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TP53 R249S mutation, genetic variations in *HBX* and risk of hepatocellular carcinoma in The Gambia

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In regions with high prevalence of chronic hepatitis B virus (HBV) infection and dietary aflatoxin B_1 (AFB_1) exposure, hepatocellular carcinomas (HCCs) often contain *TP53* mutation at codon 249 (*R249S*). Furthermore, a C-terminal truncated HBx protein expressed from hepatocyte integrated HBV is associated with HCC development. This study evaluates the association between *R249S* and *HBX* status in relation to HCC in West African population. *HBX* (complete or 3′-truncated) and *HBS* genes were assessed by PCR in cell-free DNA (CFDNA) from plasma of subjects recruited in a hospital-based case–control study (325 controls, 78 cirrhotic patients and 198 HCC cases) conducted in The Gambia. These samples had been previously analyzed for *R249S* and HBV serological status. Complete *HBX* sequence was frequently detected in CFDNA of HCC-*R249S* positive (77%, 43/56) compared with HCC-*R249S*-negative cases (44%, 22/50). Conversely, the proportion of 3′-truncated *HBX* gene was significantly higher in HCC-*R249S* negative than positive cases (34%, 17/50, compared with 12%, 7/56) ($\chi^2 = 12.12$; $P = 0.002$; distribution of *R249S* negative and positive according to *HBX* status). Occult HBV infection (detected by PCR) was present in 24% of HCC previously considered as negative by HBV serology. Moreover, HBV mutation analysis revealed that double mutation at nucleotides $1762^T/1764^A$ was associated with diagnosis of cirrhosis or HCC {cirrhosis: odds ratio (OR): 9.50 [95% confidence interval (CI) 1.50–60.11]; HCC: OR: 11.29 [95% CI 2.07–61.47]}. These findings suggest that in HCC from The Gambia, complete *HBX* sequences are often associated with the presence of *TP53 R249S* mutation.

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer mortality and morbidity in many parts of the world. Overall, it is the seventh most common cancer globally and the third leading cause of cancer-related deaths (1). Over 80% of HCC occurs in sub-Saharan Africa and Southern and Eastern Asia. In The Gambia, West Africa, incidence rates are 32.84 per 100 000 person-years in men and 14.9 cases per 100 000 in women (2). The main risk factors are chronic hepatitis B virus (HBV), which is endemic in the Gambian population. The proportion of carriers among non-HBV vaccinated Gambians is 15% and aflatoxin B_1 (AFB_1)-contaminated foodstuffs are commonly consumed by >95% of the population (3–5). Other risk factors such as HCV and alcohol are found to play a relatively minor role, particularly in cases arising <50 years of age (4,6). AFB_1 is a mycotoxin produced by the fungus *Aspergillus flavus* that contaminates the staple diet in many tropical areas, in particular after storage under hot and humid conditions. After ingestion and metabolism in the liver, metabolites of AFB_1 can form a DNA adduct at the third base of codon 249 in the *TP53* tumor suppressor gene, inducing a G to T transversion (AGG to AGT, arginine to serine; *R249S*), a mutation that is detected in >75% of HCC from areas with high AFB_1 exposure (7–10).

Chronic HBV infection is the dominant global attributable risk factor for HCC, accounting for 55% of cases worldwide and ≥80% in sub-Saharan Africa and South-East Asia (11). The mechanisms by which HBV causes malignant transformation remain uncertain; however, many studies have highlighted a pathogenic role for HBx, the product of the HBV gene *HBX*, which encodes a 154 amino acid, 17 KDa factor that enhances viral replication and modulates multiple cellular growth signaling pathways (12). One of the molecular hallmarks of HBV-induced HCC is integration of the viral genome into the host cellular genome detected in 80%–90% of these cancers (13,14). Integration of viral DNA may lead to insertional mutagenesis (15). In addition, integration often results in a 3′-terminal truncation of *HBX*, generating a shorter form of the HBx protein that is deleted at the C-terminal region by 20–40 amino acids. Integration is also often accompanied by multiple point mutations in the *HBX* sequence. It has been suggested that both truncation and point mutations may enhance the oncogenic activation processes. In experimental models, the C-truncated HBx proteins were shown to transform immortalized liver cell lines (16) and to interact with the mutant p53 protein p.R249S to alter genetic stability and proliferation of non-transformed hepatocytes (17). Other studies found that the mutant p53 protein p.R249S and HBx formed stable complexes in transformed hepatocytes, further suggesting that the two factors could cooperate in hepatocarcinogenesis occurring in a HBV/AFB_1 etiological setting (18).

