Published in final edited form as:

J Med Virol. 2011 July ; 83(7): 1142–1150. doi:10.1002/jmv.22090.

Hepatitis B Virus (HBV) X Gene Diversity and Evidence of Recombination in HBV/HIV Co-Infected Persons

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Abstract

The high frequency of mutation during hepatitis B virus (HBV) infection has resulted in 8 genotypes (A–H) with varying effects on disease severity and treatment efficacy. However, analysis of inpatient HBV diversity is limited, especially during HIV co-infection. Therefore, a preliminary study was performed to analyze HBV X gene diversity in 17 HBV/HIV co-infected individuals. Phylogenetic analysis revealed HBV genotype A in 13 individuals (76.5%) or genotype E in 1 individual (5.9%). Additionally, 3 individuals were dually infected with HBV genotypes A and G (17.6%). Overall, higher genetic distance and entropy were observed in the X region and overlapping polymerase (Pol(X)) regions when compared to the PreS, S, and overlapping polymerase (Pol(PS) and Pol(S)) regions analyzed in the same patients as part of a previous study. In addition, multiple viral variants from 2 individuals with dual HBV infection did not group with either genotype A or G by phylogenetic analysis, indicating possible recombination. SimPlot bootscan analysis confirmed recombination breakpoints within the X gene in both individuals. Recombination between HBV genotypes may represent an important evolutionary strategy that enhances overall pathogenic potential and/or alters the downstream effects of the HBV X protein.

Keywords

inpatient diversity; dual HBV infection; HBV/HIV co-infection; hepatitis B virus X protein (HBx); quasispecies; recombination

INTRODUCTION

Hepatitis B virus (HBV) is one of the leading causes of liver cirrhosis, as well as hepatocellular carcinoma (HCC). Currently, at least 350 million individuals are living with chronic HBV infection [Lavanchy, 2004]. Compared to most DNA viruses, HBV has a high

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This work was presented at the 6th International Workshop on HIV & Hepatitis Co-infection in Tel Aviv, Israel, May 31–June 2, 2010 and at the HIV & Liver Disease Conference 2010 in Jackson Hole, Wyoming, September 24–26, 2010.

mutation rate—approximately 2×10^{-5} nucleotide substitutions per site per year [Okamoto et al., 1987], although this is lower than most RNA viruses. Thus far, 8 distinct HBV genotypes (A–H) have been identified, which differ by at least 8% at the nucleotide level [Stuyver et al., 2000]. Dual infection with multiple distinct HBV genotypes is also common [Ramos et al., 2007; Sanchez et al., 2007; Kurbanov et al., 2010], and recombination between HBV genotypes has been observed [Bollyky et al., 1996; Morozov et al., 2000; Cui et al., 2002; Sugauchi et al., 2002; Kato et al., 2002b; Kurbanov et al., 2005; Suwannakarn et al., 2005; Bekondi et al., 2007; Osiowy et al., 2008]. While the overall number of HBV infections due to recombinant viruses is unknown, recombinant variants may be more prevalent than initially thought and are capable of becoming the dominant variants of HBV within a population, as observed in Tibet [Cui et al., 2002]. In the United States, genotype A is common, although all HBV genotypes have been reported [Stuyver et al., 2000; Moriya et al., 2002; Chu et al., 2003]. Evaluating HBV genotypes is clinically relevant, as genotype can impact both treatment efficacy and severity of liver disease [Fung and Lok, 2004; Schaefer, 2005]. Nonetheless, few studies have been performed to date that directly assess inpatient HBV genetic diversity.

The severity of disease and the development of cirrhosis and HCC have been linked to the HBV X protein (HBx) [Bouchard and Schneider, 2004; Zhang et al., 2006]. HBx is a 154 amino acid regulatory protein whose functions include direct and indirect modulation of protein degradation, apoptosis, signal transduction, transcription, and cell cycle progression [Bouchard and Schneider, 2004]. HBx has also been implicated in transcriptional activation of nuclear factor- κ B, constitutive activation of extracellular signal-related kinases and c-Jun N-terminal kinases, as well as exit from G₀ in arrested cells [Mahé et al., 1991; Madden and Slagle, 2001; Nijhara et al., 2001]. Furthermore, HBx is associated with the development of HCC in patients [Zhang et al., 2006], although this association remains controversial [Lee et al., 1990; Kim et al., 1991].

