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Mutations Associated With Occult Hepatitis B in HIV-Positive South Africans

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Abstract

Occult hepatitis B is characterized by the absence of hepatitis B surface antigen (HBsAg) but the presence of HBV DNA. Because diagnosis of hepatitis B virus (HBV) typically includes HBsAg detection, occult HBV remains largely undiagnosed. Occult HBV is associated with increased risk of hepatocellular carcinoma, reactivation to chronic HBV during immune suppression, and transmission during blood transfusion and liver transplant. The mechanisms leading to occult HBV infection are unclear, although viral mutations are likely a significant factor. In this study, sera from 394 HIV-positive South Africans were tested for HBV DNA and HBsAg. For patients with detectable HBV DNA, the overlapping surface and polymerase open reading frames (ORFs) were sequenced. Occult-associated mutations—those mutations found exclusively in individuals with occult HBV infection but not in individuals with chronic HBV infection from the same cohort or GenBank references—were identified. Ninety patients (22.8%) had detectable HBV DNA. Of these, 37 had detectable HBsAg, while 53 lacked detectable surface antigen. The surface and polymerase ORFs were cloned successfully for 19 patients with chronic HBV and 30 patients with occult HBV. In total, 235 occult-associated mutations were identified. Ten occult-associated mutations were identified in more than one patient. Additionally, 15 amino acid positions had two distinct occult-associated mutations at the same residue. Occult-associated mutations were common and present in all regions of the surface and polymerase ORFs. Further study is underway to determine the effects of these mutations on viral replication and surface antigen expression in vitro.

Keywords

HBV polymerase; HBV surface antigen; HBV/HIV co-infection; immune selection; occult hepatitis B virus

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Introduction

Globally, two billion people have been exposed to hepatitis B virus (HBV) (family *Hepadnaviridae*, genus *Orthohepadnavirus*, species *Hepatitis B virus*), and 240 million people have chronic HBV [World Health Organization, 2013]. Chronic HBV is defined as the presence of hepatitis B surface antigen (HBsAg) in the serum for at least six months. In contrast, occult infection is defined by the presence of HBV DNA in the absence of HBsAg [Chevrier et al., 2007]. The detection of antibodies against hepatitis B core (anti-HBc) alone was used previously as a diagnostic marker [Torbenson and Thomas, 2002], although occult HBV infections with other serological profiles have been identified [Mphahlele et al., 2006; Lukhwani et al., 2009]. In up to 20% of occult HBV cases, all serological markers are absent [Kleinman et al., 2005]. As current testing algorithms for HBV diagnosis rely on HBsAg detection alone or in combination with antibodies against HBV, occult HBV is undiagnosed frequently.

Occult HBV is clinically significant in several contexts. First, occult HBV has been associated with an increased risk of developing hepatocellular carcinoma compared to healthy controls [Kew et al., 2008; Chen et al., 2009; Ikeda et al., 2009; Wong et al., 2011]. In vitro studies also suggest that occult HBV may contribute to cellular stress and DNA damage in peripheral blood mononuclear cells (PBMCs) [Bhargava et al., 2010]. Furthermore, occult HBV is a concern during immune suppression during which viral replication may increase up to 100-fold before serological markers appear [Hui et al., 2006; Coppola et al., 2011]. The immune response to these increasing viral loads—either during immunosuppression or upon restoration of the immune system—can lead to acute hepatitis, fulminant hepatic failure, and/or death [Coppola et al., 2011]. Finally, occult HBV can be transmitted via blood transfusion or liver transplant resulting in the establishment of chronic HBV infection or fulminant hepatic failure [Chazouillères et al., 1994; Levicnik-Stezinar et al., 2008; Yuen et al., 2011].

Multiple mechanisms may explain the low viral replication and lack of detectable surface antigen that characterize occult HBV infections, including epigenetic modifications of the HBV genome [Vivekanandan et al., 2008; Kaur et al., 2010], sequestration of HBsAg in immune complexes [Yotsuyanagi et al., 1998; Yotsuyanagi et al., 2001], polymorphisms in host cytokine and chemokine receptors [Arababadi et al., 2010; Hassanshahi et al., 2010; Ahmadabadi et al., 2012], and suppression of viral replication via modulation of cytokine levels [Martin et al., 2009]. Additionally, viral mutations play a significant role. Occult-associated mutations—those that appear exclusively or with higher frequency in occult HBV infections as compared to chronic HBV infection—have been identified throughout the HBV genome. While occult-associated mutations have been identified in the X preCore/ Core, and Pol regions most studies to date have focused on mutations within the PreS/S reading frame [Hou et al., 2001; Chan et al., 2002; Kao et al., 2002; Chaudhuri et al., 2004; Jeantet et al., 2004; Wagner et al., 2004; Khatib et al., 2005; Pogány et al., 2005; Datta et al., 2006; Awerkiev et al., 2007; Banerjee et al., 2007; Araujo et al., 2008; Candotti et al., 2008; Gonza et al., 2008; Henke-Gendo et al., 2008; Miyagawa et al., 2008; Vivekanandan et al., 2008; Bremer et al., 2009; Chen et al., 2009; Kumar et al., 2009; Song et al., 2009; Tamori et al., 2009; Cheung et al., 2010; El Chaar et al., 2010; Martin et al., 2010; Martin et

al., 2012; Hamkar et al., 2010; Motta et al., 2010; Thedja et al., 2010; Xu et al., 2010; Yuan et al., 2010; Fujise et al., 2011; Launay et al., 2011; Mello et al., 2011; Bes et al., 2012; Shahmoradi et al., 2012; Svicher et al., 2012; Cassini et al., 2013; Kim et al., 2013]. In several studies, the functional importance of these mutations has been evaluated [Schories et al., 2000; Hass et al., 2005; Martin et al., 2012; Svicher et al., 2012].

Multiple studies suggest that HIV is risk factor for occult HBV infection. The prevalence of chronic HBV in HIV-positive South Africans is 8.7%–22.9%, while the prevalence of occult HBV is 8%–23% [Mphahlele et al., 2006; Firnhaber et al., 2009; Lukhwareni et al., 2009; Bell et al., 2012]. A previous study found that occult HBV is more common in HIV-infected South Africans than their HIV-negative counterparts [Mphahlele et al., 2006]. The aim of this study was to identify occult-associated mutations in HIV-positive South Africans.

Materials and Methods

Patient Population

Serum samples were collected from 394 HIV-positive South Africans receiving care at the Dr. George Mukhari Hospital (DGMH) at the University of Limpopo (Medunsa Campus) in Pretoria between September 2004 and December 2009. To avoid unnecessary bleeding of study participants, leftover blood samples were used after routine testing was completed. All the samples were de-linked from personal identifiers. Ethical clearance was obtained from the Research and Ethics Committee at the University of Limpopo (Project number: MP 07/2005) and from the DGMH.

HIV and Liver Function Testing

HIV serostatus was determined as described previously [Lukhwareni et al., 2009; Mphahlele et al., 2006]. HIV viral load was determined by the NucliSens EasyQ assay (Biomerieux, Durham, North Carolina). HIV treatment status was not available for many patients due to the cross-sectional nature of this analysis; therefore, those patients with undetectable viral loads (<175 copies/ml) were considered to be treated. CD4 T cell count was determined using the Flow Care PLG CD4 count (Beckman Coulter, Pasadena, California). ALT values were determined using the SYNCHRON CX System (Beckman Coulter, Pasadena, California). As data were taken from testing performed at the time of sample collection, values were not available for all samples.

