Analysis of hepatitis B virus X gene phylogeny, genetic variability and its impact on pathogenesis: Implications in Eastern Indian HBV carriers

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A B S T R A C T

HBx genetic variability was explored in the Eastern Indian population with low HCC incidence. DNaSe 1 sensitive HBV DNA was detected in 53% samples, which differed significantly between clinical groups (P<0.001). HBV genotypes A (Aa/A1), C (Cs/C1) and D (D1, D2, D3, D5) were detected in 37.5%, 18.7% and 43.7% samples respectively. Population specific signature HBx residues A36, V88, S101 in Aa/A1 and residues P38, Q96, D98 in D5 were detected. Mutations T127, M130 and I131 were detected in 66.7%, 91% and 75% of genotype A, C and D5 samples respectively. Very low occurrence of HCC associated mutations (V5M/L, P38S, and H94Y) and absence of C-terminal deletions were observed. Our study shows that HBV genotype associated clinically important HBx variations may evolve and act distinctly in different geo-ethnic populations. Further studies on HBx functions from the perspective of genetic variability are essential for the better understanding of the clinical significance of HBV.

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Introduction

Hepatitis B virus (HBV) infection is a global health problem with more than 2 billion infected individuals. HBV infection leads to diverse outcomes ranging from acute to fatal fulminant hepatitis, and chronic hepatitis (CHB), which may result in severe complications as liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) is one of the important human DNA viruses having strong oncogenic potential (De Oliveira, 2007).

Genetic and phylogenetic analysis of HBV DNA sequences has lead to the definition of eight genotypes designated A through H, based on more than an 8% diversity in the whole genome, and further into their subgenotypes (more than 4%, but less than 8% intragenotype diversity), having distinct ethno-geographical distribution (Schaefer, 2005). Recent studies have clearly demonstrated that genotype variability of different HBV genetic regions can influence the clinical manifestation, and even response to therapy (Schaefer, 2005).

The highly compact, HBV genome encodes four overlapping ORFs (PreS/S, PreC/C, Pol and X). Considering their clinical significance in diagnostics, immune evasion, vaccine development, immune activation functions and drug resistance, studies on the genetic variability of the PreS/S, PreC/C and Pol genetic regions have remained at the foci of earlier investigation. However, data on the clinical impact of HBx genetic variability is still sparse (Kim et al., 2008). The X ORF is the smallest, but expresses a 154 amino acid multifunctional molecule (HBx), with an N-terminal negative regulatory/antiapoptotic and a C-terminal proapoptotic/transactivation domain, and has widely been associated with modulation of a wide range of normal cell functions, leading to HCC (Bouchard and Schneider, 2004; Schuster et al., 2002; Tang et al., 2006). In view of its strong involvement in HBV related pathogenesis, recent genetic analysis studies from our group and others (Datta et al., 2007; Sung et al., 2008), and in vitro functional analysis studies (Lin et al., 2005; Tan and Chen, 2005) have highlighted the clinical substance of HBx genetic variability. We have earlier demonstrated that HBx genetic variability can enlighten the differential oncogenic potential of HBV genotypes (Datta et al., 2007).

In India, more than 10% of the global chronic HBV population reside (Sarin et al., 2001). HBV genotypes A and D are known to circulate in different parts of India (Thakur et al., 2002; Gandhe et al., 2003; Vivekanandan et al., 2004). In addition to these two genotypes, we have recently documented, characterized and found that HBV...
genotype C has recently emerged in this part of India and thus has remained confined in the Eastern India population (Datta et al., 2006; Banerjee et al., 2006a, 2006b). Based on full-length genome analysis, we have also revealed that HBV genotypes A and C circulating in the eastern part of India are phylogenetically related to HBV strains prevalent in Sub-Saharan Africa and East Asia respectively (Banerjee et al., 2006a). Interestingly, despite this similarity in circulation of HBV genotypes, the incidence of HCC is very low in India (average annual incidence of ≤3.3 HCC cases/100,000 individuals), compared to Sub-Saharan Africa and East Asian countries (average annual incidence of ≤98.9 HCC cases/100,000 individuals) (Hussain et al., 2007). Regardless of such an interesting facet of HBV epidemiology, the clinical relevance of the HBx genetic variability of Indian HBV strains has not been sought previously.

