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## Observational Study

# Prevalence of hepatocarcinoma-related hepatitis B virus mutants in patients in grey zone of treatment

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## Abstract

### BACKGROUND

Antiviral treatment of patients with chronic hepatitis B (CHB) in the grey zone of treatment commands risk management in order to optimize the health outcome. In this sense, the identification of HBV mutants related with an increased risk of hepatocellular carcinoma (HCC) could be useful to identify subpopulations with potential indication of antiviral treatment.

### AIM

To analyze the prevalence/persistence of hepatitis B virus (HBV) preS and basal core promoter (BCP)/precore/core variants associated to HCC development in CHB patients in the grey zone.

### METHODS



Edition of the Gilead Fellowship Program, No. GLD13/00046 and Modificaciones de los niveles de expresión génica mediada por mutantes naturales de la región PreS del virus de la hepatitis B, y asociación con genes implicados en el desarrollo de hepatocarcinoma Efecto del tratamiento antiviral.

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Work was designed as a longitudinal retrospective study, including 106 plasma samples from 31 patients with CHB in the grey zone of treatment: Hepatitis B e antigen negative, HBV-DNA levels between 12-20000 IU/mL, normal or discordant transaminase levels during follow up and mild/moderate necro-inflammatory activity in liver biopsy or Fibroscan (up to 9.5 kPa). Serum HBV-DNA was tested using the Abbott Real Time HBV Assay and the BCP/precore/core and the hepatitis B surface antigen (HBsAg) coding regions were analyzed in positive samples by PCR/bulk-sequencing to identify the HCC-related HBV mutants.

#### RESULTS

High-risk HCC related mutants were detected in 24 (77%) patients: 19 (61%) in the BCP/precore/core, and 7 (23%) in the HBsAg coding region (2 preS1 and 5 preS2 deletions). The prevalence of preS deletions was genotype-dependent: 3/5 (60%) patients with preS2 deletions and 1/2 with preS1 deletions were infected with the HBV-E genotype. Since HBV-E was the most prevalent in sub-Saharan patients, a correlation between preS deletions and ethnicity was also found: 6/8 (75%) sub-Saharan *vs* 1/19 (5%) Caucasian patients had preS deletions ( $P = 0.00016$ ). Remarkably, this correlation was maintained in those patients infected with HBV-A, a minor genotype in sub-Saharan patients: 2/2 patients infected with HBV-A from West Africa *vs* 0/6 of Caucasian origin had preS deletions. The HCC related variants were the major strains and persisted over time (up to 48 mo). Patients with preS deletions had a significant higher prevalence of F2 fibrosis stage than the negatives (57% *vs* 10%,  $P = 0.0078$ ).

#### CONCLUSION

HBV genetic analysis of selected populations, like sub-Saharans infected with HBV-E/A genotypes, will allow identification of subpopulations with risk of HCC development due to accumulation of high-risk HBV variants, thus commanding their increased clinical surveillance.

**Key words:** Hepatitis B virus; Hepatocellular carcinoma; PreS deletions; Hepatitis B virus treatment; Grey zone

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**Core tip:** The antiviral treatment in patients with chronic hepatitis B in the “grey zone” of treatment is controversial and not clearly indicated. The genetic analysis of hepatitis B virus (HBV) basal core promoter/precore/core and preS regions has shown a high prevalence and persistence of preS deletions in the sub-Saharan population infected with HBV-E/A genotypes. By contrast, Caucasian patients, who have shown a good clinical evolution in previous studies, were negative for these variants. The recognition of these subpopulations warrant to increase the clinical surveillance in order to minimize the risk of liver cancer development due to accumulation of hepatocellular carcinoma-related HBV genetic variants.

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#### INTRODUCTION

In most cases, patients with chronic hepatitis B (CHB) treated with nucleos(t)ide analogues (NAs) such as tenofovir or entecavir achieve a maintained virological suppression<sup>[1-3]</sup>. Although the inhibition of viral replication has an evident beneficial effect on the progression of the liver disease, the antiviral treatment does not achieve eradication of the hepatitis B virus (HBV) infection due to the persistence of

intracellular covalently closed circular DNA in the nucleus of infected hepatocytes<sup>[4]</sup>. Thus, therapy is generally recommended for prolonged periods of time and the discontinuation of NAs is controversial because the early interruption of treatment is usually associated with a reactivation of viral replication that may induce severe flares if not controlled by the host immunity, requiring the re-start of the antiviral treatment<sup>[5]</sup>. According to EASL guidelines treatment may be discontinued in hepatitis B e antigen (HBeAg) negative patients only when hepatitis B surface antigen (HBsAg) loss is achieved or after 3 years of virological suppression<sup>[1]</sup>. Conversely, AASLD guidelines still recommends lifelong antiviral treatment in patients with HBeAg negative CHB<sup>[2]</sup>.

The indications for CHB treatment are mainly based on the combination of three criteria: serum HBV-DNA levels, alanine aminotransferase (ALT) values and severity of liver disease. Patients should be considered for treatment when they have HBV-DNA levels above 2000 IU/mL, persistent or intermittent serum ALT abnormal levels and severity of the liver disease assessed by liver biopsy (or non-invasive markers validated in HBV-infected patients) showing moderate to severe active necroinflammation and/or at least moderate fibrosis using a standardised scoring system<sup>[1,2]</sup>. Conversely, there are HBeAg-negative patients with maintained minimal or mild fibrosis, for whom the treatment is not clearly indicated: A) patients with maintained normal ALT levels and low or undetectable serum HBV-DNA; B) patients with marginally elevated ALT and/or HBV-DNA titers ranging 2000-20000 IU/mL; and C) persistently normal ALT and HBV-DNA titers higher than 2000 IU/mL<sup>[6,7]</sup>.

The indication of treatment in these patients, in the so named “grey zone” of treatment, requires to balance the risks and benefits for health outcomes. Although low levels of HBV replication and the absence of significant liver damage are good prognostic factors in the natural history of CHB, it cannot be ruled out that some of these patients may be infected with viral strains with mutations associated with high-risk of hepatocellular carcinoma (HCC) development, which are an additional risk of progression for liver disease<sup>[8,9]</sup>. There are several studies that associate certain mutations in the HBV genome with an increased risk of HCC development, but the results are controversial because the HBV genotypes or even different HBeAg status have different mutation patterns. One of the mechanisms by which HBV can promote direct carcinogenesis is the ability of wild-type and mutated/truncated viral proteins like hepatitis B x-protein, hepatitis B core-protein (HBc) and preS region (preS) to affect cell functions, activate oncogenic pathways and sensitize liver cells to mutagens<sup>[10]</sup>. The most common HBV specific mutations are at the preS2 start codon, preS deletions, and point mutations in the basal core promoter (BCP) and in the precore/core coding region<sup>[11-13]</sup>.