The genotypes/subgenotypes of HBV show heterogeneity in their global distributions and these varied strains have differences in their biological properties (19–21). It is now evident that the heterogeneity in this distribution of HBV genotypes/subgenotypes may account not only for differences in the prevalence of HBV mutations in the different populations but may also be responsible for variation in the clinical outcomes of HBV infections and the response to antiviral therapy (22,23).

In this study, we have explored circulating cell-free DNA (CFDNA) from Gambian HCC patients to further address the contribution of *R249S* and *HBX* to cancer risk. Previous studies have shown that plasma or serum of cancer subjects (in particular HCC patients) as well as healthy subjects contains detectable amounts of CFDNA (range: 1–500 ng/ml). This CFDNA often contains mutations and epigenetic alterations identical to those detected in tumor tissues (24). In a case–control study of HCC in The Gambia, we have used CFDNA to demonstrate the presence of *R249S* in up to 40% of HCC patients while low levels of mutant DNA were also detected in non-cancer controls, in particular among high-risk HBV chronic carriers (25,26). Although the molecular mechanisms of release and the stability of CFDNA are still unclear, this resource appears to represent a convenient minimally invasive repository of biomarkers for HCC in regions where liver biopsies are not routinely available. Using CFDNA from the plasma of subjects recruited in a hospital-based case–control study conducted in The Gambia and previously analyzed for *R249S* status and for HBV serological status, we have examined HBV genotype by sequencing the *HBS* gene

Abbreviations: AFB_1, aflatoxin B_1; CFDNA, cell-free DNA; CI, confidence interval; HBsAg, HBV surface antigens; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio; RFLP, restriction fragment length polymorphism; SOMA, short oligonucleotide mass analysis.

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(encoding HBsAg) and we have characterized *HBX* integrity and mutational status.

Materials and methods

Study participants

This study has been conducted using specimens and data from The Gambia Liver Cancer Study, for which design, ethical approval, recruitment procedures and laboratory testing methods have been previously reported (4). Briefly, The Gambia Liver Cancer Study was a hospital-based case–control study in which incident cases of HCC and cirrhosis were recruited from three tertiary hospitals sites in The Gambia from September 1997 to January 2001. The diagnosis of HCC was based on concordant clinical and ultrasonography findings and on serum levels of α-fetoprotein of ≥100 ng/ml. Cirrhosis was diagnosed using clinical parameters and ultrasonography (27). A minority of the cases was confirmed by histopathology of liver biopsies (4). Hospital controls with no clinical evidence for liver disease were frequency matched by age (within 10 years), gender and study site. All study participants provided informed consent and both Gambian and international institutional review boards approved the study protocol.

HBV serology, DNA extraction and quantitation of R249S mutation in CFDNA

Methods and results for HBV serology, DNA extraction and quantitation of *R249S* mutation in CFDNA have been reported previously (4,25,26). Briefly, HBV surface antigens (HBsAg) and HBV e antigens (HBeAg) were determined as markers of chronic HBV carriage or viral replication, respectively, using standard laboratory kits (Murex Diagnostics Ltd, Dartford, UK and Sorin Biomedica Diagnostics, Vercelli, Italy). CFDNA was extracted from 200 µl of plasma using QiAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's blood and body fluid spin protocol. Purified CFDNA was eluted from the QiAmp Silica column with two volumes of water (2 × 50 µl) (PCR-grade, Sigma, St Louis, MO). Quantitation of extracted DNA was performed by fluorimetry using picogreen (Molecular Probes, Eugene, OR). *R249S* was detected using two methods, restriction fragment length polymorphisms (RFLP) (25) or short oligonucleotide mass analysis (SOMA). All samples were analyzed by RFLP and the status of 38% was confirmed by SOMA. The latter method allowed the quantitation of *R249S* against a synthetic, internal standard as described previously (26) and results were considered as '*R249S*-positive' at a cutoff number of 67 copies of mutant DNA/ml. Overall, RFLP and SOMA results were highly concordant.

