

HEPATITIS B VIRUS X GENE MUTATIONS AMONG HBV INFECTED INDIVIDUALS IN SOUTHWEST NIGERIA

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ABSTRACT

Despite the availability of efficient vaccines against hepatitis B, it still poses a great public health issue especially in places with less developed healthcare facilities and buoyant economy to cater for the infected and conduct relevant studies that give directions to more efficient prevention and control of the disease. Each ORF of the hepatitis B virus plays key roles in the perpetuation and pathogenesis of the virus but not many studies have been carried out on the X ORF especially in Nigeria. The resulting multifunctional protein which is strongly associated with the development of HCC has not been well studied in Nigeria. The study was, hence, designed to analyze the HBx ORF in sick and asymptomatic HBV carriers in southwest Nigeria. Venous blood samples collected from consenting apparently healthy and sick individuals presenting with fever and mild abdominal discomfort were pre-screened for the HBsAg. Samples positive for this marker had their HBV DNA extracted, HBx gene amplified, sequenced, genotyped and their sequences analyzed using appropriate tools. In all, 19 HBV isolates were amplified and confirmed to be genotype E. Observed in them all was S123L mutation. Also observed were S62A (4/19; 21.1%) and F73*, T74K, S75V, R77W, and R78C all having 10.5%; 2/19 each. Mutations, I88F and V131I, whose roles in the pathogenesis of hepatitis B have been reported were also observed. Multiple mutations on the N- and C- termini whose association with advanced liver diseases and predisposition to HCC need to be studied were observed in seven (7/19; 36.6%). Individual studies of the HBV ORFs may be necessary, especially in Africa where there seems to be more asymptomatic carriers of HBV for a better understanding of the evolution of the virus which will in-turn enhance the prevention and control of the disease.

Keywords: Genes, Mutations, Carcinoma, Virus, Hepatitis, Open reading frame.

INTRODUCTION

The liver is a crucial organ in the human body with inestimable functions ranging from digestion, detoxification, metabolism, and immunity among others. These functions can be impaired if the organ is damaged or diseased as a result of obesity, alcoholism and viral hepatitis. The damage can start from hepatitis to fibrosis, cirrhosis and end-stage liver disease (liver failure). Alcoholism and chronic hepatitis B and C commonly cause cirrhosis. Despite being preventable and treatable, viral hepatitis remains one of the leading infectious disease killers worldwide and the goal of eliminating viral hepatitis by 2030 is off-track (WHO, 2026). The World Health Organization estimated that 240 million people lived with chronic hepatitis B (CHB) infection in 2024 and 900,000 new infections each year (WHO, 2026). In 2024, hepatitis B, caused by the hepatitis B virus (HBV), caused an estimated 1.1 million deaths mostly from cirrhosis and hepatocellular carcinoma (HCC), a form of primary liver cancer (WHO, 2026). As at 2020, CHB was the leading cause of HCC globally while primary liver cancer was the third leading cause global cancer death as

HCC accounts for 75-85% primary liver cancer (Sagnelli *et al.*, 2020; Sung *et al.*, 2020). Among the most common cancers in Africa, HCC ranks the 4th and the second most common cause of cancer-related mortality while in Sub-Saharan Africa, it occupies the second and third most common malignancy in men and women respectively (Abou-Alfa *et al.*, 2023; Bray *et al.*, 2024; Okeke *et al.*, 2020).

The HBV genome, 3.2 kb in size, contains four overlapping open reading frames (ORFs) encoding the surface (S), core (C), polymerase (P), and X proteins for specific functions in the virus. Of significance in the HCC formation is the hepatitis B virus X (HBx) gene which codes for a regulatory protein, HBx protein, 154 amino acids long, which interacts with numerous host cellular proteins and signaling pathways. The roles of HBx protein in the HBV include the stimulation of cell cycle progression of quiescent cells and has the ability to activate HBV promoter and enhancer and a wide range of other viral promoters thus making it a pleiotropic transactivator (Spandau and Lee, 1988; Twu *et al.*, 1989; Zahm *et al.*, 1988; Koike *et al.*,

1994). Considering the significant roles of HBx in the replication and signaling pathways, mutations on this gene can cause deleterious effects. A study on the tumorigenic effects of combo HBx mutations was conducted by Pu *et al.* (2022) to elucidate the mechanism by which HBx mutants promote HBV-induced carcinogenesis but also reveals effective therapeutic options for HBV-HCC.

