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Anti-HBV treatment induces novel reverse transcriptase mutations with reflective effect on HBV S antigen

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Summary   *Introduction:* The identification of novel reverse-transcriptase (RT) drug-resistance mutations is critical in predicting the probability of success to anti-HBV treatment. Furthermore, due to HBV-RT/HBsAg gene-overlap, they can have an impact on HBsAg-detection and quantification.

Methods: 356 full-length HBV-RT sequences from 197 drug-naive patients and 159 patients experiencing virological-breakthrough to nucleoside/nucleotide-analogs (NUCs) were analyzed. Mutants and wild-type HBs-antigens were expressed in HuH7-hepatocytes and quantified in cell-supernatants and cell-lysates by Architect HBsAg-assay.

Results: Ten novel RT-mutations (rtN53T-rtS78T-rtS85F-rtS135T-rtA181I-rtA200V-rtK212Q-rtL229V/F-rtM309K) correlated with specific NUC-treatments and classical drug-resistance mutations on divergent evolutionary pathways. Some of them reduced RT-binding affinity for anti-HBV drugs and altered S-antigen structure. Indeed, rtS78T (prevalence: 1.1% in drug-naïve and 12.2% in adefovir-failing patients) decreased the RT-affinity for adefovir more than the classical adefovir-resistance mutations rtA181 T/V (WT:−9.63 kcal/mol, rtA181T:−9.30 kcal/mol, rtA181V:−7.96 kcal/mol, rtS78T:−7.37 kcal/mol). Moreover, rtS78T introduced a stop-codon at HBsAg-position 69, and completely abrogated HBsAg-quantification in both supernatants and cell-lysates, indicating an impaired HBsAg-secretion/production. Furthermore, the HBsAg-mutation sP217L, silent in RT, significantly correlated with M204V/I-related virological-breakthrough and increased HBsAg-quantification in cell-lysate.

Conclusions: Mutations beyond those classically known can affect drug-binding affinity of mutated HBV-RT, and may have potential effects on HBsAg. Their cumulative effect on resistance and HBV-pathogenicity indicates the importance of preventing therapeutic failures.

Introduction

Although a growing arsenal of nucleos(t)ide-analogues (NUCs) has promoted HBV treatment, prolonged mono-therapies directed against a single target may result in the emergence of viral resistance.[1−3] So far, the following HBV resistant mutants have been identified: rtV173L, rtL180M, rtM204V/I, and rtA181 V/T for lamivudine (LMV); rtN236T, and rtA181 V/T for adefovir (ADF); rtI169T, rtT184G, rtS202G/I, and rtM250V for entecavir (ETV); rtA181 T/V and rtN236T are also associated with a decreased susceptibility to tenofovir (TDF) *in-vitro*, and reduced response *in-vivo*. The main LMV resistant mutants are cross-resistant with telbivudine, and partially to ETV. Finally, the A194T has been associated with TDF-resistance *in vitro*, but to date no conclusive data on its role *in vivo* are available.[2−4]

Beyond these classical mutations, there is an increasing evidence that additional mutations play a role in mechanisms underlying HBV drug-resistance, and therefore may affect a successful anti-HBV therapy.[5−7] These additional mutations can either restore impaired viral-fitness after the development of primary resistance mutations, or they can confer drug-resistance themselves, especially in combination with primary drug-resistance mutations. In some cases, they also may be responsible for therapeutic failure in patients without evidence of major drug-resistance mutations.

A proper definition of drug-resistance profiles critically contributes to effective anti-HBV treatments, particularly in patients failing long-term antiviral-treatment and/or experiencing sequential anti-HBV therapies and multiple virological failures.

Furthermore, anti-HBV drug-resistance mutations outline clinically complex scenarios, due to the genetic overlap of RT and HBV Surface Antigen (HBsAg) coding regions. Some RT drug-resistance mutations alter the sequence of the "a-determinant" domain and, hence, reduce the binding affinity for neutralizing antibodies (including vaccine-induced ones).[8−12] Other drug-resistance mutations, such as those at RT-positions 181 and 204, can introduce stop-codons in the HBsAg reading-frame, leading to the production and intracellular retention of truncated HBsAg forms.[13] This may in turn induce oxidative stress and transactivation of oncogene promoters, favoring the neoplastic transformation of the hepatocytes and liver cancer.[14,15]

Thus, the issue of HBV drug-resistance should also be considered in the context of overlapping reading-frames to determine in which patients the continuous replication of resistant variants can have the higher impact on HBV pathogenicity and progression of liver disease.

In this light, using extensive sequence analyses, computational and structural methods, and phenotypic characterization, this study is aimed at investigating: i) the correlation of novel mutations with anti-HBV treatment in a large population of chronically HBV-infected patients, failing long-term anti-HBV treatment, ii) their impact on RT-affinity for the drugs; iii) and their impact on HBsAg quantification in the cell-supernatants and cell-lysates of cell culture experiments.

Materials and methods

Patients

This study included 356 chronically HBV-infected patients, enrolled in different clinical centers in Italy ($N = 266$), France ($N = 66$) and Germany ($N = 24$). Of them, 197 were naïve for treatment with antiviral-drugs, while 159 were failing their last antiretroviral regimen containing

LMV (N = 106), ADF (N = 35) or ETV (after LMV-failures, N = 18). Among the 35 patients included in the "ADF-treated" group, 22 were under ADF-monotherapy (after LMV-failure), while 13 received ADF + LMV combination therapy.