Analysis of HBX truncation status

Detection of truncated versus complete integrated *HBX* was performed by PCR and sequencing based upon a method initially described by Ma *et al.* (16) and modified as described previously (28). Briefly, CFDNA was used to produce four overlapping amplicons from *HBX* gene of 139, 192, 334 or 425 bp. The 425 bp amplicon encompasses the entire *HBX* sequence and the three shorter amplicons correspond to fragments initiated at the 5′-end of *HBX* and covering progressive lengths of its sequence. Amplification of all four fragments signals the presence of a complete *HBX*, whereas amplification of one or several shorter fragments signals the presence of 3′-truncated *HBX*. The same forward primer X1F was used in each reaction (5′-GGGACGTCCTTTGTCTACGT-3′). The four reverse primers were X1R (5′-GGGAGACCGCGT-AAAGAGAG-3′), X2R (5′-GTGCAGAGGTGAAGCGAAGT-3′), X3R (5′-CCCAACTCCTCCCAGTCTTT-3′) or X4R (5′-GGCAGAGGT-GAAAAAGTTGCA-3′). PCR was carried for 50 cycles after activation with GoTaq Hot Star Polymerase at 94°C for 2 min (0.5 U, Promega). PCR cycles included denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 45 s followed by a final extension at 72°C for 10 min. Five microliters of PCR products were purified using standard ExoSap-IT (Usb Corporation, Cleveland, OH) and nucleotide sequences were determined for both strands by automated dideoxy sequencing (sequencer AbiPrism 3100, Applied Biosystems, Foster city, CA) using the same primers as for PCR. Sequences (nucleotide and predicted protein) were analyzed against *HBX* reference genes from GenBank using MEGA5 software (29). Because of the presence of a gap and of the covalent binding of the viral polymerase at the 5′ end of this gap, this PCR method does not readily amplify the partially double-stranded viral genome. Rather, it amplifies *HBX* sequences released into CFDNA either as HBV replication intermediates or as integrated viral DNA in the host's genome. The identity of all PCR products was confirmed by a second PCR and sequencing.

HBV genotyping

HBV genotyping was performed by sequencing *HBS* after amplification using a heminested PCR as described elsewhere (30). For the first reaction, 2 µl of DNA were used with primers S_HBVpol1 (5′-CCTGCTGGTGGCTCCAGTT-CA-3′) and S_HBVporv2 (5′-AAAGCCCAAAAGACCCACAAT-3′); round setting was 95°C (15 min); 40 cycles of 95°C (30 s), 60°C (30 s), 72°C (1 min); followed by extension at 72°C for 7 min. The second step used 2 µl of the first reaction and primers S_HBV123s (5′-TCGAGGATTGGGGACCCTG-3′) and S_HBVporv2; round setting was 95°C (15 min); 45 cycles of 95°C (30 s), 58°C (30 s), 72°C (1 min); followed by extension at 72°C for 7 min. Five microliters of PCR products were purified using standard ExoSap-IT (Usb Corporation) and both strands were directly sequenced by automated dideoxy sequencing (sequencer AbiPrism 3100; Applied Biosystems) using primers S_HBV123s, S_HBVporv2 and S_HBV778r (5′-GAGGTATAAAGGGACTCAAG-3′). HBV genotypes and subtypes were determined as previously described (31) and phylogenetic trees were built using HBV references from GenBank and the software MEGA5 (29).

Statistical analyses

Odds ratios (ORs), χ^2 and *P* values were calculated using STATA 11.1 (College Station, TX). All ORs were adjusted for age and gender.

Results

Characteristics of study participants

In this study, we analyzed a total of 325 controls, 78 patients with cirrhosis and 198 patients with HCC recruited in the Gambia Liver Cancer Study (4). Characteristics of study participants are presented in Table I. Despite frequency matching within 10 years age strata, HCC patients were significantly older than controls and more frequently male than patients with cirrhosis or than controls. HBV chronic carriage (positivity for HBsAg) was significantly more frequent in HCC and in cirrhosis than in controls. HBsAg was detected in 56% (44/78) and in 54% (106/198) of cirrhosis and HCC, respectively; compared with 13% (43/325) of controls {cirrhosis: OR 13.36 [95% confidence interval (CI) 1.08–165.04]; HCC: OR 12.19 [95% CI 1.29–115.59]}. Among HBsAg-positive subjects, 32% of cirrhosis and 16% of HCC were HBeAg-positive, compared with 5% of controls [OR for HCC versus controls: 0.77 (95% CI 0.14–4.34); for cirrhosis versus controls: 5.66 (95% CI 1.03–31.06)]. When compared with patients with cirrhosis, patients with HCC were less frequently positive for HBeAg [OR: 0.30 (95% CI 0.12–0.76)]. Median viral loads were higher in cirrhosis and HCC

