# No Resistance to Tenofovir Disoproxil Fumarate Detected After up to 144 Weeks of Therapy in Patients Monoinfected With Chronic Hepatitis B Virus

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**Tenofovir disoproxil fumarate (TDF) is a nucleotide analogue with potent activity against human immunodeficiency virus type 1 and hepatitis B virus (HBV). To date, no reports of HBV clinical resistance to TDF have been confirmed. In two phase 3 studies (GS-US-174-0102 and GS-US-174-0103), 375 hepatitis B e antigen–negative ($HBeAg^-$) patients and 266 $HBeAg^+$ patients with chronic hepatitis B (some nucleoside-naive and some lamivudine-experienced) were randomized 2:1 to receive TDF (n = 426) or adefovir dipivoxil (ADV; n = 215) for 48 weeks. After week 48, eligible patients received open-label TDF with no interruption. The studies are being continued through week 384/year 8; week 144 data are presented here. Per protocol, viremic patients (HBV DNA level $\geq$ 400 copies/mL or 69 IU/mL) had the option of adding emtricitabine (FTC) at or after week 72. Resistance analyses of HBV polymerase/reverse transcriptase (pol/RT) were based on population dideoxy sequencing. Phenotypic analyses were conducted in HepG2 cells with recombinant HBV derived from patient serum. Most patients maintained TDF monotherapy treatment across both studies (607/641, 95%). A resistance analysis of HBV pol/RT was performed at the baseline for all patients, for viremic patients at week 144 or at the last time when they were on TDF monotherapy (34 on TDF and 19 on ADV-TDF), and for patients who remained viremic after the addition of FTC (7/20 on TDF and 5/14 on ADV-TDF). No patient developed amino acid substitutions associated with resistance to TDF. Virological breakthrough on TDF monotherapy was infrequent over 144 weeks (13/426, 3%) and was attributed to documented nonadherence in most cases (11/13, 85%). Persistent viremia ($\geq$400 copies/mL) through week 144 was rare (5/641, 0.8%) and was not associated with virological resistance to TDF by population or clonal analyses. *Conclusion:* No nucleoside-naive or nucleoside-experienced patient developed HBV pol/RT mutations associated with TDF resistance after up to 144 weeks of exposure to TDF monotherapy. (HEPATOLOGY 2010;000:000-000)**

*Abbreviations: ADV, adefovir dipivoxil; ADV-R, adefovir dipivoxil–associated resistance; AS-PCR, allele-specific polymerase chain reaction; CHB, chronic hepatitis B; $EC_{50}$, 50% effective concentration; FTC, emtricitabine; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; LAM-R, lamivudine-associated resistance; N/A, not applicable; ND, not determined; OL-TDF, open-label tenofovir disoproxil fumarate; PCR, polymerase chain reaction; pol/RT, polymerase/reverse transcriptase; TDF, tenofovir disoproxil fumarate; WT, wild type.*

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Hepatitis B virus (HBV) constitutes a major global health threat with an estimated worldwide population of 400 million chronic HBV carriers. Worldwide, approximately 1 million people die annually of complications of chronic hepatitis B (CHB).[1]

The goal of CHB therapy is to decrease the risk of complications such as cirrhosis and hepatocellular carcinoma by potent and durable suppression of viral replication. Long-term therapy requires acceptable tolerability, minimal toxicity, potent activity, a dosing regimen that facilitates adherence, and minimal selection for drug resistance. Long-term treatment with oral antiviral therapies eventually leads to some level of resistance: up to 70% after 4 years with lamivudine[2]; up to 29% in hepatitis B e antigen–negative ($HBeAg^-$) patients after 5 years with adefovir dipivoxil (ADV)[3];

25.1% and 10.8% in $HBeAg^+$ and $HBeAg^-$ patients, respectively, after 2 years with telbivudine[4]; and 1.2% after 5 years with entecavir.[5]

Tenofovir disoproxil fumarate (TDF) was approved at the dosage of 300 mg once daily for the treatment of human immunodeficiency virus type 1 infection in 2001 and was approved at the same dosage in 2008 for the treatment of CHB. Clinical studies have demonstrated that TDF has high potency against HBV, with 76% of $HBeAg^+$ patients and 93% of $HBeAg^-$ patients achieving complete viral suppression (HBV DNA $<$ 400 copies/mL) after 48 weeks of treatment.[6] There was no difference in the efficacy of TDF between treatment-naive patients and lamivudine-experienced patients and between patients with different HBV viral genotypes (A-H).[7] Hepatitis B surface antigen (HBsAg) loss was observed in 3.2% of $HBeAg^+$ patients at week 48,[6] and cumulatively, 8% of $HBeAg^+$ patients experienced HBsAg loss through week 144.[8] No $HBeAg^-$ patient experienced HBsAg loss through week 144. To date, there have been no reports of virological resistance to TDF among HBV-monoinfected patients. One study reported the development of the rtA194T substitution in combination with preexisting rtL180M and rtM204V lamivudine-associated resistance (LAM-R) mutations for two human immunodeficiency virus type 1/HBV–coinfected patients receiving antiviral treatment including TDF.[9] In this study, the rtA194T substitution was associated with reduced susceptibility to tenofovir *in vitro*. However, these results have not been reproduced,[10] and more recently, clinical data showed that the rtA194T substitution did not have an impact on the TDF response in CHB-monoinfected patients.[11] *In vitro*, the rtN236T ADV-associated resistance mutation resulted in cross-resistance to tenofovir.[12] Clinical studies evaluating the use of TDF in ADV-treated patients have yielded conflicting results with respect to the activity of TDF in this patient population.[13,14]

Studies GS-US-174-0102 and GS-US-174-0103 evaluated the safety and efficacy of TDF (300 mg once daily) in patients with $HBeAg^-$ or $HBeAg^+$ CHB. Patients in the comparison arm of the studies were treated with ADV (10 mg once daily) for 48 weeks. All eligible patients with a week 48 liver biopsy sample were switched to open-label tenofovir disoproxil fumarate (OL-TDF) without treatment interruption for up to 7 additional years. Per protocol, the patients had the option of adding emtricitabine (FTC; 200 mg once daily) to their OL-TDF regimen [via Truvada, a fixed-dose combination of FTC (200 mg) and TDF (300 mg)] for confirmed viremia (HBV DNA $\geq$400 copies/mL) at week 72 or beyond. Resistance surveillance and genotypic and phenotypic evaluations are being conducted annually for the duration of these studies for viremic patients. This report summarizes the cumulative year 3 genotypic and phenotypic results for both studies.

