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HAL Authorization

Primary resistance of hepatitis B virus to nucleoside and nucleotide analogues

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Summary

Nucleoside and nucleotide analogues (NUCs) targeting hepatitis B virus are capable of selecting resistant viruses upon long-term administration as monotherapies. The prevalence of resistance-associated substitutions (RASs) and fitness-associated substitutions at baseline of NUC therapy and their impact on treatment responses remain unknown. A total of 232 treatment-naïve patients chronically infected with hepatitis B virus (HBV) consecutively referred for the first time to one of French reference centres were included. The nearly full-length HBV reverse transcriptase was sequenced by means of deep sequencing, and the sequences were analysed. RASs were detected in 25% of treatment-naïve patients, generally representing low proportions of the viral quasispecies. All amino acid positions known to be associated with HBV resistance to currently approved NUCs or with increased fitness of resistant variants were affected, except position 80. RASs at positions involved in lamivudine, telbivudine and adefovir resistance were the most frequently detected. All patients with RASs detectable by next-generation sequencing at baseline who were treatment-eligible and treated with currently recommended drugs achieved a virological response. The presence of pre-existing HBV RASs has no impact on the outcome of therapy if potent drugs with a high barrier to resistance are used.

KEYWORDS

antiviral treatment, hepatitis B, next-generation sequencing, primary resistance

Abbreviations: cccDNA, closed circular DNA; CHMP, Committee for Medicinal Products for Human Use; EIA, enzyme immunoassay; EMA, European Medicines Agency; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; IQR, interquartile range; NUCs, nucleoside and nucleotide analogues; pegIFN, pegylated interferon; RASs, resistance-associated substitutions; TAF, tenofovir alafenamide.

*A complete list of the Hepatology Reference Centers and Laboratories NETWORK for chronic hepatitis B surveillance is provided in the Data S1.

1 | INTRODUCTION

Chronic hepatitis B can be treated either with pegylated interferon (pegIFN) alpha, administered for a finite duration, or with nucleoside or nucleotide analogue that must most often be administered lifelong.^{1,2} An important feature of hepatitis B virus (HBV) nucleoside and nucleotide analogues is their ability to select resistant viruses upon their long-term administration as monotherapies. Indeed, lamivudine and adefovir were shown to be associated with virological breakthroughs due to the selection of resistant viruses in 70% and 29% of patients after 5 years of administration, respectively, while telbivudine selected resistant viruses in 17% of cases after 2 years.¹ In contrast, entecavir and tenofovir have a high barrier to resistance and exceptionally selected resistant viruses over up to 6-7 years of administration.^{1,3-5}

Hepatitis B virus resistance is characterized by the on-treatment outgrowth of viral populations carrying resistance-associated substitutions (RASs) on their genome. Resistant viruses are not generated by the antiviral drugs. They pre-exist before the start of treatment, as a result of the quasispecies distribution of HBV populations and RAS archiving in covalently closed circular DNA (cccDNA) that acts as an intra-hepatic reservoir and a source for production of resistant HBV variant populations that are subsequently selected during treatment.^{6,7} RASs confer substantially reduced susceptibility to antiviral drugs with a low barrier to resistance, whereas drug susceptibility is poorly altered with compounds with a high barrier to resistance. The fitness of these "primary" RASs may gradually increase during therapy through the occurrence and subsequent selection of compensatory (or "secondary") substitutions, also called fitness-associated substitutions.

Because they are less fit than wild-type virus in the absence of treatment, naturally occurring resistant variants are generally undetectable at treatment baseline with classical sequencing techniques, such as population sequencing.^{8,9} Thus, the actual prevalence of HBV RASs and fitness-associated substitutions at baseline of nucleoside/nucleotide analogue therapy remains unknown.

The goals of this study were to use a highly sensitive sequencing method based on deep sequencing to characterize the natural prevalence of HBV RASs and fitness-associated substitutions in a large cohort of treatment-naïve patients chronically infected with HBV, to analyse the clinical and biological correlates of the

presence of HBV RASs and to assess their impact on antiviral treatment responses.

2 | MATERIALS AND METHODS

2.1 | Patients

Santé publique France (the French national public health agency) conducted a prospective cohort study of all patients with chronic hepatitis B referred for the first time to one of the 33 French reference centres between 2008 and 2012. The first 607 patients included were selected for the virological study. As shown in Figure 1, 345 patients met the selection criteria for inclusion in the present study, that is, being treatment-naïve with an HBV DNA level ≥ 2.5 log international units (IU)/mL (minimum amount of HBV DNA for efficient PCR amplification). Among them, the amount of serum or plasma available was insufficient in 50 cases, initial PCR amplification failed in two cases and emulsion PCR failed in 61 cases. Thus, 232 patients were available for analysis (Figure 1).

The study was approved by the appropriate ethics committee. All patients gave written informed consent.

2.2 | Assessment of liver fibrosis

The fibrosis stage was assessed by means of liver biopsy and/or transient elastography (FibroScan®, Echosens, Paris, France) and/or FibroTest (BioPredictive, Paris, France). Cirrhosis was defined by a METAVIR score F4 on liver biopsy, a FibroScan score ≥ 14.6 kPa or a FibroTest score ≥ 0.85 .