In HBV/HIV co-infected individuals, an increase in HBV replication may be observed [Gilson et al., 1997; Colin et al., 1999]. In contrast, HBx acts as a nuclear co-activator capable of inducing transcriptional activity of the HIV long terminal repeat [Balsano et al., 1993; Gomez-Gonzalo et al., 2001], resulting in increased HIV replication and further impairment of the host immune function. However, few studies have analyzed inpatient HBV quasispecies diversity in HBV/HIV co-infected individuals, especially in the X region. Therefore, we conducted a preliminary analysis of X gene variability in HBV/HIV-infected individuals.

MATERIALS AND METHODS

Patient Population

We previously determined the prevalence of HBV infection in a cross-sectional analysis of HIV-positive individuals being followed at the University of Cincinnati Infectious Diseases Center [Shire et al., 2007]. HBV DNA levels were quantified in patient serum samples using a real-time PCR assay with a lower limit of detection of 100 IU/ml. Serologic markers of HBV infection—HBsAg, anti-HBc, and anti-HBs—were measured by ELISA [Shire et al., 2007]. In a previous report, we quantified inpatient HBV diversity within the PreS, S, and

overlapping polymerase regions—denoted Pol(PS) and Pol(S)—within this population [Martin et al., 2010]. For the current analysis, the X gene was amplified from a convenience sample of 17 individuals with HBV/HIV co-infection.

PCR Amplification and Cloning

Two hundred to 400 µl of patient serum was used to extract HBV DNA, of which 5 µl served as the template for amplification of full-length HBV genomes [Günther et al., 1995]. One microliter of the full-length PCR product was then utilized as the template for a second round X gene-specific PCR [389 bp; nucleotides (nt) 1294–1683 according to X97848] using primers P198-S ([Uchida et al., 1994]; nt 1294–1316) and CDR1-AS ([Kannangai et al., 2004]; nt 1683–1661). Amplification conditions were as follows: 94°C for 5 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) was used for amplifications according to the manufacturer's instructions. HBV DNA-negative serum samples and a reaction without template served as negative controls, while a previously amplified HBV DNA-positive serum sample served as a positive control. All PCR products were gel purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI). A median number of 10 viral variants were sequenced per individual and submitted to GenBank under accession numbers HM484887–HM484967.

Phylogenetic Analysis

Sequence alignments were initially created using the neighbor-joining method in Clustal X [Thompson et al., 1997] for both the X region and the overlapping segment of the polymerase open reading frame (Pol(X)). The statistical robustness and reliability of the branching order were assessed 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) v1.5.0 program [Drummond and Rambaut, 2007] under an uncorrelated log-normal relaxed molecular clock and the generalized time reversible (GTR) model with nucleotide site heterogeneity estimated using a gamma distribution. The BEAST MCMC analysis was run for a chain length of 50,000,000 with sampling every 5,000th generation. Results were visualized in Tracer v1.4 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 was selected from the posterior tree distribution after a 10% burn-in using Tree Annotator v1.5.0. Posterior probabilities >90% were considered statistically significant.

Shannon entropy was calculated using the following equation: $S_n = - (p_i \ln p_i) / \ln N$, where p_i is the frequency of each distinct nucleotide sequence, and N is the total number of sequences analyzed per patient. Intrapatient genetic distances (GD) were calculated by pairwise comparison of nucleotide sequences using the Kimura method, while non-synonymous (dN) and synonymous (dS) mutations were calculated via the Nei–Gojobori method [Nei and Gojobori, 1986] as implemented in the MEGA 4 software [Tamura et al., 2007].

Recombinant Analysis

To assess intergenotypic recombination, consensus HBx sequences from each patient were aligned with non-recombinant GenBank references representing all HBV genotypes. Individual HBx viral variants that were outliers by phylogenetic analysis were further compared to the consensus sequences of non-recombinant variants for that patient. Similarity plots and bootscan analyses were then performed in SimPlot version 3.5.1 [Lole et al., 1999] using the Kimura 2-parameter with a 100 bp window and a 10 bp step. GenBank reference X75657 (genotype E) was utilized as an outlier. Additionally, principal coordinate analysis [Higgins, 1992] was performed to assess patterns in sequence data using the PCOORD program, accessible at <http://www.hiv.lanl.gov/content/sequence/PCOORD/PCOORD.html>.