HBV Testing

HBV DNA was extracted from 200 µL of patient serum using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Indianapolis, Indiana). All samples were evaluated for the presence of HBV DNA using real-time PCR with the LightCycler® 2.0 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN) with a lower limit of detection of 250 copies/ml [Ho et al., 2003]. HBV DNA is detected frequently in samples with lower viral loads, but sensitivity testing has not been performed below 250 copies/ml. Elecsys 2010 diagnostic assays (Roche Diagnostics, Penzberg, Germany) were used to evaluate the presence of HBsAg in each sample. Patients that were positive for HBV DNA and HBsAg

comprised the *chronic* HBV infection group, while those positive for HBV DNA but negative for HBsAg comprised the *occult* HBV infection group.

PCR Amplification and Cloning

The overlapping surface and polymerase reading frames were amplified for each sample with one of four sets of primers (Supplementary Table SI). Pico-Maxx High Fidelity PCR system (Agilent, Santa Clara, California) was used for amplification as follows: 94°C for 2 min initially, 40 cycles of 94°C for 40 sec, 60°C for 90 sec, and 68°C for 3 min, 2 min at 68°C every ten cycles, and ending with 68°C for 8 min. PCR products were separated by gel electrophoresis, purified, and cloned into the pGEM T Easy vector (Promega, Madison, Wisconsin). To identify the dominant viral sequences in each patient, three clones per PCR product were sequenced bidirectionally using the primers described in Günther et al. [1995]. For this initial analysis, sequencing was limited to three clones, as a previous study found less inpatient diversity than expected when 10 clones per patient were sequenced, and because we were most interested in the dominant mutation present at each position [Martin et al., 2010].

Phylogenetic Analysis

Sequence chromatographs were assembled and edited using CodonCode Aligner (CodonCode, Centerville, Massachusetts). Sequences were deposited in the NCBI database with accession numbers KF475982-KF476030. Sequences were aligned in ClustalX with full-length GenBank references and trimmed to the same length (1.99 kb). The statistical robustness and reliability of these trees was determined using bootstrap analysis with 1,000 replicates. Additional phylogenetic inference was performed using a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the Bayesian Evolutionary Analysis by Sampling Trees (BEAST) v1.7.3 program [Drummond and Rambaut, 2007] under an uncorrelated log-normal relaxed molecular clock and the Hasegawa, Kishino and Yano (HKY) model with nucleotide site heterogeneity estimated using a gamma distribution. The BEAST MCMC analysis included a chain length of 10,000,000 with sampling every 1,000th generation. Results were visualized in Tracer v1.5 to confirm chain convergence, and the effective sample size (ESS) was calculated for each parameter. All ESS values were >500 indicating sufficient sampling. The maximum clade credibility tree (Fig. 1) was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator v1.7.3. Posterior probabilities >90% were considered statistically significant. The association between HBV genotype and occult versus chronic HBV status was evaluated using Fisher's exact test. Genetic distances were calculated by pairwise comparison of nucleotide sequences using the Kimura method of MegAlign (DNASTAR, Madison, Wisconsin).

Recombination Analysis

Simplot software (version 3.5.1) was used to assess for recombination by comparing South African HBV sequences to references representing genotypes (A-H), with a window size of 200 base pairs and a step size of 20 base pairs. Subgenotypes A1, A2, and D3 were given specific attention as the major subgenotypes represented in the region.

Mutational Analysis

Patient-derived HBV sequences were aligned with 233 genotype-matched GenBank references (Supplementary Table SII). BioEdit was utilized to translate sequences and to compare patient-derived HBV sequences with references in both the surface and polymerase ORFs. All clonal sequences were compared to each of the 233 GenBank references as described previously [Martin et al., 2010]. Those mutations that occurred exclusively in occult HBV patient samples and not in chronic HBV patient samples or chronic HBV sequences retrieved from GenBank were considered occult-associated mutations. This comparison to individual sequences rather than a consensus sequence minimizes the potential of misclassifying naturally occurring polymorphisms as occult-associated mutations. Occult-associated mutations were compared to previously published mutations [Hou et al., 2001; Chan et al., 2002; Kao et al., 2002; Chaudhuri et al., 2004; Jeantet et al., 2004; Wagner et al., 2004; Khattab et al., 2005; Pogány et al., 2005; Datta et al., 2006; Awerkiew et al., 2007; Banerjee et al., 2007; Araujo et al., 2008; Gonza et al., 2008; Henke-Gendo et al., 2008; Miyagawa et al., 2008; Vivekanandan et al., 2008; Bremer et al., 2009; Kumar et al., 2009; Song et al., 2009; Tamori et al., 2009; Cheung et al., 2010; El Chaar et al., 2010; Hamkar et al., 2010; Martin et al., 2010, Martin et al., 2012; Motta et al., 2010; Thedja et al., 2010; Xu et al., 2010; Yuan et al., 2010; Launay et al., 2011; Mello et al., 2011; Bes et al., 2012; Shahmoradi et al., 2012; Svicher et al., 2012; Cassini et al., 2013; Kim et al., 2013].

Immune Selection Pressures

Individual codons under positive or negative selection were detected via the fixed effects likelihood (FEL) method as implemented in the DataMonkey program [Pond and Frost, 2005]. Briefly, this method estimates the rate of synonymous and non-synonymous mutations for each codon. When the rate of synonymous mutations is greater than the rate of non-synonymous mutations, negative selection—or purifying selection—is said to occur. When the rate of non-synonymous mutations is greater than the rate of synonymous mutations, positive selection has occurred. As this analysis is performed on a codon-by-codon basis, the surface and polymerase ORFs were examined separately. Chronic and occult sequences were examined separately as well to determine codons under positive and negative selection for each infection group.

Statistical Analysis

Fisher's exact test was utilized for group-to-group differences in race, sex, and HBV serology. Kruskal–Wallis one-way analysis of variance was used to analyze age, ALT, AST, HIV viral load, CD4 count, and HBV viral load. For all analyses, *P*-values of less than 0.05 were considered statistically significant.

Results

Patient Population

Of the 394 patients evaluated, 90 (22.8%) had detectable HBV DNA. Of these, 37 patients—9.4% of original cohort—also had detectable HBsAg (chronic HBV infection). Fifty-three

patients (13.5%) had detectable HBV DNA but lacked detectable HBsAg (occult HBV infection). PCR amplification of the polymerase ORF was successful for 49 of 90 patients, including 30 patients with occult HBV and 19 patients with chronic HBV. Samples were tested with at least two different sets of primers before being considered PCR negative (Supplementary Table SI).

Age, race, AST, and CD4 count did not vary significantly between the HBV negative, chronic HBV, and occult HBV groups (Table I). ALT levels were elevated slightly but not significantly in patients with chronic HBV. Though the percentage of patients with undetectable viral loads was approximately the same for all groups (65.9%, 67.8%, and 62.9% for HBV negative, chronic HBV, and occult HBV, respectively), there was a statistically difference in HIV viral load ($P = 0.0062$) between chronic HBV and the other groups. No significant difference was found in HIV viral load or HBV viral load between the HBV negative and occult HBV groups. HBV serology also differed between the chronic HBV group and the HBV negative and occult HBV groups ($P = 0.0348$). Those in the chronic HBV group had a higher occurrence of the anti-HBc positive anti-HBs negative profile commonly associated with chronic HBV infection than the HBV negative or occult HBV groups.

