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Genotype-specific mutations in the polymerase gene of hepatitis B virus potentially associated with resistance to oral antiviral therapy ☆

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ABSTRACT

The evolution of hepatitis B virus (HBV) and the role of different variants during antiviral therapy may be influenced by HBV genotype. We have therefore analysed substitutions potentially related to nucleos(t)ide analogues (NAs) resistance at 42 positions within RT-region in a cohort of patients with chronic hepatitis B in relation to HBV-genotype. RT mutations analysis was performed by direct sequencing in 200 NAs-naïve patients and in 64 LAM or LAM + ADV experienced patients with NAs resistance, infected mainly by HBV-genotypes D and A. 27 polymorphic-sites were identified among the 42 positions analysed and 64 novel mutations were detected in 23 positions. Genotype-D displayed the highest mutation frequency (6.4%) among all HBV-genotypes analysed. Single or multiple mutations were detected in 80% of naïve patients. Overall, the most frequent single mutations were at residues rt54, rt53 and rt91 which may associate with significantly lower HBV-DNA levels ($p = 0.001$). Comparison with sequencing data of patients failing LMV or LAM + ADV therapy revealed an higher frequency of novel genotype-specific mutations if compared with naïve patients: 3 mutations under LAM monotherapy in HBV-D (rtS85F; rtL91I; rtC256G) and 3 mutations under ADV therapy in HBV-A (rtI53V; rtW153R; rtF221Y). In HBV-D treated patients the dominant resistance mutation was rtL80V (31.4%) and rtM204I (60%) in LAM + ADV group while LAM-treated patients showed a preference of rtM204V (51.9%). Interestingly, none of HBV-A patients had mutation rtM204I under ADV add-on treatment but all of them had the "V" AA substitution. These results suggested that in patients with CHB, HBV-genotype might be relevant in the evolution and development of drug resistance showing also different mutation patterns in the YMDD motif between HBV genotype D and A.

1. Introduction

The mechanism of viral persistence in infected hepatocytes and the risk of emergence of drug resistance are still main concerns in the treatment of chronic hepatitis B (CHB) with nucleos(t)ide analogues (NAs) as failure of viral clearance may associate with progression of liver disease (Di Marco et al., 2004; Liaw et al., 2004; Hadziyannis et al., 2000; Papatheodoridis et al., 2002). To date, interferon alpha and five NAs (LAM-lamivudine, ETV-entecavir, LdT-telbivudine, ADV-adefovir, TDF-tenofovir) have been approved for the treatment of chronic HBV infection (European Association For The Study of The Liver, 2012).

Mutations conferring resistance to NAs therapy occurs in the RT region of HBV polymerase gene and 16 amino acid substitutions at 11 positions are known to associate with either reduced susceptibility to antiviral drugs (primary mutations) or with restoration of replicative fitness (compensatory mutations) (Zoulim and Locarnini 2009; Lok et al., 2007; Keeffe et al., 2008; Ghany and Doo, 2009). HBV resistance mutations were recently identified in 5% of treat-

Abbreviations: AA, amino acid; ADV, adefovir-dipivoxil; CHB, chronic hepatitis B; ETV, entecavir; HBeAg, hepatitis B e Antigen; HBV, hepatitis B virus; HBV-A-D, hepatitis B genotype A-D; HBV-non D, hepatitis B genotype non D; LAM, lamivudine; LdT, telbivudine; NAs, nucleos(t)ide analogues; NAr, nucleos(t)ide analogues resistance; RT, reverse transcriptase; TDF, Tenofovir.

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ment naïve patients by using INNO LiPA line probe analysis (Mirandola et al., 2011). These results are consistent with those of other previous studies as they confirm the existence of HBV variants bearing primary NA resistance (NAr) mutations in patients never treated before with these drugs (Amini-Bavil-Olyaee et al., 2008; Akarsu et al., 2006; Pastor et al., 2009; Pollicino et al., 2009; Han et al., 2009; Margeridon-Thermet et al., 2009; Kobashi et al., 2009; Guo et al., 2009; Günther et al., 1999). Many other AA changes within the RT region have been described in NAr studies but their possible association with primary drug resistance or with replication fitness is still uncertain (Rhee et al., 2010). During the phylogenetic evolution of HBV, eight genotypes (A to H) have been identified and they differ from each other by more than 8% in the whole sequence of the genome (McMahon, 2009). The HBV genotype might have a potential role in the evolution and selection of mutations associated (or potentially associated) with NAr as described by Liu et al. who analysed AA variations in 42 positions of RT region in a cohort of untreated patients infected with HBV-genotype B and C (Liu et al., 2010). Previous reports have shown that in patients treated with NAs, HBV genotypes influence response to long-term LAM treatment and development of YMDD mutants in patients with CHB (Kobayashi et al., 2006; Orito et al., 2006; Inoue et al., 2011). In a recent study it was reported that mutation pattern of LAM resistance differs among major HBV-genotypes as HBV-A typically selects rtM204V at higher rates compared to rtM204I (Damerow et al., 2010) In this study we have characterised the AA substitutions at 42 RT-positions in a cohort of NAs-naïve CHB patients, in order to define the prevalence of naturally occurring variants potentially affecting treatment with NAs, also in relation to HBV-genotype. The type and frequency of these mutations in naïve patients were then analysed in comparison to a cohort of drug-resistant/suboptimal-responder patients (treated with LAM or LAM + ADV), in order to identify new genotype-specific mutations with the potential to contribute to reduced response to oral antiviral therapy.

