Infection, Genetics and Evolution xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Naturally occurring deletion/insertion mutations within HBV whole genome sequences in HBeAg-positive chronic hepatitis B patients are correlated with baseline serum HBsAg and HBeAg levels and might predict a shorter interval to HBeAg loss and seroconversion during antiviral treatment

Ran Hao [a], Kuanhui Xiang [a], Yaqin Peng [a], Jinlin Hou [b], Jian Sun [b], Yao Li [a], Mingze Su [a], Ling Yan [a], Hui Zhuang [a,*], Tong Li [a,*]

[a] Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Haidian District, Beijing 100191, China
[b] Institute of Hepatology, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, China

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form 10 May 2015
Accepted 11 May 2015
Available online xxxx

Keywords:
Hepatitis B virus
Deletion
Insertion
Whole genome
HBeAg-positive
Nucleos(t)ide analogue

ABSTRACT

Objectives: Deletion/insertion (Del/Ins) throughout hepatitis B virus (HBV) genome has not been well studied for HBeA-positive chronic hepatitis B (CHB) patients. This study aimed to characterize the HBV Del/Ins mutations in full-length genome quasispecies sequences in such patients at antiviral baseline and to reveal their potential impacts on HBV serological markers and responses to nucleos(t)ide analogue (NUC) treatment.

Materials and methods: A total of 30 HBeAg-positive CHB patients with genotype C infection receiving a 104-week lamivudine (LMV) and adefovir dipivoxil (ADV) combination therapy were enrolled. HBV whole genome sequences in serum samples at baseline were clone sequenced and analyzed using bioinformatics tools.

Results: Among 306 unspliced clone sequences, 61.8% (189/306) had Del/Ins mutations, 38.2% (117/306) were full-length genomes without any Del/Ins. Due to different combinations of 125 deletion types and 45 insertion types, we identified 55 Del/Ins-harboring HBV genome patterns, which affected a single or several functional genomic regions. Importantly, the proportion of Del/Ins-harboring clones was found to be significantly negatively correlated with HBsAg ($r = -0.3985$, $P = 0.0292$) and HBeAg ($r = -0.3878$, $P = 0.0342$) at baseline. Higher percentage of Del/Ins-harboring clones at baseline was found to predict a shorter interval to HBeAg loss and seroconversion.

Conclusion: Del/Ins mutations within HBV whole genome were prevalent in HBeAg-positive CHB patients prior to antiviral treatment. A higher detection rate of these mutations at baseline might correlate with a better response to LMV and ADV combination therapy.

1. Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus, which causes chronic infections for more than 350 million people worldwide. It has been estimated that the liver cirrhosis, liver failure and hepatocellular carcinoma (HCC) associated with chronic HBV infections account for 620,000 deaths annually (Harrison, 2006; Lavanchy, 2012).

The replication cycle of HBV comprises an error-prone reverse transcription step mediated by a virus-encoding reverse

Abbreviations: aa, amino acid; ADV, adefovir dipivoxil; anti-HBe, antibodies to hepatitis B e antigen; BCP, basic core promoter; C, core; CHB, chronic hepatitis B; CP, core promoter; Del/Ins, deletion/insertion; DR, direct repeat; EN, enhancer; S, surface; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LHBsAg, large hepatitis B surface protein; LMV, lamivudine; MHBsAg, middle hepatitis B surface protein; nt, nucleotide; NUCs, nucleos(t)ide analogues; P, polymerase; PgRNA, pregenomic RNA; PreC, precore; RH, RNase H; RT, reverse transcriptase; SHBsAg, small hepatitis B surface protein; SP, S promoter; TP, terminal protein; URR, upstream regulatory region; XP, X promoter.

* Corresponding authors.
E-mail addresses: zhuangbmu@126.com (H. Zhuang), tongli08@vip.sina.com (T. Li).

http://dx.doi.org/10.1016/j.meegid.2015.05.013
1567-1348/

transcriptase (RT) (Lauring and Andino, 2010). Consequently, HBV circulates in vivo as a viral population with a spectrum of genetically distinct but closely related variants, known as quasispecies. The quasispecies property of HBV implies that any newly generated mutant with a selective advantage in a given environment will overtake other variants and become the dominant quasispecies following a classical Darwin evolutionary process (Domingo and Gomez, 2007). In addition, the previously reported spliced variants have been suggested to be derived from packaging of the spliced RNA pregenomes and the reverse transcription (Günther et al., 1997; Huang et al., 2013). The HBV genome possesses the same signals as the host at fairly specific positions, which allow it to utilize the human cell splicing mechanism to produce the spliced RNA transcripts from its pregenomic RNA (pgRNA) (Günther et al., 1997). Thus, despite its small genome size of 3.2 kb, HBV displays surprising complexity in its genotypic and phenotypic characteristics, and host–virus interactions. It is known that the viral mutants with the higher fitness levels may predominate by competitive replication, although the predominant mutants may differ in the changing environments.

The HBV genome contains four partly or completely overlapping open reading frames (ORFs), i.e., preC/C that encodes for e antigen (HBeAg) and core (C) antigen (HBcAg); P for polymerase with RT activity, preS/S for surface proteins [three forms of HBsAg, small (S), middle (M) and large (L)], and X for a transcriptional trans-activator protein (Glebe and König, 2014). Mutations have been identified in each of these ORFs.

