

Signature LMV-resistance mutations were observed in three samples from the co-infected patients (who had all had prior experience with LMV) and one sample from a mono-infected individual who was treatment naive. It has been previously reported that LMV-resistance mutations can occur naturally in treatment-naïve patients with chronic HBV infection (Kirishima et al., 2002; Kobayashi et al., 2001).

The HBV subgenotype distribution within the cohort conformed to previously identified geographic distribution (Huy et al., 2004; Norder et al., 2004). That is, most genotype A sequences were subtype A2, and HBV sequences from Thailand and Hong Kong were predominantly subtype C1. Despite a high degree of sequence identity, the C1 sequences from Hong Kong and most of the sequences from Thailand separated into different clades on the tree, indicative of their different geographical origins (Huy et al., 2004).

A positive HBeAg status was associated with a decrease in mutation frequency in both the total cohort and the co-infected patients alone. Lower mutation counts in HBeAg positive patients has been observed previously using different methodology and may be a consequence of reduced immune pressure in HBeAg non-seroconverters (Lim et al., 2007). Whilst we detected a number of HBV mutations in key immunological epitopes, overall the frequency of mutations under selection pressure was low. This is consistent with the low HBV-specific T-cell response observed in both HBV mono-infection and HIV-HBV co-infection (Chang et al., 2005, 2007; Crane et al., 2009). A number of mutations were identified undergoing negative selection pressure, which supports previous findings that negative selection predominates in HBV evolution (Osiowy et al., 2006). The presence of a small number of codons under negative selection within immunological epitopes in the core, polymerase and envelope genes of the HIV-HBV co-infected patients implies functional constraints on the encoded amino acids. Although we identified 3 codons under positive selection in the HIV-HBV co-infected patients, we did not identify positive selection in the HBV core/Precore gene as previously reported in HBeAg seroconverters (Lim et al., 2007).

