Infection, Genetics and Evolution 43 (2016) 232–238

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Evidence of susceptibility to lamivudine-based HAART and genetic stability of hepatitis B virus (HBV) in HIV co-infected patients: A South African longitudinal HBV whole genome study

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A R T I C L E I N F O

Article history:
Received 2 February 2016
Received in revised form 9 May 2016
Accepted 25 May 2016
Available online 28 May 2016

Keywords:
HBV
HIV
Whole genome
Molecular evolution
Genetic variability
3TC-based HAART

A B S T R A C T

Background: Reports on the concomitant impact of HIV co-infection and long term highly active anti-retroviral therapy (HAART) on the genetic stability and molecular evolution of HBV are limited in sub-Saharan Africa.
Materials and methods: This retrospective study investigated the molecular evolution of chronic HBV in HIV co-infected patients on lamivudine (3TC)-based HAART over a 5 year period. Four HIV co-infected patients, consecutively recruited and followed-up, were screened for hepatitis B serological markers, and their viral loads determined. The HBV genome was amplified from longitudinal samples and characterized by Bayesian inference, mutational analysis, and identification of immune selection pressure.
Results: All patients exhibited persistent chronic HBV infection at baseline, as well as over the course of follow-up despite exposure to 3TC-based HAART. The polymerase gene in all isolates was relatively variable prior to HAART initiation at baseline and during the course of follow-up, although primary drug resistance mutations were not detected. All but one patient were infected with HBV subgenotype A1. The divergence rates between baseline and the last follow-up sequences ranged from 0 to 2.0×10^{-3} substitutions per site per year (s/s/y). Positive selection pressure was evident within the surface and core genes.
Conclusion: Despite persistent HBV infection in the HIV co-infected patients exposed to long term 3TC-based HAART, the molecular evolution of HBV over a 5 year period was unremarkable. In addition, HBV exhibited minimal genetic variability overtime.

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1. Introduction

The global prevalence of hepatitis B virus (HBV) infection in human immunodeficiency virus (HIV) positive individuals is estimated at 10% (Soriano et al., 2010). This rate is elevated in regions like sub-Saharan Africa where HBV and HIV are endemic (Burnett et al., 2005). In South Africa, the prevalence of chronic HBV infection in the HIV positive population ranges from 0.4%–23% (Firnhaber et al., 2008; Lukhwareni et al., 2009; Boyles and Cohen, 2011; Mayaphi et al., 2012). The frequency with which HBV–HIV co-infections occur is expected, especially when considering the shared risk factors and routes of transmission.

Of significance is the poor prognosis during HBV–HIV co-infection, with HIV known to cause immune-suppression and negatively alter the natural course of HBV infection (Burnett et al., 2005; Mphahlele et al., 2006). The HBV infection in HIV co-infected individuals is characterized by a persistent increase in HBV replication and expression of antigens which hastens the progression to chronic hepatitis B and the development of liver cirrhosis, end stage liver disease and hepatocellular carcinoma (HCC) (Cheruvu et al., 2007; Soriano et al., 2010). The effects of the HIV co-infection may impact HBV diversity, leading to the development of HBV variants with clinically relevant properties different from the wild type virus, although there is paucity of data (Audsley et al., 2010; Cassino et al., 2012; Pal et al., 2013).

HBV genetic diversity may be compounded by therapeutic pressure. Although highly active anti-retroviral therapy (HAART) is tailored to manage HIV infection, a number of drugs – lamivudine (3TC), Tenofovir disoproxil (TDF), Emtricitabine (FTC), Adefovir dipivoxil (ADV) and Entecavir (ETV) – have antiviral effects on HBV replication (Benhamou, 2004). It is suggested that the potential for emergence of

Abbreviations: HBV, Hepatitis B virus; HIV, Human immunodeficiency virus; HBsAg, Hepatitis B surface antigen; 3TC, Lamivudine; HAART, Highly active anti-retroviral therapy.

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http://dx.doi.org/10.1016/j.meegid.2016.05.035
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HBV drug resistance and virological failure is substantial due to increased HBV replication in HIV co-infected individuals on long term HBV-active HAART regimens with low resistance barriers (Sheldon et al., 2005; Hoffmann and Thio, 2007; Soriano et al., 2015). Resistance mutations within the HBV polymerase (*pol*) gene could further select for mutations within overlapping gene regions of the viral genome, advancing genetic variability (Soriano et al., 2015).

The concomitant effect of HIV co-infection and long term cross-reactive HAART on the genetic stability and molecular evolution of HBV has not been adequately investigated, especially in settings that are hyperendemic for both HBV and HIV, and with one of the largest anti-retroviral treatment (ART) programmes, like South Africa (Lukhwareni et al., 2009; Johnson, 2012; Evans, 2013). This retrospective study investigated the molecular characteristics of chronic HBV in HIV co-infected patients on 3TC-based HAART over a 5 year period.