For these reasons, the aim of this work is to analyse the prevalence and persistence over time of HBV mutants that predispose to the development of HCC in patients with CHB but without clear indication of treatment, and to analyze their role as a tool in the selection of suitable subpopulations for antiviral treatment.

## MATERIALS AND METHODS

### Study population

This work was designed as an observational, longitudinal, retrospective study including 106 plasma samples from 31 CHB patients in the grey zone of treatment: HBeAg negative, HBV-DNA levels between 12-20000 IU/mL, normal or discordant ALT levels during follow up and mild/moderate necro-inflammatory activity in liver biopsy or Fibroscan (up to 9.5 Kpa). Patients with chronic hepatitis C, D or HIV coinfection, or with toxic, alcoholic or autoimmune hepatitis were excluded. The average number of analysed samples per patient was [mean  $\pm$  standard deviation (SD)]  $3.2 \pm 1.2$  (ranging: 2-6 samples) and the follow-up time period per patient was (mean  $\pm$  SD)  $2.1 \pm 2.2$  years (ranging: 0.5-8.4 years). No patient was under antiviral treatment for HBV at the time in which samples were collected. The clinical features of the patients, obtained from the full clinical charts, with a follow-up time (mean  $\pm$  SD) of  $6.1 \pm 3.9$  years (ranging: 0.7-12.8 years) are shown in Table 1. The study was approved by the Ethical Committee of the Hospital Universitario La Paz/Carlos III in Madrid, according to the ethical guidelines of the 1975 Declaration of Helsinki. All the participants received and signed written consent for their participation.

### Serum HBV-DNA analysis

Serum HBV-DNA was quantified using the Abbott Real Time HBV Assay (Abbott Laboratories, Abbott Park, IL, United States). In positive samples, the viral genome was purified from 200 L of serum using the QIAmp DNA Kit (QIAGEN GmbH,

**Table 1** Demographic and clinical features of patients

Demographic data	Value
Male/female	16/15
Caucasian/sub-Saharan/chinese <sup>1</sup>	22/8/1
Age (years: mean $\pm$ SD)	45.1 $\pm$ 10.2
Virological data	
HBV-DNA positivity (persistent/fluctuant) <sup>2</sup>	28/3
HBV-DNA titers (IU/mL: Mean $\pm$ SD) <sup>3</sup>	1.4 $\times 10^3 \pm 3.5 \times 10^4$
Clinical data <sup>4</sup>	
Persistently normal/marginally altered AST or ALT	18/13
Persistently normal/marginally altered AST	25/6
Persistently normal/marginally altered ALT	20/11
AST (IU/L: mean $\pm$ SD)	25.6 $\pm$ 9.5
ALT (IU/L: mean $\pm$ SD)	28.5 $\pm$ 14.3
Fibrosis stage (Fibroscan)	
F0-F1	22
F2	6
Not available	3

<sup>1</sup>Origin country of sub-Saharan patients: 3 Equatorial Guinea, 2 Senegal, 1 Ivory Coast, 1 Ghana, and 1 Cameroon;

<sup>2</sup>Persistent/fluctuant: Positive results in more/less than the 90% of samples;

<sup>3</sup>In 16 samples from 8 patients, point flares of HBV-DNA titers ( $> 20000$  IU/mL) were observed;

<sup>4</sup>Persistently normal/marginally altered: Normal values in more/less than the 90% of the samples. IU: International units; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Standard deviation.

Hilden, Germany), and BCP/precore/core and HBsAg coding regions were amplified using the PCR Master Mix (Promega Corporation, Fitchburg, WI, United States) using genotype-specific primers.

### DNA sequencing

PCR products were purified using the Illustra Exo ProStar (GE Healthcare Life Sciences, Fairfield, Connecticut, United States), sequenced using Bright Dye Terminator Cycle Sequencing (NimaGen, Nijmegen, Netherlands) and analyzed with the DNA sequencer ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, United States).

### Sequence analysis

Analysis of the DNA sequences was done using Lasergene SeqMan Pro V7.1.0 (ADNSTAR, United States) software. The phylogenetic relations were established using the MEGA version 4.0 software (Center for Evolutionary Medicine and Informatic, Tempe, AZ, United States).

Assignment of the HCC risk development associated to each HBV mutation was performed according to the previously published data<sup>[11,13]</sup> (Table 2).

### HBV genotyping

HBV genotype was analyzed in all cases using the line probe assay INNO-LiPA HBV Genotyping assay (Innogenetics N.V., Ghent, Belgium). Patients with negative results in this technique were typed by the sequence analysis of HBsAg coding region using the Geno2pheno[HBV] open access of the Genafor website (www.genafor.org).

### Statistical analysis

Data were analyzed using the SPSS v13 software (SPSS Inc. North Chicago, IL, United States) on an intention to treat (ITT) basis. All parameters expressed as absolute number or percentages were analyzed using the Spearman's ran-correlation, the Wilcoxon matched-pairs and the *U* Mann-Whitney systems. The mean comparisons were performed using the Student-*t* test.



**Table 2 Assignment of risk of hepatocellular carcinoma development associated to each hepatitis B virus genetic variants**

HCC risk	HBV region	Genetic variant
High	PreS2	PreS2 deletions
	BCP	C1653T, T1753V (A/C/G), A1762T + G1764A
	Precore	G1896A, G1899A
Suspect	PreS1	PreS1 deletions
	PreS2	T53C
	BCP	C1773T
	Precore	A1846T
	Core	C1914G, C2289A
Minor	PreS1	T3098C, T3139A
	PreS2	PreS2 start codon
	S	T766A, T791A
	BCP	T1674C, G1727, C1741, C1761, C1766T, T1768A
	Precore	C1858T, G1862T
	Core	C1909, A1934T, C2002T, T2003A, C2100A, A2159G, A2189C, A2246C

High evidences: HBV mutants with positive data for HCC correlation evidenced in at least 3 different papers, or in meta-analysis studies; Suspected correlation: Positive data for HCC correlation evidenced in at least 2 works or without meta-analysis evidences; Minor evidences: Positive data for HCC correlation in single works. HCC: Hepatocellular carcinoma.

