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Case Report

**Selection of a multiple drug resistant hepatitis B virus strain in a liver  
transplanted patient**

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**Abbreviations:** cccDNA, covalently closed circular DNA; IC<sub>50</sub>, 50% inhibitory concentration; LT, liver transplantation; S, surface; wt, wild-type; YMDD, tyrosine, methionine, aspartate, aspartate.

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**Abstract**

**Background & Aims:** Sequential anti-HBV therapy may lead to the selection of complex mutants. We analysed the genetic and phenotypic evolution of the viral quasispecies of a patient who received successively lamivudine, add-on adefovir+lamivudine, followed by lamivudine+adefovir+Hepatitis B Immunoglobulins (HBIG) after liver transplantation (LT).

**Methods:** For genotypic analysis, a 1310 bp region of the polymerase gene was amplified, cloned, and sequenced. HuH-7 cells were transfected to compare the replication fitness of HBV mutants and their susceptibility to drugs.

**Results:** At baseline, all HBV genomes carried a wild-type (wt) RT gene but 22 % harboured the sP120S and 55% the sC107stop mutations within the S gene associated with vaccine escape. Following viral breakthrough to lamivudine monotherapy, a complex mixture of lamivudine-resistant HBV strains prevailed. Interestingly, among these mutants emerged a population harbouring only the rtL180M+A181V mutations, conferring lamivudine-resistance *in vitro*. After addition of adefovir to the ongoing treatment, viral load dropped, the patient underwent a LT and received HBIG. As viral load rose again, a single viral population was progressively selected, harbouring the rtV173L+L180M+A181V+N236T and sP120S mutations. *In vitro*, this last mutant showed a level of replication reduced by only 30% compared to wt HBV and a strong resistance to both lamivudine (>1000-fold) and adefovir (>10-fold). It remained sensitive to tenofovir both *in vitro* and *in vivo*.

**Conclusions:** We report the selection of a complex HBV mutant that escaped the antiviral pressure of lamivudine, adefovir and HBIG, and provide insight on the process of selection *via* genotypic and phenotypic analysis.

## Introduction

Chronic hepatitis B virus (HBV) infection remains a major health problem worldwide with 400 million chronic carriers exposed to the risk of liver cirrhosis and hepatocellular carcinoma development<sup>1</sup>. Although the number of approved antiviral agents has increased in recent years, the treatment of chronic hepatitis B remains a clinical challenge. Administration of standard or pegylated interferon alpha (IFN $\alpha$ ) induces a sustained response in approximately 30% of patients and is associated with dose-dependent side effects<sup>2, 3</sup>. The other alternative is the use of nucleos(t)ide analogues such as lamivudine, adefovir dipivoxil or entecavir. Lamivudine is a nucleoside analog of L-deoxycytidine, adefovir is a phosphonate nucleotide analog of adenosine monophosphate, and entecavir is a deoxyguanosine analog. They target the HBV reverse transcriptase (RT) activity, thus inhibiting viral replication. They are powerful antivirals and monotherapy with these agents results in significant improvement in virologic, biochemical, and histologic status in most patients<sup>4-6</sup>. However, long-term therapy with nucleoside analogs is required because of the persistence of infected cells and intrahepatic viral covalently closed circular (ccc) DNA<sup>7</sup>, which may result in reactivation of viral replication after treatment cessation. Due to the spontaneous error rate of the viral polymerase and the accumulation of viral genome mutations during the natural history of infection, the viral quasi-species may undergo significant changes under the selective pressure of antiviral therapy with the selection of drug resistant mutants<sup>8</sup>.

Drug resistance is associated with the appearance of polymerase gene mutations, followed by an increase in viral load, i.e. virologic breakthrough, and subsequently an increase in ALT levels and worsening of liver disease<sup>9</sup>. Genotypic resistance occurs in approximately 20% of patients per year in lamivudine treated patients<sup>10, 11</sup>, and at a

lower rate in adefovir treated patients, i.e. 3% at year two with a progressive increase to 29% at year five of therapy<sup>12</sup>.