Virological-breakthrough was defined by a rebound of serum HBV-DNA of >1 log IU/ml from the nadir value confirmed by 2 consecutive determinations. Only patients infected with HBV-genotype A (N = 104) or D (N = 252) were included.

HBV-sequencing

Sequencing of HBV-RT (344 amino acids) and HBsAg (226 amino acids) was performed on plasma-samples as previously described[16–18] (Supplementary Text SI).

Computational analysis

Mutation prevalence
The frequency of each RT-mutation was determined in the following groups of patients: (i) drug-naïve patients versus LMV-treated patients, (ii) drug-naïve patients versus ADF-treated patients, and (iii) drug-naïve patients versus ETV-treated patients. We performed chi-squared tests of independence (based on a 2 × 2 contingency-table) to verify statistically significant differences in frequency between the two groups of patients. We used the Benjamini–Hochberg method to correct for multiple-hypothesis testing at a false discovery rate of 0.05. Frequencies were calculated considering the number of mutated sequences on the total of sequences covering the amino acid position considered.

To assess the association of HBsAg-mutations with anti-HBV treatment, we calculated their frequencies in isolates from 197 drug-naïve patients and 159 drug-treated patients.

Mutation covariation
In the set of 159 anti-HBV treated patients, we exhaustively analyzed patterns of pairwise interactions among mutations associated with treatment. Specifically, for each pair of mutations and corresponding wild-type residues, Fisher's exact test was performed to assess whether co-occurrence of amino acid replacements differed significantly from what would be expected under assumption of independence. Then, to analyze the covariation structure of mutations in more detail, we performed average linkage hierarchical agglomerative clustering, as described previously.[18]

Mutagenetic trees
The evolutionary pathways of resistance development under selective LMV or ADF selective pressure were reconstructed performing a mutagenetic tree modeling. This approach employs mixture probabilistic models for describing evolutionary processes, characterized by the accumulation of genetic changes.[19] In particular, a single mutagenetic tree is a weighted directed tree in which the genetic events are represented by nodes and weights on the edges correspond to the conditional probability of the child event happening given that the parent event has occurred. This tree structure provides a probabilistic model that annotates evolutionary paths of disease progression.

Dependencies between events from the joint probabilities between all pairs of events were reconstructed by using the Mtreemix software.

Modeling of proteins, ligand-docking and affinity analyses
A three-dimensional model of HBV RT was constructed by i-TASSER,[20] based on homology with the HIV-RT (PDB entry 1jle chainA). The impact of mutations on RT binding-affinity for the drugs was assessed by docking-analysis using Patch-Dock,[21] PEARLS[22] and Chimera V1.6[23] software. Details are reported in Supplementary Material (SII).

Site-specific mutagenesis and HBsAg detection assay
The methodology used for the generation of mutants and for HBsAg-quantification was previously described[17] and reported in Supplementary Material (SIII). Results were obtained in three independent experiments.

Results

Novel RT-mutations and their association with treatment

The study included 197 drug-naïve patients and 159 patients experiencing virological-failure to anti-HBV therapy after a median (interquartile range, IQR) time of 2.4 (1.3–4.5) years and with a median log serum HBV DNA of 4.1 (3.0–5.7) log IU/ml (Table 1). Stratifying the 159 drug-treated patients according to the type of antiviral regimen, we identified (throughout RT-sequence) 10 novel mutations significantly correlated with failure of anti-HBV treatment (Fig. 1, panel A). By logistic regression analysis, the presence of at least one of these newly identified RT-mutations was per se predictive for failure to NUCs-treatment (Odds-ratio, OR [95% confidence interval, CI]: 8.481[3.847–18.695]; $P < 0.001$). Even after adjustment for the presence of major resistance-mutations, their presence remained significantly associated with failure (OR [95% CI]: 5.364[1.937–14.848]; $P = 0.001$).

In particular, 5 novel mutations (rtS85F, rtS135T, rtA200V, rtL229 F/V) at 4 RT positions were significantly correlated with LMV-treatment. Among them, rtS85F, rtA200V, rtL229F were completely absent (0% prevalence) in drug-naïve patients. Their prevalence increased under LMV-treatment up to 3.9% prevalence for rtS85F ($P = 0.017$) and 2.9% for rtA200V and rtL229F ($P = 0.037$). The rtS135T mutation was present in 0.5% of drug-naïve patients and in 3.8% of LMV-treated ($P = 0.050$), while rtL229V was detected in 1.6% of drug-naïve and in 7.7% of LMV-treated patients ($P = 0.019$).

Four novel RT-mutations (rtN53T, rtS78T, rtA181I, rtK212Q) were significantly correlated with ADF-containing regimens. rtA181I is a novel mutation at RT position 181 known to be involved in resistance to LMV, ADF and partially to TDF. rtN53T, rtA181I and rtK212Q were absent (0%) in drug-naïve patients and reached a prevalence of 7.7% ($P = 0.041$), 5.7% ($P = 0.014$) and 5.7% ($P = 0.014$) in ADF-treated patients, respectively. The rtS78T was detected in 1.1% of drug-naïve patients and its prevalence raised up to 11.8% in ADF-treated patients ($P = 0.007$).

The prevalence of patients failing LMV- or ADV-containing regimens with major drug-resistance was of 81.1% and 74.3%,

Table 1 Demographic and virological characteristics of the study population at the time of virological breakthrough.

