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Genotypes B and C hepatocellular carcinoma–associated hepatitis B virus pre-S mutants: their detection among F1b and A2 – but not F4 – isolates from Argentina

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SUMMARY. Prevalence rates of hepatocellular carcinoma (HCC)-associated hepatitis B virus (HBV) pre-S mutants among most genotypes are still lacking. In this study, viral (sub)genotypes of 70 Argentine nucleotide sequences (33 newly obtained) were determined by phylogenetic analysis, and the presence of such mutants was assessed in the American continent for the first time. Nucleotide substitutions of the pre-S2 start codon were observed in 10% of the HBV/A2 sequences. Ten per cent of the HBV/A2 and 12.5% of the HBV/F1b – but none of HBV/F4 – exhibited a deletion in the pre-S1/pre-S2 region. The contribution of these variants to liver cirrhosis (LC) and/or HCC development among HBV/F and HBV/A isolates deserves further prospective clinical studies.

Keywords: Argentina, hepatitis B virus, hepatitis B virus genotypes, hepatocellular carcinoma, pre-S mutants.

INTRODUCTION

Chronically infected patients with hepatitis B virus (HBV) are at risk of developing liver cirrhosis (LC) and hepatocellular carcinoma (HCC), the third most common cause of cancer mortality. Detecting HCC-associated HBV mutations is useful for the development of HCC screening and prevention strategies.

Nine HBV genotypes (HBV/A-HBV/H and HBV/J) and a controversial tenth (HBV/I?) have been reported based on the differences in full-length genomes [1]. They show different geographical distributions [1], displaying distinct mutation patterns at the pre-S and precore regions, which are considered independent risk factors for HCC [2]. In some studies, however, an increased risk of HCC was associated with specific HBV mutations in such regions, irrespective of their genotypes [2].

HBV pre-S region consists of pre-S1 [nucleotides (nt) 2848–3204] and pre-S2 (nt 3205–154) domains, which contain B- and T-cell epitopes and several functional and structural sites essential for the viral life cycle. The most frequently reported pre-S mutations are deletions, followed by pre-S2 start codon mutations [3]. These mutants correlate with the severity of HBV liver disease, because its emergence and selection become increasingly more prevalent as chronic HBV infection (CHB) progresses from the inactive carrier state to LC or HCC. Pre-S mutants might worsen liver diseases by causing cytotoxicity, suggesting they are a significant risk factor for HCC.

The prevalence of pre-S mutants was reported to be highly correlated with HBV/C compared to other genotypes [2–4]. However, all these studies were performed in Asia where HBV infection is endemic and genotypes B and C prevail. A common drawback in the published reports is the absence of samples assigned to other genotypes. Only one of them [4] determined the prevalence of pre-S deletions in samples ascribed to genotypes A–H, but they included a limited number from the most prevalent genotypes in Latin America: 32 samples of HBV/A and five of HBV/F (n = 387 samples encompassing the whole population). Owing to the strong association between HBV/F1b (sub)genotype and the early development of HCC, and the lack of association between such disease and precore mutants in these samples [5], it seems to be essential to determine the prevalence of pre-S mutants among those isolates ascribed to this genotype. Therefore, the

Abbreviations: CHB, chronic HBV infection; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

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aim of this study was to retrospectively assess the prevalence of pre-S deletions and pre-S2 start codon mutations among the HBV genotypes circulating in Argentine patients.

MATERIALS, PATIENTS AND METHODS

The presence of pre-S mutants was initially assessed among all nucleotide HBV pre-S1/pre-S2 sequences of Argentine origin deposited in GenBank (n = 37 until February 2012). While 29 of them (78.4%) were obtained from Buenos Aires city, the remaining 8 (21.6%) were from the Northern region of Argentina. All samples had been collected between 2000 and 2009. To increase the sample size (up to n = 70), serum and epidemiological data were collected from 33 nonconsecutive randomly selected Argentine patients diagnosed with CHB [HBsAg and anti-HBc total antibodies (+)] during the period 2000–2010, who had been referred to our Institute. Twenty patients (85% male; mean age ± standard deviation = 37.5 years ± 13.1; elapsed time of infection = 3.4 years ± 4.2) were from Buenos Aires city, which has been described as a HBV low-prevalence area. The remaining 13 (69% male; 22.2 years ± 3.4; elapsed time of infection = 10.8 years ± 1.2) were residents of Gualeguay, a town suspected to be a high-prevalence area, where most infections appears to occur at an early age [6]. The demographic, clinical and histological characteristics of the 33 patients recruited by this study are shown in Table 1.