2.3 | Laboratory measurements

Serum HBV DNA levels were measured by means of the real-time PCR assay Cobas Ampliprep/Cobas TaqMan HBV version 2 (Roche Molecular Systems, Pleasanton, CA), according to the manufacturer's instructions. Serum HBsAg levels were quantified by means of the Architect® HBsAg assay. The HBe status was determined

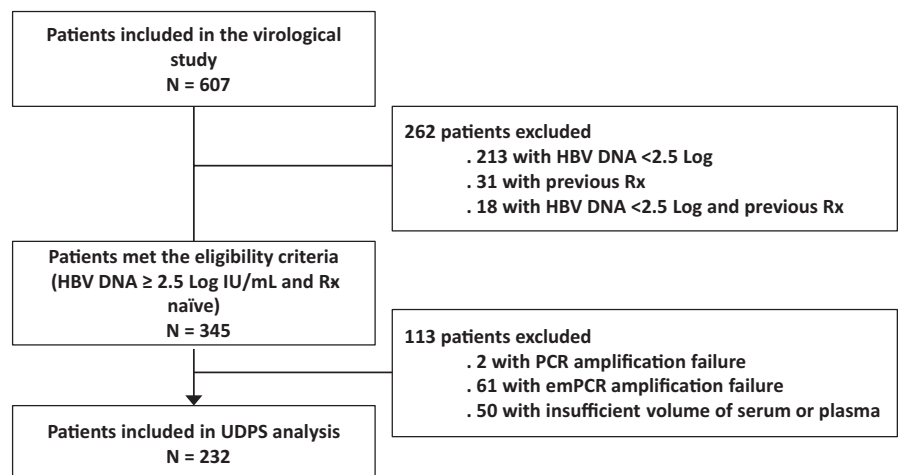


FIGURE 1 Patient disposition (Rx: treatment; UDPS: ultra-deep pyrosequencing; emPCR: emulsion PCR)

by means of a commercial enzyme immunoassay (EIA) (VIDAS™ HBe/Anti-HBe, Biomérieux, Marcy-l'Étoile, France). Serological markers for hepatitis D virus (HDV) were assessed by means of EIA (ETI-AB-DELTA-K-2, Bio-Rad Laboratories, Hercules, CA). The HBV genotype was determined by directly sequencing a portion of the overlapping genes encoding HBsAg and the B and C subdomains of the HBV reverse transcriptase, as previously described.⁸ Hepatitis C virus (HCV) antibodies were sought by means of the VITROS Eci/ECiQ immunodiagnostic system (Ortho-Clinical Diagnostics, Raritan, Franklin, NJ), while the Architect automated device (HIV Ag/Ab Combo, Abbott Diagnostic, Chicago, IL) was used for simultaneous detection of HIV-1 p24 antigen and HIV-1 and HIV-2 antibodies.

2.4 | Deep sequencing of the HBV reverse transcriptase region

The nearly full-length HBV reverse transcriptase gene was PCR-amplified and deep-sequenced by means of the GS FLX Titanium sequencing kit on an FLX Genome Sequencer (454 Life Sciences, Roche Diagnostics, Branford, CT), as previously described.⁹ The sequences generated were analysed by means of our in-house PyroPack® software package (Créteil, France).⁹ Briefly, the sequences were ascribed to each patient by means of identity tags and PyroClass®. Then, the sequences were quality filtered and aligned, and substitutions were analysed statistically by means of PyroMute® to eliminate low-quality data and errors due to the procedure and keep only relevant sequences for final analysis. The consensus amino acid sequence was built and used as a reference to map genetic variability using PyroDyn®.

The error rate of the technique at each amino acid positions was calculated using different dilutions of plasmid as described previously.⁹ Only sequences with frequencies above 1% were taken into account in the analysis.

2.5 | Statistical analysis

Results are presented as median with interquartile range [IQR] for continuous data and number (percentage) for categorical data. Factors associated with the presence of RASs were analysed by means of the Student's t test for continuous data or Fisher's exact test for categorical data.

Statistical analysis was performed with Stata® 11.0 (StataCorp LP, College Station, TX). *P* values <0.05 were considered as statistically significant.

3 | RESULTS

3.1 | Characteristics of the study patients

Table 1 shows the baseline characteristics of the 232 patients included in the primary HBV resistance study. The median age was 46 years, and 61.2% of them were males. Approximately one-third

of patients were born in France and another third in Sub-Saharan Africa, while 12.8% originated from Asia. Most patients (87.1%) were HBeAg-negative. At baseline, the mean HBV DNA and HBsAg levels were 4.12 ± 1.65 and 3.60 ± 0.70 Log IU/mL, respectively. The most frequent HBV genotypes were A, D and E. Sixty-one of the 174 patients who had fibrosis assessment (35.1%) had significant fibrosis, and 18 patients (10.3%) had cirrhosis. Six patients (2.6%) were coinfecting with HDV, 2 (0.9%) with HIV and 4 (1.8%) with HCV.

