Antiviral Research 87 (2010) 74–77

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short communication

Naturally occurring hepatitis B virus (HBV) variants with primary resistance to antiviral therapy and S-mutants with potential primary resistance to adefovir in Argentina

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ARTICLE INFO

Article history:
Received 5 January 2010
Received in revised form 8 April 2010
Accepted 12 April 2010

Keywords:
HBV
Antivirals
Naturally occurring resistance
S-mutants
Argentina

ABSTRACT

Hepatitis B virus (HBV) variants may either emerge in patients with chronic hepatitis B (CHB) as a result of positive selection pressure exerted by their own immune response, or during therapy with nucleos(t)ide analogues (NAs). Naturally occurring HBV variants with primary antiviral resistance are rarely observed. The aim of this study was to retrospectively analyze the (eventual) circulation of HBV variants with natural resistance to NAs currently used as therapy for CHB in Argentina. This study reports 13 cases of CHB-infected patients with natural antiviral resistance to at least one NA. Five of them were also carriers of S-variants that might escape the humoral immune system recognition with potential resistance to adefovir. In addition to the already reported A2 HBV subgenotype association to NAs natural resistance, E and F genotypes association to such resistance is described for the first time. These findings suggest that sequence analysis of the HBV reverse transcriptase might be an essential tool before starting antiviral therapy, in order to choose the proper NAs for optimizing the therapeutic management of chronically infected patients. Moreover, the circulation and transmission of S-mutants with resistance to such antiviral drugs should be of public health concern as they may represent an additional risk for the community.

Treatment with pegylated interferon-alpha as well as with the NAs lamivudine (LMV), adefovir (ADV), entecavir (ETV) and telbivudine (LdT) are good options for patients with chronic hepatitis B (CHB) (Ghany and Liang, 2007; Niesters et al., 2005). However, these therapeutic tools exert strong selection pressures on HBV, which may result in the emergence of viral variants or resistance mutants (Locarnini, 1998).

In 2002, a report from Germany showing 2 patients with CHB with natural resistance to ADV, associated to the viral polymerase point mutation rtL217R, heralded the era of naturally occurring HBV variants with primary resistance to NAs (Schildgen et al., 2004). Since then, several cases have been reported (Bottecchia et al., 2008; Lai and Yeh, 2008; Liu et al., 2010; Schildgen et al., 2004). The rtL217R variant was observed in most HBV strains ascribed to genotype A, subgenotype A2, but not in strains ascribed to other genotypes (Schildgen et al., 2004). This means that HBV subgenotype A2 harbours the polymorphism L217R associated with primary resistance to ADV (Bottecchia et al., 2008). Naturally occurring LMV-resistant mutants as well as HBV variants related to natural ETV resistance have also been detected (Alvarado-Esquivel et al., 2006; Jardí et al., 2007; Lai and Yeh, 2008).

HBV exhibits high rates of viral replication that, due to the lack of proofreading, results in high rates of mutation. As a consequence, quasispecies (including variants with mutations associated with drug resistance) already exist in an individual before antiviral chemotherapy. The subsequent selection of drug-resistant HBV is dependent on many factors, including viral load, virus replication rate, and the potency or availability of the antiviral agent, but since monotherapy is usually directed at a single viral target, rapid

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doi:10.1016/j.antiviral.2010.04.005

Table 1
Main features of the patients studied.

