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REVIEW

Antiviral-resistant hepatitis B virus: can we prevent this monster from growing?

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SUMMARY. Despite the recent progress in antiviral therapy of chronic hepatitis B, clinical experience has shown that antiviral drug resistance is inevitable with the administration of nucleoside analog monotherapy. The long-term persistence of the viral genome in infected cells and the high rate of spontaneous mutation is the basis for the selection of HBV mutants that are resistant to polymerase inhibitors. Selection of antiviral-resistant mutations leads to a rise in viral load and progression of liver disease. The incidence of antiviral resistance depends on the potency and genetic barrier to resistance of the antiviral drug, highlighting the importance of the choice if first line therapy. The determination of cross-resistance profile of each drug has allowed the design of rescue therapy for patients with virologic breakthrough. Early diagnosis and treatment intervention allow the majority of patients to maintain in clinical remission despite the occurrence of drug resistance. Clinical studies are ongoing to determine the best strategy to prevent or delay antiviral drug resistance and of its impact on liver disease.

Keywords: antiviral drugs, antiviral therapy, chronic hepatitis, drug resistance, hepatitis B virus, viral genome variability.

INTRODUCTION

Despite the development of new nucleoside analogues that effectively inhibits hepatitis B virus (HBV) replication, antiviral therapy of chronic hepatitis B remains a clinical challenge mainly because of the slow kinetics of viral clearance and the subsequent emergence of drug-resistant mutants [1]. In this manuscript, we review the clinical impact of drug resistance, the new concepts of viral resistance management based on genotypic and phenotypic analysis of HBV drug-resistant mutants and measures to prevent antiviral-resistant HBV.

Nucleoside analogues mainly target the different viral polymerase activities, i.e. RNA-dependent DNA synthesis (reverse transcription) and DNA-dependent DNA synthesis [1]. This results in the inhibition of infectious virion production and a decreased rate of infection of new hepatocytes. However, none of the available polymerase inhibitors have been shown to prevent infection of uninfected hepatocytes and the *de novo* formation of covalently closed circular (ccc) DNA [2]. Antivirals have a modest indirect effect on cccDNA by inhibiting the intracellular recycling of nucleocapsids, but the long half-life of infected hepatocytes and cccDNA necessitates very long duration of therapy placing patients at risk of developing drug resistance [1,3].

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HIV, human immunodeficiency virus.

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HOW DO ANTIVIRAL-RESISTANT HEPATITIS B VIRUS MUTANTS ARISE?

Hepatitis B virus is a DNA virus that replicates via reverse transcription of pregenomic RNA. Unlike DNA polymerase, reverse transcriptase does not have proofreading ability. Therefore, HBV has a higher error rate than other DNA viruses. The high error rate and the large amount of virions produced (10^{12}–10^{13}/day) mean that every possible mutation is generated daily [4]. Because of the overlapping open reading frames, many of the spontaneous mutations may be detrimental to the virus and would not be propagated. Nevertheless, some spontaneously occurring mutations may persist and antiviral-resistant HBV mutants may be generated spontaneously before exposure to nucleos(t)ide analogue treatment [5].

Antiviral-resistant HBV mutants are selected in the presence of antiviral therapy because they can replicate better than wild-type virus in the presence of antiviral drug. Various factors are associated with an increased likelihood of antiviral-resistant mutant selection. Patients with high pretreatment HBV DNA levels, those with slow or inadequate viral suppression, and those who had resistance to prior treatment that are cross-resistant to the current therapy are at increased risk of developing antiviral resistance [6,7]. The likelihood of antiviral resistance is higher if the treatment has intermediate antiviral activity (enough to exert selective pressure on the virus but not enough to result in rapid suppression of HBV replication) and a low genetic barrier to resistance (marked decrease in susceptibility because of a single amino acid substitution not affecting viral genome replication capacity). Host factors that contribute to an increased likelihood of antiviral resistance include noncompliance with medications, immunosuppression (resulting in enhanced replication by loss of immune control of infected cells) [8], abundant replication space (large number of uninfected hepatocytes as in the newly transplanted liver) [9] and possibly polymorphisms in kinases responsible for converting prodrugs to active drugs or for phosphorylation of nucleos(t)ide analogues to the active triphosphates.