The main aim of this study was to investigate the genetic heterogeneity of HBx sequences in our population. We analyzed and compared the HBx sequence from our study population with related GenBank sequences and published literature related to clinically relevant HBx variations. In this manuscript we discuss the clinical significance of the genetic variability of HBx and also present the HBx genetic variability in our study population with interesting HBV epidemiology.

Results

Amplification of X gene

During this study, we observed a discrepancy in amplification results of the surface gene and the X gene region amplification PCR assays, although these assays had similar sensitivity, as calibrated with an international HBV DNA standard (NIBSC code 97/750; National Institute of Biological Standards and Control, Hertfordshire, UK). Of the 102 HBV DNA samples that were positive by partial surface gene PCR (amplifying nts 184 to 731 from the EcoRI site), the HBV genome region corresponding to nts 1092 to 1972 including the entire X gene region could be amplified from a total of only 48 (47%) samples, suggesting the absence of this region in the rest of the samples. Among the rest of the 54 samples in which full-length X gene could not be amplified, our attempts of amplifying partial X gene using previously published primers also failed repeatedly. However the extracts were repeatedly found to be positive for PCR assays amplifying different regions of human genomic DNA and HBV core gene/preC and PreS/S regions, ruling out the possibility of faulty DNA extraction in these samples. Further, false positivity in the surface gene, precore/core gene PCR due to cross contamination was also excluded by the results obtained with controls included during extraction and amplification stages.

Fascinatingly, we observed that full-length X gene could only be amplified in the extracts that had full-length DNase I resistant virion HBV DNA. Such intact virion HBV DNA was detected in a lower but almost similar percentage of the acute hepatitis B (ACU), inactive carriers (IC) and LC patients (37.5%, 34.4% and 33.3% respectively), while the percentage of detection of intact viral genome was significantly higher (P<0.001) in CHB and HCC patients (68.7% and 66.6% respectively) (Fig. 1). In the rest of the extracts, the dominant proportion of HBV DNA was found to be defective HBV DNA, lacking detectable HBx genetic region.

Phylogenetic analysis, genotypes and subgenotypes

The phylogenetic analysis (Fig. 2) of the 48 X gene sequences along with GenBank reference sequences showed seven distinct clusters corresponding to the HBV genotypes (A–D and F–H), however genotype E, clustered with the genotype D sequences. Among the 48 sequences generated in this study, genotype D (43.7%) was most frequently detected followed by genotype A (37.5%) and genotype C (18.7%). The genotypes determined with HBx sequences in this study matched exactly with RFLP patterns obtained with surface gene region sequences (data not shown). No recombinants were observed in this study. Among the isolates from the present study, all the 18 genotype A isolates clustered with reference subgenotype Aa/A1 clade, all the 9 genotype C isolates clustered with subgenotype Cs/C1 clade. However, 21 genotype D isolates showed the most diverse topology, clustering with clade corresponding to subgenotypes D1 (4 isolates), D2 (3 isolates), D3 (10 isolates) and D5 (4 isolates) (Fig. 2).

Inter-genotypic and inter-subgenotypic divergence

The phylogenetic clusters observed with the X gene region in this study exactly matched with previously described genotype/subgenotype clusters obtained with whole genome sequences (Norder et al., 2004). This suggested a high inter- and intra-genotypic variation in the 465 nt encoding the X gene region, comparable to that in the full genome of HBV. The mean inter-genotypic distances at the nucleotide and amino acid level are shown in Table 1. The mean inter-subgenotypic distance among the subgenotypes of genotype A, B, C, D and F in the full-length X gene, and its two distinct functional domains (N-terminal and C-terminal) are shown in the Table 2. Comparison of the consensus of reference subgenotype sequences showed a high divergence at both the nucleotide and amino acid level, inter-genotypically and inter-subgenotypically. However the N-terminal showed an exceptionally high sequence divergence, compared to the C-terminal, indicating an exceptionally high type specific variability in the N-terminal domain.

