

Discovery of the Novel Entecavir-Resistant Hepatitis B Virus Reverse Transcriptase A181C Substitution From an Integrated Genotypic Analysis

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Entecavir (ETV) is a first-line therapy for chronic hepatitis B virus (HBV), demonstrating potent suppression of HBV DNA and a high barrier to viral resistance. Previous studies revealed that ETV-resistant (ETV_r) HBV DNA resulted from substitutions in the HBV reverse transcriptase (RT) at positions rtT184, rtS202, or rtM250 in combination with lamivudine-resistance (LVD_r) substitutions rtM204I/V±rtL180M. *In vitro*, viral variants exhibit varying degrees of ETV susceptibility and replication capacity depending on specific resistance substitutions. To explore the potential for additional pathways to ETV_r, HBV RT sequences from 982 evaluable patients enrolled in 17 ETV clinical studies were analyzed. Thirty novel emergent substitutions at amino acid positions not previously associated with HBV nucleos(t)ide drug resistance were observed in at least 2 patients and were identified in patient-derived HBV with a wild-type, LVD_r, or ETV_r RT sequence. Phenotypic analysis of these substitutions indicated that they had no effect on ETV susceptibility. Phenotypic analysis was also performed on patient-derived HBV RT sequences from 10 LVD-naive and 13 LVD-experienced patients with virologic breakthrough and emergent novel substitutions while on ETV treatment. One LVD-experienced patient-derived HBV RT harboring LVD_r substitutions rtL180M+rtM204V with rtA181C displayed reduced ETV susceptibility (122-fold greater than wild-type HBV) and remained susceptible to adefovir and tenofovir. HBV harboring the rtA181C substitution without LVD_r substitutions rtL180M+rtM204V remained susceptible to inhibition by ETV, adefovir, and tenofovir, although cross-resistance to LVD and telbivudine was observed. *Conclusion*: An integrated genotypic analysis of HBV RT sequences from patients with chronic HBV treated with ETV led to the discovery of the novel ETV_r substitution rtA181C. This substitution was always detected in combination with LVD_r substitutions rtL180M+rtM204V in ETV-treated patients. (*Hepatology Communications* 2018;2:1123-1135)

There are several approved nucleos(t)ide analog therapies available for the treatment of patients with chronic hepatitis B virus (HBV) infection, including entecavir (ETV), lamivudine (LVD), adefovir dipivoxil (ADV), telbivudine (LdT), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). These nucleos(t)ide analogs are converted to nucleoside triphosphate mimics that are selectively recognized by and inhibit the HBV reverse transcriptase (RT), resulting in premature chain

Abbreviations: ADV, adefovir dipivoxil; EC50, median effective concentration; ETV, entecavir; HBV, hepatitis B virus; LdT, telbivudine; LVD, lamivudine; PCR, polymerase chain reaction; Pt, patient number; r, resistant/resistance; RT, reverse transcriptase; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; WT, wild-type.

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termination of viral replication. Successful therapy can result in the reduction of circulating HBV, halting of liver disease progression, promotion of seroconversion to viral antigens, and immune control of the virus⁽¹⁾ and facilitate the reversal of liver fibrosis⁽²⁾ and cirrhosis.⁽³⁾ A major consideration with prolonged antiviral therapy and incomplete viral suppression, however, is the emergence of drug-resistant viral variants that diminish the benefits of antiviral therapy. HBV treatment guidelines have recommended treatment with potent nucleos(t)ide analogs with a high barrier to resistance, including ETV.⁽⁴⁻⁷⁾

A robust cell culture system for the selection of HBV resistance to antiviral agents remains elusive; therefore, elucidation of resistance pathways to these antivirals requires analysis of isolates from patients failing therapy in clinical studies. Genotypic evaluation of patient-derived isolates is performed to identify novel emergent substitutions, while phenotypic evaluation of these substitutions by *in vitro* cell culture drug susceptibility testing confirms their role in drug resistance. Signature resistance substitutions have been identified for the approved nucleos(t)ide analogs LVD, ADV, LdT, and ETV.⁽⁸⁾ Signature substitutions associated with TDF or TAF resistance have not been identified in HBV-infected patients treated with TDF or TAF for up to 8 years⁽⁹⁾ or 2 years,⁽¹⁰⁾ respectively.

The molecular nature of ETV resistance (ETV_r) is complex and requires multiple substitutions in the HBV RT to result in high-level resistance and virologic breakthrough. ETV_r results from substitutions in the HBV RT at positions rtT184, rtS202, or rtM250 in combination with LVD-resistant (LVD_r) substitutions rtM204I/V±rtL180M.⁽¹¹⁾ Analyses of isolates from ETV-treated patients have revealed that ETV_r substitutions exhibit varying levels of ETV susceptibility depending on the specific resistance substitutions.⁽¹²⁾ ETV_r is rarely seen in nucleoside treatment-naïve patients (1.2% in a 5-year study) due to the requirement of three HBV RT substitutions for high-level resistance.⁽¹¹⁾ In patients with LVD_r HBV,

the emergence of ETV_r is more frequent (51% in a 5-year study) due to the presence of the required LVD_r substitutions.⁽¹¹⁾

To explore the potential for additional pathways to ETV_r, we analyzed HBV RT sequences in ETV-treated patients from additional studies conducted by Bristol-Myers Squibb in which HBV sequence information was available (total of 11 new studies). Results from these new analyses were integrated with those from previous studies. Here, we report a comprehensive meta-analysis of emerging genotypic changes that occurred during treatment with ETV (total of 17 studies).

Patients and Methods

STUDY SAMPLES

Patients from 17 ETV-based clinical studies were monitored for resistance; these studies are summarized in Supporting Table S1. All patients provided written informed consent, and study protocols conformed to the 1975 Declaration of Helsinki and were approved by the appropriate institutional review boards.

