ORIGINAL RESEARCH

Genotyping of HBV and tracking of resistance mutations in treatment-naïve patients with chronic hepatitis B

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Background and aims: Resistance mutation analogs to nucleos(t)ides have been described in treatment-naïve patients with chronic hepatitis B (CHB), with clinical implications. The aim of this study was to investigate primary resistance mutations and genotypes circulating in patients naïve to chronic hepatitis B, in the Northern and Northeastern regions of Brazil.

Methods: We conducted a study of resistance mutations and genotypic characterization of hepatitis B virus (HBV) in 189 treatment-naïve patients chronically infected with HBV.

Results: Drug resistance-associated mutations located in the RT domain of the P gene (rtHBV) were found in 6% of the treatment-naïve patients from the Northeastern Region. The mutations were rtA194T, rtL180M + rtM204V, rtS202I, rtM204I, and rtA181S. No patient in the Northern Region had the resistance mutation. In the gene S region, the frequency of vaccine escape mutations was 2.4% in the Northeastern Region and 8.6% in the Northern Region.

Conclusion: This information before the start of treatment may contribute to clinical decision making, reducing treatment failure and the risk of progression to cirrhosis and hepatocellular carcinoma for CHB.

Keywords: Northeast, North, Brazil, direct sequencing, treatment failure

Introduction

Although there is a safe and effective vaccine against hepatitis B virus (HBV) since the early 1980s, chronic hepatitis caused by this pathogen is far from being eradicated and continues to constitute a severe public health problem in many parts of the world, and was considered recently by the World Health Organization as a pandemic. There are 400 million people infected worldwide and, in Brazil, 2 million are chronically infected. Brazil has an ethnic, economic, and regional diversity that would be no different for the epidemiology of HBV infection, but also presents a very heterogeneous distribution.[1–4] These individuals are exposed to the risk of developing severe complications including cirrhosis and hepatocellular carcinoma (HCC).[5,6]

In the last 20 years in Brazil, significant advances in the treatment for chronic hepatitis B (CHB) have occurred, initially with the use of interferon (IFN)-alpha, allowing to alter the natural progression of the disease. Currently, through the Clinical Protocol of Therapeutic Guidelines for Hepatitis B (2011), which directs the therapeutic conduct for HBV, five drugs are available for treatment, IFN-alpha and the nucleos(t)ide analogs (NAs) lamivudine (LAM), adefovir dipivoxil, entecavir (ETV), and tenofovir (TDF).[6,7] However, the use of NAs for a long time can determine the emergence of strains resistant to the drug.

The emergence of antiviral resistance has a close association with NA amino acid substitutions in the reverse transcriptase (RT region of the HBV genome), which are classified as primary and secondary self-compensatory resistance mutations.[6,8] The NA therapy can induce certain resistance mutations, and it is noticed that natural HBV RT mutations exist even in treatment-naïve patients.[9,10]

In addition, the HBV genome is complicated and has been classified into eight genotypes (A–H) based on differences of ≥8% in its whole genome sequence. The HBV genotypes have distinct geographic distribution: genotype A has a universal distribution, being the predominant genotype in Europe, North and Central America, sub-Saharan Africa, and India. Genotypes B and C are predominant in Southeast Asia, China, Japan, and Australia. Genotype D is mainly found in the Middle East and the Mediterranean countries. Genotype E seems to be predominant in West Africa, whereas genotype G is distributed throughout the USA, Mexico, and France. Genotype F is mainly found in Central and South America and Alaska. Finally, genotype H is unique to Central America and the USA.[11–13] Genotypes A, D, and F circulate among Brazilian HBV carriers.[4]

The aim of this study is to investigate primary resistance mutations in the genotypes circulating in patients naïve to CHB in the Northern and Northeastern regions, represented by the State of Acre and Bahia, and their implications in the clinical treatment of CHB.