Patient	Sex	Age (years-old)	Anti-HIV antibodies	Previous treatment	HBV vaccination	ALT/AST (IU/L)	Viral load (copies/mL)	HBV serology		
								HBsAg/anti-HBs	HBeAg/anti-HBe	Total anti-HBc
P1	F	17	–	LMV	No	28/37	$>4 \times 10^7$	+/–	+/–	+
P2	M	43	–	–	No	242/98	1.87×10^7	+/+	+/–	+
P3	M	36	+	LMV	No	83/63	3.7×10^6	+/–	+/–	+
P4	M	52	–	LMV	No	80/50	3.2×10^6	+/–	+/–	+
P5	M	41	–	LMV	No	75/54	2.3×10^6	+/–	–/+	+
P6	M	49	–	–	No	115/64	N.D.	+/+	+/–	+
P7	F	27	–	–	No	130/76	1.7×10^6	+/+	+/–	+
P8	M	49	N.D.	LMV	No	50/33	2×10^7	+/–	+/–	+
P9	M	36	+	LMV	No	44/25	$>4 \times 10^7$	+/–	+/–	+
P10	F	5	–	–	Yes	75/66	$>4 \times 10^7$	+/+	+/–	+
P11	F	13	–	–	No	44/36	3.85×10^7	+/–	+/–	+
P12	M	10	–	–	No	25/24	$>4 \times 10^7$	+/+	+/–	+
P13	M	37	–	LMV	No	138/128	1.6×10^5	+/–	+/–	+

F = female; M = male; N.D.: not determined due to serum sample shortage.

selection of resistant strains is frequently inevitable (Jardí et al., 2007).

Serum samples from 13 Argentinean patients with CHB (P1-P13) were referred to the CEVH to undergo viral resistance studies. This study was approved by the Ethics Committee on Research, Faculty of Medicine, UBA. None of them had ever undergone antiviral therapy with ADV, ETV or LdT. Patients P1, P3-P5, P8-P9 and P13 had only received LMV as the sole anti-HBV therapy for a period longer than 12 months. Furthermore, patients P2, P6-P7, P10 and P11 were referred to such Center for molecular biology studies due to the co-circulation of HBsAg and anti-HBs antibodies. Of them, only P10, a 5-year-old girl had been vaccinated. Thus, this study presents a retrospective analysis of HBV variants previously detected in the above mentioned center within the period 2002–2007.

Serological tests for HIV and for HBsAg, anti-HBs, HBeAg, anti-HBe and total anti-HBc were tested with commercially available kits (Abbott Laboratories, USA) and are depicted in Table 1.

Viral DNA was extracted from serum and the near-full length S-gene (and the overlapping polymerase gene) was amplified by PCR (nucleotide positions 158–832), by using the sense primer HBs1 (5′-CAGGGACCCGGTGAGAACATCACATCAGG-3′; underlined letters correspond to targeted specific HBV DNA sequences) and the antisense primer HBs2 (5′-GGCACCAGAGCGTTTATGTATACCCA-3′), both of them used for further cloning and expression studies (data not shown). The PCR profile was an initial 3 min denaturation step at 94 °C, followed by 40 cycles of amplification including denaturation for 45 s at 94 °C, annealing for 60 s at 53 °C, and extension for 90 s at 72 °C. Final elongation was completed at 72 °C for 7 min.

Amplicons of expected size were bidirectionally sequenced by using Big-Dye Termination chemistry (Applied Biosystem, USA) with the same primers used for PCR amplification.

Appropriate precautions and procedures were strictly followed to avoid cross-contamination (Borst et al., 2004; Kwok and Higuchi, 1989; Mathet et al., 2003).

The obtained nucleotide sequences were aligned with GenBank sequences corresponding to HBV isolates assigned to genotypes A–H by using the Clustal X program.

Phylogenetic trees were constructed by using the neighbour-joining analysis included within the PHYLIP package version 3.5 c (Felsenstein, 1993). As shown in Table 2 HBV isolates recovered from patients P1, P2-P9, P10-P11, P12, and P13 were assigned to A1, A2, E, F1b, and F4 (sub)/genotypes, respectively.

In the present study, carried out in Argentina, we report the circulation of naturally occurring HBV variants with resistance to current NAs approved for the treatment of CHB. Among them, the potential rtL217R ADV-resistance variant was present in 9 of the

Table 2
Polymerase novel substitutions and resistant mutations to LMV, ADV, ETV and LdT observed in the HBV isolates from the patients studied.

