Sequence Analysis of PreS2 Region of Hepatitis B Virus Genotype D Isolates

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ABSTRACT

Hepatitis B virus (HBV) is a well-known agent of liver diseases. HBV disease burden varies across the globe with regions from low to high endemicity. Pakistan lies in the intermediate endemic zone, with high rate of mortality due to liver disease, cirrhosis and hepatocellular carcinoma. There is a wide range of heterogeneity in relation to HBV genotypes and sub-genotypes and in their patterns of pathogenesis, virulence and response to antiviral therapy. A large number of HBV genomic variations are associated with clinical outcomes such as hepatocellular carcinoma and liver cirrhosis. Thus, the present study aims to analyze PreS2 gene sequences from HBV isolates and their phylogeny. To investigate this, a study was conducted on twenty one HBV chronically infected individuals, serum samples were subjected to PCR with specific primers for PreS2 region of HBV genotype D and then sequenced. Point mutations; A39V, P41H and L42I were found in cell permeability domain of PreS2 protein. However, MHC class I and II epitopes were conserved in all sequences. Phylogenetic analysis was carried out by comparing the nucleotide sequence with 22 reference sequences of HBV sub-genotype D retrieved from the GeneBank. Phylogenetic analysis showed that two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2.

Key words: Hepatitis B virus; Phylogenetic analysis; PreS2 region; Genotype D; Pakistan

1. INTRODUCTION

Hepatitis B virus (HBV) infection is a threat to the public health worldwide. Incidences of HBV infections are increasing at an alarming rate due to unavailability of effective vaccines and improved antiviral therapies for low income population. According to the current estimates of World Health Organization (WHO) 400 million people are chronically infected worldwide (Locarnini, 2002). In Pakistan, there are estimated 7-9 million carriers. Recently, a study reported 28.87% and 22% in HBV positive patients suffering from liver cirrhosis and hepatocellular carcinoma respectively Ali et al. (2011).

On the basis of genetic variability of (i.e. > 8%), HBV can be classified into eight genotypes including genotypes A to H Okamoto et al. (1988). All genotypes exhibit distinct geographical distributions, depicting ethnographic patterns of disease transmission Kramvis et al. (2005). Further, these eight genotypes have been grouped into 49 sub-genotypes on the basis of nucleotide variability of 4% to 8% Schaefer et al. (2005). In Pakistan, Genotype D (63.71%) is the most prevalent genotype. Moreover, sub-genotypes D1 and D3 have been reported to be prevalent in Pakistani population Baig et al. (2009). In addition, presence of D2 has also been reported Ahmed et al. (2009). However, Genotype C prevails with 7.55% in local population and is considered as an emerging genotype Ali et al. (2009).

HBV is a member of Hepadnaviridae family Pungpapong et al. (2007). The virus genome is double stranded circular virus of 3.2 kilobase pairs, which is organized in four overlapping open reading frames ORF S, C, P and X. ORF S encodes surface protein, ORF C encodes either HBV e antigen or viral capsid protein that has the ability of self assembly into the viral capsid. ORF P and ORF X encode large polymerase protein and 16.5 kDa HBV X protein respectively. ORF S is divided into three in frame AUG start codons which encodes three different surface proteins (Schadler and Hildt, 2009). Large surface protein (LHBs) encompasses PreS1 (108-119aa), PreS2 (55aa) and Surface (226aa) domains. Middle protein (MHBs) includes PreS2 and surface domain. Moreover, Small protein (S) consists of only Surface domain. PreS2 domain consists of human serum albumin binding site (aa3-16), N-glycosylation site (aa 4), transactivator domain, cell permeability domain (aa 41-52) Lin et al. (2012) and several T and B cell epitopes Barnaba et al. (1989). Numerous mutations have been reported in PreS2 gene mutations which add complication in the diagnosis and treatment of HBV infected individuals (Weber, 2006; Gao et al., 2007; Su et al., 2007). In addition, PreS2 mutations are also associated with clinical outcomes. Point mutations; L36P, A39V, L42I, P41H, P52R were detected in Occult HBV isolated from patients with Hepatocellular Carcinoma Pollicino et al. (2007).

