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Characterization of the basal core promoter and precore regions in anti-HBe-positive inactive carriers of hepatitis B virus

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SUMMARY

Background: The study of hepatitis B virus (HBV) genomic heterogeneity has become a major issue in investigations aimed at understanding the relationship between HBV mutants and the wide spectrum of clinical and pathological conditions associated with HBV infection. Although most chronically infected HBV patients are inactive carriers, several virological aspects of this state remain unclear.
Methods: In order to determine the prevalence and clinical significance of mutations in the basal core promoter (BCP) and precore (pC) regions among inactive carriers, the nucleotide sequences from 41 inactive carriers were analyzed and compared with those from 29 individuals with chronic active hepatitis.
Results: Genotypes A (24.3%), D (37.1%), F1b (12.9%), and F4 (18.6%) were the most prevalent. Mutations in the BCP/pC regions were observed in most of the inactive carriers (92.7%) and in most of the patients with chronic active hepatitis (93.1%). The prevalence of mutation 1764^{A} was significantly higher in patients with chronic active hepatitis (65.5%) than in inactive carriers (36.6%) ($p = 0.038$), whereas the prevalences of mutations at the other positions analyzed were not significantly different. Older patients (>50 years) showed BCP/pC patterns with a higher number of substitutions. Mutations were found to be biased by genotype: the 1896^{A} mutation was highly prevalent in genotypes D and F4, while alternative substitutions in the pC region were more prevalent in genotypes A and F1b.
Conclusions: Mutations in the BCP/pC regions are the hallmark of chronic anti-HBe-positive individuals; nevertheless, the even distribution of mutations in active and inactive carriers suggests that BCP/pC mutations may occur during HBV infection not strictly related to the HBV infection activity.

1. Introduction

Hepatitis B virus (HBV) infection is a challenging global health problem, affecting an estimated two billon people worldwide.[1,2] Of those infected, 400 million remain infected chronically and it has been estimated that one million die annually from HBV-related liver diseases.[3,4] Infection with HBV causes a wide spectrum of disease manifestations, ranging from acute self-limiting to chronic infection, with disease activity varying from an asymptomatic infection to chronic active hepatitis. Patients infected chronically may eventually develop liver failure, cirrhosis, or hepatocellular carcinoma.[5–7]

During the course of chronic infection, a large proportion of HBV surface antigen (HBsAg) carriers seroconvert from HBV e antigen (HBeAg) to anti-hepatitis B e antibody (anti-HBe)-positive, which represents a late phase in the natural history of chronic infection.[8,9] The great majority of anti-HBe-positive patients, who are characterized by persistently normal alanine aminotransferase (ALT) levels (<40 U/l), low HBV-DNA levels (<4 $\log_{10}$ copies/ml), and the absence of histological liver injury (necroinflammation score ≤4), are considered inactive carriers[10,11] and appear to have favorable clinical outcomes in the vast majority of cases.[12,13] The inactive HBV carrier state is one of the most intriguing and common conditions observed in HBsAg carriers.

Mutations reducing or abrogating HBeAg production may occur either at the transcriptional level, in regulatory elements, or at the translational level, blocking HBeAg synthesis because of nucleotide insertions, deletions or mutations that abolish precore region (pC) expression.[14]

The basal core promoter (BCP) region (nucleotides (nt) 1742–1849) plays a central role in both HBV replication and

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morphogenesis,[15] directing the transcription of precore RNA (pC-mRNA) and pre-genome RNA (pg-mRNA) messengers. Mutations occurring in this region may influence both viral replication and HBeAg secretion.[16–20] On the other hand, the pC region (nt 1814–1900) contains the encapsidation signal, whose stability is important for the packaging of pg-RNA and for the priming of genomic replication.[21]