Mutations conferring resistance to lamivudine have been mapped in the conserved YMDD motif within the C domain of the viral RT (rtM204I/V)<sup>10</sup>. They are frequently associated with compensatory mutations in the conserved B domain (rtV173L, rtL180M) that restore partially the replicative capacity of YMDD mutant strains *in vitro*<sup>13, 14</sup>, and are associated with a 1000-fold reduction in the susceptibility to the drug<sup>15</sup>. Adefovir-resistance is associated with the selection of the rtN236T mutation within the D domain of the viral enzyme or with a rtA181V amino acid change in the B domain of the RT<sup>16-18</sup>. The rtN236T mutation induces a reduction in the susceptibility to adefovir by 3 to 6 fold compared to wild-type HBV (wt) *in vitro*, but retains some level of susceptibility to lamivudine<sup>16, 17, 19</sup>.

In this report, we describe the case of a patient who failed lamivudine therapy, and subsequently failed lamivudine, adefovir and hepatitis B immunoglobulins (HBIG) combination therapy after LT. A genotypic analysis of HBV strains isolated from the patient revealed the complexity in the evolution of viral quasi-species all along therapy, with the selection of HBV strains harbouring resistance mutations to both drugs and HBIG. *In vitro* assays were performed to analyze the replication capacity and drug susceptibility of the major HBV strains and to gain insight in the mechanism involved in the mutant selection process.

## **Material and Methods**

### **Patient**

A 43 years-old haïtian man with HBeAg positive chronic hepatitis B and severe cirrhosis was started on a lamivudine monotherapy (100 mg daily) in June 1996. After 12 months of lamivudine treatment, serum HBV DNA had decreased to  $1,00E+03$  IU/ml, but re-increased to its initial level 5 months later, i.e.  $1,80E+07$  IU/ml. Thirty-one months later, adefovir dipivoxil 10 mg daily was added to the ongoing treatment. After eight months of bitherapy, the patient was still HBsAg positive, HBeAg positive, anti-HBe negative and HBV DNA undetectable by the Digene I assay used in the institution at that time (limit of detection:  $2.5E+05$  IU/ml). The patient then underwent an orthotopic liver transplantation (OLT). Post-transplantation, he received intramuscular HBIg in association with lamivudine and adefovir dipivoxil. Thirty ml of HBIg (9,500 IU) were administrated intra-muscular to the patient during the anhepatic phase of OLT, then he received 15 ml daily for 10 days, 15 ml every 2 weeks for the first 3 months, and 10 ml monthly afterward. Eighteen months after LT, HBsAg remained negative and HBV DNA was consistently undetectable ( $< 200$  copies/ml) by quantitative PCR (Roche diagnostic). However, a viral breakthrough occurred 31 months after transplantation. HBsAg and HBeAg became positive again and serum HBV DNA levels rose to  $> 10E+07$  IU/ml. Nine months later, adefovir was replaced by tenofovir 300 mg daily with ongoing lamivudine therapy. Subsequently, viral load decreased to approximately  $1,00E+03$  IU/ml. The evolution of viral load over time, requantified by bDNA assay (Versant HBV DNA 3.0 assay; Bayer Corporation, Tarrytown, N. Y.), is shown in Figure 1.

### **Clonal analysis of the HBV polymerase gene**

HBV DNA was extracted from 200 to 500  $\mu$ l of serum sample by using QIAamp Ultrasens virus kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The HBV polymerase gene domain between amino acid (a.a.) position 323 to 770<sup>20</sup>, encompassing the whole RT domain, was PCR amplified using primer pair L1 5'-TTCCTGCTGGTGGCTCCAGTTC-3' (nucleotide position 54-75) and B1 5'-GGCAGCACASCCTAGCAGCCATGG-3' (nucleotide position 1395-1372), and the expand high fidelity PCR system according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). PCR amplification consisted of 40 cycles of 94°C for 30s, 50°C for 30s and 72°C for 1min. PCR products were cloned into the pGEMt easy vector system (Promega, Madison, WI) according to the manufacturer's protocol. Following transformation into DH5 $\alpha$  cells (Promega, Madison, WI), 20 colonies per serum sample were screened for the presence of pGEMt-HBV polymerase plasmids, and the PCR products were sequenced with the RV5 primer (5'-GGTTGCGTCAGCAAACACTTG-3') located downstream the RT domain. Amino-acid residues are numbered according consensus nomenclature developed by Stuyver et al..<sup>20</sup>

### **HBV constructs for phenotypic analysis**

A replication competent 1.1 HBV genome unit length vector containing HBV genome isolated from the baseline clinical sample was constructed. HBV DNA was extracted from the patient's baseline serum sample using QIAamp Ultrasens virus kit (Qiagen, Hilden, Germany). Two PCR fragments were obtained and cloned in one step into a modified pTriEX vector (Novagen, Madison, WI), as previously described in detail<sup>21</sup>. The sequence and the replication capacity of these vectors containing HBV genome were verified.

