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Article

Prevalence of Hepatitis B Virus Mutations Associated with Hepatocellular Carcinoma in HBV and HIV Co-infected Adults in Botswana

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Abstract: Mutations within the hepatitis B virus (HBV) genome have been associated with rapid progression to hepatocellular carcinoma (HCC); however, there is limited information regarding the prevalence and impact of these mutations in most of sub-Saharan Africa, including Botswana. We aimed to determine the prevalence of HBV mutations known to be associated with progression to HCC using a retrospective, cross-sectional analysis of 48 previously generated HBV sequences from adults with concomitant HBV/HIV initiating HIV antiretroviral therapy in Botswana. The sequences were aligned with reference sequences, and HCC-associated mutations were manually identified using BioEdit. Sixteen (33.3 %) of 48 participant samples had 20 HCC-associated mutations. Seven HCC mutations were present in the core region, 4 in the preCore region, 7 in the X region, and one mutation in the surface region, as well as deletions within the preSurface 1 region. Seven of the 16 participants (43.8%) had multiple HCC-associated mutations. There were also previously uncharacterized mutations at positions with known HCC-associated mutations. HCC-associated mutations were common in this cohort; hence, some participants may require close clinical monitoring as they might be more prone to rapid disease progression. Other functionally uncharacterized polymorphisms were also detected and require characterization in future studies.

Keywords: hepatocellular carcinoma (HCC); hepatitis B virus (HBV); mutations; HBV/HIV co-infection; Botswana; Africa

1. Introduction

Hepatitis B virus (HBV) is a significant global health problem which results in approximately 820,000 deaths per annum due to hepatocellular carcinoma (HCC) and cirrhosis [1]. HBV is endemic in Africa and Western Pacific, with prevalence rates of ~6% among adults [1]. In Botswana, the HBV prevalence ranges from 3.1% to 10.6% in people with human immunodeficiency virus (HIV) (PWH) [2-5]. In HIV-uninfected pregnant

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women and blood donors, the HBV prevalence was 1.1% and 1.0%, respectively, in Botswana [6,7].

HCC is the fifth most common cancer in men and the second leading cause of cancer deaths globally [8]. Regions with the highest HCC incidence in the world are East and Southeast Asia, and sub-Saharan Africa, with incidence rates of >20 per 100,000 individuals [9,10]. HCC has also been reported in people with occult HBV (OBI) [11-13]. OBI is the presence of HBV DNA in serum or liver of participants who are negative for HBV surface antigen (HBsAg) [14]. Studies have reported an increase in HCC incidence among PWH, resulting in accelerated progression of liver disease to HCC [15,16]. The reason for this high HCC incidence among PWH remains unclear. There is limited data on whether HIV directly accelerates HCC pathogenesis or whether the rising incidence is a secondary effect of highly active antiretroviral therapy (HAART) for HIV [17]. A risk factor for high HCC incidence and mortality among PWH is coinfection with chronic hepatitis B (CHB), as well as lack of biomarkers for early detection and limited treatment options for late-stage HCC [18]. Early diagnosis and surgical resection remain the key to possible curative treatment. Nonetheless, most HCC participants present with late-stage tumors and have poor prognosis especially in resource limited settings [19]

The HBV genome consists of four overlapping open reading frames (ORFs) [20]. The longest ORF is the polymerase with 832 amino acids (aa) long, which codes for the polymerase/reverse transcriptase (Pol/RT) [20]. The second largest ORF is the pre-surface1/pre-surface2/surface (preS1/preS2/S), which codes for the large, middle and small envelope proteins respectively, collectively forming the HBsAg [20]. The third ORF is Precore/Core (preC/C), which codes for HBV e antigen (HBeAg) and HBV core antigen (HBcAg), respectively [20]. The shortest ORF codes for the X protein (HBx), a 154 aa multifunctional protein [20]. Several mutations have been reported to lead to a rapid progression of liver disease to HCC [21-26]. Mutations T1762/A1764 and A1896 found in the basal core promoter (BCP) and preC region respectively, have been associated with HBeAg seroconversion and persistent viral replication [27]. PreC/C mutations that occur within the epitopes restricted to major histocompatibility complex (MHC) class II molecules are the major drivers of rapid HCC progression [21].