## RESULTS

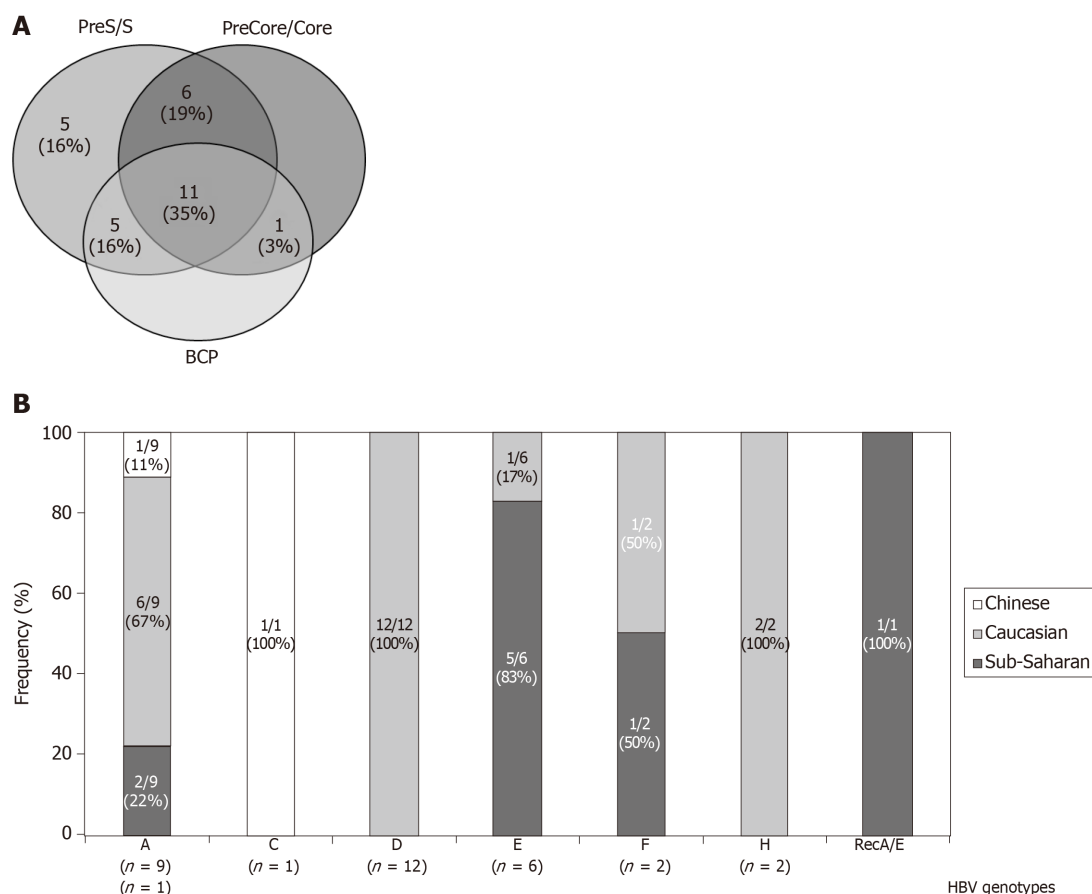
### HBV genotyping analysis

In only 3 (10%) patients no amplification of any of the HBV analyzed regions was achieved. In the remaining 28 patients the efficacy of the HBV-DNA amplification was different depending on the region of the HBV genome analyzed. Thus, we have data from the complete HBsAg and BCP/precure/core regions in at least one sample of the follow-up in 11 (35%) patients, and partial information in 17 (55%) patients (Figure 1A). HBV-DNA titers were significantly higher in the samples positive for the in-house PCR techniques than in the negatives (mean  $\pm$  SD:  $7.6 \times 10^3 \pm 1.3 \times 10^4$  IU/mL vs  $3.1 \times 10^3 \pm 4.6 \times 10^3$  IU/mL, respectively.  $P = 0.016$ ).

HBV genotype was determined in 30 (97%) patients [12 (39%) by LIPA and 27 (87%) by HBsAg sequence analysis]: 9/31 (29%) were infected with HBV-A genotype, 1 (3%) with HBV-C, 12 (39%) with HBV-D, 6 (19%) with HBV-E, 2 (6%) with HBV-F, 2 (6%) with HBV-H and 1 (3%) with a recombinant HBV-A/E. Serial changes of the genotyping results during the follow-up were observed in 4 patients: One sub-Saharan patient (who evolve from E to F genotypes), one Chinese (A to C), and 2 Caucasians (from D to A and from A to H, respectively). Differences in the genotype distribution among ethnic groups, especially in the frequency of D and E genotypes, were observed (Figure 1B). In only one case, corresponding to the A/E recombinant, discrepancies between LIPA and HBsAg analysis were remarkably noted.

### Identification of mutants related with HBeAg negative status

In 21/23 (91%) patients in whom the BCP and/or precure/core regions were amplified, the presence of the most common genetic variants related with the HBeAg negative/anti-HBe status was confirmed. Only partial sequence data (BCP or precure/core sequences) were available for the remaining 2 patients lacking confirmation of these variants. The frequency of mutants related to the HBeAg negative status was higher in the precure/core region than in the BCP in all genotypes, especially in genotypes D and E (Figure 2A). Thus, in 10 patients with mutations in the precure/core region no changes were observed in the BCP region. In addition, coexistence of several mutations in the BCP/precure/core region was observed in the 21 patients (Figure 2B), with special importance for the combination of the A1846T + C1858T + G1896A mutations, which was present in the majority of the patients irrespective of the genotype (Figure 2C).