Characteristic	Drug-naive patients, $N = 197$	Drug-treated patients, $N = 159$
Male, N(%)	150 (76.1)	120 (75.5)
Italian nationality, N(%)	91 (46.2)	140 (88.1)
Age (years), Median (IQR)	45.0 (35.0–58.0)	49.5 (41.0–62.0)
Plasma HBV-DNA (logIU/ml), Median (IQR)	4.6 (3.2–6.5)	4.1 (3.0–5.7)
ALT (IU/ml), Median (IQR)	40 (27–77)	44 (30–77)
AST (IU/ml), Median (IQR)	36 (26–54)	32 (23–50)
Viral co-infections, N(%)		
Hepatitis C virus	24 (12.2)	5 (3.1)
Hepatitis D virus	4 (2.0)	0 (0.0)
Human immunodeficiency virus	72 (36.5)	31 (19.5)
HBV-genotype, N(%)		
A	73 (37.1)	31 (19.5)
D	124 (62.9)	128 (80.5)
Type of anti-HBV treatment, N(%)		
Adefovir therapy[a]	—	35 (22.0)
Entecavir mono-therapy	—	18 (11.3)
Lamivudine mono-therapy	—	106 (66.7)
Time to virological breakthrough (years), Median (IQR)[b]	—	2.4 (1.3–4.5)

IQR, interquartile range.
[a] Among the 35 patients included in the "ADF-treated" group, 22 were under ADF monotherapy (all after LMV-failure), while 13 received ADF + LMV combination therapy.
[b] Virological-breakthrough was defined by a rebound of serum HBV-DNA of >1 log IU/ml from the nadir value confirmed by 2 consecutive determinations.

respectively, while the prevalence of patients failing exclusively with novel mutations was of 0% and 8.6% (due to rtS78T), respectively. In addition, 12/18 (66.7%) of ETV-treated patients after lamivudine failures, were failing without evidences of any major drug-resistance mutation. Four of the six remaining ETV-failing patients without major drug-resistance mutations presented the novel rtM309K mutation, that significantly increased in prevalence under ETV-treatment. The rtM309K mutation was indeed detected in 2.8% of drug-naïve patients and in 16.7% ETV-failing patients ($p = 0.039$). All associations were confirmed when HIV co-infected patients were excluded from the analysis (data not shown). Furthermore, the analysis of an independent dataset from Stanford HBV Database (http://hivdb.stanford.edu/HBV/DB/cgi-bin/MutPrevByGenotypeRxHBV.cgi) involving 3764 HBV chronically-infected patients (3216 drug-naïve and 548 drug-treated) supported the correlation with virological failure for rtS78T (0.4% in drug-naïve versus 2.4% in drug-treated, $p < 0.001$), rtA200V (0.0% versus 1.6%, $p = 0.001$), rtL229 F/V (2.0% versus 6.4%, $p < 0.001$), rtM309K (4.4% versus 13.5%, $P < 0.001$), and for position 85 (0.4% versus 2.0%, $p < 0.001$).

As a final step, we investigated the correlation of HBsAg-mutations with virological failure to anti-HBV treatment. In this analysis, we identified two HBsAg-mutations (silent in the RT-frame) significantly correlated with anti-HBV treatment (Fig. 1, panel B).

The sS154L mutation, localized in the post "a"-determinant of HBsAg protein, was absent in drug-naïve patients and present in 2.5% of drug-failure cases ($p = 0.039$). This mutation corresponds to residues rtI162/rtI163 in RT-protein.

The sP217L, located in the C-terminal HBsAg-domain, had 0.5% prevalence in drug-naïve patients and 3.8% prevalence in therapy-failing patients ($p = 0.049$). This mutation corresponds to residues rtT225/rtN226 in RT-protein.

By logistic regression analysis, the presence of at least one of these newly identified synonymous HBsAg-mutations was significantly correlated with failure to antiviral treatment (OR[95%CI]: 9.841[2.878–33.642]; $p < 0.001$).

Co-occurrence of novel mutations with the classical HBV drug-resistance mutations

The above mentioned results prompted us to investigate whether the novel mutations participate in the same evolutionary pathways of the classical drug-resistance mutations.