Studies have reported HBV prevalence and drug resistance mutations among different populations in Botswana [6,28-34], but no study has determined the prevalence of HCC-associated HBV mutations in HBV/HIV co-infection. Therefore, we sought to determine the prevalence of the known HCC-associated mutations among HBV/HIV co-infected individuals in Botswana, as well as their association with markers of HBV disease progression.

2. Materials and Methods

2.1. Study participants

This was a retrospective, cross-sectional study utilizing previously generated HBV sequences from the Bomolemo study conducted between 2009 and 2012 in Botswana [35]. Briefly, the Bomolemo study evaluated the efficacy and tolerability of tenofovir and emtricitabine as the nucleoside reverse transcriptase inhibitor (NRTI) backbone for first-line HAART in adults in Botswana. The study participants were treatment-naïve PWH initiating HAART and aged 18 years and older. Additional eligibility criteria included the presence of an AIDS-defining condition and a CD4+ T-cell count <250 cells/μL per the national guidelines at the time. Female participants were excluded if they were pregnant or had received single-dose nevirapine for prevention of perinatal transmission within the 6 months preceding enrollment. After study entry and HAART initiation, participants were scheduled for evaluations at one month and then every three months thereafter until the final study visit at week 96.

This study utilized near full-length HBV genome sequences previously generated from 48 participants – accession numbers MH464807 to MH464856 [34]. Twenty-four

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belong to subgenotype A1 and the other 24 belonged to subgenotype D3. There was an equal number of CHB and OBI per subgenotype (12 each) [34].

2.2 Ethical considerations

The study was conducted at Botswana Harvard HIV Reference Laboratory (BHHRL), and study participants provided written informed consent for the parent Bomolemo study, as well as for the storage of residual samples. The study was approved by the ethics review committee of the University of Botswana and the Health Research Development Committee (HRDC) at the Botswana Ministry of Health and Wellness.

2.3 Mutational analysis

HBV genotypes A and D sequences from Botswana and the corresponding reference sequences were retrieved from National Center for Biotechnology Information (NCBI) GenBank. Multiple sequence alignments (MSAs) for both genotype A and D were generated using AliView (version 1.18.1) [36]. Babylon Translator tool (http://hvdr.bioinf.wits.ac.za/babylon/) was used to extract all the seven HBV proteins [37]. Multiple sequence alignments from each ORF were stratified by genotype, and a custom Python script was used to curate all mutations. Mutation profiles were compared to the known HCC-associated mutations listed in Table 1 including those reviewed by Kim *et al*. [21]. Drug resistance mutations (DRMs) were also confirmed using online tools, geno2pheno (https://hbv.geno2pheno.org) and HBVseq embedded in Stanford Drug resistance database (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html). Mutations in known immunodominant areas of the HBV core region were also searched for as they have been linked with disease progression [24].

2.4 Statistical analysis

To determine liver injury, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and AST platelet ratio index (APRI)was calculated as 100 × (AST/40) / platelet (PLT), while the FIB-4 value was calculated as age [years] × AST [IU/L] / √ (PLT [10^9/L] × (ALT [IU/L]) [38]. The Wilcoxon rank-sum test was used to compare continuous variables between participants with and without HCC-associated mutations. Chi-squared test was used to compare categorical data. Statistical analysis was performed using STATA v 14.0 with p-values < 0.05 considered statistically significant.

