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X GENE VARIABILITY AND GENOTYPING OF HEPATITIS B VIRUS (HBV) IN SUDANESE PATIENTS WITH LIVER DISEASES, KHARTOUM, SUDAN

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ABSTRACT

Background: HBx gene mutations may have a role in the progression of liver disease from chronic infection to liver cirrhosis and/or hepatocellular carcinoma. These mutations affect the biological functions of HBx protein, which may influence the development of liver disease. The aim of this study was to describe the variability of HBx gene among hepatitis B patients, find out the prevalent genotypes and possible association of different genotypes with HBV infection outcome. **Methods:** A total of 185 hepatitis B patients, 88 asymptomatic carriers(ASC), 39 chronic hepatitis(CH), 38 liver cirrhosis(LC) and 20 with hepatocellular carcinoma(HCC) were recruited to participate in this study. HBx gene positive products were sequenced and genotyped. Nucleotide and amino acid variability were determined. **Results:** HBx DNA of nineteen patients was successfully amplified. HBx30, HBx127, HBx130 and HBx131 were the most detected mutations in HCC and LC patients. Double mutations K130M/V131I and F30L/V mutation were associated with high risk of LC and HCC development. Our study found that the most prevalent genotype was genotype D (47.4%), followed by E (42.1%) and A (10.5%). Patients with genotype E had ALT elevation exceeding 1.5 times than those with genotype D and A. **Conclusions:** HBx30, HBx130 and HBx131 mutations were associated with liver disease

progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma. These mutations may be useful markers for predicting the clinical course of patients with chronic hepatitis B.

KEYWORDS: HBV, genotyp, HBx gene, hepatocellular carcinoma, asymptomatic carriers, chronic hepatitis B.

INTRODUCTION

Hepatitis B is a virus that attacks the liver and can cause both acute and chronic disease. It is estimated that 257 million people are living with hepatitis B infection and overwhelming epidemiological evidence indicates that they are at high risk for the development of liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC).[1,2,3] HBV has a compact genome with partially double stranded DNA of approximately 3200 bases in length.[4] The genome encodes four overlapping open reading frames (ORFs), overlapping each other and covering the entire genome**.** The ORFs include: core (C) [pre-core/core], surface (S) [(pre-S1/S2/S)], polymerase (P) and HBX-encoding (X) regions.[5]

HBV-X is a 154 aminoacid multifunctional protein with transcriptional transactivator activity on a number of cellular and viral promoters. It has been associated with the pathogenesis of HBV related diseases, especially in the occurrence of hepatocellular carcinoma in chronic patients.[6,7,8,9]

Mutations in the X region can involve the regulatory elements that control replication, such as the basal core promoter and Enhancer II. Because the basal core promoter encompasses nt 1742-1802 and overlaps with the X gene in the concomitant reading frame. The A1762T plus G1764A core promoter mutations also cause changes in the X gene at xK130M and xV131I.[10] The aim of this study was to describe the variability of HBx gene among hepatitis B patients study groups infected with different genotypes, in relation to liver diseases.

MATERIALS AND METHODS

A cross-sectional, hospital based study was conducted at the Hepatology clinic at Ibn Sina Hospital, Khartoum Isotope canter and Military hospital in Khrtoum state, between November 2013 to October 2017. Ethical approval was obtained from Research Ethics Committee, Sudan University of Science and Technology and the National Ethics Committee at Ministry of Health, Khartoum State. A written informed consent to participate in this study was obtained from all the patients. A total of 185 hepatitis B patients with different liver

diseases were recruited to participate in this study, blood specimens were collected from HB patients in plain containers, allowed to clot then the sera seperated then stored at -20°C until analysed. ALT levels were determined. Patients were grouped into asymptomatic carriers, chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma according to their clinical recorded data.

Serological markers

The hepatitis B serological markers included HBsAg, HBcAb and HBeAg, were performed using Enzyme Linked Immunosorbant Assay Kits (AccuDiagTM, Ca, USA).