All patients receiving ETV for ≥12 weeks were monitored for resistance using HBV DNA quantification and nucleotide sequence analysis. HBV DNA was quantified using the Roche COBAS Amplicor polymerase chain reaction (PCR) system (version 2; lower limit of quantification 300 copies/mL). In earlier phase 2 and 3 studies, HBV DNA was measured in copies/mL, and we therefore converted this to IU/mL, using the conversion factor 1/5.82.⁽¹³⁾ Isolates were sequenced at baseline (i.e., on or before the start of ETV treatment) and on ETV treatment from patients with PCR-detectable HBV DNA (≥50 IU/mL) at the end of each yearly interval or at the end of ETV dosing. These patients included those experiencing a virologic breakthrough (defined as ≥1 log₁₀ increase in HBV DNA from the on-treatment nadir, confirmed by two sequential measurements or unconfirmed for

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the last on-treatment assessment at the end of dosing). When multiple samples were available at the end of the yearly interval, the sample closest to the end of each year was used (i.e., weeks 48, 96, 144, 192, and 240).

For the earlier phase 2 and 3 studies, annual visits for years 1 through 5 occurred at 48-week intervals as described.⁽¹¹⁾ For the later studies, including post-ETV approval studies, the windows for years 1 through 3 were defined as follows: year 1 (42- to \leq 54-week intervals), year 2 (90 to \leq 102), and year 3 (138 to \leq 150). For patients monitored through to year 8, the windows for years 6 through 8 were defined as follows: year 6 (276- to \leq 300-week intervals), year 7 (324 to \leq 348), and year 8 (372 to \leq 396).

HBV POLYMERASE SEQUENCING

HBV DNA genotyping of patient serum samples to identify novel emergent substitutions was performed as described.^(14,15)

HBV EXPRESSION CONSTRUCTS

Patient HBV RT domains were introduced into the laboratory wild-type (WT) plasmid (p) cytomegalovirus-HBV expression vector p180B3 as described,^(14,15) and clones were selected for phenotypic analysis. The laboratory WT aligned with HBV strains representing genotype D. Site-directed mutagenesis, using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), was employed to introduce substitutions into p180B3 or patient-derived RT domains, and the sequences of the constructs were confirmed by DNA sequencing as described.⁽¹⁴⁾

HBV CELL CULTURE SUSCEPTIBILITY

Cell culture susceptibility assays were performed by transfection of HepG2 hepatoma cells with HBV expression plasmids in the presence of serially diluted antiviral agents, as described.⁽¹⁴⁾ In general, ETV was titrated using nine 4-fold dilutions from a starting concentration of 15 μ M. ADV was titrated using 2-fold dilutions and TDF was titrated using 3-fold dilutions from a starting concentration of 50 μ M, while LVD and LdT were each titrated using 2-fold dilutions from a starting concentration of 100 μ M.

Following 5 days of culture, replicated, released, immunocaptured HBV DNA was quantified using

real-time quantitative PCR as described^(14,16) with the following modifications: HBV DNA was extracted from immunocaptured nucleocapsids by the addition of 100 μ L per well of QuickExtract DNA Extraction Solution (Epicentre Technologies Corp. Chicago, IL) and incubated at 65°C for 1 hour, followed by a 3-minute incubation at 95°C. TaqMan Fast Advanced Master Mix (Life Technologies, Carlsbad, CA) was used to quantify 5 μ L of extracted DNA, with 0.9 μ M forward primer 5'-CTCTGC ACG TCG CAT GGA-3', 0.9 μ M reverse primer 5'-AAG ACC TTG GGC AAC ATT CG-3', and 0.25 μ M probe 5'-FAM-CAC CGT GAA CGC CCA-MGBNFQ-3' in a final volume of 25 μ L. The PCR reaction was incubated at 50°C for 2 minutes, then 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Copies of DNA versus antiviral agent concentration were used to determine the half maximal effective concentration (EC₅₀) values for ETV, ADV, TDV, LVD, and LdT and were calculated using XLfit version 2.0 (ID Business Solutions Ltd., Boston, MA) and a four-parameter logistic equation (Model 205).

Results

EVALUABLE PATIENTS

Previous resistance surveillance analyses of six phase 2 and 3 studies in adult patients established the resistance profile for ETV in adults through 5 years of treatment.⁽¹¹⁾ These analyses indicated that ETVr results from HBV RT substitutions at positions rtT184, rtS202, or rtM250 in combination with LVDr substitutions rtM204I/V \pm rtL180M. To confirm and extend these findings and explore the potential for novel pathways to ETVr, an integrated genotypic analysis was performed using available HBV RT sequences from ETV-treated patients from 11 additional Bristol-Myers Squibb-conducted clinical studies. The results from these new analyses were integrated with those from six previously reported studies in a comprehensive meta-analysis of emerging genotypic changes that occurred during treatment with ETV (Supporting Table S1).

A total of 3,357 patients received the recommended daily dose (0.5 or 1 mg) of ETV in 17 clinical studies; 1,532 of these patients met genotypic resistance testing criteria, and 982 of these had evaluable ETV on-treatment isolates (Supporting Table S2). Baseline characteristics for these 982 evaluable patients are summarized in Supporting Table S3. Over half (53%)

of the evaluable HBV RT sequences were derived from Asian patients, while the most frequently (42%) observed HBV genotype was C. In total, 550 nonevaluable patients were excluded from these analyses due to not having either on-treatment isolates available for testing or resultant sequencing data. Thirty-two (16 LVD-naive patients and 16 LVD-experienced patients) of these nonevaluable patients experienced virologic breakthrough; 16 (7 LVD-naive patients and 9 LVD-experienced patients) were included in studies performed in China.

RESISTANCE SURVEILLANCE IN ETV-TREATED, LVD-NAIVE, AND LVD-EXPERIENCED PATIENTS

HBV RT sequencing results confirmed previous findings that ETV_r substitutions at rtT184, rtS202, or rtM250 emerged most frequently in patients treated with ETV and more specifically in LVD-experienced rather than LVD-naive patients. Overall, 5% (21/382) of LVD-naive and 39% (235/599) of LVD-experienced patients had sequenced isolates harboring ETV_r substitutions. ETV_r substitutions were not observed at baseline in LVD-naive patients but were observed at baseline in 39 LVD-experienced patients. The ETV_r substitution rtT184 with LVD_r emerged more frequently in patients harboring HBV genotypes A or C, whereas the ETV_r substitution rtS202 with LVD_r emerged more frequently in patients harboring HBV genotypes B, D, or F (Supporting Fig. S1). One patient infected with HBV genotype B experienced virologic breakthrough with detection of a novel substitution rtS202V in combination with rtT184G and LVD_r substitutions rtL180M+rtM204V. Although not previously reported in a patient, the phenotype of this substitution has been described.⁽¹²⁾