2. Materials and methods

2.1. Study patients

From a total of 181 participants of an overarching study (ethics approval number: MP 07/2005) which consecutively recruited and followed-up HIV positive patients due for HAART initiation from 2004 to 2008, five patients were found to persistently test positive for the HBV surface antigen (HBsAg), from baseline to last follow-up. Of the five, one patient was excluded on the basis of their sera not being viable for further nucleic acid testing. Further exclusion criteria for the current study included patients with no follow-up sera, insufficient sera volumes (<600 μl), having initiated HAART prior to collection of the baseline sample, or testing positive for HBV DNA but without detectable HBsAg. The demographics of the study population in the overarching study have been previously reported (Lukhwareni et al., 2009).

The current study included two males and two females, with baseline ages of 23, 23, 44 and 61 years (Table 1). Based on the persistent detection of HBsAg, these four patients presented a unique cohort for investigation of chronic HBV during HIV co-infection and long term 3TC-based HAART. Following HIV baseline evaluations, all four patients were started on a HAART regimen consisting of Stavudine (d4T), Efavirenz (EFV), and lamivudine (3TC) as the only anti-HBV active drug. The patients were bled at baseline before HAART initiation, and then 2 or more serial bleeds were obtained from each patient during the course of therapy, bringing the sera tally for this study to 17 samples. Patients' HIV viral load, $CD4^+$ T cell count, and liver aminotransferase levels were monitored before and after initiation of HAART. Ethics approval to conduct this study was granted (MREC/P/315/2014) by the University Research Ethics Committee.

2.2. HBV serology

For all study patients, longitudinal serological testing was carried out for the following hepatitis B serological markers: HBsAg and antibodies to HBsAg (anti-HBs), antibodies to hepatitis B core antigen (total anti-HBc and IgM anti-HBc), hepatitis B e antigen (HBeAg), and antibodies to HBeAg (anti-HBe), using the Elescys® 2010 Immunoassay Analyzer (Roche, Hitachi, Japan) and Elecsys® test kits (Roche Diagnostics, Penzberg, Germany).

2.3. HBV viral load determination

HBV DNA was manually extracted from 200 μl of patient serum and eluted in 50 μl of elution buffer using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany). Longitudinal viral loads were then determined based on an optimized standard curve using an in-house quantitative real time polymerase chain reaction (qPCR) with a lower limit of detection (LDL) of 10^1 copies/ml that targets a 98 base pair (bp) conserved region within the HBV surface (S) gene (Garson et al., 2005).

2.4. HBV whole genome amplification

HBV DNA was amplified using two different PCR assays targeting the HBV whole genome. The first was a one-step PCR assay (LDL = 10^6 copies/ml) using primers P3 (5′-CTCGCTCGCCCAAATTTTTCACCTCTGCCTAATCA-3′) and P4 (5′-CTGGTTCGGCCCAAAAAGTTGCATGGTGCTGG-3′), and cycling conditions as previously described by Günther et al. (1995) with slight modifications. When the HBV viral load was $<10^6$ copies/ml, as was the case during follow-up after therapy exposure, a more sensitive nested PCR assay was employed to amplify the HBV genome in two overlapping fragments (Fragment A = 1670 bp and Fragment B = 1866 bp) (Enomoto et al., 2007) using cycling conditions described previously by Mphahlele et al. (2006) with minor modifications. All PCR amplified products were directly sequenced with the ABI3500XL Genetic Analyzer (Applied Biosystems, CA, USA).

2.5. Sequence analyses

Sequences were compared with subgenotype-matched reference strains from GenBank. Full length *pol* sequences were submitted to two separate online algorithms – the HIV–GRADE HBV Tool v0.8 (http://www.hiv-grade.de/cms/grade/explanations/hbv-tool/) and the Geno2Pheno [HBV] v2.0 (http://hbv.geno2pheno.org/index.php) – for prediction of phenotypic drug resistance and escape mutations within the reverse transcriptase (RT) domain and HBsAg gene frame, respectively (Neumann-Fraune et al., 2014). Genetic recombination within study isolates was also investigated using SimPlot v1.3 (Lole et al., 1999).

2.6. Bayesian inference and evolutionary rate analysis

Phylogenetic inference was performed using a Bayesian Markov chain Monte Carlo (MCMC) approach in the BEAST v1.8.0 program (Drummond et al., 2012) under an uncorrelated exponential relaxed molecular clock using the general time-reversible model with nucleotide site heterogeneity. The MCMC analysis was run twice for a chain length of a 100 million each until convergence of all parameters was

Table 1
Patients' baseline demographic data and clinical data before and after HAART initiation.

Patient	Sex	Age	No. of years of follow-up	Number of serial bleeds	HIV viral load[a] [After HAART]	CD4[d] [After HAART]	ALT[e] [After HAART]
ZADGM1486	F	61	2	3	NA[b] [LDL[c]]	190 [329]	NA [21]
ZADGM8787	F	23	3	4	NA [1.0×10^4]	NA [283]	NA [32]
ZADGM1163	M	44	4	5	3.9×10^4 [LDL]	271 [325]	43 [14]
ZADGM4643	M	23	5	5	7.5×10^5 [4.9×10^3]	51 [165]	24 [38]

[a] HIV viral load in RNA copies/ml.
[b] NA = Data not available.
[c] Lower than detectable limit (<50 copies/ml).
[d] $CD4^+$ T cell count in cells/mm^3.
[e] Serum alanine amino transferase (ALT) in IU/l; baseline cut-off value = 40 IU/l.

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