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Evolution of hepatitis B virus during long-term therapy in patients with chronic hepatitis B (♦)

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ABSTRACT

Background. Long-term lamivudine (LAM), adefovir (ADV) and entecavir (ETV) treatment induce the emergence of drug-resistant hepatitis B virus (HBV) in patients with chronic hepatitis B infection. **Aim.** To evaluate the LAM, ADV and ETV resistance mutations detected in our patient group. **Materials and methods.** Twenty patients who had received at least two years of treatment with nucleoside/tide analogues were enrolled in this study. Patients with detectable HBV DNA were analyzed in order to detect resistance mutations and in this group of patients treatment was change. **Results.** Three patients developed LAM resistance mutations (2 presented rtM204I and one rtL180M+rtM204V/I) and one patient showed rtN236T ADV resistance mutation. During ADV and LAM treatment, one patient developed ADV plus LAM resistance mutations (rtI163V+rtL180M+rtA181V+rtN236T), in this case, HBV strains harbouring polymerase mutations did not develop LAM associated rtM204V/I primary mutation. In addition, ETV resistance mutations (rtL180M+rtT184A+rtS202G+rtM204V) were detected in one patient. **Conclusions.** These findings suggest that monotherapy resulted in a limited virological response and combination strategies including potent antiviral agents should be recommended for patients with resistant mutations.

Key words. ETV. LAM. ADV. Mutations. Resistance.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection leads to long-term sequel such as chronic liver disease, cirrhosis and hepatocellular carcinoma.[1,2] The goals of therapy in patients with chronic HBV are sustained in DNA suppression, normalization of serum aminotransferase levels, and improvement in liver necroinflammation. In recent years, treatment of chronic HBV has been improved, with the availability of nucleoside/tide analogues, such as lamivudine (LAM), adefovir (ADV), telbivudine (TBV), entecavir (ETV) and tenofovir (TDF).[3,4] Nucleoside/tide analogues target the HBV reverse transcriptase, thus inhibiting viral replication and leading to virologic, biochemical and histological improvement in most patients.[5,6]

However, prolonged therapy with nucleoside/tide analogues often results in the selection of mutations in the target gene, which confer drug-resistance and treatment failure.[7] To detect drug-resistance mutations the direct sequencing of the viral *pol* gene is the most useful method, which allows the identification of all substitutions, including novel, primary and secondary or compensatory mutations. Thus, early detection of antiviral resistant mutants is of clinical importance for choosing new treatment strategies.[8]

The present study evaluated the antiviral efficacy and emergence of resistance in patients with chronic hepatitis B who showed evidence of resistance during prolonged therapy with nucleoside/tide analogues and underwent to changing treatment.

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MATERIALS AND METHODS

Samples from twenty patients with chronic hepatitis B, undergoing treatment with nucleoside/tide analogues for at least two years, were analyzed. Patients proceeded from Basurto Hospital in Basque Country, North of Spain. For therapy monitoring purposes, samples were tested for genotype, HBV DNA levels and HBV drug-resistance.

Serum HBV DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and HBV genotype was determined by commercially INNO-LiPA HBV Genotyping assay (Innogenetics N.V., Ghent, Belgium).

HBV DNA extraction and real-time PCR quantification from serum was determined by the fully automatic system COBAS Ampli-Prep-COBAS TaqMan (Roche Diagnostics, Germany). Virologic breakthrough was defined as a confirmed rise in HBV DNA levels, and confirmed HBV DNA rises were those in which the next treatment visit also had rice in HBV DNA levels from the nadir.

The sequencing of the HBV *pol* gene was performed on plasma samples as follows. Extracted HBV DNA was amplified with AmpliTaq Gold™ DNA polymerase enzyme (Applied Biosystems) using the following primer pairs:[9]

- 5'-GGATGTGTCTGCGGCGTTT-3'.
- 5'-ACCCCATCTCTTTGTTTTGTTAGG-3'.

PCR conditions were:

- One cycle at 94 ºC for 10 min.
- 40 cycles (94 ºC, 30 s; 55 ºC, 30 s; 72 ºC, 40 s).
- A final cycle at 72 ºC for 10 min).

The PCR product of 486-bp was visible after agarose gel electrophoresis and was purified (Qiagen "QIAquick® PCR Purification kit") prior to cycle sequencing with a BigDye terminator v.3.1 kit (Applied Biosystems) and an automated sequencer (ABI-3100). Sequencer software (BLAST) was used to align the sequences to determine changes that emerged during therapy. The sequences were also compared to those in database genomes from Gen Bank reference sequences.

