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RESEARCH ARTICLE

Prevalence of Genotypic Resistance Mutations in Patients with Chronic Hepatitis B (HBV) Treated with Entecavir and Tenofovir in Two Reference Centers in the North and Northeast of Brazil

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ABSTRACT

Mutations of genotypic resistance to nucleotide analogues Entecavirand Tenofovirhave been described in patients undergoing treatment and virgins for hepatitis B virus. The present study demonstrated in a sample of 263 patients with chronic HBV from the North and Northeast of Brazil, a mutation rate of resistance to nucleotide analogues of 3.8% (10). Of the 10 patients who had genotypic resistance mutations, only 1 had no genotypic resistance mutation for the first line treatment for hepatitis B, entecavir and tenofovir.Due to the emergence of vaccine escape mutations and resistance mutations to antiviral treatment, and the severity of liver disease caused by HBV, screening for genetic mutations is important due to the impact on therapeutic management.

Keyword: Hepatitis B, genotypic mutations, entecavir, tenofovir

Introduction

Hepatitis B virus (HBV) is considered one of the biggest public health problems in the world. Approximately 400 million people are chronically infected with HBV, accounting for more than 600,000 death records per year[1,2]. HBV infection has a major personal, social and economic impact, and is associated with significant morbidity and mortality, and can progress to liver cirrhosis, hepatic decompensation and hepatocellular carcinoma [3-5].

Despite the existence of safe and effective vaccines and antiviral treatment for HBV, mutations in the HBV genome have been described in the scientific literature, with biological and clinical implications. Among these mutations, mutations in the S gene are related to vaccine escape and mutations in the P gene, precisely those in the reverse transcriptase (RT) region, are related to resistance mutations to antiviral treatment, Prevalence of genotypic resistance mutations in patients with chronic Hepatitis B (HBV) treated with Entecavir and Tenofovir in two reference centers in the North and Northeast of Brazilthe nucleotide analogues.

The nucleotide analogues entecavir (ETV) and tenofovir (TDF) are the first line of treatment since the approval and incorporation into the Clinical Protocol of Therapeutic Guidelines since 2009 by the Brazilian Ministry of Health, have demonstrated, in clinical studies, activity in controlling HBV replication, and are

associated with minimal risk of developing antiviral resistance mutations, due to the high genetic barrier, even in long treatment, as in the case of treatments by nucleotide analogues [6]

Resistance mutations ETV have been mapped in the B domain, rtI169T, rt1L80M e rtS184G, in the C domain, rtS202I and rtM204V, in the E domain, the rtM250V mutation, these mutations were described in salvage therapy with patients with prior treatment with LAM, which had treatment failure, being the most frequent mutations. ETV is considered among the low-resistance therapeutic options, i.e., with high genetic barrier, in 6 years of therapy with treatment virgin patients the rate was 1.2% of resistance mutations [7] So far no resistance mutations for TDF have been found in patients on monotherapy, only in HIV co-infected patients, or patients previously treated with LAM, the mutation found in these cases was rtA194T, it is also considered together with ETV of high genetic barrier [8]

In Brazil few studies have been conducted aiming to screen mutations with antiviral therapy in patients with chronic hepatitis B treated with AN or treatment virgins. Despite the large number of studies addressing worldwide the different therapeutic strategies and their clinical and virological consequences, only a few studies report the situation of antiviral treatment in Brazil for patients with chronic hepatitis B.The present work aimed to track vaccine escape mutations and antiviral resistance to first-line treatment ETV e TDF, and HBV genotypes and by applying molecular epidemiology in patients with chronic hepatitis B.

Metodology

Research Participants

Cross-sectional, analytical study involving 263 patients with chronic hepatitis B, samples collected during the period 2011-2015. All patients were AgHBs positive for more than six months. The research participants were from the Specialized Service of Hepatitis Outpatient Clinic of the Hospital Fundação do Acre - FUNDHACRE (Hospital das Clínicas) in Rio Branco, Acre, northern region of Brazil and from the Complexo Hospitalar Professor Edgard Santos, Ambulatório Magalhães Neto in Salvador, Bahia, northeastern region of Brazil.