The compact nature of the viral genome and the error-prone reverse transcriptase lacking proofreading capability during replication contribute to high mutation rates and because the ORFs overlap, a mutation on one may affect another one by altering the nucleotides and hence the amino acids. Despite substantial progress in vaccination and antiviral therapy, HBV continues to pose a significant public health challenge, particularly in sub-Saharan Africa, where the burden of chronic infection remains among the highest globally. Therefore, mutations occurring on the HBx will have significant effect on its roles and the overlapping ORFs. Studies relating to HBx are important to the understanding of viral evolution, identifying molecular determinants of disease progression, and improving risk stratification for chronic liver disease and hepatocellular carcinoma. Although whole genome sequencing would have been more appropriate for this study, paucity of fund has limited a number similar studies to one ORF at a time. While studying mutations of the C ORF of HBV, Adesina *et al.* (2024) reported different mutations whose significance to the virus and the carriers have not been discovered. Studies like this can help understand the HBV genome if it is hard to study the whole genome at once. This study was designed to identify and discuss mutations within the HBx gene of HBV isolates circulating in the Southwestern Nigeria.

MATERIALS AND METHODS

Study location and selection of participants

The study covered five States, Ekiti, Lagos, Ondo, Osun and Oyo, in the southwest Nigeria. The region falls to the tropical rainforest region and is bounded in the East by Edo and Delta states, in the North by Kwara and Kogi States, in the West by the Republic of Benin and in the South by the Gulf of Guinea, lies between longitude 2°31' and 6°00' East and Latitude 6°21' and 8° 37'N with a

total land area of 77,818 km². The climate of the zone is characterized by wet and dry seasons.

Participants for the study included randomly selected apparently healthy individuals, pregnant women on routine medical check and those presenting with any of fatigue, fever, abdominal disturbance, nausea and vomiting from randomly selected primary and secondary healthcare facilities, Private blood bank, and an institutional health service centre across the five states. Some apparently healthy and few sick individuals outside the health facilities in Ogbomoso and Oyo communities were also enlisted for the study after proper explanation and obtaining their consent.

Ethical Considerations and sample collection

Ethical approval (OSHREC/PRS/569T/3) for the study was obtained from the Research and Ethical Committee, Seventh Day Adventist Hospital, Ile-Ife while permissions were obtained from the private health facilities and the Obafemi Awolowo University Health Centre, Ile-Ife before the commencement of sample collection.

About 3 ml of venous blood was collected from each consenting individual visiting the healthcare facilities used in the states. The blood samples were separated and then serum stored at -20°C until analyzed.

HBsAg serology

Due to paucity of funds, 223 randomly selected samples out of the 787 originally collected for the study were screened for HBsAg. The ELISA kit (Beijing WANTAI Biological Pharmacy Enterprises Co. Ltd. China) was used for HBsAg screening. The samples and the kits were brought to room temperature. According to the manufacturer's instructions, 20 µl of the specimen diluent was dispensed into each micro-plate well followed by the addition of 100 µl of each sample into the appropriately identified well. Other steps including, incubation, addition of other reagents, washing, and stopping the reaction were followed as stated in the protocol. The results were read at a wavelength of 450nm not later than 30 minutes after the completion of the test. All HBsAg positive samples were prepared for molecular analysis.

Nucleic acid extraction

Viral nucleic acid was extracted using QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) for simultaneous purification of viral DNA and RNA from plasma, serum, and cell-free body fluids. Following the manufacturer's instructions from lysis to the final elution in buffer AVE, a yield of 60 µl of purified viral nucleic acid from each sample was extracted and ready for use.