## Patients and Methods

***Patient Population.*** Study GS-US-174-0102 enrolled 375 $HBeAg^-$ patients (250 and 125 in the TDF and ADV arms, respectively), and study GS-US-174-0103 enrolled 266 $HBeAg^+$ patients (176 and 90 in the TDF and ADV arms, respectively). The studies were conducted in accordance with international scientific and ethical standards (including but not limited to the International Conference on Harmonization Guidelines for Good Clinical Practice and the Declaration of Helsinki). The studies were approved by independent ethics committees or institutional review boards at the study sites. Written informed consent was obtained from all patients before any procedures were performed. Inclusion criteria and patient demographics have been previously described.[6]

***Genotypic Analysis.*** A resistance analysis of the reverse-transcriptase domain of the HBV polymerase gene [polymerase/reverse transcriptase (pol/RT)] was attempted with stored serum samples for all patients at the baseline and annually if they were viremic (HBV DNA $\geq$ 400 copies/mL), at the time of early discontinuation, or at the time of FTC addition. Briefly, HBV DNA was extracted from serum with the QIAamp DNA blood mini kit (Qiagen, Germantown, MD) and amplified via nested polymerase chain reaction (PCR) with custom primers (available upon request). The lower limit of quantitation for the PCR amplification was 400 copies/mL. The PCR product served as the template in fluorescence-based cycle sequencing reactions with Big-Dye Terminator version 3.1 (Applied Biosystems, Foster City, CA) with custom primers designed to provide double-stranded coverage for amino acids 1 to 344 of the pol/RT. Samples were analyzed with the 3100 ABI-Prism genetic analyzer (Applied Biosystems). Minor species could be detected if they were present in the population at a frequency of approximately 25%. Based on an alignment of amino acid sequences from all patients with available baseline data in these studies, conserved sites in the pol/RT were defined as those positions at which only one amino acid was found or at which two amino acids were present but the prevalence of the minority amino acid was less than 1%. All other positions within the

pol/RT were considered polymorphic sites. Post-baseline pol/RT sequences were aligned to their respective baseline sequences (or the last sequences during the previous treatment for those who switched treatments).

***Phenotypic Assays.*** *In vitro* phenotypic analyses of tenofovir susceptibility were attempted with serum HBV samples obtained from patients who developed emerging amino acid substitutions at conserved sites of pol/RT, patients for whom substitutions developed at polymorphic sites (observed in more than one patient), and patients who experienced virological breakthrough while they were on the study drug. Virological breakthrough was defined as two consecutive HBV DNA values $\geq$ 400 copies/mL if the HBV DNA value was previously $<$400 copies/mL or a confirmed increase $\geq$ 1 $\log_{10}$ copies/mL from the HBV DNA nadir while a patient was on the study drug.

Phenotypic analyses were conducted as previously described[15] with HepG2 cells transiently transfected with plasmid DNA derived from patient serum HBV pol/RT quasispecies. A plasmid pool containing the baseline pol/RT population from the same patient was also tested. If a recombinant virus containing the change of interest could not be obtained, the mutation was created by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) with either the pHY92 genotype A laboratory strain of HBV or the pCMVHBV genotype D laboratory strain of HBV. The interassay variability for susceptibility according to these assays was $\leq$2-fold of the mean values.

***rtN236T Mutant Detection With Allele-Specific Polymerase Chain Reaction (AS-PCR).*** For patients on TDF shown to harbor the rtN236T by population sequencing, the rtN236T mutant percentage was determined with MultiCode RTx AS-PCR modified for HBV rtN236T detection.[16,17] Briefly, HBV DNA was isolated, PCR-amplified, and then diluted for AS-PCR. Standard curves were generated via the mixing of rtN236T and rtN236N plasmids at different ratios ranging from 0.1% to 50%, which were then diluted and PCR-amplified with the same protocol used for plasma sample amplification. The AS-PCR assays were carried out with the Roche LightCycler 480 (Roche, Indianapolis, IN). AS-PCR primer sequences and cycling parameters are available upon request. The rtN236T percentage was determined on the basis of standard curves generated with SigmaPlot (Systat Software, San Jose, CA); the lower cutoff for rtN236T quantification was 0.5%.

***Adherence to Study Medications.*** To assess adherence for patients who qualified for resistance analysis, plasma tenofovir levels were evaluated by liquid chromatography/mass spectrometry. Also analyzed were drug accountability records associated with case report forms and physician-reported drug accountability records included in clinical deviation logs.

## Results

***Baseline Genotypic Findings.*** Baseline genotypic data were obtained for 628 of 641 patients randomized and treated with at least one dose of the study drug across both studies (415 and 213 in the TDF and ADV arms, respectively). Among the 13 patients who could not be evaluated (5 were $HBeAg^+$, and 8 were $HBeAg^-$), the median HBV DNA level was 7.3 $\log_{10}$ copies/mL (range = 3.5-10.3 $\log_{10}$ copies/mL), the median age was 48 years, 11 were male, and 5 were treatment-experienced; the baseline alanine aminotransferase levels were elevated in all cases.