3.2 | Natural variability of the HBV reverse transcriptase

Figure 2 shows plots of variability per reverse transcriptase amino acid sequence position (positions 66 to 275) relative to the genotype-specific consensus sequence: 80.5% (169/210) of the positions had a less than 10% average amino acid variability as compared to the consensus sequence. Among the 41 remaining

TABLE 1 Baseline characteristics of the study population (N = 232)

Age, y [median (range)]	46 (26-89)
Sex, male [n (%)]	142 (61.2)
Country of birth [n (%)]	
France	75 (33.2)
Europe, excluding France	25 (11.1)
North Africa	20 (8.8)
Middle East	16 (7.1)
Asia	29 (12.8)
Sub-Saharan Africa	61 (27.0)
HDV coinfecting [n (%)]	6 (2.6)
HBe-negative [n (%)]	202 (87.1)
HBV DNA level (Log IU/mL), median (IQR)	3.5 (2.9-5.1)
HBsAg level (Log IU/mL), median (IQR)	3.7 (3.2-4.1)
HBV genotype [n (%)]	
A	84 (36.2)
B	12 (5.2)
C	18 (7.8)
D	78 (33.6)
E	39 (16.8)
G	1 (0.4)
Fibrosis stage distribution [n (%)]	
F0-F1	113 (48.7)
F2	33 (14.2)
F3	10 (4.3)
F4	18 (7.8)
Not done	58 (25.0)
ALT level (U/L) [median (IQR)](range)	36 (24-56) (8-496)
ALT > ULN [n (%)]	86 (37.1)

positions, 17 had a variability comprised between 10% and 20%, 16 between 20% and 30%, five between 30% and 40%, and three between 40% and 50% (Table 2). Three hot spots of variability, spanning amino acid positions 121-153, 211-224 and 256-273 were identified outside of reverse transcriptase domains A to E. These results indicate that HBV reverse transcriptase sequences are conserved across HBV isolates and quasispecies variants, regardless of the HBV genotype.

3.3 | Natural variability of HBsAg

The same sequence data set was analysed in the alternative open reading frame coding for HBsAg. Figure 3 shows plots of variability per HBsAg amino acid sequence position (positions 107 to 226) according to the HBV genotype. Overall, 80.8% (97/120) of the positions had an average variability of less than 10% as compared to the genotype-specific consensus sequence. Among the 23 remaining positions, 12 had a variability comprised between 10% and 20%, nine between 20% and 30%, and two between 30% and 40%. Of the 23 variable HBsAg positions, six (positions 126, 127, 131, 134, 140 and 143) resided within the "a" determinant of HBsAg major hydrophobic region (amino acids 124-147). Among them, all but two (positions 126 and 140) have already been described to be polymorphic (<http://hbv.bioinf.mpi-inf.mpg.de/index.php>). Amino acid changes at position 126 were reported to be associated with active or passive immunization escape.¹⁰ No

differences were observed between the different HBV genotypes (Figure 3).

3.4 | Prevalence of HBV RASs and fitness-associated substitutions

Among the 232 treatment-naïve patients, 58 (25.0%) had detectable RASs in their serum or plasma by deep sequencing with a cut-off of 1%. They represented 1%-5% of the viral quasispecies in 43 of 232 patients (18.5%), 5%-10% in 5 of 232 patients (2.2%), 10%-20% in 6 of 232 patients (2.6%) and >20% in 4 of 232 patients (1.7%). Among these 58 patients, 41 (70.7%) had only RASs, whereas the remaining 17 (29.3%) had fitness-associated substitutions in addition to their RASs. Finally, 53 additional patients (22.8%) had detectable fitness-associated substitutions not associated with RASs (Figure 4).

With a cut-off of 1%, RASs or fitness-associated substitutions were found at all amino acid positions known to be associated with resistance to currently approved nucleoside/nucleotide analogues or with increased fitness of resistant variants (including amino acid positions 163, 169, 173, 180, 181, 184, 186, 202, 204, 236 and 250), with the exception of position 80 (Table 3). Only five positions (rtI163, rtL180, rtA181, rtR202 and rtM204) harboured substitutions present in >20% of the patients' HBV variant populations. Substitutions at other positions were less prevalent in the quasispecies when present (Table 3).