Methods
Patients

A study of resistance mutations and genotypic characterization of HBV was conducted in 189 naïve patients chronically infected with HBV, 84 (Northeastern Region) and 105 (Northern Region), during the period of 2011–2015. All patients tested positive for the hepatitis B surface antigen (HBsAg) antigen within at least 6 months, and tested negative for HIV, and were naïve for the treatment of HBV, in two centers of reference for viral hepatitis, at the Northeastern Region, in the Hospital Universitário Professor Edgard Santos – HUPES and, at the Northern Region, in the Fundação Hospital do Acre – FUNDHACRE, Brazil. This study was reviewed and approved by the Ethics Committee of FUNDHACRE, Rio Branco, Acre, and the Ethics Committee of HUPES, Salvador, Bahia, Brazil. All participants provided written informed consent and the study followed the Declaration of Helsinki and Good Clinical Practice guidelines.

HBV quantification

HBV DNA was detected and quantified by the real-time polymerase chain reaction (PCR) assay. The test procedures followed the manufacturer's recommendations and were performed automatically at the laboratory facilities of the Public Health Central Laboratory (Laboratório Central de Saúde Pública) of the State of Acre and Bahia (LACEN).

PCR

The HBV DNA was extracted from 200 μL of serum samples with High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The RT region of the *HBV* gene (1032 pb) was amplified by a nested PCR. Primers were described by Krekulova et al[14] and modified to increase sensitivity to PCR. In the first reaction, the primers used were HB-1 5′ TAT TTC CCT GCT GGT GGC TCC 3′ (position 50–71) and HB-4 5′ ACT TTC CAA TCA ATA GG 3′ (position 969–986). In the second reaction, the primers used were HB-5 5′ GAA CAG TAA ACC CTG CTC CG 3′ (position 78–98) and HB-8 5′ TGT ACA ATA TGA TCC TGT GG 3′ (position 909–929) (Thermo Fisher Scientific, Waltham, MA, USA).

For the amplification of DNA, 5.0 μL of the sample was used for the first reaction and put into a mixture containing 2.5 μL 10× PCR buffer, 0.5 μL of deoxynucleotide triphosphate (dNTP) mix (10 mM), 1.25 μL of $MgCl_2$ (50 mM), 0.5 μL of each primer, and 0.2 μL of platinum Taq DNA polymerase (5 U/μL) (Thermo Fisher Scientific, Waltham, MA, USA), resulting in a final volume of 25 μL. The cycle conditions used initially were 94°C for 2 minutes for denaturing, followed by 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 2 minutes, and a final extension of 72°C for 7 minutes in a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany).

The second reaction used 1.0 μL of the PCR product, which was put into a mixture containing 5 μL 10× PCR buffer, 1.0 μL of dNTP mix (10 mM), 2.5 μL $MgCl_2$ (50 mM), 5 μL of each primer, and 0.2 μL of platinum Taq DNA polymerase (5 U/μL), resulting in a final volume of 50 μL. The cycle conditions used initially were 94°C for 2 minutes for denaturing, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes, and a final extension of 72°C for 7 minutes in a Mastercycler Gradient Thermal Cycler.

Sequencing of the HBV RT region

The samples were purified with a QIAquick PCR Purification Kit (Qiagen NV, Venlo, the Netherlands) and sequenced with forward and reverse primers using a BigDye Terminator 3.1 Cycle Sequencing kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The reactions were performed in an automatic ABI PRISM 3100 Genetic Analyzer Sequencer (Thermo Fisher Scientific). The sequencing project coverage was only twofold to sixfold corresponding to the sequence obtained from each primer product (HB-2, HB-3, HB-5, HB-6, HB-7, and HB-8, data not shown).

Sequence analysis

To confirm the specificity of each amplicon, the sequences obtained were analyzed using the GenBank Basic Local Alignment Search Tool. Subsequently, sequences from the same patient were assembled to a reference sequence and conflicting sites were corrected by visual inspection (CLC Main Workbench v.5, Qiagen, Aarhus, Denmark). Consensus sequence of each HBV isolate was submitted to a web-based software for subtyping and prediction of phenotypic resistance to genotype-specific mutations in the polymerase gene (RT mutation) and escape mutation (HBs mutation) (Max-Planck-Institut für Informatik, Germany, at http://hbv.geno2pheno.org/index.php). One hundred and eighty-nine sequences of these analyses were submitted to the GenBank (accession number: KU847525-KU847634; KU847635-KU847735)

Statistical analyses

Statistical analyses were performed using the software SPSS 20.0 (IBM Corporation, Armonk, NY, USA). Comparison between groups was performed using a one-way analysis of variance t-test or a chi-square test, as appropriate. The HBV DNA concentrations were expressed on a logarithmic scale. A P-value of <0.05 was considered statistically significant.

