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DIFFERENT MUTATION RATES IN THE BASAL CORE PROMOTER AND PRECORE REGIONS BETWEEN HEPATITIS B VIRUS SUBGENOTYPE F1B CLUSTERS

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INTRODUCTION

Hepatitis B virus (HBV) is the main etiological agent of hepatocellular carcinoma (HCC) worldwide. HBV has been classified into nine genotypes (designated A to I) and several subgenotypes, with uneven global distribution. There is growing evidence that the viral genotype influences the course and outcome of chronic hepatitis B infection. In particular, subgenotype F1b is the most prevalent in Argentina, Chile and Peru, and is also present in the Alaska Native population. Numerous studies carried out in Alaska and Peru have shown a strong association of subgenotype F1b infection with a higher risk of progression to HCC [1,2]. The age-standardized incidence rate (ASR) per 100 000 people of HCC in Alaska and Peru is higher than 5; while in Argentina and Chile it is lower than 3 [3]. Recently, two evolutionarily different clusters of the subgenotype F1b, called basal and cosmopolitan, have been described which differ by eight nucleotides throughout the genome [4]. Phylogenetic analysis of the subgenotype F1b clusters showed that the basal cluster included isolates mainly from Alaska and Peru, while the cosmopolitan cluster is mostly constituted by sequences from Argentina, Chile and Uruguay or areas where the subgenotype F1b seems to be foreign.

On the other hand, several studies have shown that mutations that arise during the course of chronic infection are closely associated with the progression of liver disease [5]. The most widely

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described are detected in the basal core promoter (BCP) and preCore regions (pC), which modulate the HBV e-antigen (HBeAg) expression. In particular, the G1896A mutation creates a premature stop codon (28th codon) that prevents the production of HBeAg, and the A1762T and G1764A double mutation in the BCP region down regulate the synthesis of HBeAg and enhance viral replication. The HBeAg abrogation is a late event in the course of chronic infection and may lead to HBeAg-negative chronic infection (formerly known as inactive carriers) or HBeAg-negative chronic hepatitis.

AIM

To characterize the frequency of HBeAg negativity and the mutation patterns in the BCP/pC region of the basal and cosmopolitan clusters of the subgenotype F1b.

METHODS

From a blood bank of 247 samples from patients with chronic HBV infection, 68 cases infected with the F1b subgenotype were identified and included in the study.

HBeAg status was determined using the Elecsys HBeAg on a Cobas e801 instrument (Roche, Mannheim, Germany). HBV DNA was extracted from 200 µl of serum and the BCP and pC regions were amplified by nested PCR (nt 1700-2350). PCR products were purified, and direct sequencing was carried out in a 3500xl Genetic Analyzer (Applied Biosystems) in both amplification senses.

The subgenotype F1b cluster was assessed by phylogenetic analysis. Nucleotide sequences of the BCP/pC region representing the basal and cosmopolitan clusters were retrieved from GenBank and used as references. Phylogenetic relationships were evaluated using the maximum likelihood method.

Fisher's two-tailed exact test and the corrected X^2 test were used to compare qualitative data. ANOVA and non-parametric tests (Mann-Whitney U and Kruskal-Wallis H) were used to compare quantitative variables. Data analysis was performed by the statistical software package SPSS (version 10.0, SPSS, Inc., Chicago, USA).

RESULTS

Of the 68 cases included in this study, 22 belonged to the subgenotype F1b basal cluster and 46 to the cosmopolitan cluster (Table 1). No differences were observed in terms of gender or age of the patients between both clusters.

The HBeAg negative rate was slightly higher in the basal cluster in relation to the cosmopolitan one. Remarkably, significant differences were observed in the rate of mutations associated with the loss of HBeAg between the two clusters.

Among the HBeAg-negative patients, 22 were chronic infections (persistently normal ALT levels and HBV-DNA below 2,000 IU/ml) and 14 chronic hepatitis (abnormal and/or fluctuating ALT levels and viral load > 20,000 IU/ml). Four cases with cirrhosis (3 HBeAg positive and 1 HBeAg negative) and one case of HCC (HBeAg positive from the basal cluster) were identified in the cohort.

The A1762T/G1764A and G1896A mutations were more frequently found in the basal samples compared to the cosmopolitan ones (85.7 and 92.9 % vs 50 and 18.2%, respectively). Interestingly, no HBeAg loss-associated mutations were observed in 7.1 and 36.4% of the basal and cosmopolitan cases, respectively.

Table 1. Frequency of mutations in the BCP/pC region according to the subgenotype F1b cluster.