Several mutations located in these regions have been associated with the abrogation of HBeAg expression.[14] In particular, HBV variants carrying the double $1762^{T}/1764^{A}$ mutation in the BCP region and/or the 1896^{A} mutation in the pC region have been presumed to be strictly associated with forms of progressive liver disease.[20,22,23]

The study of the genomic heterogeneity of the BCP/pC regions is the key to understanding the relationships between major HBV mutants and the wide spectrum of clinical and pathological conditions associated with HBV infection.[24–26]

Since most of the studies in this field have been performed in patients with chronic active hepatitis, there is a paucity of information about genomic heterogeneity in the inactive carriers. This aspect is worthy of elucidation considering the apparent importance of viral variability in the outcome of HBV-related liver diseases.[20]

The purpose of this study was to determine the prevalence and clinical significance of mutations in the BCP/pC regions among inactive carriers and to compare them with control anti-HBe-positive patients with chronic active hepatitis.

2. Patients and methods

2.1. Patients

This retrospective study included 70 untreated HBeAg-negative/anti-HBe-positive chronic HBsAg carriers (median age 46.8 years, range 21–77; 40 males and 30 females) admitted to the Hepatology Unit of the Hospital Italiano Buenos Aires and to the Hospital de Infecciosas "F. Muñiz", Buenos Aires, Argentina, between 2004 and 2009. For inclusion, patients had to be: HBsAg-positive, negative for antibodies against hepatitis C virus (HCV) and human immunodeficiency virus (HIV), and with no history of autoimmune liver disease or high alcohol intake (more than 10 g/day). The patients were examined at least three times a year and followed for at least 2 years in order to define their clinico-pathological profiles. At the end monitoring, HBsAg carriers were classified as inactive or active according to the presence or absence of the following biochemical and virological profiles: persistence of ALT serum levels below normal values (<40 U/l) and persistence of serum HBV-DNA <4 $\log_{10}$ copies/ml. The study was performed according to the principles of the Declaration of Helsinki and was approved by the local ethics committee.

2.2. Serology and histology

Liver function tests included the evaluation of aspartate aminotransferase and ALT, gamma-glutamyl transpeptidase, alkaline phosphatase, albumin, globulins, total bilirubin, prothrombin time, and α-fetoprotein; tests were routinely performed. HBsAg, antibody to the hepatitis B core antigen (anti-HBc), HBeAg, and anti-HBe were detected by commercially available immunoassays (MEIA Axsym System; Abbott, Chicago, IL, USA).

Serum HBV DNA levels were quantified by COBAS Amplicor Monitor 2.0 HBV assay (Roche Diagnostic Systems Inc., Mannheim, Germany), with a lower detection limit of 2.48 $\log_{10}$ copies/ml and a linearity range from 2 $\log_{10}$ copies/ml to 7.6 $\log_{10}$ copies/ml (Roche Diagnostic Systems Inc.). Liver specimens were obtained with patient informed consent and processed using standard methods; grading of inflammation and staging of fibrosis were assessed by METAVIR scoring system.[27]

2.3. HBV-DNA amplification

DNA was extracted from serum samples according to the proteinase K protocol. Briefly, 200 μl of serum was added to 450 μl of mix containing 1 mg/ml proteinase K, 5 mM Tris HCl (pH 8.5), 2.0% sodium dodecyl sulfate (SDS) and 25 mM ethylenediaminetetraacetic acid (EDTA) and incubated at 37 °C for 4 h. DNA was precipitated with 1 volume of absolute isopropanol in the presence of 20 μg of Dextran T500 and 1/10 volume of 3 M NaAc (pH 4.7). DNA was recovered by centrifugation at 20 000 *g* for 15 min; pellets were washed with 70% ethanol, dried, and dissolved in 20 μl of water.