Genotype specific residues

Comparison of the consensus sequences, calculation of $d_{N}$ and $d_{S}$ at each codon position indicated the amino acid positions that were significantly different among the consensus sequences, representing genotype specific residues (Fig. 3). These predicted sites were further confirmed by manually comparing with the reference sequence from different countries, retrieved from the GenBank. As expected from the divergence data, most of the specific residues were located in the N-terminal (first 50 amino acids) compared to the C-terminal regions. Among the variable positions in the N-terminal domain, residues at 6, 12, 26, 30, 38, 40, and 42 were found to specify the genotypes and the rest of the positions were well conserved within subgenotype reference sequences. At the C-terminal, residues at 78, 91, 101, 102, 118 and 119 were shown to be genotype dependent (Fig. 3).

Genetic variability in the present sequences

Among the 48 HBx sequences generated in the present study, the mean similarity between the sequences (in percentage) at nucleotide
level was 95.67±0.015 and mean similarity between sequences (at amino acid level) was 92.92±0.033.

Compared to the respective reference genotype consensus sequence, the variability in the sequences generated in the present study was determined. Sporadic mutations were observed at some sites, although in the majority, the sequences were similar to the consensus reference sequences, indicating low genetic variability in these samples. However region specific variability (substitution at a particular site in 50% or more of the isolates, compared to the corresponding consensus reference sequence) was observed in subgenotypes Aa/A1 (at positions 36, 88, 101), in Cs (at position 30), in D1 (in position at 47) and in D5 (at positions 104, 75 and 104) are well conserved among all the genotypes. One PKC and CK II phosphorylation sites in the HBx C-terminal (at sites 54, 75 and 104) are well conserved among all the genotypes. One PKC (S11SR) in the N-terminal was present only in Aa/A1 and Ac/A3, while subgenotype Ae/2 had P11SR. The rest of the other genotypes had either P11TR or P11AR.

The HBx protein was predicted to be a compact globular domain (with 91 residues predicted to be exposed) by the GLOBE program. The results of the prediction of helix, extended sheet and coiled loop by PHD and PROF programs are presented in Supplementary data 3. The random coiled structure was observed in the N-terminal region corresponding to the hypervariable region, while the helical structure was predicted in the C-terminal binding domains. Relative solvent accessibility (in 10 states) as predicted by PROFacc was found to be higher in residues corresponding to the hypervariable region of the N-terminal.

Discussion

Using an advanced algorithm for protein structure prediction, in contrast to a previous study (Colgrove et al., 1989), we in this study detected non-alpha, non-sheeted, coiled-coil structure with relatively high solvent accessibility of the N-terminal residues, suggesting a dominant B-cell epitope region (Park et al., 2000). Further analysis of the sequences of different HBV genotypes also showed that most of the type specific variability is concentrated in the N-terminal of the HBx. The presence of HBx genotype variability in the immunodominant region supports the role of immune selection in evolution of different HBV genotypes (Osiowy, 2006). It is well known that the N-terminal domain of HBx is indispensable for dimerization, transforming activity, anchorage-independent proliferation activity, negative regulation of proapoptotic activity and can overcome oncogene induced senescence (OIS) (Bouchard and Schneider, 2004; Tang et al., 2006). On the other hand, due to the overlap with several regulatory elements (Tang et al., 2006), the C-terminal domain of HBx sequence and its functions are relatively well conserved (Schuster et al., 2002). Taken together, it is evident from the present analysis that the HBx N-terminal domain and its variability is involved in significantly contributing to differential pathogenic potentials of HBV genotypes/subgenotypes, which clearly supports a recent study showing that effects of the same C-terminal HBx mutations are genotype differentiated (Lin et al., 2005).

The present study reveals important facets of HBV genetic variability in our study population. The detection of DNase I sensitive

