

"Dinucleotide-pattern" G→A Hypermutations in the pre-Core 5'-GGGG Tetrad of HBeAg Negative Variants of Hepatitis B Virus

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Received: May 12, 2013 Revised: May 27, 2013

Accepted: May 29, 2013

Published online: September 21, 2013

ABSTRACT

The natural G→A substitutions in the HBV pre-Core gene with HBeAg negative phenotype is implicated in the chronic hepatitis and disease severity. Liver 'apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC)' protein mediated G→A "mononucleotide" hypermutations have been reported in HBV pre-Core gene that prefer 5'GGGG tetrad substrate, as an antiviral-innate immune mechanism. The pre-Core nucleotide sequence analysis of twelve HBe negative viral isolates from chronic hepatitis B patients, showed classical G1896A mutations in 3 samples. Of these, one viral sequence showed an additional G1897A substitution, representing a 'dinucleotide-pattern' hypermutation resulting in pre-Core stop codon (UGG→UAA) in the 5'GGGG tetrad. In another sample, a second G1899A substitution was also identified in the same tetrad stretch, but in the next codon (UGGGGC→UAGGAC). These results therefore, suggest that the pre-C 5'GGGG stretch appears as a favorable spot for 'dinucleotide-pattern' G→A hypermutation that could have been introduced by APOBEC enzyme(s) in HBe negative HBV variants.

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Key words: Hepatitis B virus; HBV; Pre-Core; HBe negative; G→A hypermutations

Parvez MK. "Dinucleotide-pattern" G→A hypermutations in the pre-Core 5'-GGGG Tetrad of HBeAg Negative Variants of Hepatitis B Virus. *Journal of Gastroenterology and Hepatology Research* 2013; 2(9): 795-797 Available from: URL: <http://www.ghrnet.org/index.php/joghr/article/view/485>

Hepatitis B virus (HBV) is one of the most widespread viral infections, estimated to affect about 500 million individuals with an annual 1.2 million death, worldwide^[1,2]. Chronic hepatitis B is a serious public health issue because it causes broad spectrum of liver diseases like, fulminant liver failure, cirrhosis and hepatocellular carcinoma (HCC)^[3]. HBV has a partially double-stranded, circular DNA genome (~3.2 kb) containing four overlapping open reading frames (Surface, S; Polymerase/Reverse-transcriptase, pol/RT; Core/pre-Core, C/pre-C and X) that replicates via a unique RNA intermediate step^[4]. A specific pattern of mutation is observed in human retroviruses, like HIV and HBV, the pararetrovirus with extensive, monotonous guanosine (G)→adenosine (A) base substitutions^[5]. Such hypermutations often result in premature stops when tryptophan (UGG) changes to a termination codon (UAG, UAA or UGA). Two mechanisms are therefore, proposed to explain this hypermutation. The first is the viral pol/RT enzyme with error-prone reverse-transcription that misincorporates adenosines for guanosines and generates genetically related viral quasispecies in a host^[6]. In earlier studies, HBV genomes with G→A hypermutation have been detected at low frequency in infected human serum^[7]. The second explanation is the human APOBEC-family proteins that play a crucial anti-viral role in the host innate-immune system^[8-10]. The host liver-specific APOBEC proteins, if incorporated into viral nucleocapsids, deaminate cytidine (C) bases in the nascent viral (-)strand DNA during reverse-transcription. The deaminated cytidine then, base-pairs to thymidine (T) and consequently, guanosines are replaced by adenosines during synthesis of viral complementary (+)strand DNA. Liver APOBEC proteins-induced G→A hypermutations in HBV genome has been already reported^[11-13]. Moreover, both interferons (IFNs), IFN- α and IFN- γ are shown to elevate levels of APOBEC mRNA in cultured HepG2 cell that promote hypermutations in HBV DNA^[14-16]. Further, the exacerbations of hepatitis B reflected by an increase in the number of mutant viral genomes were associated not only with a fall in the serum viremia but also with HBeAg clearance and subsequently, anti-HBe seroconversion^[17,18]. The seronegativity of HBeAg often results from a G→A substitution at first or second position in the 5'-GGGG tetrad (nts. 1896-1899) stretch in the pre-C coding region (Figure 1). It is also postulated that this pre-C 5'-GGGG stretch is the preferred substrate for the APOBEC enzymatic editing. Importantly, such substitution(s) in this 5'-GGGG tetrad should result in the multiple stop codons in pre-C coding sequences. In this report, I therefore, looked into the occurrence of G→A hyper-