Genotype and Recombinant Analysis

Clonal sequences were aligned with references for each genotype and subgenotypes when relevant. Phylogenetic analysis identified genotype A1 in four of the occult patients and eight of the chronic HBV patients. Genotype A2 was present in 24 occult patients and 10 chronic patients. The association between genotype A2 and occult HBV was statistically significant ($P = 0.038$). Two patients with occult HBV and one patient with chronic HBV were classified as genotype D3. Due to the low number of patients with genotype D, only genotype A sequences were included in subsequent mutational analyses.

With only two exceptions, all clonal sequences are unique. In one case, the set of two identical sequences are clones from the same patient. In another, the two identical sequences are from different patients. Overall, the median genetic distance between sequences was 3.52% with a range of zero (the two sets of identical sequences) to 14.39%. Comparison of patient samples against non-recombinant references representing genotypes A through H showed no evidence of recombination. No samples displayed significant boot scan values greater than 70% for any reference sequences other than their subgenotype references.

Occult-Associated Mutations

In total, 235 occult-associated mutations were identified throughout the overlapping surface and polymerase ORFs. The surface ORF contained 84 occult-associated mutations (Table II). Eighteen mutations were found in the preS1 region, nine in the preS2 region, and 57 in the surface region. Three mutations were found in clones from more than one patient, and six amino acid residues had more than one distinct occult-associated mutation at that residue (i.e., two different mutations occurred at the same site). Eighteen occult-associated mutations were identified previously in other studies. Additionally, 27 newly identified

mutations occurred at amino acid residues where different occult-associated mutations had been identified previously (Table II).

In the polymerase ORF, 151 occult-associated mutations were found (Table III). Occult-associated mutations were identified in all regions of the polymerase gene: 35 in the terminal region, 27 in the spacer region, 86 in the reverse transcriptase region, and three in the 59 amino acids of the RNase H region that were analyzed. Seven mutations were found in more than one patient, and nine amino acids positions had more than one occult-associated mutation at that residue. Four mutations had been identified previously, while five newly identified mutations occurred at an amino acid residue where a different occult-associated mutation were reported previously.

Treatment-Associated Mutations

Six sequences from three distinct treatment-naïve patients had mutations associated with HBV drug resistance [Lok et al., 2007]. All three clones for patient A831 (chronic HBV infection) had rtM204I, a mutation associated with resistance to lamivudine and telbivudine. Two clones from patient B18661137 with chronic HBV infection also had this mutation, although the third clone did not. Finally, one clone from patient A227 with occult HBV infection had rtM204V, a mutation also associated with resistance to lamivudine.

Immune Selection Pressures

In both the surface and polymerase ORFs, a number of codons were under positive or negative selection in both the occult and chronic HBV infection groups. In the surface ORF, one positively selected codon and five negatively selected codons were identified in the occult sequences (Table IV). In the chronic sequences, six positively selected codons and 38 negatively selected codons were identified. One of the negatively selected codons was shared between the two groups. Furthermore, occult-associated mutations were located at seven of the negatively selected codons in the chronic infection group, as well as one positively selected codon each in the occult and chronic infection group and one negatively selected codon in the occult infection group. One negatively selected site in the occult group was located in the “a” determinant. Additionally, two sites potentially involved with drug resistance were identified as sites of negative selection, with rt180 identified in the chronic group and rt204 identified in both the chronic and the occult group.

In the polymerase ORF, two positively selected codons and 23 negatively selected codons were identified in the occult infection group (Table V). In the chronic infection group, one positively selected codon and 55 negatively selected codons were identified. Four of the negatively selected codons were common between the two groups. In addition, occult-associated mutations were located at nine of the negatively selected codons identified in the chronic infection group.

Discussion

Occult HBV infection has been associated with increased risk of hepatocellular carcinoma, reactivation during immune suppression, and transmission via blood transfusion and liver transplantation. While the mechanism underlying the low levels of viral replication and the

lack of detectable HBsAg are not fully understood, viral mutations play a significant role. To our knowledge, this is only the third study to examine the virologic differences between occult and chronic HBV infection in South Africa and the second to focus on HIV-positive South Africans. In this previous study, HBsAg negative/HBV DNA positive blood donors from three blood centers across South Africa were evaluated [Allain et al., 2009]. While this population may have included a small number of HIV-positive individuals, it did not focus specifically on HIV-positive patients. An additional study evaluated virologic differences in HBV-monoinfected and HIV-HBV co-infected patients, while including analysis of HBsAg-negative versus HBsAg-positive individuals [Makondo et al., 2012]. To our knowledge, this is the first study to address sites of positive and negative selection pressures in occult HBV-HIV co-infection. As the prevalence of occult HBV is much higher among HIV-positive people than HIV-negative people (8%–23% in HIV-positive people compared to <1% to 2.4% in HIV-negative people) [Mphahlele et al., 2006; Allain et al., 2009; Firnhaber et al., 2009; Lukhwareni et al., 2009; Bell et al., 2012], it is necessary to examine occult-associated mutations in HIV-positive South Africans to best understand the disease in this unique population.

Here, we report the presence of genotypes A1, A2, and D in South Africa. Previous studies have also reported these genotypes, although A1 was the predominant genotype in other studies [Kimbi, 2004; Makondo et al., 2012]. In this study, genotype A2 was more common than A1 in both patients with occult (24 vs. 4) and chronic HBV infection (10 vs. 8), and the association between genotype A2 and occult HBV infection was statistically significant. A previous study characterizing virologic characteristics of occult HBV in South Africa found none of the 30 occult patients and only four of the 73 chronic patients had genotype A2, as compared to 23 occult patients and 62 chronic patients with genotype A1 virus [Allain et al., 2009]. In another study, genotype A2 HBV infections were reported [Makondo et al., 2012]. The reasons for the predominance of A2 in our patient population are unclear. Since this study included patients from a single hospital setting, it is possible that the increased prevalence of A2 is specific to the particular patient population served by this hospital. Thus, the statistical association between occult HBV and genotype A2 should be viewed with caution and confirmed in a larger, more geographically diverse population. While the subgenotype of South African genotype D viruses has rarely been determined, genotype D3—one of the genotypes found in this study—has been reported previously in South Africa [Norder et al., 2004].

Within the 28 occult HBV patients with genotype A, 235 occult-associated mutations were identified. These mutations were found exclusively in the occult HBV patient samples in the cohort. Comparison of occult-associated mutations to those observed in chronic HBV patients from the same cohort and to genotype-matched reference sequences excluded most of the natural polymorphisms found in both our population and genotype A HBV. As there were no significant differences between the chronic and occult patient groups in ALT, AST, age, gender, treatment rate, or HBV viral load, these mutations are unlikely to be related to these factors. Despite these strict criteria, a small minority of the identified mutations could represent natural HBV variation instead of mutations specifically contributing to the occult HBV phenotype. Thus, in vitro analysis is underway to determine the functional relevance of these mutations.