In addition to well-characterized HBV RT amino acid positions associated with nucleos(t)ide resistance,⁽⁸⁻¹⁰⁾ 30 novel emergent substitutions at 24 amino acid positions were identified in ETV-treated patients that were each observed in at least 2 patients and were not previously analyzed.^(15,17) The 30 novel emergent substitutions and the background selected for phenotypic analysis are summarized in Supporting Table S4. In addition, novel emergent HBV RT substitutions were identified in 10 LVD-naive and 12 LVD-experienced patients with virologic breakthrough. There was also 1 LVD-experienced patient with

virologic breakthrough with an ETV_r substitution and a novel substitution at rtI169, an amino acid position previously associated with ETV_r.⁽¹⁴⁾

PHENOTYPIC ANALYSIS OF NOVEL EMERGENT SUBSTITUTIONS IN AN HBV RT WT BACKGROUND

Substitutions conferring ETV_r in an HBV RT background without LVD_r substitutions have not been observed.⁽¹⁷⁾ In our analysis, there were nine novel emergent HBV RT substitutions at eight amino acid positions identified in a WT background for phenotypic analysis. Three of the substitutions, rtR242S, rtL247F, and rtR289S, were not detected by clonal analysis and were therefore not phenotyped whereas the remaining six substitutions were phenotyped (Table 1). Phenotypic analysis of recombinant clones harboring these substitutions revealed that none reduced ETV susceptibility. These substitutions appeared to be the result of natural genetic drift and unrelated to resistance development.

NOVEL EMERGENT SUBSTITUTIONS IN AN LVD_r HBV RT BACKGROUND

Previous analyses have shown that the primary LVD_r substitutions (rtL180M+rtM204V/I) reduce ETV susceptibility by approximately 8-fold and that patients harboring LVD_r substitutions display varying levels of ETV susceptibility (3.1-fold to 20-fold greater than WT).⁽¹⁸⁾ Nine novel emergent substitutions at eight HBV RT amino acid positions were identified in an LVD_r HBV RT background for phenotypic analysis. One substitution, rtV214L, was not detected by clonal analysis and was therefore not phenotyped in the LVD_r HBV RT background. This substitution was phenotyped in an HBV RT WT background and did not reduce ETV susceptibility (1.0-fold compared with WT; Table 1). The rtV214P substitution was observed in the ETV_r background in 1 patient by population-based sequencing; however, clonal analysis revealed that this substitution was linked to LVD_r substitutions rather than signature ETV_r substitutions. Phenotypic analysis in the LVD_r background revealed ETV susceptibility similar to LVD_r HBV (6.6-fold greater than WT; Table 2).

The ETV susceptibility for the remaining seven identified novel substitutions in the LVD_r HBV

TABLE 1. NOVEL EMERGENT SUBSTITUTIONS IN A WT OR LVD_r HBV RT BACKGROUND

Patient Background Genotype	Novel Emergent HBV RT Substitutions*	Other Novel Emergent HBV RT Substitutions [†]	HBV RT LVD _r Substitutions	ETV Phenotype (Fold/Control ± SD) [‡]
WT	H13L	Q271V, F273L	None	0.5 ± 0.2
WT	H13S	V214L	None	1.0 ± 0.3
WT	R153Q	None	None	0.6 ± 0.2
WT	A186S	I290L, C332Y	None	0.5 ± 0.1
WT	V191I	A62T, Q215R	None	0.2 ± 0.1
WT	E263A	None	None	0.9 ± 0.2
WT	R242S [§]	None	None	nd
WT	L247F	None	None	nd
WT	R289S [¶]	None	None	nd
LVD _r	H9D	V173L	L180M, M204I	3.0 ± 1.5
LVD _r	H9L	None	L180M, M204V	5.7 ± 3.6
LVD _r	V27A	None	M204I	7.1 ± 1.2
LVD _r	V84M, V191I	Y124C, V208I	M204I	3.1 ± 0.5
LVD _r	S117Y	N76K, V142N	M204I	17 ± 1.5
LVD _r	T118H, N139T	L80V, N337D	M204I	7.4 ± 1.0
LVD _r	V214L [#]	None	M204I	nd

*Novel amino acid substitutions that emerged on ETV therapy that were not observed in >99% of 250 WT HBV sequences from GenBank.

[†]The novel emergent substitution was only observed in 1 patient or previously phenotyped.⁽¹⁶⁾

[‡]ETV EC₅₀ of patient clone/EC₅₀ of laboratory reference clone ± SD; mean of ≥3 independent experiments.

[§]rtR242S was not found in 24 or 28 individual clones from 2 patients, and therefore the isolates were not tested.

^{||}rtL247F was not found in 19-27 individual clones from 3 patients, and therefore the isolates were not tested.

[¶]rtR289S was not found in 22 or 27 individual clones from 2 patients, and therefore the isolates were not tested.

[#]rtV214L was not found in 23 individual clones from 1 patient, and therefore the isolates were not tested.

Abbreviation: nd, not determined.

TABLE 2. NOVEL EMERGENT SUBSTITUTIONS IN AN HBV RT ETV_r BACKGROUND

Novel Emergent HBV RT Substitutions*	Other Novel Emergent HBV RT Substitutions [†]	HBV RT LVD _r and ETV _r Substitutions	ETV Phenotype (Fold/Control ± SD) [‡]
H13L	I187V	L180M, T184L, M204V	>758
R18K	K318R	L180M, S202G, M204V	>758
H35N	None	L180M, M204V, M250V	>758
N53K	W257H	L180M, S202G, M204V	>758
N53H	None	L180M, S202G, M204V	>758
N123H	S78C, Q125K, V142G	I169L, L180M, T184A, M204V	>758
V142A	None	L180M, T184A, M204V	>758
C188L	V173L	L180M, M204V, M250V	>758
V191I	None	L180M, T184S, M204V	15 ± 3.4
V214A	None	L180M, T184A, M204V	>758
V214A	None	L180M, S202G, M204V	>758
V214I	None	L180M, T184L, M204V	>758
V214P	V84M, V142G, V191I	L180M, M204I	6.6 ± 0.5
L220I	None	L180M, S202G, M204V	>758
A329S	None	None	0.4 ± 0.1
N337D	S40A	L180M, T184L, M204V	>758

*Novel amino acid substitutions that emerged on ETV therapy that were not observed in >99% of 250 wild-type HBV sequences from GenBank.