Subsequently, the HBV RT and S gene mutants identified by the clonal analysis were inserted in the baseline viral genome by replacing the 1250bp XhoI-NcoI fragment of the vector containing HBV from baseline by the corresponding XhoI-NcoI fragment from selected pGEMt-HBV polymerase plasmids. The newly obtained plasmids were verified by DNA sequencing prior to cell transfection.

### **Analysis of HBV genome replication and drug susceptibility**

Huh-7 cells were cultured in DMEM (Eurobio, Courtaboeuf, France) supplemented with 10% FBS. Lamivudine, adefovir, and tenofovir were obtained from Gilead Sciences, entecavir from Bristol-Myers.

HBV genome replication capacity was measured by co-transfecting transiently recombinant pTriex-HBV clones with a luciferase reporter plasmid (pGL3, Promega, Madison, WI) to normalize transfection efficiency. Huh-7 cells at 70-80% confluence, in twelve well plates, were co-transfected with 0.5 µg of recombinant HBV clones and 0,1 µg of pGL3 reporter plasmid using Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Five days after transfection, purification of HBV DNA from intracellular core particles was performed following the protocol described by Summers et al.<sup>22</sup>. The luciferase activity was measured by mixing 10 µl of the lysis mixture supernatant and 50 µl of luciferin LAM reagent (Promega, Madison, WI). The reading was performed in the luminoskan Ascent (Thermo Lab systems, GMI, Inc, Ramsey, Minnesota). Intracellular HBV DNA was submitted to *Southern blot* analysis, quantified using PhosphorImager analysis, and adjusted for the efficiency of transfection according to the results of the luciferase assay.

The analysis of drug susceptibility was performed after transient transfection of HuH-7 cells with recombinant wt or mutant pTriex-HBV clones. Sixty hours after transfection, the medium was changed and transfected cells were reincubated with drug-free medium or medium containing 0.01, 0.1, 1, 10, 100  $\mu$ M of lamivudine or entecavir, or 6.25, 12.5, 25, 50, 100  $\mu$ M of adefovir or tenofovir. Treatments were renewed every day for 5 days. Then, intracellular HBV DNA was purified and subjected to *Southern blot* analysis as described above. For each drug, the concentration inhibiting by 50% the amount of intracellular viral DNA detected in treated cells at the end of the treatment, compared with untreated cells (IC50) was determined by phosphorImager analysis.

For analysis of HBsAg expression and recognition by anti-HBs antibodies, supernatant of HuH-7 transfected cells were harvested 5 days after transfection, and secreted HBsAg was detected with Monolisa HBsAg Ultra kit (BioRad, Hercules, CA) and Enzygnost HBsAg (Dade Behring, Marburg, Germany) following a 50-fold dilution of supernatant.

## Results

Viral genome analysis was performed for serum samples 1 to 13 throughout the clinical course of the patient (Figure 1). The DNA sequence from 20 clones per serum sample were analyzed according to the procedures described above and the major mutants were tested for their *in vitro* replication capacity and drug susceptibility.

### Evolution of Surface (S) gene sequences in the viral quasi-species

We mainly focused our analysis on the immunodominant loop encompassing a.a. 101 to 163 of the S protein, including the “a” determinant (a.a. 124-147) which is the major target of neutralizing anti-HBs antibodies, due to its exposition at the surface of viral particles<sup>23</sup>. Surprisingly, sequencing of the S gene revealed on the baseline sample the presence of mutations in the immunodominant loop for many clones. 22% of HBV clones harboured a sP120S mutation, and 55% a sC107stop mutation leading to a truncated envelope protein deleted for the “a” determinant. These mutations within S protein lead to rT128I and rS116T substitutions respectively in RT protein (Table 1). Interestingly, 80% of sC107stop HBV clones also harboured the nucleotide mutation inducing the sP120S a.a. change located after the stop codon.