Statistical Analysis

For quasispecies parameters (GD, entropy, and dN–dS), each dependent variable was rank-ordered, and analysis of variance (ANOVA) tests were performed between regions—including PreS, S, X, Pol(S), Pol(PS), and Pol(X)—with the Tukey correction for multiple comparisons. Spearman's correlations between quasispecies parameters and age, race, alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBV DNA, and CD4 cell count were performed. *P*-values <0.05 were considered significant.

RESULTS

Patient Population Characteristics

For the HBV/HIV co-infected individuals in this study, 15 were HBsAg+/HBV DNA+, 2 were HBsAg–/HBV DNA+. All were male; 65% were African-American and 35% were Caucasian, with a median age of 36.1 years (range = 21.2–51.3 years). HBV DNA levels ranged from 2.8×10^2 to 7.6×10^8 IU/ml with a median of 1.5×10^7 IU/ml. The median ALT and AST were 52 U/L (17–114) and 54 U/L (24–78), respectively. The median CD4 cell count was 265 cells/ml (7–665), and the median HIV RNA level was 5.8×10^4 copies/ml (7.2×10^2 – 2×10^5).

HBV Genotypes

The HBV genotypes determined by analysis of the X gene were consistent with those previously identified based on analysis of the PreS and S regions [Martin et al., 2010] and included genotypes A (n = 13, 76.5%) and E (n = 1, 5.9%). Additionally, 3 individuals (17.6%) were dually infected with HBV genotypes A and G (Fig. 1A). Importantly, all 3 A +G dual infections (HBsAg+/HBV DNA+) were previously characterized as genotype G single infections based on analysis of the PreS and S regions [Martin et al., 2010]. In addition, several HBx variants—10 from patient 241571 and 4 from patient 243541—appeared as outliers when compared to the consensus A or consensus G sequences for that particular individual (underlined, Fig. 1B). Interestingly, these viral variants clustered with different genotypes when the alternate open reading frames (ORFs) were analyzed. For instance, 8 of 10 outlier variants for patient 241571 and 1 of 4 outlier variants for patient 243541 clustered with genotype G reference sequences in the Pol ORF (5' end) and with

genotype A reference sequences for the X ORF (3' end). The remaining 2 outlier variants for patient 241571 and 3 outlier variants for patient 243541 clustered with genotype A reference sequences for the Pol ORF (5' end) but with genotype G reference sequences for the X ORF (3' end; data not shown).

Quasispecies Diversity

Overall, inpatient median GD (Fig. 2A) and entropy (Fig. 2B) values were slightly higher for the X and Pol(X) regions compared to those previously reported for the PreS, S, and the corresponding Pol(PS) and Pol(S) regions. Median GD was similar between X and PreS (0.0035 vs. 0.0019; $P = 0.128$) and approached significance when comparing X and S (0.0035 vs. 0; $P = 0.009$). Median GD for Pol(X) was 0.0032 compared to 0.0019 for the Pol(PS) region ($P = 0.134$) and also approached significance when compared to 0 for the Pol(S) region ($P = 0.011$). For the X region, median entropy was 0.4088 compared to 0.4084 for the PreS region ($P = 0.987$) and 0.1412 for the S region ($P = 0.026$). For Pol(X), entropy was significantly higher compared to Pol(S) (0.5931 vs. 0.1412; $P = 0.002$), but not when compared to the Pol(PS) region (0.5931 vs. 0.4084; $P = 0.441$).

Median dN–dS values above 0 were not observed for either the X or Pol(X) regions, indicating that positive immune selection pressures were not consistently acting upon this region in the majority of patients (Fig. 2C). Although dN–dS values for X were less than 0 for all patients, it is interesting to note that positive dN–dS values were observed for the genotype A variants of patient 241571 and for the genotype G variants of patient 243541, indicating that positive immune selection pressure is present in those individuals with putative recombinant viruses. No significant associations were observed between the various indicators of quasispecies diversity and CD4 cell count, HIV viral load, or HBV DNA level.