Serological tests for HBeAg and anti-HBe antibodies were carried out in all collected samples (AxSYM; Abbott, North Chicago, IL, USA). Viral DNA from the pre-S1/pre-S2 region was amplified by a previously described hemi-nested PCR (nt 2814–256) [7]. Amplicons – even those with an unexpected different size (Supplementary Figure S1) – were purified from the agarose gel (QIAGEN Gel Extraction Kit; QIAGEN, Hilden, Germany)) and bidirectionally sequenced (Applied Biosystems, Foster City, CA, USA). Viral (sub)genotypes were determined by neighbour-joining phylogenetic analysis using the PHYLIP package version 3.5c (Joseph Felsenstein, Department of Genome Sciences and Department of Biology, University of Washington, Seattle, WA, USA). The presence of deletions and/or nucleotide substitutions at the Pre-S2 start codon was analysed in all sequences by alignment with wild-type isolates using BioEdit Sequence Alignment Editor, version 7.0 (Thomas Hall, Ibis Biosciences, Carlsbad, CA, USA).

RESULTS

A statistically significant difference was revealed when the results of the serological tests for HBeAg and anti-HBe antibodies were compared according to their geographical location: 75% of the samples from Buenos Aires were HBeAg (+), while only 15.4% of those from Gualeguay exhibited this marker ($P < 0.01$), a difference that might be associated with an earlier exposure to the virus in the latter group (Table 1).

Thirty-nine of the 70 sequences were ascribed to HBV/F (55.7%), 24 to subgenotype F1b and 15 to F4; 24 to HBV/A (34.3%), 20 to A2 and 4 to A1; 2 to subgenotype D1 (2.9%); 2 to HBV/H (2.9%); one sequence was a recombinant D3/A2 strain (1.3%) (Supplementary Figure S2); and the remaining two corresponded to mixed A2-F1b infections (2.9%). The latter result was confirmed by RFLP of the HBV S gene PCR products [8]. No statistically significant differences were observed when genotype prevalence was analysed in Buenos Aires and Gualeguay cities.

Pre-S mutants were observed in six of the 33 samples collected in this study (18.2%): four of them from Buenos Aires (4/20; 20%) and the remaining two from Gualeguay (2/13; 15.4%; $P > 0.05$). Interestingly, no pre-S mutants were observed in the group of Argentine sequences from GenBank (0/37; 0%; newly obtained *vs* downloaded sequences $P < 0.01$). Of the 70 analysed sequences, pre-S deletions spanning from the 3′ terminus of pre-S1 to the 5′ terminus of pre-S2 were observed in three of 24 samples assigned to HBV/F1b (12.5%) and two of 20 to HBV/A2 (10%; $P > 0.05$). Only two samples – ascribed to HBV/A2 (10%) – showed nucleotide substitutions at the pre-S2 start codon (Table 1).

Interestingly, mutant HBV isolates were present together with wild-type ones forming a mixed viral population (Figure S1 and Table 1) in three samples (BUE7, GU7 and GU8). In contrast, sample BUE10 (Supplementary Figure S1 and Table 1) revealed a mixed viral population consisting of two mutated variants that exhibited: (i) a deletion at the pre-S1/pre-S2 region; and (ii) a conserved nucleotide extension with a point mutation at the pre-S2 start codon.

No statistically significant differences were observed when the presence of pre-S mutants was analysed with regard to the gender, age, geographical, clinical and/or histological features of the patients. Despite the differences in age and elapsed time of infection ($P < 0.01$ for both characteristics) observed between patients from Buenos Aires and Gualeguay, there was not statistical difference between the detection of pre-S mutants in both areas. Besides, the presence of these mutants among patients from Buenos Aires was significantly associated with the HBeAg minus phenotype [three HBeAg (−)/four showing pre-S mutants *vs* two HBeAg (−)/16 without such mutants; $P < 0.01$].