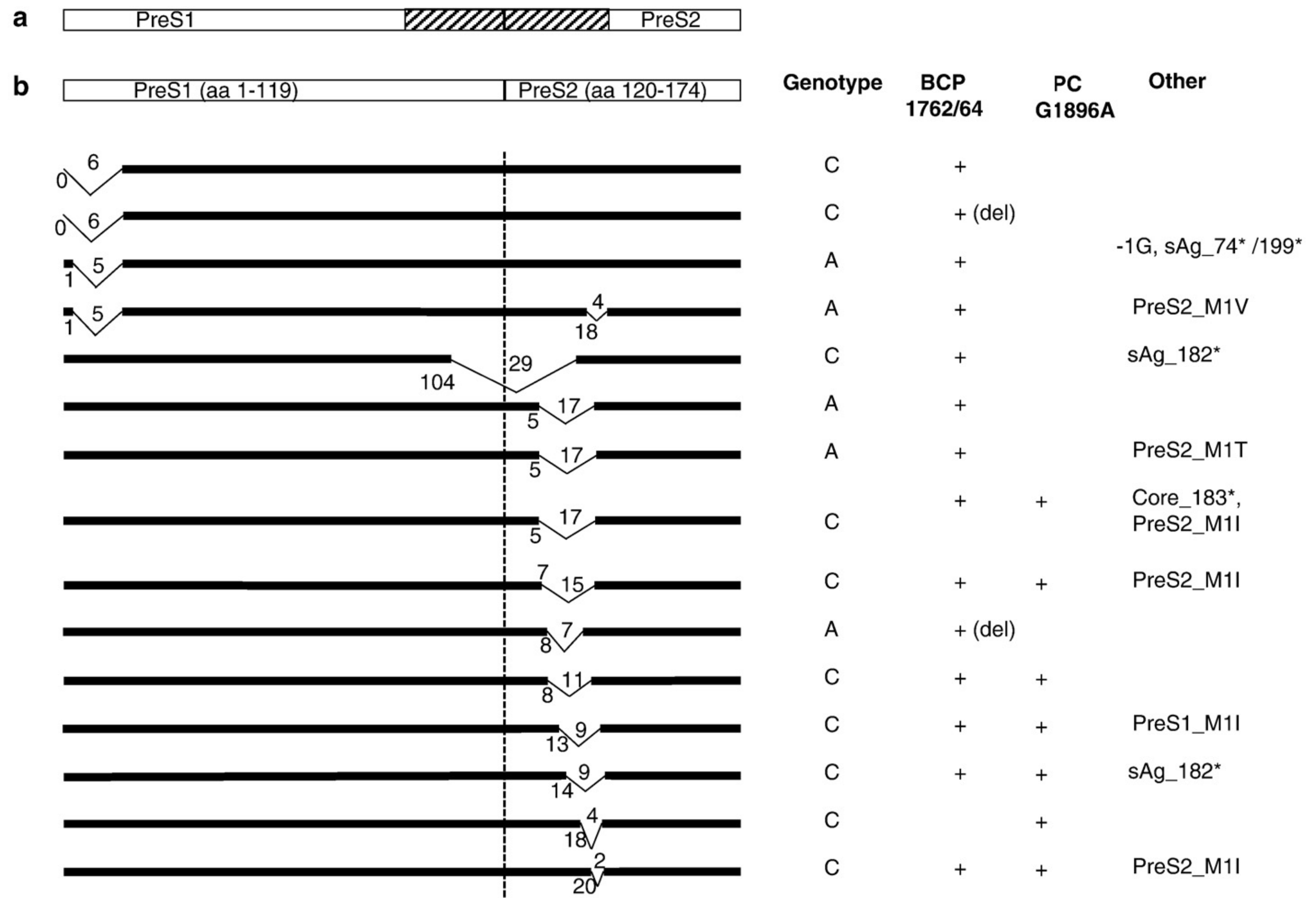

Fig. 2. PreS deletions observed in the co-infection group. (a) Regions of the PreS sequence where deletions have been predominantly detected previously in mono-infection (Abe et al., 2009; Chen et al., 2006, 2008; Fan et al., 2001; Preikschat et al., 2002). (b) Position of PreS deletions observed in the co-infection group. Mapping of the wild type preS1/preS2 DNA regions shown at the top. Deletions are indicated by the interruption; the number prior to each deletion indicates the position in the PreS1 or preS2 gene and the number within the interruption indicates the length of each deletion. The genotype for each sequence is shown to the right with the presence of other clinically significant mutations detected from the same viral sequence. -1G = -1G frameshift mutation; sAg_/182*/183*/74* and 199* = stop codon in HBV surface antigen at amino acid positions 182, 183 or 74 and 199; PreS2_M1V/T/I = mutation in HBV PreS2 at amino acid position 1, substitution of M (methionine) for either V (valine), T (threonine) or I (isoleucine); Core_183* = stop codon in HBV Core at amino acid position 183.

Table 2b
Number and percentage of co-infected and mono-infected individuals with mutations at previously reported clinically significant locations according to genotype.

Location	Co-infected			Mono-infected		
	Number mutations by genotype			Number mutations by genotype		
	A	B	C	A	B	C
	$n=42$	$n=3$	$n=35$	$n=13$	$n=17$	$n=33$
BCP A1762T	15 (35.7%)	0 (0%)	17[a] (48.6%)	5 (38.5%)	5 (29.4%)	25[a] (75.8%)
BCP G1764A	15 (35.7%)	0 (0%)	17[b] (48.6%)	5 (38.5%)	5 (29.4%)	26[b] (78.8%)
PreS2 deletions	4 (9.5%)	0 (0%)	8 (22.8%)	0 (0%)	0 (0%)	2 (6.1%)
-1G frameshift	8[c] (19.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[a] Statistically significant for genotype C ($p=0.008$).
[b] Statistically significant for genotype C ($p=0.004$).
[c] Statistically significant for total genotype A compared to total genotype B combined with total genotype C ($p=0.001$).

However, it must be noted that while many of the patients in our cohorts were HBeAg negative, their anti-HBe status was unknown. In addition, our study was restricted to HBV genotype C, whereas Lim *et al* studied HBV genotype B (Lim et al., 2007). It was not possible to measure evolution rates in this cross-sectional study. However, longitudinal samples available from this cohort will enable us to address this in future studies.

It is surprising that we did not observe a statistically significant difference in HBV DNA level between the mono- and co-infected patients. HBV DNA is generally higher in co-infection (Colin et al., 1999; Gilson et al., 1997), however these studies were conducted in cohorts of European origin and therefore likely to be HBV genotype A or D, not C. In addition, it is well described that HBV DNA levels are higher in HBeAg positive patients (Fattovich et al., 2008), and the co-infected group had a higher percentage of HBeAg positive patients. The lack of difference in our study may be due to the predominance of genotype C patients in both groups. Similar cohorts have not been examined previously.

This study had a number of limitations. Direct comparisons between the mono-infected and co-infected patients are inherently problematic due to different genotype distribution in the groups. Patients with HBV genotype C from Asia are much more likely to have acquired their HBV by perinatal transmission. They are therefore more likely to have a longer duration of HBV infection, resulting in a longer time period for mutations to accumulate and for virus evolution. In contrast, patients infected with HBV genotype A in Western countries are much more likely to have acquired their HBV infection as adults through sexual contact or exposure to contaminated blood; hence their duration of HBV infection will be shorter. However, in this study these potential differences were minimised since genotype-specific analyses were performed, genotype was an independent variable at the multivariate level and analysis of positive and negative selection was only completed for patients with HBV genotype C, where there were approximately equal numbers in the mono-infected and co-infected patients. Age data and risk factors for HBV infection were not available for the mono-infected patients. Although the co-infected genotype C participants were recruited from Thailand and the mono-infected from Hong Kong, the subgenotype distribution in the Asian patients (Thailand and Hong Kong) was similar, with B2 and C1 subgenotypes predominant. Another limitation was the use of different HBV DNA assays. However, all HBV DNA results were converted into the standard international units for comparison, which should limit assay variability. HBV DNA comparisons between the mono-infected and co-infected patients may have been affected by the in-house assay used for quantitation of samples from participants in Hong Kong ($n=42$). In a subset of 11 patients from Hong Kong, the plasma HBV DNA was repeated using the commercial RealArt HBV Light Cycler PCR assay, and the two sets of results were similar. Finally, as a cross sectional study, we only assessed a single sample from each individual and therefore could not evaluate rates of mutation evolution.