In this analysis, the major LMV-resistance mutation rtM204I significantly correlated with either the novel mutation rtS85F (phi = 0.22) and the classical rtL80I/V (phi = 0.41) (Fig. 2, panel A). The association of rtS85F with rtM204I was specific: indeed, all patients harboring rtS85F also displayed rtM204I. Of note, rtL80I/V and rtS85F never occurred together, suggesting the existence of 2 distinct evolutionary and compensatory pathways involving rtM204I. This hypothesis was supported by mutagenetic-tree analysis, which highlighted an ordered accumulation of LMV-resistance mutations along two pathways rtM204I + rtL80I/V and rtM204I + rtS85F (Fig.S1, panel A). Of note, this accumulation was associated with a longer time to virological-failure, which was statistically significant for rtS85F ($p = 0.023$), and with a progressively
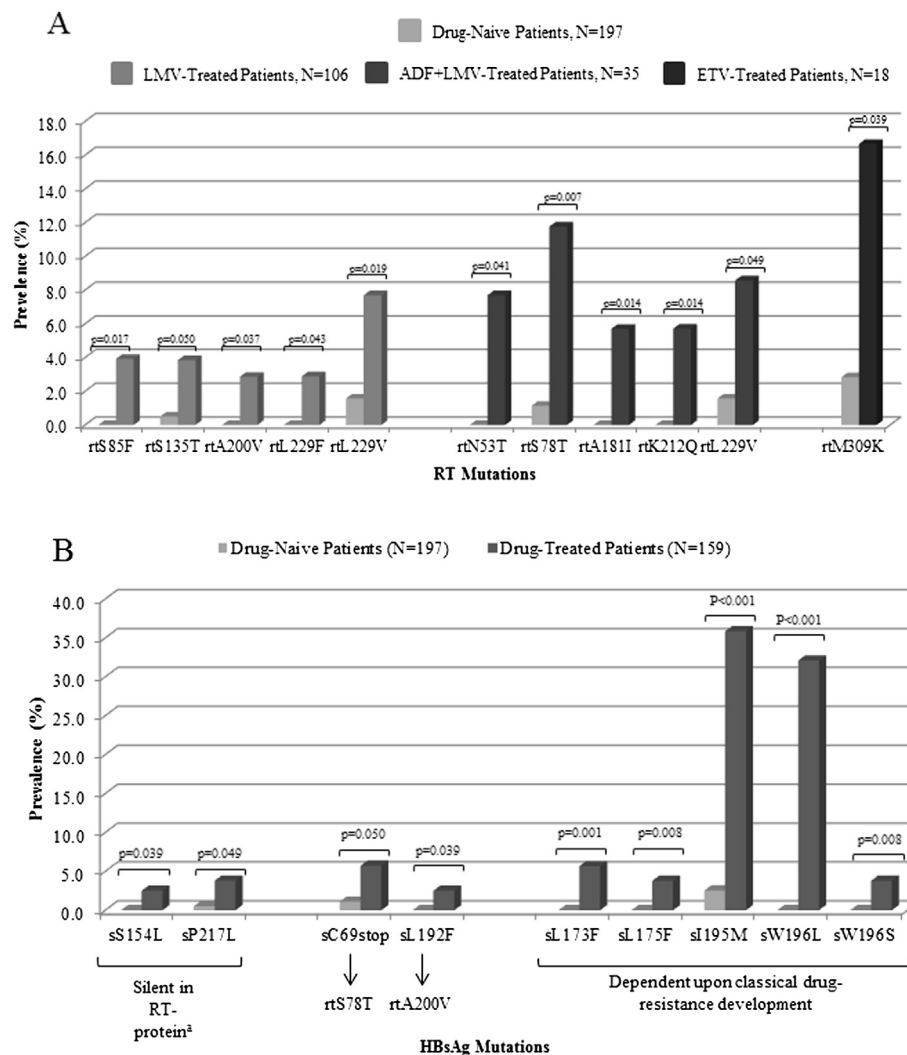
Figure 1 *Prevalence of amino acidic RT-protein (A) and HBsAg protein (B) mutations associated with virological failure to NUC-treatment*. P-values were determined by Fisher exact test applying a Benjamini-Hochberg correction for multiple comparisons at a false positive rate of 0.05. [a]The HBsAg mutations sS154L and sP217L involved residues rtI162-rtI163 and rtT225-rtN226 in RT protein, respectively.

increased viremia, indicating that prolonged LMV-failure boosts HBV genetic complexity, eventually increasing the level of cross-resistance or viral fitness.

At the novel resistance-associated position rt229, the F was found exclusively with the pattern of resistance dependent upon the rtM204V mutation (Fig. 2, panel B), while the V occurred with either rtM204I (5/10) or rtM204V (5/10). Again, the mutagenetic-tree suggested an accumulation of rtL229F variants following rtL180M + rtM204V development (Fig.S1, panel B).

Among ADF-related novel mutation, rtS78T occurred either without any major drug-resistance mutations in 3/7 patients failing ADF-treatment, or in combination with the classical ADF-resistance mutations rtA181 T/V (Fig. 2, panel C). Also in this case, the mutagenetic-tree confirmed this association pattern (Fig.S1, panel C), highlighting that rtS78T can be developed following rtA181 T/V, irrespective of rtN236T mutation presence.

Lastly, the novel mutation rtM309K was found alone without any other classical drug-resistance mutations in

4/10 ETV-treated patients, while it was detected with rtM204V or rtM204I in 2 and 4 patients, respectively.

Among the 10 patients with HBsAg-mutations sS154L and sP217L (silent in RT), 7 showed rtM204I, 2 rtM204V and 1 patient treated with ADF + LMV also had the rtA181T, indicating that the prolonged failure to anti-HBV can induce additional mutations in the overlapping S-ORF even without directly affecting RT-structure.

