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Short Communication

Characterization of drug-resistance mutations in HBV D-genotype chronically infected patients, naïve to antiviral drugs

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ABSTRACT

Presence of drug-resistance mutations in drug-naïve hepatitis B virus (HBV) infected patients can seriously compromise response to antiviral treatment. Therefore, our study was aimed at defining the prevalence of HBV drug-resistance in a population of 140 patients, all infected with HBV-D-genotype (the most common HBV-genotype in Eastern Europe, Mediterranean countries and Middle East) and naïve to antiviral therapy. HBV reverse-transcriptase (RT) region was sequenced and analyzed for 20 mutations, confirmed by *in vitro* studies as associated with resistance to nucleos(t)ide HBV-RT inhibitors (rtL80I/V-rtI169T-rtV173L-rtL180M-rtA181T/V/S-rtT184A/S/G/C-rtA194T-rtS202C/G/I-rtM204V/I-rtN236T-rtM250V). Amino acid changes at other six RT positions, potentially associated with resistance, were also analyzed (rtV84M-rtV191I-rtV207L-rtV214A-rtQ215S-rtI233V). Overall, only 2/140 (1.4%) patients carried primary drug-resistance mutations [rtA181V (0.7%), and rtA194T (0.7%)], while 3/140 (2.1%) patients harbored the secondary mutations rtV173L (1.4%) and rtL180M (0.7%). Additionally, five polymorphic mutations, with a suggested role in drug resistance, were detected [rtQ215S (12.8%), rtI233V (4.3%), rtV214A (3.6%), rtV191I (0.7%), rtV207L (0.7%)]. Notably, no YMDD mutations, namely rtM204V/I, were found. Taken together, the rate of important drug resistance mutations in naïve HBV D-genotype infected patients is today very low, and suggests the potential full efficacy of new-generation antiviral drugs used in first line therapy. Whether such low rate can be extrapolated to non HBV-D subtypes, requires a detailed investigation to be performed in a different cohort of patients.

The discovery and clinical use of antiviral agents targeted at the reverse transcriptase (RT) have revolutionized the treatment of patients chronically infected with the hepatitis B virus (HBV) (Durantel et al., 2005). To date, five nucleos(t)ide RT inhibitors have been approved for the treatment of chronic HBV infection: three NsRTIs [LMV (lamivudine), ETV (entecavir), TBV (telbivudine)] and two NtRTIs [ADV (adefovir), TDF (tenofovir)] (Morgan and Keeffe, 2009). These drugs efficiently suppress HBV replication in most patients and delay disease progression (Nevens et al., 1997; Nguyen and Keeffe, 2009), though, when antiviral therapy fails to be sufficiently suppressive, new viral variants can emerge conferring resistance to the drugs. This in turn is associated with disease progression (Locarnini and Zoulim, 2010).

The circulation of HBV-resistant strains in drug-experienced patients can represent a serious concern for public health, since it can lead to a risk of transmission of resistant HBV strains to drug-naïve patients, increasing chances of failure to first-line antiviral treatment.

Reports about resistance in naive patients have been recently published (Ntziora et al., 2009; Pastor et al., 2009; Solmone et al., 2009; Han et al., 2009), though the results are quite contradictory. This can be due to a number of factors: (1) The limited number of patients studied; (2) The technology used for the assessment of resistance: gene sequencing has been used in a limited number of studies, despite its superiority over in situ hybridization, which is perhaps more sensitive but carries a greater risk of unreliable and/or incomplete data. (3) The geographic distribution: the results in different continents cannot be extrapolated to Europe, because of many epidemiological differences (4) The HBV genotype: different HBV genotypes show a remarkable variability, that

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can affect the natural presence of mutations/polymorphisms associated with resistance.

Thus, and despite its clinical relevance, the rate of HBV drug-resistant strains in European naive patients carrying D-genotype is still poorly known. Therefore, our study aims at defining the prevalence of drug-resistance mutations in a large cohort of patients, all infected with HBV D-genotype, naive to antiviral drugs, followed between 2007 and 2010 in several clinical centers in Central Italy. To our knowledge, this is the largest cohort of HBV-infected patients, naive to antivirals, all carrying the same D-genotype.

Drug resistance mutations and polymorphic changes at positions related to resistance were assessed comparing each patient's HBV sequence with a HBV D-genotype sequence (genbank id: V01460), as reference strain.

Among the 187 patients sequenced at baseline, we focused our attention on 140 drug-naïve HBV D-genotype infected patients (Table 1). The median (IQR) age was 47.6 (36.3–61.0) years. The majority of patients (70.0%) was of Italian origin. At the time of HBV sequencing, median (IQR) log HBV-viremia was 4.0 (2.3–5.4) log IU/L, while median (IQR) ALT and AST were 47 (28–105) IU/L and 37 (26–83) IU/L, respectively. The majority of patients (63.9%) were exclusively infected with HBV. Among co-infected patients, HIV-1 infection was the most common (34.2%). None of HIV co-infected patients were previously treated with anti-HIV drugs.