For BCP/pC region amplification, 10 μl (corresponding to 100 μl of serum) of extracted DNA was used for PCR in a 100-μl reaction volume containing buffer 1 × (67 mM Tris–HCl (pH 8.7), 16 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 U Hot Start Taq polymerase (Qiagen, Germany) and 0.4 μM of each primer; HBVpC1 (sense, 5′ ATA AGW GGA CTC TTG GAC T 3′, 1654–1672) and HBVpC2 (antisense, 5′ CGT CTG CGA GGC GAG GGA GT' 3′, 2467–2448). Thermal profile amplification: pre-heating at 94 °C for 15 min, 35 cycles including denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, followed by 5 min at 72 °C. Five microliters of PCR product was analyzed by ultraviolet fluorescence after ethidium bromide staining in a 2% agarose gel. A nested PCR was performed with primers HBVpC3 (sense, 5′ AAT GTC AAC GAC CGA CCT TG 3′, 1677–1696) and HBVpC4 (antisense, 5′ TGA GCA ATG CTC AGG AGA CTY TAA 3′, 2452–2429) using the same thermal profile and reaction conditions as those of the first PCR round. The same protocol was followed to amplify the S region with the primers HBVS1 (sense, 5′ CTG CTG GTG GCT CCA GTT C 3′, 57–75) and HBVS2 (antisense, 5′ AGA AAA TTG GTA ACA GMG GYA 3′, 814–794) for the first round, and HBVS3 (sense, 5′ GCG GKG TKT TTC TTG TTG ACA A 3′, 205–226) and HBVS4 (antisense, 5′ GGG ACT CAA GAT GYT GYA CAG 3′, 789–769) for the second round.

2.4. HBV-DNA sequencing

PCR products covering the BCP/pC and S regions were purified using Qiagen columns (Qiagen, Germany), and direct sequencing was carried out using a 3730xl DNA Analyzer (Applied Biosystems, USA) in both amplification senses by Macrogen Inc. (Seoul, Korea).

2.5. HBV-DNA typing

After amplification, nucleotide sequences of the PreS/S region were compared with reference strains representing each of the genotypes A–H, obtained from GenBank. Genotyping of HBV was then determined by phylogenetic analysis, and full genome sequences representing the different HBV genotypes were used as references. The HBV sequences were aligned with ClustalX v1.83 software.[28] The model of nucleotide substitution and the parameters that best fit the data (GTR+I+G) were estimated by the Modeltest 3.7 program[29] according to the Akaike information criterion (AIC). Using this model, a maximum likelihood tree was inferred with PAUP* software version 4.0b10.[30] The numbers at each node correspond to neighbor-joining bootstrap values obtained with 10 000 replicates, using the same model.

2.6. Statistical analysis

Fisher's two-tailed exact test and the corrected Chi-square test were used to compare qualitative data. Analysis of variance (ANOVA) and non-parametric tests (Mann–Whitney U and

Kruskal–Wallis H) were used to compare quantitative variables. Results are expressed as mean ± standard error of the mean (SEM). Data analysis was performed using SPSS software (version 10.0; SPSS Inc., Chicago, IL, USA). Significance was set at a p-value of less than 0.05. For statistical comparisons, an arbitrary value of 7.6 $\log_{10}$ copies/ml was assigned to samples with HBV-DNA levels higher than the upper limit.

3. Results

A total of 70 anti-HBe-positive HBsAg chronic carriers, none of whom had previously been on antiviral therapy, were included in this study. Based on biochemical and virological markers, 41 of them (22 females and 19 males), all of whom had persistently normal ALT levels and HBV-DNA titers below 4 $\log_{10}$ copies/ml, were defined as inactive carriers. Liver biopsy specimens were available for 21 of these 41 individuals, and all of them showed a histological activity index of ≤4 and a fibrosis stage of ≤1 (Table 1).

Twenty-nine carriers (eight females and 21 males) with abnormal ALT levels and HBV-DNA higher than 4 $\log_{10}$ copies/ml, were defined as having HBV-related ongoing disease with chronic active hepatitis and were included as the control group. Six out of these 29 individuals (20.7%) had clinical signs of cirrhosis; the liver biopsy specimens of the remaining 23 patients indicated that 19 had chronic hepatitis and four had cirrhosis.