Detection of HBV by qPCR

The detection of HBV DNA was carried out using a LightCycler Multiplex DNA Master (Roche Diagnostics, Mannheim, Germany). A 15 µl mix containing 9.25 µl PCR grade water, 4 µl qPCR reaction mix – 5x concentration, 0.25 µl HBV probe TM-5 – 10 µM and 0.75 µl each of 10 µM sense primer HBV-61 (5'-GGA CCC CTG CTC GTG TTA CA) and antisense primer HBV-62 (5'-GAG AGA AGT CCA CCA CGA GTC TAG A) was prepared and dispensed into the labeled qPCR plate. To complete the 20 µl, 5 µl each of the DNA template, HBV standards of concentrations from 1E1 to 1E6, positive and negative controls were dispensed into their appropriate wells. Following a short spinning of 200 rpm for 2 minutes, the detection was done following the protocol of pre-incubation at 50°C for 2 minutes and 95°C for 10 minutes; and 45 cycles of amplification at 95°C for 10 seconds, 60°C for 34 seconds. Samples with low and medium qPCR Ct values were prepared for HBV X gene amplification.

HBV X gene amplification

Only 31 samples with good viral DNA yield from the qPCR were prepared for the HBx gene nested PCR using HotStart kit master mix (QIAGEN, Hilden, Germany). The 12.5 µl reaction consisted of 1 µl of the template with 4.25 µl of PCR grade water, 6.25 µl of HotStart master mix, 0.5 µl each of newly synthesized primer sets HBV-123, CATGGCTGCTAGGCTGTGCTG (sense) and HBV-124 CACAGCTTGGAGGCTTGAACA (anti-sense) for first round of PCR conditioned at 10 minutes at 95°C; 40 cycles of 30 secs at 94°C, 30 secs at 54°C and 40 secs at 72°C then, 10 mins at 72°C and holding at 8°C. For the second round PCR, 0.25 µl of the primers HBV-123 and HBV-125

GAGATGATTAGGCAGAGGTGAAAAAG were used. The conditions for the amplification of the second round was 10 mins at 95°C 40 cycles for 30 secs at 94°C, 30 secs at 54°C and 40 secs at

72°C then 5 mins at 72°C, and holding at 8°C. Expected band size for the two reactions was 500 bp.

Detection of amplified PCR products in gel electrophoresis

The amplified PCR products were visualized using 1.5 % agarose gel electrophoresis stained with gel red using TAE buffer. Samples were loaded into appropriately identified wells after mixing 2.5 µl sample with 2.5 µl of the loading buffer. Positive, with expected molecular weight and negative controls as well as a 100bp ladder (Invitrogen, Germany) were also loaded on the gel. The system was set at 100V, 100mA for 35 minutes. Visualization under the UV light source was done using Biometra Analytic image viewer (Jena, Germany).

Cleaning and Gene Sequencing

Sanger sequencing was done in Germany using 0.5 µl BigDye version 3.1 (Life Technologies, Applied Biosystems, Darmstadt, Germany), 1.5 µl buffer and 1 µl of the cleaned PCR2 product in an automated DNA sequencer ABI 3130xl (Applied Biosystems, Foster City, CA).

Mutation analysis

Sequence alignment of the isolated strains was performed using Muscle algorithm and the default parameters on the National Center for Biotechnology Information (NCBI) server. Mutation analysis was done using Geneious 11, BioEdit version 7.2.6, MEGA 7.0.26 and geno2pheno software. Genomic sequences obtained for the HBx gene were subjected to phylogenetic analysis and then translated into amino acid (aa) sequences to compare them with HBV reference sequences on the NCBI database.

RESULTS

The serologic screening showed that 83 (37.2%) of the 223 samples had detectable HBsAg. Only 31 (37.3%) of the 83 HBsAg positive samples had low to medium qPCR Ct values. However, HBx gene was successfully amplified and sequenced in 19 (61.3%) of the 31 samples. The 19 sequenced samples belonged to the genotype E. According to the State of sample collection, 3 (15.8%) of the 19 were from Ekiti, 1 (5.3%) from Lagos, 8 (42.1%) from Osun and 7 (36.8%) from Oyo States respectively. The sequences have been deposited in the GenBank with the accession numbers

MT570147 -- MT570165. The phylogenetic tree (Figure 1) shows 22 sequences because three samples had both 210 and 459 bp but those of 459 bp were submitted. Sequences with accession numbers MT570147 - MT570161, MT570165 had

210 bp while those with accession numbers MT570162 - MT570164 had 459 bp. The amino acid sequences showing the mutations observed in the samples after aligning with a reference sequence is shown in Figure 2.

