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

Mohammad Khalid Parvez

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[12]. 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)[13]. 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 hypermutations in HBV 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 HB 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 (U UGG→AAAA 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 (UGGGG→UAGGIC). 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.

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 HB 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 (U UGG→AAAA 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 (UGGGG→UAGGIC). 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

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 batch 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, Brxctel, 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[10]. Briefly, viral DNA was isolated from 100 mL serum using a 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-isooamyl 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 HBsAg 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 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 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’GGGTTGGGCTTTAAGGCATGGGAC-3’. 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 ethedium bromide and detected as a 603 bp band under UV light. For direct automated DNA sequencing, ampli-

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 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[18].

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

Figure 2 The HBV pre-C hypermutation region sequence analysis. A: Multi alignment of the three pre-C 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 G1997A 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→TAAG), resulting into a presumably forced, ‘double-stop’ mutation (Figure 2B). We however, could not find any significant clinicopathological 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[10], the liver APOBEC-mediated G→A hypermutation has been subsequently reported in other retroviruses[18] as well as HBV[15,18,20]. The current finding of G→A double-substitution (TGG→TAAG) 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[18].

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REFERENCES