DEFINITION OF HEPATITIS B VIRUS DRUG RESISTANCE

Clinically, HBV drug resistance manifests at different levels [10,11]: (i) *genotypic resistance* corresponds to the detection of mutation(s) in the HBV genome which have been found to be selected during antiviral therapy and to confer resistance to the antiviral agent; i.e. the detection of specific HBV polymerase gene mutations; (ii) *virologic breakthrough* corresponds to a confirmed increase (two consecutive occasions) in serum HBV DNA levels by >1 $\log_{10}$ copies/mL during therapy, compared with nadir, in a medication compliant patient; (iii) *biochemical breakthrough* is defined as an increase in serum aminotransferase levels that accompanies a virologic breakthrough. In most patients, these three levels of drug resistance present successively with intervals of months or years. However, progression from genotypic resistance to biochemical breakthrough can be abrupt particularly in patients who are immunosuppressed or have underlying cirrhosis.

Another situation of treatment failure is a *suboptimal treatment response and primary nonresponse.* The definition is still debated but there is some agreement to define it by the inability of antiviral treatment to reduce serum HBV DNA by ≥2 $\log_{10}$ IU/mL and to a level <4 $\log_{10}$ IU/mL after the first 6 months of therapy. This phenomenon is likely to be due to pharmacologic issue including the pharmacodynamics of the drug and its antiviral potency as well as patient's compliance. Recently, it was suggested that virological factors may be involved as pre-existing mutations, i.e. rtI233V, were associated with primary nonresponse to adefovir in three patients [12].

Cross-resistance is defined as a mutation(s) that confers resistance to more than one antiviral drug by *in vitro* testing. This usually translates into failure of the other drugs as rescue therapy in the clinical setting. The knowledge of cross-resistance is therefore critical in developing treatment

Table 1 Results of *in vitro* cross-resistance analysis

	Resistance mutation			
	L180M + M204V M204I	N236T	A181V	S202G/I* M250I*
Drugs with reduced activity	Lamivudine Lamivudine Entecavir Telbivudine Emtricitabine Emtricitabine Clevudine Entecavir Telbivudine Elvucitabine	Adefovir Tenofovir	Adefovir Lamivudine	Entecavir Lamivudine Telbivudine
Drugs that remain active	Adefovir Tenofovir	Lamivudine Emtricitabine Entecavir Telbivudine	Tenofovir Entecavir	Adefovir Tenofovir

Results were obtained after *in vitro* phenotypic analysis of the resistant mutants [15,17–19,21,22,38].
*In the presence of primary resistance mutations (L180M + M204V).

strategies after treatment failure to the first antiviral drug. The results of cross-resistance analysis for the most common HBV antiviral-resistant mutants are summarized in Table 1.

PHENOTYPIC ASSAYS

Phenotypic assays are crucial in confirming if HBV polymerase mutations observed during antiviral therapy confer resistance to the drug and to determine if those mutations have cross-resistance to other drugs. Several assays has been used to determine the *in vitro* phenotype of antiviral-resistant mutants and have been reviewed recently [13]: (i) site-directed mutagenesis of laboratory strains cloned in appropriate vectors and transient transfection of hepatoma cell lines; (ii) exchange of polymerase gene fragments between HBV genomes from clinical isolates and the laboratory strain; (iii) subcloning of the whole HBV polymerase gene; (iv) amplification of the whole HBV genome and its transfection in hepatoma cell lines, either directly or after a cloning step; (v) cloning of 1.1 unit length HBV genome from clinical isolates and their transfection in cell lines. In each case, viral genome replication capacity is determined in the presence or absence of drugs. Other approaches have been used such as the cloning of HBV mutants in recombinant baculovirus vectors and the transduction of hepatoma cell lines by these baculoviruses.

Phenotypic assays are tedious but they have been instrumental in characterizing the mutations that confer resistance to lamivudine, adefovir, entecavir and telbivudine; the susceptibility of these mutants to other antiviral agents; and the differentiation between primary resistant mutations and secondary compensatory mutations [14–20]. Phenotypic assays have demonstrated that combination of antiviral drugs does not adequately suppress multi-drug-resistant mutants selected after sequential monotherapies [21,22].