In conclusion we found two major differences in the frequency of HBV mutations in co-infection and mono-infection, even when only comparing patients with the same genotype. BCP mutations A1762T and G1764A were more frequent in HBV genotype C mono-infection and the -1G frameshift was only observed in HBV genotype A co-infection. In addition, PreS2 deletions were more common in co-infected patients. Further work is needed to determine the effect of these mutations on hepatocyte pathology. These co-infected patients are being followed prospectively to determine any relationship between these preHAART HBV mutations and disease progression.

Subjects and methods

Study participants

This study included a total of 151 patients, 88 who were HIV-HBV co-infected and 63 HBV mono-infected, who were recruited from October 2004 to September 2008. Co-infected patients were recruited from sites in Australia, the United States (US) and Thailand; and mono-infected patients were recruited from sites in Australia and Hong Kong. Inclusion criteria for the cohort included documented chronic HBV infection (presence of hepatitis B surface antigen (HBsAg) on two occasions a minimum of 6 months apart); and HCV negative, which was defined as HCV antibody (anti-HCV) negative or anti-HCV positive and HCV RNA negative. For patients who were anti-HCV negative with a CD4 count <200 cells/μl, HCV RNA was tested regardless of anti-HCV status to look for occult hepatitis C (Bonacini et al., 2001). Additional inclusion criteria for the co-infected patients were documented positive HIV antibody, HAART-naïve (defined as a minimum of three antiretrovirals from at least two different classes), and a stored serum sample prior to the initiation of HAART. For the mono-infected patients, documented negative HIV antibody was required as well as being naïve to any anti-HBV medication. Written, informed consent was obtained from all participants, and the study was approved by the relevant Human Research Ethics Committees in Australia, the United States, Thailand and Hong Kong.

HBV DNA quantification

HBV DNA quantification at all sites, except Hong Kong, was performed using either the HBV Digene Hybrid Capture II microplate assay (Roche, Branchburg, N.J.; lower limit of detection (LLOD) 2.3×10^4 IU/mL), the Versant HBV DNA 3.0 assay (Bayer HealthCare-Diagnostics, Tarrytown, N.Y.; LLOD 357 IU/mL), or the RealART^{TM} HBV LC PCR (QIAGEN), LLOD 20 IU/ml in accordance with the manufacturer's instructions. HBV DNA quantification in Hong Kong was determined using an in-house Taqman PCR assay, which amplified the HBV core region (linear range 1×10^2 - 1×10^9 copies/ml). All results were converted into international units for analysis.

HBV sequencing

HBV DNA was extracted from 200 μL of serum using the semi automated Roche MagNA Pure System (version 2.1, Roche, Branchberg, NJ), performed according to the operators manual. Extracted DNA was eluted in a final volume of 50 μL of supplied elution buffer. Full-genome HBV sequences were amplified and sequenced as previously described (Gunther et al., 1995). Full genomic sequences could not be amplified from samples with low HBV DNA, in which case we used alternate primers to amplify the HBV polymerase (pol) (Ayres et al., 2004). HBV consensus sequences were constructed using the DNA sequence analysis program *SeqScape* (ABI Prism, Applied

Table 3a
Multivariate Poisson regression analysis of clinical parameters associated with various HBV genome region mutations in the total cohort (co-infected and mono-infected patients). Statistically significant ($p<0.05$) or near significant results only.

HBV region	Variable	Univariate analysis			Multi-variate analysis		
		Poisson co-eff[a]	95% CI	p-value	Poisson co-eff	95% CI	p-value
Precore	HBeAg + ve	−1.2	−1.74, −0.67	<0.001	−1.33	−1.89, −0.79	<0.001
	HBV mono-infection	0.49	−0.02, 1.00	0.058	0.72	0.20, 1.24	0.007
BCP	HBeAg positive	−0.42	−0.62, −0.22	<0.001	−0.36	−0.57, −0.16	0.001
	HBV Genotype C	0.43	0.23, 0.63	<0.001	0.41	0.20, 0.62	<0.001
Upstream (aa1–109) of the *a* determinant of S protein	HBeAg positive	−0.59	−0.81, −0.37	<0.001	−0.48	−0.71, −0.26	<0.001
	HBV Genotype B	−0.94	−1.42, −0.47	<0.001	−0.61	−1.11, −0.10	0.019
	HBV Genotype C	0.76	0.53, 0.98	<0.001	0.51	0.26, 0.76	<0.001
downstream (aa161–226) of the *a* determinant of S protein	HBeAg positive	−0.45	−0.66, −0.24	<0.001	−0.33	−0.55, −0.12	0.002
	HBV Genotype C	0.96	0.68, 1.12	<0.001	0.84	0.61, 1.07	<0.001
Pol rt D	Mono-infection	1.08	0.32, 1.83	0.005	0.91	0.15, 1.66	0.019
	HBV Genotype C	1.63	0.74, 2.52	<0.001	1.52	0.62, 2.41	0.001
Env PreS1	HBeAg positive	−0.39	−0.55, −0.23	<0.001	−0.27	−0.43, −0.10	0.002
	HBV Genotype A	−0.29	−0.48, −0.11	0.002	0.69	0.37, 1.00	0.001
	HBV Genotype C	0.74	0.57, 0.90	<0.001	1.12	0.84, 1.40	<0.001
Env PreS2	HBeAg positive	−0.49	−0.69, −0.29	<0.001	−0.46	−0.66, −0.25	<0.001
	HBV Genotype B	−0.54	−0.89, −0.19	0.002	−0.49	−0.84, −0.14	0.006