DNA extraction and PCR

Chelex extraction method was used to extract DNA from serum samples under sterile technique to prevent the contamination of DNA extractions. The study followed protocol of nested PCR as described by (Han Lee *et al.,* 2011). Which used two sets of forward and reverse primers. In each reaction, positive and negative control was used for quality control. Primers used for the first amplification were (5-CATGCGTGGAACCTTTGTG-3; positions 1233 to 1251) and (5-CTTGCCTKAGTGCTGTATGG-3; positions 2072 to 2053). For the second PCR amplification (5-TCCTCTGCCGATCCATACTG- 3; positions 1254 to 1263) and (5-CAGAAGCTCCAAATTCTTTA TA-3; positions 1937 to 1916). First round PCR was performed with 2 µL of genomic DNA in a 25 µL reaction mixture containing 10X buffer, 200 µM dNTPs, 20 mM Cl2Mg, 10 pmol of each primer and 1 U Taq polymerase (Intron Biotechnology. South Korea). PCR cycling for the first round was performed as follows: 94 C 5 min; 94°C 1 min, 55°C 1 min, 72°C 2 min for 35 cycles; and final extension 72°C for 10 min. For the second round, 1-2 µL of the first round PCR product was re-amplified using the same reaction mixture composition, except that internal primers were used. Second round PCR was performed as follows: 94°C 5 min; 94°C 30 sec, 51°C 1 min, 72°C 2 min for 30 cycles; and finally 72°C for 10 min.

Detection of the amplified HBV X gene PCR products

PCR products were run on agarose gel for 30 min, stained with ethidium bromide, and evaluated under UV light. Detection of the amplified PCR products was performed using two microliters (µl) of the second round PCR product on 1.5% agarose gel electrophoresis with ethidium bromide. The gel was run for 30 minutes in a 1x Tris-acetate-EDTA (TAE) buffer at 95 Voltage with positive and negative controls. The expected bands (683bp) were detected against 1000bp DNA ladder using ultraviolet (UV) BDA system (Biometra Inc) (figure1).

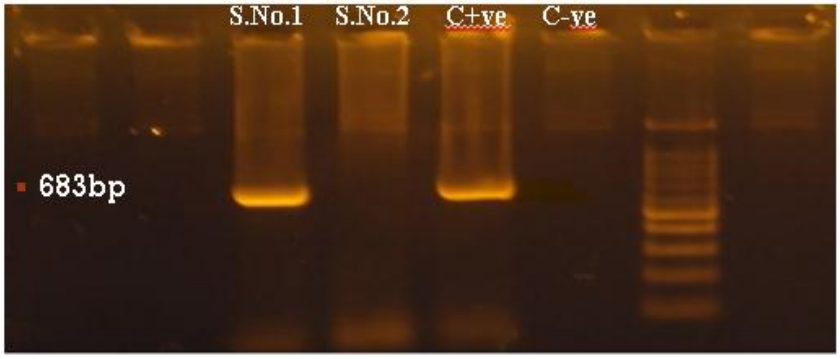

Figure 1: Agel electrophoresis diagram showing X gene positive products with 100bp DNA ladder. The PCR amplification generated a DNA fragment of expected size (683 bp). Positive control(c+ve), Negative control(c-ve), Sample number (S.No).

Nucleotide Sequence Determination of HBV x Gene

The nucleotide sequences of the HBx gene were determined using sanger DNA sequencing in both directions using the primers used in the second PCR. Determination of the nucleotide sequence of PCR products was performed at Macrogen Inc (Canada).

HBV genotyping

Deduced X gene base pairs were compared to several consensus sequences of HBV genotypes, and a phylogenetic tree was constructed by the neighbor-joining method. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 7(Molecular Evolutionary Genetic Analysis version 7). The genetic distances were estimated by Kimura's two-parameter method. The reliability of the phylogenetic tree analysis was assessed by the interior branch test with 1000 replicates.

Statistical analysis

Data were expressed as means. All statistical analyses and tests were performed with the SPSS statistical package (SPSS 17.0, Chicago, IL, USA) and $p<0.05$ was regarded as statistically significant. Significant differences were determined using t tests.