Load Viral HBV-DNA

HBV-DNA was detected and quantified by real-time PCR assay. The procedures adopted followed the manufacturer's recommendations and were performed automatically at the facilities of the Central Public Health Laboratory of the State of Acre (LACEN).

Polymerase Chain Reaction – PCR

HBV-DNA was extracted from the patient samples from 200 ul of each serum sample using the High Pure kit of viral nucleic acid (Roche, EUA) according to the manufacturer's instructions. The HBV RT gene region (1032 bp) was amplified by polymerase chain reaction (PCR). The primers were described by Krekulova [9] and modified to increase PCR sensitivity. In the first reaction, the primers used were HB-1 5 'TAT TTC CCT GCT GGT GGC TCC 3' (50-71 position), and HB-4 5 'ACT TTC CAA ATA TCA GG 3' (969-986 position). In the second reaction, the primers used were HB -5 5 'TAA GAA CAG ACC CTG CTC CG 3' (78-98 position) and HB-8 5 'ACA ATA TGT TGA TGT TCC GG 3' (909-929 position) (Life Technologies, EUA).

For DNA amplification, 5.0µL of the sample was used for the first reaction with the following reagents: 2.5 ul of 10 × PCR buffer, 0.5 uL of dNTP mix (10 mM), 1,25 ul of MgCl2 (50 mM), 0,5 ul of each primer, and 0,2 ul of DNA-polimerase Taq Platinum (5 U / mL) (Invitrogen, San Diego, CA, EUA), resulting in a final volume of 25 uL. The cycling conditions used initially were 94°C for 2 min

denaturation, followed by 45 cycles of 94°C for 30 s, 56.0°C for 30 s, 72°C for 2 min, and a final extension of 72°C for 7 min in the thermal cycler Mastercycler Gradient Termociclador (Eppendorf, em Hamburgo, Alemanha).

For the second reaction, 1.0µL of PCR product was used, with the following reagents: 5 ul of 10 × PCR buffer, 1.0 uL of dNTP mix (10 mM), 2.5 mL of MgCl2 (50 mM), 5 uL of each primer, and 0.2µL of platinum DNA-polymerase Taq (5 U / mL) (Invitrogen, San Diego, CA, EUA), resulting in a final volume of 50 uL. The cycling conditions used were 94°C for 2 min denaturation, followed by 35 cycles of 94°C for 30 s, 60.0°C for 30 s, 72°C for 2 min, and a final extension of 72°C for 7 min in the thermal cycler Mastercycler Gradient Termociclador (Eppendorf, Hamburgo, Alemanha).

Sequencing of the HBV RT region

Samples were purified using the QIAquick PCR Purification kit (Qiagen, EUA), and sequenced with the primers, using the Big Dye Terminator Cycle Sequencing 3.1 kit (Applied Biosystems, Foster City, CA, EUA) according to the manufacturer's instructions. The reactions were performed on the automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The analysis of the sequence

To confirm the virus type, the obtained sequences were analyzed using BLAST GenBank. HBV genotypic analysis was performed by comparing reference sequences with sequences obtained from HBV-DNA isolated from patients with HBV consensus sequences. Subsequently, nucleotide sequences were analyzed using the softwares DNA Star 5.0 and 4.0 MEGA. Resistance mutations (RT), genotypes, subgenotypes and mutations in the S gene were determined by phylogenetic analysis using published sequences and virtual phenotyping was performed by two algorithms, available on the Stanford University website (http: // hivdb. stanford.edu/HBV/HBVseq/development/HBVseq. html) and on the website Max-Planck-Institut für Informatik, Germany (http://hbv.geno2pheno.org/index.php). The 156 sequences from these analyses have been submitted to GenBank (accession number:)

Statistical Analysis

Variables were analyzed using the statistical package SPSS 20.0 (IBM Corporation, Armonk, NY, USA). Events of interest were described using frequencies and measures of central tendency (mean and median) and dispersion (standard deviation). HBV DNA concentrations were expressed on a logarithmic scale.