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Table 1. Functionally characterized HCC-associated mutations

ORF	Mutation	Descriptions and biological impact of mutation [Reference]	Genotype/ subgenotype
X	V5M	Associated with disease progression [22,39,40]	C
	T36A/S/P	Enhances viral genome integration into the host cell resulting in insertion mutations and a 3′-terminal truncation of HBx [41]	A, C,D
	P38S	Risk factor for HCC [42]	B/C
	A44V	Located in B epitope [43]	
	G50R	Associated with HCC [44]	A/D
	H94Y	Modulates the transactivation property of HBx that is known to be associated with hepatocarcinogenesis [45]	B/C
	S101*	Located in enhancer region [46]	B/C
	S106T	Located in enhancer region [47]	C2
	L/V116V/L	Survival after operation in HCC participants, located in enhancer region, downregulation of glycogen synthase kinase 3 beta [41,47]	NS
	L123S	Located in enhancer region [48]	D1
	I127T/L/N/S	Increases transactivation activity in genotype B [27,40,42,43,47,49-52]	A, B, C and D
	K130M	Upregulated the HIF-1α expression and transcriptional activity [43,53,54]	A, B, C and D
	V131I	Upregulates the HIF-1α expression and transcriptional activity [43,53]	B and C
	C143R	Located in the core promoter [41]	C
Precore	M1L	Leads to a failure of HBeAg production [55]	A
	W28*	It is a stop codon in the HBeAg sequence which halts protein synthesis [23]	A and D
	V17F	V17F is responsible for the HBeAg-negative serostatus and much lower viremia titers in participants infected with HBV genotype A [56]	D
	G29D	Associated with HBeAg serostatus [57,58]	D
Core	P5T/L/H /R	Activates endo reticulum stress [57,58]	A, C, D
	F24Y	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	P50A/H	Associated with HBeAg serostatus in chronic participants infected with subgenotype C2 [24]	A
	E64D	Reduces T-cell proliferation in vitro when it occurred with mutation T67N [21]	A
	L65V	Uncharacterized	A
	T67N	Reduces T-cell proliferation in vitro when it occurred with mutation E64D [59]	A
	E77Q/D	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	A80I/T/V	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	E83D	Associated with progression to HCC [57]	C
	V91S/T	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	I97F/L	Associated with progression to HCC by immune evasion [57,60,61]	C
	L100I	Associated with progression to HCC [57]	C
	L116I	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	P130Q/T	Predictor of HCC [58,62]	C, D
	A131P	Associated with HBeAg serostatus in chronic participants infected with subgenotype C2 [[21]	A
	T147C	Predictor of HCC [55]	D
	E180A	Associated with progression to HCC and cirrhosis [24]	A, B, C, D, E
	Q182*/K	Significantly associated with progression to HCC [57]	C
	Start codon mutations (M1 V/T/I /A)	Associated with increased risk of HCC [25,63]	B/C
	C7A	Associated with increased risk of HCC [64,65]	C
PreS2	F 22 L	Associated with increased risk of HCC [63]	B/C
	Deletions	Associated with increased risk of HCC [63]	B/C
	T53C	Associated with increased risk of HCC [65]	C
	F141L	Increase cell proliferation and transformation [66]	B, C
	W4P/R	Increases progression to HCC [25,67]	B, C
	K7T/N	Increases progression to HCC [25]	B, C
PreS1	Q10L	Associated with HCC [68,69]	C

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S	A81T	Increases progression to HCC [25]	B, C
	S98T	Predictor of HCC [62]	D
	W36L	Reduce HBsAg secretion and promotes cell apoptosis. [26,70-72]	C
	T47K,	Reduce HBsAg secretion and promotes cell apoptosis [70,71]	C
	N52D	Reduce HBsAg secretion and promotes cell apoptosis [70,71]	C
	W74L	Reduces HBsAg secretion [71,73]	C
	L77R	Reduces HBsAg secretion [71,73]	C
	sL95*	Increased cell proliferation and transformation [26]	NS
	sW172*	Promotes retention of HBsAg [74]	NS
	sW182*	Increased cell proliferation and transformation [26,35]	C
	V184A	Reduce HBsAg secretion and promotes cell apoptosis [[70,71]	C
Polymerase	P203Q	Promotes retention of HBsAg and cell proliferation [72]	D, A
	S210R	Promotes retention of HBsAg and cell proliferation [72]	D, A
	sL216*	Increased cell proliferation and transformation [26]	C
	sF220L	Reduce HBsAg secretion and promotes cell apoptosis [70,71]	C
	sW172*	Increases risk of HCC [74,75]	B, C
	rtA181T	Increases risk of HCC [74,75]	B, C