2. Materials and Methods

2.1. Patients

HBV genomes were isolated from sera of 200 consecutive NAs-naïve patients with CHB recruited in 13 centres of the North-East of Italy between 2007–2009. All these patients belonged to the cohort of 255 naïve patients who were previously tested for HBV polymerase mutations by InnoLiPA DR v2 and DR. v3 (Mirandola et al., 2011). Mean interval after initial diagnosis of HBV infection was of 37 ± 22 months and all patients were diagnosed and followed by a single clinical centre. In addition, we examined viral genomes from 64 CHB-patients undergoing monotherapy with LAM ($N = 43$) or ADV add-on LAM therapy ($N = 21$) all being recruited from the same clinical centres mentioned above. These treated patients were selected on the basis of failure to LAM therapy due to emergence of drug resistance and/or suboptimal response to ADV, with viral load exceeding 200 IU/ml and negative HCV, HIV and HDV serology. Mean duration of LAM treatment differed between LAM and LAM + ADV groups (49.1 ± 36.8 months and 83.4 ± 52.0 months respectively; $p = 0.005$); mean duration of combination therapy was 29.9 ± 22.2 months. Exclusion criteria included hepatitis C, hepatitis D and human immunodeficiency virus co-infection. The study protocol was approved by our local Ethics Committee and informed consent was obtained from each patients.

2.2. HBV DNA extraction, quantification, and sequencing

HBV-DNA was extracted and quantified from 1 ml of serum by using m2000 Abbott Real Time HBV technology. (Abbott, IL, USA; limit of detection 10–10^9 IU/ml). Extracted DNA (20 μl) was amplified by using primers specific for the RT region of HBV (nucleotides 65 to 1277 from the theoretical EcoRI site of the 3221-nucleotide HBV sequence). The sense primer was 65F (5'-GGC TCC AGT TCA GGA ACA-3'), and the antisense primer was 1277R (5'-TCC ACA GTA TGG ATC GGC A-3'). Thermal cycling parameters involved 50 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for s. PCR products were analysed by gel electrophoresis in 1.0% agarose stained with ethidium bromide. The expected product of 1212 bp was purified with Microcon columns (Millipore,) prior to cycle sequencing with an ABI Prism 377 DNA sequencer by standard dye terminator chemistry. Sequencing chromatograms were examined for nucleotide heterogeneity using Chromas 2.23v (http://www.technelysium.com.au/chromas.html). Sequence data were then aligned with RT sequences from among 102 GenBank sequences of known genotype representing the HBV genotypes (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Each nucleotide sequence was translated (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) and aligned with the corresponding consensus reference HBV- genotype amino acid sequence using BoxShade software (http://www.ch.embnet.org/software/BOX_form.html). Based on the alignment of amino acid sequences from all patients with the consensus genotype-reference sequence, conserved sites in the RT region were considered those positions at which only one amino acid (consensus or wild-type AA) was found or at which two amino acids were present but the prevalence of the minority amino acid was less than 0.5%. All other positions within the RT region were defined polymorphic sites and the minority AA residues were considered to represent a mutation.

2.3. Statistical Analysis

Continuous variables were provided as means ± SD or median (range) and categorical variables as frequency and percentage. Comparison among different groups was performed using ANOVA Kruskal–Wallis or Mann Whitney U test for continuous variables. Fisher exact test was used for categorical data. Statistical associations between mutations and NAs- treatment were assessed using the Fisher's Exact test combined with the method of Benjamini and Hochberg to control the false-discovery rate (FDR) at 0.05 (Benjamini and Hochberg, 1995). Statistical analyses were performed using Statistica software (version 6.0) (StatSoft, OK).