Figure 1 Schematic representation of HBV pre-Core (pre-C) gene. Location of the 5'GGGG tetrad stretch is shown.

mutations in the pre-C coding sequences in HBeAg negative HBV isolates.

HBV sequences from twelve north-Indian HBeAg seronegative hepatitis B patients with clinically diagnosed chronic liver disease were selected for this study. Every source host fulfilled the following inclusion criteria: HBeAg seronegativity, persistence of HBsAg and anti-HBe seropositivity for at least 12 months, HBV DNA seropositivity, liver alanine transaminase >1.5× upper limit of normal, and seronegativity for HCV and HDV markers. The commercial ELISA based-serological tests were performed on at least two occasions for all patients: HBsAg and HBeAg (Organon Teknika, Broxton, The Netherlands), total anti-HDV (Abbot Laboratories, IL, USA) and anti-HCV (Third generation ELISA, UBI 4.0, NY, USA). HBV DNA was extracted from the patient's serum, using the standard phenol-chloroform method^[19]. Briefly, viral DNA was isolated from 100 mL serum using sera lysis buffer (20 mM Tris, pH 7.5; 10 mM EDTA; 150 mM NaCl), 1% SDS, and proteinase K (1 mg/mL) at 37 °C for 3 h, followed by extraction with Tris-saturated phenol (pH 7.9) and chloroform-isoamyl alcohol and finally DNA precipitation with 3 M NaOAc (pH 5.2) and absolute ethanol. The DNA pellets were air-dried in sterile condition, dissolved in 30 mL of 1×TE buffer (10 mM Tris and 1 mM EDTA) and stored at -20°C for further use. Two microgram of viral DNA was subjected to HBV-specific diagnostic polymerase chain reaction (PCR) (GeneAmp PCR System 2400, Perkin Elmer, USA), as described elsewhere^[20]. Serum from a chronic hepatitis B patient (positive for HBeAg and HBV DNA) was used as the positive control while that from a healthy subject (negative for HBV, HCV and HDV) and molecular-grade sterile water served as negative controls. The HBV positive samples were further subjected to a second PCR step to amplify the pre-C/C coding region, using pre-C/C specific primer sets: forward (nts. 1779-1798), 5'GGGTGTAGGCATAAATTGGT3' and reverse (nts. 2376-2400), 5'GTGCGAGGCGAGGGAGTTCTTCTTC3'. A 2.5 µL of DNA template and 10 pmol of each primer were used in a 50 µL PCR-reaction volume. Of the 35 thermal cycles, each cycle comprised of 94°C- 1 min, 55°C- 1.5 min and 72°C- 2 min. Proper positive as well as negative controls (described, above) were also included. Ten micro liter of each PCR product was subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and detected as a 603 bp band under UV light. For direct automated DNA sequencing, amplicons were column purified (Qiagen PCR Purification Kit, Germany). In a 10 µL reaction volume, each of forward and reverse sequencing reactions were set with 1 µL (5 pmol) of each primer and 5 µL (~250 ng) purified template plus 4 µL sequencing mix (Perkin Elmer, CA, USA). The PCR was carried out for 30 thermal cycles, conditioned for 96°C- 30 s, 55°C- 30 sec and 60°C- 4 min per cycle. The purified amplicons were ethanol-precipitated and sequenced (ABI Prism-377 DNA Sequencing System, Perkin Elmer, CA, USA). The nucleotide sequences of HBe negative HBV variants were analyzed with that of a wild type HBV, using the online software MULTALIN version 5.4.1 (<http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>).