The rtM204V/I$\pm$rtL180M LAM-R mutations were observed in seven patients (five in the TDF arm and two in the ADV arm). The widely accepted viral genotypes A to H were observed across both studies, with viral genotype D being predominant[6]; viral genotypes I and J were not observed among the patients in these studies. A frequency distribution analysis demonstrated that among $HBeAg^-$ patients, 124 of the 344 amino acid positions of the pol/RT (36%) were considered to be polymorphic versus 98 of the 344 positions (28%) among the $HBeAg^+$ patients. There were no significant differences in the week 48 response to TDF according to the baseline characteristics of LAM-R, viral genotype, or polymorphic site substitutions.[6,18]

***Virological Findings Among Patients Receiving up to 144 Weeks of TDF Monotherapy.*** Thirty-four of the 426 patients (8%) originally randomized to the TDF arms were viremic after up to 144 weeks of TDF monotherapy. Among these 34 patients, 10 discontinued TDF between weeks 32 and 120 (median = 52 weeks), 20 patients added FTC to OL-TDF between study weeks 72 and 96 (median = 81 weeks), and 4 patients had HBV DNA levels $>$ 400 copies/mL at week 144. The reasons for discontinuation included withdrawn consent for three (two refused the week 48 biopsy), loss to follow-up for six, and discontinuation due to compliance for 1. The majority of the patients (20/34, 59%) showed no change in their pol/RT versus the baseline, 7 of 34 (21%) harbored polymorphic site changes, and 3 of 34 (9%) harbored distinct conserved site changes; PCR amplification failed for 4 patients primarily because of their low viral load (Table 1). The conserved site changes occurred in the absence of virological breakthrough at the following

**Table 1. Amino Acid Substitutions Among Patients With HBV DNA Levels ≥ 400 Copies/mL (≥69 IU/mL) Through Week 144 of TDF Monotherapy**

| Study | Treatment Group | Patient ID | Baseline HBV DNA ($log_{10}$ Copies/mL)* | Disposition | Genotypic Findings at Last On–TDF Monotherapy Visit† |
|---|---|---|---|---|---|
| GS-US-174-0102 | TDF | 002 | 7.90 | Discontinuation at week 48 | rtR332R/S |
| GS-US-174-0102 | TDF | 001 | 7.05 | Discontinuation at week 56 | rtI91I/L, rtA118A/T, rtN238H/N, and rtR319Q/R |
| GS-US-174-0103 | TDF | 005 | 8.76 | Discontinuation at week 96 | rtF221F/Y |
| GS-US-174-0103 | TDF | 009 | 10.70 | Added FTC at week 72 | **rtL101F/L**, rtR120G/R, and rtL231L/V |
| GS-US-174-0103 | TDF | 010 | 9.55 | Added FTC at week 72 | rtN123N/T, **rtV173L**, **rtL180M**, and **rtM204V**‡ |
| GS-US-174-0103 | TDF | 007 | 9.83 | Added FTC at week 80 | rtL145L/M |
| GS-US-174-0102 | TDF | 003 | 4.38 | Added FTC at week 80 | rtP215P/S |
| GS-US-174-0103 | TDF | 006 | 9.85 | Added FTC at week 96 | rtN131D/N and rtN238D/N |
| GS-US-174-0103 | TDF | 008 | 8.30 | Week 144 | **rtR51K** |
| GS-US-174-0102 | TDF | 004 | 7.20 | Week 144 | rtT16I/T, rtT128N/T, rtF221Y, and rtT322S/T |
| GS-US-174-0103 | ADV-TDF | 011 | 7.35 | Added FTC at week 72 | rtA214A/V |
| GS-US-174-0103 | ADV-TDF | 016 | 6.66 | Added FTC at week 72 | **rtA307A/T** |
| GS-US-174-0103 | ADV-TDF | 012 | 7.56 | Added FTC at week 120 | rtR110G |
| GS-US-174-0103 | ADV-TDF | 017 | 7.01 | Week 144 | rtY124D/Y, rtT128N/T, rtS223A/S, rtH234H/Q, **rtN236N/T**,§ and **rtR274Q/R** |
| GS-US-174-0103 | ADV-TDF | 015 | 6.01 | Week 144 | rtT128N, **rtG152E**, and rtV191I |
| GS-US-174-0103 | ADV-TDF | 014 | 5.42 | Week 144 | rtT213S/T |
| GS-US-174-0103 | ADV-TDF | 013 | 4.76 | Week 144 | rtD134D/E |

*For the ADV-TDF group, the value corresponds to the week 48 (last on-ADV) visit.

†Substitutions shown in bold represent changes at conserved sites.

‡LAM-R mutations observed as a viral subpopulation (6.5%) at the baseline.

§ADV-associated resistance mutations observed as a viral subpopulation (9.3%) on ADV before the initiation of TDF.

loci: rtR51K; rtL101F/L; and rtV173L, rtL180M, and rtM204V (Fig. 1A-C). Clonal analysis of the baseline sample from the lamivudine-naive patient with lamivudine resistance mutations demonstrated the presence of the rtV173L, rtL180M, and rtM204V mutational pattern at a frequency of 6.5% with individual mutations present in up to 15% of the clones.

Phenotypic analysis of the baseline and post-baseline isolates was performed for the three patients with post-baseline conserved site changes. Because the rtL101 change was observed as a mixture, a clone containing the full rtL101F change was also phenotyped to evaluate the impact of this substitution. The pHY92 laboratory strain and the laboratory isolate containing the rtA181V and rtN236T ADV-associated mutations were used as controls for tenofovir sensitivity and reduced susceptibility, respectively. Overall, there was no change in tenofovir susceptibility within the three patients who developed conserved site changes in the pol/RT (Table 2).

***Virological Findings Among ADV-Treated Patients Receiving up to 96 Weeks of TDF Monotherapy.*** Among the 215 patients originally randomized to the ADV arms of the studies, 196 entered the OL-TDF phase. Nineteen of the 196 patients (9.7%) remained viremic after up to 96 weeks of OL-TDF; 1 discontinued TDF monotherapy at week 80, 14 patients added FTC to TDF between study weeks 72 and 120 (median time of TDF monotherapy = 30 weeks), and 4 patients received 96 weeks of TDF monotherapy. The majority of the patients (11/19, 58%) showed no change in the pol/RT versus the week 48 results (the last on-ADV results), 4 of 19 (21%) harbored polymorphic site changes, and 3 of 19 (16%) harbored distinct conserved site changes; PCR amplification failed for 1 patient (Table 1). The conserved site changes occurred at the following loci: rtG152E; rtA307A/T; and rtN236N/T and rtR274R/Q. Only rtG152E was observed in the context of confirmed virological breakthrough (Fig. 1D-F).