3. Results

3.1. Patients' characteristics

200 nucleos(t)ide (NAs) treatment-naïve patients with CHB and 65 LAM or LAM + ADV-experienced individuals were included. The main characteristics of the three groups of patients are described in Table 1. Most patients were of male sex, Caucasic and HBeAg-negative. NAs-naïve patients differed significantly from treated-groups being younger ($p < 0.0001$; ANOVA Kruskal Wallis) and having higher serum HBV-DNA levels ($p = 0.01$; ANOVA Kruskal Wallis. NAs-naïve patients were infected with six different genotypes (A, B, C, D, E, F) of which genotype D was the most frequent one (69.0%). LAM and LAM + ADV-treated patients were infected mainly with HBV-genotypes D and A which are the most represented genotypes in Europe (McMahon, 2009).

3.2. Analysis of AA substitutions in 42 positions of the RT region amplified from HBV genomes obtained from NAs-naïve patients

For each of the 200 NAs-naïve patients, AA sequence of the RT region of HBV polymerase was aligned and compared with the

Table 1
Baseline characteristics in 200 NAs -naïve patients and 64 LAM and LAM + ADV-treated patients with chronic hepatitis B.

Characteristic	NAs naïve group (n = 200)	LAM treated group (n = 43)	LAM + ADV treated group (n = 21)
Age (mean ± SD). y	43.0 ± 12.9	55.2 ± 10.7	56.0 ± 11.0
Gender (% of male)	147/200 (73.5)	37/43 (86.0)	18/21 (85.7)
HBeAg- negative (% patients)	139/176 (79.0)	30/36 (83.3)	15/17 (88.23)
HBV-DNA (log IU/ml)			
Median (range)	4.7 (1.8–9.0)	3.4 (2.3–8.5)	3.1(2.1–8.0)
Geographic origin (% patients)			
Caucasic	172/200 (86.0)	40/43 (93.0)	21/21 (100)
Asiatic	16/200 (8.0)	3/43 (7.0)	–
Black	12/200 (6.0)	1/43 (2.3)	–
HBV-genotype (% of patients)			
A	26/200 (13.0)	5/43 (11.6)	4/21 (19.0)
B	5/200 (2.5)	–	–
C	12/200 (6.0)	3/43 (7.0)	–
D	138/200 (69.0)	35/43 (81.4)	17/21(81.0)
E	10/200 (5.0)	–	–
F	9/200 (4.5)	–	–

corresponding genotype consensus reference AA sequence. For each isolated viral genome, all the sequences (1 or more) encoding each possible amino acid at a single RT-position were included in the analysis.

AA residues at 42 RT-positions potentially associated with NAs-resistance were then analysed and the prevalence of each mutation was calculated in relation to the HBV-genotype. In details, we have analysed AA residues at 11 well-characterised resistance associated positions: rt80, 169,173,180,181, 184, 194, 202, 204, 236 and 250 (Lok et al., 2007; Keeffe et al., 2008) and also any substitutions at 31 additional positions that have been reported as potentially related to NAr: rt38, 53, 54, 82, 84, 85, 91, 124, 126, 128, 134, 139, 153, 166, 191, 200, 207, 213, 214, 215, 217, 218, 221, 224, 229, 233, 237, 238, 242, 245, 256 (Liu et al., 2010).

Genotype-dependent polymorphic-sites were found at 27 out of the 42 RT-positions analyzed. The AA substitutions we have observed, including those previously reported (Liu et al., 2010), are described in Table 2. and found here in 33.5% (67/200) of naïve patients, with 24 different AA residues in a total of 20 positions (putative and pre-treatment mutations), and 64 novel AA mutation types in 23 positions of the 42 screened AA sites.

3.3. Mutations distribution and frequency according to HBV-genotypes

Analysis of mutations distribution among HBV different genotypes revealed an high number of viral genomes carrying RT-mutations particularly in genotype D (89.1%) with a statistically significant difference compared to genotype A (61.5%,), to genotype E (50%) and to genotype F (33.3%) whereas no significant differences emerged comparing genotype D to genotype B r genotype C (Table 3). Moreover, genotype D displayed the highest mutation frequency (6.4%) among all HBV-genotypes analysed with a statistical significant difference compared to all other HBV-genotypes except genotype B (Table 3). Most of the polymorphic sites had AA substitutions which occurred in two or more different HBV genotypes (Table 2) whereas other RT positions displayed AA substitutions only in a single genotype including rt169 (0.7%), rt215 (21%), rt221 (8.7%), rt229 (6.5%), rt233 (1.4%), rt236 (0.7%) and rt245 (2.2%) for genotype D and rt200 (3.8%) for genotype A.