Many studies demonstrated nucleotide point mutations were related to the nucleos(t)ide analogues (NUCs) resistance, liver disease progression, immune escape of virus and etc. (Locarnini and Zoulim, 2010; Perrillo et al., 2004; van Bömmel and Berg, 2014; Wu et al., 2010; Xu et al., 2013). Meanwhile, the detection of deletion/insertion (Del/Ins) mutations has been reported as well. Deletion mutations in HBV preS/S region were frequently detected in the patients with chronic hepatitis B (CHB)-related HCC than in the patients with CHB (Gao et al., 2007; Liu et al., 2011; Xu et al., 2011). Presence of preS/S variants negatively correlated with HBsAg titers and variants with deletions in preS/S appeared to be not associated with HBV DNA replication and HBsAg synthesis/secretion (Pollicino et al., 2012; Ren et al., 2010). Emergence of C ORF deletion variants has been detected before seroconversion to antibodies against HBeAg (anti-HBe) and may block the appearance of a signal for the transport or the maturation of the capsid that is linked to viral DNA synthesis and required for envelopment (Ferns et al., 2007; Koschel et al., 2000). Deletions of nucleotides (nt) 1762 and 1764 were reported to be commonly found in HCC patients (Liu et al., 2008; Zhu et al., 2007). Märschenz et al. (2006) found out that in the variant carrying 11 bp insertion (at position 1776/1777), the level of preS1 mRNA was similarly diminished to approximately 60% of the wt level. Northern blots of total RNA demonstrated that the 11-nt insertion of CP contributed to the increased 3.5-kb pgRNA and reduced preS2/S mRNA levels. In fulminant hepatitis patients, the single nucleotide insertion in the precore region leading to a frameshift was found frequently. The insertion abrogated HBeAg and could enhance the HBV replication in vitro and heightened the replication capacity via changing sequence of the core protein (Inoue et al., 2011). Gérolami et al. (2005) found an 11-bp insertion in the core promoter region in a fulminant hepatitis patient, which created a novel hepatocyte nuclear factor-1 binding site and led to enhanced viral replication. Hamkar et al. (2010) investigated single nucleotide insertion in S gene from patients with occult HBV infection and found that the mutation resulted in nonfunctional HBsAg and caused frameshift, single-nucleotide replacement, and premature stop codons which caused failure in detection of HBsAg with routine diagnostic tests.

Thus far, complexity analysis of Del/Ins mutations occurring throughout the whole HBV genome in HBeAg-positive CHB patients prior to NUC treatment is very limited and its virological or clinical significance is not defined yet. In this study, HBV whole genome sequences at treatment baseline were analyzed by clone sequencing in 30 HBeAg-positive CHB patients with genotype C infection, who received a 104-week lamivudine (LMV) and adefovir dipivoxil (ADV) combination therapy. The results showed that the baseline Del/Ins mutations throughout HBV whole genome sequences were prevalent. The associations of naturally occurring Del/Ins mutations with baseline serological markers of HBV infection and their potential impacts on antiviral responses were analyzed.

2. Materials and methods

2.1. Patients

In this study the studied subjects were designed as genotype C infected HBeAg-positive CHB patients accomplishing a 104-week LMV and ADV combination therapy from March 2010 to February 2013. Patients were enrolled from a registered clinical study (NCT01088009) (Liang et al., 2014). The informed consents were obtained. The clinical diagnosis of CHB was according to the guidelines of prevention and treatment for CHB (Li and Jia, 2011). None of the patients had hepatitis C virus or human immunodeficiency virus co-infection, autoimmune liver disease, or alcohol or drug abuse. The patients had no history of antiviral treatment at least six months prior to receiving a LMV and ADV combination therapy. Due to insufficient serum samples or failure in HBV whole genome clone sequencing, the resulting cohort of 30 patients was carefully studied.

2.2. Laboratory tests

The serologic markers of HBV infection and viral load were tested before and during the treatment, including baseline, 12-week to 104-week with intervals of eight or 12 weeks. The serum HBsAg, HBeAg and anti-HBe were determined by chemiluminescent microparticle immunoassay (CMIA) using ARCHITECT i2000 analyzer (Abbott Diagnostics, North Chicago, IL). The level of serum HBsAg was detected by ARCHITECT HBsAg kit (Abbott Diagnostics) with a detection range of 0.05–250 IU/mL. Samples with HBsAg level higher than the upper limit of detection were diluted with ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics). The signal to cut-off (S/CO) value was used for HBeAg and anti-HBe. For HBeAg, S/CO values $\geqslant$1.0 were considered positive, whereas values <1.0 were negative. For anti-HBe, S/CO values $\leqslant$1.0 were considered positive, whereas values >1.0 were negative.

HBV DNA levels were quantified by TaqMan® 48 automatic florescence quantitative PCR kits using Roche COBAS® AmpliPrep®/COBAS® TaqMan® 48 Analyzer (Roche Diagnostics, Mannheim, Germany). The linear range of detection was 12–1.7×10^8 IU/mL of HBV DNA in plasma. All the samples with HBV DNA level higher than the upper limit of detection were diluted with nucleic acid testing dilution matrix (AcroMetrix, Benicia, CA). HBV genotyping was performed as described in our previous studies (Liu et al., 2010).

2.3. HBV DNA extraction, amplification and whole genome clone sequencing

HBV DNA was extracted from 200 µl baseline serum sample using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). HBV whole genome amplification was performed using LA Taq (Takara,

Please cite this article in press as: Hao, R., et al. Naturally occurring deletion/insertion mutations within HBV whole genome sequences in HBeAg-positive chronic hepatitis B patients are correlated with baseline serum HBsAg and HBeAg levels and might predict a shorter interval to HBeAg loss and seroconversion during antiviral treatment. Infect. Genet. Evol. (2015), http://dx.doi.org/10.1016/j.meegid.2015.05.013