DISCUSSION

In this study, the prevalence of reportedly known genotypes B and C HCC-associated HBV pre-S mutants was reported among patients from Argentina. Moreover and most importantly, they were detected for the first time among 12.5% of HBV/F1b – but not F4 – and 10% of HBV/A2 isolates. Interestingly, four of the six isolates exhibiting pre-S mutants (BUE7, BUE10, GU7 and GU8; Table 1) showed mixed populations (wild-type and mutated pre-S/S genomes, or combination of mutated variants), likely reflecting a viral strategy of immune evasion.

Table 1 Characteristics of the studied patients with CHB

Sample*	Gender	Age (in years)	Years of infection†	HBeAg/ anti-HBe Ab	Transaminases	HBV genotype	Pre-S deletions and/or mutations	Liver biopsy‡
BUE1	F	43	1	+/−	Normal	D1	No	Necroinflammatory activity. 5/18; Stage 0/6; slight hemosiderosis I/IV
BUE2	M	31	1	+/−	Elevated	A2	No	Necroinflammaotory activity. 7/18; Stage 2/6; METAVIR Score F1A1
BUE3	M	23	2	+/−	Elevated	A2	No	Necroinflammatory activity. 7/18; Stage 2/6; METAVIR Score F1A1; 35% macrovesicular diffuse steatosis
BUE4	M	34	2	+/−	Normal	F1b	No	Chronic hepatitis; Stage 2 Perls (−)
BUE5	M	26	1	+/−	Elevated	F1b	No	METAVIR Score F2A1
BUE6	M	45	2	+/−	Elevated	F1b	No	Necroinflammatory activity 14/18; Stage 3/6; METAVIR Score F2A3
BUE7	M	25	4	+/−	Normal	A2	Two viral populations detected: (i) pre-S2 start codon deleted (nt 3070 to nt 12) and (ii) wild type	Necroinflammatory activity 10/18; Stage 2/6; Knodell Score 9
BUE8	M	44	2	−/+	Elevated	A2	Pre-S2 start codon mutated (ATG → GTC)	Necroinflammatory activity 14/18; Stage 3/6; METAVIR Score F2A3
BUE 9	M	29	2	+/−	Elevated	F4	No	Necroinflammaotory activity. 7/18; Stage 2/6; METAVIR Score F1A1
BUE10	M	72	2	−/+	Normal	A2	Two viral populations detected: (i) pre-S2 start codon mutated (ATG → ACG) and (ii) pre-S2 start codon deleted (nt 3069 to nt 10)	Necroinflammatory activity 7/18; Stage 6/6; hemosiderosis II/IV
BUE11	M	28	4	−/+	Normal	F1b	Pre-S2 start codon deleted (nt 3167 to nt 35)	Necroinflammatory activity 13/18; Stage 3/6
BUE12	M	34	4	+/−	Elevated	A2	No	METAVIR Score F1A1
BUE13	M	46	3	+/−	Elevated	A2	No	Strong necroinflammatory activity, moderate fibrosis