3.4. RT mutations in relation to HBV replication and genotype

Single or multiple secondary mutations in the RT region of HBV polymerase were detected in 160 naïve patients (160/200, 80%). Overall, serum HBV DNA levels were significantly lower in patients with multiple mutations compared to patients without mutations and to those with single mutation (p = 0.001; ANOVA Kruskal Wallis) (Table 4). Similar results in terms of viral replication were obtained stratifying patients according to HBV-genotypes (Table 4) even though statistical significance was reached among the three groups only in HBV-non D patients (p = 0.03; ANOVA Kruskal Wallis). In HBV-D patients, viral load of the group without mutations was significantly lower only if compared with the multiple mutations group (p = 0.04; Mann Whitney U-test).

Moreover frequency of HBeAg positivity was higher in the absence of mutations (52.5%) compared with the presence of a single substitution (22.2%; p = 0.004; Fisher's exact test) or of multiple mutations (8.4%; p < 0.0001; Fisher's exact test).(Table 4) Patients with multiple mutations were mainly infected with genotype D (83.3%) while patients with a single mutation or with no mutations had a lower frequency of this genotype (63.5, p = 0.005 and 37.5%, p < 0.0001 respectively; Fisher's exact test) (Table 4). Among patients with a single mutation, substitutions at residues rt 54, rt53, and the rt91 were the most prevalent ones being detected in 14 out of 52 cases (27%), 7 cases (13.5%) and 9 cases (17.3%) respectively whereas each of the other mutated residues, rt38, rt213, rt214, rt215, rt229, rt233, rt238 and rt256, occurred only in one or two cases.

3.5. Comparison of the prevalence of RT mutations between naïve patients and NAs-treated patients

Table 5 shows the results on the prevalence of AA substitutions in the RT region of HBV polymerase observed in naïve patients compared to 64 patients who failed treatment with LAM or LAM plus ADV. Comparison was performed only in genotype D and genotype A infected patients as they were the most representative groups.

In genotype D infected patients, seven mutations at five positions (rtL80I/V, rtV173L, rtL180M, rtT184I, rt204I/V) were significantly associated with LAM therapy (Fisher's exact test; Benjamini–Hochberg adjusted p value < 0.05). Differences in the rtL80 V/I and rtM204 V/I substitution pattern were identified between the two groups of treatment-experienced patients. The dominant resistance mutation was rtL80V (31.4%) and rtM204I (60%) in LAM + ADV group while LAM-treated patients showed a preference of rtM204V (51.9%) (see Table 5; Fisher's exact test; Benjamini–Hochberg adjusted p value < 0.05). In both groups of treated patients each mutation was associated with longer duration of treatment with LAM (rtM204I: 35.44 ± 24.2 vs. 56.9 ± 36.5 months; rtM204V: 57.5 ± 35.9 vs. 64.0 ± 33.7 months; rtL80 V: 55.3 ± 28.9 vs. 61.5 ± 35.2 months in LAM vs. LAM + ADV

Table 2
Characterization of AA residues of the 27 polymorphic sites found in 200 NAs-naïve patients with chronic hepatitis B according to HBV-genotype.