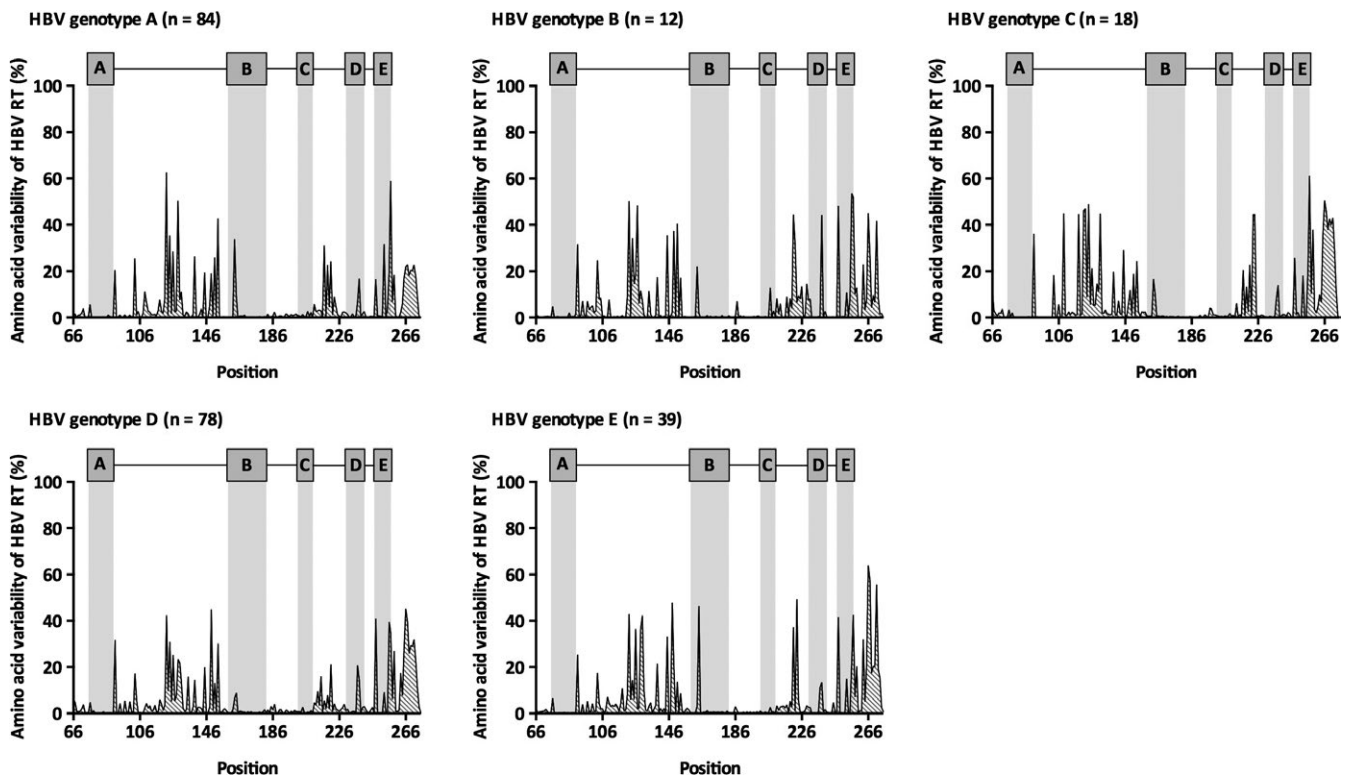


FIGURE 2 Natural amino acid variability of the HBV RT (amino acid positions 66 to 275) in 232 treatment-naïve patients chronically infected with HBV genotype A (n = 84), genotype B (n = 12), genotype C (n = 18), genotype D (n = 78) and genotype E (n = 39). Functional domains A (aa 75-90), B (aa 159-182), C (aa 201-210), D (aa 230-241) and E (aa 247-257) of the HBV RT are shown in grey

Degree of variability	Amino acid positions
10%-20%	S109; N118; I121; G127; Q130; N131; N139; Y148; I163; L164; L217; S219; V224; V253; D263; C272; F273
20%-30%	L91; V103; H126; M129; L145; Q149; F151; R153; F221; A223; H238; S259; I265; K268; I269; K270
30%-40%	H124; H248; C256; Q267; E271
40%-50%	L122; Y257; I266

TABLE 2 Natural variability of the HBV reverse transcriptase: the degree of variability is indicated for specific HBV reverse transcriptase positions