RESULTS

Viral genotype was determined showing the presence of genotype D in eleven (55%) patients, genotype A in seven (35%) patients, genotype C in one (5%) case, and one patient presented two genotypes A + F (5%).

Among the 20 patients in treatment with nucleoside/tide analogues, HBV DNA was not detected (< 1.08 log copies/mL) in 13 patients (53%) after prolonged therapy. Seven patients (35%) had a detectable level of HBV DNA, six patients showed genotype D and one genotype A, and samples from these patients were sequenced. With the direct sequencing, six patients showed mutations in the HBV polymerase gene, while one carried wild type sequence (Table 1).

In addition to the primary mutations known to confer resistance to LAM, ADV and ETV, emerging HBV reverse transcriptase (RT) substitutions, absent from an assembled database of wild type HBV, were found in patients with evidence of resistance.

Patient 1 was previously treated with LAM for eight months, developed LAM resistance (rtM204V + rtL180M) despite good compliance. ADV monotherapy was started, and the viral load dropped rapidly with restoration of YMDD wild type. After 21 months of ADV therapy, ADV resistance (rtA181V + rtN236T) resulted in viral breakthrough. ADV was replaced by ETV resulting in a significant drop in viral load, but after 11 months of therapy, resistance mutations (rtM204V + rtL180M + rtT184A + rtS202G) resulted in viral breakthrough.

Patient 4, after 17 month of ADV therapy, ADV resistance (rtN236T) resulted in viral breakthrough. LAM was added to ADV, however, after 29 months of therapy resistance mutations to both ADV and LAM (rtI163V + rtL180M + rtA181V + rtN236T) developed with hepatitis B flare-up. In this patient, HBV strains harbouring polymerase mutations did not develop LAM associated rtM204V/I primary mutation.

HBV DNA negative conversion was found in four patients after ADV plus LAM treatment. These patients were resistant to one of the nucleoside/tide analogues before the combination therapy. Patient 2 was switched to ADV plus LAM combination therapy after LAM breakthrough. At the start of combined ADV plus LAM therapy, HBV from this patient had LAM resistant mutations (rtL180M + rtM204V/I). After 45 months of continued treatment with ADV plus LAM combined, patient had undetectable HBV DNA levels (< 1.08 copies/mL). Patient 3 received a combination of LAM plus ADV after LAM breakthrough. At the start of combinatory therapy, HBV had LAM resistant primary mutation (rtM204I) and two secondary or novel mutations (rtI187L + rtA200V). Thirty months after treatment,

Table 1. Virological data for the seven patients with positive viral load samples in whom resistance-related variations were detected by direct sequencing.

Patient	Genotype	HBeAg anti-HBe	Baseline HBV DNA level[1]	HBV DNA level*	Antiviral treatment	Treatment duration (month)	Mutation by direct sequencing RT substitution**
1	D	HBeAg	> 5.3	> 5.3	LAM	8	L180M + M204V
		HBeAg		4.34	ADV	21	A181V + N236T
		HBeAg		6.47	ETV	11	L180M + T184A + S202G + M204V
2	D	anti-HBe	4.98	4.1	LAM	10	L180M + M204V/I
		anti-HBe		< 1.08	LAM+ADV	3	
		anti-HBe		< 1.08		45	
3	A	anti-HBe	> 5.3	3.39	LAM	21	I187L + A200V + M204I
		anti-HBe		< 1.08	LAM+ADV	3	
		anti-HBe		< 1.08		30	
4	D	anti-HBe	> 5.3	4.84	ADV	17	N236T
		anti-HBe		< 1.08	ADV+LAM	2	
		anti-HBe		2.39		29	I163V + L180M + A181V + N236T
5	D	anti-HBe	5.3	1.48	ADV	36	A181V + Q215S
		anti-HBe		< 1.08	ADV+LAM	3	
		anti-HBe		< 1.08		28	
6	D	anti-HBe	5.32	2.6	LAM	18	M204I + Q215S
		anti-HBe		< 1.08	LAM+ADV	3	
		anti-HBe		< 1.08		18	
7	D	HBeAg anti-HBe	8.74	2.61	ADV	46	Wild type

*log copies/mL. **Known resistance mutations are in bold.

Table 2. Characteristics of patients with undetectable HBV DNA serum samples (< 1.08 log copies/mL).