Following the beginning of antiviral therapy, the proportion of clones harbouring the sC107stop mutation rapidly decreased. Noteworthy, 22 months after transplantation, this mutant re-emerged transiently in 17% of clones isolated (serum sample # 7). This mutant was not detected subsequently in our clonal analysis (Figure 2). In contrast, the number of clones harbouring the sP120S substitution increased during lamivudine and adefovir dipivoxil therapy with a peak at the time of LT. Clones

harbouring the sP120S mutation then decreased to 30% of the HBV population, and reincreased progressively to become the dominant species (100% of clones) when the viral load reached its highest level. Noteworthy, despite cessation of HBIg administration after HBV recurrence, the sP120S mutation was maintained as the major species (100% of clones) until the end of follow-up.

### **Evolution of the viral RT gene in the viral quasi-species**

Evolution of the viral quasi-species during lamivudine monotherapy : The sequence of the viral RT gene at the baseline of lamivudine therapy was wt but a strong polymorphism was distributed all along this gene. Twelve months after the beginning of lamivudine monotherapy, HBV DNA titers were very low but the rtM204V lamivudine-resistance mutation was already detected in 10% of HBV clones (Figures 1 and 3). By month 18, the rise in viral load was associated with the emergence of multiple mutations in the RT gene: 15% of HBV clones harboured the rtL180M+T184I+M204V mutations, 20% the rtL180M+A200V+M204V mutations, and interestingly 40% of the viral population harboured the rtL180M+A181V mutations without mutation at the rtM204 codon. At month 24 of lamivudine treatment, while viremia level remained high, the viral quasi-species continued to evolve. Although the rtL180M+A200V+M204V mutant was detected in the same proportion as at month 18, the rtL180M+A181V mutant disappeared and was replaced by the rtM204I and rtL180M+M204V mutants in 27% and 45% of clones respectively.

Noteworthy, the sequence analysis revealed a complex mixture of variants at each time point, but we reported here only the mutants representing at least 5% of the viral quasi-species (for more details see Table 1).

Evolution of the viral quasi-species during lamivudine+adefovir combination therapy:

A complex evolution of the viral quasi-species was observed following the addition of adefovir dipivoxil to lamivudine therapy, and subsequently LT and the administration of HBIg. Eight months after the addition of adefovir dipivoxil to lamivudine, i.e. at the time of transplantation, HBV DNA titers were significantly decreased but the rtA181V mutation re-appeared in 70% of clones in association with lamivudine resistance mutations; 60% of the quasi-species was composed of the rtV173L+L180M+A181V+M204V mutant (Figure 4). This mutant persisted but declined progressively during the following 32 months. At month 24 of lamivudine+adefovir treatment (month 16 post-LT), while HBV DNA titers were maintained at low levels, the rtN236T adefovir resistance mutation emerged alone or in combination with the rtV173L+L180M+A181V mutations. The rtV173L+L180M+A181V+N236T mutant was progressively selected to become the major variant species when the viral load re-increased significantly 30 months post-LT, and subsequently represented 100% of the viral population (Figure 4). Interestingly, this variant did not harbour the rtM204V/I lamivudine-resistance mutation although lamivudine treatment was maintained after the addition of adefovir dipivoxil. The presence of viral strains harbouring five resistance mutations rtV173L+L180M+A181V+M204V+N236T was detected in 5% and 19% of clones at month 38 and 40 of the combination therapy, but this mutant was not further selected although it harboured all the mutations conferring resistance to lamivudine and adefovir (Figure 4). Therefore, many complex mutants co-existed before the outgrowth of the rtV173L+L180M+A181V+N236T mutant which became dominant suggesting that this mutant had an intrinsic selective advantage in this patient.

### **Viral genome replication capacity of the major HBV mutants**

To gain insight in the selection process of the different mutants, the replication capacity of the major HBV variants identified at each time point was analyzed in a transient transfection cell culture assay system as described in Material and Methods.

We selected the five main mutants that emerged after 18 and 24 months of lamivudine monotherapy. The replication capacity of these mutants was compared to that of a mixture of five clones obtained from the pre-therapeutic serum sample and considered as wt. As shown in figure 5A, the rtM204I mutant had the lowest replicative capacity (5-fold less than wt HBV) in the HuH-7 cell line. The rtL180M+M204V mutant replication capacity was reduced by 40% compared to wt HBV. The addition of the rtT184I mutation to the rtL180M+M204V mutant resulted in weak replication impairment, whereas the addition of the rtA200V substitution increased slightly the replication capacity of the same mutant. The rtL180M+A181V mutant had a similar replication level as wt HBV (88% of the wt HBV).