NS = Not specified; * = stop codon

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3. Results

3.1. Participant baseline demographics

Sixteen of 48 participants (33.3% [CI: 21.7 – 47.5]) had HCC-associated mutations. Twelve participants with HCC-associated mutations were HBsAg positive, while 4 were OBI positive. There were no statistically significant differences between participants with HCC-associated mutations and those without HCC-associated mutations with respect to all demographics except chronic versus occult HBV status. Participants with HCC-associated mutations mostly had CHB compared to participants without HCC-associated mutations (p = 0.030) (Table 2). Among participants that had HCC-associated mutations, there was no association between age and HBV subgenotype (p=0.135, Fisher's exact test). There was also no association between the number of mutations (single versus multiple) and age category (≤35years versus >35 years) (p=0.358, Fisher's exact test).

Table 2. Participant baseline demographics.

Characteristic	With HCC-associated mutations N = 16	Without HCC-associated mutations N = 32	P-value
Gender, N (%)			
Female	10 (62.5)	24 (75.0)	0.503
Male	6 (37.5)	8 (25.0)	
HBV phenotype, N (%)			
CHB	12 (75.0)	12 (37.5)	0.030*
OBI	4 (25.0)	20 (62.5)	
HBV sub-genotype, N (%)			
A1	9 (56.3)	15 (46.9)	0.760
D3	7 (43.7)	17 (53.1)	
Age, years, median (IQR)	36 (32 – 39)	34 (31 – 41)	0.974
Age categories, years			
≤35	7 (43.7)	18 (56.3)	
>35	9 (56.3)	14 (43.8)	0.543
BMI, median (IQR), n = 37	19.8 (18.9 – 21.1)	21.1 (19.9 – 23.2)	0.194
Haemoglobin, g/dL, median (IQR), n = 46	11.5 (10.1 – 13.0)	10.7 (8.8 – 12.3)	0.297
CD4 cells/mm³, median (IQR), n = 45	248 (84 – 305)	190 (112 – 238)	0.209
Log HIV VL, copies/mL, median (IQR), n = 44	4.7 (4.5 – 5.0)	5.5 (4.5 – 5.8)	0.126
Log HBV VL, IU/mL, n = 44	3.1 (1.4 – 4.7)	1.4 (1.3 – 3.0)	0.192
HBV VL category, n = 44			
<20,000	8 (57.1)	26 (86.7)	0.051
≥20,000	6 (42.9)	4 (13.3)	
Platelet count, mcL, median (IQR), n = 46	241 (210 – 300)	244 (188 – 280)	0.519
ALP, IU/mL, median (IQR), n = 33	84.40 (68.16 – 101.34)	67.13 (57.28 – 105.66)	0.428
Albumin, g/dL, median (IQR), n = 33	38.43 (36.53 – 40.55)	37.45 (32.66 – 41.97)	0.524
ALT, IU/L, median (IQR), n = 45	27.4 (18.1 – 35.7)	17.5 (12.8 – 23.6)	0.120
AST, IU/L median (IQR), n = 45	33.5 (22.4 – 43.8)	26.9 (21.4 – 33.8)	0.459
APRI, median (IQR), n = 45	0.34 (0.17 – 0.53)	0.29 (0.23 – 0.35)	0.933
FIB-4, median (IQR)	1.23 (0.53 –1.42)	1.01 (0.78 –1.26)	0.725