Recombinant Analysis

For patients with potential X gene recombination, separate viral DNA extractions, PCRs, and cloning were performed to confirm the presence of recombination. Additional variants sequenced were included in the recombinant analysis to further explore intergenotypic recombination. HBV nucleotide sequences for all viral variants from patients 241571 and 243541 were compared to GenBank genotype references using SimPlot. For patient 241571, 7 viral variants belonged to genotype A (23.3%), 13 viral variants belonged to genotype G (43.3%), and 10 viral variants showed evidence of A/G recombination (33.3%). For patient 243541, 15 viral variants belonged to genotype A (48.4%), 12 viral variants belonged to genotype G (38.7%), and 4 viral variants showed evidence of A/G recombination (12.9%).

BootScan analyses indicated that 8 of 10 recombinant variants from patient 241571 clustered with genotype G at the 5' end of the amplified region but clustered with genotype A at the 3' end. The breakpoints for three viral variants were located at nucleotide 1397 (according to X97848) in the overlapping polymerase and X ORFs, while the other 5 had breakpoints corresponding to nucleotides 1497–1587 (representative variant shown in Fig. 3). BootScan analyses indicated that the remaining 2 variants for patient 241571 displayed the opposite recombination pattern with the 5' end of the amplified region clustering with genotype A, but clustering with genotype G at the 3' end with breakpoints at nucleotides

1407–1417 (all breakpoints shown in Fig. 5). Similarly, recombination analysis for patient 243541 indicated that 3 viral variants clustered with genotype A at the 5' end, but with genotype G at the 3' end. Breakpoints were observed at nucleotides 1417, 1547, and 1577 within the Pol/X overlap region (representative variant shown in Fig. 4). The remaining variant from patient 243541 displayed recombination with the 5' end clustering with genotype G, the 3' end clustering with genotype A, and a breakpoint at nucleotide 1527 (all breakpoints shown in Fig. 5). Phylogenetic trees constructed for the 5' end or the 3' end of each recombinant sequence provided clear evidence of relatedness between the recombinant variants and the consensus sequence of the genotype indicated by BootScan analysis (data not shown).

Using principal coordinate analysis, the 10 recombinant variants for patient 241571 clustered into two distinct groups between genotype A and genotype G reference sequences. One group of 2 recombinants was located adjacent to genotype G, while the other group consisting of 8 recombinants was located adjacent to genotype A (Supplemental Figure 1A). This distribution highlights the variability in breakpoints observed by BootScan analysis. In contrast, the remaining non-recombinant viral variants from patient 241571 grouped closely with reference sequences for either genotype A ($n = 7$) or G ($n = 13$). For patient 243541, PCOORD analysis demonstrated that the 4 recombinant variants clustered as one large grouping between the genotype A and the genotype G reference sequences. The remaining viral variants from patient 243541 grouped closely with references for either genotype A ($n = 15$) or G ($n = 12$; Supplemental Figure 1B).

DISCUSSION

To date, most HBV studies have focused mainly on genotypic diversity within a given population [Amini-Bavil-Olyaei et al., 2006; Datta et al., 2008] and limited data are available on X gene variability despite a growing number of studies suggesting its importance in the HBV life cycle and regulation of several cellular processes [Mahé et al., 1991; Madden and Slagle, 2001; Nijhara et al., 2001; Zhang et al., 2006]. However, the current preliminary study aimed to investigate inpatient HBV diversity within the X and overlapping polymerase genes. Overall, the X and Pol(X) regions tended to have higher GD and entropy values. Significantly higher entropy values were observed in the Pol(X) region when compared to the Pol(S) ($P = 0.002$) region, previously analyzed in the same patients [Martin et al., 2010].