Another important aspect of phenotypic assays is the assessment of replication fitness of the drug-resistant mutants, i.e. their capacity to spread in the liver and outgrow wild type virus [9]. The principle relies on the production of HBV mutants and the inoculation of susceptible cells, i.e. primary human or *Tupaia* hepatocytes and/or the highly differentiated HepaRG cell line [13]. However, the number of infected cells and virus production in these systems are low. Another approach is to inoculate humanized chimeric mice with these mutants to study their infectivity *in vivo* [23]. These assays to study viral fitness are being improved to provide new insight in the selection process of the HBV drug-resistant mutants.

DIAGNOSIS OF HEPATITIS B VIRUS DRUG RESISTANCE

Direct sequencing is the most convenient method for identifying resistance mutations to new treatments. However, direct sequencing is insensitive. Whereas direct sequencing can detect mutants that constitute 10–20% of the virus population, studies in patients with human immunodeficiency virus (HIV) infection who have received highly active antiretroviral treatment have shown that resistant mutants are not consistently detected unless they constitute ≥40% of the virus population. For nucleos(t)ide analogues with well-characterized resistance mutations, more sensitive methods that can consistently detect mutants when present in ≥10% of the virus population should be used [24]. These more sensitive methods permit earlier detection of resistance mutants, prior to biochemical breakthrough [25,26]. Recent studies showed that institution of rescue therapy at this early stage is more effective. Early detection of antiviral resistance is particularly important for patients with cirrhosis and in liver transplant recipients. Restriction fragment length polymorphism and reverse hybridization using strips coated with oligonucleotide probes (line probes) are the most common methods used for detecting antiviral-resistant HBV mutations. They are simple and can provide results within 1 day; however, they can only detect previously characterized mutations, and adaptation of these methods to detect mutants that confer resistance to a growing list of nucleos(t)ide analogues will be challenging [27].

INCIDENCE OF ANTIVIRAL-RESISTANT HEPATITIS B VIRUS

Although antiviral-resistant HBV mutations can occur spontaneously, these mutants are present as a minor virus species (<0.1%) in a large pool of viruses in most HBV carriers who have not been exposed to nucleos(t)ide analogues. Thus, unless ultra-sensitive assays are used to selectively amplify the mutants, antiviral-resistant HBV cannot be detected in patients who have not received nucleos(t)ide analogue treatment [5]. Among patients receiving a specific nucleos(t)ide analogue, the frequency of detection of antiviral-resistant HBV mutants correlates with pretreatment serum HBV DNA level, rapidity of viral suppression and duration of treatment [7,28–30]. The incidence of genotypic resistance also varies with the sensitivity of the methods used for the detection of resistant mutations and the patient population being studied.

Clinical studies of antiviral resistance have varied from testing all patients with detectable HBV DNA by polymerase chain reaction to only patients with confirmed virologic breakthrough, those with viral rebound (increase in HBV DNA to >5 log copies/mL), and those with biochemical breakthrough. The latter approaches result in underestimation of the rate of genotypic resistance, particularly if this is coupled with the use of an insensitive method for detection of antiviral-resistant mutation such as direct sequencing. These differences in methodology make it difficult to compare the incidence of genotypic resistance to various HBV therapies

across clinical trials. Variations in patient characteristics (such as pretreatment HBV DNA level, prior treatment and liver transplantation) further confound these comparisons, emphasizing the need for standardized definitions and assays for viral resistance.