Phenotypic evaluations of a site-directed mutant containing the rtG152E substitution demonstrated that the virus remained susceptible to inhibition by tenofovir *in vitro* (Table 2), and the corresponding patient achieved undetectable HBV DNA levels with continued TDF monotherapy (Fig. 1D). Repeated attempts to obtain phenotypic results from either the patient pool or a clone containing the rtA307T substitution were unsuccessful, and the substitution was not observed upon subsequent genotypic testing. For patient 017, because the conserved site changes at rtN236 and rtR274 were observed as mixtures, individual clones containing the full changes were phenotyped. Phenotypic analysis of the viral pool remained sensitive to inhibition by tenofovir, as did a clone containing the single rtR274Q substitution. In contrast, a clone containing the single rtN236T substitution had reduced susceptibility to tenofovir (Table 2). Clonal analysis demonstrated that the rtN236T and rtR274Q substitutions detected at week 144 were not present on the same genome.

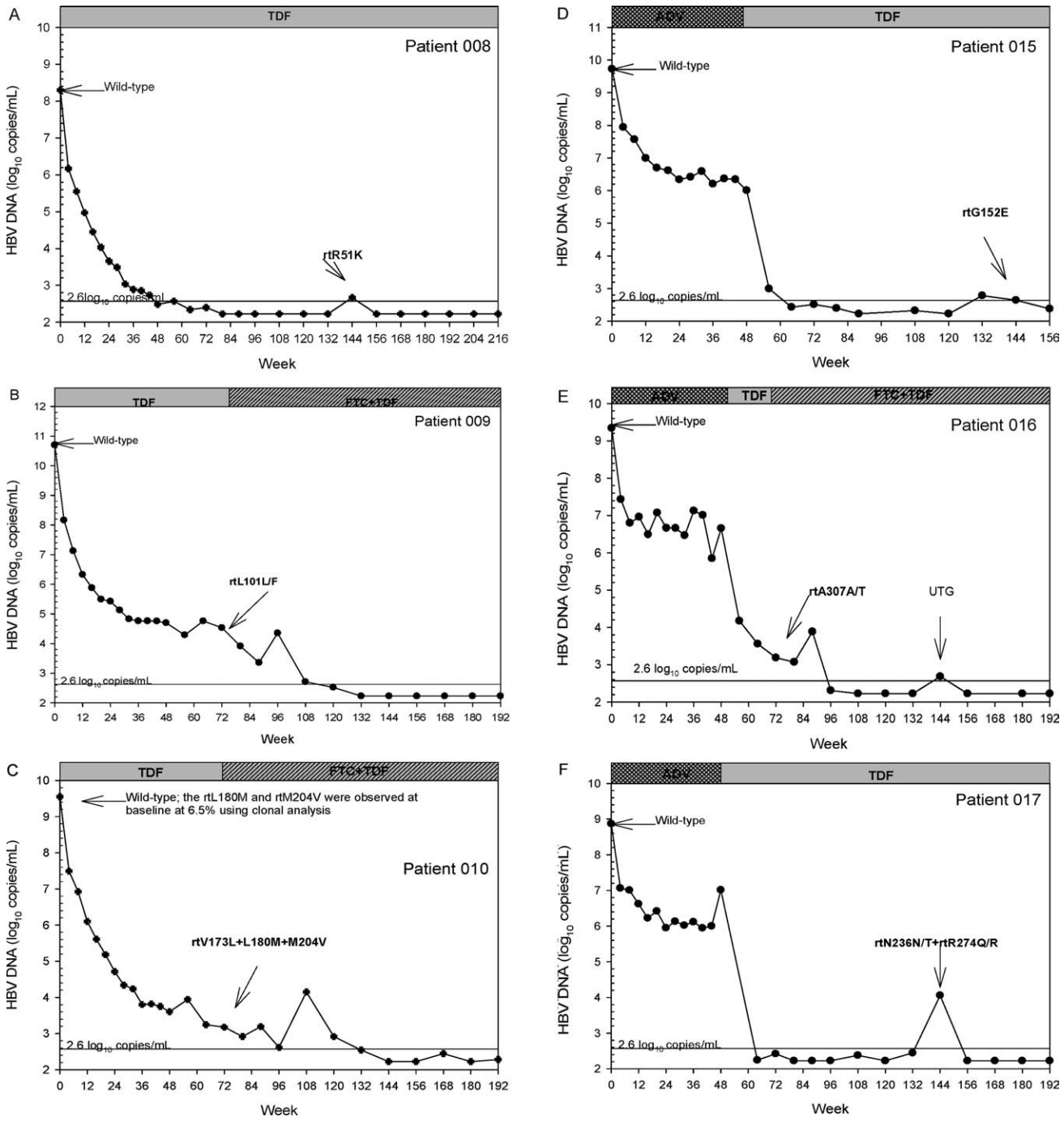


Fig. 1. HBV DNA responses through week 144 or the last on-treatment visit for patients with post-baseline conserved site changes: (A-C) patients originally randomized to TDF and (D-F) patients originally randomized to ADV.

The rtN236T was detected as a subpopulation in clinical isolates obtained from this patient between weeks 32 and 48 of ADV therapy by AS-PCR. The frequency of rtN236T was shown to increase from 0.6% at week 32 to 9.3% at week 48; HBV DNA levels became undetectable at the next study visit (week 64) after the initiation of TDF monotherapy. According to the week 48 HBV DNA level, the rtN236T mutant strain was present at a level of 5.98 $\log_{10}$ copies/mL; therefore, a switch to TDF resulted in a 3.8 $\log_{10}$ decline of the rtN236T mutant population. The rtN236T and rtR274Q substitutions were observed by population sequencing at week 144 during a period of patient-initiated drug interruption. Reintroduction of TDF monotherapy resulted in complete suppression of HBV DNA at week 156 (Fig. 2).