Patient	HBV genotype	HBV subgenotype	LMV-resistant mutants	ADV-resistant mutants/novel substitutions	ETV-novel substitutions	LdT-resistant mutants/novel substitutions
P1	A	A1	rtM204L	–	–	rtM204L
P2	A	A2	–	**rtL217R**[a] **rtY221F**	**rtS202N**[b]	
P3	A	A2	rtV173L + L180M + M204V	**rtL217R**[a]	–	rtV173L + L180M + M204V
P4	A	A2	rtL180M + M204V/I	**rtL217R**[a]	–	rtL180M + M204V/I
P5	A	A2	rtN238K[b]	**rtL217R**[a] rtN238K[b]	–	–
P6	A	A2	–	**rtL217R**[a]	–	–
P7	A	A2	**rtM204I, rtS213T**	**rtL217R**[a]	–	**rtM204I**
P8	A	A2	rtA200S[b], rtS213T	**rtS85F**[b]**/T**[b] **rtL217Q**[b] **rtS219P**[b]	–	–
P9	A	A2	rtV173G[b] + L180M + M204V/L rtT150A, rtL164V[b], rtS213C[b], rtA200P[b]	**rtL217H**[b]**/I**[b]**/N**[b]	**rtS202R**[b]	L180M + M204V/L
P10	E	N.A.	–	**rtL217R**[a]	–	–
P11	E	N.A.	–	**rtL217R**[a]	–	–
P12	F	F1b	**rtL180M + M204V**	**rtL217R**[a]	–	**rtL180M + M204V**
P13	F	F4	rtL80V, rtM204I	**rtV84G**[b] **rtS85L**[b]	**rtS202R**[b]	rtM204I

Naturally occurring antiviral-resistant mutants are in bold letters. Patients P1, P3, P4, P5, P8, P9 and P13 received LMV as therapy, selecting the LMV-resistant mutants shown in the table. The rtM204I LMV-resistant mutant is reportedly known to produce cross-resistance to LdT; N.A., not applicable.

[a] Potential ADV-resistant mutant.

[b] Changes in the indicated positions were reported as resistant mutants, but exhibiting dissimilar amino acidic substitutions, thus, they cannot be regarded as definitively "drug resistant", since in vitro phenotypic testing is still lacking.

13 patients studied (7 of them had undergone LMV treatment). Remarkably, six of these samples were ascribed to the reportedly known subgenotype A2 polymorphism (Bottecchia et al., 2008). Unexpectedly, two of them were ascribed to genotype E (Mathet et al., 2006) and the remaining one to genotype F, subgenotype F1b. To our knowledge, the latter two HBV genotypes are reported to be associated to natural (reportedly known) potential resistance to ADV for the first time. Interestingly, in all cases, these variants were present at high proportion within each given mixed viral population, as shown in the chromatogram and confirmed in selected cases by restriction fragment length polymorphism (RFLP; data not shown, available upon request). Although pre-existing NA resistant variants have been reported in patients prior to antiviral therapy, it is reportedly known that they usually contribute with a minor proportion to the whole viral population in a given host. It might be speculated that this was not the case among A2 infected patients, since the above mentioned rtL217R is a genotype-specific polymorphism. However, the same replacement is shown for the first time among genotype E isolates. Since this genotype was exceptionally detected in Argentina (Mathet et al., 2006) as well as in America, at present we do not know if this is a characteristic pattern of a viral (sub)lineage circulating in the American continent, as compared with African isolates, from which this HBV genotype derives. Interestingly, P12's mother exhibited an HIV-HBV coinfection, due to which she had received antiviral treatment, possibly explaining the mother-to-child transmission of a predominant viral population with LMV-resistant mutants.

Pretreatment with LMV in these patients may have led to some resistance mutants that do not only mediate LMV but other drugs resistances (i.e. ADV). The lack of such treatment in patients P10-12 did not preclude the detection of the same amino acidic (aa) replacement in these strains harbouring other genotypes.

Naturally occurring HBV variants with point mutations in positions associated to ADV-resistance, such as [rtL217H/I/N], [rtS85F/T + L217Q + S219P], and [rtV84G + S85L], were also identified in 3 of the 13 patients studied (at low proportion within each given mixed viral population). Clearly, phenotypic studies should be performed to determine if these novel mutations detected in the same position of reportedly known resistant mutants are also associated with antiviral drug resistance.