![Fig. 2. Phylogenetic relatedness of the full-length X gene (465 nucleotides corresponding to positions 1374 to 1838 from the unique EcoRI site) sequences of HBV isolates from Eastern Indian patients (taxa name starting with ‘E’ or ‘EIX’, marked with grey solid rounds), with reference sequences representing different genotypes and subgenotypes retrieved from the GenBank. Reference sequences are denoted by accession numbers and the country of origin. Reference sequences of genotypes (B, E, F, G, H) not detected in this part of India were kept at a minimum for clarity of the figure. Genotypes, subgenotypes and bootstrap values are marked at the nodes.](image-url)
HBV DNA in our study subjects is in accordance with a previous study (Cabrerizo et al., 1997). Moreover, similar to the reports of the frequent loss of surface and polymerase genetic regions of HBV in other populations (Gunter et al., 1997), the frequent loss of HBx genetic region appears to be a unique feature of HBV strains circulating in our population. The phenomenon of the detection of HBV genome lacking important HBV genetic regions has been suggested to occur due to splicing (Gunter et al., 1997; Lee et al., 2008) or due to the circulation of HBV DNA integrated in human chromosomes (Fleischacker and Schmidt, 2007). Recent studies have established that the HBx C-terminal domain exercises a proapoptotic activity to counter balance the proliferative and transforming activities of the HBx N-terminal domain (Tang et al., 2006), which explains the frequent development of HCC in patients with truncation in the HBx C-terminal region (Tu et al., 2001; Liu et al., 2008). On the other hand, the abundance of HBV DNA, lacking the entire HBx genetic region in this population and extremely low occurrence of solely C-terminal truncated HBV in our patients well correlate with low incidence of HCC in our population. Furthermore the higher prevalence of intact virion DNA, in CHB patients in this study underscores the importance of intact HBx in persistently inducing immune response leading to chronic liver injury (Bouchard and Schneider, 2004; Tang et al., 2006). On the other hand, the higher prevalence of LC patients with HBV variants in our study clearly supports a recent finding that HBV variants with deletions are important in the pathogenesis of cirrhosis (Marschenz et al., 2006). Thus, in addition to supporting previous findings, our study also discloses other interesting results, which encourages further investigations.

In this study, we detected three distinct HBV genotypes (A, C and D) from the phylogenetic analysis of the HBx gene sequences that confirmed our previous results (Banerjee et al., 2006a; Banerjee et al., 2006b). The Eastern Indian population thus provides an opportunity to compare between three distinct HBV genotypes. Although due to the detection of a number of patients with defective HBV genome and low occurrence of HCC, we could study the variability of the HBx in a relatively small number of patients, but our analysis presents the very first picture of the HBx genetic variability patterns in a population of the Indian subcontinent.

In order to verify the clinical relevance of the mutations documented in this study, we collected the available literature focused on the functional significance of different HBx substitutions and sought their associations with genotypes/subgenotypes of HBV (Table 3). We noted that most of the HBx substitutions associated with severe liver disease, studied until now are either genotype specific or prevalent among certain genotypes only (Table 3) and involved a change from a hydrophilic to hydrophobic residue or vice versa at a particular site. This also suggests that a small number of substitutions emerging at particular positions in particular HBV genotypes/subgenotypes may alter the local configuration of HBx, which might lead to altered functional properties of HBx. Moreover, the review of the literature on this particular aspect seem to indicate that clinically relevant substitutions may emerge differently in distinct populations even with the circulation of similar strains of HBV. In a recent study on Korean patients (Kim et al., 2008), where subgenotype Ce/C2 is prevalent, a particular mutation M/L5 was found to be significantly associated with HCC development. In our analysis we observed that in HBx of subgenotype Ce/C2, the naturally occurring residue is V5, while in most other genotypes the naturally occurring residues are M/L5 (Table 3). Thus, in the Korean population, the substitution of the normal V5 with M/L5 appears to increase the risk for HCC development in subjects infected with Ce/C2. Similarly, in a previous study on Taiwan (Yeh et al., 2000), the A31 mutation of HBx was significantly associated with HCC. Interestingly, we detected a high prevalence of A31 in non-HCC patients in our study population.