Of the 84 identified surface mutations, eighteen were previously identified, and twenty-seven occurred at amino acid residue where occult-associated mutations had previously been identified (Tables II and III). However, the majority of the mutations identified in the current study had not been reported previously. This may be due to the large number of occult HBV patients evaluated in this study. Alternatively, these occult-associated mutations may be specific to South Africa and not characteristic of other countries. Fewer of the identified polymerase mutations were previously identified or are located where mutations have been identified previously (7 of 160), although this is likely due to the much smaller number of studies examining occult-associated mutations in the polymerase ORF as compared to the surface ORF.

Many of the mutations identified were in areas of the surface and polymerase proteins where the immune system may assert significant selection pressure. In the surface protein, the “a” determinant is the region of HBsAg targeted by neutralizing antibodies and spans amino acids 124–147 (Supplementary Figure S1). Seven of the 56 mutations identified in the surface gene were found within this region. Such mutations have been shown to decrease HBsAg expression in in vitro and in vivo studies [Huang et al., 2012]. These mutations may also decrease the ability of commercial assays to detect HBsAg, suggesting HBsAg may have been produced but not detected in these patients [Huang et al., 2012]. Occult-associated mutations were identified in epitopes targeted by cytotoxic T lymphocytes (CTLs) as well. Twelve CTL epitopes were described previously in the surface gene, all from the HLA-A2 locus [Chisari and Ferrari, 1995; Westover and Hughes, 2007]. In total, 25 mutations were found in these epitopes (Supplementary Figure S1). Four CTL epitopes have been described in polymerase (also all HLA-A2) [Westover and Hughes, 2007], and seven mutations were identified in the three epitopes included in our analysis (Supplementary Figure S2). Mutations in these regions may allow the virus to evade immune detection, although they could potentially decrease viral replicative capacity as well. Additionally, 15 mutations were found in the region of HBsAg that interacts with the viral capsid (S amino acids 96–122 and 169–195) [Van Hemert et al., 2008].

In both the surface and polymerase ORFs, more negatively selected codons were identified in the chronic sequences than the occult sequences. This suggests that less purifying selection is occurring in patients with occult HBV. Functional constraints in the viral genome are unlikely to differ between the two groups; however, immune selection pressures may vary between occult and chronic HBV. One potential contributing factor may be the typically low viral loads of patients with occult HBV, as the virus may be replicating at sufficiently low levels to partially evade immune selection. This may also account for the increase in positively selected codons in the surface ORF of the chronic HBV group. More exposure to the immune system may favor both non-synonymous mutations and synonymous mutations, as mutations facilitating immune escape would be favored, while those that allow better immune control would be selected against. Sixteen negatively selected codons in the chronic HBV sequence occurred at sites of occult-associated mutations. These mutations may be occurring in areas of functional significance, potentially affecting polymerase activity and contributing to the occult HBV phenotype.

The current study has several potential limitations. First, analysis was limited to the surface and polymerase ORFs. While this represented 2.2 kb of the 3.2 kb genome, the X and preC/C ORFs were not analyzed. As occult-associated mutations have previously been identified in both of these ORFs [Kao et al., 2002; Chaudhuri et al., 2004; Candotti et al., 2008; Chen et al., 2009; Fujise et al., 2011], the total number of occult-associated mutations through the HBV genome is likely underestimated. Additionally, we were most interested in the dominant viral variant in each patient. Thus, additional minor variants may have been missed. Finally, the surface and polymerase ORFs could not be amplified from all patients with HBV DNA. The in-house PCR is likely less sensitive than the diagnostic assay, so samples from patients with lower HBV viral loads may not have been amplified in all instances.

In conclusion, occult-associated mutations have been identified in all regions of the surface and polymerase open reading frames of HIV-positive South Africans. These mutations may play a role in decreasing viral replication and HBsAg expression. While some mutations are found in immunologically active regions, many are not, suggesting additional pathways that regulate the development of occult-associated mutations. Identification of distinct virologic factors of occult HBV in HIV positive South Africans aids in the understanding of occult HBV pathogenesis in this population. Functional analysis is ongoing to assess the effect of these mutations on HBsAg expression and viral replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Ahmadabadi BN, Hassanshahi G, Arababadi MK, Leanza C, Kennedy D. The IL-10 promoter polymorphism at position -592 is correlated with susceptibility to occult HBV infection. *Inflammation*. 2012; 35:818–821. [PubMed: 21901441]
- Allain JP, Belkhir D, Vermeulen M, Crookes R, Cable R, Amiri A, Reddy R, Bird A, Candotti D. Characterization of occult hepatitis B virus strains in South African blood donors. *Hepatology*. 2009; 49:1868–1876. [PubMed: 19434719]
- Arababadi MK, Pourfathollah AA, Jafarzadeh A, Hassanshahi G, Rezvani ME. Association of exon 9 but not intron 8 VDR polymorphisms with occult HBV infection in south-eastern Iranian patients. *J Gastroenterol Hepatol*. 2010; 25:90–93. [PubMed: 19793172]
- Araujo NM, Branco-Vieira M, Silva ACM, Pilotto JH, Grinsztejn B, de Almeida AJ, Trepo C, Gomes SA. Occult hepatitis B virus infection in HIV-infected patients: Evaluation of biochemical, virological and molecular parameters. *Hepatol Res*. 2008; 38:1194–1203. [PubMed: 18624719]
- Awerkwiew D, Däumer M, Reiser M, Wend UC, Pfister H, Kaiser R, Willems W, Gerlich WH. Reactivation of an occult hepatitis B virus escape mutant in an anti-HBs positive, anti-HBc negative lymphoma patient. *J Clin Virol*. 2007; 38:83–86. [PubMed: 17134939]