RESULTS

Demographic and Clinical characteristics

A total of 185 patients were recruited in this study. The participants mean age was 43.5 ±16.02. The majority of the patients (75.7%). Patients were grouped into ASC (n=88), CH(n=39), LC(n=38) and HCC(n=20) according to their clinical recorded data. The HCC patients were significantly older than patients in the other groups (*P*.value=0.01) with mean

age 53.76±16.91. All the participants were reactive for HBsAg and HBcAb. Patients those positive for HBeAg were 34(18.4%) (Table1).

HBx gene positive products

Out of 185 hepatitis B patients, 19(10.3%) patients was successfully amplified using nested PCR. Of the 19 patients, 16 (84.2%) were males. The mean age of the 19 patients was 45.7 ±14.8 years. For the purpose of mutation analysis the 19 patients were classified as group 1 which included 7 patients with LC plus 3 patients with HCC, and Group2 which included 6 patients with ASCs plus 3 patients with CH. Out of the 19 positive PCR samples, HBeAg was positive in 4(21%) patients and 15(79%) were HBeAg-negative.

HBVx gene mutations according to the clinical status

The most common detected HBx mutations were HBx30, HBx88, HBx127, HBx130 and HBx131. HBx30 and HBx130/131 mutations were significantly (*P*.value=0.04 and 0.01 respectively) associated with HCC and LC. HBx 30 mutation was detected in 9 (90%) patients of group 1 while in group 2 this mutation was detected in just 3(33.3%) patients (Table2). Double mutations of HBx130/131 were detected in 8(80%) patients of group 1, but in group 2 we detected these mutations in 1(11.1%) patient (Table3). HBx127 was found in 6(60%) patients of HCC and LC group and 3(33.3%) patients of CHB group with no significant association (*P*.value:0.27) of this mutation with HCC and LC diseases. HBx 88 mutation was detected in 5(50%) patients of HCC and LC group with no association (Table2). Several other mutations were detected in this study, which include HBx 31, 94, 101, 69 and 73, but in a low frequencies.

Table 1: Clinical and demographic characteristics of 185 patients.

Parameter	Liver diseases			
	HCC(20)	LC(38)	CH(39)	ASC(88)
Age mean±SD	53.7±16.91	48.4±13.96	38.5±13.26	39.2±17.22
ALT mean	46.2	52.9	68.1	22.1
HBeAg+	2	7	11	14
HBcAb	20	38	39	88

HCC: hepatocellular carcinoma, LC: liver cirrhosis, CH: chronic hepatitis, ASC: Asymptomatic carriers.

Table 2: Detected mutations according to the liver diseases.

Mutation	Group 1 (HCC+LC)n=10	Group2 (CH+ASC)n=9	Total frequencies
HBx130/131	8(80%)	1(11.1%)	9
HBx30	9(90%)	3(33.3%)	12
HBx127	6(60%)	3(33.3%)	9
HBx88	5(50%)	7(77.8%)	12

HBV genotyping and phylogenetic analysis

HBV genotyping was done using amplification and sequencing of the complete X gene followed by phylogenetic analysis. The genotype distribution in the 19 samples was Genotype D:9(47.4%), genotype E:8(42.1%) and genotype A:2(10.5%). A rooted phylogenetic tree of 19 complete X sequences of HBV obtained from Sudanese liver disease patients and reference HBV sequences, using neighbour-joining. Bootstrap statistical analysis was performed using 1000 datasets. The sequences are labeled by their accession numbers and country(Figure 2).

Prevalence of mutations according to the genotype

Out of the 3 patients with HCC, genotype A was found in 2 (66.7%) patients and genotype E 1(33.3%)patients. Out of the 7 patients with LC, genotype E was detected in 4(57%) patients, while genotype D was detected in 3 (43%) patients. In the group of patients with CH, genotype D was found in 2(66.7%) patients and genotype E was found in 1(33.3%) patients. In ASC group, Genotype D was found in 4(66.7%) and genotype E was found in 2(33.3%) patients. The mean of ALT level in patients with genotype E was 63, compared with ALT level mean in patients with genotype D which was 31.7. Patients with genotype E had ALT elevation exceeding those with genotype D (Table3).

Table 3: Distribution of genotypes in relation to Liver diseases.