Abbreviation: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; BMI, body mass index; ALP, alkaline phosphatase; IQR, interquartile range; HIV, human immunodeficiency virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; APRI, AST to platelet ratio index; FIB-4, Fibrosis-4 index

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3.2. Mutation profiling

Mutational analysis identified 20 HCC-associated mutations. Eight HCC-associated mutations were observed in subgenotype A1 participants, six mutations in subgenotype D3 participants, and six mutations occurred in both subgenotypes as shown in Table 3. Seven mutations –

P50A, P50H, E64D, L65V, T67N, E77D, and A131P – were present in the core region. Four mutations – M1L, V17F, W28Stop, G29D – were present in the preCore region while 7 mutations T36A, H94Y, I127T/L/R, K130M, and V131I were in the X region. There were no known HCC mutations in the PreS2 region, whereas one patient had deletions (position 92-119) in PreS1, and one patient had V184A mutation in the surface region which had been associated with HCC [71]. The most common mutations were K130M and V131I which occurred in 5 participants each. They appeared as dual mutations in 4 of these 5. The second most common mutations were E64D and A131P occurring in 4 participants each followed by L65V and I127L which were each found in 3 participants each. Seven of the 16 participants (43.8%) with HCC-associated mutations had multiple mutations (Table 3, Figure 1). There were uncharacterized mutations in positions with known HCC-associated mutations in the core region, (E64K, A80G, V91L, I116L) and X region (L5V, S101P, L116V). The I116L was the most common, occurring in 3 participants. Uncharacterized mutations in positions with known HCC-associated mutations in the PreS2 region were C7T, F22T/P/H, and A53V. The mutations within Pres2 position 22 occurred in 4 participants. A53V occurred in combination with R48T. In the PreS1 region, two participants had an uncharacterized mutation (K10N) in a position with known HCC-associated mutations. The distribution of the mutations by genotype and HBV type are shown in Table 3.

Table 3. Distribution of HCC-associated mutations in participants.

	HBV Clinical Outcomes				HCC-associated mutations in HBV ORFs				
ID	**HBV Type**	**Genotype**	**HBeAg status**	**HBV VL (IU/mL)**	**Core**	**PreCore**	**X**	**S**	**PreS1/PreS2**
1	OBI	D3	NT	57.4	P50H	-	-	-	-
2	CHB	A1	NEG	<20	P50A, A131P, L65V, T67N	-	V131I	-	Deletions (position 92-119)
3	CHB	D3	NEG	1,420	A131P	-	H94Y, K130M, V131I	-	-
4	OBI	A1	NT	<20	A131P	-	-	-	-
5	CHB	A1	NEG	2,660,000	A131P	W28*	-	-	-
6	CHB	D3	NEG	21,900	E64D, E77D	W28*, G29D	H94Y, K130M, V131I, I127R	-	-
7	CHB	A1	NEG	48,700	E64D, L65V, T67N, E77D	-	I127T, K130M, V131I	-	-
8	CHB	A1	POS	41,300	E64D, E77D	-	K130M, V131I	V184A	-
9	CHB	A1	NEG	871	E64D, L65V,	-	-	-	-
10	CHB	A1	NEG	TND	-	M1L	-	-	-
11	CHB	D3	NT	TND	-	V17F	-	-	-
12	OBI	A1	NT	<20	-	-	T36A	-	-
13	CHB	A1	POS	>170,000,000	-	-	K130M	-	-
14	CHB	D3	-	-	-	-	I127L	-	-
15	OBI	D3	-	-	-	-	I127L	-	-
16	CHB	D3	-	-	-	-	I127L	-	-

Abbreviations: HBV: hepatitis B virus; OBI: occult hepatitis B infections; CHB: chronic hepatitis B infections; HBeAg: hepatitis B e antigen; DNA: deoxyribonucleic acid; NT: not tested; VL: viral load; TND: target not detected; IU/mL: International Units per milliliter; Neg: Negative; Pos: Positive; * = stop codon.