CLINICAL IMPACT OF HEPATITIS B VIRUS DRUG RESISTANCE

The emergence of antiviral-resistant strains of HBV leads to viral, and subsequently biochemical breakthrough, negating any benefits achieved during initial antiviral treatment. The clinical outcome for patients with antiviral resistance is related to their age, the severity of the underlying liver disease and the intensity of the hepatitis flare. One study investigated the use of lamivudine for a period of up to 6 years in nearly 1000 patients found that the proportion of patients who experienced hepatitis flares increased from 33% for those with lamivudine-resistant HBV for 1 year to 77% for those who had lamivudine-resistant HBV for more than 4 years [30]. Furthermore, hepatic decompensation occurred in only 0–2% of patients with lamivudine resistance during the first 4 years, but thereafter the risk of developing hepatic decompensation increased to 6%. The favourable outcome in this study could be related to the relatively young age of the patients (mean: 32 years) and the fact that most patients had early-stage liver disease (only 10% had cirrhosis) [30]. Another study examined the efficacy of lamivudine *vs* placebo in the prevention of disease progression for patients with advanced liver fibrosis/cirrhosis [31]. After a median of 32 months, 49% of the patients in the lamivudine-treated group had genotypic resistance. The Child-Pugh scores were more likely to be increased in these patients than in those without lamivudine-resistant mutants (7% *vs* <1%, $P < 0.001$). Eight of the 14 deaths in the lamivudine-treated group were related to emergence of lamivudine resistance [31]. Despite these complications, patients with lamivudine resistance were less likely to develop progressive liver disease than the placebo-treated patients indicating a clinical benefit of lamivudine therapy, during the study period.

However, there have been many reports that emergence of lamivudine resistance was associated with severe hepatitis flare, liver failure and death, especially in older patients and those with cirrhosis or immunosuppression [26,30,32]. Several studies have also demonstrated that histologic improvements achieved during the first year of treatment are negated with the development of lamivudine resistance. In one study, patients underwent liver biopsies at baseline, year 1 and year 3. Among the patients with lamivudine resistance for more than 2 years, the proportion of patients with reduction in necroinflammation decreased from 56% at year 1 to 36% at year 3, and progression to bridging fibrosis was observed in approximately 27% of patients. By contrast, 83% of the patients without lamivudine resistance experienced a reduction of liver fibrosis in the same period [33].

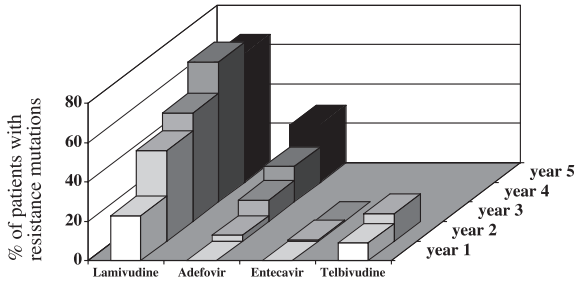

Fig. 1 Incidence of genotypic resistance in nucleoside naïve patients. Results were obtained from clinical studies [5,7,29,30,34,45]. For entecavir, Lamivudine resistance mutations are considered as primary entecavir resistance mutations.

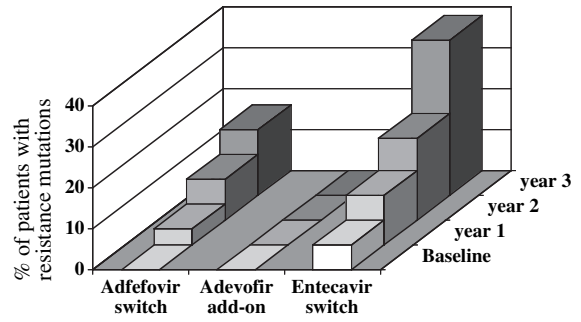

Fig. 2 Incidence of genotypic resistance in lamivudine-resistant patients. Results of selected studies are presented [35,42].

Patients who develop lamivudine resistance are less likely to undergo hepatitis B e antigen (HBeAg) seroconversion. One study of 58 patients found that after 4–5 years of continued lamivudine therapy, HBeAg seroconversion was achieved in 38% of those with and 75–80% of those without lamivudine resistance mutations [7].

Most of the data on the clinical impact of antiviral-resistant HBV have been derived from studies of lamivudine therapy. Limited data indicate that resistance to other antiviral drugs such as adefovir is associated with the same consequences: viral rebound, hepatitis flares and rarely liver failure [34]. The rate of resistance to adefovir dipivoxil and to entecavir is lower than that to lamivudine in nucleoside-naïve patients [5,34] (Fig. 1). However, in lamivudine-experienced patients, the risk of resistance to adefovir dipivoxil (when prescribed in monotherapy) or to entecavir increases significantly [6,35,36] (Fig. 2).