The demographic, clinical, and serological data of the patients included in the study are shown in Table 1. There were no significant differences in age between the two groups, however the gender distribution was uneven (p = 0.019).

3.1. HBV-DNA typing

The prevalence of HBV genotypes was determined by phylogenetic analysis of preS/S nucleotide sequences (GenBank accession numbers **HM216215** to **HM216257**, **HM216259** to **HM216276** and **HM216278** to **HM216286**). Six out of the eight genotypes described were found in the population studied. The overall prevalences of these genotypes were: A 24.3%, B 4.3%, C 1.4%, D 37.1%, F1b 12.9%, F4 18.6%, and H 1.4%. Although genotype A was more slightly prone to be present on the inactive carriers group as compared to genotype D, F1b, and F4, there were no significant differences in the prevalence of HBV genotypes between the inactive carriers and the chronic active hepatitis group (Figure 1).

Table 1
Epidemiological biochemical, virological, and histological characteristics of the anti-HBe-positive patients studied

	Inactive carriers	Active carriers	p-Value
n	41	29	
Age (years)			
Mean	48.4	48.4	NS
SD	11.6	11.6	
Median	48.2	45.2	
Gender (M/F)	19/22	21/8	0.019
ALT (U/l)			
Mean	21.8	125.0	
SD	7.2	68.2	
Minimum	7.0	48	
Maximum	39.0	299	
HBV-DNA ($\log_{10}$ copies/ml)			
Mean	3.1	5.9	
SD	0.6	1.3	
Minimum	2.5	3.5	
Maximum	4.0	7.6	
Histology (n)	21	23	
HAI			
Mean	2.6	5.8	
SD	1.3	3.5	
Minimum	0	1.0	
Maximum	4	16.0	
Fibrosis stage			
Mean	0.6	2.2	
SD	0.5	1.7	
Minimum	0	0	
Maximum	1	5	

anti-HBe, antibody to the hepatitis B e antigen; SD, standard deviation; M/F, male/female; ALT, alanine aminotransferase; HBV, hepatitis B virus; HAI, histological activity index.

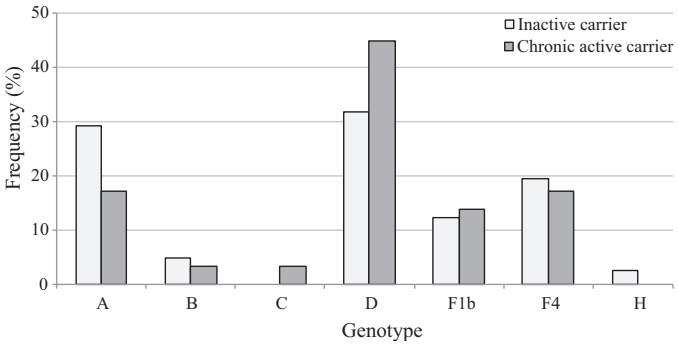

Figure 1. Prevalence of HBV genotypes in active and inactive anti-HBe-positive patients.

3.2. Correlation between BCP and pC mutations and active/inactive state

BCP/pC sequences were characterized by direct sequencing in 70 HBsAg carriers in whom HBV-DNA was successfully amplified by PCR (GenBank accession numbers **HM216287** to **HM216329**, **HM216331** to **HM216348** and **HM216350** to **HM216358**). Mutations modulating HBeAg expression were observed in most of the inactive carriers (92.7%) and in most of the patients with chronic active hepatitis (93.1%). The mutations most frequently observed were: 1753^{C} in 26 cases (37.1%), 1762^{T} in 30 cases (42.9%), 1764^{A} in 34 cases (48.6%), 1766^{T} in seven cases (10%), and 1896^{A} in 39 cases (55.7%). The prevalence of mutation 1764^{A} was significantly higher in patients with chronic active hepatitis than in inactive carriers (65.5% and 36.6%, respectively; p = 0.038), whereas the prevalences of mutations at the other positions analyzed were not significantly different (Figure 2).