	NAs-naïve Patients (by Genotype)					
	HBV-A (N=26)	**HBV-B (N=5)**	**HBV-C (N=12)**	**HBV-D (N=138)**	**HBV-E (N=10)**	**HBV-F (N=9)**
Position						
38	**A** T 4.4	**T**	**T**	**A** E 7.0 T 14.0 K 0.5	**A**	**T**
53	**I** V 6.7 S 4.4 N 2.2 P 2.2 D 2.2	**N** I 20	**S**	**N** K 5.1 D 5.1 E 2.8 S 0.9 I 0.5 T 0.5 H 0.5 Y 0.5	**S**	**T** N 44.5
54	**T** N 2.2 S 2.2	**T**	**T**	**H** Y 26.0 D 2.3	**N**	**T**
91	**I**	**L** I 20.0	**I** L 23.5	**L** I 16.7	**L** I 12.5	**L**
124	**N** H 2.2 R 2.2	**N** H 20.0 D 20.0	**Y** H 11.8	**H** Y 0.5 N 0.9	**H**	**H**
126	**Y** H 6.7	**H**	**H**	**H** R 23.7 Y 1.4 Q 0.5	**Y**	**H**
128	**T** N 2.2 A 2.2 S 2.2	**T**	**T** A 5.9	**T** I 1.4 N 1.4 A 0.5 S 0.5	**T**	**T**
134	**D**	**N** D 20.0 S 20.0	**D** N 11.8 S 5.9	**D** E 0.5 S 0.5	**D** N 6.2	**N**
139	**Q** E 2.2	**N**	**N**	**N** D 0.9 K 2.3 Q 0.9	**N**	**N**
153	**W** R 6.7	**R**	**R**	**R** W 0.5 K 0.5	**R** P 6.2	**R**
169	**I**	**I**	**I**	**I** M 0.5	**I**	**I**
191	**V** I 6.7	**I**	**I**	**V** I 0.5	**I**	**I**
194	**A** D 2.2	**A**	**A**	A V 0.5	**A**	**A**
200	**A** V 2.2	**A**	**A**	**A**	**A**	**A**
207	**V** M 2.2	**V**	**V** I 5.9	**V**	**V**	**V**
213	**S** T 6.7	**S**	**S**	**S** T 4.2 N 1.4	**S** F 6.2	**S**
214	**V** E 2.2 T 2.2	**V**	**V** A 5.9	**V** A 2.3 E 1.9 P 0.9	**V**	**V**
215	**Q**	**Q**	**Q**	**Q** P 2.8 S 4.2 H 5.6 E 0.5 Y 0.5	**Q**	**Q**
217	**R** L 6.7	**L**	**L**	**L** R 0.9	**L**	**L**
221	**Y**	**Y**	**F**	**F** Y 5.1 V 0.5	**Y**	**Y**
229	**L**	**L**	**L**	**L** V 1.4 W 0.5 M 2.3	**L**	**L**
233	**I**	**I**	**I**	**I** V 0.9	**I**	**I**
236	**N**	**N**	**N**	**N** K 0.5	**N**	**N**
237	**P**	**P**	**P**	**P**	**P** S 6.2	**P**
238	**N** D 2.2 T 2.2	**H**	**N** H 5.9	**N** D 1.4 H 7.0 K 0.5	**N**	**S** A 33.3
245	**Y**	**Y**	**Y**	**Y** H 1.4	**Y**	**Y**
256	**S** C 2.2	**S** G 20.0	**S** C 5.9	**C** G 3.7 S 13.9	**S**	**S**

This table shows the 27 genotype-dependent polymorphic sites found in the RT region of HBV-polymerase amplified in viral genomes obtained from 200 NAs-naïve patients with chronic hepatitis B. The first column indicates the RT position and columns 2 through 7 indicate the percent prevalence of mutation at these 27 positions in NAs naïve patients according to virus genotype.
In each column, the consensus AA is shown in bold at the top of each cell and the RT-mutations along with their percent prevalence (shown as superscript) are indicated below the consensus. The three different colours indicate the mutation category according to Liu et al.: RED = putative mutations; GREEN = pretreatment mutations; BLUE = novel mutations found in this study.

Table 3
Mutations distribution among the different HBV-genotypes analysed.

HBV-Genotype	No. of viral genomes with mutations (%)	p	Mutation frequency (%) *	p
D (*N* = 138)	123/138 (89.1)		369/5796 (6.4)	
A (*N* = 26)	16/26 (61.5)	0.0003[a]	41/1092 (3.75)	0.0008[a]
B (*N* = 5)	4/5 (80)	NS[b]	7/210 (3.3)	NS
C (*N* = 12)	9/12 (75)	NS[c]	14/504 (2.8)	0.0012
E (*N* = 10)	5/10 (50)	0.004[d]	6/420 (1.4)	<0.0001
F (*N* = 9)	3/9 (33.3)	0.003[e]	7/378 (1.9)	0.0004

Differences between groups was performed by Fisher's exact test.
Mutation frequency: total number of viral mutations detected/ (42 studied site x *N*).
[a] D vs A.
[b] D vs B.
[c] D vs C.
[d] D vs E.
[e] D vs F.
* Some viral genomes displayed two or more AA residues in one RT position.

Table 4
Pattern of viral parameters in the 200 NAs-naïve patients according to the number of mutations in the RT region and HBV-genotype.

	No mutations	Single mutation	Multiple mutations
All NAs-naïve patients (*N*)	(40)	(52)	(108)[a]
HBV-DNA (log10 IU/ml)			
Median (range)	6.9 (2.61–9.50)	4.9 (4.2–4.4)	4.3 (2.0–9.0)
HBV-D Nas-naïve patients (*N*)	(15)	(33)	(90)
HBV-DNA (log10 IU/ml)			
Median (range)	5.9 (2.6–9.0)	4.6 (2.2–7.8)	4.3 (1.8–9.0)
HBV-non D Nas-naïve patients (*N*)	(25)	(19)	(18)
HBV-DNA (log10 IU/ml)			
Median (range)	7.7 (2.8–9.0)	4.9 (3.0–8.7)	4.4 (2.0–8.6)
HBV-D (% of naïve patients)	15/40 (37.5)	33/52 (63.5)	90/108 (83.3)
HBeAg + (% of naïve patients)	21/ 40 (52.5)	11/52 (22.2)	9/108 (8.4)