<table>
<thead>
<tr>
<th>Nucleotide position (s)</th>
<th>HBx residue (s)</th>
<th>Reported effects of the HBx residues and/or corresponding nucleotides</th>
<th>Genotype/subgenotype specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1386† / A31</td>
<td>LI/M5</td>
<td>Suggested to be a major target of host immune response against HBx and also affects regulatory functions of HBV. Significantly associated with HCC in Korean patients (Kim et al., 2008).</td>
<td>†L5 – genotypes E, G, H, all the subgenotypes of A, B, D, and also affect subgenotypes C1/C1, C3, C4, C5, F1</td>
</tr>
<tr>
<td>1467† A31</td>
<td>S18</td>
<td>Low gene expression, escape from immune surveillance, reduced apoptosis, promotion of HCC (Yeh et al., 2000). Independent risk factor for development of HCC in genotype C (Muroyama et al., 2006).</td>
<td>†A31 – subgenotype Aa/A1</td>
</tr>
<tr>
<td>1485† S18</td>
<td>Y94</td>
<td>1653 changes the α box binding site that enhances the binding affinity and activity of the enh II and core Promoter (Shinkai et al., 2007) leading to HCC.</td>
<td>†Y94 – low expression of p21, increases cell proliferation (Kwun and Jang, 2004).</td>
</tr>
<tr>
<td>1497† P26</td>
<td>S101</td>
<td>High expression of p21, reduction of cell cycle and increased apoptosis (Kwun and Jang, 2004).</td>
<td>†S101 – reduction of cell cycle and cell proliferation (Kwun and Jang, 2004).</td>
</tr>
<tr>
<td>1653† Y94</td>
<td>P101</td>
<td>Low expression of p21, increase in cell cycle and thus cell proliferation (Kwun and Jang, 2004).</td>
<td>†P101 – high expression of p21</td>
</tr>
<tr>
<td>1672† M130</td>
<td>M130</td>
<td>Strong inhibitor of p21, increased cell proliferation (Kwun and Jang, 2004), 1762‡ prevent binding of LEP, suppress only preC mRNA (HBcAg expression), enhance progeny virus synthesis (Kramvis and Kew, 1999).</td>
<td>†M130 – reduced apoptosis, promotion of HCC in Korean patients (Kim et al., 2008).</td>
</tr>
<tr>
<td>1753‡ T127</td>
<td>T127</td>
<td>These residues affect anti-proliferative activity (Lin et al., 2007).</td>
<td>†T127 – anti-proliferative activity (Lin et al., 2007).</td>
</tr>
<tr>
<td>1762‡ M130</td>
<td>T127</td>
<td>These residues affect anti-proliferative activity (Lin et al., 2007).</td>
<td>†M130 – reduced apoptosis, promotion of HCC in Korean patients (Kim et al., 2008).</td>
</tr>
</tbody>
</table>

The genotype/subgenotype specificity of these mutations is also presented.

a Numbers and superscripted alphabets indicate nucleotide positions and bases respectively.

b Alphabets and subscripted numbers indicate amino acid residues and their positions respectively.

c Hydrophobic residue.

d Hydrophilic residue.

e Liver enriched factors.

Fig. 3. Different features of the HBx. (a) Two functional domains (N-terminal and C-terminal), the Proline-Serine Rich (PSR) hypervariable region and the split Kunitz domain like (KI) highly conserved regions. (b) Sequence diversity among different subgenotypes of HBV genotypes, corresponding to the functionally important regions. (c) Genotype (†) and subgenotype (‡) specific sites. (d) Codon-wise rate of synonymous (dS, green bars) and non-synonymous (dN, brown bars) substitutions, showing high inter-genotype variability at specific positions in the N-terminal domain. (e) Hydropathy plot showing altered hydrophobicity and/or antigenicity at genotype/subgenotype specific positions.
Our analysis revealed that A31 is specific for subgenotype Aa/A1. Moreover examination of genotype specific residues, as reported by Kidd-Ljunggren et al. (1995), we found that all the HBx sequences reported by Yeh et al. (2000) were of genotype A. Interestingly based on the analysis of surface gene region, prevalence of genotypes B and C in Taiwanese HCC patients (Kao, 2003), while detection of genotype A specific HBx sequences by Yeh et al. (2000) seem to indicate that the prevalent strains of HBV in Taiwan might possibly be B/A, C/A recombinants, which might have higher oncogenic potentials. Thus the HBx with a A31 substitution found in this study, is a part of the natural variability of non-recombinant genotype Aa/A1 strains in our population and was not associated with higher occurrence of HCC.

Recently, Muroyama et al. (2006) reported HBx substitution S58H in genotype C to be significantly associated with HCC patients. However the association of S58 with HCC remains unclear as in another recent study from the same country (Japan), such association with HCC was not documented (Shinkai et al., 2007). Another residue, Y94, has been shown to induce clonal outgrowth of cells and inhibit apoptosis (Poussin et al., 1999). The prevalence of these residues (S58H and Y94) was found to be very low in our study population. In addition, most of the present sequences had S101, which induces high expression of p21 (Kwun and Jang, 2004), leading the reduced cell proliferation and increased apoptosis of the HBV infected cells.