[†]The novel emergent substitution was only observed in 1 patient or previously phenotyped.⁽¹⁶⁾

[‡]ETV EC₅₀ of patient clone/EC₅₀ of laboratory reference clone ± SD; mean of three independent experiments.

RT background is presented in Table 1. Phenotypic analysis of recombinant clones harboring these substitutions revealed that they had reduced ETV susceptibility (3.0-fold to 17-fold greater than WT), similar to LVD_r HBV. These substitutions appeared to be the result of natural genetic drift and unrelated to resistance development. Nevertheless, ETV susceptibility analyses were performed on a patient-derived sequence harboring a substitution (rtS117Y) conferring 17-fold reduction in susceptibility greater than WT HBV. The tyrosine at rt117 was changed to serine by site-directed mutagenesis in the patient-derived HBV RT clone. Phenotypic analysis of the rt117S clone indicated an ETV susceptibility (10-fold greater than WT) similar to the parental rtS117Y clone. Four LVD-naïve patients each harbored the rtS117Y substitution at baseline and all achieved undetectable HBV DNA while on ETV treatment. In addition, rtS117Y emerged in 1 LVD-naïve patient on ETV treatment at a time point with HBV DNA <50 IU/mL. Taken together, these data suggest that the rtS117Y substitution does not contribute to reduced ETV susceptibility.

NOVEL EMERGENT SUBSTITUTIONS IN AN ETV_r HBV RT BACKGROUND

HBV substitutions at rtT184, rtS202, or rtM250 confer resistance to ETV when associated with LVD_r substitutions rtM204V/I±rtL180M and display varying levels of ETV susceptibility depending on the specific resistance substitutions.⁽¹²⁾ Fifteen novel emergent substitutions at 12 HBV RT amino acid positions were identified in an ETV_r HBV RT background for phenotypic analysis. For patients with two of these substitutions, clonal analysis of patient-derived isolates revealed that the rtV214P and rtA329S substitutions were not linked with ETV_r substitutions. The rtV214P substitution linked with LVD_r substitutions rtL180M+rtM204I (described above). The rtA329S substitution was only identified in a WT HBV RT background with ETV susceptibility similar to WT HBV (0.4-fold greater than WT; Table 2). The phenotypic data suggest that the rtV214P and rtA329S substitutions are the result of natural genetic drift and unrelated to ETV_r.

ETV_r substitutions at rtT184 (rtT184C/G/I/S) have been shown to exhibit low-level ETV_r and have only been observed in combination with other

ETV_r substitutions in patients with virologic breakthrough.⁽¹²⁾ The novel emergent rtV191I substitution was phenotyped in an ETV_r HBV RT background of rtL180M, rtT184S, and rtM204V and displayed reduced ETV susceptibility (15-fold greater than WT; Table 2) within the range observed for LVD_r HBV. The rtV191I substitution in a WT, LVD_r, or ETV_r HBV RT background did not reduce ETV susceptibility.

Phenotypic analysis of the remaining 12 of 15 identified novel emergent substitutions indicated a reduced susceptibility to ETV (>758-fold greater than WT; Table 2) due to the presence of high-level ETV_r HBV RT substitutions rtT184L, rtT184A, rtS202G, or rtM250V (Table 2). Therefore, the contribution of these novel emergent substitutions could not be determined due to the existing reduced susceptibility to ETV related to the presence of known ETV_r substitutions. Ten of the 12 HBV RT substitutions preexisted in other patients, suggestive of natural genetic drift and not related to ETV_r, while two substitutions (rtH35N and rtC188L) did not preexist. Phenotypic analyses of rtH35N and rtC188L were each assessed in the context of other substitutions detected at virologic breakthrough (see patient [Pt]21 and Pt25, described below).

NOVEL EMERGENT SUBSTITUTIONS IN TREATMENT-NAIVE PATIENTS WITH VIROLOGIC BREAKTHROUGH

Ten LVD-naïve patients experienced virologic breakthrough with novel emergent HBV RT substitutions for phenotypic analysis; results are summarized in Table 3. As described above, the rtL247F substitution (observed in patient Pt1) was not detected by clonal analysis and was not phenotyped.

ETV treatment in nucleoside-naïve patients can infrequently select for LVD_r HBV.⁽¹¹⁾ Two patients (Pt2 and Pt3) had emergent LVD_r substitutions at virologic breakthrough in addition to the novel emergent substitutions rtA27V+rtA200V or rtL229F/V (Table 3). Phenotypic analysis of recombinant clones from these 2 patients showed reduced ETV susceptibility similar to LVD_r HBV (5.5-fold to 9.4-fold greater than WT; Table 3), suggesting that the novel HBV substitutions rtA27V+rtA200V, rtL229F, and rtL229V did not contribute to the reduced ETV susceptibility.

Two patients (Pt4 and Pt5) who experienced virologic breakthrough on ETV treatment harbored the novel

TABLE 3. NOVEL EMERGENT HBV RT SUBSTITUTIONS IN TREATMENT-NAIVE PATIENTS WITH VIROLOGIC BREAKTHROUGH

Patient Number	Novel Emergent HBV RT Substitutions*	ETV Phenotype (Fold/Control \pm SD) [†]
1	L247F [‡]	nd
2	V27A, A200V, M204I	5.5 \pm 2.0
3	L180M, M204V, L229F	6.3 \pm 0.8
3	L180M, M204V, L229V	9.4 \pm 4.9
4	L164F, A329S	1.0 \pm 0.2
5	S135F, R280G	0.8 \pm 0.3
6	Q267N	0.9 \pm 0.4
7	Q125K	0.8 \pm 0.1
8	R153Q	0.6 \pm 0.2
9	H13L, Q271V, F273L	0.5 \pm 0.2
10	V214I	0.4 \pm 0.1

*Novel amino acid substitutions that emerged on ETV therapy that were not observed in >99% of 250 WT HBV sequences from GenBank.

