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Short communication

Dynamics of hepatitis B virus resistance to entecavir in a nucleoside/nucleotide-naïve patient

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ABSTRACT

The genotypic evolution of HBV quasi-species was analyzed in a nucleoside/nucleotide-naïve patient who developed resistance to entecavir. The lamivudine resistant quasi-species (rtM204V ± rtL180M), absent at baseline, were emerged as early as 48 weeks after entecavir administration. Entecavir-resistant quasi-species (rtM204V ± rtL180M plus S202G) were found after week 112 and gradually became the predominant mutations afterwards. The lamivudine- and entecavir-resistant mutations emerged closely in combination with the rtV207L, rtA222T, rtP237T or rtI163V substitutions. Our results indicated that the lamivudine-resistant mutations were developed first and may serve as a prequisite for subsequent entecavir-resistant mutations in this nucleoside/nucleotide-naïve patient.

Entecavir (ETV) is a novel deoxyguanosine analog with potent activity against HBV in vitro and in vivo (Chang et al., 2005, 2006; Colonno et al., 2006; Genovesi et al., 1998; Lai et al., 2006; Levine et al., 2002; Sherman et al., 2006; Sims and Woodland, 2006; Yao, 2007). However, as a result of covalently closed circular DNA persistence in host cells, the subsequent selection of drug-resistant mutants from the viral quasi-species was considered inevitable with nucleoside/nucleotide monotherapy, as clearly shown by lamivudine (LMV) and adefovir (ADV) studies (Angus et al., 2003; Lai et al., 2003; Villeneuve et al., 2003). Most cases of entecavir resistance (ETV^r) were observed in patients receiving entecavir when failings on lamivudine. Evaluations of ETV^r in LMV refractory patients demonstrated a requirement for pre-existing lamivudine resistance (LMV^r) mutations at sites of rtL180 and rtM204, and at least one additional mutation at site of rtT184, rtS202, or rtM250 in the viral reverse transcriptase (RT). These ETV^r mutations have not been reported in any ETV-treated patient in the absence of LMV^r mutations, and when introduced alone into recombinant clones, have a minor impact on ETV susceptibility. It seems that, for nucleoside/nucleotide-naïve individuals treated with ETV, LMVr quasi-species are necessary prior to emergence of ETV^r variants. A two-hit model was suggested for the development of ETV resistance in that LMV^r mutations were selected initially and one or two additional ETVr mutations were acquired in the same virus afterwards (Colonno et al., 2006; Tenney et al., 2004, 2007; Villet et al., 2007; Yim et al., 2006; Zoulim, 2006). However, there was no evidence supporting this model in nucleoside/nucleotide-naïve individuals with ETV monotherapy. Colonno et al. (2006) reported one case of ETV^r mutation accompanied by viral rebound in a nucleoside/nucleotide-naïve patient upon ETV administration. The patient was further shown to have baseline LMV^r mutations prior to treatment. Here, we describe another case of a nucleoside/nucleotide-naïve patient who developed resistance to entecavir after more than 3 years of ETV therapy.

The nucleoside/nucleotide-naïve patient, a 32-year-old male Chinese, has had chronic HBeAg-positive (genotype B) HBV infection and was HCV and HIV seronegative. He was enrolled in a registrational clinical study using daily 0.5 mg ETV. He received ETV monotherapy for more than 3 years and was the only one developing resistance to ETV from 11 patients. Serum samples were taken at various time points before and during ETV therapy. The serum aspartate aminotransferase, alanine aminotransferase and

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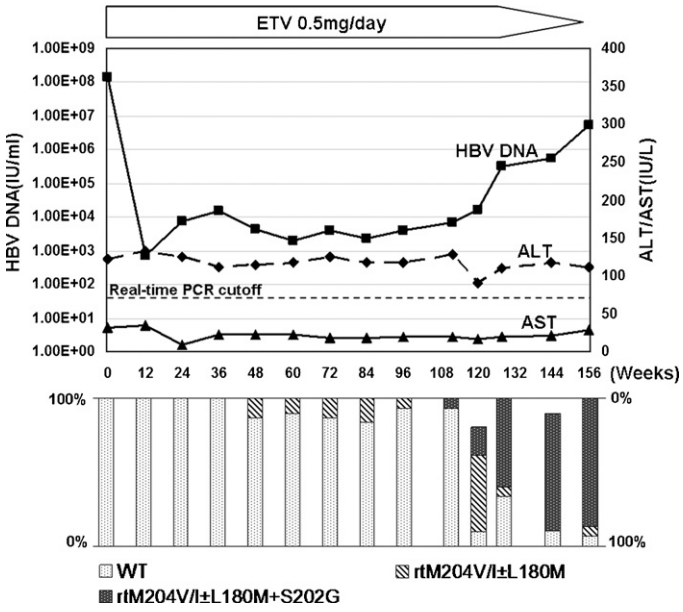

Fig. 1. Dynamics of serum HBV DNA levels, serum aminotransferase activities, and wild type, LMV and ETV signature-resistant viral populations before and during entecavir therapy. The dynamics of HBV DNA and aminotransferase levels are shown at the top, while the dynamics of HBV variants are shown at the bottom as respective proportions of wild type, LMV signature-resistant (rtM204V/I ± rtL180M) and ETV signature-resistant (rtM204V/I ± rtL180M + rtS202G) variant populations within the viral quasi-species at each time point. Antiviral therapy is shown above the graph. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; WT, wild type.