- Banerjee A, Chandra PK, Datta S, Biswas A, Bhattacharya P, Chakraborty S, Chakrabarti S, Bhattacharya SK, Chakravarty R. Frequency and significance of hepatitis B virus surface gene variant circulating among “antiHBc only” individuals in Eastern India. *J Clin Virol.* 2007; 40:312–317. [PubMed: 17997353]
- Bell TG, Makondo E, Martinson NA, Kramvis A. Hepatitis B virus infection in human immunodeficiency virus infected southern African adults: occult or overt—that is the question. *PLoS One.* 2012; 7:e45750. [PubMed: 23049685]
- Bes M, Vargas V, Piron M, Casamitjana N, Esteban JI, Vilanova N, Pinacho A, Quer J, Puig L, Guardia J, Sauleda S. T cell responses and viral variability in blood donation candidates with occult hepatitis B infection. *J Hepatol.* 2012; 56:765–774. [PubMed: 22173156]
- Bhargava A, Khan S, Panwar H, Pathak N, Punde RP, Varshney S, Mishra PK. Occult hepatitis B virus infection with low viremia induces DNA damage, apoptosis and oxidative stress in peripheral blood lymphocytes. *Virus Res.* 2010; 153:143–150. [PubMed: 20667493]
- Bremer CM, Saniewski M, Wend UC, Torres P, Lelie N, Gerlich WH, Glebe D. Transient occult hepatitis B virus infection in a blood donor with high viremia. *Transfusion.* 2009; 49:1621–1629. [PubMed: 19413737]
- Candotti D, Grabarczyk P, Ghiazza P, Roig R, Casamitjana N, Iudicone P, Schmidt M, Bird A, Crookes R, Brojer E, Miceli M, Amiri A, Li C. Characterization of occult hepatitis B virus from blood donors carrying genotype A2 or genotype D strains. *J Hepatol.* 2008; 49:537–547. [PubMed: 18602718]
- Cassini R, De Mitri MS, Gibellini D, Urbinati L, Bagaglio S, Morsica G, Domenicali M, Verucchi G, Bernardi M. A novel stop codon mutation within the hepatitis B surface gene is detected in the liver but not in the peripheral blood mononuclear cells of HIV-infected individuals with occult HBV infection. *J Viral Hepat.* 2013; 20:42–49. [PubMed: 23231083]
- Chan HLY, Tsang SWC, Leung NWY, Tse CH, Hui Y, Tam JSL, Chan FKL, Sung JJY. Occult HBV infection in crypto-genic liver cirrhosis in an area with high prevalence of HBV infection. *Am J Gastroenterol.* 2002; 97:1211–1215. [PubMed: 12014730]
- Chaudhuri V, Tayal R, Nayak B, Acharya SK, Panda SK. Full-length genome and analysis of mutant surface promoter. *Gastroenterology.* 2004; 127:1356–1371. [PubMed: 15521005]
- Chazouillères O, Mamish D, Kim M, Carey K, Ferrell L, Roberts JP, Ascher NL, Wright TL. “Occult” hepatitis B virus as source of infection in liver transplant recipients. *Lancet.* 1994; 343:142–146. [PubMed: 7904004]
- Chen CH, Changchien CS, Lee CM, Tung WC, Hung CH, Hu TH, Wang JH, Wang JC, Lu SN. A study on sequence variations in pre-S/surface, X and enhancer II/core promoter/ precore regions of occult hepatitis B virus in non-B, non-C hepatocellular carcinoma patients in Taiwan. *Int J Cancer.* 2009; 125:621–629. [PubMed: 19431214]
- Cheung WI, Chan HLY, Leung VKS, Tse CH, Fung K, Lin SY, Wong A, Wong VWS, Chau TN. Reactivation of hepatitis B virus infection with persistently negative HBsAg on three HBsAg assays in a lymphoma patient undergoing chemotherapy. *J Clin Virol.* 2010; 47:193–195. [PubMed: 20031483]
- Chevrier MC, St-Louis M, Perreault J, Caron B, Castilloux C, Laroche J, Delage G. Detection and characterization of hepatitis B virus of anti-hepatitis B core antigen-reactive blood donors in Quebec with an in-house nucleic acid testing assay. *Transfusion.* 2007; 47:1794–1802. [PubMed: 17880603]
- Chisari, FV.; Ferrari, C. Hepatitis B virus immunopathology. Vol. 17. Springer Semin Immun; 1995. p. 261–281.
- Coppola N, Tonziello G, Pisaturo M, Messina V, Guastafierro S, Fiore M, Iodice V, Sagnelli C, Stanzione M, Capoluongo N, Pasquale G, Sagnelli E. Reactivation of overt and occult hepatitis B infection in various immunosuppressive settings. *J Med Virol.* 2011; 83:1909–1916. [PubMed: 21915865]
- Datta S, Banerjee A, Chandra PK, Chowdhury A. Genotype, phylogenetic analysis, and transmission pattern of occult hepatitis B virus (HBV) infection in families of asymptomatic HBsAg carriers. *J Med Virol.* 2006; 78:53–59. [PubMed: 16299727]

- Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol.* 2007; 7:214. [PubMed: 17996036]
- El Chaar M, Candotti D, Crowther RA, Allain JP. Impact of hepatitis B virus surface protein mutations on the diagnosis of occult hepatitis B virus infection. *Hepatology.* 2010; 52:1600–1610. [PubMed: 20815025]
- Feng B, Wei L, Chen M, Wang L. Dynamic changes of hepatitis B virus polymerase gene including YMDD motif in lamivudine-treated patients with chronic hepatitis B. *Microbiol Res.* 2008; 163:487–492. [PubMed: 17317127]
- Firnhaber C, Viana R, Reyneke A, Schultze D, Malope B, Maskew M, Bisceglie AMD, MacPhail P, Sanne I, Kew M. Occult hepatitis B virus infection in patients with isolated core antibody and HIV co-infection in an urban clinic in Johannesburg, South Africa. *Int J Infect Dis.* 2009; 13:488–492. [PubMed: 19081280]
- Fujiise K, Tatsuzawa K, Kono M, Hoshina S, Tsubota A, Niiya M, Namiki Y, Tada N, Tajiri H. A mutation of the start codon in the X region of hepatitis B virus DNA in a patient with non-B, non-C chronic hepatitis. *World J Hepatol.* 2011; 3:56–60. [PubMed: 21423595]
- Gonza L, Zaijier HL, Torres P, Ontan A, Lelie PN, Boot HJ. Multiple surface antigen mutations in five blood donors with occult hepatitis B virus infection. *J Med Virol.* 2008; 80:1344–1349. [PubMed: 18551607]
- Günther S, Li BC, Miska S, Krüger DH, Meisel H, Will H. 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.* 1995; 69:5437–5444. [PubMed: 7636989]
- Hamkar R, Aghakhani A, Soufian S, Banifazl M, Ghavami N, Nadri M, Sofian M, Ahmadi F, Razeghi E, Eslamifar A, Ramezani A. Surface gene mutations of hepatitis B virus among high-risk patients with occult hepatitis B virus infection. *Diagn Microbiol Infect Dis.* 2010; 66:285–291. [PubMed: 19903586]
- Hass M, Hannoun C, Kalinina T, Sommer G, Manegold C, Günther S. Functional analysis of hepatitis B virus reactivating in hepatitis B surface antigen-negative individuals. *Hepatology.* 2005; 42:93–103. [PubMed: 15962285]
- Hassanshahi G, Arababadi MK, Khoramdelazad H, Yaghini N, Zarandi ER. Assessment of CXCL12 (SDF-1 α) polymorphisms and its serum level in posttransfusion occult HBV-infected patients in Southeastern Iran. *Arch Med Res.* 2010; 41:338–342. [PubMed: 20851290]
- Henke-Gendo C, Amini-Bavil-Olyae S, Challapalli D, Trautwein C, Deppe H, Schulz TF, Heim A, Tacke F. Symptomatic hepatitis B virus (HBV) reactivation despite reduced viral fitness is associated with HBV test and immune escape mutations in an HIV-coinfected patient. *J Infect Dis.* 2008; 198:1620–1624. [PubMed: 18847320]
- Ho SKN, Yam WiC, Leung ETK, Wong LP, Leung JKH, Lai KN, Chan TM. Rapid quantification of hepatitis B virus DNA by real-time PCR using fluorescent hybridization probes. *J Med Microbiol.* 2003; 52:397–402. [PubMed: 12721315]
- Hou J, Wang Z, Cheng J, Lin Y, Lau GK, Sun J, Zhou F, Waters J, Karayiannis P, Luo K. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology.* 2001; 34:1027–1034. [PubMed: 11679975]
- Huang CH, Yuan Q, Chen PJ, Zhang YL, Chen CR, Zheng QB, Yeh SH, Yu H, Xue Y, Chen YX, Liu PG, Ge SX, Zhang J, Xia NS. Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors. *J Hepatol.* 2012; 57:720–729. [PubMed: 22634131]
- Hui CK, Cheung WWW, Zhang HY, Au WY, Yueng YH, Leung AYH, Leung N, Luk JM, Lie AKW, Kwong YL, Liang R, Lau GKK. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology.* 2006; 131:59–68. [PubMed: 16831590]
- Ikeda K, Kobayashi M, Someya T, Saitoh S, Hosaka T, Akuta N, Suzuki F, Suzuki Y, Arase Y, Kumada H. Occult hepatitis B virus infection increases hepatocellular carcinogenesis by eight times in patients with non-B, non-C liver cirrhosis: a cohort study. *J Viral Hepat.* 2009; 16:437–443. [PubMed: 19226331]