Table I. Characteristics of the study participants

Clinical status	Controls, *n* (%)	Cirrhosis, *n* (%)	HCC, *n* (%)
Number of subjects	325	78	198
Age			
≤30	84 (26)	20 (25.5)	23 (12)
31–40	62 (19)	17 (22)	36 (18)
41–50	54 (17)	21 (27)	54 (27)
≥51	125 (38)	20 (25.5)	85 (43)
Sex			
Men	224 (69)	50 (64)	160 (81)
Women	101 (31)	28 (36)	38 (19)
HBsAg			
Positive	43 (13)	44 (56)	106 (54)
Negative	274 (85)	34 (44)	88 (44)
N/A	8 (2)	0 (0)	4 (2)
HBeAg[a]			
Positive	2 (5)	14 (32)	17 (16)
Negative	39 (90)	27 (61)	87 (82)
N/A	2 (5)	3 (7)	2 (2)
Viral loads[a]			
> Copies/ml[b] (*n*)	$2.5.10^3$ (22)	$9.5.10^6$ (29)	$3.4.10^5$ (76)
> <200, *n* (%)	21 (49)	15 (34)	30 (28)

N/A: not available.
[a]only for HBsAg-positive subjects.
[b]Median.

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($9.5.10^6$ and $3.4.10^5$ copies/ml, respectively) than in controls ($2.5.10^3$ copies/ml).

Presence of R249S in CFDNA of HCC patients is associated with detection of complete HBX sequence

Integration of *HBX* in the host cell genome is a hallmark of HCC and this integration often involves truncation of the portion of *HBX* encoding the C-terminus of the protein. This truncation is thought to somehow activate the oncogenic potential of HBx. Since previous results have shown that the mutant protein p.R249S interacts with HBx protein and since this interaction appears to involve the C-terminus of HBx, we reasoned that integration of the complete *HBX* sequence may occur more frequently in HCC with *R249S* mutations than in HCC without such *R249S* mutation. To test this hypothesis, we have analyzed *HBX* gene status by PCR amplification of four fragments covering overlapping portions of the gene, from the 5′-end to the complete sequence, in the CFDNA of HBV chronic carriers (HBsAg-positive subjects). This strategy allowed to distinguish between absence of *HBX* sequence (no PCR amplification), presence of truncated *HBX* (amplification of 1–3 PCR fragments covering regions of progressive length) and presence of complete *HBX* (amplification of all four PCR fragments, the longer one containing the region encoding the C-terminus).

Table II shows *HBX* status (undetectable, 3′-truncated or complete) in HBV carriers in relation with detection of *R249S* mutation in CFDNA. The detection of *R249S* in these subjects using two methods, RFLP or SOMA, has been reported previously (25,26). *HBX* sequences were detected in 14/43 HBV carrier controls (33%), in 32/44 HBV carrier patients with cirrhosis (73%) and in 89/106 HBV carrier patients with HCC (84%). Among each group, complete *HBX* sequence was detected in 19% of controls, in 57% of patients with cirrhosis and in 61% patients with HCC. However, when subjects in each group were classified according to *R249S* status, a clear difference emerged between HCC patients and other groups (Table II). In HCC patients, complete *HBX* sequences were detected in 43/56 (77%) of *R249S*+ subjects, as compared with 22/50 (44%) in *R249S*− subjects. In contrast, the number of HCC patients with truncated *HBX* was significantly higher among *R249S*− HCC patients (17/50; 34%) than among *R249S*+ subjects (7/56, 12%) (test for distribution of *R249S*+ and *R249S*− subjects according to *HBX* status among HCC cases: $\chi^2 = 12.12$; $P = 0.002$). It should be noted, however, that whatever the mutation status, the group of patients with complete *HBX* is larger than with 3′-truncated *HBX* (44% versus 34% in *R249S*-negative patients and 77% versus 12% in *R249S*-positive patients). Overall, these results suggest that integration of complete *HBX* sequence is more common in HCC cases with *R249S* mutation than in cases without this mutation. In the latter cases, 3′-truncation of *HBX* upon integration appears to be a more common event.