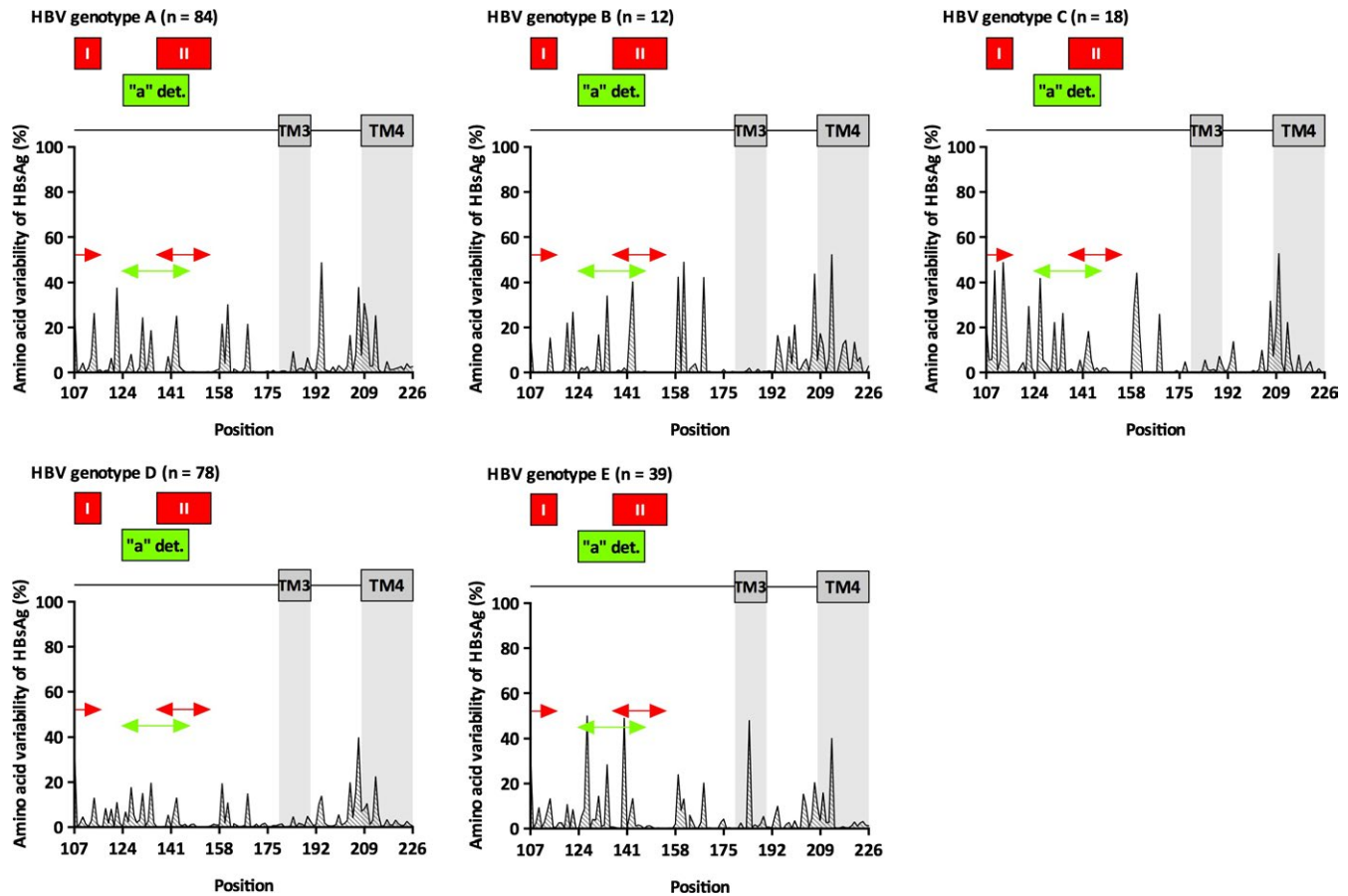


FIGURE 3 Natural amino acid variability of HBsAg (amino acid positions 107 to 226) in 232 treatment-naïve patients chronically infected with HBV genotype A ($n = 84$), genotype B ($n = 12$), genotype C ($n = 18$), genotype D ($n = 78$) and genotype E ($n = 39$). The positions of HLA I epitope (I, aa 97-107) and HLA II epitope (II, aa 163-174) are shown in red. HBsAg "a" determinant (aa 124-147), the main B cell epitope, is shown in green. Transmembrane domains 3 (TM3, aa 179-190) and 4 (TM4, aa 208-226) are represented in grey

3.5 | HBV resistance profiles

Table 4 shows the individual profiles of RASs and/or fitness-associated substitutions in the 58 patients with detectable RASs by deep sequencing. rtM204I/V \pm rtL180M were present in 22 of the 58 patients in proportions ranging from 1.2% to 22% of the viral quasispecies. In most cases, the resistance profiles were complex, with the presence of multiple additional substitutions at positions rt163, rt173, rt202, rt250 and/or rt181 (Table 3). Of the 58 patients, only four displayed an entecavir resistance profile, characterized by the presence of substitutions at positions 202 and 250 in addition to rtM204I/V, present in small proportions (1.3% to 4.3% of the quasispecies) in all cases. A RAS at position rt186 was detected in four patients, alone or

in combination with other substitutions at positions rt184, rt181 and/or rt236, always present in low proportions. In addition, substitutions at positions rt169, rt184, rt202 or rt250 were detected in 21 patients without RASs at position rt204, in proportions ranging from 1% to 22.9% of the quasispecies. rtI163V, which increases the fitness of rtA186T-carrying variants in the presence of entecavir,¹¹ was detectable in 67 of 232 treatment-naïve patients (28.9%), generally in high proportions (over 20% of the quasispecies in 43 of them). However, rtI163V was never associated with rtA186T in our series.

Of the 58 patients with detectable RASs, 19 harboured an adefovir resistance profile, including the presence of rtA181V/T in 14 patients, rtN236T in four patients and both in one patient. rtA181V/T was generally present in low proportion, except in three patients in

whom it represented 13.0%, 13.8% and 85.2% of the viral quasispecies, respectively. rtN236T was also present in low proportion (<5%) in all but one case. In all patients but 4, adefovir RASs were detected

in combination with other substitutions at positions 163, 173, 180, 184, 186 and/or 204.

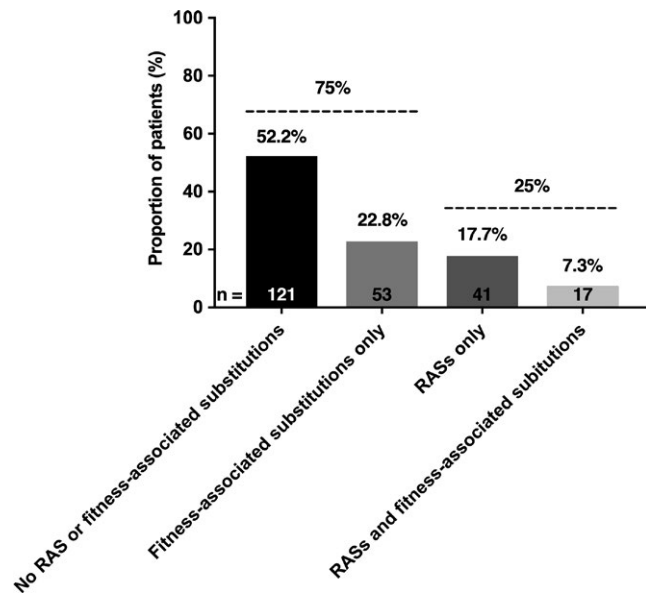


FIGURE 4 Proportion of treatment-naïve patients with and without detectable RASs or fitness-associated substitutions (n = 232)

TABLE 3 Proportions of treatment-naïve patients with various amounts of detectable resistance-associated substitutions (RASs) or fitness-associated substitutions at amino acid positions of the HBV RT known to harbour such substitutions