- Jeantet D, Chemin I, Mandrand B, Tran A, Zoulim F, Trepo C, Kay A. Cloning and expression of surface antigens from occult chronic hepatitis B virus infections and their recognition by commercial detection assays. *J Med Virol.* 2004; 73:508–515. [PubMed: 15221893]
- Kao JH, Chen PJ, Lai MY, Chen DS. Sequence analysis of pre-S/surface and pre-core/core promoter genes of hepatitis B virus in chronic hepatitis C patients with occult HBV infection. *J Med Virol.* 2002; 68:216–220. [PubMed: 12210410]
- Kaur P, Paliwal A, Durantel D, Hainaut P, Scoazec JY, Zoulim F, Chemin I, Herceg Z. DNA methylation of hepatitis B virus (HBV) genome associated with the development of hepatocellular carcinoma and occult HBV infection. *J Infect Dis.* 2010; 202:700–704. [PubMed: 20653444]
- Kew MC, Welschinger R, Viana R. Occult hepatitis B virus infection in Southern African blacks with hepatocellular carcinoma. *J Gastroenterol Hepatol.* 2008; 23:1426–1430. [PubMed: 18853999]
- Khattab E, Chemin I, Vuillermoz I, Vieux C, Mrani S, Guillaud O, Trepo C, Zoulim F. Analysis of HCV co-infection with occult hepatitis B virus in patients undergoing IFN therapy. *J Clin Virol.* 2005; 33:150–157. [PubMed: 15911431]
- Kim H, Lee SA, Kim DW, Lee SH, Kim BJ. Naturally occurring mutations in large surface genes related to occult infection of hepatitis B virus genotype C. *PloS One.* 2013; 8:e54486. [PubMed: 23349904]
- Kimbi GC. Distinctive sequence characteristics of subgeno-type A1 isolates of hepatitis B virus from South Africa. *J Gen Virol.* 2004; 85:1211–1220. [PubMed: 15105537]
- Kleinman SH, Strong DM, Tegtmeier GGE, Holland PV, Gorlin JB, Cousins C, Chiacchierini RP, Pietrelli La. Hepatitis B virus (HBV) DNA screening of blood donations in minipools with the COBAS AmpliScreen HBV test. *Transfusion.* 2005; 45:1247–1257. [PubMed: 16078909]
- Kumar GT, Kazim SN, Kumar M, Hissar S, Chauhan R, Basir SF, Sarin SK. Hepatitis B virus genotypes and hepatitis B surface antigen mutations in family contacts of hepatitis B virus infected patients with occult hepatitis B virus infection. *J Gastroenterol Hepatol.* 2009; 24:588–598. [PubMed: 19207682]
- Launay O, Masurel J, Servant-Delmas A, Basse-Guérineau AL, Méritet JF, Laperche S, Sogni P, Rosenberg aR. High levels of serum hepatitis B virus DNA in patients with “anti-HBc alone”: role of HBsAg mutants. *J Viral Hepat.* 2011; 18:721–729. [PubMed: 21914087]
- Levicnik-Stezinar S, Rahne-Potokar U, Candotti D, Lelie N, Allain JP. Anti-HBs positive occult hepatitis B virus carrier blood infectious in two transfusion recipients. *J Hepatol.* 2008; 48:1022–1025. [PubMed: 18436328]
- Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, Liaw YF, Mizokami M, Kuiken C. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology.* 2007; 46:254–265. [PubMed: 17596850]
- Lukhwari A, Burnett RJ, Selabe SG, Mzileni MO, Mphahlele MJ. Increased detection of HBV DNA in HBsAg-positive and HBsAg-negative South African HIV/AIDS patients enrolling for highly active antiretroviral therapy at a tertiary hospital. *J Med Virol.* 2009; 81:406–412. [PubMed: 19152393]
- Makondo E, Bell TG, Kramvis A. Genotyping and molecular characterization of hepatitis B virus from human immunodeficiency virus-infected individuals in southern Africa. *PloS One.* 2012; 7:e46345. [PubMed: 23029487]
- Martin CM, Welge JA, Shire NJ, Shata MT, Sherman KE, Blackard JT. Cytokine expression during chronic versus occult hepatitis B virus infection in HIV co-infected individuals. *Cytokine.* 2009; 47:194–198. [PubMed: 19625194]
- Martin CM, Welge JA, Shire NJ, Rouster SD, Shata MT, Sherman KE, Blackard JT. Genomic variability associated with the presence of occult hepatitis B virus in HIV co-infected individuals. *J Viral Hepat.* 2010; 17:588–597. [PubMed: 19889143]
- Martin CM, Welge JA, Rouster SD, Shata MT, Sherman KE, Blackard JT. Mutations associated with occult hepatitis B virus infection result in decreased surface antigen expression in vitro. *J Viral Hepat.* 2012; 19:716–723. [PubMed: 22967103]
- Mello FCA, Martel N, Gomes SA, Araujo NM. Expression of Hepatitis B Virus Surface Antigen Containing Y100C Variant Frequently Detected in Occult HBV Infection. *Hepat Res Treat.* 2011; 2011:695859. [PubMed: 21331286]