In this study apart from genotype/subgenotype specific substitutions and some regional variability, the occurrence of sporadic mutation was also low, and mutations, insertions or deletions specific for any clinical outcome were not observed, similar to previous reports (Cabrerozo et al., 1998; Terada et al., 2001). However, in accordance with published reports (Kim et al., 2008; Sung et al., 2008; Song et al., 2005; lavarone et al., 2003) we also detected substitutions at 127, 130 and 131 residue positions among all the three genotypes, with significantly higher percentage among advanced liver disease patients, supporting the clinical importance of these mutations (Kim et al., 2008). Although functional significance of the double mutations (M120 and I131) have been sought, the results are contradictory, with one report suggesting increased p21 repression activity (Kwun and Jang, 2004) while two others documenting no alteration in the transactivation function (Tu et al., 2001; Minemura et al., 2005).

Notably, these three studies were performed with HBV sequences isolated from patients from Korea (Kwun and Jang, 2004), Japan (Minemura et al., 2005) and France/South Africa (Tu et al., 2001), countries with different prevalence of HBV genotypes, indicating that the divergence in results may be due to genotype differentiated effects of these HBx mutations, clearly supporting a recent finding (Lin et al., 2005). In our study, C-terminal truncation was predicted in only one non-HCC case, while in none of the HCC cases. This is in contrast to a recent study from China, reporting that HBx C-terminal deletions are frequent in both tumor and non-tumor tissues (Liu et al., 2008). Taken together, it is evident from the present study that the variety of clinically relevant HBx mutation/deletion patterns may have their own prevalence and behavior in different geo-ethnic populations and the high incidence of HCC in certain populations may signify the presence of other risk factors, such as ethnicity, high dietary aflatoxin B1 exposure etc.

Interestingly, molecular epidemiological studies have revealed that high incidences of HBV related HCC development in Sub-Saharan African or East Asian countries is strongly associated with codon 249 mutation of p53, known to be induced by HBx expression and high dietary exposure to aflatoxin B1 (AFB1) (Hussain et al., 2007; Madden et al., 2002). We would like to mention here that in a study on p53 mutations (Datta et al., unpublished data), we could not detect the codon 249 mutation of p53 in any of our study population, which corroborate with a previous study (Katiyar et al., 2000) documenting very low prevalence of this particular HCC associated mutation in Northern Indian HCC patients, despite high Aspergillus contamination. Interestingly, among the daily Indian dieters, turmeric (active ingredient ‘Curcumin’) is widely used (Sinha et al., 2003), that has been shown to protects against AFB induced mutagenicity and hepatocarcinogenesis (Sonii et al., 1997). The preventive role of Indian spices and food additives in the low incidence of HCC and other forms of cancers thus needs further investigations.

In conclusion, compared to other regions of world with high HCC prevalence, Indian HBV isolates have very low prevalence of HBx mutations, absence of C-terminal deletions in HBx, and absence of p53 codon 249 mutations. Thus, our study shows that clinically important HBx mutations may emerge specifically in different HBV genotypes/subgenotypes that might modulate different pathogenic pathways through complex interactions with different ethnic and environmental risk factors. Therefore the clinical significance of HBV genotypes and corresponding mutations in a given population may not be always relevant to another population, even if the prevalent viral strains are similar in both the population. Taken together, evaluation of HBx functions from the perspective of genetic variability and geo-ethnic factors are thus essential for a better understanding of HBV genotype-phenotype relations. Further studies on HBx functions, from the perspective of genetic variability may help identify HBV genotype specific pathogenetic pathways, which are essential for the development of HBV genotype specific therapeutics to efficiently reduce the burden of HBV related liver complications.