CI = confidence interval.
We examined the relationship between mutation count in HBV and multiple clinical parameters. The specific regions of the HBV genome included terminal protein; spacer; pol RT F, A, B, C, and E domains; interdomain; RNAse H; envelope PreS1 and PreS2; upstream and downstream of the *a* determinant region of the surface protein; the *a* determinant region of the surface protein, epsilon; enhancer II; glucocorticoid responsive element, basal core promoter, preCore and core regions and the X protein. Regulatory elements including the promoter sequences, core upstream regulatory sequence, direct repeats 1 and 2, enhancer I, enhancer I modulator, enhancer I central and X promoter were not analysed as all such sequences were wild type in this cohort.
The clinical parameters included gender, HBeAg status, HBV DNA level, and HBV genotype.
[a] A negative Poisson co-efficient indicates a decrease in the number of mutations, and a positive co-efficient indicates an increase.

Biosystems, Foster City, CA). HBV genotype and unique HBV mutations were identified using a web-based analysis program, *SeqHepB* (www.seqhepb.com), as previously described (Bartholomeusz et al., 2004; Yuen et al., 2007).

In cases where the sequencing revealed mixed HBV genotypes, we determined the constituents of these genotype mixtures using an INNO-LiPA HBV genotyping assay (Innogenetics, Gent, Belgium).

Phylogenetic analysis

The HBV genomic length sequences obtained from the HIV-HBV co-infected ($n=74$) and HBV mono-infected ($n=55$) patients were aligned using Clustal X (Thompson et al., 1997) and MegAlign (Clewley and Arnold, 1997) and edited using Bioedit and EditSeq (Arnold and Clewley, 1997). Neighbour-joining phylogenetic trees were constructed using the Phylip Seqboot, DNADist/ProtDist, Neighbour and Consense programs (http://evolution.gs.washington.edu/phylip.html) (Revill et al., 2007). The resultant neighbour-joining tree was evaluated by bootstrap resampling with 1000 replicates and trees were edited using Treeview (Page, 1996) and Figtree (http://tree.bio.edu.ac.uk/software/figtree). Sequence identity was determined using MegAlign (Clewley and Arnold, 1997). The following reference sequences for each HBV genotype and subtype were obtained from GenBank: A1 M57663; A2 X02763; A3 AB194951; A4 AM18062; A5; B1 D00329; B2 AF121249; B3 M54923; B4 AY033072; B5 AB219429; C1 AY057947; C2 AY217378; C3 X75665; C4 AB048705; C5 AB241111. D2 Z35716; D3 J02203; D4 AB033559; F1 AY179735; G AB056515; H AY090460. Our classification of HBV subgenotype C was based on the method of Huy et al. (2004). Recombinant HBV sequences were identified using SimPlot (Lole et al., 1999).

Positive and negative selection

Positive and negative selection was analysed using the HyPhy program (www.datamonkey.org) using alignments generated in Clustal X from patients infected with HBV genotype C. Positive

Table 3b
Multivariate Poisson regression analysis of clinical parameters associated with various HBV genome region mutations in the co-infected patients. Statistically significant ($p<0.05$) or near significant results only.

HBV region	Variable	Univariate analysis			Multi-variate analysis		
		Poisson co-eff[a]	95% CI	p-value	Poisson co-eff	95% CI	p-value
Upstream (aa1–109) of the *a* determinant of S protein	HBeAg positive	−0.65	−0.94, −0.36	<0.001	−0.52	−0.82, −0.22	0.001
	HBV Genotype C	0.69	0.41, 0.97	<0.001	0.51	0.21, 0.80	<0.001
a determinant of S protein	HBeAg positive	−0.81	−1.28, −0.35	0.001	−0.66	−1.10, −0.18	0.007
	HBV Genotype C	0.82	0.39, 1.25	<0.001	−0.32	−0.61, −0.03	0.032
Downstream (aa161–226) of the *a* determinant of S protein	HBeAg positive	−0.53	−0.81, −0.25	<0.001	−0.32	−0.61, −0.03	0.032
	HBV Genotype C	0.95	0.67, 1.23	<0.001	0.86	0.57, 1.16	<0.001
Env PreS1	HBV Genotype A	−0.39	−0.61, −0.18	<0.001	0.67	0.21, 1.13	0.005
	HBV Genotype C	0.70	0.49, 0.90	<0.001	1.24	0.79, 1.68	<0.001