- Miyagawa M, Minami M, Fujii K, Sendo R, Mori K, Shimizu D, Nakajima T, Yasui K, Itoh Y, Taniwaki M, Okanou T, Yoshikawa T. Molecular characterization of a variant virus that caused de novo hepatitis B without elevation of hepatitis B surface antigen after chemotherapy with rituximab. *J Med Virol.* 2008; 80:2069–2078. [PubMed: 19040281]
- Motta JS, Mello FC, Lago BV, Perez RM, Gomes Sa, Figueiredo FF. Occult hepatitis B virus infection and lamivudine-resistant mutations in isolates from renal patients undergoing hemodialysis. *J Gastroenterol Hepatol.* 2010; 25:101–106. [PubMed: 19817965]
- Mphahlele MJ, Lukhwireni A, Burnett RJ, Moropeng LM, Ngobeni JM. High risk of occult hepatitis B virus infection in HIV-positive patients from South Africa. *J Clin Virol.* 2006; 35:14–20. [PubMed: 15916918]
- Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology.* 2004; 47:289–309. [PubMed: 15564741]
- Pogány K, Zaaijer HL, Prins JM, Wit FW, Lange JM, Beld MGHM. Occult hepatitis B virus infection before and 1 year after start of HAART in HIV type 1-positive patients. *AIDS Res Hum Retroviruses.* 2005; 21:922–926. [PubMed: 16386107]
- Pond SLK, Frost SDW. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics.* 2005; 21:2531–2533. [PubMed: 15713735]
- Schories M, Peters T, Rasenack J. Isolation, characterization and biological significance of hepatitis B virus mutants from serum of a patient with immunologically negative HBV infection. *Journal of Hepatology.* 2000; 33:799–811. [PubMed: 11097490]
- Shahmoradi S, Yahyapour Y, Mahmoodi M, Alavian SM, Fazeli Z, Jazayeri SM. High prevalence of occult hepatitis B virus infection in children born to HBsAg-positive mothers despite prophylaxis with hepatitis B vaccination and HBIG. *J Hepatol.* 2012; 57:515–521. [PubMed: 22617152]
- Song EY, Yun YM, Park MH, Seo DH. Prevalence of occult hepatitis B virus infection in a general adult population in Korea. *Intervirology.* 2009; 52:57–62. [PubMed: 19401629]
- Svicher V, Cento V, Bernassola M, Neumann-Fraune M, Van Hemert F, Chen M, Salpini R, Liu C, Longo R, Visca M, Romano S, Micheli V, Bertoli A, Gori C, Ceccherini-Silberstein F, Sarrecchia C, Andreoni M, Angelico M, Ursitti A, Spanò A, Zhang JM, Verheyen J, Cappiello G, Perno CF. Novel HBsAg markers tightly correlate with occult HBV infection and strongly affect HBsAg detection. *Antiviral Res.* 2012; 93:86–93. [PubMed: 22086128]
- Tamori A, Hayashi T, Shinzaki M, Kobayashi S, Iwai S, Enomoto M, Morikawa H, Sakaguchi H, Shiomi S, Takemura S, Kubo S, Kawada N. Frequent detection of hepatitis B virus DNA in hepatocellular carcinoma of patients with sustained virologic response for hepatitis C virus. *J Med Virol.* 2009; 81:1009–1014. [PubMed: 19382258]
- Thedja MD, Roni M, Harahap AR, Siregar NC, Ie SI, Muljono DH. Occult hepatitis B in blood donors in Indonesia: altered antigenicity of the hepatitis B virus surface protein. *Hepatol Int.* 2010; 4:608–614. [PubMed: 21063484]
- Torbenson M, Thomas DL. Occult hepatitis B. *Lancet Infect Dis.* 2002; 2:479–486. [PubMed: 12150847]
- Van Hemert FJ, Zaaijer HL, Berkhout B, Lukashov VV. Occult hepatitis B infection: an evolutionary scenario. *Virol J.* 2008; 5:146. [PubMed: 19077239]
- Vivekanandan P, Kannangai R, Ray SC, Thomas DL, Torbenson M. Comprehensive genetic and epigenetic analysis of occult hepatitis B from liver tissue samples. *Clin Infect Dis.* 2008; 46:1227–1236. [PubMed: 18444860]
- Wagner AA, Denis F, Weinbreck P, Loustaud V, Autouge F, Rogez S, Alain S. Serological pattern “anti-hepatitis B core alone” in HIV or hepatitis C virus-infected patients is not fully explained by hepatitis B surface antigen mutants. *AIDS.* 2004; 18:569–571. [PubMed: 15090814]
- Werle B, Cinquin K, Marcellin P, Pol S, Maynard M, Trépo C, Zoulim F. Evolution of hepatitis B viral load and viral genome sequence during adefovir dipivoxil therapy. *J Viral Hepat.* 2004; 11:74–83. [PubMed: 14738561]
- Westover KM, Hughes AL. Evolution of cytotoxic T-lymphocyte epitopes in hepatitis B virus. *Infect Genet Evol.* 2007; 7:254–262. [PubMed: 17140859]

- Wong DKH, Huang FY, Lai CL, Poon RTP, Seto WK, Fung J, Hung IFN, Yuen MF. Occult hepatitis B infection and HBV replicative activity in patients with cryptogenic cause of hepato-cellular carcinoma. *Hepatology*. 2011; 54:829–836. [PubMed: 21809355]
- World Health Organization. WHO | Hepatitis B Fact Sheet N204: Hepatitis B. World Health Organization; 2013. Retrieved January 23, 2013, from <http://www.who.int/mediacentre/factsheets/fs204/en/>
- Xu L, Wei Y, Chen T, Lu J, Zhu CL, Ni Z, Huang F, Du J, Sun Z, Qu C. Occult HBV infection in anti-HBs-positive young adults after neonatal HB vaccination. *Vaccine*. 2010; 28:5986–5992. [PubMed: 20637763]
- Yotsuyanagi H, Yasuda K, Iino S, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Kimura S, Koike K. Persistent viremia after recovery from self-limited acute hepatitis B. *Hepatology*. 1998; 27:1377–1382. [PubMed: 9581694]
- Yotsuyanagi H, Yasuda K, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Nojiri N, Fuji T, Hoshino H, Shimoda K, Hino K, Kimura S, Iino S, Koike K. Frequent presence of HBV in the sera of HBsAg-negative, anti-HBc-positive blood donors. *Transfusion*. 2001; 41:1093–1099. [PubMed: 11552064]
- Yuan Q, Ou SH, Chen CR, Ge SX, Pei B, Chen QR, Yan Q, Lin YC, Ni HY, Huang CH, Yeo AET, Shih JWK, Zhang J, Xia NS. Molecular characteristics of occult hepatitis B virus from blood donors in southeast China. *J Clin Micro*. 2010; 48:357–362.
- Yuen MF, Wong DKH, Lee CK, Tanaka Y, Allain JP, Fung J, Leung J, Lin CK, Sugiyama M, Sugauchi F, Mizokami M, Lai CL. Transmissibility of hepatitis B virus (HBV) infection through blood transfusion from blood donors with occult HBV infection. *Clin Infect Dis*. 2011; 52:624–632. [PubMed: 21245155]

Table 1
Demographic and Clinical Characteristics of Patient Population

	No HBV (n = 304)	Chronic HBV (n = 37)	Occult HBV (n = 53)	P-value
Age (years)	37	35	35.5	NS
ALT	23	26	20	0.076
AST	29.5	39	24	NS
Undetectable HIV viral load (%) ^a	65.9	67.8	62.9	
CD4 cell count (cells/mm ³)	229	216.5	269.5	NS
HBV viral load (IU/ml)		2597.55	1950	NS
Gender (% female)	67.8	54.5	79.5	0.067
Race (% black)	98.0	100	97.6	NS
Serology (%)				0.035
anti HBs –/anti HBc –	51.6	37.5	46.9	
anti HBs –/anti HBc +	18.8	46.9	18.4	
anti HBs +/anti HBc –	3.1	6.3	4.1	
anti HBs+/anti HBc +	26.6	9.4	30.6	

Median values for age, ALT, AST, CD4 cell count, and HBV viral load are given. NS= not significant ($P > 0.10$).

^a While all three groups had similar rates of undetectable HIV viral load, there was a significant difference ($P = 0.006$) among those with detectable viral loads, with the chronic HBV group having significantly higher HIV viral loads.