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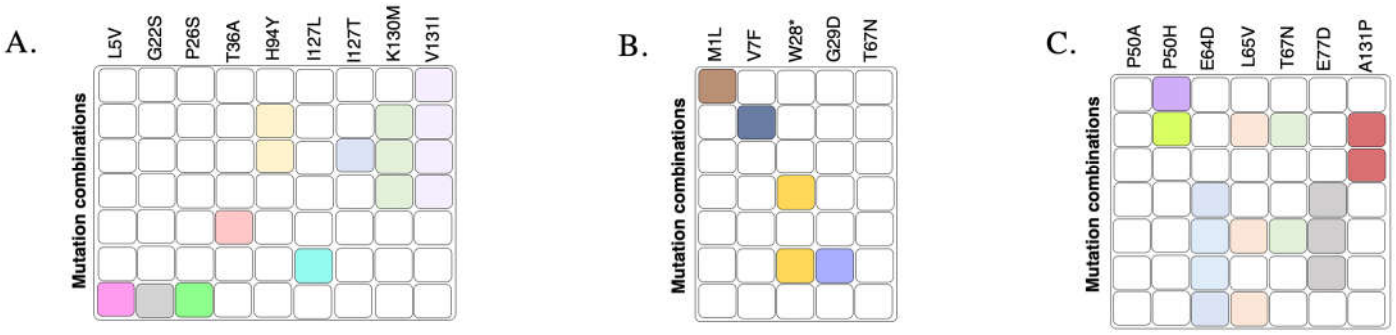

Figure 1. HCC-associated mutations arranged by regions irrespective of HBV genotype. The vertical columns of coloured squares represent the mutation within the different ORFs. The rows represent mutation combinations as they appear in the 16 patients in Table 3. While others can occur along such as V131 in X region, majority co-existence as combinations of 2 mutations and above. **A)** Combination of HCC-associated mutations in X region**. B)** Combination of HCC-associated mutations in Precore region. **C)** Combination of HCC-associated mutations in core region.

3.3. Mutations in the immunodominant areas of core region.

Several mutations within immunodominant areas have been linked with faster disease progression [24]. Nineteen participants (39.6%) had mutations in the immunodominant areas of the core region. Eleven (57.9%) of the participants with mutations in immunodominant areas also had HCC-associated mutations. Three of 4 OBI participants with HCC-associated mutations also had mutations in immunodominant areas. There were 50 mutations in immunodominant areas of core region. 20 (40%) were in T helper cells epitopes. 18 (36%) were in B cells epitopes, and 12 (24%) were in $CD8^+$ CTL epitopes. Most of the participants 12 (63.2%) were infected with HBV subgenotype D3 (Table 4).

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Table 4. Distribution of mutations in immunodominant areas of core region.