HBV DNA levels were determined. A 1050-bp fragment encompassing domains A–F of the HBV RT was amplified. When HBV DNA level was below 100,000 IU/ml, a nested PCR was used to amplify a 808-bp fragment spanning the HBV RT domains A–E. Purified PCR products were directly sequenced and cloned into the pEGFP-C1 vector. Thirty colonies per serum sample were randomly picked and the plasmid DNA inserts were sequenced. An additional 30 colonies were picked for baseline serum sampling to improve the sensitivity of colonal analysis.

As shown in Fig. 1, maximum viral suppression to 2.84 log 10 IU/ml was achieved at 12 weeks after entecavir administration. However, a relatively high residual level of HBV replication was noted between 24 and 120 weeks of therapy, ranging from 3.30 to 4.21 log 10 IU/ml, indicating that the patient had a suboptimal response to ETV. Virologic breakthrough, characterized by a significant increase in HBV DNA level by more than 1.0 log 10 IU/ml above the nadir (Lok et al., 2007), was observed at 36 and 128 weeks of therapy.

An extensive analysis of the evolution of viral quasi-species was then performed on serum samples throughout the clinical course of ETV treatment. A total of 453 clones from the 14 time points were generated and analyzed, allowing us to fully characterize HBV RT quasi-species at each time point (Table 1). Seven amino acid substitutions exhibit dynamic evolution during ETV therapy. The principal mutations in this patient were rtM204V/I (methionine to valine or isoleucine substitution) and rtL180M (leucine to methionine substitution), known as LMV signature resistance; rtS202G (serine to glycine substitution), known as ETV signature resistance. As shown in Table 1 and Fig. 1, prior to the ETV treatment, no quasi-species mutations known to confer lamivudine or enticavir resistance were detected with our clonal analysis method. Although the sensitivity of clonal analysis used in our study was expected to be 1.67–3.3% (1/60 to 1/30) based on an average of 30–60 clones analyzed from each sample, our results cannot exclude the possibility that such variants were absent. However, their prevalence in the quasi-species populations should likely be very low. Quasi-species variants bearing LMV resistance mutations, rtM204V/I and/or rtL180M (rtM204V/I ± rtL180M), emerged after 48 weeks of therapy, following the first viral rebound by 3 months but preceding the second virologic breakthrough by 18 months. These resistant variants were composed of minor populations of quasi-species at first, but these became predominant (51.61%) at week 120. Interestingly, with the appearance of the second viral breakthrough the populations of these resistant variants declined rapidly. Dual LMV- and ETV-resistant quasi-species, harboring rtM204V/I and/or rtL180M with rtS202G substitutions(rtM204V/I ± rtL180M + rtS202G), emerged as early as after 112 weeks of ETV therapy, preceding the second virologic breakthrough by 4 months. Gradually, these resistant variants, initially representing a minority of the quasi-species, almost completely replaced the wild type variants. This switch occurred in parallel to an increase in HBV DNA load, indicating a significant increase in the absolute amount of circulating resistant variants. The HBV RT quasi-species resistant to LMV emerged far earlier than those resistant to ETV during the prolonged ETV treatment, suggesting that the LMV-resistant mutants may be at the basis of developing ETV-resistant mutations.

Four additional substitutions such as rtI163V (isoleucine to valine substitution), rtV207L (valine to leucine sustitution), rtA222T (alanine to threonine substituion) and rtP237T (proline to threonine substituion) were also found and selected in the mutant strains (Table 1). They have not been previously reported as signature LMV or ETV resistance mutations but were apparently associated with the LMV^r and ETV^r mutations in this patient. Serum HBV DNA levels increased above 1 log 10 IU/ml when these mutations dominated the HBV populations. They may help to restore viral replication efficiency of the rtL180M + rtM204V dual mutant strains or the rtL180M + rtM204V + rtS202G triple mutant strains. Nonetheless, the significance of these mutations may need to be validated in a larger cohort of ETV-treated patients.

The patient reported here showed a suboptimal response to ETV treatment. The mechanism behind the high level of residual HBV replication in the presence of an inhibitor may be more complicated. As shown in Fig. 1, the selection of the LMV^r mutant may possibly be associated with the first viral rebound at week 36 and the residual HBV replication afterward. Further increase in HBV DNA level at week 128 is likely related to the additional selection of rtS202G substitution. Biochemical breakthrough often coincides with a viral rebound, but serum ALT may remain normal for a few weeks or a few years after virologic breakthrough in part of the patients (Lok et al., 2007). In this patient ALT elevation was not observed following the two viral rebounds. Maybe the cytotoxic T lymphocytes did not recognize the new viral strains.