For each patient, HBV-RT full-length sequencing (344 amino acids) was performed on plasma samples as follows. HBV-DNA was extracted using a commercially available kit (QIAmp DNA blood mini-kit, Qiagen Inc., USA), and amplified with Amplitaq-Gold-polymerase using the following primer pairs: 5′GGTCACCATATTC TTGGGAA and 5′GTGGGGGTTGCGTCAGCAAA. PCR conditions were: one cycle (93 °C 12 min), 40 cycles (94 °C 50 s, 57 °C 50 s, 72 °C 1 min and 30 s). PCR-products were sequenced by using eight different sequence-specific primers, BigDye-terminator-v.3.1 sequencing kit (Applied-Biosystems FosterCity) and an automated sequencer (ABI-3100). The sequences were analyzed using SeqScape-v.2.0 software. The quality endpoint was ensured by a double sequence coverage for each nucleotidic region. The full sequencing methodology has been described in details in a previous publication (Svicher et al., 2010).

We analyzed 20 mutations known to be associated with drug-resistance (rtL80I/V-rtI169T-rtV173L-rtL180M-rtA181T/V/S-rtT 184A/S/G/C-rtA194T-rtS202C/G/I-rtM204V/I-rtN236T, rtM250V) (Locarnini, 2008; Ghany and Liang, 2007; Sheldon et al., 2008; Reynaud et al., 2009; Amini-Bavil-Olyaee et al., 2009; Bartholomeusz and Locarnini, 2006; Zoulim and Locarnini, 2009). The other six mutations, involved in reduction of drug effectiveness, were also analyzed (rtV84M-rtV191I-rtV207L-rtV214A-rtQ215S-rtI233V), despite definitive data on their involvement in drug resistance are not yet available (Ghany and Liang, 2007; Sheldon et al., 2008; Keeffe et al., 2008; Chotiyaputta and Lok, 2009; Ghany and Doo, 2009; Locarnini, 2008; Zöllner et al., 2005). HBV RT sequences have been submitted to Genbank (Accession No.: JN225963–JN226099).

Table 1
Main characteristics of the study population.

Characteristics	*N* = 140 (%)
Country of origin	
Italy	98 (70)
Other countries	42 (30)
Age (years), Median (IQR)	47.6 (36.3–61.0)
Median ALT (IQR), IU/L	47 (28–105)
Median AST (IQR), IU/L	37 (26–83)
Median Viremia (IQR), log IU/L	4.0 (2.3–5.4)
HBV monoinfection	69 (49.3)
Coinfections	
HIV–HBV coinfection	37 (26.4)
Others (HCV, HDV)	5 (3.6)
Unknown	9 (6.4)

IQR, interquartile range.

The results show that one drug-naïve patient carried the primary drug-resistance mutations rtA181V (*N* = 1[0.7%]), while another patient carried rtA194T (*N* = 1[0.7%]). rtA181V *in vitro* affects at different levels the efficacy of four antiviral drugs, since it decreases the susceptibility to lamivudine/telbivudine (10-fold), adefovir (8-fold), tenofovir (3-fold) (Villet et al., 2008). The other primary mutation found in our population, rtA194T, is reported to be relevant for its implication in a poor response to tenofovir (Sheldon et al., 2005, 2008).

The primary drug resistance mutations rtM204I/V, associated with cross-resistance to lamivudine, telbivudine and entecavir (common in patients experiencing virological breakthrough during treatment with these drugs), were completely absent.

Three out of 140 patients carried secondary mutations, known to restore viral fitness in resistant HBV: 2 (1.4%) had rtV173L, while 1 (0.7%) had the rtL180M. Interestingly, the patient carrying rtL180M showed no evidence of any other drug-resistance mutation. rtL180M usually occurs in patients with primary lamivudine/telbivudine resistance (Lok et al., 2007), in order to rescue the rtM204I/V-mediated loss of HBV fitness (Sheldon et al., 2006). The presence of rtL180M might be due to natural HBV genetic evolution in the absence of drug-pressure, or might have been transmitted along with M204V/I from an anti-HBV drug treated patient. In this latter case, it is conceivable that the absence of drug-pressure has led to a loss of M204I/V (due to their impairment in viral fitness), and to the maintenance of L180M in the predominant viral species. Thus, we cannot exclude, in this drug-naïve patient, the presence of rtM204I/V mutations as minority species or archived in cccDNA (serving as sole transcriptional template for HBV replication in the hepatocytes), and thus not detectable by population-based sequencing. This point is also crucial since the presence of rtM204V and rtL180M could at least partially compromise entecavir usage (Table 2).