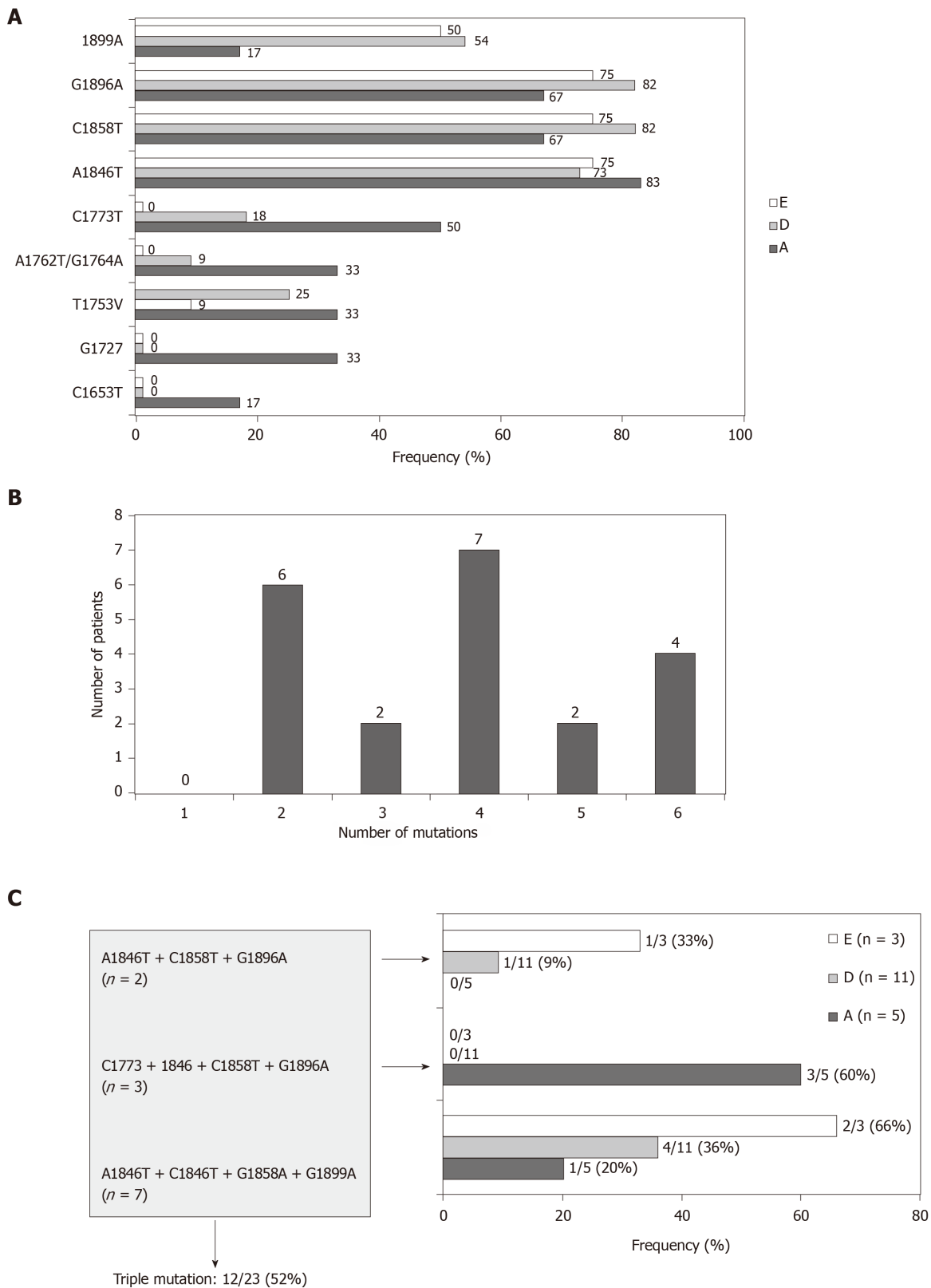


**Figure 1 Features of samples analyzed.** A: Number of patients with HBV-DNA amplification positive results in the different genomic regions analysed; B: Ethnic group distribution among the different HBV genotypes. The 4 patients in whom were confirmed sequential changes of the genotyping results during the follow-up were included in both genotype categories. BCP: Basal core promoter; HBV: Hepatitis B virus.

### Identification of HCC related mutants

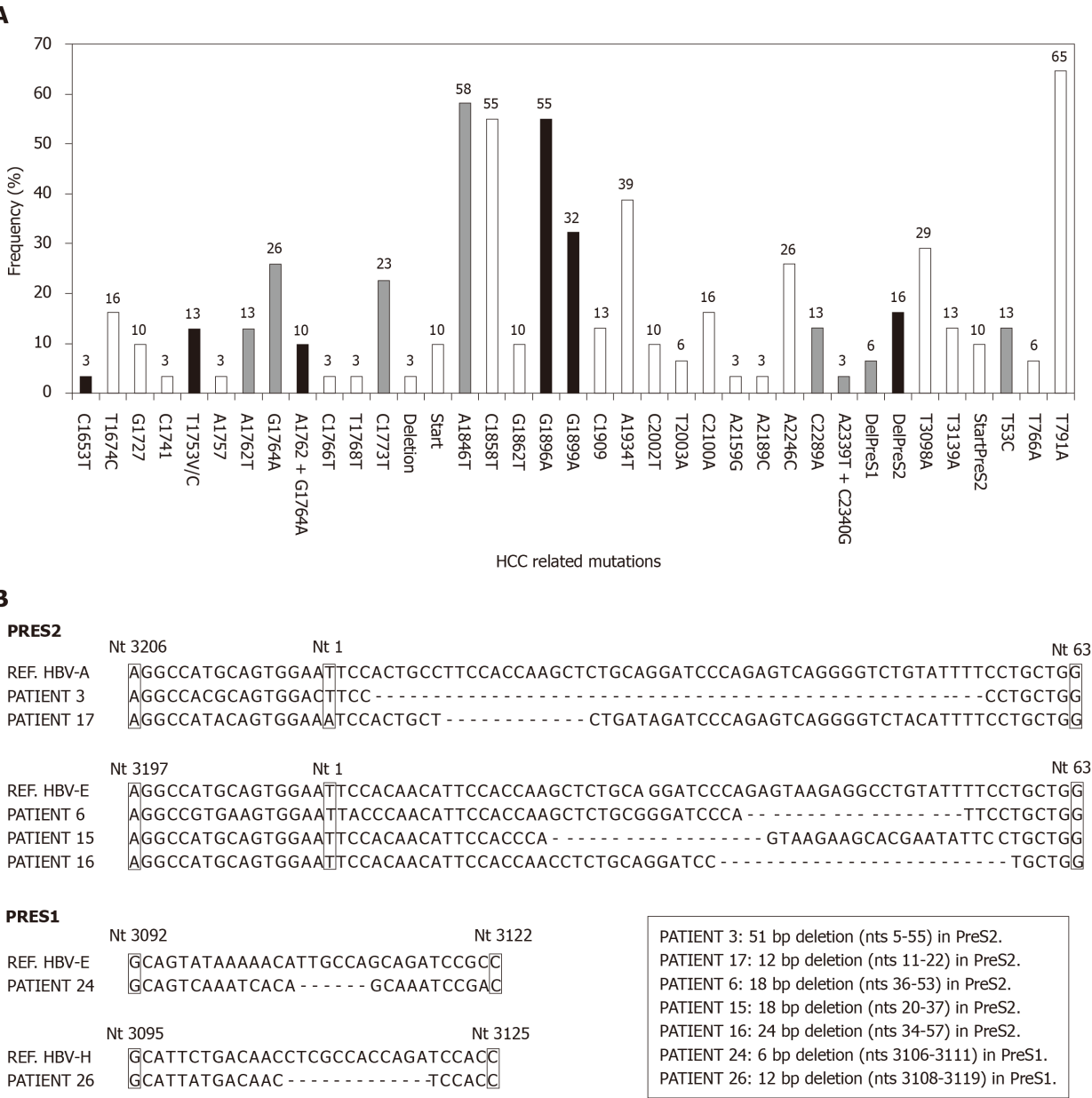
BCP/precore/core region: Taken globally, 19 (61%, on an ITT basis) patients have any high-risk variant (C1653T, T1753V, A1762T/G1764A, G1896A and G1899A) and this percentage increases up to 21 (68%) patients by including the genetic variants with suspect correlation with HCC (C1773T, A1846T, C1914G, C2289A and A2339T+C2340G) in the BCP or precore/core regions. The distribution of the individual mutations in the study population is shown in Figure 3A. Although the presence of single high-risk mutations was the most frequent situation (9 patients), the existence of coinfection with several high-risk mutations was also a common event (Figure 4A).