The replication capacity of the four main mutants which emerged under lamivudine+adefovir combination therapy was also analyzed. These four mutants also co-existed 40 months following the beginning of the bitherapy prior to the selection of the final mutant. As shown in Figure 5B, the clone harbouring the five mutations rtV173L+L180M+A181V+M204V+N236T had the weakest replication capacity. HBV viruses harbouring rtV173L+L180M+A181V and M204V or N236T mutations in the polymerase gene had similar replication capacities (68% and 71% respectively) compared to wt HBV. The rtV173L+L180M+A181V mutant without change at codon positions 204 and 236, showed a similar replication capacity to wt

HBV and to the rtL180M+A181V mutant transiently selected during lamivudine therapy.

### **Drug susceptibility of the major HBV mutants**

The five main mutants previously described during lamivudine monotherapy were analyzed for their susceptibility to lamivudine and adefovir. Results showed that all the tested mutants, except rtL180M+A181V mutant, were strongly resistant to lamivudine with IC<sub>50</sub>s >1000-fold higher than that of wt HBV, but retained susceptibility to adefovir (Table 2). Interestingly, we demonstrated that the rtL180M+A181V mutant had a reduced susceptibility to lamivudine (800-fold) despite the absence of the rtM204V substitution and a slightly reduced susceptibility to adefovir (2.7-fold).

We also asked whether the rtV173L+L180M+A181V+N236T which became the dominant strain during the lamivudine+adefovir combination therapy displayed the highest resistance to these nucleos(t)ide analogues. We analyzed the drug susceptibility of the four main mutants emerging after LT. As shown in Table 3A, all the tested mutants, except rtV173L+L180M+A181V mutant, were strongly resistant to lamivudine with IC<sub>50</sub>s >100 μM. The rtV173L+L180M+A181V mutant, as previously described for the rtL180M+A181V double mutant, had an IC<sub>50</sub> of 100 μM. All these mutants had an increased adefovir IC<sub>50</sub> compared to wt HBV indicating a 4 to 7.7-fold resistance except for the rtV173L+L180M+A181V+N236T mutant which showed a >10-fold resistance to adefovir.

Since the patient was treated with tenofovir following lamivudine+adefovir breakthrough, we analyzed the susceptibility of the final mutant rtV173L+L180M+A181V+N236T to this drug and found that this mutant showed only

a slight decrease in susceptibility to tenofovir compared to wt HBV. Similar results were obtained with entecavir which had only a 4-fold decreased inhibitory activity on this mutant *in vitro* (Table 3B).

### **Reactivity of HBV Surface antigen to anti-HBs antibody**

To investigate the effect of the S gene mutations on HBsAg affinity to anti-HBs antibody, supernatants of HuH-7 cells transfected by the different mutants were collected and assessed with two commercial ELISA kits. Table 4 describes the mutations in S gene for the different mutants including S mutations due to RT changes. Clones obtained from the pretherapeutic sample were tested individually since they presented significant heterogeneity within the S gene. Results showed a high HBsAg titer for all the clones not harbouring sP120S or sC107stop mutations in S gene using both kits (Table 4) except for the rtM204I mutant. This mutation in RT gene leads to a truncated S protein at position 196, suggesting that this 30 a.a. C-terminal deletion decreased the binding to anti-HBs antibodies from the kits.

HBs proteins expressed from the clones harbouring the sC107stop mutation were, as expected, not recognized by anti-HBs antibodies included in the two kits. The same results were obtained with the clone harbouring a 10 a.a. deletion at the beginning of the immunodominant loop. The sP120S mutation appeared to be recognized preferentially by the Monolisa plus kit, with a 2.3 to 26-fold decrease in recognition by Enzygnost kit compared to Monolisa kit anti-HBs antibodies.

## Discussion

In this report, we describe the case of a cirrhotic patient who received a liver graft and developed HBV resistance to lamivudine, adefovir and HBIg.