HBV Type	Genotype	T_h (HBcAg 35–45)	T_h (HBcAg 48–69)	B-Cell (HBcAg 7689)	B-Cell (HBcAg 105 - 116)	B Cell (130-135)	CTL ($CD8^+$) (HBcAg 18–27)	CTL ($CD8^+$) (HBcAg 74–83)	CTL ($CD8^+$) (HBcAg 141–151)
OBI	A	A41S	-	-	-	P130Q, Y132P	-	-	R151C
CHB	A	S35A	C48G, S49T, E64D, L65V, T67N	E77D	E113D	-	-	N74S	R151C
CHB	A	A41S	-	-	-	Y132P	-	-	R151C
OBI	A	S35A, P45S	I59L	-	-	-	S26P	-	-
CHB	A	-	C48G, E64D, L65V	-	-	-		N74T	-
CHB	A	-	E64D	E77D	-	-	S21P	-	-
CHB	A	-	P50A, L65V, M66T, T67N	L84Q,	I105V, L108I, T109C	Y132P, P135Q	-	-	T142I, T147S, R151C
OBI	D	E40D	I59V	-	-	P130Q	-	V74A	
OBI	D	E40D	I59V	-	-	P130Q	-	V74A	V149I
OBI	D	-	E64K	-	-	-	-	-	-
OBI	D	-	P50H	-	I116L	-	-	-	-
CHB	D	-	-	S87G	-	-	-	-	-
CHB	D	-	-	V85G	-	-	-	-	-
OBI	D	-	-	-	I105V	-	-	-	-
CHB	D	Y38F	-	V85G	I116L	A131P	-	V74A	T147C
CHB	D	S35K, E4OD	E64D, M66I, A69S	E77D, P79Q, A80G, L84R	T109M	-	S21A	-	-
OBI	D	-	-	S87N	I116L	-	-	V74N	-
CHB	D	-	C48S	-	-	-	-	-	-
CHB	D	-	-	-	-	-	-	V74A	-

Abbreviations: HBV: hepatitis B virus; OBI: occult hepatitis B infections; CHB: chronic hepatitis B infections; CTL: cytotoxic T lymphocytes

3.4. Mutations in participants with coexistence of HBsAg and anti-HBs

Coexistence of HBsAg and anti-HBs has been linked with faster disease progression. There were 3 participants with HBsAg and anti-HBs in this study. None of the 3 participants had mutations associated with HCC or faster disease progression.

4. Discussion

This is the first study to report on the prevalence of HBV mutations linked to HCC and disease progression in Botswana. The present study analyzed all HBV ORFs for the mutations associated with the rapid progression of liver disease to HCC among people with concomitant HBV/HIV. We report a 33.3% prevalence of HCC-associated mutations among people with concomitant HBV/HIV. Most studies on HCC-associated mutations worldwide have not reported their prevalence rates; hence more studies focusing on the prevalence of HCC-associated mutations should be conducted in different risk groups.

Twenty HCC-associated mutations were detected in our study, with eight of those mutations occurring in subgenotype A1 participants, six in subgenotype D3, and 6 occurring in both subgenotypes. Genotypes affect the severity of HCC-associated

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mutations, with genotype C and D mutations more likely to lead to cirrhosis and HCC than those caused by genotypes A and B [76]. In our study, the number of HCC-associated mutations did not differ by subgenotype. The difference might be because studies used patients with HCC while the current study is using HCC-associated mutations as a proxy as the participants did not have HCC [77,78]. In our study, there were significantly more CHB participants with HCC when compared to OBI participants as reported previously in the literature [79]. The presence of HCC-associated mutations in some OBI participants also agrees with previous reports [79,80].

There was no difference in the number of HCC-associated mutations by region in our study. Some studies reported that HCC-associated mutations are more common in the PreC/C region than in other genomic regions [21]. The difference in the observations might be because there were fewer PreC regions analyzed compared to the core and X regions. HCC-associated mutations in the core region also show that E64D and A131P had the highest frequency, occurring in 4 participants each followed by L65V occurring in 3 participants. These mutations in the BCP region are caused by deletions or insertions in the C-terminal leading to severe clinical outcomes in chronic participants. Previously, a study of HBV core variability among HAART participants, as in our study, suggested that the presence of the T67N mutation significantly reduced T-cell proliferation in vitro when it occurred with mutation E64D [59]. In our study, these mutations occurred both in combination and independent of each other. Other core mutations detected include P50A, P50H, and A131P which have been previously associated with HBeAg negative status in participants with subgenotype C2 [21]. Indeed, all participants with these mutations and available HBeAg results were HBeAg-negative in our study. E77D mutation was reported in 3 participants and has previously been linked with disease progression [24]. In our study, it appeared as a double mutation with E64D suggesting a possible synergistic action. Mutations A80/I/T/V, V91S/T, and L116I have been linked with disease progression [24]. In our study we reported mutations A80G, V91L, and I116L in the same positions. A80G was also reported in blood donors in Mexico, while I116L was also reported in other studies including in participants with liver cirrhosis [81-83].