Results

The samples from 189 treatment-naïve CHB patients were enrolled in this retrospective study. Their clinical features are summarized in Table 1. There were 84 treatment-naïve patients at HUPES/Salvador-Bahia (Northeastern Region), consisting of 50.6% males and 49.4% females, with a median age of 44 years (the range was 18–72 years). A high alanine aminotransferase (ALT) serum level (reference value is >41 UI/mL) was found in 38.7% of the patients, with a median of 45.65 UI/mL. The HBV DNA level in the

Table 1 Clinical features of treatment-naïve patients for CHB attending two reference centers for viral hepatitis between 2011 and 2015

Characteristics	Northeast		North	
	n=84	Percentage	n=105	Percentage
Age* (min–max, years)	18–72	44.3±12.3	18–72	40.5±12.3
Gender (male/female)	40/39	50.6/49.4	39/66	37.1/62.9
ALT* (IU/L)	7–231	45.65±38.9	12–414	42.63±52.3
RV >41 IU/L	29	38.7	28	28.3
HBeAg (+/−)	10/62	13.9/86.1	07/91	7.1/92.9
HBV DNA log 10 >4	12	15.6	14	13.3
HBV DNA level*	1.51–8.34	3.3±1.47	1.75–8.04	3.12±1.4
HBV genotype				
A	75	89.3	62	59
D	2	2.4	22	21
F	6	7.1	21	20
C	1	1.2	0	0
Coinfection				
HBV HCV	1	1.2	2	1.9
HBV HDV			4	3.8
Mutation				
Vaccine escape (sHBV)	2	2.4	9	8.6
Mutation resistance NA	5	6	0	0

Note: *Percentage column shown in median and SD and n column shown in min–max.
Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; NA, nucleoside/nucleotide analogs; RV, reference value; sHBV, surface antigen gene of hepatitis B virus.

serum had a median of log 3.3 (the range was 1.51–8.34) while 86.1% of that was hepatitis B e antigen (HBeAg) sero-negative. The HBV genotype distribution found was: A (89.3%), D (2.4%), F (7.1%), and C (1.2%). Coinfection with hepatitis C virus (HCV) was found to be 1.2%.

There were 105 treatment-naïve patients at FUND-HACRE/Rio Branco-Acre (Northern Region), consisting of 37.1% males and 62.9% females, with a median age of 40 years (the range was 18–72 years). A high ALT serum level (reference value is >41 UI/mL) was found in 28.3% of the patients, with a median of 42.63 UI/mL. The HBV DNA median load in serum was 3.12 log copies/mL (the range was 1.75–8.04) while 92.9% of that was HBeAg sero-negative. The HBV genotype distribution found was: A (59%), D (21%), and F (20%). Coinfection with HCV (HBV HCV) was found to be 1.9%, and with hepatitis D virus (HBV HDV) was found to be 3.8%.

Only five treatment-naïve patients of the Northeastern Region (n=84) had mutations at positions that may be associated with viral resistance, as described in Table 2. No patient

Table 2 Virological and resistance mutation profile of CHB-naïve patients to analog nucleos(t)ides

Patient	Age (years)	Gender	Viral load (log UI/mL)	Genotype	Mutation rtHBV	sHBV	HBeAg status	Resistance profile LAM	ADV	ETV	TDF
H:351	56	M	3.72	A1	S202I		No reagent	S	S	R	S
H:105	62	M	8.6	A1	A194T		No reagent	S	S	S	R
H:117	48	M	2.21	A1	M204I	Q129H	Reagent	R	S	I	S
H:123	27	M	6.11	F2	A181S		Reagent	R	R	S	S
H:226	30	M	8.3	A2	L180M + M204V	I195M	Reagent	R	S	R	S

Abbreviations: ADV, adefovir dipivoxil; CHB, chronic hepatitis B; ETV, entecavir; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; I, intermediate/reduced susceptibility; LAM, lamivudine; R, resistant; S, susceptible; TDF, tenofovir.