Materials and methods

Study subjects

The present study was done on 102 sera from HBsAg positive, HBV surface gene PCR or HBV core gene PCR positive subjects who presented with different clinical manifestations. These patients were referred to our unit from different liver clinics, for detailed HBV DNA analysis. All the subjects were screened for HCV or HIV infection, chronic alcoholism or other concomitant causes of liver diseases, before including in the study. Of the sera included for X gene amplification, sequencing and analysis, 32 sera were from inactive carriers (IC), 32 were from chronic Hepatitis B (CHB) and 24 were from liver cirrhosis (LC) patients. Due to low incidence in this region, we could study sera from only 6 well diagnosed HCC cases. Sera from only 8 acute hepatitis B (ACU) patients, satisfying the study inclusion criteria were available for detailed analysis.

This work was the part of a study approved by the institutional Ethics Committee. Informed consent was obtained from the subjects or their guardians before blood collection.

Serological markers

Commercial ELISA based kits were used for HBsAg (Biomerieux, Boxtel, The Netherlands), HBeAg/anti-HBe (Biomerieux, and EQUIPAR, Soronno, Varese, Italy), anti-HCV (Ortho-Clinical Diagnostics, New Jersey, USA) and anti-HIV (Biomerieux) detection. ALT was estimated by Ecoline ALAT kit (Merck Specialties, Mumbai, India).

DNA extraction, amplification and sequencing

HBV DNA was extracted from serum by Proteinase K–Phenol/ chloroform method. Amplification of the partial promoter region of the human interleukin-1β gene was done for verification of the DNA extraction procedure and HBV DNA was initially detected by in-house nested HBV surface or precore/core gene amplification PCRs. Strict precautions were followed to avoid cross contamination and appropriate negative and positive controls were included during DNA extraction and PCR amplification steps.

HBV X gene region was amplified by a hotstart PCR protocol (AmpliTaq Gold DNA Polymerase, Applied Biosystems, Foster City, CA, USA), and the primers HBx1093F [5′-accttctggcaacttacagggcattt-3′, 190–198]
Detection of circulating virion free HBV DNA

Although all the sera samples included in this study had HBV surface gene sequences amplifiable by nested PCR, HBV X gene sequences could not be amplifiable in a number of these samples, despite similar amplification efficiencies of both the PCR assays. This observation prompted us to probe for the presence of DNaI sensitive HBV genomes in these cases. To distinguish between DNaI sensitive HBV DNA and virions associated intact HBV DNA, 400 μl of sera samples were divided into two parts. One part was digested with DNaI [New England Biolabs, Beverly, USA] at 37 °C for 30 min following an earlier described method (Cabreroz et al., 1997). The DNaI digested and undigested parts of each serum sample were then subjected to DNA extraction, and amplification by nested PCR assays as described previously.

Molecular evolutionary analyses

All the HBx sequences generated in this study were edited manually by visual inspection and were multiply aligned with reference full genome HBV sequences (retrieved from the GenBank) of different genotypes and subgenotypes (Norder et al., 2004), using ClustalW program incorporated in Bioedit version 7.0 (Hall, 1999). Genetic distances were estimated by Kimura's two-parameter method of evolutionary comparison and phylogenetic tree analysis was assessed by boot-neighbor-joining (NJ) method. The reliability of the pairwise method and the phylogenetic tree was constructed by the Genetic distances were estimated by Kimura's two-parameter method (Nei and Gojobori, 1986) and VESPA (Korber and Myers, 1992) were calculated using sequence analysis tools — CONSENSUS, SNAP (Nei and Gojobori, 1986) and VESP&A (Korber and Myers, 1992) respectively, available at the Los Alamos National Laboratory website (http://hcv.lanl.gov/).

Prediction of structural characteristics of HBx

To identify potential antigenic sites of HBx, hydrophilicity profile was created employing the Kyte and Doolittle (1982) method, implemented in Bioedit version 7.0. Predictions of different structural characteristics of HBx were done, utilizing the PredictProtein server (Rost et al., 2004; http://www.predictprotein.org/).

Statistical comparisons

Statistical differences were calculated using the χ² test or Fisher's exact probability test and Student's t-test where applicable. Two tailed P values ≤0.05 were considered statistically significant. For statistical calculations, Microsoft Office Excel (Microsoft Corporation, USA) or StatCalc (EpiInfo, version 6.0, CDC, USA and WHO, Switzerland) were used.

GenBank accession numbers

The GenBank accession numbers of Eastern Indian HBV X gene sequences analyzed in this study are EF594748 through EF594786 and DQ315776 through DQ315786.

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Appendix A. Supplementary data


References