With regard to the less frequent substitutions in the pC region, mutations in the precore initiation codon (PIC) were present in eight out of the 70 cases (11.4%) in a polymorphic manner, involving the three positions of the codon (1814^{C}, 1815^{C}, 1816^{A}, and 1816^{T}). Interestingly, we observed a two-fold difference in the mutation

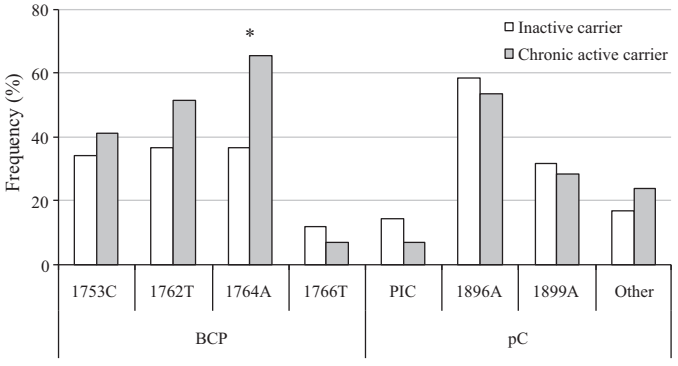

Figure 2. Correlation between BCP/pC mutants and active or inactive HBV infection. Other includes: 1817^{T} (n = 1), 1847^{T} (n = 2), 1897^{A} (n = 1), nucleotide insertion in 1846 (n = 4), 1939 (n = 3), 1839 (n = 1), 1847 (n = 1), and deletion in nucleotide 1845 (n = 1); $^{*}p < 0.05$.

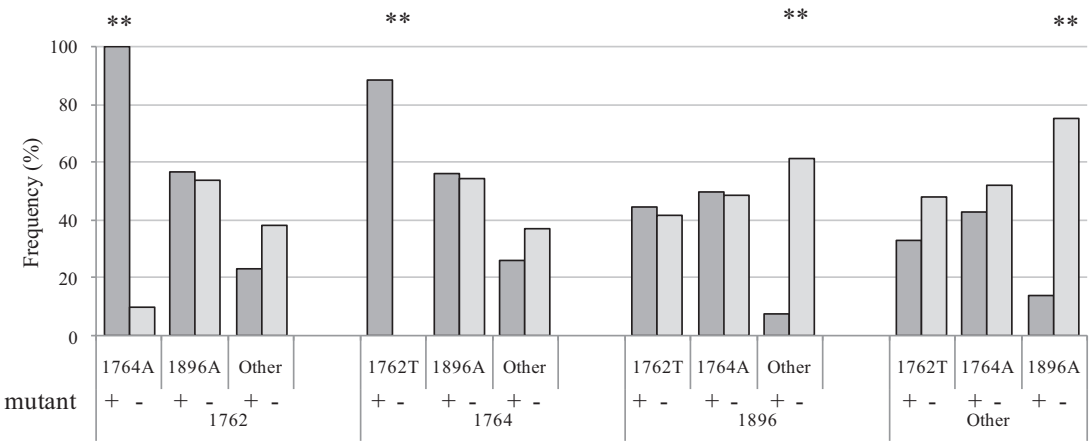

Figure 3. Relationship between nucleotides affecting HBeAg expression. Each bar represents the frequency of mutation of a given nucleotide according to the status of another nucleotide. Other means mutants in the precore region excluding nucleotide 1896; $^{**}p < 0.01$.

frequency between inactive carriers and patients with chronic active hepatitis (14.6% vs. 6.7%, respectively). The PIC variants were biased by genotype, being more prevalent in genotype A (23.5%) than in genotypes D (11.5%), F1b (0%), and F4 (0%). Furthermore, the PIC variants were frequently associated with a wild-type background in positions 1762^{A}, 1764^{G}, and 1896^{G}.