Patient	Age	Sex	Genotype anti-HBe	HBeAg	Antiviral treatment	Baseline HBV DNA level*	Time of treatment (month) HBV DNA became undetectable	Treatment duration (month)
8	45	F	D	anti-HBe	ADV	2.7	3	56
9	58	M	D	anti-HBe	ADV	4.79	4	56
10	49	F	A	anti-HBe	ADV	3.02	7	55
11	37	M	A	anti-HBe	LAM	5.88	12	44
12	45	M	A	anti-HBe	ADV	4.28	28	44
13	51	M	D	anti-HBe	ADV	6.93	14	43
14	46	F	D	anti-HBe	ADV	7.16	11	32
15	31	F	C	anti-HBe HBeAg	ADV	4.17	8	32
16	34	F	A + F	anti-HBe	ADV	2.65	6	31
17	46	M	D	anti-HBe	ETV	5.02	11	30
18	52	F	A	anti-HBe	ADV	5.14	5	26
19	28	M	A	anti-Hbe HBeAg	ETV	8.04	12	24
20	48	M	A	anti-HBe	ETV	6.56	9	24

*log copies/mL.

patient presented undetectable viral load (< 1.08 copies/mL). Patient 5 presented mutations conferring resistance to ADV (rtA181V + rtQ215S), after of 36 months of ADV monotherapy treatment. LAM was added and after 28 months of ADV plus LAM combination therapy patient remained viremic undetectable. Patient 6 had received LAM for 18 months before viral breakthrough occurred, at this time, resistant mutations to LAM (rtM204I + rtQ215S) were detected. LAM was continued and ADV was added. Serum HBV DNA levels decreased and 18 months after therapy, this patient had undetectable serum HBV DNA (< 1.08 copies/mL).

The only patient (patient 7) who did not clear HBV DNA after 46 months of therapy presented wild type strain of HBV confirmed by direct sequence. Serum HBV DNA levels from this patient with persistent viremia continued to decline progressively.

The characteristics of the study population with undetectable viral load are shown in table 2. Serum HBV DNA became undetectable in 13 patients after a long-term (24 to 56 months) monotherapy treatment (nine with ADV, three with ETV and one with LAM). Among these 13 patients, five showed genotype D, six genotype A, one genotype C and another one A + F.

DISCUSSION

Nucleoside/tide analogues have revolutionized the treatment of chronic HBV infection because sustained suppression of HBV replication and a remission of liver disease. However, long-term therapy is associated with selection of HBV polymerase mutants and emergence of resistance.[10-12]

Many published data showed that the appearance of resistance mutations may precede the increase in viral load and could be a prognostic marker of viral breakthrough.[13,14] Therefore, all patients receiving nucleoside/tide analogues therapy for HBV should be closely monitored for virology response and breakthrough during treatment to maximize the success of early therapeutic changes in failed patients. Serum HBV DNA should be tested prior to treatment and then every 3 months during treatment.[15] In this context, the direct sequencing method, which is able to detect HBV *pol* mutations, would allow the identification of patients who develop genotypic resistance early during their treatment, enabling early intervention and prevention of treatment failure.

To date, insufficient information on the molecular epidemiology of HBV in Basque Country was available and only few HBV were analyzed from patients with chronic hepatitis B in this country. This study showed the main prevalence of HBV genotype D (55%) followed by genotype A (35%). Genotype C detected as minority genotype was found exclusively among a Chinese immigrant and in our study one Colombian patient showed A + F genotypes. These findings are in accordance to the geographical distribution of genotypes D and A in Europe, especially in the western part of Europe.[16,17] In conclusion, the most common HBV genotypes in Basque Country are D and A, in addition, this study reveals the circulating of exotic HBV genotypes C and F in Basque Country.

In contrast with other authors, LAM drug-resistance isolates were observed among 50% of HBV genotype A and 100% of HBV genotype D after one year therapy.[16,18] In our study, two patients with genotype D developed mutations conferring resistance to ADV before two years of therapy. In contrast, there were four patients with genotype D and ADV therapy with undetectable viral load after two years of treatment.

Combination therapy of LAM plus ADV has been shown to be associated with lower rates of virologic breakthrough compared to LAM or ADV monotherapy, but resistance was not completely prevented.[15,19] In this study, we demonstrated that among patients with LAM plus ADV therapy resistant HBV conferring resistance to both antiviral agents could be detected.