CI = confidence interval.
We examined the relationship between mutation count in HBV and multiple clinical parameters. The specific regions of the HBV genome included terminal protein; spacer; pol RT F, A, B, C, and E domains; interdomain; RNAse H; envelope PreS1 and PreS2; upstream and downstream of the *a* determinant region of the surface protein; the *a* determinant region of the surface protein; epsilon; enhancer II; glucocorticoid responsive element, basal core promoter, preCore and core regions and the X protein. Regulatory elements including the promoter sequences, core upstream regulatory sequence, direct repeats 1 and 2, enhancer I, enhancer I modulator, enhancer I central and X promoter were not analysed as all such sequences were wild type in this cohort.
The clinical parameters included gender, HBeAg status, HBV DNA level, genotype, HIV duration, preHAART AIDS defining illness, nadir CD4 and age at the preHAART sample.
[a] A negative Poisson co-efficient indicates a decrease in the number of mutations, and a positive co-efficient indicates an increase.

selection occurs when an aa alteration confers a selective advantage (adaptation), whereas negative selection is the elimination of aa alterations that confer a disadvantage. Amino acids can also be altered purely by chance (neutral selection). Positive and negative selection can be estimated using codon-based models that estimate the relative amounts of synonymous (d*S*) and non-synonymous (d*N*) mutations in different populations of sequences. If d*N* is greater than d*S*, the sequences are undergoing positive selection, i.e., the mutation has increased the fitness of the sequence. Conversely, if d*S* is greater than d*N*, the sequences are undergoing negative selection to remove deleterious sequences from the gene pool. Datamonkey (www.datamonkey.org, (Pond and Frost, 2005) is a web based engine that enables analysis of positive and negative selection in aligned sequences using the HyPhy program (Pond et al., 2005). HyPhy enables statistical comparison of the number of synonymous and non-synonymous mutations at each site in an aligned group of sequences. To enable comparison of the degree of positive and negative selection in HBV sequences from HIV-HBV co-infected and HBV mono-infected patients we analysed separate alignments of each individual reading frame (core, precore, polymerase, envelope and X) from co-infected and mono-infected sequences, using the single likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) algorithms (Pond et al., 2005). Briefly, SLAC is a rapid method that enables detection of non-neutral selection, based on the Suzuki–Gojobori counting approach. FEL by contrast is a likelihood based method that fits an independent d*N* and d*S* to every site and tests whether $dN \neq dS$ (Kosakovsky Pond and Frost, 2005; Pond et al., 2005). Only sites that were identified by both the SLAC and FEL algorithms ($p \leq 0.01$ significance) were included in the final result. In our study, analysis was restricted to sequences from patients infected with HBV genotype C, since this was the most frequent genotype in the cohort and because there were similar numbers of HIV-HBV co-infected ($n = 33$) and mono-infected ($n = 29$) patients infected with genotype C.

Statistical methods

Categorical variables were compared using the Chi-square test, or, where cell numbers were small, Fishers Exact test. Continuous variables were compared using the Wilcoxon Rank Sum test. Data from the co-infected patients were compared with the data from the mono-infected patients, and where statistically significant differences were found the data was subgrouped by genotype and the analysis was repeated to examine genotype effect. Poisson regression analysis at the univariate and multivariate levels was used to investigate predictors of the number of mutations per HBV genome region. Univariate Poisson regression was used to identify candidate predictors for inclusion in the multivariate model. The final model for each analysis was derived through a process of backwards elimination where non-significant variables were sequentially removed until a subset was left with wholly significant predictors. Overall model fit was assessed using a Goodness of Fit Pearson Chi Square test. In all analyses $p < 0.05$ was considered significant. Poisson regression is a log-linear model, with the polarity of raw Poisson co-efficients providing an indication of either an increase or decrease in the count data for each outcome. The co-efficients must be exponentiated to obtain the linear equivalent of the increase or decrease. The raw co-efficients are presented in the tables, and the exponentiated factor increases or decreases of significant outcomes are provided in the results section. Analysis was completed using STATA software (Version 10.1, StataCorp, TX, USA).

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References

Abe, K., Thung, S.N., Wu, H.C., Tran, T.T., Le Hoang, P., Truong, K.D., Inui, A., Jang, J.J., Su, I.J., 2009. Pre-S2 deletion mutants of hepatitis B virus could have an important role in hepatocarcinogenesis in Asian children. Cancer Sci. 100 (12), 2249–2254.

Allen, M.I., Deslauriers, M., Andrews, C.W., Tipples, G.A., Walters, K.-A., Tyrrell, D.L., Brown, N., Group, L.C.I., Condreay, L.D., 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Hepatology 27 (6), 1670–1677.

Arnold, C., Clewley, J.P., 1997. From ABI sequence data to LASERGENE's EDITSEQ. Meth. Mol. Biol. 70, 65–74.

Ayres, A., Locarnini, S., Bartholomeusz, A., 2004. HBV genotyping and analysis for unique mutations. Meth. Mol. Med. 95, 125–149.

Bartholomeusz, A., Locarnini, S., Ayres, A., Thompson, G., Sozzi, V., Angus, P., Sievert, W., Sasadeusz, J., Chalmers, D., Cooper, M., 2004. Molecular modelling of hepatitis B virus polymerase and adefovir resistance identifies three clusters of mutations. Hepatology 40 (S4), 246A.