Implementation of combination prophylaxis of nucleos(t)ide analogues and hepatitis B immune globulin has decreased HBV recurrence postliver transplant to less than 10%. Currently, antiviral-resistant HBV is the main cause of HBV recurrence, accounting for over 90% of recurrent HBV postliver transplant [37].

Another consequence of the development of antiviral-resistant HBV is the presence of mutations that confer cross-resistance with other nucleos(t)ide analogues that are approved or currently under development, limiting future treatment options. This is of particular concern in young patients. Switching from one treatment to another may result in the selection of multi-drug-resistant mutations, especially if sequential monotherapy is used. Recent data indicate that multi-drug-resistant mutations collocate in the same HBV genome and are refractory to combination therapy [21,38,39]. Thus, patients may eventually run out of treatment options.

Finally, a potential concern is the transmission of antiviral-resistant HBV and *de novo* infection with these resistant strains [40]. Although antiviral-resistant HBV mutants are supposed to have decreased replication fitness compared with wild-type HBV and are expected to be outgrown by wild-type HBV in a nucleos(t)ide analogue-naïve host, transmission is possible and vigilance is necessary.

PREVENTION OF ANTIVIRAL-RESISTANT HEPATITIS B VIRUS

As with other areas of medicine, prevention is better than treatment. Judicious use of nucleos(t)ide analogues in patients with chronic hepatitis B is the most effective prophylaxis against the development of antiviral-resistant HBV. Thus, patients with minimal disease (such as inactive carriers) and those who are unlikely to achieve sustained response (such as HBeAg-positive patients in the immune tolerant phase) should not be treated with nucleos(t)ide analogues, particularly if they are young (<30 years of age). When possible, the most potent nucleos(t)ide analogue with the lowest rate of genotypic resistance should be administered and compliance reinforced. Although combination therapy has been shown to prevent antiviral resistance in patients with HIV infection, the promise of combination therapy has not yet been fulfilled for patients with HBV infection. Sequential therapy with drugs that are cross-resistant will lead to stepwise selection of strains harbouring increasing number of polymerase gene mutations conferring resistance to the first drug and then to both medications, as in the case of entecavir therapy for patients with lamivudine-resistant HBV. Thus, the benefits of antiviral treatment must be balanced against the risk of drug resistance during long-term treatment. Studies combining drugs with high genetic barriers to resistance and no cross resistance should be conducted to determine the optimal combination therapy for hepatitis B.

MANAGEMENT OF PATIENTS WITH ANTIVIRAL-RESISTANT HEPATITIS B VIRUS (FIG. 3)

Hepatitis B patients receiving antiviral therapy should be closely monitored for virologic breakthroughs. Recent studies indicate that initiating rescue therapy early, at the time of virologic breakthrough, is more effective than delaying until the patients have developed biochemical breakthrough [41].

Patients with lamivudine resistance can be managed by switching to adefovir or by adding adefovir. Several studies found that addition of adefovir is associated with a lower risk of subsequent adefovir resistance. This is confirmed in a large multicentre Italian cohort study involving 588 HBeAg-negative patients who had developed lamivudine resistance and in whom treatment with adefovir was initiated either in combination with or as a substitution for lamivudine. Over a period of up to 36 months, 11% of patients switched to adefovir monotherapy developed genotypic resistance to adefovir, compared with none of the patients who received add-on adefovir. Similarly, virologic breakthrough was only