Ethical considerations

This study was reviewed and approved by the Ethics Committee of FUNDHACRE, Rio Branco, Acre, and also by the CEP-HUPES, Salvador, Bahia.

Results

Table 1. Epidemiological characteristics, treatment, and mutation profile of patients with chronic HBV, seen at the Hospital das Clínicas do Acre, in the period 2011-2015.

	North Region		Northeast Region		Total	
	n= 156	%	n=107	%	n=263	%
Age † (min-max)	18-72	40,9±12,4	18-76	44,5±12,9	18-76	42,2±12,7
> 40 years old	77	49,4	58	65,2	135	55,1
Gender (male/female)	64/92	41/59	55/43	56,1/43,9	119/135	46,9/53,1
ALT level						
RV > 41 UI/L	36	30,3	42	44,2	78	36,4
HBsAb status						
HBsAb (+/-)	20/127	13,6/86,4	21/70	23,1/76,9	41/197	172,2/82,8
HBV-DNA						
DNA-HBV log10>4	37	23,7	22	22,4	59	22,4
DNA-HBV level †	1,74-8,4	3,47±1,5	1,71-84	3,43±1,6	254	3,45±1,6
Treatment profile						
Untreated (virgins)	81	59,5	84	80,8	175	66,5
In treatment	62	40,5	20	19,2	82	32,5
Cirrhosis	11	7,1	6	5,6	17	6,5
Coinfection						
HCV	3	1,9	1	0,9	4	1,5
HIV I/II			2	1,9	2	0,75
HDV	18	11,5	–	–	18	6,8

† values : mean and SD(standard deviation) * values vary according to data availability

Table 2. Detection of genotypes and subgenotypes, resistance mutations in polymerase rt and mutations in the S gene, in chronic HBV patients.

	North Region		Northeast Region		Total	
	n= 156	%	n=107	%	n=263	%
HBV genotype						
A	89	57	97	90,6	186	71
C			1	0,9	1	0,4
D	31	19,9	3	2,8	34	12,9
F	36	23,1	6	5,6	42	16
Mutations						
Vaccine escape (sHBV)	11	7,1	9	8,4	20	7,6
Resistance mutation NA	2	1,2	8	7,4	10	3,8

Frequencies of resistance mutation patterns at the sites of the polymerase rt domains were estimated, and were related to the genotype. The most frequent mutations were: rtN246S (23,7%), rtH248N (5,1%), rtI233Ve rtN238H/S/T (3,8%), rtI187L/N, rtI187L/N e rtS219A/S (3,2%), rtL217R/M, rtL220L/V, rtN248H, rtQ215H/Q, rtV214E/A (2,5%), rtL229M/L/V (1,9%), rtK212N e rtL207M (1,3%), rtA194S, rtL235S, rtN226H e rtT237P/T (0,6%).

Table 3. Frequency of mutation patterns, nucleotide substitutions in RT polymerase in chronic HBV patients.