Bläckberg, J., Kidd-Ljunggren, K., 2003. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. J. Med. Virol. 71 (1), 18–23.

Bock, C.T., Tillmann, H.L., Maschek, H.J., Manns, M.P., Trautwein, C., 1997. A preS mutation isolated from a patient with chronic hepatitis B infection leads to virus retention and misassembly. Gastroenterology 113 (6), 1976–1982.

Bonacini, M., Lin, H.J., Hollinger, F.B., 2001. Effect of coexisting HIV-1 infection on the diagnosis and evaluation of hepatitis C virus. J. Acquir. Immune Defic. Syndr. 26 (4), 340–344.

Bruss, V., Ganem, D., 1991. Mutational analysis of hepatitis B surface antigen particle assembly and secretion. J. Virol. 65 (7), 3813–3820.

Buckwold, V.E., Xu, Z., Chen, M., Yen, T.S., Ou, J.H., 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. 70 (9), 5845–5851.

Carman, W.F., Hadziyannis, S., McGarvey, M.J., Jacyna, M.R., Karayiannis, P., Makris, A., Thomas, H.C., 1989. Mutation preventing formation of Hepatitis B e antigen in patients with chronic Hepatitis B infection. Lancet 334 (8663), 588–591.

Chang, J.J., Wightman, F., Bartholomeusz, A., Ayres, A., Kent, S.J., Sasadeusz, J., Lewin, S. R., 2005. Reduced hepatitis B virus (HBV)-specific CD4+ T-cell responses in human immunodeficiency virus type 1-HBV-coinfected individuals receiving HBV-active antiretroviral therapy. J. Virol. 79 (5), 3038–3051.

Chang, J.J., Alexander, J.V.T., Kumar, V., Stephen, J.K., Paul, U.C., Fiona, W., Paul, D., Stephen, A.L., Sharon, R.L., 2007. The phenotype of hepatitis B virus-specific T cells differ in the liver and blood in chronic hepatitis B virus infection. Hepatology 46 (5), 1332–1340.

Chen, C.-H., Lee, C.-M., Lu, S.-N., Changchien, C.-S., Eng, H.-L., Huang, C.-M., Wang, J.-H., Hung, C.-H., Hu, T.-H., 2005. Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. J. Clin. Microbiol. 43 (12), 6000–6006.

Chen, B.-F., Liu, C.-J., Jow, G.-M., Chen, P.-J., Kao, J.-H., Chen, D.-S., 2006. High Prevalence and Mapping of Pre-S Deletion in Hepatitis B Virus Carriers With Progressive Liver Diseases. Gastroenterology 130 (4), 1153–1168.

Chen, C.-H., Hung, C.-H., Lee, C.-M., Hu, T.-H., Wang, J.-H., Wang, J.-C., Lu, S.-N., Changchien, C.-S., 2007a. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. Gastroenterology 133 (5), 1466–1474.

Chen, C.-H., Lee, C.-M., Hung, C.-H., Hu, T.-H., Wang, J.-H., Wang, J.-C., Lu, S.-N., Changchien, C.-S., 2007b. Clinical significance and evolution of core promoter and precore mutations in HBeAg-positive patients with HBV genotype B and C: a longitudinal study. Liver Int. 27 (6), 806–815.

Chen, C.H., Changchien, C.S., Lee, C.M., Hung, C.H., Hu, T.H., Wang, J.H., Wang, J.C., Lu, S.N., 2008. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. J. Infect. Dis. 198 (11), 1634–1642.

Clewley, J.P., Arnold, C., 1997. MEGALIGN. The multiple alignment module of LASERGENE. Meth. Mol. Biol. 70, 119–129.

Colin, J., Cazals-Hatem, D., Loriot, M., Martinot-Peignoux, M., Pham, B., Auperin, A., Degott, C., Benhamou, J., Erlinger, S., Valla, D., Marcellin, P., 1999. Influence of human immunodeficiency virus infection on chronic hepatitis B in homosexual men. Hepatology 29 (4), 1306–1310.

Crane, M., Oliver, B., Matthews, G., Avihingsanon, A., Ubolyam, S., Markovska, V., Chang, J.J., Dore, Gregory J., Price, P., Visvanathan, K., French, M., Ruxrungtham, K., Lewin, Sharon R., 2009. Immunopathogenesis of hepatic flare in HIV/hepatitis B virus (HBV) coinfected individuals after the initiation of HBV active antiretroviral therapy. J. Infect. Dis. 199 (7), 974–981.

Desmond, C.P., Bartholomeusz, A., Gaudieri, S., Revill, P.A., Lewin, S.R., 2008. A systematic review of T-cell epitopes in hepatitis B virus: identification, genotypic variation and relevance to antiviral therapeutics. Antivir. Ther. 13 (2), 161–175.

Du, H., Li, T., Zhang, H.-Y., He, Z.-P., Dong, Q.-M., Duan, X.-Z., Zhuang, H., 2007. Correlation of hepatitis B virus (HBV) genotypes and mutations in basal core promoter/precore with clinical features of chronic HBV infection. Liver Int. 27 (2), 240–246.