To our knowledge, this is the first description of failure of a combination of lamivudine, adefovir and HBIg, due to the selection of an HBV strain harbouring a complex pattern of mutations. An HBV strain resistant to both lamivudine and HBIg has been previously observed in a patient who underwent a liver transplantation<sup>24</sup>. This multidrug resistance phenomenon was not observed in previous cohorts of cirrhotic or transplanted patients receiving adefovir dipivoxil for lamivudine failure<sup>25</sup>.<sup>26</sup>. This may be due to the relatively short term follow-up in these cohort studies, as resistance occurred 31 months after LT in our patient. The incomplete viral suppression with high HBV DNA titers observed at the time of LT, that were not detected by the Digene assay but by newer and more sensitive assays, may have also contributed to the subsequent emergence of HBV mutants. Moreover, in contrast to recent studies suggesting that combination of lamivudine and adefovir may prevent emergence of resistance in patients with lamivudine-resistance, our patient developed resistance despite the maintenance of lamivudine following LT<sup>27</sup>. Noteworthy, the switch to tenofovir together with lamivudine allowed to rescue antiviral drug resistance and therefore confirms previously published data on the favorable cross-resistance profile of this antiviral agent<sup>19</sup> and its *in vivo* antiviral potency<sup>28</sup>.

A detailed clonal HBV genome analysis was performed on sequential serum samples throughout the course of the disease, as it was recently done in four patients who failed lamivudine therapy<sup>29</sup>. These data allowed to show the complex evolution of the viral quasi-species under the different antiviral pressures.

The analysis of the surface gene sequence revealed some unexpected results. Indeed, sequencing of clones from the pre-treatment serum sample showed that more than 75% of HBV clones harboured mutations in the S gene, with a stop codon at position 107, leading to a truncated S protein and a deleted antigenic “a” determinant, or a sP120S substitution located just upstream of the “a” determinant. This latter mutation has been previously reported to decrease the binding of HBsAg to anti-HBs antibodies, through alteration of the conformation of the second loop (aa 139-147) of the “a” determinant<sup>30-32</sup>. Serologic assays performed on the supernatant of transiently transfected cells with the sP120S mutant showed a defect in recognition depending on the anti-HBs antibodies used. The lack of antibody binding, observed with Enzygnost kit, was not due to a defect in HBsAg secretion, since this mutant was detected as well as the wt clones with the Monolisa plus kit. This high proportion of immune escape variants in the pre-therapeutic viral quasi-species may be due to a higher fitness of this mutant compared to wt strains and/or to virus transmission by an HBV carrier who failed vaccination. Previous studies also reported the presence of mutations in the HBV S gene prior to LT and antiviral treatment. These pre-existing mutations may have contributed to the development of HBV recurrence post-LT in these patients<sup>33, 34</sup>.

Sequence analysis of HBV clones isolated from the patient's sera during lamivudine monotherapy revealed a complex evolution of the viral quasi-species, but unexpectedly the rtL180M+M204V mutant frequently observed in patients who failed lamivudine<sup>10</sup> was not detected. These rtL180M and rtM204V mutations were always found to be associated to a third mutation at codon position 184 or 200. The most surprising result was the emergence of a mutant harbouring the rtL180M+A181V substitutions representing 40% of the viral population. Indeed, the rtA181V mutation

has been described only in patients who failed adefovir dipivoxil therapy<sup>18</sup>. The selection of the rtL180M+A181V mutant during lamivudine breakthrough was explained by our *in vitro* phenotypic analysis which showed a susceptibility decrease by 800-fold to lamivudine and by 2.7-fold to adefovir compared to wt HBV. Moreover, this mutant had a conserved viral genome replication capacity compared to wt HBV genome. These findings support the clinical relevance of genotypic assays to identify the profile of resistance mutations and tailor antiviral therapy to the virologic evolution.

Twenty-four months after the start of lamivudine therapy, the rtA181V mutation was not found among the isolated HBV mutants but the rtM204I and rtL180M+/-A200V+M204V mutants prevailed. Thus, in a short period of therapy, a switch in the HBV population was observed with the emergence of the most fit variants and the elimination of other variants.

During lamivudine+adefovir bitherapy, the complexity of the viral quasi-species increased with the emergence of mutants harbouring resistance mutations to both drugs. Indeed, mutants harbouring rtA181V, rtN236T or both mutations in addition to lamivudine resistance mutations were rapidly selected. The role of LT in the selection of these mutants has to be discussed. Indeed, transplantation was performed when viral load was still high due to the low sensitivity of the DNA quantification assay used at that time, and the liver graft may have provided a new replication space allowing the spread and outgrowth of these mutants<sup>35</sup>. The spreading of these resistant mutants was however slow as HBV recurrence became clinically detectable only 26 months post-LT, most likely because of the strong antiviral pressure of antiviral therapy and HBIg administration.