PreS1/preS2/S region: PreS1 deletions were found in 2 (6%), and preS2 deletions in 5 (16%) and the T53C mutation in 4 (13%) patients, respectively. The distribution of the individual mutations in the study population is shown in Figure 3A and the sequences of the deletions of the preS region in Figure 3B. In only 1/10 patient the simultaneous presence of preS1 deletion and the T53C mutation was confirmed. Moreover, the previously described mutation in the preS2 start codon was identified in 3 patients (1 HBV-A, 1 HBV-E and 1 HBV-F). A high correlation between lack of start codon and the preS2 deletion was found, with coexistence of both changes in 2 of the 3 patients with the preS2 start codon mutation. The distribution of HCC related mutations in the HBsAg coding region was genotype-dependent. In this way, 3/5 (60%) patients with preS2 deletions and 1/2 patient with preS1 deletions were infected with the HBV-E genotype. Moreover, it is very remarkable the correlation of these preS deletions with ethnicity: 6/8 (75%) sub-Saharan had preS deletions *vs* 1/19 (5%) of the Caucasian patients ( $P = 0.00016$ ). This correlation was observed not only in the HBV-E, which is the predominant genotype in sub-Saharan population, but also in the HBV-A genotype, that is minoritarian within this ethnic group. So, among HBV-A infected patients, only those from West Africa ( $n = 2$ ) had preS deletions, but none of the patients ( $n = 6$ ) with Caucasian origin (Figure 4B). In 5/10 (50%) patients with high or medium risk mutations in the HBsAg, data of genetic analysis of the



**Figure 2 Analysis of basal core promoter/precore/core mutants related with the e-antigen negative status.** A: Prevalence of individual mutations of the in the 3 most frequent HBV genotypes analyzed; B: Frequency of coexistence of more than one BCP/precore/core mutants; and C: Frequency of the most common combination of mutations in the different genotypes. The frequency calculation was performed including only the 23 patients with precore/core sequences data, not in on intention to treat basis. BCP: Basal core promoter; HBV: Hepatitis B virus; A: HBV-A; D: HBV-D; E: HBV-E.

precore/core region were also available. In 2/5 (40%) patients with high-risk deletion in the preS2 coding region, and in 3/4 (75%) patients with the T53C substitution, were simultaneously detected high-risk mutations in the BCP/precore region. Especially significant was the sub-Saharan patient infected with an HBV-E genotype who had a