Occult HBV infection based on HBX detection

Although the analysis described above was focused on those subjects identified as HBV carriers solely on the basis of HBsAg status, we investigated whether *HBX* DNA could be detected among HBsAg-negative subjects as a possible marker of occult HBV infection. Of a total of 396 HBsAg-negative subjects, 34 (9%) were found to harbor *HBX* sequences in CFDNA (Table III). These 34 subjects included 8/274 controls (3%), 5/34 patients with cirrhosis (15%) and 21/88 (24%) in patients with HCC.

To further assess the significance of *HBX* sequence detection in HBsAg-negative subjects, we performed a PCR analysis to detect the presence of the *HBC* gene and correlated it with positivity for anti-HBc antibodies (Table III). Of the 34 *HBX*-positive subjects, 33 could be analyzed for *HBC*, among which 29 (85%) were found to be positive for *HBC* gene or anti-HBc antibodies, thus showing a remarkable correlation between *HBX* and *HBC* and anti-HBc antibodies positivity. Overall, these results suggest that detection of *HBX* sequence is a marker for occult infection and there is a trend toward more frequent occult infection in chronic liver disease and in HCC.

Predominance of HBV genotype E

In previous studies, genotype E was found to be the most common HBV genotype in subjects from The Gambia as well as other parts of West Africa (20,30). In the present study, the *HBS* gene could be amplified from CFDNA by PCR in 66 of 170 *HBX*-positive subjects (39%) and sequenced to determine subtypes and establish a phylogenetic tree. Genotype E was detected in 75.5% of these subjects, including 2/4 controls, 15/19 cirrhosis and 33/43 HCC, with a majority of *ayw4* subtype (88%) (Supplementary Table 1, available at *Carcinogenesis* Online). Twenty-three percent of subjects carried genotype A with 93% of subtype *ayw1* and only one subject (1.5%; HCC patient (28)) carried genotype D subtype *ayw2*. *HBS* sequences of genotype E were heterogeneously distributed among the existing phylogenetic tree of this genotype, with no relation to disease status (Supplementary Figure 2A, available at *Carcinogenesis* Online). In contrast, all 15 *HBS* genotype A sequences were distributed into a single group corresponding to subgenotype A3 (Supplementary Figure 2B, available at *Carcinogenesis* Online). There was no difference in the distribution of genotypes and subgenotypes according to either presence of *R249S* or clinical status.

HBX sequences and mutations

Since *HBS* sequences were not available for 104 subjects, we used *HBX* sequences to identify HBV genotypes. *HBX* sequences from the 66 subjects genotyped based on *HBS* sequences were used to establish a phylogenetic tree (Supplementary Figure 3, available at *Carcinogenesis* Online). This tree separates *HBX* sequences associated with genotypes A, D or E. By identifying their closest neighbor *HBX* sequence, it thus became possible to assign HBV genotypes in 76 of the 104 subjects for whom *HBS* sequences were not available. This analysis further established the presence of genotype E, A and D in 66%, 16.5% and 1% of the subjects, respectively. The remaining 16.5% of *HBX* sequences could not be clearly classified.

Table II. Number and percentage of subjects according to *HBX* and *R249S* in HBsAg-positive subjects

Status	*HBX* status, *n* (%)				Total
	R249S	Complete	3′-truncated	Undetectable	
Control	−	8 (21)	5 (13)	25 (66)	38
	+	0 (0)	1 (20)	4 (80)	5
Cirrhosis	−	16 (52)	5 (16)	10 (32)	31
	+	9 (69)	2 (15.5)	2 (15.5)	13
HCC	−	22 (44)	17 (34)	11 (22)	50
	+	43 (77)	7 (12)	6 (11)	56

Table III. Number and percentage of subjects according to *HBX* and *HBC* in HBsAg-negative subjects

Status	*HBX*, *n* (%)			Total
	HBC[a], *n* (%)			
	Complete	3′-truncated	Undetectable	
Control	1 (0.4)	7 (3)	266 (96.6)	274
	1 (100)	*5 (71)*	N/A	
Cirrhosis	0 (0)	5 (15)	29 (85)	34
	N/A	*4 (80)*	N/A	
HCC	3 (3)	18 (20)	67 (77)	88
	3 (100)	*16 (89)*	N/A	

N/A: not applicable.
[a]Positive for anti-HBc antibody or *HBC* gene, percentages were calculated on *HBX*-positive subjects.