Two ADV-treated patients harbored a polymorphic site change (rtT128N) that was also observed in a TDF-treated patient at week 144. The virus from one

**Table 2. Phenotypic Evaluations of Patients Who Developed Conserved Site Changes in HBV pol/RT***

| | | | Tenofovir | |
|---|---|---|---|---|
| **Patient Isolate** | **Treatment Group** | **pol/RT** | **$EC_{50}$ ($\mu$M)** | **Fold Change*** |
| 008: baseline | TDF-TDF | Wild type | 7.8 ± 3.0 | |
| 008: week 144 | | rtR51K | 11.3 ± 3.0 | 1.4 |
| 009: baseline | TDF-TDF | Wild type | 12.4 ± 3.6 | |
| 009: week 72 | | rtL101L/F | 13.8 ± 0.6 | 1.1 |
| 009: week 72 | | rtL101F (clone) | 10.0 ± 6.2 | 0.8 |
| 010: baseline | TDF-TDF | Wild type | 9.9 ± 3.4 | |
| 010: week 72 | | rtV173L, rtL180M, and rtM204V | 12.5 ± 6.3 | 1.3 |
| 015: week 144 | ADV-TDF | rtG152E (pHY92)† | 28.4 ± 5.3 | 1.8 |
| 017: week 40 | ADV-TDF | Wild type (pool) | 6.7 ± 2.7 | 1.0 |
| 017: week 144 | | rtN236T ± rtR274Q (pool) | 12.5 ± 3.4 | 1.9 |
| 017: week 144 | | rtR274Q (clone 11) | 13.0 ± 0.3 | 1.9 |
| 017: week 144 | | rtN236T (clone 3) | 55.3 ± 15.4 | 8.2 |
| 017: week 144 | | rtT128N (clone1) | 7.3 ± 5.0 | 1.1 |
| 017: week 144 | | rtR274Q ± rtT128N (clone 11) | 13.0 ± 0.3 | 1.9 |
| 006: baseline | TDF-FTC/TDF | Wild type | 14.3 ± 1.5 | |
| 006: week 144 | | rtA181T | 9.7 ± 1.6 | 0.7 |
| 026: baseline | TDF-FTC/TDF | Wild type | ND | |
| 026: week 144 | | rtR192H | Replication-defective | N/A |
| 026: week 144 | | rtR192H (pCMVHBV) | Replication-defective | N/A |
| | | | Tenofovir | |
| | | | $EC_{50}$ ($\mu$M) | Fold Change* |
| pHY92 | | Wild type | 14.0 ± 3.7 | |
| ADV-R | | rtA181V and rtN236T | 43.3 ± 9.4 | 3.5 |

Results are presented as means and standard deviations from three independent tests.

Abbreviations: N/A, not applicable; ND, not determined/unable to amplify the hepatitis B virus genome.

*Defined as $EC_{50}$ of the last on-treatment sample/$EC_{50}$ of the baseline sample. Values ≤2-fold are within the assay variability.

†The fold change was calculated on the basis of the pHY92 wild-type laboratory strain due to the creation of the site-directed mutant.

of these patients also harbored the rtN236T and rtR274Q conserved site changes; rtT128N was observed alone and in clones containing rtR274Q, and it was never observed in conjunction with rtN236T. Phenotypic analysis of clones containing rtT128N alone or in conjunction with rtR274Q demonstrated no change in tenofovir susceptibility (Table 2). The rtT128N substitution was observed in approximately 2% of patients at the baseline across studies GS-US-174-0102 and GS-US-174-0103, and this polymorphism did not have an impact on the TDF treatment response ($P > 0.05$).

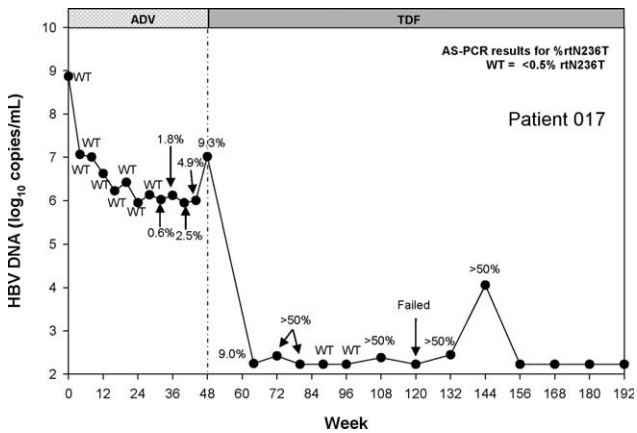


Fig. 2. Evolution of low levels of rtN236T with ADV-TDF therapy: case study of patient 017 by AS-PCR. Abbreviation, WT, wild type.

***Virological Findings Among Patients Who Added FTC to OL-TDF.*** Per the clinical protocol, patients with confirmed viremia at or after week 72 were eligible to add FTC to their OL-TDF regimen. Of the 641 randomized and treated patients, 51 (29 and 22 in the TDF and ADV-TDF arms, respectively) met this criteria before week 144; 17 of 51 (33%; 9 and 8 in the TDF and ADV-TDF arms, respectively) continued on TDF monotherapy; and 34 of 51 (67%; 20 and 14 in the TDF and ADV-TDF arms, respectively) added FTC between weeks 72 and 120. The addition of FTC did not appear to affect the subsequent decline in HBV DNA because 12 of 17 patients (71%) who remained on TDF monotherapy versus 22 of 34 patients (65%) who added FTC had HBV DNA levels < 400 copies/mL at week 144 or at their last study visit (Fig. 3A). Interestingly, for those patients with declining HBV DNA levels on TDF monotherapy who added FTC, there was no apparent change in the rate of HBV DNA decline versus the rate before FTC addition (Fig. 3B,C). Because the addition of FTC to OL-TDF was at the investigator's discretion, there were instances when patients had similar HBV DNA profiles but one patient maintained TDF monotherapy