Structural analysis of novel RT-mutations

Localization of novel mutations in the 3D-structure of the RT-enzyme

The location and spatial orientation of the novel mutations associated with virological-failure to anti-HBV drugs is reported in Fig. 3 (panel A). In this RT-model, residue 85 (associated with LMV-treatment) was found in the highly conserved A domain, within the hydrophobic region of the RT-palm involved in nucleotide-binding. This residue is
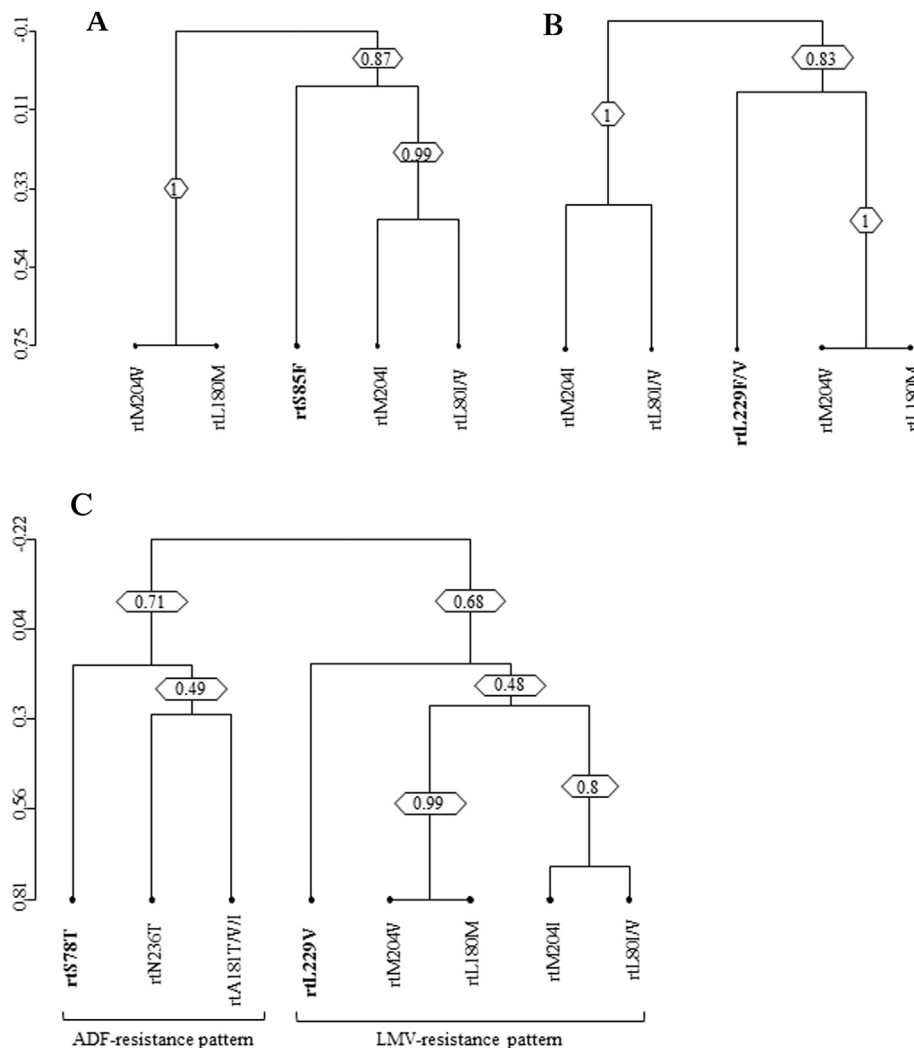
Figure 2 *Hierarchical clusters analysis of novel RT-mutations identified*. The dendograms report the hierarchical clusters analysis performed including the novel lamivudine-mutations rtS85F (A) and rtL229 F/V (B) in combination with classical lamivudine resistance mutations, or the novel adefovir-mutations rtS78T and rtL229V together with classical adefovir and lamivudine resistance mutations (C). Bootstrap values are reported into boxes.

also in close proximity to the YMDD motif (containing the classical drug-resistance position rt204, and 2 catalytically essential aspartic acid residues at positions rtD205 and rtD206) and to the catalytically essential aspartic acid residue at position 83 (rtD83) (Fig. 3, panel B). Indeed, the calculated distance of rtS85 with rtD83 and rtD205 was of only 6.38 Å and 5.87 Å, respectively, and rtS85-rtM204 distance was of 8.912 Å.

These distances were shorter than those observed for the classical drug-resistance associated position rtL80 (9.05 Å for rtL80-rtD83, 13.69 Å for rtL80-rtD205, and 13.485 Å for rtL80-rtM204). Finally, residue rt85 cooperates with residue rt204 in the formation of LMV-RT complexes by VanDerWaals interaction.

Similar to rtS85, the residue rt78 also localizes within RT-palm domain, in a hydrophobic region that also contains the rtK212, another residue associated with ADF-treatment (Fig. 3, panel B). Both residues rt85 and rt78 were within 10.1—7.45 Å of the methyl group of an essential threonine residue (rtT240) within motif D. Among all the newly-

identified mutations only rtM309K, associated with ETV-treatment, was located in the thumb domain of the RT-enzyme (Fig. 3, panel C).