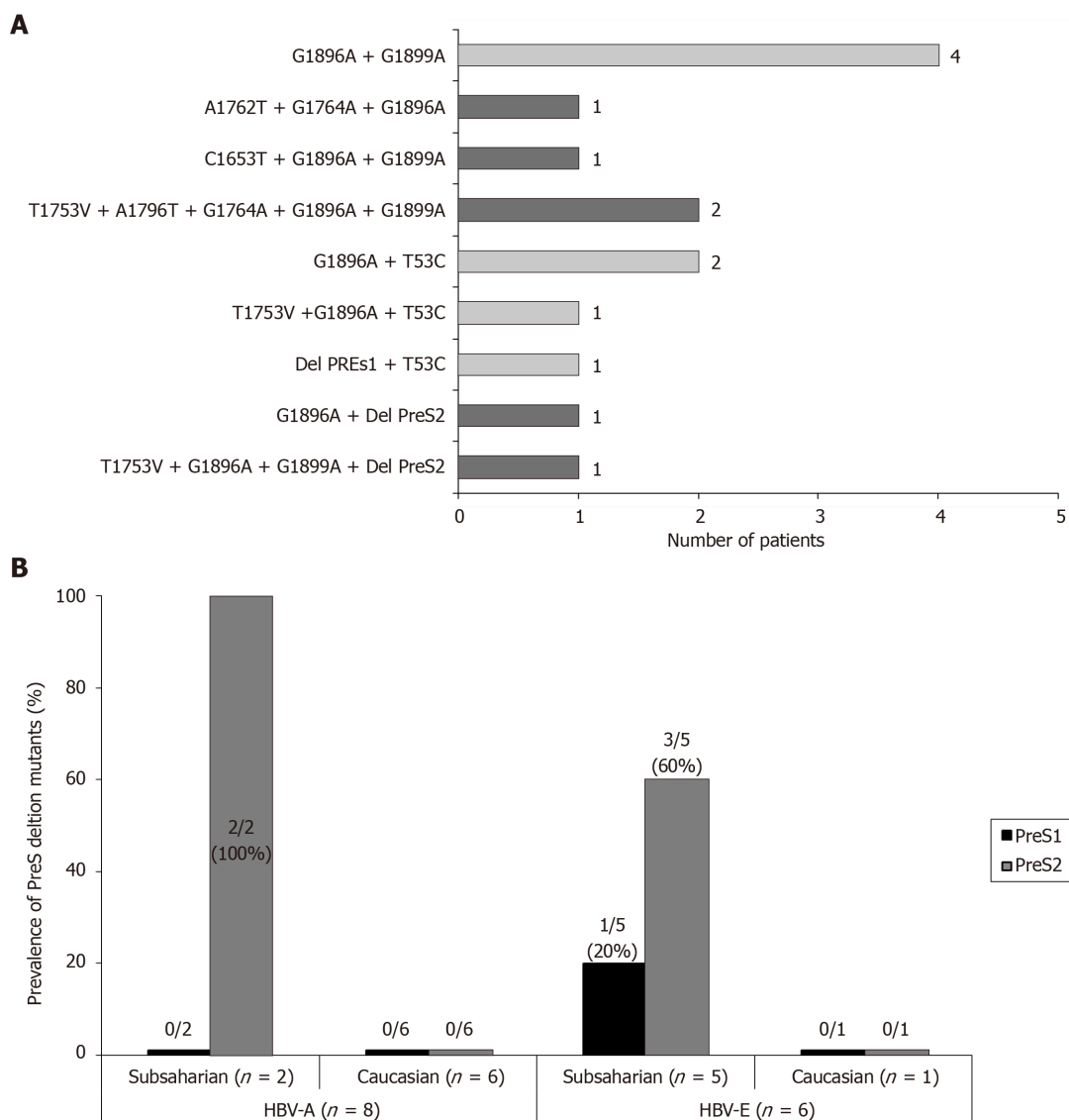


**Figure 3 Analysis of basal core promoter/precore/core region mutants related with the hepatocellular carcinoma risk.** A: Frequency distribution of BCP/precore/core and HBsAg mutations (Black, grey and white bars show the viral variants with the highest, median and lowest evidences of association with HCC, respectively); B: Sequences of the 7 patients with preS deletions. BCP: Basal core promoter; HCC: Hepatocellular carcinoma.

preS2 deletion and was simultaneously positive for 3 high-risk mutations in the BCP/precore/core region (T1753V, G1896A and G1899A) (Figure 4A).

Persistence of HCC related HBV strains: In 12 patients with HCC related mutations, it was possible to analyze the persistence of these changes over time by using bulk sequencing [follow-up time (mean  $\pm$  SD):  $24.9 \pm 24.0$  mo; ranging: 5-96 mo]. In 11 cases, the mutations persist during the complete follow-up period, or punctually disappear to latter re-appear in the last samples tested. In the remaining case, the G1899A mutation disappears in stable manner. No changes in the pattern of preS2 deletions were observed in none case (Figure 5).

Liver function tests: Although all the patients are in the grey zone of treatment, with low-moderate fibrosis levels (F0-F2, according the selection criteria of patients), it was observed a significant higher percentage of patients with F2 stage of fibrosis in patients with preS deletions than in the negative ones (Figure 6A). In addition, the behaviour of aspartate aminotransferase (AST) and ALT was quite different between patients with or without preS2 deletions. So, the percentage of patients of patients with fluctuant AST or ALT trend to be higher in patients with preS2, although these differences reached significant values only when analyzing AST behaviour (Figure 6B). Only one patient, with sub-Saharan origin, infected with HBV-E genotype, and



**Figure 4 Analysis of preS deletion mutations related with the hepatocellular carcinoma risk.** A: Frequency of coexistence of hepatocellular carcinoma related mutations in both BCP/precore and preS regions (Dark bars indicate the patients with theoretical highest risk of hepatocellular carcinoma development, based in the coexistence of mutations in the HBsAg and precore region, or the simultaneous detection of 3 or more mutation irrespective of the genomic region); B: Prevalence of preS deletions in different genotypes and ethnic groups. Only a Caucasian patient infected with HBV-H genotype had a preS1 deletion. BCP: Basal core promoter.

with a preS2 deletion, reported alpha fetoprotein abnormal values at the end of follow-up, although at the moment of its inclusion in this work no radiologic evidences of any neoplastic event was confirmed.

## DISCUSSION

In this work we performed a longitudinal retrospective study to analyze the prevalence and persistence over time of HBV mutants related with risk of HCC development in HBeAg-negative/anti-HBe-positive patients within the grey zone of treatment, in order to identify subpopulations susceptible to be treated.

Our results confirm the presence of high-risk HCC mutations in BCP/precore or preS regions, in 19 (61%) patients, using a statistical analysis of ITT, with a higher prevalence in the BCP/precore than in the preS region. The most frequently detected high-risk mutations in the BCP/precore region were the G1896A and G1899A. Indeed, finding these mutations in this group of patients is common and frequently related with the Mediterranean variants in patients with HBeAg-negative serological profile<sup>[14]</sup>. Thus, the G1896A mutation introduces a stop codon at position 28 of the HBc protein that prevents the synthesis of HBeAg<sup>[15,16]</sup>. The simultaneous presence of A1762T and G1764A mutations that are related to a decrease in HBeAg levels<sup>[17-19]</sup> and