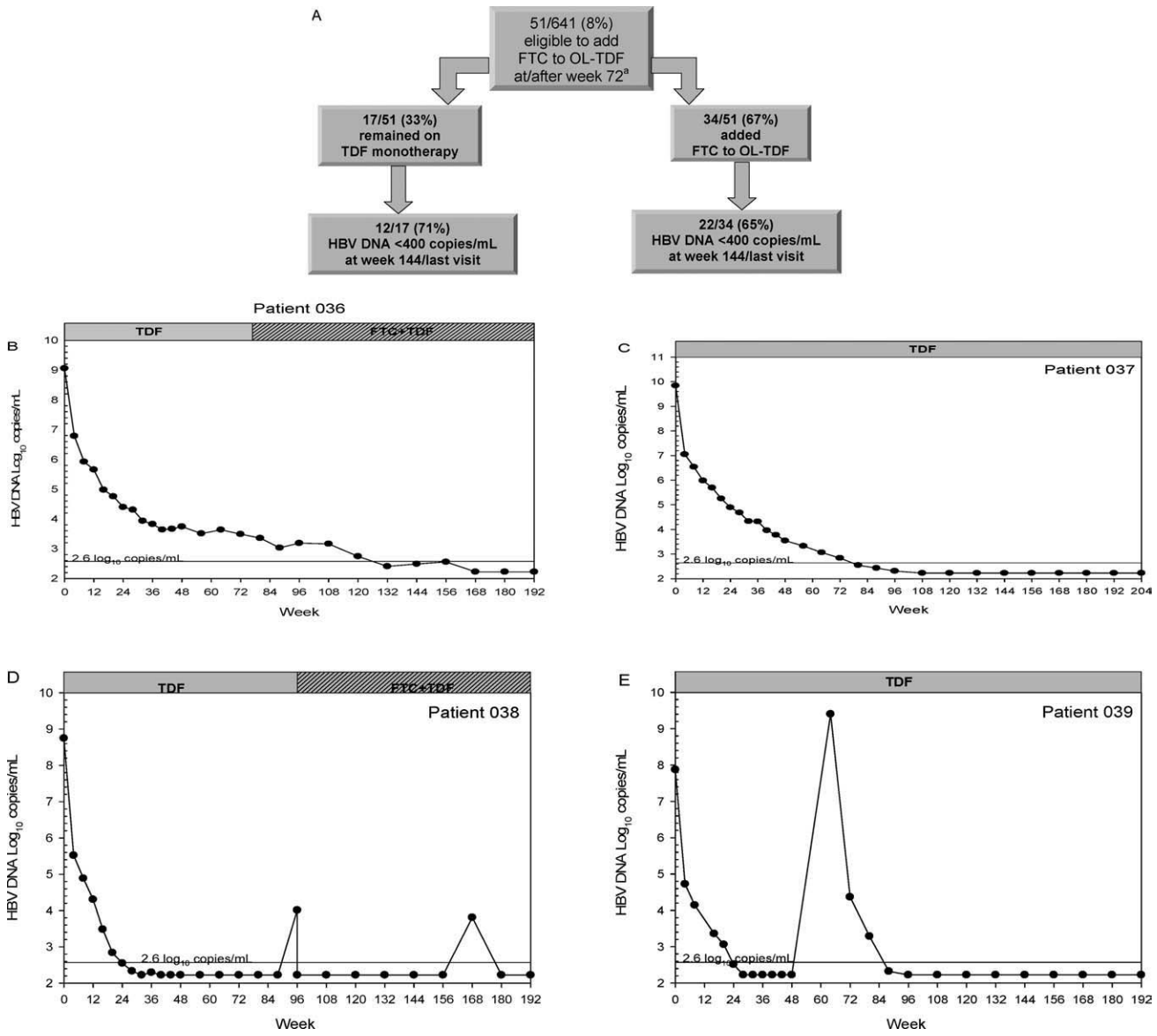


Fig. 3. The addition of FTC to TDF between weeks 72 and 144 did not affect the subsequent decline in HBV DNA in comparison with maintenance of TDF monotherapy. (A) Disposition of viremic patients who opted to add or not add FTC to OL-TDF between weeks 72 and 144. [a]Patients had the option, at the discretion of the investigator, to add FTC (200 mg) to OL-TDF (300 mg) if they were confirmed to be viremic ($\geq$400 copies/mL) at week 72 or beyond. No patient was found to have resistance to tenofovir before the addition of FTC. (B-E) HBV DNA profiles of patients who added FTC or maintained TDF monotherapy.

and the other switched to combination therapy (Fig. 3D,E). Regardless, both patients achieved undetectable HBV DNA levels whether they maintained TDF monotherapy or switched to combination therapy.

Among the 34 patients who added FTC, 12 remained viremic on their last evaluable visit through week 144 (median duration of combination therapy = 59 weeks, range = 25-70 weeks); PCR amplification failed for 1; 5 showed no change in the pol/RT versus the last observation while they were on TDF monotherapy; 4 harbored distinct polymorphic site changes; and 2 developed conserved site changes (rtL180L/M, rtA181T, and rtM204M/V and rtR192H; Table 3). Clonal analysis of the baseline sample for the patient harboring the rtL180L/M, rtA181T, and rtM204M/V mutations demonstrated the presence of these mutations as subpopulations on separate genomes (rtL180M and rtM204V at 3.7% and rtA181T at 7.4%). Phenotypic analysis of the viral pool containing the rtA181T mutation demonstrated that the virus was fully sensitive to inhibition by tenofovir (Table 2). Clones containing the rtL180M and rtM204V mutations could not be obtained with the PCR primers used for phenotyping. For patient 026, the viral