RASs or fitness-associated substitutions	Distribution of RASs and/or fitness-associated substitution frequencies in the viral quasispecies (%)				
	<1%	1%-5%	5%-10%	10%-20%	>20%
rtL80V	100	0	0	0	0
rtL80I	100	0	0	0	0
rtI163V	71.1	5.6	2.2	2.6	18.5
rtI169T	98.7	0.9	0	0.4	0
rtV173L	97.8	1.3	0.4	0.4	0
rtL180M	98.7	0.9	0	0	0.4
rtA181V	98.3	1.3	0	0	0.4
rtA181T	94.8	3.0	1.3	0.9	0
rtT184S	98.7	0.9	0.4	0	0
rtT184A	98.7	1.3	0	0	0
rtT184I	100	0	0	0	0
rtT184L	100	0	0	0	0
rtT184G	100	0	0	0	0
rtA186T	98.3	1.7	0	0	0
rtR202G	96.1	3.5	0	0	0.4
rtR202I	99.1	0	0.9	0	0
rtM204V	95.7	3.0	0.4	0	0.9
rtM204I	94.8	4.3	0.9	0	0
rtN236T	97.8	1.7	0	0.4	0
rtM250V	95.7	3.9	0	0.4	0

3.6 | Factors associated with the presence of HBV RASs and fitness-associated substitutions

The age, gender, country of birth, HBV genotype, ALT level and fibrosis stage were not associated with the presence of one or several substitutions (including RASs and fitness-associated substitutions). In contrast, the HBsAg level was significantly higher in patients with at least one detectable substitution than in those without any substitution (3.7 ± 0.7 Log IU/mL versus 3.5 ± 0.7 Log IU/mL; $P = 0.047$). In addition, the HBV DNA level was significantly higher in patients with at least two substitutions, as compared to those without any substitution (5.4 ± 1.8 Log IU/mL vs 4.0 ± 1.7 Log IU/mL; $P < 0.001$). The country of birth (Europe, North Africa and Asia; $P < 0.001$) and the HBV genotype (genotypes A, B and C; $P = 0.017$) were also significantly associated with the presence of the rtI163V RAS, whereas an HDV coinfection was significantly associated with the presence of substitutions at position rt181 ($P = 0.044$).

3.7 | Influence of baseline RASs on subsequent treatment response

Among the patients who had detectable RASs at baseline and were eligible to antiviral treatment, 22 were treated. Treatment was based

TABLE 4 Profiles of resistance-associated substitutions and/or fitness-associated substitutions observed in 58 treatment-naïve patients by means of deep sequencing

	Resistance profile	Number of patients	Number of treated patient
Resistance to LAM, FTC, LdT (n = 22)			
rtM204I/V	rtM204I/V	6	1 (TDF) 1 (ETV)
	rtM204I + <i>rtI163V</i>	2	
	rtA181T + rtM204I/V	4	1 (TDF)
	rtM204I + rtN236T	1	
	rtM204I + rtM250V	1	1 (TDF)
	rtA181T + rtM204V + <i>rtL180M</i>	1	
	rtA181T + rtM204I + <i>rtI163V</i>	1	
	rtA181T + rtM204I + <i>rtV173L</i>	1	
	rtM204V + <i>rtL180M</i> + <i>rtI163V</i>	1	
	rtR202G + rtM204V + <i>rtI163V</i>	2	
	rtA181V + rtM204V + <i>rtI163V</i> + <i>rtL180M</i>	1	
	<i>rtI163V</i> + rtM204V + rtM250L + <i>rtV173L</i>	1	
Resistance to ETV (n = 29)			
rtM204V/I ± <i>rtL180M</i>	rtM204I/V + rtM250V	2	
	rtR202G + rtM204I/V	2	1 (ETV)
rtA186T ± <i>I163V</i>	rtA186T	2	
	rtT184A + rtA186T	1	1 (TDF + FTC)
	rtA181V + rtT184A + rtA186T + rtN236T	1	
	<i>rtI169T</i>	2	1 (ETV)
	<i>rtI169V</i> + <i>rtI163V</i>	1	
	rtT184A	1	
	rtR202G	9	3 (TDF)
	rtM250V	8	2 (TDF)
Resistance to ADV, TDF and TAF (n = 19)			
rtA181T/V	rtA181V/T	2	1 (TDF)
	rtA181V + rtM204I/V	4	
	rtA181V + <i>rtI163V</i>	3	
	rtA181V + rtM204I + <i>rtI163V</i>	1	
	rtA181T + rtM204I + <i>rtV173L</i>	1	
	rtA181V + rtT184S + <i>rtV173L</i>	1	
	rtA181V/T + rtM204V + <i>rtI163V</i> + <i>rtL180M</i>	2	
	rtN236T	rtN236T	3
rtM204I + rtN236T		1	
rtA181V + rtT184S + rtA186T + rtN236T		1	

rtM204I/V confer resistance to lamivudine (LAM), emtricitabine (FTC) and telbivudine (LdT), and reduced susceptibility to entecavir (ETV), while *rtL180M* improves the fitness of rtM204V-carrying variants.