In addition, other mutations creating stop codons or frame shifting were observed: 1817^{T} in one case (1.4%), 1847^{A} in two cases (2.9%), 1897^{A} in one case (1.4%), a one-base-pair insertion in nine cases (12.9%), and a one-base-pair deletion in one case (1.4%).

HBeAg expression was mostly affected either by a $1762^{T}/1764^{A}$ double-mutation in the BCP region or mutations in the pC region; nevertheless, there were 30 (42.9%) patients who presented more than one mutation justifying the anti-HBe-positive phenotype.

By analyzing the relationships between the different nucleotides that affect HBeAg expression, it was possible to observe that mutations in the BCP region did not affect the prevalence of substitutions in the pC region and vice versa (Figure 3). The substitution at position 1762^{T} was usually present concurrently with the substitution at position 1764^{A} and vice versa.

In contrast, the 1896^{A} mutation seemed to circumvent other substitutions affecting HBeAg expression in the pC region. In addition, the prevalences of substitutions at positions 1762, 1764, and 1896 among patients carrying PIC mutations were remarkably lower than in those carrying wild-type PIC.

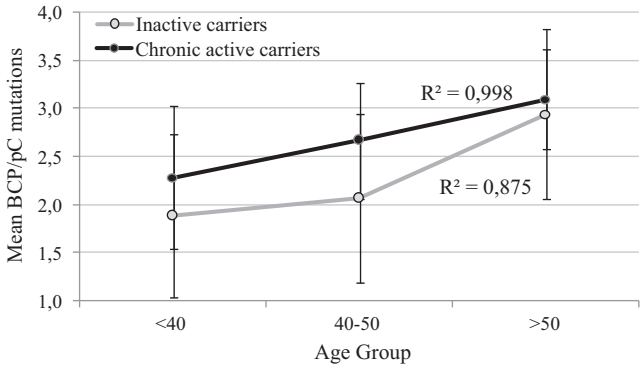

Figure 4. Mean number of mutations in the BCP/pC regions by age group.

Finally, the analysis of mutations affecting HBeAg expression by age showed an increasing prevalence of multiple mutated profiles in carriers older than 50 years as compared to carriers younger than 50 years old. The overall number of mutations affecting HBeAg expression increased across the decades, both in inactive carriers and patients with chronic active hepatitis (Figure 4). The mean number of mutations in patients younger than 40 years of age ($n = 16$) was 2.1, whereas that for patients aged between 40 and 50 years ($n = 26$) was 2.3 and for patients older than 50 years