[†]ETV EC₅₀ of patient clone/EC₅₀ of laboratory reference clone \pm SD; mean of three independent experiments.

[‡]rtL247F was not found in 19 individual clones from this patient, and therefore the isolates were not tested.

Abbreviation: nd, not determined.

HBV RT substitutions rtL164F+rtA329S or rtS135F+rtR280G and achieved HBV DNA levels <50 IU/mL subsequent to virologic breakthrough. Phenotypic analysis of recombinant clones from these 2 patients did not show reduced ETV susceptibility (\leq 1.0-fold compared with WT; Table 3), suggesting that these two combinations of novel HBV substitutions did not impact ETVr.

Five patients (Pt6, Pt7, Pt8, Pt9, and Pt10) had emergent novel HBV RT substitutions rtH13L+rtQ271V+rtF273L, rtQ125K, rtR153Q, rtV214I, or rtQ267N, respectively, at virologic breakthrough to ETV treatment. Phenotypic analysis of recombinant patient clones harboring these novel emergent substitutions resulted in ETV susceptibilities (<1-fold greater than WT; Table 3) similar to that observed for the WT HBV clone, suggesting that rtH13L+rtQ271V+rtF273L, rtQ125K, rtR153Q, rtV214I, and rtQ267N did not impact ETVr development.

NOVEL EMERGENT SUBSTITUTIONS IN LVD TREATMENT-EXPERIENCED PATIENTS WITH VIROLOGIC BREAKTHROUGH

One patient (Pt11) harbored novel emergent substitutions (rtH13S, rtS78T, and rtQ267R/M) without LVDr substitutions. Phenotypic analysis of recombinant clones from this patient harboring either rtS78T+rtQ267R or rtH13S+rtQ267M did not show reduced susceptibility to ETV (\leq 1.0-fold WT; Table 4), suggesting no impact on ETVr.

Two patients (Pt12 and Pt13) harbored the novel emergent substitution rtV27A with the LVDr substitution rtM204I. In both these patients, rtV27A was not maintained at subsequent time points on ETV treatment, and 1 patient (Pt12) achieved undetectable HBV DNA subsequent to virologic breakthrough. Phenotypic analysis of recombinant clones from these 2 patients showed susceptibility to ETV (7.1-fold and 8.4-fold greater than WT; Table 4) similar to LVDr HBV. The phenotypic and virologic outcomes data for these patients with rtV27A suggested that this substitution did not impact ETVr development.

The rtL80I substitution has been reported to be a compensatory substitution selected by LVD treatment to enhance the replication efficiency of the LVDr substitution rtM204I without affecting LVDr.⁽¹⁹⁾ Two patients (Pt14 and Pt15) harbored rtL80I with LVDr substitutions rtM204I \pm rtL180M, while 1 patient (Pt16) harbored rtL80I with the novel emergent substitutions rtN131G+rtQ316H and rtM204I. One patient (Pt14) harboring rtL80I+rtM204I achieved undetectable HBV DNA subsequent to virologic breakthrough. Phenotypic analysis of recombinant clones from these 3 patients showed susceptibility to ETV (1.8-fold to 6.7-fold greater than WT; Table 4) similar to LVDr HBV. The phenotypic and virologic outcomes data suggested that rtL80I \pm rtN131G+rtQ316H did not impact ETVr development.

Two patients (Pt17 and Pt18) harbored the novel emergent substitution rtA186T with LVDr

TABLE 4. NOVEL EMERGENT HBV RT SUBSTITUTIONS IN LVD TREATMENT-EXPERIENCED PATIENTS WITH VIROLOGIC BREAKTHROUGH

Patient Number	Novel Emergent HBV RT Substitutions*	HBV RT LVD _r Substitutions	ETV Phenotype (Fold/Control \pm SD) [†]
11	S78T, Q267R	None	1.0 \pm 0.1
11	H13S, Q267M	None	0.2 \pm 0.4
12	V27A	M204I	7.1 \pm 1.2
13	V27A	M204I	8.4 \pm 0.7
14	L80I	M204I	1.8 \pm 0.2
15	L80I	L180M, M204I	4.1 \pm 1.1
16	L80I, N131G, Q316H	M204I	6.7 \pm 3.0
17	T128N, A186T	L180M, M204V	2.5 \pm 0.8
18	A186T	L180M, M204V	2.5 \pm 1.4
19	V173L	L180M, M204V	6.3 \pm 0.8
19	V207I	L180M, M204V	4.7 \pm 0.1
20	L229V	L180M, M204V	3.8 \pm 0.7
20	L229W	L180M, M204V	3.8 \pm 0.8
21	V173L, C188L	L180M, M204V	9.7 \pm 5.7
22	I169V, V173L	L180M, T184L, M204V	>758
23	A181C	L180M, M204V	122 \pm 34

*Novel amino acid substitutions that emerged on ETV therapy that were not observed in >99% of 250 wild-type HBV sequences from GenBank.

[†]ETV EC₅₀ of patient clone/EC₅₀ of laboratory reference clone \pm SD; mean of three independent experiments.

substitutions rtL180M+rtM204V. Patient 17 also harbored the novel emergent substitution rtT128N, and Pt18 harbored rt186T+rtL180M+rtM204V and achieved undetectable HBV subsequent to virologic breakthrough on ETV treatment. Phenotypic analysis of recombinant clones from these 2 patients showed susceptibility to ETV (2.5-fold greater than WT; Table 4) similar to LVD_r HBV. The rtA186T substitution has been reported to be associated with ETV_r.⁽²⁰⁾ Two additional LVD_r patients also harbored emergent rtA186T. One patient responded to ETV treatment, and phenotypic analysis of a recombinant clone harboring substitutions rtI163V+rtL180M+rtA186T+rtM204V from this patient showed susceptibility to ETV (4.8-fold greater than WT) similar to LVD_r HBV. By contrast, the other patient experienced a virologic breakthrough harboring the novel substitutions rtF88F/Y, rtT184A/S, rtA186A/T, and rtF201F/Y, with LVD_r substitutions L180M+M204V that persisted on ETV treatment. Due to the presence of ETV_r substitutions rtT184A/S in this patient, ETV susceptibility was not assessed. Taken together, our phenotypic and virologic outcomes data for patients harboring the rtA186T substitution suggested that rtA186T did not contribute to ETV_r. These results are inconsistent with those reported by Hayashi et al.⁽²⁰⁾