Together with rtM204 ± *rtL180*, substitutions at positions *rtI169*, *rtI184*, *rtR202* or *rtM250* increase the level of resistance to ETV.

rtA186T is associated with ETV resistance, and substitutions at position *rtI163* improve the fitness of the corresponding variants.¹¹

rtA181V/T and rtN236T confer reduced susceptibility to adefovir (ADV) and, to a lesser extent, to tenofovir or tenofovir alafenamide (TDF or TAF), at least in vitro.

rtA181V/T also confer reduced susceptibility to LAM and LdT. Fitness-associated substitutions are in italic.

on pegylated interferon in three cases and on nucleoside/nucleotide analogues in 19 cases, including lamivudine in one patient, entecavir in six patients and tenofovir in 12 patients (in combination

with emtricitabine in one case). A virological response, defined as an undetectable HBV DNA 48-96 weeks after the start of treatment, was achieved in the 14 patients receiving a nucleoside/nucleotide

analogue regimen for whom follow-up was available (two patients receiving entecavir, two patients receiving tenofovir and one patient receiving lamivudine were lost to follow-up). The three IFN-treated patients all had undetectable HBV DNA 48 months after the start of treatment.

4 | DISCUSSION

Drug resistance is the principal cause of treatment failure in patients treated with nucleoside or nucleotide analogues who are adherent. However, little information is available on the prevalence of resistant HBV variants in treatment-naïve patients. Classical techniques, such as population sequencing, reverse DNA hybridization, restriction fragment length polymorphism or cloning of PCR products followed by sequencing of the clones, have been widely used for HBV resistance testing in both *in vitro* and *in vivo* research studies.^{12,13} These molecular methods have limitations relating to their specificity, sensitivity, ability to detect novel mutations and reporting of qualitative results. Population sequencing- and reverse hybridization-based assays are commercially available and have been used in clinical practice.^{14,15} These methods are useful to identify highly prevalent resistant populations in patients who failed on therapy. In contrast, population sequencing is not sensitive enough to detect minor populations because of their poor fitness compared to wild-type viruses, while hybridization-based assays are unable to detect unknown RASs.¹⁶ Next-generation sequencing (NGS) techniques based on deep sequencing were recently developed to increase the sensitivity and accuracy of classical techniques. They are highly sensitive to detect minor viral populations and well-suited to viral resistance studies. Despite the potential clinical utility of NGS in the monitoring of resistance during antiviral therapy, there remain barriers to its use, including high cost, the lack of molecular expertise and bioinformatics/statistical capabilities.^{12,17} However, NGS-based techniques provide invaluable tools for molecular studies of viral resistance.

In the present study, we used a sensitive NGS-based approach to show that HBV RASs pre-exist in a substantial proportion (~25%) of patients with chronic HBV infection never exposed to nucleoside/nucleotide analogues. This proportion was higher in our study using NGS than in previous works using less sensitive sequencing methods,¹⁸ and in the same order as in another study using NGS with a cut-off of 1% to longitudinally monitor a small number of treated patients.¹⁹ All positions associated with reduced susceptibility to nucleoside/nucleotide analogues were found to carry substitutions in one to several cases in our study. RASs were generally present in low proportion within the viral quasispecies, a finding in keeping with their reduced fitness in the absence of drug administration. However, RASs at positions 181 and 204 were frequently found (15 and 22 patients, respectively) and were present in moderate to high proportions, explaining the high incidence of virological breakthroughs due to the selection of resistant HBV variants when low-barrier to resistance drugs, such as lamivudine, adefovir or telbivudine, are used.

Currently, these compounds are no longer recommended as first-line treatments for hepatitis B in international guidelines. Instead, compounds with a high barrier to resistance should be used, including entecavir or tenofovir.^{1,2} In our study, most of the patients with RASs detectable at baseline who were treatment-eligible were treated with entecavir, tenofovir, the combination of tenofovir and emtricitabine or pegylated IFN. All of them experienced a virological response characterized by an undetectable HBV DNA 48-96 weeks after the start of antiviral treatment. Thus, our study shows that the presence of pre-existing HBV RASs has no impact on the outcome of therapy if potent drugs with a high barrier to resistance are used, at least on the mid-term.

Our study has a few limitations. First, the least frequent genotypes (G, H and I) were not represented. Secondly, the rate of deep sequencing failure was high, in the order of 20%, similar to what has been observed in other studies with the method used in the present study.²⁰ However, no difference was found on the other parameters between these patients and those whose deep sequencing was successful.

Only few baseline parameters were associated with the presence of RASs. Not surprisingly, a higher HBV DNA level was associated with a greater HBV variability. The relationship with the HBsAg level is difficult to explain and suggests a confounding factor, such as the genotype, although their relationship was not found in our series, possibly because the number of patients was too small in the different genotype groups. A relationship was found between the presence of certain RASs at baseline and the country of birth, that could also reflect a role for the HVB genotype, the route of transmission and/or the duration of infection. A relationship with the HDV status was also found, which could also be explained by different epidemiologies.