Viral DNA sequence analysis revealed multiple mutations in the pre-C region of all the twelve HBV DNA samples. Three sequences

(pcTetG-2, pcTetG-4, and pcTetG-5) showed a classical G1896A substitution in the 5'GGGG tetrad, converting 'tryptophan' to 'stop' (TGG →TAG) at codon 28 (Figure 2A). The nine HBe negative viruses had G→A 'mononucleotide' mutations, converting the respective codons into pre-mature 'stops' in the flanking regions of 5'GGGG tetrad (data not shown). The viral isolate, pcTetG-4 had a second G1899A 'mononucleotide' mutation at the fourth position in the 5'GGGG tetrad, in addition to G1896A change. Interestingly, the viral pcTetG-5 variant was found to have a tandem G1897A substitution at second position, following G1996A in the 5'GGGG stretch. This represented a novel 'dinucleotide' G→A hypermutation in pre-C codon 28 (TGG →TAA), resulting into a presumably forced, 'double-stop' mutation (Figure 2B). We however, could not find any significant clinico-pathological correlate of this 'double-stop' mutation in the respective patient compared to those harboring a 'single-stop' mutation in the tetrad. Further, we did not observe a G1998A change, alone or in combination with G1896A mutation in any of the viral sequences, included in this report. After its first discovery in HIV^[19], the liver APOBEC-mediated G→A hypermutation has been subsequently reported in other retroviruses^[10] as well as HBV^[12-14,21,22]. The current finding of G→A double-substitution (TGG→TAA) in HBV pre-C gene is in agreement with the 'dinucleotide-pattern' substrates preferred by human APOBEC family of enzymes^[18]. However, unlike HIV, HBV does not show a consistent 'dinucleotide context', is also supported by other HBe negative pre-C sequences. Further, compared to HIV, such hypermutation in HBV could be extensively induced by six (A3A-C and F-H) of the seven enzymes in the APOBEC family^[15].

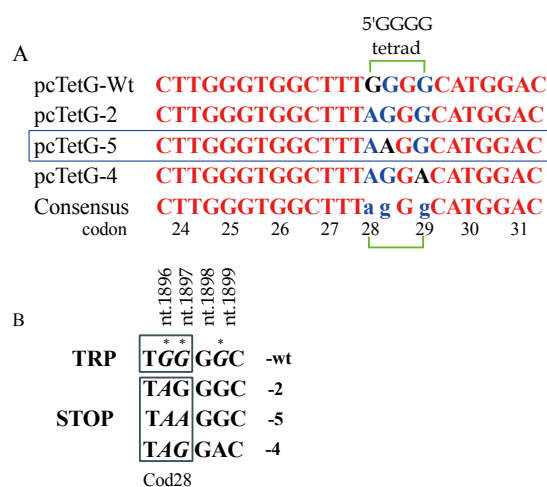


Figure 2 The HBV pre-C hypermutation region sequence analysis. A: Multi alignment of the three pre-C sequences (pC-2, -5, -4) with wild type (wt), showing G→A hyper mutation in the 5'GGGG tetrad; B: 'Dinucleotide-pattern' tryptophan (Trp) to Stop codon mutations in the 5'GGGG stretch resulting into HBe negative phenotype.

The above results suggest that the pre-C 5'GGGG stretch appears as a favorable spot for G→A hypermutations in HBV genome. It could be therefore, assumed that the detected 'dinucleotide-pattern' (GG→AA) hypermutations could have been introduced by APOBEC family enzymes that resulted into HBeAg negative phenotype. Nevertheless, molecular studies like, in vitro expression levels of APOBEC as well as its genetic polymorphism in study-patients are further required.

ACKNOWLEDGMENTS

Supports from the Research Center, College of Pharmacy, KSU, Riyadh, Saudi Arabia is acknowledged. The author is very grateful to

Dr. S. N. Kazim, G. B. Pant Hospital, New Delhi, India for his help and assistance in nucleotide sequencing.

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