Impact on novel mutations on binding-affinity with the drugs

Ligand docking analysis showed that the introduction of rtS78T in RT three-dimensional model caused a decrease in RT binding-affinity for ADF from −9.63 kcal/mol for wild-type RT, to −7.37 kcal/mol (Fig. 4). This decrease was stronger than that observed for the classical ADF-resistance mutation rtA181T or rtA181V (−9.30 kcal/mol and −7.96 kcal/mol, respectively). Combination of mutations further exacerbated ADF affinity estimates (rtS78T + rtA181T:−4.33 kcal/mol or rtS78T + rtA181V:−5.43 kcal/mol, Fig. 4). Likewise, a decrease was observed in the LMV binding-affinity of RT carrying both the novel mutation rtL229V and the classical rtM204V (−5.96 kcal/mol for rtM204V + rtL229V *vs.* −6.50 kcal/mol for wild-type RT). Docking-affinity analysis indicated a considerable reduction of ADF-RT stability
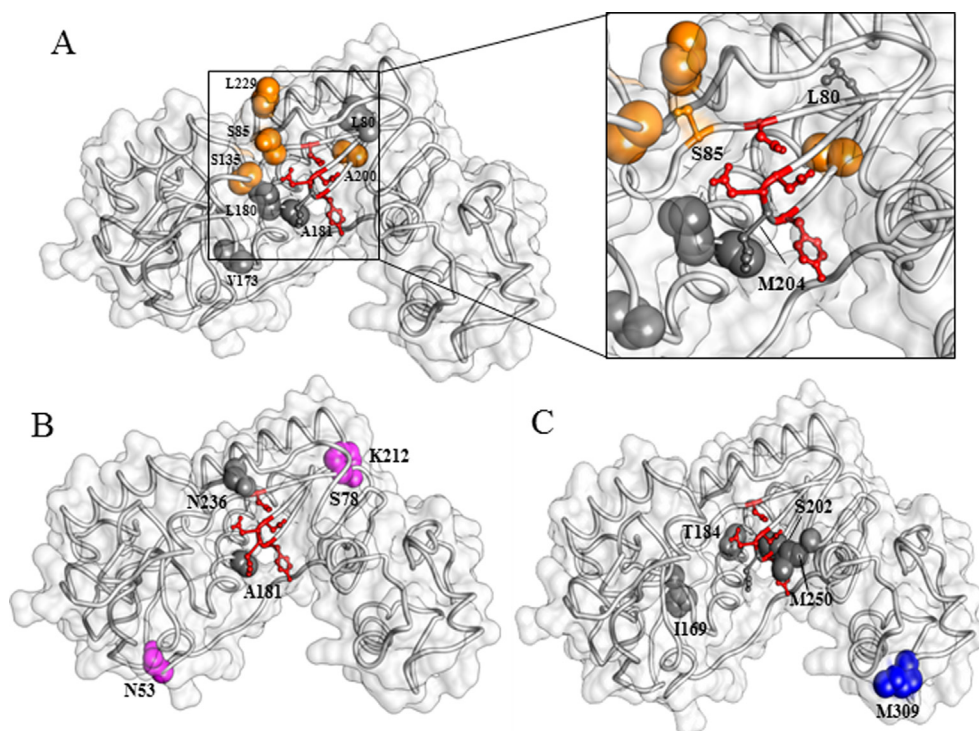
Figure 3 *Localization of novel RT-mutations associated with lamivudine (A), adefovir (B) and entecavir (C) in 3D models of RT.* The YMDD motif and the catalytic D83 are highlighted in red. RT positions associated with classical resistance development to each drug are reported in space-filled format and colored in grey. RT positions 80, 200, 212, 229 are included in the palm domain, positions 53 and 135 in the finger-domain, while position 309 is included within thumb domain.

from -9.63 kcal/mol (wild-type RT) to -2.46 kcal/mol (rtM204V + rtL229V), thus explaining the association of this pair of mutations with failure to LMV + ADF-treatment.

Localization of novel RT-mutations on HBsAg structure

Due to RT/HBsAg overlap, changes in RT can cause mutations in HBsAg. In particular, rtA200V and rtL229V corresponded to the HBsAg-mutations sL192F and sF220L. Residue s192 resides in HBsAg-region involved in capsid-interaction,[24] while residue s220 localizes in the C-terminal membrane-embedded HBsAg-domain, important for ZHBsAg-secretion.[25] Notably, similar to rtA181T, rtS78T always introduces a stop-codon at the HBsAg-position 69, a position critical for HBsAg-secretion from infected hepatocytes.[16]

Phenotypic analysis of novel HBsAg-mutations associated with anti-HBV treatment

To investigate in more detail the role of novel HBsAg-mutations associated with anti-HBV treatment, their ability to hamper HBsAg production and detection was investigated in cell-culture using the ABBOTT Architect HBsAg (Quant) assay (Fig. 5). Architect antibodies target the second loop of HBsAg (aa 137–151).

Notably, the introduction of the stop-codon at HBsAg-position 69 abrogated full-length wt HBsAg quantification in both cell-supernatants (mean[SD] HBsAg-quantification = 1.1[±0.5]IU/ml vs. 44.8 [±13.4]IU/ml for wild-type HBsAg, $p < 0.001$) and in cell-lysates (mean [SD] HBsAg in cell-

lysates = 0.0[±0.0]IU/ml vs. 25.8[±2.2]IU/ml for wild-type HBsAg, $p < 0.001$) (Fig. 5).

An increase of HBsAg-quantification in cell-lysates was observed (though not statistically significant), for sP217L (74.9 [±12.7]IU/ml *vs.* 44.8 [±13.4]IU/ml for wild-type HBsAg), suggesting a potential impact on HBsAg-secretion. Conversely, no modifications were detected for sL192F, sF220L and sS154L (Fig. 5).

Discussion

Our study indicates that additional HBV RT-mutations, beyond those currently known as classical resistance mutations, can contribute to the development of drug-resistance *in vivo*. In fact, 10 novel mutations have been identified on basis of their association with currently used anti-HBV therapies. Some of them, similar to classical resistance-mutations, showed a direct impact also on HBsAg-protein, inducing the development of non-synonymous mutations or, in case of rtS78T, stop-codons that might potentially affect HBV pathogenicity and oncogenic potential.

Although we cannot totally exclude the presence of these novel mutations before the beginning of treatment due to the unavailability of pre-treatment samples, their limited presence/absence in drug-naïve patients supports that they are mostly associated with treatment-failure.