As it is described, LAM resistance patients with ADV monotherapy or ADV plus LAM suppress viral replication during the first year of treatment.[20] We found that combination therapy with ADV plus LAM can maintain virological remission for at least two years in patients with LAM resistance. In contrast, in one LAM resistance patient who had been treated with ADV monotherapy, mutation conferring resistance to ADV appeared before two years of treatment. As recent study reported,[21] our study shows that adding ADV to LAM is more effective than switching to monotherapy in patients with resistant mutations. In addition, ADV plus LAM combination is well tolerated and prevents the emergence of resistance, although not entirely. Therefore, it represents the treatment of choice in nucleoside/tide analogue resistant patients.[22]

The different preceding nucleoside/tide analogues treatment strategies, depends on the level of HBV DNA at the time of treatment modification.[23,24] In LAM resistant patients with HBV DNA > 10^6-10^8 copies/mL, the probability of achieving undetecta-

ble levels by adding ADV is low.[13] In our case we detected mutations conferring resistance at early stage, so they presented low viral loads at the rescue therapy. In contrast, in patient 1 with LAM resistance mutations, the treatment was switched to ADV monotherapy, resistance to ADV emerged before 21 months of therapy, in this case baseline HBV DNA level was high and it is possible that the higher viral load attenuated the effectiveness of ADV.

Previous reports described the primary drug-resistant mutations cause an amino acid substitution in the polymerase that result in reduced susceptibility to nucleoside/tide analogues while secondary compensatory mutations cause amino acid substitutions that restore the polymerase activity. As described, primary LAM resistance associated changes occur at codon 204 and result in amino acid changes rtM204V/I/S. The most common compensatory mutation associated with LAM resistance, rtL180M restores replication fitness of HBV polymerase that harbours the rtM204V/I/S mutation.[15,25,26] Since LAM is effective against ADV resistant HBV mutants, we introduced LAM therapy in two patients. One of these patients showed a significant decrease in HBV DNA levels; however, the other patient whose baseline HBV DNA was higher, did not respond and developed rtI163V + rtL180M + rtA181V + rtN236T mutations which confer resistance to ADV and developed secondary mutation to LAM. The rtL180M mutation alone does not confer resistance to LAM.[25] In this case by direct sequencing, we did not detect the presence of rtM204V/I/S resistance mutation. It is supposed that rtL180M is compensatory or secondary mutation, but in our case the presence was previous to rtM204V/I/S.

In patients with primary ADV resistance mutation, isolates of HBV with the rtN236T change are susceptible to LAM while isolates with rtA181T/V changes have decreased susceptibility to LAM.[15] By contrast, in our study group of patients with previous ADV resistant mutations LAM was added and a patient (Patient 4) who had rtN236T mutation developed earlier resistant mutation to LAM, than patient (Patient 5) who had rtA181V resistant mutation.

Moreover, the results indicate that direct sequencing is suitable for the detection of mutations at the very early signs of treatment failure and in addition this method is suitable for the identification of drug-resistance mutations, as well as those that are novel, and require further investigations to determine their significance.

The rtQ215S mutation has been widely described as a secondary mutation for ADV and LAM[27,28] but other study reveals that rtQ215S substitutions in the polymerase frequently occur in chronic hepatitis B patients, even without exogenous selection pressures.[29] We found that the genotype D associated rtQ215S is present in two patients one in LAM failed patient and the other with ADV resistance, in both patients rtQ215S mutation was an additional mutation to a primary mutation. However, further investigations need to evaluate if the combination with primary resistances impacts the replication capacity or drug susceptibility of these viral strains.[30,31] One limitation of this study is the unavailability of prior treatment samples, thus we cannot totally exclude that some amino-acid variants analyzed might have been already present before the beginning of treatment.

Sequential antiviral therapy leads to selection of resistant HBV and evolution of mutations may select for mutants with increased replication or maximal viral resistance. In this study, LAM resistant HBV mutation was no detected in patient 1 by direct sequence analysis 21 months after LAM was stopped. Nevertheless, LAM resistant mutations remerged within 11 months after introduction of ETV. Persistence of LAM resistant mutations in patients who are switched to ETV is worrisome because resistance to ETV is greatly enhanced in the presence of LAM resistant mutations.[20] Our finding raises concerns about the long-term efficacy of ETV in patients with LAM resistant HBV. It has been demonstrated that the baseline presence of rtM204V + rtL180M favours the appearance of mutations associated with ETV resistance at position 184 and 202,[32-35] this is consistent with our study.

The recent introduction of more efficacious drugs, together with a better understanding of the activities of drug combinations, suggests that, in future, the careful design of combination strategies should lead to improved outcomes, particularly with respect to long-term viral suppression and consequent minimization of drug-resistance.

CONCLUSION

The increase in viral load is a prognostic marker of viral breakthrough. Antiviral therapy should be administered after careful study of genotype HBV and viral response should be carefully monitored. Therefore it is important to detect resistance mutations at early signs of a failing treatment, to maximize the success of early therapeutic changes in failed patients.

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