**Table 3. Amino Acid Substitutions Among Viremic Patients Who Added FTC to OL-TDF**

| Study | Original Treatment | Patient ID | Genotypic Findings at Last On–TDF Monotherapy Visit | Week 144 Genotypic Findings (on FTC/TDF) |
|---|---|---|---|---|
| GS-US-174-0103 | ADV-TDF | 016 | **rtA307A/T** | Unable to genotype |
| GS-US-174-0103 | ADV-TDF | 018 | No change | No change |
| GS-US-174-0103 | ADV-TDF | 019 | No change | No change |
| GS-US-174-0103 | TDF | 020 | No change | No change |
| GS-US-174-0103 | TDF | 021 | No change | No change |
| GS-US-174-0103 | ADV-TDF | 022 | Unable to genotype | No change |
| GS-US-174-0103 | ADV-TDF | 023 | No change | rtR18K/R and rtL91I/L |
| GS-US-174-0103 | TDF | 024 | No change | rtY122S/Y |
| GS-US-174-0103 | TDF | 025 | No change | rtN139K/N |
| GS-US-174-0103 | TDF | 007 | rtL145L/M | rtT78S, rtV191I, and rtN238H |
| GS-US-174-0103 | TDF | 006 | rtN131D/N and rtN238D/N | rtY124H/Y, rtN139N/T, **rtL180L/M**, **rtA181T**, **rtM204M/V**, rtS213S/T, rtS317L/S, and rtN337H/N |
| GS-US-174-0103 | TDF | 026 | Unable to genotype | **rtR192H** |

Substitutions shown in bold represent changes at conserved sites.

quasispecies pool, individual clones (n = 7), and an rtR192H site-directed mutant in the pCMVHBV backbone were all replication-defective in a cell culture (Table 2).

***Virological Breakthrough on TDF Therapy.*** Thirteen patients experienced a confirmed virological breakthrough (10 and 3 in the TDF and ADV-TDF arms, respectively) during the 144 weeks of cumulative exposure to TDF monotherapy; nonadherence to the study medication contributed to the majority of the virological breakthrough events (11/13, 85%), and all patients experiencing virological breakthrough remained phenotypically sensitive to inhibition by tenofovir (Table 4). Four patients experienced virological breakthrough while they were on combination FTC/TDF therapy (three and one in the TDF and ADV-TDF arms, respectively), virological breakthrough could be attributed to nonadherence in two of the four patients, and the virus obtained from these patients remained phenotypically sensitive to inhibition by tenofovir and FTC (Table 4).

## Discussion

We performed extensive genotypic and phenotypic analyses of 641 $HBeAg^+$ and $HBeAg^-$ patients who received up to 144 weeks of TDF therapy. We identified six previously undescribed conserved site changes in the HBV pol/RT. These novel conserved site changes were located in areas of high variability within the HBV genome.[19] None of these changes appeared to be related to tenofovir resistance, as demonstrated by the lack of phenotypic resistance to tenofovir *in vitro*, nor were they associated with a confirmed virological breakthrough. Phenotypic analysis was also performed for patients who experienced virological breakthrough because this can be a hallmark of resistance development. All of the viruses tested remained phenotypically sensitive to inhibition by tenofovir; this is consistent with the genotypic findings, which demonstrated no changes in the pol/RT among these patients. Up to 30% of virological breakthroughs observed in clinical trials may be related to medication nonadherence.[20] In our studies, virological breakthrough was infrequent and occurred in only 3% of patients on TDF monotherapy; the vast majority of these patients (85%) were shown to have a documented history of nonadherence.

In our analysis of baseline samples from $HBeAg^-$ and $HBeAg^+$ patients in these studies, the frequency of HBV pol/RT polymorphic sites was determined to be approximately 35%,[18] which is comparable to the findings of other analyses.[19] According to our week 48 analysis, no naturally occurring baseline polymorphisms were associated with a reduced virological response to TDF in either $HBeAg^+$ or $HBeAg^-$ patients.[18] Only one polymorphic site change (rtT128N) was observed to develop in more than one patient on TDF monotherapy. This substitution did not result in phenotypic resistance to tenofovir, nor did it have an impact on the TDF treatment response, as observed among the 2.7% of patients who had this baseline polymorphism across both studies. This change corresponds to the sP120T substitution in the overlapping S gene and is considered a vaccine escape mutation.[21] This substitution has also been studied in the context of lamivudine resistance in previous studies showing that the rtT128N/sP120T substitution partially restores the *in vitro* replication phenotype of lamivudine resistance.[21]

The clinical study design allowed viremic patients to add FTC to their OL-TDF regimen at or after week 72. This option was put in place at a time when data

**Table 4. Cumulative Summary of TDF-Treated Patients Who Experienced Virological Breakthrough**

| Study | Patient ID | Baseline HBV DNA ($log_{10}$ Copies/mL)†‡ | TDF Nadir HBV DNA ($log_{10}$ Copies/mL) | Treatment | Breakthrough Week | Documented Nonadherence? | Changes in HBV pol/RT Versus the Baseline†‡ | Fold Change in $EC_{50}$ for Tenofovir* |
|---|---|---|---|---|---|---|---|---|
| GS-174-0102 | 027 | 7.45 | 3.64 | TDF | Week 16 | Yes | rtD134N, rtT213S, rtI220L, and rtH221Y | 0.7 |
| GS-174-0102 | 001 | 7.06 | 3.74 | TDF | Week 28 | Yes | None | 0.9 |
| GS-174-0102 | 028 | 6.89 | 2.23 | TDF | Week 28 | Yes | None | ND |
| GS-174-0103 | 029 | 8.87 | 4.24 | TDF | Week 28 | Yes | None | 1.2 |
| GS-174-0103 | 005 | 8.76 | 2.63 | TDF | Week 64 | Yes | rtF221F/Y | 1.0 |
| GS-174-0102 | 033 | 7.55 | 2.23 | TDF | Week 80 | Yes | None | 0.9 |
| GS-174-0102 | 003 | 4.38 | 2.23 | TDF | Week 80 | Yes | rtP215P/S | 0.8 |
| GS-174-0103 | 020 | 9.90 | 3.70 | TDF | Week 80 | Yes | None | 1.0 |
| GS-174-0102 | 034 | 7.87 | 2.23 | TDF | Week 96 | Yes | None | 1.1 |
| GS-174-0102 | 035 | 7.65 | 3.13 | TDF | Week 132 | No | Unable to genotype | ND |
| GS-174-0103 | 023 | 8.87 | 6.09 | ADV-TDF | Week 64 | Yes | None | N/A§ |
| GS-174-0103 | 015 | 6.01 | 2.64 | ADV-TDF | Week 132 | No | rtT128N, **rtG152E**, and rtV191I | 1.8 |
| GS-174-0103 | 014 | 5.42 | 8.56 | ADV-TDF | Week 144 | Yes | rtT213S/T | N/A‖ |
| GS-174-0103 | 021 | 6.43 | 9.16 | TDF-FTC/TDF | Week 108 (on FTC/TDF) | Yes | None | ND |
| GS-174-0103 | 024 | 3.28 | 3.60 | TDF-FTC/TDF | Week 120 (on FTC/TDF) | No | rtY122S/Y | ND |
| GS-174-0103 | 025 | 3.14 | 2.75 | TDF-FTC/TDF | Week 132 (on FTC/TDF) | Yes | rtN139K/N | ND |
| GS-174-0103 | 018 | 5.96 | 3.05 | ADV-TDF-FTC/TDF | Week 120 (on FTC/TDF) | No | None | 0.7 |