[a] Double mutations (*N* = 48); 3 mutations (*N* = 23); 4 mutations (*N* = 20); 5 mutations (*N* = 10) ; 6 mutations (*N* = 4) ; 7 mutations (*N* = 2).

treated patients respectively) even though these differences were not statistically significant. In order to understand if substitution pattern was influenced by the duration of ADV therapy mean time of ADV treatment was compared between different mutations. Patients carrying AA "V" at position rt80 had received ADV for longer than those with the wild-type AA (V: 43.0 ± 25.8 months vs. L: 19.7 ± 20.6 months; $p = 0.04$). Moreover mean duration of ADV treatment was similar for cases carrying rtM204I/V substitutions (I: 26.9 ± 25.2 months vs. V: 22.7 ± 16.7, p = NS) but lower if compared to patients with wild type AA (M: 54 ± 8.5, $p = 0.03$). In genotype A, three mutations at two positions were associated with the nucleoside analogue LAM: rtL180M and rtM204V/I. Surprisingly at position 204 none of the patients in the combined-treatment group had AA "I" but all of them had AA "V" (100%) (mean time of ADV treatment was 38.5 ± 22.12 months).

Table 6 shows the prevalence of mutations at some of the RT-positions which are potentially associated to NAs resistance (Liu et al., 2010). For HBV genotype D, the prevalence of three mutations (rtS85F, rtL91I and rtC256G) was significantly higher in the LAM-group if compared with both naïve and LAM + ADV treated patients (Fisher's exact test; Benjamini–Hochberg adjusted p value < 0.05). Notably, in the LAM group the prevalence of rtL91I was significantly influenced by the time of treatment with this drug as patients with the wild type AA were exposed to a shorter treatment (L: 27.6 ± 26.1 vs. I: 55.6 ± 33.7 months; $p = 0.04$). Moreover, mean time of LAM therapy for I substitution did not differ between the two on- treatment groups (55.6 ± 33.7 months vs. 54.8 ± 31.1 months) and a longer time of treatment with ADV was associated with wild type AA if compared with the I mutation (37.5 ± 20.7 months vs. 20.7 ± 17.7 months, $p = 0.04$). In genotype A, the prevalence of three AA substitutions (rtI53V; rtW153R and rtF221Y) was higher under LAM + ADV therapy if compared to naïve patients.

4. Discussion

The understanding of molecular evolution of HBV and of drug resistance during antiviral therapy requires analysis of the RT sequence changes before and during treatment.

In this study we firstly aimed to characterise AA substitutions in 42 positions of RT region within HBV-polymerase in a cohort of NAs naïve patients infected with the different HBV-genotypes. Primary drug resistance mutations and compensatory mutations were not seen in the sequences of the 200 NAs naïve viral genomes. This is apparently in contrast with previous results obtained in a larger cohort of NAs naïve patients in which HBV mutations associated with anti-viral drug resistance were detected in the 5% of patients by using a more sensitive method such as the INNO LiPA assay (Mirandola et al., 2011). However, these AA substitutions probably represent < 20% of the viral quasispecies and direct sequencing is not enough sensitive to detect them.

Interestingly 27 genotype-dependent polymorphic sites were identified among the 42 positions analyzed. As data are lacking on the relative prevalence of RT-mutations in untreated individuals, this analysis has provided an accurate representation of the distribution and prevalence of AA substitutions according to HBV genotype. These results might therefore help to interpret genotypic mutations data during NAs treatment thus discriminating natural occurring mutations from newly acquired AA substitutions in response to the pressure of the drug. Moreover, sequence analysis revealed that genotype D seemed to have the highest RT-mutation frequency (6.4%) among the HBV-genotypes here analyzed. Overall,

Table 5
Prevalence of mutations in the 10 positions of HBV-RT region known to confer resistance to NAs therapy according to HBV-genotype and treatment.