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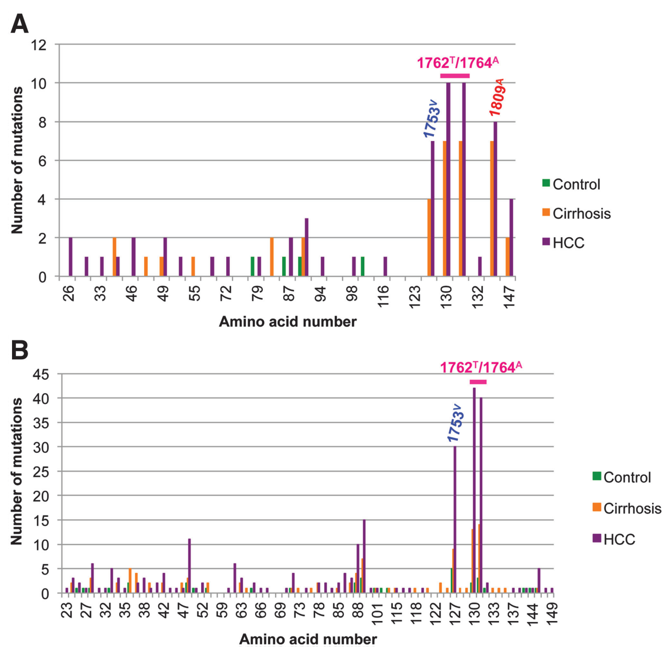

Fig. 1. Number of variations and mutations along *HBX* gene. The two mutation points 1753^V and $1762^T/1764^A$ are commonly found in genotype A (**A**) and E (**B**), whereas the mutation 1809^A is genotype A specific.

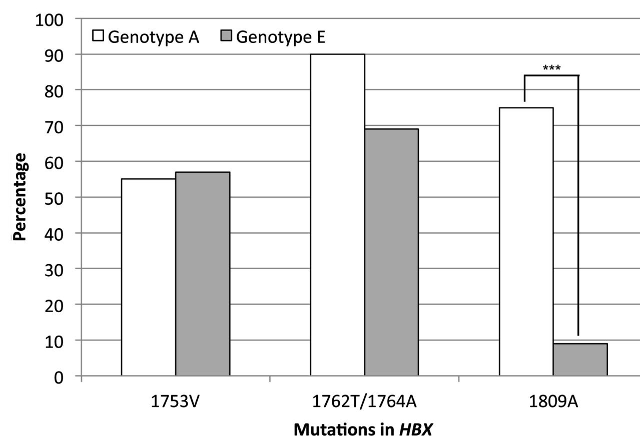

Fig. 2. Frequency of the three specific *HBX* mutations 1753^V, $1762^T/1764^A$ and 1809^A in genotypes A and E. Mutations 1753^V and $1762^T/1764^A$ are present in >50% and 70% of subjects with no difference between genotypes. On the contrary, a higher proportion of 1809^A mutations was observed in genotype A compared with genotype E ($^{***}P < 0.001$).

Further analysis of *HBX* sequences was performed to identify recurrent point mutations (Figure 1). Three mutation sites were detected in genotypes A and E, including 1753^V, the double mutation $1762^T/1764^A$ and 1809^A. However, the relative frequency of these mutations varied among genotypes (Figure 2). Although 1753^V and $1762^T/1764^A$ were equally found in both genotypes, 1809^A was more common in genotype A than E (75% versus 9%), suggesting a possible genotype-specific effect. An association was observed between the HBV $1762^T/1764^A$ double mutations and clinical status. Carriers with this double mutation had an elevated risk of cirrhosis or HCC when compared with carriers without this mutation [cirrhosis: OR: 9.50 (95% CI: 1.50–60.11); HCC: OR: 11.29 (95% CI: 2.07–61.47)]. No significant association was detected between mutations and *R249S* positivity.

Discussion

In this study, we have used circulating cell-free DNA (CFDNA) from the plasma as a surrogate material to analyze the associations between the status of complete sequences of *HBX* and the presence of the *TP53* mutation *R249S* in individuals from The Gambia, a country with high incidence of HCC in West Africa. Previous studies have shown that CFDNA in patients with HCC or chronic liver disease was a good source of DNA originating from the liver (24).