There is insufficient data regarding the mutations in the PreS2 region in local HBV genotype D infected individuals. Therefore; this study aimed to isolate PreS2 ORF gene and analyze the PreS2 sequence for mutations. In addition, a phylogenetic analysis was conducted to determine origin of PreS2 origin of HBV genotype D isolates.
2. MATERIALS AND METHODS

Blood Sample collection and Viral DNA extraction

A total of 21 blood samples of HBV positive patients were collected from patients residing in Islamabad during January to April, 2012 at Atta-ur-Rahman School of Applied Biosciences’s Diagnostics Lab. Consent of the patients was taken and they were informed about the research study. Out of 21 samples only three samples corresponded with the inclusion criteria. Accordingly, samples having HBV infections other than genotype D and either Human immunodeficiency virus or Hepatitis C virus infections were excluded from the study; demography of the samples is shown in Table 1. Total of 3ml of freshly drawn blood was taken in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and centrifuged at 8000 rpm for 3 minutes to separate the serum. Genomic DNA was purified from the serum by using Nucleospin kit (Germany) according to the manufacturer's protocol. Primers were designed by retrieving the already published sequences of HBV PreS2 gene of genotype D from National Centre for Biotechnology Information (NCBI) (Available at http://www.ncbi.nlm.nih.gov/). The PreS2 gene was amplified by the following primers: TGAAGCCCATCACCAGCA (forward) and GTAACACGCGGAGGTCTT (reverse).

Table 1. Demography of patients

| Accession no. | Sex | Age | HBsAg | HBeAg |
|---------------|-----|-----|-------|-------|
| ASAB1 (2266)  | M   | 41  | POSITIVE | POSITIVE |
| ASAB2 (HF2)   | F   | 18  | POSITIVE | POSITIVE |
| ASAB3 (PIMS7) | M   | 33  | POSITIVE | POSITIVE |

PCR Amplification of HBV PreS2 gene and Sequencing

Polymerase Chain Reaction (PCR) was carried out to amplify PreS2 gene using the extracted Genomic DNA. The reaction mixture was prepared in 0.2 ml tubes (Axygen, California, USA) by adding 12.5 μL (800 ng) sample DNA template along with 2.5 μL each of gene specific forward and reverse primer (2 pmol), 5 μL of 2 mM dNTPs, 5 μL of 25 mM MgCl2, 1.25 μL of 1.5 units of Taq polymerase (1U/μL) (Fermentas, USA) along with 5 μL of 10X Taq Polymerase Buffer and nuclease free water (16.25 μL) making a total reaction volume of 50 μL. PCR mixture was vortexed and then placed in Thermal Cycler (Swift™ MaxPro Thermal Cycler, Esco, Singapore), under the following PCR conditions: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 58.5°C for 45 s, 72°C for 60 s and a final extension at 72°C for 10 min. Reactions was held at 4°C. Amplified PCR products were analyzed by electrophoresis on 1 % agarose gel. DNA fragments were purified from agarose gel by using Silica Bead DNA Gel Extraction Kit (Fermentas, USA). Purified DNA products were sent to Macrogen Korea for sequencing along with 10μl of forward HB2250P3F and HB200P3R reverse primer.

Sequence and Phylogenetic Analysis

PreS2 isolates were sequenced from each patient by using both sense and antisense primers. These sequences were aligned in CLC workbench software (CLC Inc, Aarhus, Denmark) to draw a consensus sequence for each isolate and then consensus sequences were submitted to Genebank under accession number KF482899, KF482900, KF470787. Sub-genotype D sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) : AY741798-IRAN D1, AY1611159-INDIA D1, AY796032-TURKEY D1, AB583680-PAKISTAN D1, FJ562309.1-CHINA D1, FJ386590.1-CHINA D1, JN642163.1-LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2, JN642163.1-LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2, EI00615-EAST INDIA D3, X85254-ITALY D3, AB583679.1-PAKISTAN D3, AY373430-INDIA D3, JF815648.1-BRAZIL D4, GQ922005.1-CANADA D4, GQ922003.1-CANADA D4, GQ205386.1-INDIA D5, GQ205387.1-INDIA D5, JF815661.1-BRAZIL D6, JF815606.1-BRAZIL D6, FJ904444.1-TUNUSIA D7, FJ904440.1-CHINA D7, shown in Table 2. Consensus sequences were generated for sub-genotype D1 to D7. Then subsequent consensus and our PreS2 isolates sequences were aligned using CLC workbench software 6.5.3 (www.clebio.com). Further CLC tool was used to translate nucleotide sequence into protein sequence. B and T cell epitopes, along with other protein domains were analyzed. Phylogenetic analysis of our three PreS2 isolates with PreS2 sequences of sub genotype D was performed by CLC workbench software.