Substitutions at positions rtV173,⁽²¹⁾ rtV207,⁽²²⁾ and rtL229⁽²³⁾ in the HBV RT are compensatory

substitutions selected by LVD treatment to enhance the replication efficiency of HBV LVD_r substitutions. Two patients (Pt19 and Pt20) harbored only novel emergent LVD_r-associated compensatory substitutions. Patient 19 harbored the LVD_r substitutions rtL180M+rtM204V with either rtV173L or rtV207I while Pt20 harbored the LVD_r substitutions rtL180M+rtM204V with either rtL229V or rtL229W, as determined by clonal analysis. Phenotypic analysis of recombinant clones from these patients showed reduced susceptibility to ETV (3.8-fold to 6.3-fold greater than WT; Table 4) similar to LVD_r HBV, suggesting that the LVD_r-associated compensatory substitutions rtV173L, rtV207I, and rtL229L/W did not impact ETV_r.

One patient (Pt21) harbored the novel substitution rtC188L with the LVD_r-associated compensatory substitution rtV173L. Phenotypic analysis of a recombinant clone from this patient showed susceptibility to ETV (9.7-fold greater than WT; Table 4) similar to LVD_r HBV, suggesting that the rtV173L and rtC188L substitutions did not impact resistance development.

Substitutions at rtI169 have been considered as secondary changes not specific for ETV that may contribute to the growth efficiency of ETV_r HBV.⁽¹⁴⁾ In our analysis, 7 LVD-experienced patients were infected with HBV genotype C with the novel substitution rtI169V; 6 of these patients had virologic breakthrough. The ETV susceptibility for a clone

from Pt22 harboring HBV substitutions rtI169V+rtV173L+rtL180M+rtT184L+rtM204V resulted in high-level HBV ETVr ($EC_{50} >15,000$ nM) due to the presence of the ETVr substitution rtT184L. Therefore, the contribution of rtI169V could not be determined in this patient due to the existing reduced susceptibility to ETV; however, rtI169V did not appear to impact susceptibility to ETV in a baseline treatment isolate from Pt24 also harboring the LVDr substitutions rtL180M+rtM204V (Table 5; see below). Therefore, rtI169V may have a compensatory role in ETVr HBV as has been described for the rtI169T substitution.⁽¹⁴⁾

HBV rtA181V/T and rtA181V/T+rtN236T substitutions, in addition to conferring reduced susceptibility to ADV and TDF,^(24,25) have also been reported to confer reduced susceptibility to LVD and LdT.^(26,27) One patient (Pt23) harbored the novel emergent substitution rtA181C in combination with LVDr substitutions rtL180M+rtM204V. Phenotypic analysis of a recombinant clone harboring HBV substitutions rtL180M+rtA181C+rtM204V revealed a notable reduction in susceptibility to ETV (122-fold greater than WT; Table 4). Reverse genetics was employed to change rt181C to WT rt181A in this clone. Phenotypic analysis of this clone showed susceptibility to ETV (8.2-fold greater than WT; Table 5) similar to LVDr HBV, confirming that the presence of rtA181C in addition

to the LVDr substitutions rtL180M+rtM204V further reduced ETV susceptibility.

The HBV rtA181C substitution was detected in 0.5% (5/982) of evaluable HBV RT sequences. All 5 patients with the rtA181C substitution were LVD experienced; all except 1 had virologic breakthrough during ETV treatment with rtA181C detected with previously reported ETVr substitutions. The 1 LVD-experienced patient without virologic breakthrough showed a decline in HBV DNA that remained detectable ($\geq 3 \log_{10}$ IU/mL) on ETV therapy, with the emergence of rtA181C/G and ETVr substitutions at the last available visit.

HBV rtA181C emerged in 4 LVD-experienced patients each with preexisting LVDr substitutions before ETV treatment. The rtA181C substitution required a two-nucleotide change. Three patients had rtA181C encoded by a GCT to TGT change, while rtV181C was encoded by a GTT to TGT change in 1 patient. One LVD-experienced patient with rtA181C had no available baseline sample for analysis; this substitution was encoded by TGT. The infrequent occurrence (0.5%) of the rtA181C substitution was most likely due to the requirement for a two-nucleotide change.

ETVr substitutions rtT184, rtS202, and rtM250 were detected by direct sequencing of PCR amplicons

TABLE 5. PHENOTYPIC ANALYSIS OF rtA181 SUBSTITUTIONS

Patient Number	HBV rtA181 Substitution	HBV RT LVDr Substitutions	ETV Phenotype (Fold/Control \pm SD)*	Fitness (% of Control \pm SD)
19	C [†]	V173L, L180M, M204V	25 \pm 9.5	109 \pm 26
19	A	V173L, L180M, M204V	6.3 \pm 0.8	nd
23	C	L180M, M204V	122 \pm 34	19 \pm 15 [‡]
23	A [§]	L180M, M204V	8.2 \pm 3.1	13 \pm 6 [‡]
24	C	I169V, V173L, L180M, M204V	16 \pm 6.3	53 \pm 40
24	G	I169V, V173L, L180M, M204V	3.9 \pm 0.3	52 \pm 40
24	V	I169V, V173L, L180M, M204V	4.8 \pm 2.0	40 \pm 46
25	C	H35N, V173L, L180M, M204V	79 \pm 51	89 \pm 47 [‡]
25	A [§]	H35N, V173L, L180M, M204V	1.4 \pm 0.8	59 \pm 15 [‡]
26	C	L180M, M204V	nd	nd
27	N	L180M, M204V	7.0 \pm 2.7	52 \pm 13
28	S	L180M, M204V	10.8 \pm 6.5	11 \pm 4
29	T	V173L, L180M, M204V	0.8 \pm 0.2	33 \pm 18
Laboratory	C	V173L, L180M, M204V	43 \pm 8.9	116 \pm 32 [‡]
Laboratory	C	None	1.7 \pm 0.2	98 \pm 20

*ETV EC_{50} of patient clone/ EC_{50} of laboratory reference clone \pm SD; mean of ≥ 3 independent experiments.