Our interpretation is based on the assumption that *HBX* sequences detected in CFDNA are representative of the integrated status of *HBX* in hepatocytes. Plasma DNA has proven to be a convenient source to access and analyze liver CFDNA. In previous studies, we have shown that there was an excellent overall concordance between presence of *TP53 R249S* mutation in the plasma and in HCC tissues of patients from The Gambia (32). This excellent performance of CFDNA as a surrogate for liver DNA provides a convenient approach for studying the molecular pathology of chronic liver disease and HCC in low-resource countries where liver biopsies are not routinely available. Moreover, the use of plasma DNA is well adapted to case–control study designs in which blood samples can be equally obtained from patients and controls. To detect *HBX* and assess its truncation status in CFDNA, we have adapted a PCR-based method previously described by Ma *et al.* (16), which generates products of different lengths, the longest one corresponding to full-length *HBX* sequence. The structure of HBV genome is such that the viral DNA is partially double-stranded, not covalently closed and contains the site of covalent binding of the RNA polymerase in a region overlapping with *HBX*. This structure precludes the amplification of viral genomes using the PCR approach we have used. Thus, this method preferentially, if not exclusively, detects double-stranded *HBX* sequences released from liver cells in the bloodstream either in the form of fragmented HBV replication intermediates or in the form of fragments of cellular genomic DNA containing integrated viral sequences. In agreement with this interpretation, we did not observe a higher proportion of complete *HBX* sequences in patients with the highest viral loads (cirrhosis), which should have been expected if viral genomes were detected.

We have analyzed *HBX* gene status in the CFDNA of subjects with HCC, cirrhosis or controls (601 samples in total). To our knowledge, this is the first systematic study on *HBX* genetic status in subjects from West Africa, one of the areas of the world with the highest population carriage of HBV. We found that complete *HBX* is highly associated with HCC-*R249S*+ compared with *R249S*− ($P = 0.002$), whereas 3′-truncated *HBX* appears to be more frequent in *R249S*− HCC, defining an inverse relationship between 3′-truncated *HBX* and presence of *R249S* mutation. A study performed in a Chinese population using the same approach has identified 3′-truncated *HBX* in >70% of HCC cases although it did not examine *R249S* mutation status (16). Thus, our results suggest that entire *HBX* is often retained in HCC from this West African population, in particular when the tumor also contains *R249S*. This observation suggests that HCC in this population may develop according to different mechanism, perhaps corresponding to different mechanism of interplay between the major etiological risk factors, chronic HBV infection and exposure to AFB_1. We propose that the association between complete *HBX* and *R249S* may reflect a particular mechanism of cooperation between the HBx protein and the mutant p.R249S protein.

Previous studies have shown that the p.R249S mutant protein forms stable complexes with full-length HBx protein (18). It has also been reported that the C-terminus of HBx contains a domain with antiproliferative proapoptotic effects (33). Thus, we propose that interaction between p.R249S and HBx somehow favors the retention and integration of a full-length *HBX*, perhaps because the mutant protein neutralizes a suppressive activity located in the C-terminus of HBx, thus making its truncation dispensable during hepatocarcinogenesis (Figure 3). This interaction may contribute to explain the strong association between p.R249S and chronic HBV infection in causing HCC. Indeed, presence of p.R249S may bypass the need for generating a truncated *HBX* product and therefore accelerates hepatocarcinogenesis. Thus, presence of p.R249S may facilitate the maintenance of complete *HBX* gene until late stages of HCC progression. This

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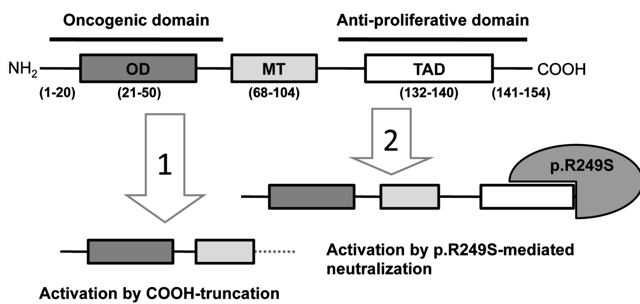

Fig. 3. Two hypothetic mechanisms for HBx protein activation in HCC. The protein contains three domains from N- to C-terminal region: an oligomerization domain (OD; aa 21-50); a mitochondria association domain (MT; aa 68-104), which is essential for cell death but not for transactivation function; and a transactivation domain (TAD; aa 132-140). Left: activation by C-truncation (due to integration of 3′-truncated *HBX* sequence in host genome). Right: activation by neutralization (due to binding of p. R249S to the antiproliferative C-terminal domain of HBx protein encoded by a complete integrated *HBX* gene).

hypothesis remains to be further assessed in patients with HCC from other areas such as parts of China where aflatoxin-induced *R249S* mutations are also common in HCC.