3. RESULTS

Nucleotide Sequence Alignment of PreS2 gene

A total of 22 Sub-genotypes (D1-D7) of genotype D HBV PreS2 sequences were retrieved from Genebank as shown in Table 2. PreS2 sequences for every genotype D sub-genotypes were aligned and a consensus sequence was generated. Consensus sequences were then compared with our isolates: ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7). Point mutations at C28T, T125C and C122A were found in PreS2 sequences as shown in Figure 1, which were further analyzed via protein alignment.

Protein Sequence Alignment of PreS2 protein

Amino acid sequences were predicted for ASAB1 (2266), ASAB2 (HF2), ASAB3 (PIMS7) and PreS2 consensus sequences using CLC Work Bench 6.5.3. Later, amino acid sequences were aligned. Mutations were found in cell permeability domain of three of our isolates as shown in Figure 2. In ASAB1 (2266) and ASAB2 (HF2) isolates, ALA at position 39 is substituted with VAL, where both are neutral and non polar in nature. Moreover, in all three isolates at position 42, LEU is replaced by another neutral and non polar amino acid ILE. However, PRO at position 41 which is neutral and non polar, is substituted by HIS a basic and polar amino acid ILE. Moreover, in all three isolates at position 42, LEU is replaced by another neutral and non polar amino acid ILE. However, PRO at position 41 which is neutral and non polar, is substituted by HIS a basic and polar amino acid ILE.

Phylogenetic analysis

Phylogenetic tree was constructed by using three different PreS2 gene sequences of HBV reported in this study along with twenty two PreS2 sequences obtained from Genebank.
with different sub-genotypes. Hepatitis B virus sub-genotypes were grouped in individual clusters. Two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2 as shown in Figure 3.

Table 2. Retrieved PreS2 sequences with sub-genotype of D from NCBI

| SUB-GENOTYPE | NCBI ACCESSION NO | ORIGIN |
|--------------|-------------------|--------|
| D1           | AY741798-IRAN, AY161159-INDIA, AY796032-TURKEY, AB583680.1-PAKISTAN, FJ562309.1-CHINA, FJ86590.1-CHINA |
| D2           | JN642163.1-LEBANON, EU594382.1-RUSSIA, JF815677.1-BRAZIL |
| D3           | E100615-EAST INDIA, X85254-ITALY, AB583679.1-PAKISTAN, AY737340-INDIA |
| D4           | JF815648.1-BRAZIL, GOQ22005.1-CANADA, GOQ22003.1-CANADA |
| D5           | GQ205386.1-INDIA, GQ205387.1-INDIA |
| D6           | JF815661.1-BRAZIL, JF815606.1-BRAZIL |
| D7           | FJ904444.1-TUNUSIA, FJ904440.1-CHINA |

Figure 1. Multiple sequence alignment (MSC) of HBV PreS2 gene sequences of sub-Genotype D strains with ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7) isolates by means of CLC workbench 6.5.3 (http://www.clcbio.com).
Figure 2. Protein sequence alignment of 3 of our isolates with other protein consensus sequences of sub-genotype D mentioned in Table 2. The conserved bases are shown as dots whereas the mutated residues are marked with the single letter code of that amino acid. Colored squares represent respective domains in the PreS proteins (Lin et al., 2012). Used software CLC workbench 6.5.3 (http://www.clcbio.com).

Figure 3. Phylogenetic tree of HBV PreS2 gene sequences. Tree was constructed by UPGMA algorithm. Bootstrap values are mentioned at the nodes. Tree shows a phylogenetic relationship between three of our isolates and sub-genotypes from the rest of the world. References sequences are labeled by Genebank accession number, Country name and by their respective sub-genotypes.
4. DISCUSSION

In Pakistan, the prevalence of HBV infections has increased beyond the reported findings. There are estimated 7-9 million carriers with carrier rate of 3-5%. In Pakistan, HBV infected population is distributed among general population including healthy blood donors, military recruits, prisoners and healthcare persons Ali et al. (2008). There are 20 to 30% HBV infected patients with cirrhosis and hepatocellular carcinoma Ali et al. (2011). Thus, HBV is a real challenge for scientist community.