HBV genotype	HCC(3)	LC(7)	CH(3)	ASC(6)	ALT mean
D(%)	0	3(43%)	2(66.7%)	4(66.7%)	63.0
E(%)	1(33.3%)	4(57%)	1(33.3%)	2(33.3%)	31.7
A(%)	2(66.7%)	0	0	0	30.0

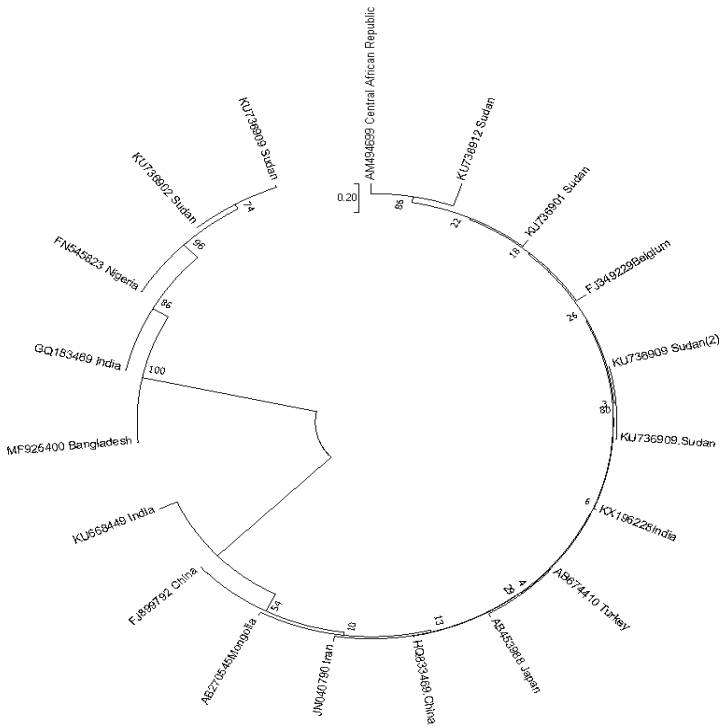

Figure 2: A rooted phylogenetic tree of 19 complete X sequences of HBV obtained from Sudanese liver disease patients reference HBV sequences, using neighbour-joining. Bootstrap statistical analysis was performed using 1000 datasets. The sequences are labeled by their accession numbers and country.

DISCUSSION

In this study it was found that the most common detected HBx mutations in LC and HCC patients were HBx30, HBx127, HBx130 and HBx131.

The double mutation A1762T/G1764A is well known to be associated with HCC and LC.[11] These mutations transforming Lys to Met at region 130 and Val to I1e at position 131 of the X protein. This mutation decrease the expression of P21, which is important regulators of cell cycle, by inhibiting the P53 proto-oncogene. Active P53 protein increase the transcription of P21 gene. Given that the P21 protein inhibits the function of G1/S cyclins, the cell stops at the G1 phase. Thus, in infected liver cells, P53 protein is deactivated by the X protein and activates the E/Cdk2 cyclin by reducing the P21 protein expression, which ultimately leads to passage of cell from phase G1 to S. This process leads to uncontrolled proliferation of HBVinfected hepatocytes. In our study, HBx130/131double mutations was closely associated with liver cirrhosis and hepatocellular carcinoma rather than chronic hepatitis which agree with previous studies.[12,13,14,35]

In this study, F30L/V mutation was found in all hepatocellular carcinoma patients, 85.7% of patients with liver cirrhosis and 22.2% of chronic hepatitis patients. This result support the study that done by Xiaodong and his co workers (2012) which focused on the significance of novel mutations of HBx in the tumor tissues in the development of HCC from China.[15] Accordingly, our study suggested that this mutation may play significant role in HCC and LC progression.

The change of HBx127amino acid may affect the function of HBx and therefore it may predict the development of hepatocellular carcinoma in patients with cirrhosis.[16,17,18] In addition, this mutation may alter the binding activity of the ubiquitous transcription factor Sp1 and affect the core promoter activity. HBx including T1753V mutant induced a higher increase in the transactivational activity than wild-type HBx.[36,37] These changes may contribute to the multistep process of hepatocarcinogenesis. In our study, (I127T/ N/P/G) substitutions occurred more frequently (60%) in patients with hepatocellular carcinoma and liver cirrhosis.