[†]Reverse genetics to change rtA181 to rtA181C.

[‡]Replication fitness of respective HBV RT constructs with rtA181C or rtA181 were comparable in the phenotypic assay.

[§]Reverse genetics to change rtA181C to rt181A.

Abbreviation: nd, not determined.

from patient isolates with the rtA181C substitution in HBV RT populations from 4 of the 5 patients (Pt2, Pt24, Pt25, and Pt26; Supporting Table S5). Clonal analysis of the isolate with the rtA181C substitution from Pt23 identified the ETVr S202G substitution in the HBV RT population not observed by direct sequencing of the PCR amplicon (Supporting Table S5). Sequence linkage of the rtA181C substitution with ETVr and LVDr substitutions was detected in 2 patients (Pt23 and Pt25); however, the ETVr combination was not the major HBV RT species. In Pt25, linkage with an additional novel HBV RT substitution (rtH35N) was also observed. The combination of rtA181C with LVDr substitutions was the major HBV RT species in Pt26.

ETV ANTI-HBV ACTIVITY AGAINST HBV rtA181C AND OTHER rtA181 SUBSTITUTIONS

To investigate the role of the HBV rtA181C substitution in reduced ETV susceptibility, this substitution was introduced into a WT laboratory clone, an LVDr laboratory clone harboring LVDr substitutions rtL173V+rtL180M+rtM204V, and a recombinant clone from a patient (Pt19 harboring LVDr substitutions rtL173V+rtL180M+rtM204V). Furthermore, recombinant clones for 2 additional patients (Pt24 and Pt25) harboring rtA181C with LVDr substitutions were constructed. In addition, recombinant clones were also constructed from patient isolates harboring other rtA181 substitutions, including rtA181G (Pt24), rtA181V (Pt24), rtA181N (Pt27), rtA181S (Pt28), and rtA181T (Pt29). The phenotypic results are summarized in Table 5.

Phenotypic analysis of four patient-derived recombinant clones harboring the HBV rtA181C substitution with LVDr substitutions showed reduced ETV susceptibility ranging from 16-fold to 122-fold greater than WT (Table 5). Reverse genetics was used to change the cysteine to WT alanine at amino acid position rt181 in clones from Pt23 and Pt25. A comparison of ETV susceptibilities of these patient-derived clones harboring rt181C versus rt181A as well as clones from Pt19 displayed a 15-fold, 56-fold, and 4-fold enhancement in ETV susceptibility by changing rt181C to rt181A for Pt23, Pt25, and Pt19, respectively (Table 5). Comparison of ETV susceptibilities to rt181C and rt181A in Pt25 confirmed that the novel emergent rtH35N substitution did not influence ETVr.

The HBV rtA181C substitution was introduced into laboratory clone p180B3, with and without LVDr substitutions. Phenotypic analysis of these clones resulted in ETV susceptibilities of 43-fold and 1.7-fold greater than WT for clones harboring rtV173L+rtL180M+rtA181C+rtM204V and rtA181C, respectively (Table 5). Taken together, the results from the patient-derived and laboratory-derived clones harboring the rtA181C substitution exhibited reduced susceptibility to ETV but only in combination with LVDr substitutions rtL180M+rtM204V.

ETV susceptibility of other HBV rtA181 substitutions observed in our integrated analysis of ETV-treated patients was evaluated. Phenotypic analysis of recombinant clones harboring rtA181 substitutions G, N, S, T, or V in combination with LVDr substitutions rtL180M+rtM204V showed susceptibility to ETV (0.8-fold to 11-fold greater than WT; Table 5) similar to LVDr HBV, suggesting that these rtA181 substitutions G, N, S, T, or V did not confer resistance to ETV.

CROSS-RESISTANCE PROFILE OF HBV rtA181 SUBSTITUTIONS TO APPROVED ANTI-HBV DRUGS

We evaluated the cross-resistance profile of the HBV rtA181C substitution in combination with LVDr substitutions to other approved anti-HBV drugs. Phenotypic analysis of recombinant clones from Pt19 and Pt23 and a laboratory clone harboring the rtA181C substitution with LVDr HBV showed susceptibility to ADV (≤ 1 -fold compared with WT) and TDF (≤ 1.6 -fold compared with WT) similar to the WT clone. In contrast, reduced susceptibility to LVD (> 15 -fold greater than WT) and LdT (> 3.9 -fold greater than WT) resulted as expected due to the presence of LVDr substitutions (Table 6).

The cross-resistance profile of the HBV rtA181C substitution in the absence of LVDr substitutions to other approved anti-HBV drugs was also evaluated. Phenotypic analysis of a laboratory recombinant clone only harboring the rtA181C substitution showed that it remained susceptible to ADV (0.9-fold greater than WT) and TDF (1.0-fold greater than WT), although reduced susceptibilities to LVD (18-fold greater than WT) and LdT (> 7.7 -fold greater than WT) were observed (Table 6).

Phenotypic analysis revealed that recombinant clones harboring rtA181 substitutions G, N,

TABLE 6. CROSS-RESISTANCE PROFILE OF HBV rtA181 SUBSTITUTIONS

Patient Number	HBV rtA181 Substitution	HBV RT LVD _r Substitutions	Phenotype (Fold/Control ± SD)*				
			ETV	ADV	TDF	LVD	LdT
19	C	V173L, L180M, M204V	25 ± 9.5	0.4 ± 0.1	1.3 ± 0.1	>15	>3.9
23	C	L180M, M204V	122 ± 34	1.0 ± 0.2	1.6 ± 0.3	>15	>3.9
24	G	I169V, V173L, L180M, M204V	3.9 ± 0.3	0.9 ± 0.2	1.5 ± 0.2	nd	nd
24	V	I169V, V173L, L180M, M204V	4.8 ± 2.0	0.9 ± 0.1	1.1 ± 0.7	nd	nd
27	N	L180M, M204V	7.0 ± 2.7	1.0 ± 0.1	1.2 ± 0.4	nd	nd
28	S	L180M, M204V	10.8 ± 6.5	>3	2.0 ± 0.1	nd	nd
29	T	A173L, L180M, M204V	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.4	nd	nd
30	V	A181V, N236T	0.5 ± 0.2	>3	5.0 ± 0.1	>30	>7.8
Laboratory	C	V173L, L180M, M204V	43 ± 8.9	0.6 ± 0.1	0.4 ± 0.1	>15	>3.9
Laboratory	C	None	1.7 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	18 ± 12	>7.8

*ETV EC₅₀ of patient clone/EC₅₀ of laboratory reference clone ± SD. ADV and TDF values represent an average of three independent experiments; LVD and LdT values represent an average of two independent experiments. Due to the cytotoxicity of ADV, LVD and LdT at high concentrations in our cell-based assays, an absolute value for fold resistance could not always be determined. Abbreviation: nd, not determined.