HBV mutants have substantial impact to trigger pathogenesis and cause severe form of liver disease Mendy et al. (2008). Deletion mutations spanning 42-54 bp within Pre-S2 N-terminal, Pre-S2 internal deletions: 1-15 Pre-S2, 1-26 Pre-S2, 12-20 Pre-S2, 44-53 Pre-S2 have been reported (Gao et al., 2007; Su et al., 2007). Where, Pre-S2 internal deletions lead to the loss of M protein-glycosylation site (Asn-4 glycan site) in Pre-S2 protein, which impairs virion secretion (Margaret and Reinhold, 1998). Further, deletions in Pre-S2 region alter T and B cell epitopes giving Pre-S2 mutants selective advantage over wild type viruses Chisari et al. (1995). Studies report, HBV Pre-S2 deletion mutants confer an inefficient immune response, hence Pre-S2 deletion mutants persist in infected individual Barnaba et al. (1989). HBV Isolates from Ground glass Hepatocytes showed deletions at 5 terminus of Pre-S2 region (nt 2-55) and point mutation within the start codon (ATG to ATA) of middle surface protein (Fan, 2000). Recent studies found, prevalence of 18.8% Pre-S2 deletions in the HCC group and 5.8% Pre-S2 deletions in non-HCC group. In addition, Pre-S2 deletions were more prevalent in HCC patients aged < 50 years as compared with older HCC patients Yeung et al. (2011). Thus, keeping in view this scenario, study was conducted to determine Pre-S2 variants. Pre-S2 gene was successfully isolated and sequenced from three genotype D patients suffering from HBV chronic infections. Moreover, protein sequence derived from nucleotide sequences were analyzed for mutations.

Point mutations ALA to VAL, LEU to ILE and PRO to HIS were found, these have been previously reported in occult HBV isolates from patients with hepatocellular carcinoma Pollicino et al. (2007). These mutations were present in cell permeability domain which is important for translocation of viral protein and nucleic acids Hildt et al. (2002). Several MHC epitopes are reported in the entire HBV envelope protein Barnaba et al. (1989). Moreover, MHC class I and MHC class II epitopes were conserved in all sequences. Humoral and cellular immune response is directed against HBV envelope protein which leads to viral clearance, and neutralizing activity of anti-HBs. So far, only small protein (S) has been extensively used for the preparation of the vaccines and production of antibodies for therapeutic purposes. However recently, Madalinski et al. (2004) demonstrated that the third-generation pre-S1/pre-S2/S vaccine containing Pre-S2 major histocompatibility epitopes showed more rapid onset and pronounced antibody response as compared to the S-gene-derived vaccine in healthy children and newborns (Madalinski et al., 2004; Madalinski et al., 2001). Thus, third generation vaccines can be used with further validation whether Pre-S2 MHC epitopes are conserved in local HBV variants.

Moreover, recently several researches demonstrated that type of chronic outcome; prevalence of mutations and the severity of virulence vary for genotypes and sub-genotypes. Thus, for better disease management and prognosis, it is imperative to determine their global distribution. Several studies report distribution of HBV sub genotypes around the globe. Where, Genotype A is further categorized in sub genotypes A1 predominant in Asia and Africa, A2 in Northern Europe and America (Bowyer et al., 1997; Kramvis et al., 2002) and A3 (Mulders et al., 2004; Kurbanov et al., 2005) in Central Africa. Sub-genotypes B1 of genotype B dominates in Japan, whereas B2 in China and Vietnam (Sugauchi et al., 2004; Norder et al., 2004). Genotype C with C1 as sub-genotype is common in South-East Asia and Bangladesh, C2 in Japan, Korea, and China (Chan et al., 2005; Sugauchi et al., 2004), C3 in Oceania and C4 in Australia (Sugauchi et al., 2003; Norder et al., 2004). In addition, geographical distribution of sub-genotypes D designated as D1–D7 are found to be in India, Iran, and Indonesia.

In this study, a phylogenetic analysis was conducted for three of our isolates. ASAB1 (2266) and ASAB3 (PIMS7) were grouped in cluster 1 and shared common ancestors with other members of the clusters, hence belong to sub genotype D1. While, ASAB2 (HF2) showed sequence homology with sub genotype D2 in cluster 2.

5. CONCLUSION

In conclusion, mutations were detected in Pre-S2 cell permeable motif. However, the implication of mutations in this region needs to be further elucidated by in silico protein modeling. Phylogenetic study demonstrated two sequences from sub-genotype D1 and one from sub-genotype D2. By further increasing number of Pre-S2 sequence analysis of HBV infected patients a considerable statement can be made about different mutations associated with sub-genotype.

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