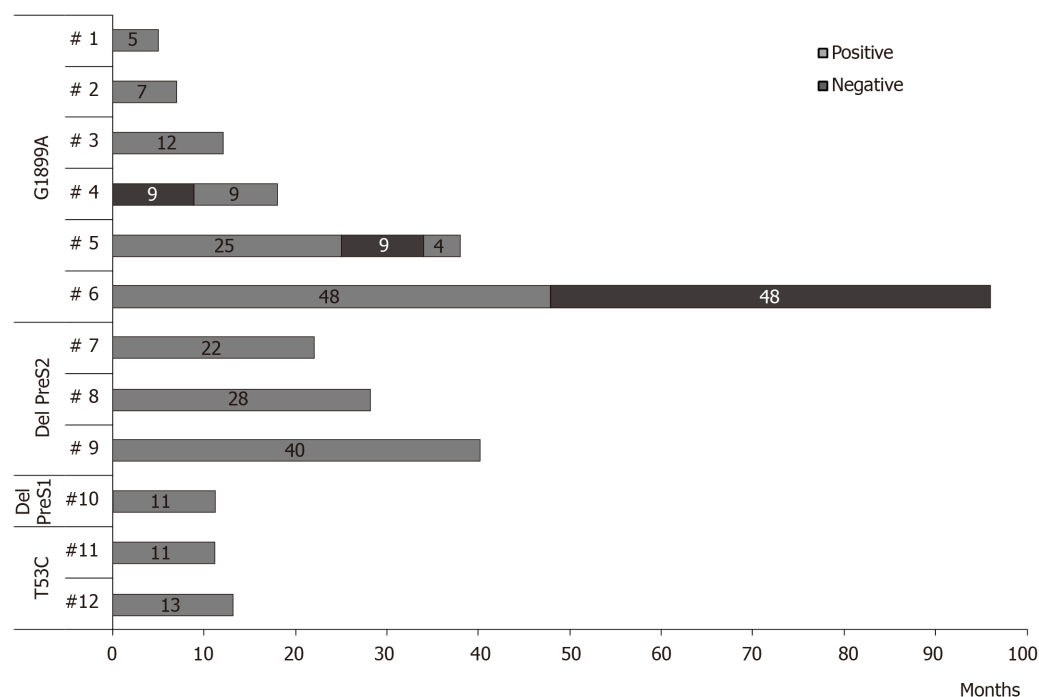


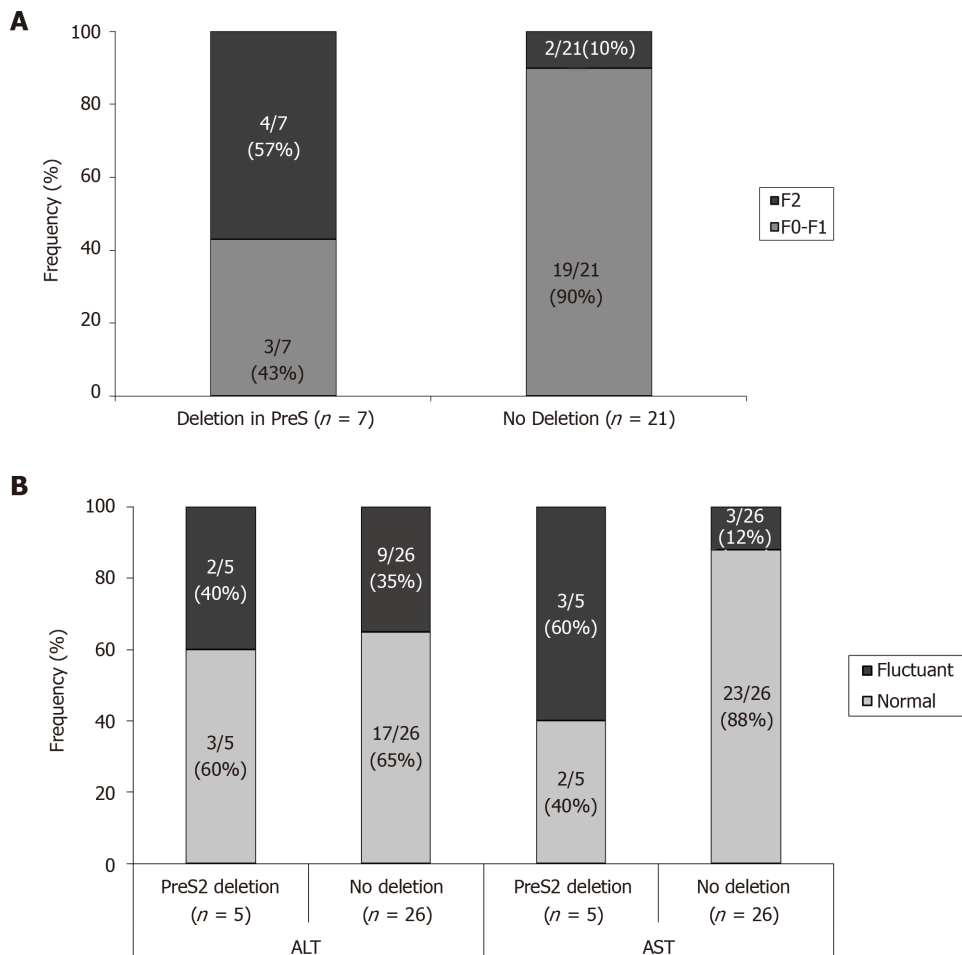
Figure 5 Persistence over time of the high-risk mutations as major population in the patients analyzed.

an increase of the HBV-DNA titers, was also confirmed in 10% of the patients<sup>[20]</sup>. The prevalence of these variants in our cohort of patients was genotype-dependent: BCP variants (C1653T, T1753V, A1762T/G1764A and C1773T) were more frequent in HBV-A genotype, as previously described, except for the C1653T mutation, which was more frequent in HBV-D. Precore/core variant G1899A was more frequent in HBV-E genotype in our cohort, tallying with the published data<sup>[21]</sup>, but also found in a high proportion of patients infected with HBV-A genotype. The prevalence of the G1896A mutation in our patients is consistent with other studies carried out in Spanish population, in which this variant was found with similar frequency in genotypes A and D<sup>[22]</sup>, contrasting with other works conducted in different geographical regions, in which no correlation with the HBV-A genotype was found<sup>[23,24]</sup>. These discrepancies probably reflect that the HBeAg-negative serological profile may present a different genetic basis in at least some HBV genotypes, with a greater representativeness than in other populations of the G1896A mutation in Mediterranean population, in which this serological pattern appears to be more related to the simultaneous presence of A1762T/G1764A double mutation<sup>[22]</sup>.

Although the BCP and precore/core variants have been usually associated to HCC development in meta-analysis studies, a recent work performed in HBeAg-negative Spanish Caucasian patients in the grey zone without antiviral treatment reflects that, in the long term, their clinical evolution is favorable, without progression of liver disease or HCC development in the vast majority of them<sup>[25]</sup>. Considering that the main objective of antiviral treatment is to prevent the fibrosis progression, these data support the idea that treatment does not provide substantial benefits in this group of patients. In this case, the low progression of HBV disease can be related with the low viral replication levels.

HBV-DNA titers have been classically considered as a prognostic marker of HCC development risk<sup>[26]</sup>. The maintenance of HBV-DNA levels undetectable or below 2,000 IU/mL can compensate the risk of harbouring HCC-related variants. However, it cannot be ruled that the accumulation of mutations in both BCP/precore/core region and other viral regions like HBsAg, maintained over time, in combination with persistence of viral replication, even at low or moderate levels are risk factors for HCC development<sup>[9]</sup>. For this reason, we have focused our work on the analysis of the prevalence and persistence over time of the BCP/precore/core and HBsAg regions mutants, present as main strains of HBV detectable by means of bulk sequencing techniques.

The prevalence of preS mutations, which have been correlated with HCC in previous meta-analysis, showed a strong correlation in our cohort with the