Some of these mutations, such as rtS85F, may emerge after the classical major drug-resistance mutations acting as compensatory mutation. Similar to the classical rtL80I/V, rtS85F localized in proximity to residue rtM204 and the 3
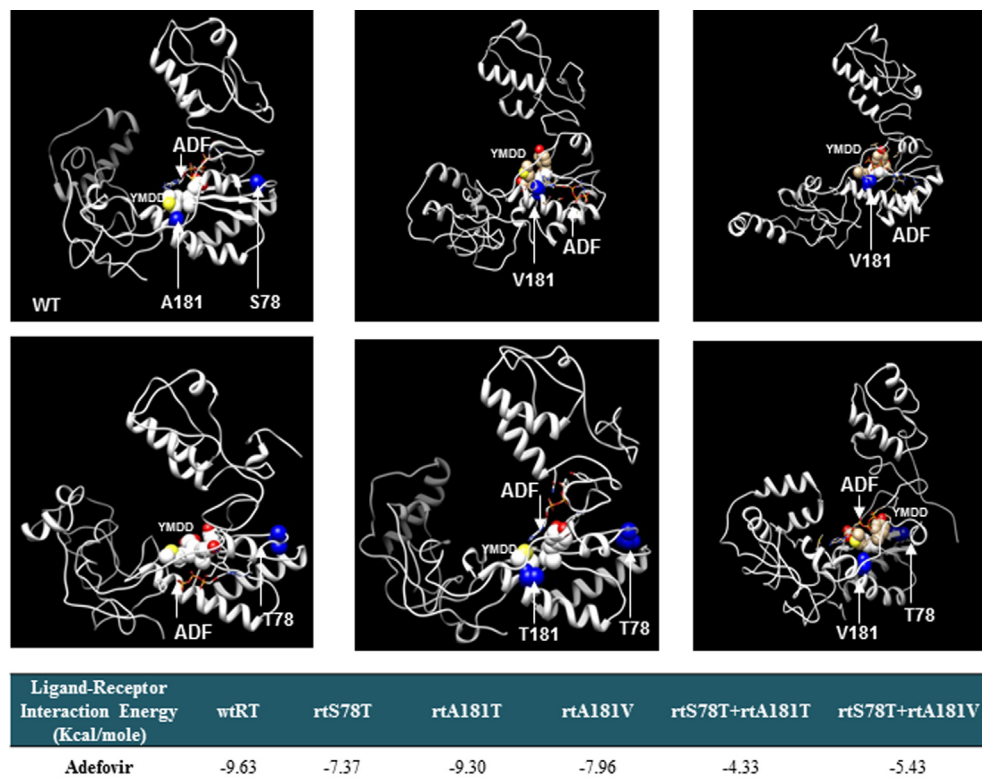
Ligand-Receptor Interaction Energy (Kcal/mole)	wtRT	rtS78T	rtA181T	rtA181V	rtS78T+rtA181T	rtS78T+rtA181V
Adefovir	-9.63	-7.37	-9.30	-7.96	-4.33	-5.43

Figure 4 *Modifications of adefovir-RT affinity following rtS78T and/or rtA181 T/V.* The ligand−receptor interaction energy (Kcal/mole) was calculated for adefovir and Mg-dATP binding to wild-type RT and the mutants rtS78T, rtA181T, rtA181V and rtS78T + rtA181 T/V. The structures of adefovir, lamivudine and Mg-dATP were taken from the RCSB-PDB database (entries 1pk0, 2noa and 3kk2, respectively). PatchDock was used for docking of ligands into the RT structure providing the YMDD sequence (or mutant variant) as the protein's binding site. The top 20 of the docking results were submitted to PEARLS for their protein-ligand energy interaction profile in order to select the complex with the lowest value for total energy interaction reflecting the highest binding affinity. The corresponding 3D-structures are displayed on top of the figure. Calculation of interatomic distances and display of structures were done via Chimera V1.6.

catalytically essential aspartic residues, and it always occurred with the well-known rtM204I. Both covariation and mutagenetic-tree analyses highlighted a negative correlation between the classical rtL80I/V mutations and the novel rtS85F. The rtS85F was recently found in LAM-monotherapy failing patient,[26] but to our knowledge, this is the first study supporting the existence of two distinct compensatory and evolutionary pathways underlying rtM204I-related drug-resistance. This is in line with a recent study showing that rtM204I/V substitution, although essential, is insufficient for establishing resistance against LMV.[27] rtM204I is the main marker of telbivudine-resistance, thus supporting a role for rtS85F in resistance to this drug.

The rtL229 F/V mutations were detected in combination with the classical LMV-resistance mutations rtM204I/V. A previous study, led in patients infected with HBV genotype B and C from China, has shown that rtL229V/F may be involved in LMV-resistance.[7] Our study supports this finding also in HBV genotype D and A. In particular, we found that this mutation might decrease the RT binding affinity, but only when present in combination with the classical rtM204V mutation. In addition, ligand docking and affinity analyses highlighted an impact of rtL229V/

F + rtM204V even in ADF-resistance, and this could explain their persistence in patients failing LMV and ADF-treatment.

A different scenario was observed for rtS78T, occurring in almost 12% of ADF-failing patients, even in absence on any other classical ADF resistance-mutations (4/7 patients). The selection of rtS78T during ADF-therapy is also supported by an independent dataset from the Stanford HBV database, which reports a prevalence of 3.4% in patients receiving acyclic nucleoside phosphonates (ADF and TDF) (http://hivdb.stanford.edu/HBV/DB/cgibin/MutPrevByGenotype-RxHBV.cgi).