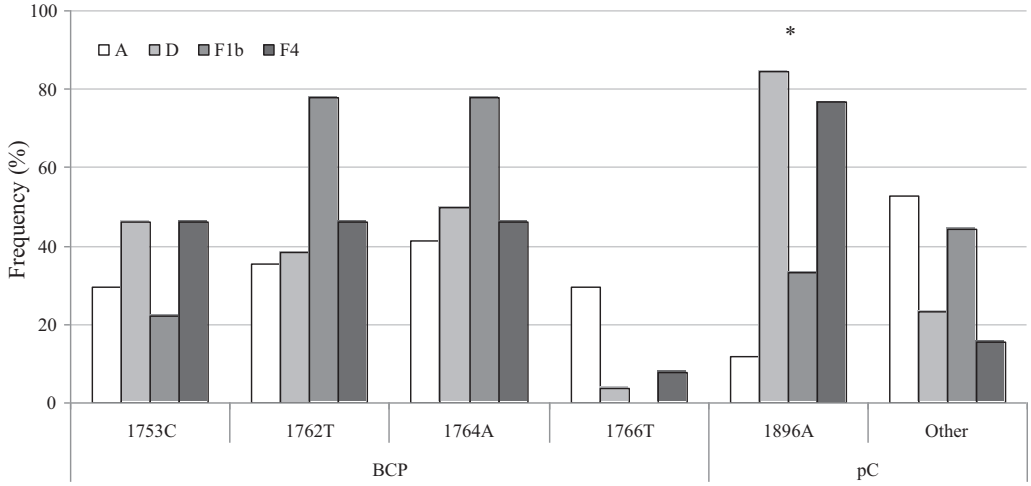

Figure 5. Prevalence of BCP/pC mutants by genotype. Other includes: precore initiation codon (PIC) mutations ($n = 8$), 1817^{T} ($n = 1$), 1847^{T} ($n = 2$), 1897^{A} ($n = 1$), nucleotide insertion in 1846 ($n = 4$), 1939 ($n = 3$), 1839 ($n = 1$), 1845 ($n = 1$), 1847 ($n = 1$), and deletion in nucleotide 1845 ($n = 1$); $^{*}p < 0.05$.

($n = 28$) was 3.0, although the differences were not significant ($p = 0.558$).

3.3. Correlation between genotypes and mutations in the BCP/pC regions

Since it has previously been described that mutations in the BCP/pC region are biased by the genotype,[31–33] we analyzed the frequencies of substitutions in the most prevalent genotypes (A, D, F1b, and F4).

The prevalence of substitutions in the BCP region was comparable in genotypes A, D, and F4, being slightly higher in genotype F1b (~40% vs. 78%, respectively) (Figure 5).

Overall, mutations in the pC region abrogating HBeAg synthesis were present in genotypes A (64.4%), D (100%), F1b (77.8%), and F4 (92.3%). However, the frequencies in different positions were unevenly distributed: substitution 1896^{A} was rare in genotype A (11.8%), intermediate in genotype F1b (33.3%), and relatively high in genotypes D (84.6%) and F4 (76.9%) ($p < 0.001$); whereas other substitutions, insertions and deletions affecting HBeAg expression were more prevalent in genotypes A (52.9%) and F1b (44.4%), as compared to genotypes D (23.1%) and F4 (15.4%), although the differences were not significant.

Position 1858 was a thymidine in all nucleotide sequences corresponding to genotypes D, F1b, and F4, whereas in genotype A, 1858^{C} was observed in all but two cases, in which it was thymidine and coupled to 1896^{A}.

4. Discussion

Over the last decades, many studies focusing on HBV heterogeneity have aimed to correlate HBV mutants and the wide spectrum of clinical and pathological conditions associated with HBV infection; nevertheless, few of them have been centered on inactive carriers. Although most of the HBV patients infected chronically are inactive carriers, several virological aspects of this state remain unclear.

In this study, the prevalence of BCP/pC variants affecting the expression of HBeAg was characterized in 70 anti-HBe-positive patients, 41 of whom were inactive carriers and 29 of whom were control patients with chronic hepatitis.

We found genotypes A, D, F1b, and F4 to be the most prevalent, as previously described in Buenos Aires.[34,35] Several attempts have been made to link a particular genotype to more severe liver disease, but results have been controversial.[36–38] In most regions of the world only two or three HBV genotypes are found, thereby limiting genotype comparisons; nevertheless, genotypes C and F have been referred to as having a worse evolution than other genotypes.[39,40]

Nonetheless, there were no significant differences in the genotype distribution between inactive carriers and chronic active hepatitis patients, perhaps because the cohort was not large enough to infer a relationship between genotype and pathogenesis.

Mutations in the BCP/pC regions are the hallmark of chronic HBeAg-negative and anti-HBe-positive HBV infection. In this study, mutations affecting HBeAg expression occurred in the great majority of individuals (92.9%). The prevalence of mutations was widely distributed, with no significant differences between inactive carriers and patients with chronic active hepatitis, except for position 1764, which was more prevalent in patients with chronic active hepatitis ($p = 0.038$). The correlation between disease severity and the presence or absence of mutations is controversial. In this study none of the variants was exclusively associated with a single clinical profile.