							Mutation			Resistence profile			
Pacient	Age	Gender	HBV DNA (logUI/mL)	Genotype	Coinfection	Treatment	rtHBV	sHBV	AgHBe/anti-Hbe status	LAM	ADV	ETV	TDF
H:105	62	M	8,6	A1		naive	A194T		–/+	S	S	S	R
H:226	30	M	8,3	A2		naive	L180M+M204V	I195M	+/–	R	S	R	S
H:351	54	F	3,72	A1		naive	S202I		–/+	S	S	R	S
H:117	48	M	2,21	A1		naive	M204I	129H	+/–	R	S	R	S
H:123	27	M	6,11	F2		naive	A181S		+/–	S	R	S	R
H:225	18	M	5,73	A1	HIV I/II	LAM+TDF	L180M+M204V	I195M	+/–	R	S	R	S
H:258		M	2,24	A2	HIV I/II	LAM+TDF	L180M+M204V	I195M	+/–	R	S	R	S
H:259				A1			L180M+M204I	W196*	–/+	R	S	R	S
A:1152	36	F	5,14	F1		TDF	A181E		–/+	R	R	S	S
A:1189	47	M	2,62	A1	HCV	IFN+RIBA	A184S		+/–	S	S	R	S

Discussion

In Brazil few studies have been conducted using molecular epidemiology applied to chronic hepatitis B, aiming to track resistance mutations with antiviral therapy in patients treated with AN or treatment virgins, or the vaccine escape mutations, correlating with genotypes and subgenotypes in the North region. Despite the large volume of studies addressing worldwide mutations in the HBV genome and their clinical and biological implications, only a few studies have been reported on the antiviral treatment situation in Brazil for patients with chronic hepatitis B. Most of these studies focus on the occurrence of resistance mutations to LAM[10] Most of these studies focus on the occurrence of resistance mutations to LAM[10-12] especially in patients co-infected with HIV [13,14 15]

The present study, carried out in the state of Acre, in the Western Amazon region, considered an area of high endemicity for HBV and co-infection with the Delta virus (DHV), showed a mean age, also found in other regions of the country,of around 40 years, with approximately 50% over 40 years, however, the highest frequency was females, which may suggest that in the state of Acre, the transmission that presents the highest frequency of HBV infection is vertical or perinatal transmission [16,17]. The clinical profile of the patients showed that approximately 90% were AgHBe negative and not cirrhotic. The patients who presented ALT reference values above the reference value were 30% and HBV-DNA levels showed that most patients maintain normal ALT and HBV-DNA levels within the values recommended by the Ministry of Health to be monitored every 6 months, reflecting the number of treatment virgin patients that were almost 60%[6] The highest frequency of co-infection was with HDV, which is already expected in this region as demonstrated in several studies [18] The highest frequency of co-infection was with HDV, which is

already expected in this region as demonstrated in several studies [18-21]

The circulating genotypes among the patients were A, D and F. Previous studies, which attempted to characterize the genotype distribution in the Northern region, also found the same distribution pattern - genotype A followed by genotype F [17,22-24]

The subgenotypes found were A1, D1, D2, D3, D4, F1 and F2. Subgenotype A1 has been identified in populations of African descent in the state of Acre, according to the 2010 census data, approximately 72% are of African descent. The D subgenotypes (1-4) have a broad non-selective geographic distribution, and the F subgenotypes are frequently described in indigenous populations in Latin America, and in Brazil, F1 and F2 are the most commonly found [25-27]

The clinical implication of genotypes in the evolution of HBV and therapeutic response is still controversial and not well defined, as in the case of hepatitis C infection (HCV). In the case of genotype A and D, which are the most prevalent in Brazil, genotype A may evolve to the more chronic form, compared to genotype D. However, other studies have shown that genotype A is more favorable to viral elimination, the "clearance", compared to other genotypes [28,29]. Genotype A may also show a better therapeutic response with nucleotide analogues than genotype D, with a lower rate of resistance mutations associated with Lamivudine treatment in the YMDD region of the HBV RT polymerase [30]. Genotype F is often associated with fulminant hepatitis, due to co-infection or superinfection with HDV [31] regarding treatment, studies should be conducted to fill this gap.

Nucleotide analogues resistance mutations, primary mutations were found in two patients under treatment (2/156) with a mutation rate of1,2%, the mutations found were the rtA181E mutation which is associated with resistance to Lamivudine (LAM) and Adefovir (ADF), and the rtT184S mutation is associated with Entecavir (ETV). However, in these two cases, one was not being treated with nucleotide analogues but with Interferon (IFN) and the other was being treated with Tenofovir (TDF). Therefore, the resistance mutation does not compromise the treatment and the therapeutic approach adopted.