This novel mutation caused a decrease in the estimated RT binding affinity for ADF, comparable or even stronger to that observed for the classical ADF resistance-mutations. The decrease in RT binding affinity for ADF is further exacerbated when the novel rtS78T mutation is present along with the classical rtA181 T/V mutations. Although *in vitro* studies are also necessary, this result suggests that rtS78T may potentially contribute by itself to ADF-resistance or increase the level of resistance when present in combination with classical ADF resistance-mutations. Similar to rtA181 T/V and rtN236T, the rtS78T mutation could slow down virological response to TDF.
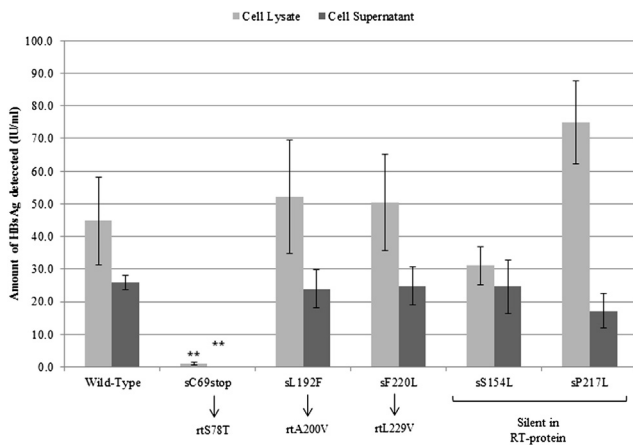
Figure 5 *Impact of novel HBsAg-mutations associated with anti-HBV treatment*. The graph reports the mean of 3 independent experiments of the HBsAg amount detected in presence of the with wild-type and mutated HBV clones. Wild type and HBsAg mutants (obtained by site-directed mutagenesis) were tested by transfection of human HuH7 cells with pCHsAg plasmids using FuGene6 (Roche diagnostics GmbH, Mannheim, Germany). The supernatant was collected after 72 h and tested using the ABBOTT Architect HBsAg (Quant) assay.

Specific experimental verification is warranted to provide insights on the impact of these mutations on drug-resistance and viral replication.

Although the expression system used in this study (containing the cytomegalovirus immediate early promoter-enhancer) may not reflect the real synthesis of HBsAg *in vivo*, the generation of a stop-codon at HBsAg position 69 caused by rtS78T in RT may have important clinical implications. Indeed, cysteine 69 is a strictly conserved residue among all *hepadnaviruses* and is essential for HBsAg secretion.[28,29] Furthermore, this stop-codon maps in the intracellular HBsAg-loop and within the "trans-activity-on region", in which 3′ deletions give rise to the generation of transactivating proteins.[30] *In vitro* analyses showed that this stop-codon completely abrogated full-length HBsAg-quantification in both cell-lysates and cell-supernatants, thus supporting a strong impairment in the production and secretion of functional, complete forms of HBsAg following sC69stop development. The fact that architect antibodies bind to a downstream region of HBsAg (137–151) further support the complete absence of S-antigen production.

The prematurely truncated form of S-antigen can accumulate into the hepatocyte and may induce transactivation of cellular promoters, including those encoding oncogenic proteins. This mechanism has already been described for sW172stop mutation, as a consequence of ADF-resistance mutation rtA181T,[14,31] thus confirming an important pathogenic implication of HBV drug-resistance, in this case specifically induced by ADF-treatment.

Furthermore, due to the peculiar characteristics of the HBV life cycle, the currently available anti-HBV drugs can only suppress viral-particle production, but not HBsAg production and secretion. In the presence of HBsAg stop-codons, truncated HBsAg continues to be produced and accumulated inside the hepatocytes, even in the setting of fully suppressive therapy,[14] and therefore the risk of liver cancer will persist even when HBV-viremia is below the limit of detection. Recently it has been reported that LMV-resistance is related to higher cumulative rates of liver cancer compared to drug-naïve patients.[31–33] Furthermore, the reduction of absolute risk of HCC development is modest in cirrhotic HBV patients under antiviral treatment (RR = 1.16; 95% CI = 0.51–2.62), and insignificant in patients without cirrhosis (RR = 0.82; 95% CI = 0.57–1.20), indicating the necessity of a close monitoring.[33,34]

Our study also highlighted a novel, putative resistance mutation to ETV-treatment. Recently, a novel pattern of mutations, observed in an ETV-failing patient (rtL80V, rtL91I, rtM204I, rtS219A, rtN238D, rtY245H), displayed 30.4 fold-resistance compared with the wild-type HBV by *in vitro* phenotyping assay.[5] In the present study, the co-presence of rtL80V, rtL91I and rtM204I mutations was observed in one ETV-failing patient (after LMV-failure). Notably, a substantial proportion of patients (11/18, 61.1%) was failing ETV-treatment without known drug-resistance mutations, underlining the need to assess the reasons, clinical or virological, of this non-response to treatment. Two of these eleven patients showed the novel rtM309K mutations. Its low-level presence also in drug-naïve patients requires a profound analysis of the role of this variant in viral sensitivity to ETV.

In conclusion, beside classical resistance-mutations, anti-HBV treatments correlates with the appearance of novel RT-mutations, that can affect the drug-binding affinity of the mutated RT-protein, and may have potential effects on HBsAg. The impact of these novel mutations in modulating ETV and TDF virological response deserves further investigation. The cumulative effect of these novel mutations on resistance, as well as their potential influence on HBV-pathogenicity, strongly support the importance of preventing therapeutic failures by using proper anti-HBV drugs and strategies.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2013.05.008.

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