We analyzed the *in vitro* phenotype of the mutants observed 32 months after LT and during viral breakthrough. Interestingly, these mutants were present in approximately the same proportions, and all harboured the sP120S mutation. While it could have been expected that the rtV173L+L180M+A181V+M204V+N236T was the mutant which had the highest chance of selection in the context of the combination therapy, the rtV173L+L180M+A181V+N236T mutant had the highest resistance to adefovir (>10-fold), a strong resistance to lamivudine (>1000-fold) without harbouring the rtM204V mutation, and a genome replication capacity at 70% of wt HBV. As this mutant also harboured the sP120S mutation, it is most likely that its viral fitness in the context of the combination of nucleos(t)ide analogs and HBV administration allowed its selection and dominance as it finally represented 100% of the viral population when viral load reached high levels. This multiple drug resistant mutant had only a slightly decreased susceptibility to tenofovir compared to wt HBV, that explains the significant decline in viremia levels observed *in vivo*. This mutant also remained sensitive to entecavir *in vitro*, which may represent an interesting treatment option in this particular clinical setting. The selection of such complex mutants inducing multidrug-resistance underlines the interest to continue the development of new anti-HBV agents.

In conclusion, we report the selection of a complex HBV mutant which escaped the antiviral pressure of lamivudine, adefovir and HBV. The originality of this study relies on the longitudinal clonal analysis of viral genomes combined with the *in vitro* phenotypic analysis of the major variants. These data allowed to gain insight in the selection process of this escape mutant. They also suggest that the prevention of drug resistance should be evaluated in clinical trials with *de novo* nucleos(t)ide

analog combinations to maintain long-term and complete viral suppression, as well as with the monitoring of antiviral treatment with viral load monitoring and genotypic assays to tailor treatment regimen to the virologic situation of the patient.

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## Figure legends

**Figure 1. Evolution of the viral load during the course of antiviral therapies.** The duration of therapies is indicated by bars above the graph and the serum samples from the patient by the numbers below the graph. Lamivudine (LAM), adefovir dipivoxil (ADV) and tenofovir (TENO) were administered to the patient. The presence of HBsAg (sera #1 to 16) and the quantification of anti-HBs antibodies (sera #6 to 8) in the sera from the patient are specified below the graph.

**Figure 2. Evolution of HBsAg substitutions during the course of therapies.** The serum samples number #1 to 13 were screened, as described in Material and Methods, for the presence of the C107stop ( $\square$ ) and P120S ( $\Delta$ ) changes in S gene.

**Figure 3. Genotypic analysis of HBV mutants evolution during lamivudine therapy.** The different bars correspond to the percentage of the viral population represented by each RT-HBV mutant. Zero, 12, 18, and 24 months of lamivudine treatment correspond to serum sample numbers #1, 2, 3 and 4 respectively. The viral rebound indicated on the right concerns serum sample numbers #3 and 4. The percentage of clones with a sP120S substitution is indicated between brackets.

**Figure 4. Genotypic analysis of HBV mutants evolution during lamivudine + adefovir bitherapy.** The different bars correspond to the percentage of the viral population represented by each RT-HBV mutant. Eight, 24, 34, 38, 40, and 42 to 50 months of lamivudine+adefovir treatment correspond to 0, 16, 26, 30, 32, and 34 to 42 months post-transplantation respectively, and to serum sample numbers #5, 6, 7, 8, 9, and 10 to 13 respectively. The viral rebound indicated on the right concerns serum sample numbers #7 to 13. The percentage of clones with a sP120S substitution is indicated between brackets.

**Figure 5. Replicative capacity in Huh7 cells of wt and RT HBV mutants isolated from patient during lamivudine therapy (A) and lamivudine+adefovir bitherapy (B).** Viral DNA synthesis was analyzed as described in Material and Methods. After phosphorimager analysis, values obtained for each mutants were divided by wt HBV value that was assumed to represent 100% of viral replication. Values are reported as the mean and standard deviation from six independent experiments.