This analysis also provided the opportunity to investigate intergenotypic recombination. Of the 17 individuals included in this study, three dual HBV infections with genotypes A+G (17.6%) were detected. HBV genotype G single infections are rare in the available literature [Alvarado-Esquivel et al., 2006; Chudy et al., 2006; Pas et al., 2008], as the vast majority of genotype G infections are found as dual infections with genotypes A, C, or H [Kato et al., 2002a; Suwannakarn et al., 2005; Sanchez et al., 2007]. It has been suggested that genotype G infections may be characterized by impaired replication and require dual infection with a functional HBV strain to maintain chronicity, although replication-competent genotype G variants have been described [Li et al., 2007]. The three dual HBV infections identified in this study had been reported previously as genotype A ($n = 2$) or G ($n = 1$) single infections

based on analysis of the PreS and S regions. However, this discrepancy is not entirely surprising given that immune selection pressures may act independently on distinct genomic regions and a lack of recombination in one region does not rule out recombination within another region [Allen et al., 2005; Harrington et al., 2007; Streeck et al., 2008].

Identification of dual HBV infection is increasingly common, and recombination has been identified between most HBV genotypes [Simmonds and Midgley, 2005]. Several recombination “hot spots” have been identified within the HBV genome, including PreS1/PreS2 (nt 3150–100), the 3' end of S (nt 650–830), and both ends of core (nt 1640–1900 and 2330–2450) [Simmonds and Midgley, 2005; Yang et al., 2006]. Osiowy et al. also identified several recombination breakpoints within A+G dually infected individuals, including several within the X gene. Here, we identified two regions within the X gene—nt 1397–1417 and nt 1497–1587—in which HBV recombination occurred. These regions are highly conserved between genotypes A and G and likely facilitate intergenotypic recombination events.

It is reasonable to suggest that decreased immune function, such as during HIV co-infection, may render HBV/HIV co-infected individuals more susceptible to dual HBV infection. Interestingly, a study investigating HBV genotypes identified more dual HBV infections in HBV/HIV co-infected individuals than expected—40% compared to 0.3% prevalence in the US [Shire et al., 2006]. In addition, dual infections also trended towards an association with increased HIV viral load. While the effects of recombination events in A+G dual infections are currently unknown, it is interesting to note that genotype G has been described as a determinant of liver fibrosis in immunocompromised individuals [Lacombe et al., 2006]. Therefore, in HBV/HIV co-infected individuals, genotype A viruses may further enhance the pathogenic potential of genotype G viruses through intergenotypic recombination. Furthermore, recombination within the X gene could result in additional functions of the HBx regulatory protein with yet unknown consequences on HBV pathogenesis, especially in HBV/HIV co-infected patients. Future studies should investigate the prevalence of dual HBV infection and HBV recombination in HBV mono-infected individuals compared to HBV/HIV co-infected individuals. Full-length HBV genomes should be sequenced to identify genomic regions that favor HBV recombination, and the potential effects these recombination events may have on pathogenesis and disease progression should be elucidated.

ACKNOWLEDGMENTS

Grant sponsor: Digestive Health Center pilot Award; Grant number: P30 DK078392; Grant sponsor: University of Cincinnati Center for Clinical and Translational Science and Training (statistical support).

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Fig. 2. Dot plots for (A) genetic distance, (B) Shannon entropy, and (C) dN-dS values. ● represents individual patient values, while (◆) represent median values for each region. Box and whisker plots indicate upper and lower quartiles, as well as outliers. The solid line in (C) represents a dN-dS value of 0.

Fig. 3.

Recombinant analysis for recombinant variant 7 from patient 241571. A representative SimPlot bootscan using the Kimura 2-parameter, with 500 replicates, a window of 100 bp, and a 10 bp step, was performed with GenBank genotype references (**A**), as well as patient-specific consensus sequences for genotypes A and G (**B**).

Fig. 4.

Recombinant analysis for recombinant variant 8 from patient 243541. A representative SimPlot bootscan using the Kimura 2-parameter, with 500 replicates, a window of 100 bp, and a 10 bp step, was performed with GenBank genotype references (**A**), as well as patient-specific consensus sequences for genotypes A and G (**B**).

Fig. 5.

Estimated breakpoints for the 10 recombinant variants from patient 241571 (A→G, n = 2 and G→A, n = 8) and 4 recombinants from patient 243541 (A→G, n = 3 and G→A, n = 1). Breakpoints grouped in two regions between nt1397 and 1417 and nt 1497–1581.