Another important lesson from our study is the identification of a high prevalence of occult HBV infection in Gambian patients. In our study, 15% and 24% of HBsAg-negative subjects with cirrhosis and HCC, respectively, were positive for *HBX* in CFDNA, suggesting that these patients may harbor viral sequences despite the absence of HBsAg expression. In recent years, occult HBV infection has emerged as an important and challenging form of persistent HBV infection. The term 'occult infection' is commonly used to characterize a heterogeneous group of patients who are HBsAg-negative and either seronegative for all HBV markers or positive for anti-HBc and/or anti-HBs. Many of these patients are positive for HBV DNA detected by PCR in the liver, the serum or both. Some of these patients have underlying liver disease, suggestive of ongoing hepatocellular injury from persistent HBV infection. A meta-analysis by Shi *et al.* has concluded that occult HBV contributes to the development of HCC. It may serve as cofactor in the development of HCV-related HCC and may also play a direct role in promoting non-B and non-C HCC growth (34). However, further studies are needed to clarify these observations. In any case, our results in Gambian subjects are consistent with the idea that occult infection may play an important part of the burden of HBV-related liver diseases in this population.

The genetic diversity of HBV genotypes is not well documented in The Gambia. In this study, sequencing of *S* gene confirmed genotype E in 76% of the subjects analyzed, with genotype A in 23% and genotype D in 1.5%, irrespective of disease status. This genotype distribution was essentially confirmed by sequencing *HBX*. Previous studies have reported over 90% genotype E in asymptomatic HBV carriers in The Gambia and in other West African countries (35). Using the same method as in the present paper, Villar *et al.* (30) have identified 90% of genotype E in carriers from three villages in rural Gambia. The higher proportion of genotype A in the present study suggests that genotype A might be more widespread than previously suspected, in particular in urban or peri-urban residential areas of the coastal region, where the majority of the participants of this case–control study were recruited.

Mutation analysis of *HBX* identified several common mutation sites in the 3′-end of *HBX*. The well-studied mutations 1753^V and the double mutation $1762^T/1764^A$ were present in >50% and 70% of subjects, respectively, with no difference between genotypes. Among HBV carriers, presence of the double mutation $1762^T/1764^A$ was associated with higher risk of cirrhosis or HCC [cirrhosis: OR: 9.50 (95% CI: 1.50–60.11); HCC: OR: 11.29 (95% CI: 2.07–61.47)], consistent with an earlier pilot study in The Gambia (36) and with results in patients from other parts of the world (36–40). The mechanisms by which these mutations enhance the risk of chronic liver disease are still a matter of debate. They may contribute to increase the rate of HBV replication and they may also result into an HBx protein with specific amino acid substitutions enhancing its oncogenic properties (17,41). In contrast with these mutations, the mutation 1809^A was more frequently associated with genotype A3 (75%) than genotype E (9%), raising the possibility that this mutation may occur in a genotype-specific manner. Previous studies have shown that mutation at positions 1809–1812 were highly associated with genotype A1 (42,43). These substitutions do not appear to be the result of adaptive changes under immune pressure, since they are also found in HBV isolates obtained from children and in acute hepatitis patients (44,45).

In conclusion, this analysis of *HBX* status in a case–control study of HCC in The Gambia has shown that there is an association between the presence of complete *HBX* sequences and the presence of the aflatoxin-induced mutation *R249S*. The significance of this association for diagnosis, patient prognosis or tumor cell activity needs to be addressed in further studies. Furthermore, this study indicates that occult HBV infection might be present in up to 24% of HCC cases, suggesting that the role of HBV chronicity as attributable risk of HCC in the Gambian population is even greater than previously suggested (46). It will be important to examine how the implementation of neonatal HBV vaccine, which has started in 1986 in the Gambian population, will affect these molecular parameters of HBV infection.

Supplementary material

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/.

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