**Table 1. Description and evolution of the different mutations observed in HBV clones isolated from the patient and their evolution in serum samples number 1 to 13.**

Pol aa change	S aa change	% of the mutation during the course of therapies												
		1	2	3	4	5	6	7	8	9	10	11	12	13
S116T	C107stop	55	10	7	9	0	5	17	0	0	0	0	0	0
T128I	P120S	66	14	27	14	88	30	96	94	89	100	100	100	100
I169V	None	0	0	5	0	0	13	0	0	0	0	0	0	0
V173L	E164D	0	0	0	5	70	26	46	94	90	100	100	100	100
L180M	None	0	0	85	68	85	56	79	89	100	100	100	100	100
A181V	L173F	0	0	40	0	70	30	50	94	95	100	100	100	100
T184I	None	0	0	25	0	5	13	0	0	0	0	0	0	0
A200V	L192F	0	0	30	18	0	0	0	0	5	0	0	0	0
M204V	I195M	0	10	40	73	65	35	54	17	33	0	5	0	0
M204I	W196stop	0	0	0	27	25	4	38	0	5	0	5	0	0
N236T	none	0	0	0	0	0	26	0	83	50	100	95	100	100

Each column numbered 1 to 13 corresponds to the percentage of each mutation in the serum samples #1 to 13 (Figure 1). The impact of each RT substitution on S protein translation and conversely is indicated in the two columns on the left.

**Table 2. *In vitro* susceptibility to lamivudine and adefovir of the mutants emerging under lamivudine therapy .**

HBV mutants	Lamivudine		Adefovir	
	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>
Wild type	0.1 $\pm$ 0.2	1	10 $\pm$ 3	1
M204I	>100	>1,000	10 $\pm$ 2	1
L180M+M204V	>100	>1,000	15 $\pm$ 6	1.5
L180M+A200V+M204V	>100	>1,000	21 $\pm$ 15	2.1
L180M+A181V	80 $\pm$ 9	800	27 $\pm$ 16	2.7
L180M+T184I+M204V	>100	>1,000	10 $\pm$ 3	1

a) Values are the mean and standard deviation of at least three independent experiments, each performed in duplicate.

b) Fold resistance = (mutant IC<sub>50</sub>)/(wt IC<sub>50</sub>).

**Table 3. *In vitro* susceptibility to lamivudine and adefovir (A), tenofovir and entecavir (B) of the mutants emerging under lamivudine and adefovir bitherapy.**

### A

HBV mutants	Lamivudine		Adefovir	
	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>
Wild type	0.1 $\pm$ 0.2	1	10 $\pm$ 3	1
V173L+L180M+A181V	100 $\pm$ 5	1,000	48 $\pm$ 19	4.8
V173L+L180M+A181V+M204V	>100	>1,000	40 $\pm$ 20	4
V173L+L180M+A181V+M204V+N236T	>100	>1,000	77 $\pm$ 20	7.7
V173L+L180M+A181V+N236T	>100	>1,000	>100	>10

### B

HBV mutants	Tenofovir		Entecavir	
	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	Fold resistance <sup>b</sup>
Wild type	10 $\pm$ 6	1	0.01 $\pm$ 0.01	1
V173L+L180M+A181V+N236T	16 $\pm$ 7	1.6	0.04 $\pm$ 0.04	4

a) Values are the mean and standard deviation of at least three independent experiments, each performed in duplicate.

b) Fold resistance = (mutant IC<sub>50</sub>)/(wt IC<sub>50</sub>).

**Table 4. Analysis of HBsAg secretion.**

RT mutations	S mutations	HBsAg Monolisa	HBsAg Enzygnost
none	none	30	22
none	C107stop	0.7	0.7
none	P120S	23	0.9
M204I	W196stop*	0.5	1.1
L180M+M204V	I195M*	30	23
L180M+A200V+M204V	L192F*+I195M*	30	21
L180M+A181V	L173F*	22	21
L180M+T184I+M204V	P120S+I195M*	31	6.6
$\Delta$ aa 111-120+V173L+L180M+A181V	$\Delta$ aa102-111*+P120S+E164D*+L173F*	0.6	0.1
V173L+L180M+A181V+M204V	P120S+E164D*+L173F*+I195M*	2.5	1.1
V173L+L180M+A181V+M204V+N236T	P120S+E164D*+L173F*+I195M*	28	2.4
V173L+L180M+A181V+N236T	P120S+E164D*+L173F*	29	2.6

HBsAg was analysed from a 1/50 fold dilution of supernatant harvested 5 days post-transfection of HuH-7 cells with the different mutants. The analyses were performed with two commercial kits, Monolisa Plus (BioRad, Hercules, CA) and Enzygnost (Dade Behring, Marburg, Germany). The first two columns correspond to mutations in RT and S genes respectively. Values below 1.0 are considered as negative samples.

\*: S gene mutations induced by changes in the RT sequence.