One of the two patients carrying the secondary rtV173L also harbored the primary rtA181V resistance mutation, an unusual pattern since rtV173L is commonly associated with primary rtM204V/I.

Besides primary and secondary drug-resistance mutations, we also assessed the prevalence of some mutations recently proposed as potentially associated with drug-resistance, that were found in 21 patients (15.0%). The rtQ215S, associated *in vitro* with low level of resistance to lamivudine and adefovir (Pastor et al., 2009; Schildgen et al., 2010), was found in 18 patients (12.9%), while rtV214A and rtI233V, both associated with adefovir resistance (Pastor et al., 2009; Schildgen et al., 2010), were found in 5 (3.6%) and 6 (4.3%) patients, respectively; rtV191I and rtV207L were found in 1 patient each (0.7%). rtV214A and rtI233V have been recently reported as associated with lower response to tenofovir (Reynaud et al., 2009).

Taken together, primary or secondary mutations associated with HBV resistance were found in only 3/140 (2.1%) patients, thus supporting that primary resistance in drug-naïve patient infected with D-genotype is very low.

Interestingly, we observed in 30 patients (21.4%) the presence of some novel amino acid substitutions at positions reported to be associated with drug resistance: rtQ215H/P/L (*N* = 17 [12.1%]), rtV214G/E/D/I (*N* = 4 [2.9%]), rtI169L (*N* = 4 [2.9%]), rtA194V/D/N/S (*N* = 3 [2.1%]), rtA181G (*N* = 1 [0.7%]), rtV173G (*N* = 2 [1.4%]), rtS202N/M (*N* = 2 [1.4%]), rtV207M (*N* = 2 [1.4%]), rtL180I (*N* = 1 [0.7%]), rtV84G (*N* = 1 [0.7%]. These polymorphisms might be directly involved in mechanisms underlying HBV drug-resistance, and/or influence the genetic barrier (defined as the number of

Table 2
Mutation analysis in study population.

Drug-resistance mutations	Resistance to	Prevalence (%), N = 140
Primary mutations		
A181T	LMV/ADV	0 (0.0)
A181V	LMV/ADV/TDF	1 (0.7)
A181S	LMV/ADV	0 (0.0)
T184A	LMV/ETV	0 (0.0)
T184S	LMV/ETV	0 (0.0)
T184C	LMV/ETV	0 (0.0)
T184G	LMV/ETV	0 (0.0)
A194T	ADV/TDF	1 (0.7)
S202C	LMV/ETV	0 (0.0)
S202G	LMV/ETV	0 (0.0)
S202I	LMV/ETV	0 (0.0)
M204I	LMV	0 (0.0)
M204V	LMV/TBV/ETV	0 (0.0)
N236T	ADV	0 (0.0)
M250V	ETV	0 (0.0)
Secondary mutations		
L80I	LMV	0 (0.0)
L80V	LMV	0 (0.0)
I169T	LMV/ETV	0 (0.0)
V173L	LMV	2 (1.4)
L180M	LMV/ETV	1 (0.7)
Potential resistance mutations		
V84M	ADV	0 (0.0)
V191I	LMV	1 (0.7)
V207L	Polymorphic	1 (0.7)
V214A	ADV/TDF	5 (3.6)
Q215S	LMV/ADV	17 (12.1)
I233V	ADV/TDF	6 (4.3)

WT, wild type; RT, reverse transcriptase.
Primary and secondary mutations should be considered as defined by Zoulim and Locarnini (2009) and Amini-Bavil-Olyaee et al. (2009).
Potential resistance mutations has been considered referring to Ghany and Liang (2007), Sheldon et al. (2008), Keeffe et al. (2008), Chotiyaputta and Lok (2009), Ghany and Doo (2009), Locarnini (2008) and Zöllner et al. (2005).

nucleotide substitutions required for the generation of a specific drug-resistance mutation) to the acquisition of drug-resistance mutations. In this regard, in the case of rtQ215P, the proline reduces, in respect to glutamine, the calculated genetic barrier for the appearance of drug-resistance mutation rtQ215S, representing an intermediate passage towards its onset. None of the other observed polymorphisms affects the genetic barrier, so they have an unknown virological and clinical relevance. Thus, further studies are necessary to investigate the impact of such determinants on virological response to anti-HBV drugs.