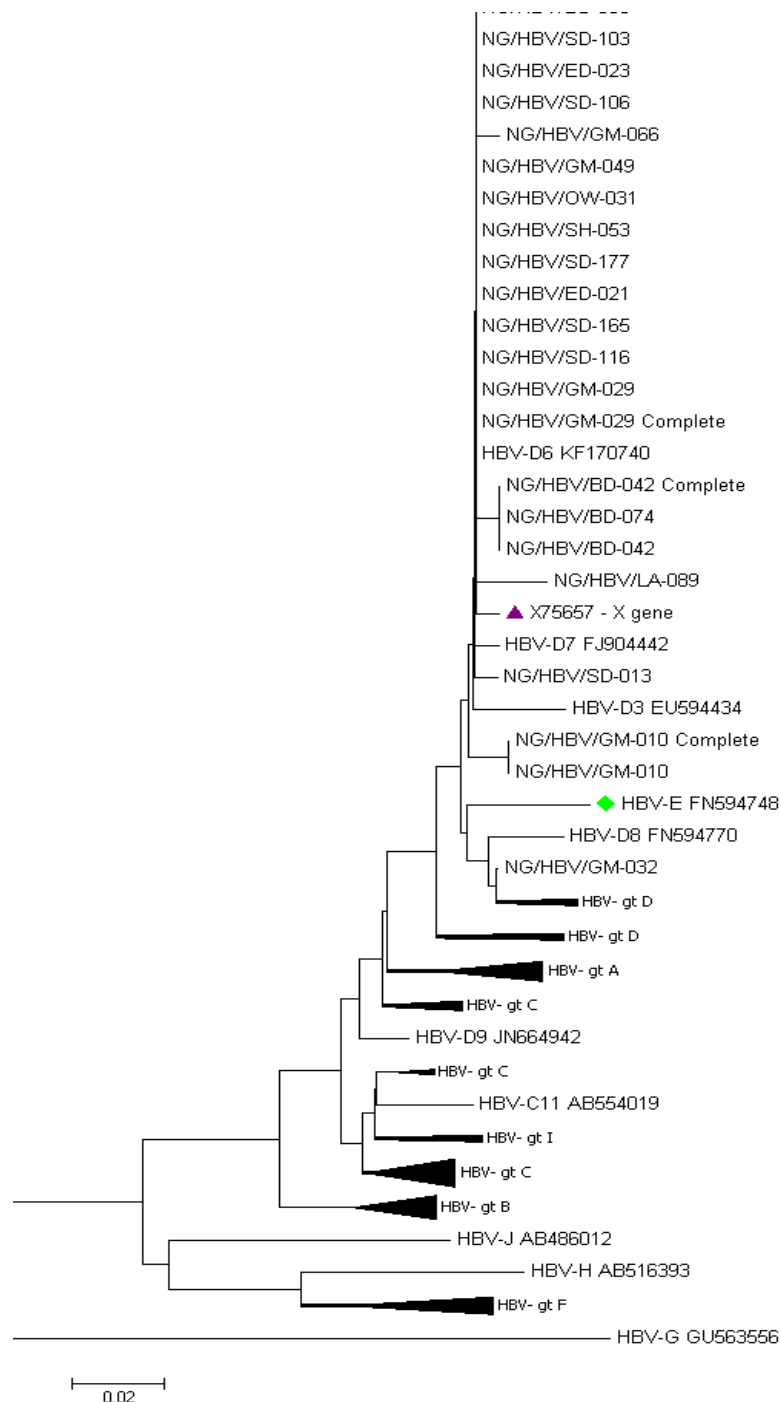


Figure 1: Phylogenetic tree showing the HBV X gene isolates from SW Nigeria and other HBV reference strains. The alignment was done on Geneious 11 and the tree was constructed using the Neighbour joining method in MEGA 7.0.26. Reference sequences were obtained from the GenBank database. The bar indicates the variation scale.