Experience with highly active antiretroviral therapy in HIV-infected patient shows that resistance to HIV RT inhibitors is acquired gradually, through the selection of new amino acid substitutions that confer stepwise increases in the level of drug resistance (Clavel and Hance, 2004). In vitro, the presence of primary LMV^r mutations, rtL180M and rtM204V, led to 8-fold reduction of ETV susceptibility. The addition of rtS202G mutation to the rtM204V + rtL180M variant could induce a 70-fold decrease in susceptibility to entecavir and the association of these three substitutions is essential for the development of entecavir resistance (Tenney et al., 2004; Yim et al., 2006). A hydrophobic pocket in the rear of the RT-dNTP binding site was predicted in a novel molecular model of ETV-HBV RT interaction (Langley et al., 2007). It has been suggested that the inhibiting potency of ETV is the result of an optimal fit into the pocket. LMV^r mutations result in restricting, but not eliminating, the ETV-binding pocket. There have been

Table 1
Evolution of ETV-resistant mutations in HBV quasi-species.

Treatment (weeks)	Mutations on direct sequencing	Mutations on clonal analysis							Numbers of clones (total)
0	WT	WT							60 (60)
12	WT	WT							30 (30)
24	WT	WT							30 (30)
36	WT	WT							30 (30)
48	WT								26
		WT	M204V	L180M					2
				L180M					2 (30)
72	WT	WT							26
			M204I						4 (30)
84	WT								25
		WT	M204V	L180M		V207L	A222T		4
			M204I						1 (30)
96	WT								28
		WT	M204I						1
				L180M					1 (30)
112	WT								28
		WT	M204V	L180M	S202G		A222T	I163V	1
			M204V		S202G	V207L	A222T		1 (30)
120	WT		M204V	L180M			A222T		15
							A222T		6
			M204V	L180M	S202G	V207L	A222T	I163V	4
		WT							3
			M204V	L180M	S202G	V207L	A222T	P237T	2
				L180M			A222T	I163V	1 (31)
128	M204V + L180M + S202G + V207L + A222T + P237T		M204V	L180M	S202G	V207L	A222T	P237T	11
									10
			M204V	L180M	S202G	V207L	A222T	I163V P237T	2
		WT	M204V	L180M	S202G	V207L	A222T	I163V	2
			M204V	L180M			A222T		1
			M204V	L180M		V207L	A222T		1
			M204V	L180M	S202G	V207L	A222T		1
			M204V	L180M	S202G	A222T	I163V	1	
			M204I						1 (30)
144	M204V + L180M + S202G + V207L + A222T + P237T		M204V	L180M	S202G	V207L	A222T	P237T	17
			M204V	L180M	S202G	V207L	A222T	I163V	5
		WT							3
							A222T	P237T	3
			M204V		S202G	V207L	A222T	P237T	1
			M204V	L180M	S202G		A222T		1 (30)
156	M204V + L180M + S202G + V207L + A222T + I163V		M204V	L180M	S202G	V207L	A222T	I163V	12
			M204V	L180M	S202G	V207L	A222T	P237T	6
		WT	M204V	L180M	S202G	V207L	A222T		5
									2
			M204V	L180M		V207L	A222T		2
			M204V	L180M	S202G		A222T	I163V	2
			M204V	L180M	S202G			I163V	1 (30)

Abbreviations: A, alanine; G, glycine; I, isoleucine; L, leucine; M, methionine; P, proline; S, serine; T, threonine; V, valine; WT, wild type.

several cases of virologic rebound with only LMV^r mutations in nucleoside/nucleotide naïve-patients under entecavir monotherapy (Colonno et al., 2006). These data suggest that LMV^r HBV variants may provide a partial relief to the virus replication from the inhibitory pressure of ETV and could provide a pathway toward entecavir resistance. Suzuki et al. (2007) reported another case of entecavir resistance in a nucleoside/nucleotide-naïve patient, in which the LMV^r mutation was not found at baseline sample and the rtL180 M, rtM204V and rtS202G mutations emerged simultaneously at 88 weeks of ETV treatment. However, only four time points in more than 2 years of ETV treatment were selected for clonal analysis in that report. Our results in the present study appear to support the two-step model for the development of entecavir resistance. During entecavir momotherapy the LMV^r mutations, rtM204V/I and/or rtL180M, were first selected, followed by the additional selection of rtS202G substitution, resulting in ETV resistance.

In summary, we report a case of entecavir resistance in a nucleoside/nucleotide-naïve HBV infected patient. The longitudinal genotypic analysis of HBV clones in this patient seems to support a two-step selection process of entecavir-resistant mutants. However, our results are restricted to a single chronically infected patient, and additional results in other ETV resistance patients are needed to further support the two-hit model.

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