Other mutations detected in our study which may have implications in the development of HCC and LC include mutations at positions aaHBx31, 94, 79, 73 and 101.

Yeh *et al* (2000) and Utama *et al* (2009) reported that S31A was found to induce low expression of apoptosis which could lead to reduced regeneration of hepatocytes and also result from immune surveillance escape which might contribute to the process of hepatocarcinogenesis.[19,11] In Our study, two patients with HCC out of three showed mutation S31A which agree with previous studies, and belong to genotype A. Datta *et al* (2008) reported that this mutation was also may be specific to genotype A.[20] Kim *et al.,* (2008) reported that H94Y substitution was shown to be involved in severe liver diseases in Korean patients.[21] C1653T (amino acid substitution H94Y) was detected in two patients both of them with LC. However, in our study H94Y mutation was found in genotype E but in another study done in South Africa by Malinga and Lesibana (2010)they detected this substitution in genotype D, which it means that it's not genotype specific.[22]

Kwun and Jang (2004) and Maligna (2010) reported that HBx variants with S101P may have different effects on the cell cycle progression, and eventually on the cell growth rate, implicating its biological significance. In our study, of three patients with HCC, 2 patients had S101P substitution One patient with LC showed S101L and two patients with Chronic

HB showed S101C/F. Shen *et al* (2008) reported that C69R and F73L substitutions may be responsible immune pressure escape during viral host interaction[23], we detected C69L and F73L in two patients with liver cirrhosis. 6 patients out of 9 with chronic HB showed I88F/L/V/C mutation, 4 of them belong to LC patients, one with chronic active and one with HCC, suggesting that there is no strong association between liver diseases progression and this substitution.

Progression of HBV infection may depend on the virus genotype. In the last study done by Muklid *et al.*,2013 found that patients infected with genotype E had higher frequency of hepatitis B e antigen-positivity and higher viral loads compared to patients infected with genotype D.[24] We found an association between genotype E and higher ALT levels, indicating greater necro-inflammation, as compared to genotype D. This inflammation may be a result of immune injury secondary to higher rate of viral replication.

Considering Sudan's unique position and the flux of people across its borders, diversity of genotypes are distributed in neighbouring countries:[25] genotype D in Egypt to the north[26], genotype E in the Democratic Republic of Congo to the west[27] and genotype A in Kenya[28,29] and Uganda[30] to the south. Knowledge of the genotypes prevailing in Sudanese, with and without liver disease, is important in treatment management, as well as disease prognosis because genotypes play a role in both of these aspects.[31,34]

A study among Sudanese asymptomatic blood donors, found that 57.5% were infected with genotype E, 40.5% with genotype D and 2% with subgenotype A2 (32) The last study which done by Muklid et al,. 2013 showed that the genotype distribution was 59% genotype D: 30% genotype E: 8.5% genotype A: 2.5% putative D/E recombinant. Our study showed genotypes distribution to be genotype D47.3% (9/19), Genotype E 42.1% (8/19) and genotype A10.5%(2/19) which is partially agree with the results of last study in Sudan.

Interested Mutations

Many other mutations were found in our study which include G22S, T36D, T47A, L123S. Although the biological function of most of these mutations is unknown, the development of multiple mutations may represent a strategy of HBV to escape immune surveillance and thus contribute to the process of multiple steps in hepatocarcinogenesis.[15]

CONCLUSION

In conclusion, it is thought that the mutations HBx30, HBx130 HBx131 in the X gene, were associated with HBV infection progression from chronic hepatitis to liver cirrhosis or hepatocellular carcinoma. These findings suggests that mutations may be useful markers for predicting the clinical course of patients with chronic hepatitis B. These mutations could be detected in as early as years prior to the development of hepatocellular carcinoma and liver cirrhosis which may lead to improve patients clinical management. HBx substitutions which circulate in different genotypes may affect the different pathogenic potential due to complex interactions of the virus with the host. Although genotype D was the most prevalent one but genotype E may have the sever clinical outcome through increasing ALT level which is a marker of liver damage.

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