In conclusion, our study showed that HBV RASs that confer reduced susceptibility to nucleoside/nucleotide analogues are frequently found in patients who have never been exposed to these compounds when a highly sensitive method, with a limit of detection of 1%, is used. It is likely that, if even more sensitive techniques with a detection cut-off far below 1% were used, RASs and fitness-associated substitutions would be found in nearly all patients before starting antiviral treatment. Although RASs were generally present in low proportions in the viral quasispecies, those conferring resistance to lamivudine, adefovir or telbivudine were often found in high proportions, as a result of their good fitness as compared to wild-type viruses. This provides a theoretical basis to support the use of nucleoside/nucleotide analogues with a high barrier to resistance, such as entecavir or tenofovir, as first-line therapy, as recommended by international guidelines.^{1,2} The drug patents expired in 2015 for entecavir, 2017 for tenofovir. Generic drugs for both entecavir and tenofovir disoproxil fumarate have been approved by the FDA and can thus be used in many other parts of the world, allowing for improved access to care. In addition, tenofovir alafenamide (TAF) has been approved for the treatment of chronic HBV infection with compensated liver disease by the FDA in the US and has received a positive opinion

from the Committee for Medicinal Products for Human Use, the scientific committee of the European Medicines Agency. This prodrug of tenofovir has demonstrated similar antiviral efficacy with improved renal and bone safety as compared to TDF, due to greater plasma stability and more efficient delivery of tenofovir to hepatocytes.²¹ Thus, in spite of our findings showing a high prevalence of HBV RASs at baseline in patients never treated with nucleoside/nucleotide analogues, it is possible to broadly achieve high rates of virological response, provided that the cascade of care is optimized.

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CONFLICT OF INTERESTS

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REFERENCES

- European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol.* 2017;67(2):370-398.
- Terrault NA, Lok AS, McMahon BJ, et al. Update on prevention, diagnosis, and treatment and of chronic hepatitis B: AASLD 2018 Hepatitis B Guidance. *Hepatology.* 2018;16:1560-1599.
- Buti M, Tsai N, Petersen J, et al. Seven-year efficacy and safety of treatment with tenofovir disoproxil fumarate for chronic hepatitis B virus infection. *Dig Dis Sci.* 2015;60(5):1457-1464.
- Kitrinos KM, Corsa A, Liu Y, et al. No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. *Hepatology.* 2014;59(2):434-442.
- Liu Y, Corsa AC, Buti M, et al. No detectable resistance to tenofovir disoproxil fumarate in HBeAg+ and HBeAg- patients with chronic hepatitis B after 8 years of treatment. *J Viral Hepat.* 2017;24(1):68-74.
- Pawlotsky JM. The concept of hepatitis B virus mutant escape. *J Clin Virol.* 2005;34(Suppl 1):S125-S129.
- Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology.* 2009;137(5):1593-1608. e1591-1592.
- Pallier C, Castera L, Soulier A, et al. Dynamics of hepatitis B virus resistance to lamivudine. *J Virol.* 2006;80(2):643-653.
- Rodriguez C, Chevaliez S, Bensadoun P, Pawlotsky JM. Characterization of the dynamics of hepatitis B virus resistance to adefovir by ultra-deep pyrosequencing. *Hepatology.* 2013;58(3):890-901.
- Wallace LA, Carman WF. Surface gene variation of HBV: scientific and medical relevance. *Vir Hep Rev.* 1997;3:5-16.
- Hayashi S, Murakami S, Omagari K, et al. Characterization of novel entecavir resistance mutations. *J Hepatol.* 2015;63(3):546-553.
- Chevaliez S, Rodriguez C, Pawlotsky JM. New virologic tools for management of chronic hepatitis B and C. *Gastroenterology.* 2012;142(6):1303-1313. e1301.
- Rybicka M, Stalke P, Bielawski KP. Current molecular methods for the detection of hepatitis B virus quasispecies. *Rev Med Virol.* 2016;26(5):369-381.
- Degertekin B, Hussain M, Tan J, Oberhelman K, Lok AS. Sensitivity and accuracy of an updated line probe assay (HBV DR vol 3) in detecting mutations associated with hepatitis B antiviral resistance. *J Hepatol.* 2009;50(1):42-48.
- Huh HJ, Kim JY, Lee MK, Lee NY, Kim JW, Ki CS. Analytical and clinical evaluation of the Abbott RealTime hepatitis B sequencing assay. *J Clin Virol.* 2016;85:27-30.
- Lowe CF, Merrick L, Harrigan PR, Mazzulli T, Sherlock CH, Ritchie G. Implementation of Next-Generation Sequencing for Hepatitis B Virus Resistance Testing and Genotyping in a Clinical Microbiology Laboratory. *J Clin Microbiol.* 2016;54(1):127-133.
- Gargis AS, Kalman L, Berry MW, et al. Assuring the quality of next-generation sequencing in clinical laboratory practice. *Nat Biotechnol.* 2012;30(11):1033-1036.
- Li X, Liu Y, Zhao P, et al. Investigation into drug-resistant mutations of HBV from 845 nucleoside/nucleotide analogue-naïve Chinese patients with chronic HBV infection. *Antivir Ther.* 2015;20(2):141-147.
- Jones LR, Sede M, Manrique JM, Quarleri J. Hepatitis B virus resistance substitutions: long-term analysis by next-generation sequencing. *Arch Virol.* 2016;161(10):2885-2891.
- Audsley J, Bent SJ, Littlejohn M, et al. Effects of long-term tenofovir-based combination antiretroviral therapy in HIV-hepatitis B virus coinfection on persistent hepatitis B virus viremia and the role of hepatitis B virus quasispecies diversity. *AIDS.* 2016;30(10):1597-1606.
- Murakami E, Wang T, Park Y, et al. Implications of efficient hepatic delivery by tenofovir alafenamide (GS-7340) for hepatitis B virus therapy. *Antimicrob Agents Chemother.* 2015;59(6):3563-3569.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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