LMV-resistant mutants were identified in 9 patients, 7 of whom had received LMV as sole anti-HBV therapy for a period longer than 12 months, while untreated patients P7 and P12 displayed HBV variants with natural resistance to LMV, ADV and LdT (at high levels). Remarkably, both of them were also carriers of potential S-immune escape variants (such as Y100C, T140I, K141Q, D144E; Table 3).

Regarding ETV, in 3 of the 13 patients studied (P2, P9 and P13), two unreported aa substitutions were located at position rt202, (rtS202N/R) where dissimilar changes have been previously assigned to natural resistance to this NA. Two of them were found within the context of LMV-resistant mutations.

Interestingly, of all the patients studied, 7 (P1, P3, P4, P7, P9, P12 and P13) displayed potential resistance to more than one NA. To the best of our knowledge, the M204L mutation detected in P1 has been exceptionally found (Tanaka et al., 2004) during LMV monotherapy in vivo. Moreover, this mutation had been previously analyzed in vitro in transfection studies, without conferring LMV-resistance, and postulated to emerge in vivo with replication competence (Ono-Nita et al., 1999). However, when the last study was carried out, LdT had not yet been launched for clinical development as a novel treatment of CHB, and therefore, resistance to such NA was not tested.

Finally, 5 of the 13 patients (P2, P6, P7, P10 and P12) exhibited HBsAg in the presence of anti-HBs antibodies, with natural resistance to ADV. In these five cases potential ADV resistance was related to the rtL217R mutant, which corresponds to L209 V in the overlappingly encoded HBsAg. At present, we do not know if such aa is an S-escape mutant *per se*. This position was reported as the single mutation within the S-protein in an untreated patient infected with genotype E in the presence of circulating anti-HBs antibodies (Mathet et al., 2006). Besides, such position was proposed to be subgenotype-dependent (but unrelated to an S-escape mutant), as it appears to be the case with A2 strains naturally encoding V209 (Norder et al., 2004), and as observed in P3, P4 and P5.

Table 3
Amino acidic substitutions within the major hydrophilic region[a] of the HBsAg in patients exhibiting co-circulation of HBs Ag and anti-HBs antibodies.

Patient	Substitutions
P2	C107R[b], T125A[b], M133K[b], I152F[b], Y161S[b]
P6	P120L, Q129L, C139Y[c], Y161C
P7	P120L, A128V, C138G, T140P, K141Q, D144E[d], G145E, Y161C
P10	P120L, T125S, P127C, Y161F[e]
P12	Y100C[f]

[a]This region is still not well defined. For instance, some authors postulated that it encompasses amino acids 100–160 of the S protein (Coleman, 2006), while others refer to it as the region between amino acids 100 and 169 (Shields et al., 1999). Some of the mutations shown in this Table have been previously reported either as a vaccine/HBIG or (potential) immune escape mutations (i.e. [b]Cuestas et al., 2006; [c]Devesa et al., 2004; [d]Kim et al., 2003; [e]Sánchez et al., 2002; [f]Gutiérrez et al., 2004).

In summary, – although this study does not aim to show an epidemiological view of resistant mutants in Argentina, considering that all patients had been referrals – we report for the first time the circulation in Argentina of naturally occurring HBV variants with resistance to LMV and LdT, and some of them with potential resistance to ADV. Most isolates from the latter group were assigned to A2 subgenotype, whereas two of them were ascribed to genotype E and the remaning one to F1b subgenotype. Their detection – before an antiviral therapy regimen is set up – might be a strategic way to optimize the treatment for CHB. Circulation and transmission of S-escape variants with resistance to NAs should be of public health concern as they may represent a potential risk for the community.

Acknowledgements

This study was partly supported by the following grants: BID 1728/OC-AR-PICT 38335 and BID 1201/OC-AR-PICT 00440/06 from ANPCyT, UBACyT M057 and UBACyT M612 from the University of Buenos and PIP 6065 from CONICET. Authors are grateful to Maria Victoria Illas for manuscript preparation and to Cristina Galoppo for her critical reading of the manuscript and meaningful comments.

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