	Treatment-naïve patients (N = 220 sequences)	LAM treated patients (N = 52 sequences)	LAM + ADV treated patients (N = 44 sequences)	
HBV-GENOTYPE D				
RT-position	Consensus AA AA substitution	Consensus AA AA substitution	Consensus AA AA substitution	p[a]
80	L	L I $^{7.7}$	L I $^{8.6}$	$p1$ = 0.004 $p2$ = 0.002 $p3$ = 1.0
80	L	L V $^{3.8}$	L V $^{31.4}$	$p1$ = 0.04 $p2$ < 0.0001 $p3$ = 0.0006
173	V	V L $^{11.5}$	V L $^{11.4}$	$p1$ < 0.0001 $p2$ < 0.0001 $p3$ = 1.0
180	L	L M $^{53.8}$	L M $^{54.3}$	$p1$ < 0.0001 $p2$ < 0.0001 $p3$ = 1.0
184	T	T I $^{11.5}$	T I $^{5.7}$	$p1$ < 0.0001 $p2$ = 0.02 $p3$ = 0.47
204	M	M I $^{34.6}$	M I $^{60.0}$	$p1$ < 0.0001 $p2$ < 0.0001 $p3$ = 0.03
204	M	M V $^{51.9}$	M V $^{17.1}$	$p1$ < 0.0001 $p2$ < 0.0001 $p3$ = 0.001
HBV-GENOTYPE A				
	Treatment-naïve patients (N = 45 sequences)	LAM treated patients (N = 11 sequences)	LAM + ADV treated patients (N = 8 sequences)	
RT-position	Consensus AA AA substitution	Consensus AA AA substitution	Consensus AA AA substitution	p[a]
180	L	L M $^{72.7}$	L M ^{100}	$p1$ < 0.0001 $p2$ < 0.0001 $p3$ = 0.82
204	M	M I $^{54.5}$	M	$p1$ < 0.0001 $p2$ = NS $p3$ = 0.02
204	M	M V $^{27.3}$	M V ^{100}	$p1$ = 0.006 $p2$ < 0.0001 $p3$ = 0.003

Columns 3 through 5 indicate the prevalence of mutations at different RT-positions in NAs-naïve individuals according to virus genotype D and A. In each column, the consensus AA is shown at the top of each cell and the RT-mutations along with their percent prevalence (shown as superscript) are indicated below the consensus.
Prevalence of mutations = (number of viral sequences with that mutation/ total number of viral sequences analysed) × 100. [For each viral genome, all sequences encoding each possible amino acid were included in the analysis].

[a] p_1 is naïve vs LAM treated patients; p_2 is naïve vs LAM + ADV treated patients; p_3 is LAM vs LAM + ADV treated patients (Fisher's exact test; Benjamini–Hochberg adjusted p value < 0.05).

patients carrying multiple mutations showed the lowest serum HBV-DNA levels, they were more frequently infected with HBV-D and they were mainly patients with HBeAg negative CHB. These results, together with the evidence of a higher prevalence of precore variants found in > 70% of individuals with genotype D (Chu et al., 2003) suggests that HBV-D may have the highest genetic variability among all HBV-genotypes. Moreover, in patients with a single mutation, substitutions at residues rt53, rt54 and rt91 were the most frequent single AA substitutions which may contribute to reduce the viral fitness as HBV-DNA levels significantly decreased in viral genomes with a single mutation if compared to patients with no mutations (p = 0.001). Characterization of the 42 RT-positions was also performed in a group of LAM or LAM plus ADV treated patients who were resistant to LAM and had a suboptimal response to ADV. Although the three cohorts of this study did not include the same group of patients, comparison of RT mutations among the three different cohorts may contribute to see if the occurrence of some mutations could increase or decrease under treatment. Adefovir has been widely used in the treatment of patients infected with LAM-resistants strains (Peters et al., 2004). However, up to 25% of LAM resistant patients might fail to achieve a complete virological response with ADV treatment and 30% of naïve patients develop ADV resistance at 5 years (Hadziyannis et al., 2006). Predictors of the response to ADV rescue therapy in patients with LAM resistance are not completely understood although it was previously reported that the baseline HBV DNA and ALT levels might play a role in the virological response to ADV add-on LAM therapy (Ryu et al., 2010).

Our study has shown that there is a different genotype-dependent mutation pattern between the two groups of treated patients. Indeed, in genotype-D infected patients, ADV add-on therapy seemed to decrease the prevalence of rtM204V mutation while rtM204I and rtL80V are the prevalent substitutions under combined treatment. On the other hand, none of the genotype A patients had mutation rtM204I under ADV treatment but all of them had rtM204V. Data obtained from a literature review and two databases analysing LAM-resistant patients (Damerow et al., 2010) revealed a mutation pattern similar to our cohort of LAM + ADV treated patients: genotype A favours the rtM204V mutation during emergence of LAM resistance while genotypes B-D showed a preference for rtM204I. However in this paper mean time of duration of LAM treatment is not reported as well as it is not clear if selection criteria included only LAM monotherapy or also combined therapy LAM + ADV. In our study, the prevalence of

Table 6
Significant prevalence of mutations in the polymorphic sites of HBV-RT region potentially associated with resistance to NAs therapy according to HBV-genotype and type of treatment.