**Figure 6 Differences between patients with or without preS deletion mutants.** A: Differences between patients with or without preS deletion mutants at fibrosis level; B: Differences between patients with or without preS deletion mutants at evolution of transaminase levels during follow-up. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

geographical origin and, in consequence, with the HBV genotypes: 75% of our sub-Saharan patients, the majority infected with HBV-E genotype, had deletions in the preS1 or preS2 regions. In addition, the only 2 sub-Saharan patients infected with HBV-A had also deletions in preS2, while this change was not found in any of the remaining patients infected with HBV-A, all of them with Caucasian origin. HBV-E genotype has not been extensively studied until now. Previous studies performed in Germany confirm our results showing that patients in the grey zone of treatment infected with the HBV-E genotype have a higher prevalence of preS1 and preS2 deletions in comparison with HBV-A and D genotypes<sup>[27]</sup>. However, the absolute prevalence rate of these mutations in HBeAg negative patients differs between different studies, ranging from 16% in the German series to 75% in our cohort. The phylogenetic analysis of HBV-E strains from different regions of Africa, indicates that the spread of HBV-E genotype in West Africa is a relatively recent event, as reflected by the conspicuously low genetic diversity of this genotype despite the higher prevalence in this geographical region<sup>[28,29]</sup>. This fact together with the high degree of isolation and scattering of human populations in these regions favours the appearance of viral strains relatively stable over time, limited to specific geographical locations. In this sense, it must be noted that the country origin of the sub-Saharan patients included in our study is located in the South/Central regions of West Africa, corresponding to the area in which the genetic variability of HBV-E is lower, among the countries with high prevalence of this viral genotype<sup>[30,31]</sup>. In this context, the striking high proportion of preS deletions found in sub-Saharan patients in our series could reflect the existence of isolated viral strains of HBV-E in this region with a higher prevalence than that described for neighbouring countries. It may constitute a subpopulation of patients with increased risk of developing HCC by simultaneously sharing high-risk mutations in different regions of HBV genome.

Especially significant was the persistence of the high-risk mutations over time, as

major strains of the viral *quasispecies*, as demonstrated by its identification with bulk sequencing techniques. As regards amino acid substitutions, these were generally very stable, although changes in the composition were observed. Thus, G1899A mutation remained continuously detectable during a follow-up period of up to 48 mo, with sporadic disappearances of the mutant strain in 3 patients and a definitive loss in only 1 patient. This persistence of point mutations in the BCP/precore/core region can be related to the low level of viral replication, which hinders the appearance and selection of mutations that restore the wild-type genotype in the population. Regarding deletion mutations in the preS region, these were maintained without sporadic eliminations during the follow-up in all the patients who carried them, with proven periods of up to 40 mo. The persistence, without fluctuations, of these deletions seems to influence the presence in the geographical origin of the patients in which these strains are majority. Under these conditions, exclusive infection with deleted strains would make the emergence of wild-type strains impossible due to the absence of a genetic template on which to act. The persistence over time of these mutations favours their accumulation, as major strains, in chronic infected patients. Thus, it was possible to confirm in our patients, not only the coexistence of the mutations A1762T/G1764A and G1896A/G1899A widely documented and associated with a high-risk of carcinogenesis, but also the accumulation of 3-5 risk mutations in other 5 patients, including a case that presented 3 risk mutations in the BCP/precore/core in combination with a deletion in the preS region. In any case, the persistence and accumulation over time of these mutations may have clinical relevance, since it perpetuates the molecular processes by which these viral variants favour the development of HCC.

The selection in this work of patients in the grey zone of treatment, with low-moderate fibrosis levels and normal or marginally elevated transaminase levels, makes it difficult to evaluate unless through a long-term follow-up, the possible harmful effect of the presence of these mutations on the progression of liver disease. However, and even recognizing the limitations of transient elastometry techniques for discriminate patients with low or moderate stages of fibrosis, our data showed a higher prevalence of F2 stage in patients with preS2 deletion than in those in which this mutation was not detected. On the other hand, the behaviour of AST and ALT was quite different between both types of patients, with is a higher percentage of patients with fluctuant AST or ALT levels during follow up in patients harbouring preS deletions.

These results focus an increased risk of HCC development in sub-Saharan patients. Africa represents one of the most HBV endemic regions in the world and HCC mainly due to CHB infection is a major cause of premature death, suggesting that viral strains located in Africa, which mostly belong to HBV-A, are more likely to cause HCC<sup>[32]</sup>. In this way, occult HBV infection was shown to be present in 75% of Black Africans with HCC, and genotype A was shown to be 4.5 times more likely than other genotypes to cause HCC in Black Africans, whilst tumours occurred at a significantly younger age<sup>[33]</sup>. Our findings also correlate a higher risk of HCC development with HBV-E, which almost exclusively occurs in African people. Moreover, it has been linked the distribution of HBV genotype E infection with African countries with high incidences of HCC<sup>[34]</sup>.

In conclusion our data indicate that the presence of preS mutations should be assessed in patients with sub-Saharan origin, especially if they are infected with HBV-E and HBV-A genotype, in order to identify subpopulations of patients in which the antiviral treatment can be indicated to minimize the risk of HCC due to accumulation of high-risk HBV genetic variants. In this sense, the development of detection systems for deletion mutations in preS region is technically feasible with conventional techniques of Molecular Biology available for most centers. Its use, especially if it is restricted to preselected subpopulations, can help the treatment decision in patients in grey area of treatment.

The most important limitations of this study are: (1) The number of patients. It must be increased to elucidate the significance of prevalence data of some mutations; (2) The necessity to perform longitudinal studies with longer follow-up periods to clarify the progression of liver disease in this kind of patients. However, our data confirm the accumulation and persistence of HBV mutants related with HCC in different regions of HBV genome especially in the subpopulation of sub-Saharan origin.

## ARTICLE HIGHLIGHTS

### Research background

The indication of treatment in patients with chronic hepatitis B in the grey zone is not clear, and

it is necessary to balance the risks and benefits for health outcomes, including the evaluation of the risk of hepatocellular carcinoma development.

### Research motivation

To optimize the management of patients in the grey zone of treatment in order to identify suitable subpopulations for antiviral treatment.

### Research objectives

To analyze the prevalence, and persistence over time, of hepatitis B virus (HBV) mutants that predispose to the development of hepatocellular carcinoma in patients in the grey zone of treatment.

### Research methods

We analyzed the presence of basal core promoter/precore/core and preS deletion mutants related with hepatocellular carcinoma development in 106 samples from 31 patient in the grey zone of treatment.

### Research results

A significant number of the patients analyzed in this work shows hepatocellular carcinoma related mutations. All these hepatocellular carcinoma related mutants are major viral strains and persist over time. Some patients have hepatocellular carcinoma related mutants in basal core promoter/precore/core and preS regions simultaneously. The presence of preS deletions is associated with sub-Saharan subpopulations infected with HBV-A and HBV-E genotypes.

### Research conclusions

The presence of preS mutations should be assessed in patients with sub-Saharan origin, especially if they are infected with HBV-E and HBV-A genotype, in order to identify subpopulations of patients in whom the antiviral treatment could be indicated to minimize the risk of hepatocellular carcinoma due to accumulation of high-risk HBV genetic variants.

### Research perspectives

The analysis of preS deletions could be indicated in the management of sub-Saharan patients in grey zone of treatment. Analysis of higher sample size populations during long-time longitudinal follow-up should be further performed in these patient subpopulation.

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