Table 3 Comparing the epidemiological characteristics of patients with resistance mutations and without resistance mutations in the HBV RT region

Characteristics	Group with mutation (n=5)	Group without mutation (n=79)
Genotype		
Type A	4	71
Type non-A	1	8
Gender		
Male	4	36
Female	1	38
HbeAg		
Positive	3	7
Negative	2	60
Age		
≥40 years	3	44
≤40 years	2	21
HBV DNA		
≥ log 4	2	10
≤ log 4	3	62
ALT		
≤ RV =41 IU/mL	2	44
≥ RV =41 IU/mL	3	26

Note: There was no significant association at $P<0.05$.
Abbreviations: HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; RV, reference value; IU, international unit.

had the resistance mutation at FUNDHACRE (n=105). The mutations were rtA194T, rtL180M + rtM204V, rtS202I, rtM204I, and rtA181S. In the pre S/S region, there were sI195M and sQ129H.

No significant correlation between the group with mutation and the group without mutation was found in our sample described in Table 3.

Discussion

Resistance mutations to NA have been described in treatment-naïve patients treated for CHB, with clinical implications. It is important to track changes in primary and secondary resistance mutations in patients that have not yet been processed[10]

in order to monitor, optimize, and advocate the best treatment, achieving sustained virological response (SVR) and, consequently, reducing the progression of cirrhosis and the evolution to HCC.[15]

In this study, the patients evaluated were from two reference centers for viral hepatitis in Brazil, one at the Northern Region, which has the highest prevalence rates for CHB, and one at the Northeastern Region, which is considered to have a low prevalence of CHB. In both the reference centers, the naïve patients had an average age of ~40 years, mostly females, and had the serological marker HBeAg– mostly, demonstrating a global and Brazilian trend, of the prevalence of CHB patients with HBeAg–.[16–18] However, the patients in the Northeastern Region, compared to the ones from Northern Region, showed values above the reference value for ALT (38.7% vs 28.3%).

The circulating genotypes already described for Brazil are A, D, and F.[4,19,20] Of them, the predominant is genotype A, as observed in the Northeastern Region with almost 90%, followed by the genotypes F, D, and C. In the Northern Region, genotype A was also predominant, with almost 60%, followed by the genotypes D and F. And the proportion of genotype F is more common in the indigenous population in the Amazon region, compared with other Brazilian regions.[21,22] Besides that genotype F is more frequent in the Amazon region, and coinfection with HBV HDV (delta) is also very common, with a found prevalence of 3.8%, followed by coinfection with HBV HCV, with a prevalence of 1.8% in the patients studied. Regarding the frequencies of subgenotypes found in the Northeastern Region were A1 (71%), A2 (4%), C2, D2, and D3 (1%), and F2 (6%). The frequencies of subgenotypes found in the Northern Region were A1 (59%), D1 (1%), D2 (2.9%), D3 (10.5%), D4 (6.7%), F1 (7.6%), and F2 (12.4%). The subgenotype A1 has been mostly identified in African population and its descendants. Subgenotype A2 is primarily found among Europeans.[23,24] Genotype C is the most prevalent in Asia. In Brazil, due to the intense migration of people

of Asian descent, genotype C2 was found.[27,28] Genotype D is divided into four subgenotypes (D1, D2, D3, and D4) found in different continents. The genotype F is considered indigenous to the American continents, and four subgenotypes have been described, F1, F2, F3, and F4. In Latin America, genotype F is the most prevalent, with an exception of Brazil, where genotype A.[26]