HBV-GENOTYPE D				
	Treatment-naïve patients (N = 220 sequences)	LAM treated patients (N = 52 sequences)	LAM + ADV treated patients (N = 45 sequences)	
RT position	Consensus AA AA substitution	Consensus AA AA substitution	Consensus AA AA substitution	p[a] value
85	S	SF $^{5.8}$	S	$p1 = 0.006$; $p2$ = NS; $p3 = 0.28$
91	LI $^{16.7}$	LI $^{63.5}$	LI $^{37.1}$	$p1 < 0.0001$; $p2 = 0.01$; $p3 = 0.02$
256	CG $^{3.7}$	CG $^{17.3}$	CG $^{22.9}$	$p1 = 0.001$; $p2 = 0.0004$ $p3 = 0.6$
HBV-GENOTYPE A				
	Treatment-naïve patients (N = 45 sequences)	LAM treated patients (N = 11 sequences)	LAM + ADV treated patients (N = 8 sequences)	
RT position	Consensus AAAA substitution	Consensus AAAA substitution	Consensus AAAA substitution	p[a] value
53	IV $^{6.7}$	I	IV $^{37.5}$	$p1$ = NS; $p2$ = NS; $p3 = 0.01$
153	WR $^{6.7}$	W	WR $^{37.5}$	$p1$ = NS; $p2$ = NS; $p3 = 0.01$
221	F	F	FY $^{25.0}$	$p1$ = NA; $p2 = 0.007$; $p3$ = NS

Columns 3 through 5 indicate the percent prevalence of mutations at different RT-positions in NAs-naïve individuals according to virus genotype D and A. In each column, the consensus AA is shown at the top of each cell and the RT-mutations along with the percent prevalence (shown as superscript) are indicated below the consensus.
Prevalence of mutations = (number of viral sequences with that mutation/ total number of viral sequences analysed) × 100. [For each viral genome, all sequences encoding each possible amino acid were included in the analysis].

[a] p_1 is naïve vs LAM treated patients; p_2 is naïve vs LAM + ADV treated patients; p_3 is LAM vs LAM + ADV treated patients. (Fisher's exact test; Benjamini–Hochberg adjusted p value < 0.05).

different resistance mutations under ADV add-on treatment, could have two different explanations: a) genotype-dependent selection of these substitutions may be the result of a longer exposure to LAM; b) HBV-genotype and the type of AA substitutions in the YMDD motif might influence the efficacy of ADV rescue therapy, considering that in our patients the prevalence of NAr mutations seemed not to depend on duration of ADV treatment. Taking into account this hypothesis, it might be suggested that the lack of virological response in a percentage of multidrug-resistant patients treated with the most potent available NAs (ETV and TDF) could depend on a combined effect of HBV-genotype, the type of AA substitutions in some positions associated to NAs resistance and the levels of HBV-DNA (Cassino et al., 2011; Patterson et al., 2011; van Bömmel et al., 2010). Our study has also identified new genotype-specific AA substitutions which might be relevant in the development and evolution of resistance to oral antiviral therapy as their frequency significantly increased in treated patients if compared with naïve patients (Genotype D: S85F, L91I and C256G; genotype A: rtI53V, rtW153R and rtF221Y) Interestingly, a novel mutation pattern (L80L/V, L91I, M204I, S219A, N238D, Y245H) showing 30.4-fold resistance to ETV was recently documented in a case report of multidrug-experienced patient infected with HBV-D (Karatayli et al., 2012).

5. Conclusions

Taken together, our data indicate that: (i) in NAs naïve patients with CHB, characterization of AA changes within the RT region according to HBV-genotype might help to discriminate natural occurring mutations from newly acquired mutations during NAs treatment; (ii) HBV genotype D may have the highest genetic variability among all HBV-genotypes analyzed; (iii) there is a different genotype-dependent mutation pattern in some positions related to LAM resistance between LAM and LAM + ADV patients; (iv) new genotype-specific AA substitutions might be relevant in the development and evolution of resistance to oral antiviral therapy as their prevalence significantly increased in treated patients if compared with naïve patients; (v) phenotypic studies should be performed to determine if these novel mutations, alone or in combination with others, might affect virus drug resistance or replicative fitness of the last generation oral antivirals.

6. Disclosures

Silvia Mirandola is currently an employee of Gilead Sciences but not at the time this work was conducted. Alfredo Alberti has received advisory board honorarium from Gilead, BMS, Novartis, Roche, Merck and Research Grants from Gilead, and Merck. All other Authors had nothing to disclose.

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