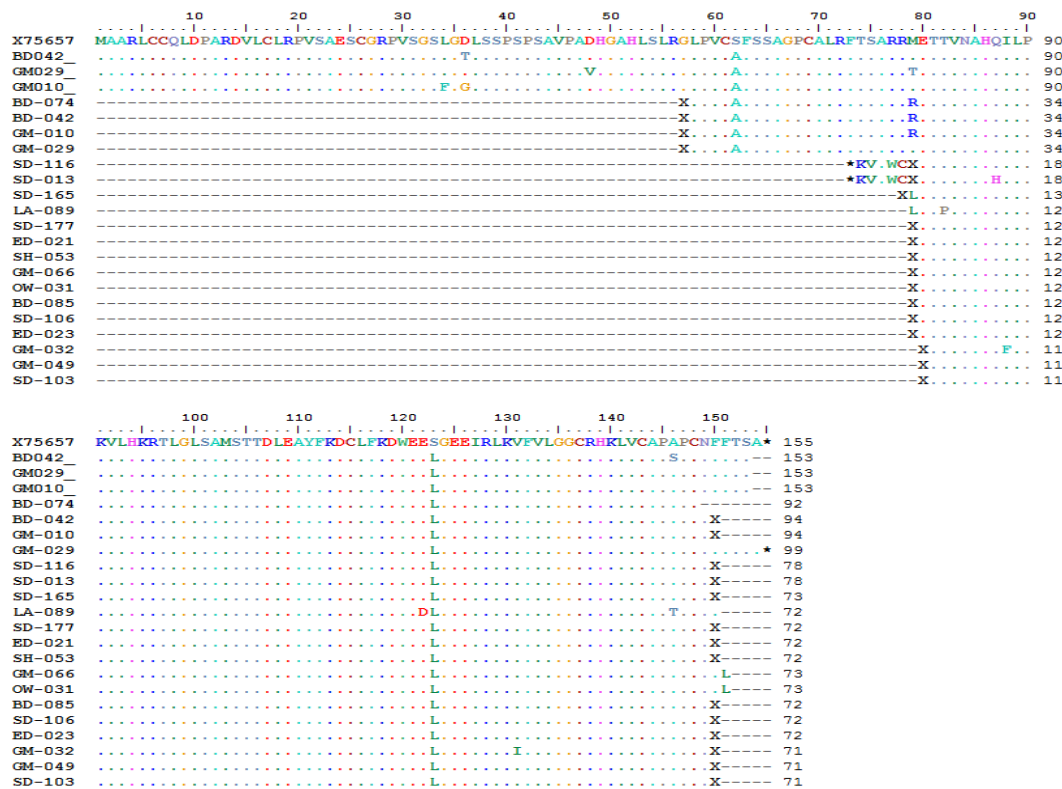


Figure 2: HBV X ORF from the study aligned with a reference X gene sequence - X75657 showing points of mutation on the X gene

Note: The first 3 samples BD042, GM029 and GM010 are 459 bp while their second appearances are 210 bp

In all, 22 different mutations were observed in the study. While some were alone, others were in combination with other types. However, S123L mutation was observed in all the isolates while mutations thus depicting no sample with single mutation. The I88F and V131I, whose roles

in the pathogenesis of hepatitis B have been reported were observed in one while a number of others whose significance have not been identified were also observed. Other observed mutations are shown in Table 1.

Table 1: Mutations observed on the HBV X ORF

X ORF mutations	Occurrence of each mutation (%) (N = 22)
L34F, D36T, D36G, D48V, M79T, M79R, M79L, T82P, Q87H, I88F, E122D, V131I, A146S, A146T	1 (4.5)
F73*, T74K, T74K, S75V, R77W, R78C	2 (9.1)
S62A	4 (18.2)
S123L	22 (100)

Multiple mutations whose combinations have not yet been reported to be associated with advanced liver diseases and predisposing to HCC were also

observed in seven (7/19; 36.6%). More details are shown in Table 2.