The presence of resistance mutations in treatment-naïve patients has shown clinical significance due to the preexisting mutations, which need to be investigated and traced. In this study, we used direct sequencing of the HBV's DNA from the RT from the polymerase region to track resistance mutations to NA. In the Northern Region, there were no primary or secondary resistance mutations to NA. In some studies, in treatment-naïve patients, they were not found too.[27,28] However, in patients in the Northeastern Region, mutations resistant to NA were found (5/84) corresponding to a prevalence of 6% of the samples. In several studies in the literature, most of them being held in the Asian population, the resistance mutation rates in naïve patients revolve ~1%–20%.[28–32,10] In Brazil, in a recently published study,[33] with samples from various regions of Brazil, the resistance rate of mutations associated with NA was found to be 1.6% in naïve patients. Expanding the prevalence for the Northern and Northeastern regions, the prevalence of resistance mutations to NA was found to be 2.6%.

One of the resistance mutations found in the Northeastern Region was rtA194T, which can be associated with resistance to TDF, which has shown in vitro reduced susceptibility to TDF when combined with the resistance mutations to LAM M204V and L180M. The clinical implication of the rtA194T mutation must be determined through long-term follow-up clinical studies.[34,35] The HBeAg– patients may have an increased risk of selecting the rtA194T mutation, and therefore, antiviral resistance, so another alternative would be an indication of ETV as a first drug of choice.[36]

The other two mutations are associated with resistance to LAM rtL180M + rtM204V and rtS202I. The rtM204V mutation is considered a primary resistance mutation due to the susceptibility of HBV to LAM, and the rtL180M mutations are considered compensatory or secondary mutations,[37] which can increase the viral replication fitness.[38] The rtM204V mutation was the most common in studies of naïve patients,[39,40] which is the resistance mutation associated with LAM. However, according to the Clinical Protocol of Therapeutic Guidelines for Chronic Hepatitis B,[7] LAM is not indicated as a first-line choice because of its low genetic barrier and antiviral resistance rate that can reach ~80% in 5 years of treatment.[6,41] The first-line choices of antiviral

treatment for treatment-naïve patients without cirrhosis are ETV and TDF, recommended by the Ministry of Health.

The resistance mutations rtL180M + rtM204V and rtS202I may be associated to the presence of mutations in the coding region of the HBsAg envelope region (S of the HBV), as found in the patterns I195M, W196L, and G145R, indicating vaccine escape. Mutations in the polymerase region associated with LAM resistance can produce changes in the envelope region (S) and, as they are superimposed, it results in a reduction of antigenicity of the HBsAg protein. Another interesting point is that these patterns of mutations found in patients with resistance mutations to NA in the S region are more described for genotype A, compared to genotype D. In the literature, there are no mutations in the S region associated with the rtA194T resistance mutation, confirming the results found.[42,43] The emergence of adefovir resistant mutant in patients with LAM resistance is more common than in treatment-naïve patients. Two major mutations of adefovir resistance are rtN236T and rtA181V/T, and in the current study we found the rtN236T mutation.

Comparing the patients with resistance mutations to NA and those without mutations, no clinical evidence was found. However, Zhang et al[44] conducted a meta-analysis to apprise the incidence of natural resistance mutations in naïve chronic hepatitis B patients. They found genotype C HBV infection, male, and HBeAg negative patients had a slightly higher natural mutation rate when compared with genotype B and D HBV infection, female, and HBeAg positve patients. The ALT serum levels showed no influence on the emergence of antiviral resistance mutations.[44] In Brazil, there are few studies that were conducted on survey resistance mutations to NA on naïve patients seeking clinical association.[45–47] However, other studies should be conducted to track and trace the changes in the mutation profile, crossing with the epidemiological characteristics of the population, with a larger number of treatment-naïve patients with CHB.

In conclusion, it is important to track the resistance mutations genotypic to NA in naïve patients due to preexisting mutations in the RT region associated with antiviral treatment. The therapeutic efficacy of the antiviral treatment can be affected by many factors including infection with drug-resistant viral strains. Since a treatment-naïve patient carries strain with resistance mutations, one must question if it is worthwhile to start the treatment with a drug for which that mutation produces resistance. This information, before the start of treatment, may contribute to clinical decision making, reducing therapeutic failure, and the risk of progression to cirrhosis and HCC for CHB.

Abbreviations

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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