	Basal	Cosmopolitan	p
Cases (n)	22	46	
Age in years (mean ± SD)			
All cases	46.6 ± 10.4	46.3 ± 16.8	0.45
HBeAg-positive	42.8 ± 12.1	42.4 ± 16.4	0.71
HBeAg-negative	50.3 ± 7.3	51.9 ± 16.4	0.39
Gender (f/m)	8/14	8/38	0.06
HBeAg-negative (%)	14 (63.6)	22 (47.8)	0.16
Chronic infection	11 (78.6)	11 (50.0)	0.08
Chronic hepatitis	3 (24.4)	11 (50.0)	
Cirrhosis or HCC	2 (9.1)	3 (6.5)	0.53
Mutations (%)†			
T1753C	4 (28.6)	6 (27.3)	0.61
A1762T/G1764A	12 (85.7)	11 (50.0)	**0.03**
G1896A	13 (92.9)	4 (18.2)	**<0.01**
G1899A	4 (28.6)	2 (9.1)	0.14
A1762T/G1764A plus G1896A	11 (78.6)	3 (13.6)	**<0.01**
Others pC	4 (28.6)	6 (27.3)	0.61
None	1 (7.1)	8 (36.4)	**0.05**

†Mutation frequencies were determined considering HBeAg-negative cases only. Others include mutations disrupting pC gene initiation codon (n: 3) or frameshift mutations (n: 7). Statistically significant differences are shown in bold.

DISCUSSION

The two subgenotype F1b clusters have constrained geographical distribution, with the particular feature that the basal cluster is present in regions of high HCC incidence associated with HBV infection, while the Cosmopolitan cluster is found in regions of low HCC incidence. Likewise, both clusters have shown *in vitro* different biological properties in terms of replication and expression of viral antigens [4].

The loss of HBeAg is a late event in the natural history of HBV infection that can lead to chronic HBeAg-negative infection or chronic hepatitis. In order to perform an in-depth characterization of the subgenotype F1b clusters, the frequency of HBeAg negativity and the mutation patterns associated with HBeAg loss were analyzed.

The difference in HBeAg negativity rate observed between both clusters suggests that the infections in the basal group occurred earlier, at a younger age than in the cosmopolitan group, or that the infections in the cosmopolitan group have delayed HBeAg seroconversion in comparison with the basal group, as has been reported when comparing infections caused by genotypes B and C.

The pathophysiology of the hepatocellular carcinoma is a multifactorial event, influenced by host factors (such as gender, genetic background, and behavior) as well as viral factors such as genotype and mutations that occur during the course of chronic infection. Particularly, both A1762T/G1764A and G1896A mutations were associated with an increased risk of developing cirrhosis and/or HCC.

Of note, it was shown that the different HBeAg abrogation-associated mutants have different implications for HBV biology, either in viral replication rate or antigen expression levels, and consequently might have different implications in the progression of chronic infection [6].

In the present study, significantly higher mutation rates in the BCP (A1762T/G1764A) and pC (G1896A) regions were observed among the sequences of the basal cluster compared to the cosmopolitan ones. This finding is further supported by sequences of the subgenotype F1b retrieved from other regions from the Genbank where 92.3 and 46.2% of the HBeAg-negative sequences belonging to the basal cluster carry the A1762T/G1764A and G1896A mutations, respectively. Interestingly, about a third of the HBeAg-negative cases in the cosmopolitan cluster did not show mutations associated with HBeAg loss. It has been shown that those cases without mutations in the pC region that achieve the HBeAg-negative stage, as consequence of the strong control of viral replication by the host's immunity, would have a better prognosis than mutation-carrying cases [7].

The molecular basis associated with the HBeAg-negative phenotype is stated to be driven by the stability of the encapsidation signal. The paradigm states that the 1858T-carrying genotypes (B, C2, D, E, F1b, and F4) abolish HBeAg expression primarily by selecting the G1896A mutation, which, in

addition to preventing HBeAg expression by replacing a residue of tryptophan at amino acid position 28 with a premature stop codon, it enhances the stability of the encapsidation signal through base pairing (1858T:A1896). Whereas, in genotypes carrying 1858C (A, C1, F2, F3, and H), instead of G1896A, which destabilizes the encapsidation signal (1858C:A1896), other mutations are usually present, such as disruption of pC gene start codon, nonsense or frameshift mutations.

Interestingly, both subgenotype F1b clusters carry 1858T. However, G1896A mutation is rarely observed in the cosmopolitan cluster. This controversy was also previously observed in some subgenotypes of genotype B [8]. Therefore, the choice of the mechanism that a given genotype uses to regulate HBeAg expression is not fully explained by the structure of the encapsidation signal, suggesting that 1858T would be necessary but not sufficient to promote the emergence and selection of G1896A. The observed difference in the mutation pattern associated with HBeAg loss between clusters suggests that some of the eight cluster-related polymorphisms outside the precore region might play a role in the mechanism of choice.

In conclusion, this study provides new insights into the pathways associated with the loss of HBeAg in the subgenotype F1b clusters. In particular, the different rate of mutations associated with a more severe course of chronic hepatitis in the basal cluster would support the difference in the HCC incidence rate in the geographical regions where the basal cluster is restricted. Moreover, the molecular pattern associated with the loss of HBeAg in the cosmopolitan cluster challenges the paradigm of the encapsidation signal stability as the sole driving force of selection.

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