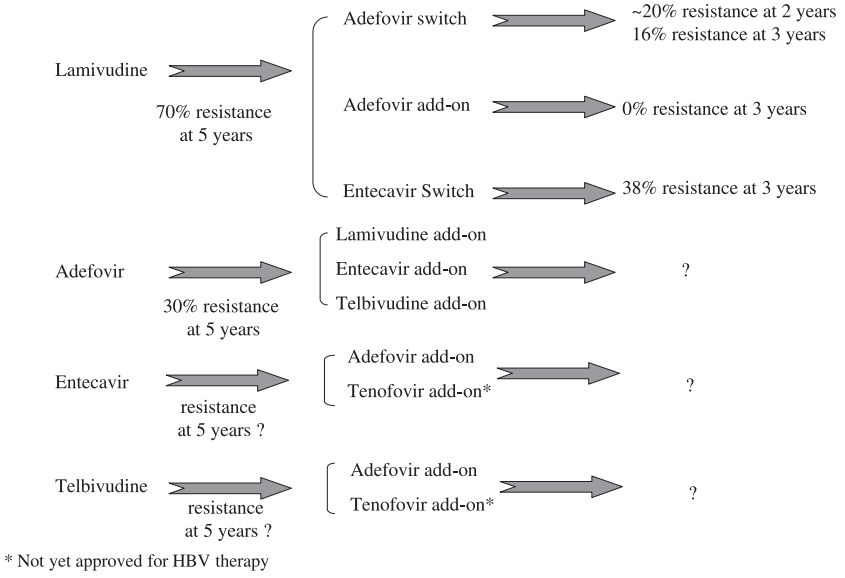

Fig. 3 Recommendations for the management of hepatitis B virus drug resistance. Resistance rate of selected studies are presented [6,35,36,42]. Recommendations are based on the results of phenotypic analysis and clinical studies.

seen in 5% of the add-on group *vs* 24% in the monotherapy group [42].

Patients with lamivudine resistance can also be managed with entecavir. However, despite the use of a higher dose, virologic rebound because of entecavir resistance was observed in 9% and 17% after 2 and 3 years of entecavir treatment [35]. Given our current understanding that pre-existing lamivudine-resistant mutations predisposes to further selection of mutations at 169, 184, 202 and 250 that confer resistance to entecavir [17,38,39], entecavir is not an optimal treatment for patients with lamivudine resistance.

Patients with adefovir resistance should be managed according to the cross-resistance profile of the mutant selected by adefovir. *In vitro* data of phenotypic assays show that the rtN236T mutation is susceptible to lamivudine and entecavir [15,18,22]. The rtA181V mutation confers a decreased susceptibility to lamivudine but remains sensitive to entecavir [21]. A few reports of clinical cases confirm that these patients should be managed according to the profile of resistance mutations [6,21,43].

To date, very few cases of entecavir resistance among nucleoside-naïve patients who have received entecavir treatment have been reported. The *in vitro* characterization of the entecavir-resistant strains showed that they are also resistant to lamivudine, but remain sensitive to adefovir and tenofovir. A recent report confirmed the initial efficacy of adefovir in decreasing serum HBV DNA level in a patient with entecavir-resistant mutants [38].

Several studies found that the risk of selection of drug-resistant mutants increases significantly when serum HBV DNA does not decline below 3 $\log_{10}$ copies/mL after 6 months of lamivudine or telbivudine therapy [28,44], or after 12 months of adefovir dipivoxil therapy [34]. These data suggest that treatment should be adapted early, in patients with partial virologic response to prevent the subsequent selection of resistant viruses.

CONCLUSION

Hepatitis B virus circulates as a pool of heterogenous viral genomes and its mutants are archived in the cccDNA; thus, HBV drug resistance is bound to occur with nucleoside analogue monotherapy. With the development of new nucleoside analogues that exhibit more potent antiviral activity and a lower rate of viral resistance, phenotypic analysis and cross-resistance data will be important for the adaptation of antiviral therapy in patients with drug resistance as well as for the design of combination trials with long-term end-points. HBV drug resistance is becoming a major clinical challenge in patients receiving long-term antiviral therapy. As many patients have now been exposed to several courses of antiviral drugs, the risk of selection of multi-drug-resistant mutants is increasingly common. Based on clinical experience and on results of detailed genotypic and phenotypic analysis, management of patients with drug-resistant HBV is evolving towards earlier add-on therapy. The availability of several antiviral drugs with different resistance patterns and excellent safety profiles offers a unique opportunity for the evaluation of *de novo* combination therapy or early add-on therapy to prevent drug resistance.

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CONFLICTS OF INTEREST

FZ serves as a consultant for Gilead and Idenix. ASL serves as a consultant and receives research support from Gilead, BMS, GSK, and Idenix. MB has declared no conflicts of interest.

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