In order to provide a comprehensive correlation between the overall degree of HBV-RT amino-acid variability and the rate of drug resistance mutations, we estimated the Shannon-Entropy of HBV-RT sequences from the 140 HBV D genotype drug-naïve patients used in this study, and also from drug-naïve patients infected with HBV-genotype A, B, and C (67 patients for A genotype, 124 for B, and 189 for C) present in our database. We found a higher degree of HBV-RT amino-acid variability in genotype D and A than in genotype B and C (mean Shannon-Entropy [sd]: 0.16 ± 0.21 for D, 0.09 ± 0.14 for A, 0.04 ± 0.11 for B, and 0.03 ± 0.07 for C). The higher degree of amino acid variability observed in D genotype is consistent with a paper previously published by another group (De Maddalena et al., 2007). By calculating, the prevalence of patients with at least 1 drug resistance mutation (both primary, secondary, and potentially associated with drug resistance), results are as follows: 24/140 (17.1%) for genotype D, 12/67 (17.9%) for A, 7/124 (5.6%) for B, 27/189 (14.3%) for C. The higher prevalence of patients with at least 1 drug resistance mutation in genotype D and A is consistent with the higher degree of genetic variability estimated by the Shannon Entropy.

Primary and secondary mutations have been reported in cohorts of drug-naïve patients from different countries but carrying different HBV genotypes (Liu et al., 2010; Pollicino et al., 2009; Sayan et al., 2010; Han et al., 2009). In particular, by population-based sequencing, the prevalence of drug resistance has been estimated in the range from 3.8% (Sayan et al., 2010) to 46% (Pollicino et al., 2009). In this latter paper (led in a cohort of patients infected by different genotypes [A, B, and D]), the prevalence of 46% can be ascribed to the presence of secondary mutations since primary mutations have not been observed. Thus, beyond differences in the set of patients analyzed, the discrepancies in the prevalence of drug resistance mutations among drug-naïve patients may also reflect the lack of a standardized list of mutations to characterize the epidemiology of HBV drug resistance in drug-naïve patients. This list may help to compare the prevalence of drug-resistance from different time-periods and regions, and facilitate meta-analyses of data collected by different groups at different times. This highlights the need of consensus guidelines for the estimation of HBV drug resistance among drug-naïve patients.

Our study shows a quite low prevalence, in drug-naïve D-genotype HBV-infected patients, of drug resistance mutations that could compromise first line treatment outcome. Primary mutations have been detected in only 2/140 (1.4%) analyzed patients; rtM204I/V mutations were completely absent in our population, while their presence has been reported with a prevalence ranging from 2% to 8% in other studies on different HBV genotypes (Han et al., 2009; Shin et al., 2003).

Secondary drug-resistance mutations were also rare in our population (2.1% prevalence), and the presence of only 1 patient with rtL180M should exclude the hypothesis of a massive presence of hidden resistant minority species.

Such limited prevalence of drug resistance, highlighted in this study, is exclusively referred to HBV D-genotype, the most prevalent in Eastern Europe, Mediterranean countries and Middle East (McMahon, 2009). In the majority of the countries in these areas, lamivudine is still used as first-line monotherapy, while most clinical practice guidelines recommend the use of tenofovir and/or entecavir, which have a higher genetic barrier to resistance. The relevance of our results therefore need to be substantiated in countries other than Italy, where the continuous use of lamivudine may create conditions for spreading of HBV drug-resistant HBV strains.

In addition, although first-line treatment with entecavir or tenofovir has been associated with very low rate of drug resistance emergence over time, the detection of drug-resistance mutations at baseline may be responsible for partial virological response to first line therapy. In particular, a recent study by Lampertico et al. (2011a,b) showed that 11% and 16% of naive patients achieves only a partial virological response with ETV and TDF respectively.

In general, these results show that drug-resistance mutations (detected by population-based sequencing) are not naturally present as predominant species in HBV D genotype infected drug-naïve patients, and that this genotype is naturally sensitive to currently used antivirals in most of the cases. However, population-based sequencing only detects variants present in at least 20% of the total viral pool where as more sensitive assays such as restriction fragment length polymorphisms (RFLP) have the ability to detect minority viral variants which may be present as low as 5%, while ultrasensitive assays such as Pyrosequencing, can detect HBV variants at less than 1% (Margeridon-Thermet et al., 2009). Although the use of these methodologies can increase the prevalence of drug resistance, the clinical relevance of minority drug resistance species has not yet been clarified and thus requires further investigation (Margeridon-Thermet et al., 2009; Solmone et al., 2009).

Based on these results, genotypic testing at the beginning of therapy seems per se not crucial for the identification of drug-naïve mutations. However, it may have other functions such as:

(i) defining HBV genotype since different genotypes are known to have different evolution to hepatocellular carcinoma and response to interferon-based regimens (Lin and Kao, 2011), and (ii) as a consequence of the overlapping between the RT and HBsAg genes, providing information regarding the presence of HBsAg immune-escape mutations and/or stop-codons (associated with an increased oncogenic HBV potential).

Thus, the routine use of resistance testing in drug-naïve patients remains an issue for future research requiring further detailed studies.

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