Table 2: Combinations of mutations observed on the X ORF

Combined mutations	Frequency (%) (N=19)
L34F + D36G + S62A + S123L	1 (5.3)
D36T + S62A + A146S + S123L	1 (5.3)
D48V + S62A + M79T + S123L	1 (5.3)

M79L + T82P + E122D + A146T + S123L	1 (5.3)
I88F + V131I + S123L	1 (5.3)
F73* + T74K + S75V + R77W + R78C + S123L	2 (10.5)
F73* + T74K + S75V + R77W + R78C + Q87H + S123L	1 (5.3)
S62A + M79R + S123L	1 (5.3)

Of the participants with observed HBx mutations, 79% (n=15/19) were females with two (13.3%) aged between 11 - 20 years. Also, 63.2% (n=12/19)

of the HBx mutant carriers were asymptomatic. Other details are shown in Table 3.

Table 3: Description of individuals with HBx mutations

Sample codes	State	Health status	Sex	Pregnancy
SD-116	Osun	Apparently healthy	Female	No
SD-013	Osun	Apparently healthy	Female	No
SD-165	Osun	Apparently healthy	Male	No
SD-177	Osun	Sick	Female	No
SD-103	Osun	Sick	Male	No
SD-106	Osun	Sick	Female	No
ED-021	Osun	Apparently healthy	Female	Yes
ED-023	Osun	Apparently healthy	Female	No
LA-089	Lagos	Apparently healthy	Female	Yes
SH-053	Oyo	Sick	Female	Yes
OW-031	Oyo	Apparently healthy	Female	Yes
GM-066	Oyo	Sick	Female	No
GM-049	Oyo	Apparently healthy	Female	No
GM-032	Oyo	Sick	Female	No
GM-010	Oyo	Apparently healthy	Male	No
GM-029	Oyo	Sick	Male	No
BD-042	Ekiti	Apparently healthy	Female	Yes
BD-074	Ekiti	Apparently healthy	Female	Yes
BD-085	Ekiti	Apparently healthy	Female	Yes

DISCUSSION

The error-prone reverse transcription process during HBV replication predisposes the genome, especially HBx to point mutations, insertions and deletions. These mutations have been found to alter viral replication capacity, disease progression, and response to antiviral therapies (Akram *et al.*, 2022). There is dearth of studies on HBx protein in Nigeria in comparison to what obtains in some other parts of the world. It is also observed that data about the prevalence of HCC and the roles of HBx in the condition is scarce in Nigeria and, more precisely, in the southwest of Nigeria. It is expedient to give more attention to this if the vision of the WHO to eradicate viral hepatitis by 2030 will be actualized. Hepatitis B virus X ORF mutation, xV131I, found to be associated with hepatocellular carcinoma (HCC), was reported from one hepatitis-treatment naive single female in Oyo State in this study. The risks associated with

this are quite clear and the calls go to both researchers and the Government to rise to the rescue of likely many of such in the population. Although some other mutations, apart from the known xV131I whose role in hepatitis B pathogenesis are not known, were observed in this study, it is worthy of note that studying these and other mutations observable in the prevalent HBV genotype in the region will be of help to the WHO goal of viral hepatitis eradication. The involvement of HBx mutations in HCC has been established by several studies: Yeh *et al.* (2000) reported that HBx codon 31 was detected more frequently in Taiwanese patients with HCC; Muroyama *et al.* (2006) reported that an HBx codon 38 change in genotype C is an independent risk factor for HCC development; Shinkai *et al.* (2007) reported that, regardless of HBeAg status, the T1653 mutation increases the risk of HCC in Japanese patients with HBV sub-genotype C2 and Choi *et al.* (2009)

reported that only the B1499 (C or G or T1499) mutation was significantly associated with HCC in hepatitis B patients with cirrhosis. Lee *et al.* (2011) have attributed the lack of established consensus on or mechanism of which HBx mutation or deletion directly affects the pathogenesis of HCC to differences in HBV genotype, the level of HBV DNA, coinfection of other viruses, and effects of host factors. Much of these have not been studied in HBV genotype E that is prevalent in West Africa.