Table II
Occult-Associated Mutations in the Surface Open Reading Frame

	Patient ID	PreS1	PreS2	Surface
A1	865	N39S ^x	L3P	G102S C147Y ^{xx}
	448	S78G	Q100R	S64P
	249	A36V	P107S ^x	W201R
	B474		S43L	L12P S61PX V96IX
			W182R ^x	W199STOP ^x
				W223STOP
A2	234	D31G	S28G ^{xx}	T23A ^x F134L ^{xx}
	300	P69S		
	311		G18E ^{*x}	F19V ^x C48R ^{xx} P70L ^{*x}
	398	I108T		C48R ^{xx} I82M ^x C137Y ^{xx}
	324	H51R ^{*xx}	S101L N37D	D14G L9p ^x M197T F219S [*]
	757		P34L ^x	P178S ^x
	764			L87P ^{xx}
	7270		N55H	M1V S132P ^{xx} N146S ^{xx}
	74		Y21H ^{xx}	W36R C138Y ^{xx} I218M
	1726296		T116A ^{xx}	F158L ^x K160R ^x F219L
	181199	S101P	I38N	L192P ^{xx} S210G ^x
	223	Q80L ^x		N146D ^{xx}
	37575		Q54STOP ^{xx}	S171F C193R ^x
	24N			L26P
	38		G44R ^x	L77V ^x F93L ^x
	227		L39P	I82V ^x L87Q ^x
	131		T37A	W191R
	393		D51G ^{xx}	H60R
	A106	Q57R	D14G	
	A1074	P79T ^{xx}		

Patient ID	PreS1	PreS2	Surface	
B313			P62S ^X	
A648	S109p ^{xx}		<u>G102D</u>	V190I [*]
A1061			L39P	M198V ^X
A274	<u>N37S</u>	A119T	C69R [*]	D99N ^X L173P L176P

Occult-associated mutations appeared only in occult HBV samples and did not appear in chronic HBV samples or references. Sample subgenotype is noted in the first column. Mutations appearing in bold were present in more than one patient. Underlined mutations indicate that more than one occult-associated mutation was found at this residue.

* Asterisks denote mutations found in more than one viral clone from the same patient.

When mutations have been previously reported, the mutation is followed by XX. Mutations followed by an X indicate that a different occult-associated mutation was previously identified at this residue.

Table III
Occult-Associated Mutations in the Polymerase Open Reading Frame

Patient ID			Terminal		Spacer		Reverse Transcriptase				RNase H		
A1	865	G59R	H100Y	Q118R*	R110K	V266A	M309V	V341A					
	448		R69G	E81G	H12Y	L72P	I103T ^{xx}	S143P	F195L				
	249	R107G	T114P	S110F	A194T	L209S	Q344R				T2P		
A2	B474	F81L			L69P	Q125STOP	V190A	V208I	G232S	W243G	K328N		
	234	N44K	E163G	R76G	Q150R ^{xx}	D31G	L102P	Q130STOP	V142A				
	300	Y135C		P72L	N93D	D134G	W243STOP						
	311	C171W	R147G	Y149C	V27G	V56A	G152R	C198R	K270R				
	398	S176P		S113P	N36D ^x	V56A	I91V	M145I					
	324	F81L		Q40R	T150A	L338P							
	757	R93G	T122A	C161R	A186V	N279S							
	764				E1G	A342V							
	7270	T55A	T78P	R108K	R6G ^x	S85P	E8A	H9R	P59T	V84A ^x	S137P	L140P	T150A
	74	I112T		S139G	V143A	N36D ^x	V44A	C198R	F227V				
1726296	K57R	P82L	K106E	A103T	V104A	S97P	N124S	F166S	F227S				
181199	N60D	I131V	I158N	Y160STOP	H197N	E218G	P237T ^{xx}						
223	T78P		E81A	S135P*	S143P*	K145R							
37575	F81L	M113V	143H	L149P	A62V	L147P	V278A			T4A			
24N	D34G			S74P	F296T	V341A							
38				G52E	S85C	A97T	L102V	A211T					
227	R116G			H90R	M309T								
131	I90V			A21T	N36D ^x	R41G	D45G	T54A	M98V	L199S			
393			Q101K ^x	R120G									
A106													
A1074	Q6R		P82H	T70A ^{xx}	S105P	T296S							
B313	K132R			T70I ^x									

Patient ID	Terminal	Spacer	Reverse Transcriptase	RNase H
A 648	N36D R162G	L112P ^{XX}	<u>C198Y</u> *	
A 1061	D89G K110R P119H		N71D D206G	
A 274	L3P	I9V G122D	L77S* G107E T322A	

Occult-associated mutations appeared only in occult HBV samples and did not appear in chronic HBV samples or references. Sample subgenotype is noted in the first column. Mutations appearing in bold were present in more than one patient. Underlined mutations indicate that more than one occult-associated mutation was found at this residue.

* Asterisks denote mutations found in more than one viral clone from the same patient.

When mutations have been previously reported, the mutation is followed by XX. Mutations followed by an X indicate that a different occult-associated mutation was previously identified at this residue.

Table IV
Immune Selection Pressure in the Surface ORF

Occult HBV (n=31)		Chronic HBV (n=18)		
Positively selected sites	Negatively selected sites pre	Positively selected sites	Negatively selected sites	
S-87*	preS1-72	preS2-35	preS1-7	preS2-13
	preS1-106	preS2-39	preS1-8	preS2-14*
	S-108	preS2-47	preS1-10	preS2-17
	S-141	S-8	preS1-19	preS2-19
	S-191*	S-109*	preS1-20	preS2-40
		S-159	preS1-29	preS2-43*
			preS1-32	S-12*
			preS1-36*	S-25
			preS1-37*	S-51
			preS1-42	S-61*
			preS1-56	S-67
			preS1-64	S-80
			preS1-68	S-91
			preS1-69*	S-94
			preS1-92	S-101
			preS1-93	S-108
			preS1-105	S-120
			preS2-7	S-180
			preS2-8	S-211

Individual codons under positive or negative selection were detected via the fixed effects likelihood (FEL) method as implemented in the DataMonkey program.

* Asterisks denote locations of occult-associated mutations.

Codons under negative immune selection in both the occult and chronic HBV groupings are denoted in bold.

Table V

Immune Selection Pressure in the Polymerase ORF

Occult HBV (n = 31)		Chronic HBV (n = 18)	
Positively selected sites	Negatively selected sites	Positively selected sites	Negatively selected sites
tp-19	tp-95	rt-73	tp-47
sp-102	tp-100	rt-47	tp-53
	tp-114	rt-78	rt-202
	tp-129	rt-89	tp-59*
	tp-157	rt-95	tp-65
	tp-168	rt-168	rt-240
	tp-175	rt-202	rt-241
	tp-178	rt-241	rt-249
	tp-183	rt-275	rt-251
	sp-34	rt-317	rt-256
	sp-46	rh-10	rt-259
rt-11			rt-260
			tp-106*
			tp-107*
			rt-263
			rt-268
			rt-274
			tp-123
			tp-124
			rt-278*
			rt-280
			tp-148
			tp-174
			tp-174
			sp-13
			rt-305*
			sp-98
			rt-318
			sp-149*
			rt-321
			sp-152
			rt-325
			sp-153
			rt-335
			sp-156
			rt-342*
			rt-6
			rh-2*
			rt-138
			rh-10
			rt-167
			rh-29
			rt-178
			rh-43

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Occult HBV (n = 31)		Chronic HBV (n = 18)	
Positively selected sites	Negatively selected sites	Positively selected sites	Negatively selected sites
		rt-180	rt-46
		rt-185	rt-54
		rt-192	

Individual codons under positive or negative selection were detected via the fixed effects likelihood (FEL) method as implemented in the DataMonkey program.

* Asterisks denote locations of occult-associated mutations.

Codons under negative immune selection in both the occult and chronic HBV groupings are denoted in bold. Polymerase regions are denoted by tp, sp, rt, and rh for terminal protein, spacer, reverse transcriptase, RNase H, respectively.