Conserved site changes are shown in bold.

Abbreviations: N/A, not applicable; ND, not determined/unable to amplify the hepatitis B virus genome for use in the phenotyping assay.

*Values $\leq$2-fold are not significant.

†For patients in the ADV arm, this value is the week 48 (last on-ADV) viral load.

‡For patients who added FTC to OL-TDF, this value is the last on–TDF monotherapy viral load.

§Virological breakthrough was not confirmed during TDF monotherapy. Phenotypic evaluations were not conducted for this patient because of unconfirmed virological breakthrough.

‖Phenotypic evaluations were not conducted for this patient because of nonadherence.

demonstrating TDF efficacy and the high threshold against developing resistance to TDF were not known. The option of adding FTC to TDF therapy for viremic patients reflected clinical practice at the time[20] and was intended to minimize the risk of resistance for those patients who remained viremic. In retrospect, the week 72 time point was perhaps too early for the change to combination antiviral therapy because the majority of patients with an incomplete virological response at week 72 who did not add FTC continued to show a decline in HBV DNA levels and achieved <400 copies/mL by week 144. Furthermore, there was no apparent change in the rate of HBV DNA decline versus the rate before the addition of FTC for those patients who did. Although the addition of FTC in patients with an incomplete response could potentially mask the development of resistance mutations, the majority of patients enrolled in these studies remained on TDF monotherapy (607/641, 95%), and resistance was not detected among any of these monotherapy patients. Furthermore, genotypic and phenotypic evaluations conducted among patients with viremia on FTC/TDF combination therapy did not demonstrate the development of TDF resistance mutations.

Both LAM-R and ADV-associated resistance mutations were observed among patients in these studies. Other studies have also described the persistence of both lamivudine-associated and adefovir-associated mutations in patients treated with TDF.[22,23] The recombinant virus containing rtL180M and rtM204V remained phenotypically sensitive to inhibition by tenofovir *in vitro*; therefore the persistence of the mutant virus population may have been due to a fitness advantage.[24] Interestingly, after a >6.0 $log_{10}$ reduction in HBV DNA on TDF monotherapy, the patient subsequently achieved an additional 1.0 $log_{10}$ reduction in HBV DNA to <169 copies/mL after the switch to FTC/TDF therapy. Because of the presence of the rtL180M$\pm$rtM204V mutations, it is unclear whether the presence of FTC (which selects for the same mutations as lamivudine) was contributing to the continued HBV DNA decline in this patient. One ADV-TDF–treated patient harbored the rtN236T mutation after 96 weeks of TDF monotherapy. The mutation was observed during a period of transient virological breakthrough while the patient was off the drug, and the reintroduction of TDF monotherapy resulted in a subsequent HBV DNA decline to undetectable levels. Individual clones containing rtN236T from this patient demonstrated a reduced susceptibility to tenofovir *in vitro*, and this is in agreement with previous studies showing cross-resistance to tenofovir *in vitro* by

the ADV-associated rtN236T mutation.[12,25] The clinical significance of these changes in the 50% effective concentration ($EC_{50}$) values is unclear because the patient subsequently achieved undetectable levels of HBV DNA on TDF monotherapy. On the basis of the level of the mutant virus within the patient's overall viral population, we estimated that TDF treatment resulted in a 3.8 $\log_{10}$ decrease in the rtN236T virus population. Furthermore, HBV DNA became undetectable within 16 weeks after the switch from ADV to TDF monotherapy and remained undetectable with TDF monotherapy through week 192. This is in contrast to recently published data on the activity of TDF in patients with ADV–associated resistance (ADV-R) mutations. In a retrospective study of 131 HBV-monoinfected patients who had experienced failure on previous nucleoside/nucleotide therapy, the presence of ADV-R appeared to impair the activity of TDF in comparison with the activity of TDF in patients without ADV-R.[13] The patients with ADV-R mutations in that study had a significantly higher viral load than the one patient in our study, and the authors pointed out that the high viral load may have had an impact on the treatment response because no particular pattern of ADV-R mutations appeared to have an impact on the TDF response. In a separate prospective study of TDF in ADV-refractory patients, the presence of ADV-R mutations did not have an impact on the response to TDF therapy.[26] In this study, baseline resistance patterns were not associated with the type of response to TDF; a rapid response to <400 copies/mL was correlated with a low baseline viral load ($P < 0.05$).[27]

In conclusion, viral resistance to nucleos(t)ide analogues during the long-term treatment of CHB can pose a major problem in patient management, with poor adherence adding to the key risk factors of treatment failure and subsequent reversal of clinical improvement. Consequently, there is a need for antiviral compounds such as TDF, which is a well-tolerated, potent therapy with a high threshold for resistance development. The addition of FTC occurred in only 5% of patients and did not appear to affect the subsequent rate of HBV DNA decline because comparable declines occurred in eligible patients who opted to remain on TDF monotherapy. Our analysis demonstrated no detectable TDF resistance among 641 $HBeAg^+$ and $HBeAg^-$ patients with CHB infection who received TDF for up to 144 weeks.

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