Much attention has been given to the genetic variability of the PreS/S, PreC/C and Pol genetic regions of HBV due to their significance in diagnostics, immune evasion, vaccine development, immune activation functions and drug resistance whereas information on the clinical impact of HBx genetic variability was adjudged scanty (Kim *et al.*, 2008), and till date, not much improvement has been seen especially in genotype E which is restricted to West Africa. Although this least studied HBV ORF is the smallest of the four ORFs expressing a 154 amino acid multifunctional molecule (HBx), with an N-terminal negative regulatory/antiapoptotic and a C terminal proapoptotic/transactivation domain, it has widely been associated with modulation of a wide range of normal cell functions, leading to hepatocellular carcinoma (HCC) (Schuster *et al.*, 2002; Bouchard and Schneider, 2004; Tang *et al.*, 2006). This HBV non-structural X protein (HBx) is a key regulatory protein of the virus that is at the intersection of HBV infection, replication, pathogenesis, and possibly carcinogenesis. Datta *et al.* (2007) demonstrated that HBx genetic variability can enlighten the differential oncogenic potential of HBV genotype.

Four promoters, *viz* preS1, preS2, core and X, and two enhancers I and II (EnhI and EnhII) in the HBV genome have been known to control the transcription of HBV proteins. These promoters and enhancers overlap with the ORFs thus making the mutations that arise from them to be crucial to the fidelity of the virus. As at date, of crucial note is the A1762T/ G1764A BCP mutation which leads to two overlapped HBx amino acid changes, K130M and V131I which is the most frequent HBV DNA mutation identified in many studies as being associated with HCC risk and outcomes (Datta *et al.*, 2008; Zhu *et al.*, 2010; Muñoz *et al.*, 2011; Lee *et al.*, 2011; Chen *et al.*, 2014). This

mutation can cause a substantial decrease in HBeAg expression and enhancement of viral genome replication, which contribute to the liver disease progression *via* increased inflammation and viral invasion (Buckwold *et al.*, 1996; Hakami *et al.*, 2013). The mutation also leads to a truncated HBx protein, which not only promotes hepatocellular proliferation but also enhances HCC cell invasion and metastasis (Yeh *et al.*, 2010; Chen *et al.*, 2012).

It is already established that HBx is a multifunctional protein composed of 154 amino acids (aa), characterized by an N-terminal negative regulatory region (residues 1–50) and a C-terminal transactivation domain (residues 51–154). Mutations in these regulatory regions can lead to amino acid substitution which can alter the structure and hence the function of the HBx (Datta *et al.*, 2008). Worthy of note is that 17 of the 21 (81%) mutations observed in this study occurred at the highly conserved C-terminal transactivation domain. Salamia *et al.*, (2017) compared the mutations in HBX ORF of some CHB with cirrhotic cases and found that the frequencies of deletion and insertion mutations in C-terminal of X-gene were more seen in cirrhotic patients than CHB cases.

Three mutations L34F, D36T and D36G (14.3%) were observed in the Proline -Serine Rich (PSR) domain of the N-terminus of the HBx. Amino acids 30 - 46 fall into this region. Mutations on this disordered unstructured region of the HBx pose a great risk of causing cirrhosis and liver cancer as such can enhance the persistence and immunoevasion of the virus in chronic carriers. The unstructured nature of the N-terminus makes it flexible to bind with different interactors (Prescott *et al.*, 2019). This unstructured region and the consequent inconsistent nature is one of the reasons why understanding the structure of the HBx is not properly understood. Better understanding of this region could open the door for the discovery of antivirals that target the HBx thus preventing the unfavourable consequences of mutations such as viral persistence and tumorigenesis.

Although, much has been reported about the role of this potentially oncogenic protein, the more precise roles of the HBx mutations on chronicity and carcinogenesis needs more attention especially

in sub-Saharan Africa where genotype E predominates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHORS' CONTRIBUTIONS

AOA - Conceptualization of the idea, sample collection and analyses, writing of the original report

OAO - Supervision of the project, report review and editing:

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