T, or V in combination with LVD_r substitutions rtL180M+rtM204V remained susceptible to ADV (<1-fold compared with WT) and TDF (≤1.5-fold compared with WT), as shown in Table 6. Phenotypic analysis of a recombinant clone harboring rtA181S with LVD_r substitutions rtL180M+rtM204V showed reduced susceptibility to ADV (>2.9-fold greater than WT) but remained susceptible to inhibition by TDF (2-fold greater than WT). Previous studies have shown that rtA181S conferred reduced sensitivity to ADV but remained susceptible to LVD, TDF, and ETV,⁽²⁸⁾ whereas rtA181S+M204I conferred reduced sensitivity to both ADV and LVD.⁽²⁹⁾ Taken together, our results from this phenotypic analysis suggest that rtA181 substitutions G, N, S, T, or V in combination with LVD_r substitutions rtL180M+rtM204V were not associated with ETV_r. In addition, they did not confer cross-resistance to ADV or TDF with the exception of the rtA181S substitution that has been reported to confer cross-resistance to ADV.⁽²⁸⁾

Discussion

We describe the results from a comprehensive genotypic meta-analysis of evaluable HBV RT sequencing data obtained from patients who experienced virologic breakthrough and/or had HBV DNA levels ≥50 IU/mL during treatment with recommended doses of ETV. The integrated analysis included HBV RT

sequences from 17 phase 2 and 3 clinical studies that were examined to establish the potential existence of novel pathways to ETV_r in addition to those already described.⁽¹¹⁾

Previous analyses have shown that the primary HBV substitutions at rtT184, rtS202, or rtM250 confer resistance to ETV when associated with LVD_r substitutions rtM204V/I±rtL180M⁽¹⁵⁾ and display varying degrees of ETV susceptibility depending on the specific resistance substitutions.⁽¹²⁾ While assessing HBV RT sequences from our integrated analysis, novel substitutions that emerged in at least 2 patients in WT, LVD_r, and/or ETV_r HBV RT backgrounds were observed. In addition, novel substitutions in patient-derived sequences from LVD-naïve and LVD-experienced virologic breakthroughs during ETV therapy were identified for phenotypic assessment.

Phenotypic analysis of 30 novel emergent HBV RT substitutions introduced into WT, LVD_r, and/or ETV_r HBV RT background sequences for ETV susceptibility indicated that these substitutions displayed an ETV phenotype similar to WT, LVD_r, and/or ETV_r HBV.

Phenotypic analysis of patient-derived HBV RT sequences from 10 LVD-naïve patients with virologic breakthrough indicated that none of the novel substitutions affected the anti-HBV activity of ETV. However, one of the patient-derived HBV RT sequences from 13 LVD-experienced patients with virologic breakthrough harbored a novel substitution (rtA181C) at an

amino acid position associated with nucleos(t)ide resistance⁽²⁴⁻²⁷⁾ in combination with LVDr substitutions rtL180M+rtM204V. The presence of this substitution appeared to have an impact on the anti-HBV activity of ETV (122-fold increase in the EC₅₀ value compared with WT HBV). Reverse genetics confirmed the role of rtA181C in ETVr, although this loss in ETV sensitivity only resulted in the presence of the LVDr substitutions rtL180M+rtM204V.

One of the novel emergent HBV RT substitutions (rtI169V) not associated with a loss in ETV susceptibility emerged in 6 LVD-experienced patients who were infected with HBV genotype C and had experienced virologic breakthrough. Interestingly, this substitution was detected with the high-level ETVr substitution rtT184L and LVDr substitutions rtL180M+rtM204V during virologic breakthrough in all cases. Previous findings have suggested that substitutions (e.g., rtI169T) at this amino acid position may act in a compensatory manner in ETVr HBV.⁽¹⁴⁾ Our data suggest that HBV rt169V may also have an adaptive role in ETVr HBV, specifically during the emergence of rtT184L with rtL180M+rtM204V in patients with virologic failure infected with genotype C HBV.

In our analysis, the only novel HBV RT substitution (rtA181C) shown to confer ETVr *in vitro* was detected in 0.5% (5/982) of evaluable patients who met the criteria for resistance testing. HBV rt181C was always detected in combination with the LVDr substitutions rtL180M+rtM204V and was observed as the major species in 3 of these patients by clonal analysis. Two nucleotide changes are required at the codon level to replace alanine with cysteine, which may explain why this substitution was observed infrequently. HBV rtA181 substitutions other than rt181C (G, N, S, T, or V) in combination with LVDr substitutions rtL180M+rtM204V showed a reduced ETV susceptibility similar to that previously observed for LVDr HBV.

Cross-resistance occurred between ETV and other approved anti-HBV drugs in analyses performed with recombinant clones expressing rt181C in combination with LVDr substitutions rtL180M+rtM204V. This combination of substitutions conferred cross-resistance to LVD and LdT but not to ADV and TDF. Clones harboring the HBV rt181C substitution in the absence of these LVDr substitutions were susceptible to inhibition by ETV, ADV, and TDF, although cross-resistance to LVD and LdT resulted.

In conclusion, we have discovered a novel pathway to ETVr. The HBV substitution rtA181C was identified from an integrated genotypic analysis of HBV RT sequences from ETV-treated patients. ETVr conferred by this substitution was always associated with the LVDr substitutions rtL180M+rtM204V. Cross-resistance by HBV rtA181C was observed to LVD and LdT but not to ADV or TDF. The HBV rtA181C substitution emerged infrequently in patients treated with ETV who had either experienced virologic breakthrough during treatment or had evidence of detectable HBV DNA throughout the course of treatment.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1231/full.