The rate of vaccine escape mutation in the S (AgHBs) gene described for patients in this study was higher compared to those with antiviral resistance mutations, a rate of 7.1%, demonstrating the importance of these results due to their clinical impact. These vaccine escape mutations raise concern because they may complicate vaccination strategies; among the vaccine escape mutations, G-145-A is the most prevalent and studied, and may become common in the coming decades [32-35]. Vaccine escape mutations may play an important role in understanding the model of HBV evolution in the future [36].

Other mutation patterns in the RT region of the HBV polymerase P gene with amino acid substitutions have also been observed, and the frequency of these mutations may accompany mutations in the S gene due to overlap with the P gene, producing mutant amino acids or truncated AgHBs proteins[37,38]. These patterns of mutations at positions in RT described and related to resistance mutations may be secondary or compensatory mutations, where some studies suggest they may be involved with resistance to nucleotide analogues. Among the mutations found in the present study, compensatory mutations were the rtL207M, rtQ215H/Q, rtL229M/L/Vand the other mutations found at the RT positions may suggest potential resistance to the nucleotide analogues ADF or ETV, due to the positions being located within the RT region. Some mutations were found in all three genotypes, such as the rtQ215H/Q and the rtN248H, with the mutation rtQ215H/Q, rtI233V, rtV214E/A e rtN238H/S/Tare more frequent in genotype D, however the rtN246S mutation was found only in patients with genotype F (23,7%) [39-43]

All this diversity of mutations described in this paper, demonstrates the genetic variability of HBV, being one of the viruses that have the highest mutation rate among the DNA viruses, this potential meets six important factors that favor this diversity, a high viral load produced during active replication, low fidelity of DNA polymerase corrective activity, selective pressure and genetic barrier of antiviral treatment, HBV viral fitness, viral replication within the hepatocyte, all these factors favor a high mutational rate. A large pool of which species of HBV is generated in which the fittest virus, i.e. the virus that has the greatest ability to replicate, becomes the dominant virus. Selective pressures, immune response result in vaccine/immunoglobulin escape mutants and antiviral resistance mutations Selective pressures, immune response result in vaccine/immunoglobulin escape mutants and antiviral resistance mutations[44]. Viruses encoding the changes associated with antiviral resistance often have reduced replication in vitro, but the accumulation of additional mutations helps restore viral phynees. Compensatory mutations can occur not only in the P gene, but also in other genes such as the S gene, due to overlap of the S gene and the P gene [8,45]

Although HBV is a chronic infection with immunization programs and antiviral treatment available, and the therapeutic management is based on protocols and clinical treatment guidelines, derived from international scientific results and expert opinion, the mutations in the HBV genome demonstrate that they should also be monitored, and screening should also be available as a diagnostic alternative, to guide clinical management, even before the patient starts treatment, allowing to offer a treatment that can achieve the Sustained Virologic Response (SVR) of chronic patients. There is a possibility of emergence of these resistance mutations and vaccine escape, treated and resistant patients, since the treatment with nucleotide analogues is long-term, there is a risk of relapse of the HBV-DNA viral load, a relapse of liver markers such as ALT, progression of liver disease such as cirrhosis, including clinical manifestations such as decompensation, elevation of viral replication activity and the possibility of increased cases of hepatocellular carcinoma [7].

Conclusion

The results presented in this paper demonstrate that even with a rate of 1.2% of resistance mutations and 7.1% of vaccine escape in a sample from a health service demand, this is a public health problem. The change in AgHBs antigenicity, which results in vaccine escape mutations and spread of resistance mutations to treatment with nucleotide analogues is a reality and an urgency to be faced in the coming years.

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