Table 1 Continued

Sample*	Gender	Age (in years)	Years of infection†	HBeAg/ anti-HBe Ab	Transaminases	HBV genotype	Pre-S deletions and/or mutations	Liver biopsy‡
BUE14	M	58	5	+/−	Normal	A2	No	Strong necroinflammatory activity; moderate fibrosis; Macrovesicular steatosis
BUE15	M	31	1	−/+	Elevated	A1	No	S. Ishak act. 9/18; Stage 2/6; METAVIR Score F1A2
BUE16	F	63	6	+/−	Normal	D1	No	Necroinflammatory activity 13/18;Stage 3/6; METAVIR Score F2A3
BUE17	M	18	1	+/−	Normal	A2	No	METAVIR Score F1A1
BUE18	M	39	3	+/−	Elevated	F1b	No	Moderate necroinflammatory activity; Stage: incomplete cirrhosis
BUE19	F	33	20	−/+	Normal	F1b	No	METAVIR Score F0A1
BUE20	M	63	2	+/−	Elevated	F1b	No	METAVIR Score F4A1
GU1	F	20	12	−/+	Normal	A2 + F1b	No	METAVIR Score F4A1
GU2	M	20	12	−/+	Normal	F1b	No	METAVIR Score F4A2
GU3	M	23	12	+/−	Normal	A2	No	METAVIR Score F3A2
GU4	M	22	11	−/+	Normal	F1b	No	METAVIR Score F1A2
GU5	M	21	11	+/−	Normal	A2	No	METAVIR Score F1A1
GU6	F	19	11	−/+	Normal	A2	No	METAVIR Score F2A1
GU7	M	21	12	−/+	Normal	F1b	Two viral populations detected: (i) pre-S2 start codon deleted (nt 3114 to nt 9) and (ii) wild type	METAVIR Score F2A1
GU8	M	18	12	−/+	Normal	F1b	Two viral populations detected: (i) pre-S2 start codon deleted (nt 3120 to nt 12) and (ii) wild type	METAVIR Score F1A1
GU9	F	23	10	−/+	Normal	A2	No	METAVIR Score F1A1
GU10	M	26	10	−/+	Normal	A2 + F1b	No	METAVIR Score F1A1
GU11	M	20	10	−/+	Normal	F1b	No	METAVIR Score F2A1
GU12	F	26	10	−/+	Normal	A2	No	METAVIR Score F2A2
GU13	M	30	8	−/+	Normal	A2	No	METAVIR Score F2A1

KNODELL Score 9: Moderate inflammatory activity. METAVIR Score: F1: Portal fibrosis without septa; F2: Portal fibrosis with few septa; A1: Mild necroinflammatory activity; A3: Severe necroinflammatory activity. Hemosiderosis II: Diffuse hemosiderosis in periportal and midzonal hepatocytes. *BUE: Buenos Aires; GU: Gualeguay. †Elapsed time taking into account the date of the initial diagnosis. ‡ISHAK Score: Stage 2/6: Minimal liver scarring around liver blood vessels; Stage 3/6: Scarring extended out from liver blood vessels; Stage 6/6: Cirrhosis.

Pre-S mutants have been detected among the newly obtained HBV Argentine sequences, but not from those downloaded from GenBank, which corresponded to samples collected at approximately the same period of time and from overlapped or adjacent geographical areas. The reason for such difference is at present unknown, as no statistical studies may be carried out because of the lack of information regarding the elapsed time of infection and/or the histological status in most of the publications.

Although a reportedly known association has been observed between pre-S mutants and HBV/C and – to a lesser extent – HBV/B in Asia [2,3], a putative relationship of these mutants with other genotypes is at present not sufficiently supported. Whether or not they are also associated with LC and/or HCC in A2 and F1b genotypes in South American patients remains to be established.

The presence of these genotypes not only in Latin America, but also in Europe and USA as a consequence of an increasing trend of human migrations highlights the global relevance of these results. The contribution of the pre-S mutants to LC and/or HCC development among HBV/F and HBV/A isolates deserves further prospective clinical studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ETHICAL APPROVAL

All patients provided their informed written consent to perform the study, which was approved by an Ethics Committee on Research (CIEI-FFYB-UBA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1: HBV pre-S1/pre-S2 PCR products were run in 1% agarose gel stained with ethidium bromide. 1: 100 base pair molecular size ladder (PBL, UNQ, Argentina); 2: PCR Negative control; 3: Sample GU6 (wild type); 4: Sample BUE6 (wild type); 5: Sample BUE10 (mixed viral population: wild type and deleted amplicons); and 6: Sample BUE11 (deletion in pre-S1/pre-S2 region).

Fig. S2: A phylogenetic neighbour-joining tree constructed by using partial HBV pre-S1/pre-S2/S region sequences (encompassing nt 2814-256) from HBV genotypes A-J. Strains isolated from CHB patients from Buenos Aires (BUE) and from Gualeguay (GU) are indicated in green and blue, respectively. The GenBank /EMBL / DDBJ accession numbers of the sequences reported in this paper are: JN393255-JN393291. Argentine-origin nucleotide sequences retrieved from GenBank are indicated in bold. Samples GU1 and GU10 were not included because they correspond to

mixed A2/F1b infection. Bootstrap values are indicated in the tree roots. The Pre-S1/Pre-S2/S region of the genome of the woolly monkey hepadnavirus, the most divergent among primate hepadnaviruses, was included as an outgroup sequence (AF046996).

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