Interestingly, no mutations associated with the modulation of HBeAg expression were found in five cases (7.1%). This result suggests that the serological shift in some patients may result from a reduction in HBeAg production due to a sustained inhibition of HBV replication with persistence of wild-type virus.[41–43]

It is well established that mutations are biased by the genotype.[31–33] The 1896^{A} mutation is commonly found in genotype D since it stabilizes the encapsidation signal and potentially stimulates viral replication, whereas substitutions in the BCP region are frequently found in genotype A. In line with these findings, we found that the 1896^{A} mutation was predominant in genotype D (84.6%). Nevertheless, other compensatory substitutions in the pC region, such as alternative mutations, insertions or deletions, were observed in genotype A (52.9%). There is a paucity of information regarding the effect of genotype F on pC substitutions. Interestingly, the two subtypes of genotype F showed an uneven pattern of mutation; subtype F1b behaved like genotype A, while F4 resembled genotype D, although both subtypes have the 1858^{T}.

Therefore, the mechanism that a given genotype selects to regulate HBeAg expression is not fully explained by the structure of the encapsidation signal.

Other mutations affecting HBeAg expression were observed; in particular, mutations in the PIC were more prevalent among the inactive carriers (14.6%) than among the patients with chronic hepatitis (6.7%). These findings agree with the study carried out in a Spanish population, where PIC mutations were found in 12.1% of the cases and particularly associated with genotype A.[44]

In contrast to other single-point mutations, the PIC variants involved the three nucleotides of the codon and the substitutions behaved in a polymorphic manner (1816^{A} or 1816^{T}), suggesting that they are targeted to avoid the initiation of protein translation. Since different mutations affecting HBeAg translation, such as 1817^{T}, 1865^{T} or 1896^{A}, have shown different impacts regarding biological properties,[45] it should be interesting to characterize the phenotypic effect of the PIC variants.

Although the BCP/pC regions have been intensively characterized in recent decades, the PIC variants have been poorly studied.[31,44,46–51] We attribute this to the fact that most of these studies have exclusively analyzed the mutations at positions 1762, 1764, 1896, and 1899, overlooking possible mutations in other BCP/pC positions.

Interestingly, 42.9% of the individuals studied had more than one mutation that justified the anti-HBe-positive phenotype. The emergence of HBV variants harboring two mutations that independently prevent HBeAg expression poses several questions regarding their selection and biological significance during the natural history of chronic HBV infection, since the selection of mutations with redundant goals is unlikely.

The analysis of the relationships between different positions showed that mutations at positions 1762 and 1764 usually coexist and do not affect mutations in the pC region, whereas 1896^{A} prevents other substitutions in the pC region. Interestingly, mutations in the PIC seem to circumvent mutations in both the BCP and pC regions. This observation suggests that the selection of a mutation may influence the subsequent selection of another substitution, thus conditioning the evolutionary pathway of the virus.

There have been many studies involving viral mutations associated with clinical features,[22,52–54] but most previous studies have either ignored age or HBV genotype/subgenotype. Studies where patients have been matched by age have shown that most of these mutations are highly frequent in older HBV carriers (≥50 years) regardless of their clinical status.[55–57]

In conclusion, mutations in the BCP/pC regions are the hallmark of chronic anti-HBe-positive individuals; nevertheless, the even distribution of mutations in active and inactive carriers suggests that BCP/pC mutations may occur during HBV infection not strictly

related to the HBV infection activity. Furthermore, the uneven distribution of mutations in the pC region observed among the genotype F subtypes suggests that the mechanism to regulate HBeAg expression hinges on a finely poised and complex interplay of several factors and is not fully explained by the structure of the encapsidation signal.

These findings prompt long-term prospective studies in large cohorts of patients.

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