The X region in HCC participants has been reported to harbor mutations associated with progression of HBV infection [84]. One participant had subgenotype A1 and 3 HCC-associated mutations (K130M, V131I, I127T) detected from the X region with elevated liver enzyme levels (ALT and AST). The participant also had the highest FIB-4 level among all participants with HCC-associated mutations, which implies significant liver damage. All these factors suggest that this participant is likely to rapidly develop HCC, as reported by Ching et al [84]. Another mutation identified in this study, H94Y, have been linked with hepatocarcinogenesis [85]. We reported T36A from an OBI participant. The mutation promotes HBV genome integration into the host cell [53]. We also identified I127L which has been linked with disease progression to HCC [22]. I127R has also been reported in Iran and plays a vital role in hepatocarcinogenesis [86,87]. There were some mutations in positions with known HCC-associated mutations some of which has been reported elsewhere. Some different mutations in the same position have been linked with the same outcome [21].

Our study identified Precore region mutations. W28* and G29D appeared as a dual mutation detected in one of the participants who had CHB, HBV VL of 21900IU/mL, normal levels of ALT and Fib4 but elevated levels of AST. V17F is responsible for the HBeAg-negative serostatus and much lower viremia titers in participants infected with HBV genotype A [56]. However, in our study, the participant had subgenotype D3 and an HBV viral load below the limit of detection suggesting that this mutation is not genotype-specific. The M1L mutation was also identified in this study, and it has previously been reported to stop HBeAg synthesis [55]. Our results support this as the patient harboring this mutation was HBeAg negative.

Several mutations in the PreS, S, and Pol regions which have been associated with HCC although these regions have not been extensively studied like the X, core, and PreC. In this study, the only HCC-associated mutations identified were V184A in the surface

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region and deletions in the PreS1 region. Lee *et al*. reported that V184A, from an OBI participant infected with genotype C induced endoplasmic reticulum-derived oxidative stress [70]. In this study, however, the V184A was identified in a CHB participant which is similar to other studies [88-90]. Deletions in both the PreS1 and PreS2 have been linked to HCC development. In this study, one participant had deletions (position 92-119) in PreS1 which is within the C terminal as reported in other studies [63]. Deletions in the PreS1 region led to HBsAg retention within the cells and development of ground glass hepatocytes which results in endoplasmic reticulum stress [91,92]. There were several uncharacterized mutations in PreS/S region which were situated in positions with known HCC-associated mutations. Therefore, it is plausible to assume that there might be different mutations associated with HCC in the preS/S regions of genotypes A and D as compared to genotypes B and C. Therefore, this study may highlight a gap in identifying and characterizing disease progression associated mutations in genotypes A and D.

This study had the advantage of assessing all the HBV regions except the Precore for most participants. In this study we reported participants with multiple HCC-associated mutations. Combinations of HCC-associated mutations have been reported to develop gradually during the course of HCC development [93,94]. Multiple mutations have also been linked to development of HCC at a younger age compared to participants with single HCC-associated mutations [93]. In our study, this association was not observed. The main limitation of our study is that HCC-associated mutations were used as proxy for disease progression. Additionally, our study was conducted on participants with concomitant HBV/HIV hence the results might not be generalizable to HIV negative individuals. In addition, there were few sequences analyzed for the preCore region hence the HCC-associated mutations might be underreported in this study.

5. Conclusions

We reported a high prevalence of HCC-associated mutations. As participants with HCC-associated mutations might be more prone to rapid disease progression, they may require closer clinical monitoring. Other polymorphisms were also detected but have not been functionally characterized; thus, future *in vitro* studies on these mutations are warranted.

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