Fan, Y.-F., Lu, C.-C., Chen, W.-C., Yao, W.-J., Wang, H.-C., Chang, T.-T., Lei, H.-Y., Shiau, A.-L., Su, I.-J., 2001. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 33 (1), 277–286.

Fang, Z.-L., Sabin, C.A., Dong, B.-Q., Wei, S.-C., Chen, Q.-Y., Fang, K.-X., Yang, J.-Y., Huang, J., Wang, X.-Y., Harrison, T.J., 2008. Hepatitis B virus pre-S deletion mutations are a risk factor for hepatocellular carcinoma: a matched nested case-control study. J. Gen. Virol. 89 (11), 2882–2890.

Fattovich, G., Bortolotti, F., Donato, F., 2008. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J. Hepatol. 48 (2), 335–352.

Funk, M.L., Rosenberg, D.M., Lok, A.S.F., 2002. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. J. Viral Hepat. 9 (1), 52–61.

Gilson, R.J., Hawkins, A.E., Beecham, M.R., Ross, E., Waite, J., Briggs, M., McNally, T., Kelly, G.E., Tedder, R.S., Weller, I.V., 1997. Interactions between HIV and hepatitis B virus in homosexual men: effects on the natural history of infection. AIDS 11 (5), 597–606.

Gunther, S., Li, B.C., Miska, S., Kruger, D.H., Meisel, H., Will, H., 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J. Virol. 69 (9), 5437–5444.

Gunther, S., Piwon, N., Will, H., 1998. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653) −>T, A(1762) −>T and G(1764) −>A mutations in the core promoter. J. Gen. Virol. 79 (2), 375–380.

Günther, S., Piwon, N., Jung, A., Iwanska, A., Schmitz, H., Will, H., 2000. Enhanced replication contributes to enrichment of hepatitis B virus with a deletion in the core gene. Virology 273 (2), 286–299.

Hsieh, Y.-H., Su, I.-J., Wang, H.-C., Chang, W.-W., Lei, H.-Y., Lai, M.-D., Chang, W.-T., Huang, W., 2004. Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Carcinogenesis 25 (10), 2023–2032.

Huy, T.T., Ushijima, H., Quang, V.X., Win, K.M., Luengrojanakul, P., Kikuchi, K., Sata, T., Abe, K., 2004. Genotype C of hepatitis B virus can be classified into at least two subgroups. J. Gen. Virol. 85 (Pt 2), 283–292.

Kellerman, S.E., Hanson, D.L., McNaghten, A.D., Fleming, P.L., 2003. Prevalence of chronic hepatitis B and incidence of acute hepatitis B infection in human immunodeficiency virus-infected subjects. J. Infect. Dis. 188 (4), 571–577.

Kirishima, T., Okanoue, T., Daimon, Y., Itoh, Y., Nakamura, H., Morita, A., Toyama, T., Minami, M., 2002. Detection of YMDD mutant using a novel sensitive method in chronic liver disease type B patients before and during lamivudine treatment. J. Hepatol. 37 (2), 259–265.

Kobayashi, S., Ide, T., Sata, M., 2001. Detection of YMDD motif mutations in some lamivudine-untreated asymptomatic hepatitis B virus carriers. J. Hepatol. 34 (4), 584–586.

Konopnicki, D., Mocroft, A., de Wit, S., Antunes, F., Ledergerber, B., Katlama, C., Zilmer, K., Vella, S., Kirk, O., Lundgren, J.D., 2005. Hepatitis B and HIV: prevalence, AIDS progression, response to highly active antiretroviral therapy and increased mortality in the EuroSIDA cohort. AIDS 19 (6), 593–601.

Kosakovsky Pond, S.L., Frost, S.D., 2005. Not so different after all: a comparison of methods for detecting amino acid sites under selection. Mol. Biol. Evol. 22 (5), 1208–1222.

Lauer, U., Weiss, L., Hofschneider, P.H., Kekule, A.S., 1992. The hepatitis B virus pre-S/S (t) transactivator is generated by 3' truncations within a defined region of the S gene. J. Virol. 66 (9), 5284–5289.

Lim, S.G., Cheng, Y., Guindon, S., Seet, B.L., Lee, L.Y., Hu, P., Wasser, S., Peter, F.J., Tan, T., Goode, M., Rodrigo, A.G., 2007. Viral quasi-species evolution during hepatitis Be antigen seroconversion. Gastroenterology 133 (3), 951–958.

Lincoln, D., Petoumenos, K., Dore, G.J., 2003. HIV/HBV and HIV/HCV coinfection, and outcomes following highly active antiretroviral therapy. HIV Med. 4 (3), 241–249.

Lindh, M., Hannoun, C., Dhillon, Amar P., Norkrans, G., Horal, P., 1999. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J. Infect. Dis. 179 (4), 775–782.

Liu, C.-J., Kao, J.-H., Shau, W.-Y., Chen, P.-J., Lai, M.-Y., Chen, D.-S., 2002. Naturally occurring hepatitis B surface gene variants in chronic hepatitis B virus infection: correlation with viral serotypes and clinical stages of liver disease. J. Med. Virol. 68 (1), 50–59.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73 (1), 152–160.

Märschenz, S., Endres, A.-S., Brinckmann, A., Heise, T., Kristiansen, G., Nürnberg, P., Krüger, D.H., Günther, S., Meisel, H., 2006. Functional analysis of complex hepatitis B virus variants associated with development of liver cirrhosis. Gastroenterology 131 (3), 765–780.

Moriyama, K., Okamoto, H., Tsuda, F., Mayumi, M., 1996. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore–core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 226 (2), 269–280.

Mun, H.-S., Lee, S.-A., Jee, Y., Kim, H., Park, J.-H., Song, B.-C., Yoon, J.-H., Kim, Y.J., Lee, H.-S., Hyun, J.-W., Hwang, E.-S., Kook, Y.-H., Kim, B.-J., 2008. The prevalence of hepatitis B virus preS deletions occurring naturally in Korean patients infected chronically with genotype C. J. Med. Virol. 80 (7), 1189–1194.

Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnius, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47 (6), 289–309.

Ockenga, J., Tillmann, H.L., Trautwein, C., Stoll, M., Manns, M.P., Schmidt, R.E., 1997. Hepatitis B and C in HIV-infected patients. Prevalence and prognostic value. J. Hepatol. 27 (1), 18–24.

Okamoto, H., Tsuda, F., Akahane, Y., Sugai, Y., Yoshiba, M., Moriyama, K., Tanaka, T., Miyakawa, Y., Mayumi, M., 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. 68 (12), 8102–8110.

Osiowy, C., Giles, E., Tanaka, Y., Mizokami, M., Minuk, G.Y., 2006. Molecular evolution of hepatitis B virus over 25 years. J. Virol. 80 (21), 10307–10314.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. CABIOS 12 (4), 357–358.

Pond, S.L., Frost, S.D., 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 21 (10), 2531–2533.

Pond, S.L., Frost, S.D., Muse, S.V., 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics 21 (5), 676–679.

Preikschat, P., Günther, S., Reinhold, S., Will, H., Budde, K., Neumayer, H.H., Krüger, D.H., Meisel, H., 2002. Complex HBV populations with mutations in core promoter, C gene, and pre-S region are associated with development of cirrhosis in long-term renal transplant recipients. Hepatology 35 (2), 466–477.

Revill, P.A., Littlejohn, M., Ayres, A., Yuen, L., Colledge, D., Bartholomeusz, A., Sasaduesz, J., Lewin, S.R., Dore, G.J., Matthews, G.V., Thio, C.L., Locarnini, S.A., 2007. Identification of a novel hepatitis B virus precore/core deletion mutant in HIV/hepatitis B virus co-infected individuals. AIDS 21 (13), 1701–1710.

Song, B.-C., Kim, S.-H., Kim, H., Ying, Y.-H., Kim, H.-J., Kim, Y.-J., Yoon, J.-H., Lee, H.-S., Cha, C.-Y., Kook, Y.-H., Kim, B.-J., 2005. Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. J. Med. Virol. 76 (2), 194–202.

Tanaka, Y., Mukaide, M., Orito, E., Yuen, M.-F., Ito, K., Kurbanov, F., Sugauchi, F., Asahina, Y., Izumi, N., Kato, M., Lai, C.-L., Ueda, R., Mizokami, M., 2006. Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. J. Hepatol. 45 (5), 646–653.

Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., Miyakawa, Y., Mizokami, M., 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J. Virol. 83 (20), 10538–10547.

Thio, C.L., Seaberg, E.C., Skolasky Jr., R., Phair, J., Visscher, B., Munoz, A., Thomas, D.L., 2002. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). Lancet 360 (9349), 1921–1926.

Thomas, D.L., Cannon, R.O., Shapiro, C.N., Hook III, E.W., Alter, M.J., Quinn, T.C., 1994. Hepatitis C, hepatitis B, and human immunodeficiency virus infections among non-intravenous drug-using patients attending clinics for sexually transmitted diseases. J. Infect. Dis. 169 (5), 990–995.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25 (24), 4876–4882.

Tong, M.J., Blatt, L.M., Kao, J.H., Cheng, J.T., Corey, W.G., 2007. Basal core promoter T1762/A1764 and precore A1896 gene mutations in hepatitis B surface antigen-positive hepatocellular carcinoma: a comparison with chronic carriers. Liver Int. 27 (10), 1356–1363.

Trautwein, C., Schrem, H., Tillmann, H.L., Kubicka, S., Walker, D., Böker, K.H., Maschek, H.J., Pichlmayr, R., Manns, M.P., 1996. Hepatitis B virus mutations in the pre-S genome before and after liver transplantatio. Hepatology 24 (3), 482–488.

Wang, H.C., Wu, H.C., Chen, C.F., Fausto, N., Lei, H.Y., Su, I.J., 2003. Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress. Am. J. Pathol. 163 (6), 2441–2449.

Xu, Z., Yen, T.S., 1996. Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. J. Virol. 70 (1), 133–140.

Yuen, L.K.W., Ayres, A., Littlejohn, M., Colledge, D., Edgely, A., Maskill, W.J., Locarnini, S.A., Bartholomeusz, A., 2007. SEQHEPB: A sequence analysis program and relational database system for chronic hepatitis B. Antivir. Res. 75 (1), 64–74.