# Lamivudine/Adefovir Treatment Increases the Rate of Spontaneous Mutation of Hepatitis B Virus in Patients

**Marianoel Pereira-Gómez[1], Juan-Vicente Bou[1], Iván Andreu[1], Rafael Sanjuán[1,2]***

**1** Institute for Integrative Systems Biology (I2SysBio), Universitat de València, València, Spain,
**2** Departament de Genètica, Universitat de València, València, Spain

\* rafael.sanjuan@uv.es

## Abstract

The high levels of genetic diversity shown by hepatitis B virus (HBV) are commonly attributed to the low fidelity of its polymerase. However, the rate of spontaneous mutation of human HBV in vivo is currently unknown. Here, based on the evolutionary principle that the population frequency of lethal mutations equals the rate at which they are produced, we have estimated the mutation rate of HBV in vivo by scoring premature stop codons in 621 publicly available, full-length, molecular clone sequences derived from patients. This yielded an estimate of $8.7 \times 10^{-5}$ spontaneous mutations per nucleotide per cell infection in untreated patients, which should be taken as an upper limit estimate because PCR errors and/or lack of effective lethality may inflate observed mutation frequencies. We found that, in patients undergoing lamivudine/adefovir treatment, the HBV mutation rate was elevated by more than sixfold, revealing a mutagenic effect of this treatment. Genome-wide analysis of single-nucleotide polymorphisms indicated that lamivudine/adefovir treatment increases the fraction of A/T-to-G/C base substitutions, consistent with recent work showing similar effects of lamivudine in cellular DNA. Based on these data, the rate at which HBV produces new genetic variants in treated patients is similar to or even higher than in RNA viruses.

## Introduction

With around 250 million people infected worldwide, hepatitis B virus (HBV) constitutes a major cause of cirrhosis, liver failure and hepatocellular carcinoma [1]. HBV has a small (3.2 kb), partially double-stranded, circular DNA virus that is primarily encapsidated as a pre-genomic RNA that undergoes reverse transcription after encapsidation [2,3]. The HBV genome is extremely compact, with four, partially or fully overlapped open reading frames (C, S, P, and X). The preC and C reading frames encode the *e* antigen (HBeAg) and core protein (HBcAg) respectively, the preS and S reading frames encode three forms of surface proteins (HBsAg) sharing the C-terminus (small, middle and large), the P gene encodes the viral polymerase, which acts both as reverse transcriptase (RT) and DNA-dependent DNA polymerase, and the X gene encodes a transcriptional trans-activator protein. HBV treatment consists of immune

modulators (interferon) combined with nucleoside analogues whose primary effect is to inhibit the viral polymerase [4,5]. However, treatment typically fails to clear the virus, which remains active in the form covalently closed circular DNA within hepatocyte nuclei [6], and long-term treatment with nucleoside analogues often selects for drug-resistant mutations in the HBV polymerase [7].

HBV shows a molecular evolutionary rate on the order of $10^{-4}$ nucleotide substitutions per site per year (s/s/y) at the epidemiological scale [8,9], and of $10^{-5}$–$10^{-4}$ s/s/y at the intra-patient level [10,11]. This rate is notably higher than those of most non-reverse transcribing DNA viruses, and similar to those of RNA viruses and retroviruses [12]. It is commonly accepted that such fast evolution is determined ultimately by the low replication fidelity the HBV polymerase which, similar to other RTs, lacks 3′exonclease proofreading activity [13] and produces frequent replication errors, resulting in highly diverse viral populations [14–17]. Despite growing interest in studying the relationship between HBV genetic variation and viral pathogenesis, as well as its implications for viral detection, prevention, treatment and prognosis, the rate of spontaneous mutation of human HBV has not been determined. Notice that, whereas the molecular evolutionary rate refers to a population genetics process, the rate of spontaneous mutation refers to a biochemical process and, therefore, these two rates should not be confused. The former describes the fixation of new alleles in a population and is determined by factors such as selection and random drift, whereas the latter is defined as the probability that new mutations appear per round of genome copying, or per cell infection cycle, and is determined by factors such as polymerase fidelity, DNA/RNA editing, and spontaneous damage. Early work with the related duck HBV estimated the reversion frequency of a single deleterious G-to-A nucleotide substitution [18] and, after correcting for several possible confounders such selection and the unknown number of rounds of replication, this yielded a mutation rate estimate of $2 \times 10^{-5}$ per nucleotide per cell infection cycle (m/n/c) [19]. However, the reliability of this value was compromised by the fact that only a single genome site was analyzed.

Another unaddressed question is the effect of treatment on the HBV mutation rate. Broad-spectrum nucleoside analogues such as ribavirin are known to mutagenize RNA viruses [20–25]. Similarly, nucleoside analogue RT inhibitors such as lamivudine and AZT have a direct mutagenic effect on HIV-1 in cell culture, in addition to their inhibitory effects [26]. Moreover, treatment with nucleoside analogues can select for resistance mutations that modify the replication fidelity of the HIV-1 RT, producing an additional, indirect effect on the viral mutation rate [27,28]. In HBV, analysis of nucleotide misincorporation kinetics in vitro showed that typical lamivudine-resistant polymerase variants such as M204I and M204V display increased replication fidelity in the absence of the drug [13], suggesting a mutagenic effect for lamivudine in patients. Nucleoside analogue RT inhibitors including lamivudine have also been found to be mutagenic in different cell lines, as shown by analysis of reporter cellular genes [29,30].

Rates of spontaneous mutation can be inferred in vivo from intra-host sequence diversity data using the lethal mutation method, which is based on the principle that the population frequency of mutations that abolish viral infectivity (lethal mutations) equals the rate at which they are produced, as these cannot be inherited [31]. Using premature stop codons in essential genes as a proxy to lethal mutations, we and others have previously used this method for estimating the mutation rate of hepatitis C virus (HCV) [21,32] and HIV-1 [33] from patient-derived sequences. Here, we applied the lethal mutation method to publicly available, full-length, molecular clone HBV sequences derived from untreated patients, as well as from patients undergoing long-term lamivudine/adefovir combination therapy. We obtained an estimated spontaneous rate on the order of $10^{-5}$ m/n/c for HBV, albeit with ample variations among HBV reading frames. Furthermore, we show that lamivudine/adefovir elevates the spontaneous HBV mutation rate by at least sixfold. Analysis of intra-host sequence diversity

indicated that lamivudine/adefovir treatment produces a shift in the HBV mutation spectrum whereby A-to-G and the complementary T-to-C base transitions are specifically elevated.

## Material and Methods

### Data

We searched molecular clone full-length sequences deposited in GenBank. Publications were first browsed in PubMed based on the following electronic search strategy: ((hepatitis b virus) AND genome*) AND (((clone*) OR cloning) OR full-length). This allowed us to retrieve a large number of articles, which were then inspected to collect only sequences that explicitly included the method used to obtain HBV molecular clones. Also, if fewer than three sequences per patient were analyzed, these were discarded. All sequences corresponded to plasma or serum samples, except for AF182805 and AF182804, which were obtained from a resected peri-tumor liver tissue and a hepatocellular carcinoma, respectively. Sequences were aligned using the MUSCLE algorithm (www.drive5.com/muscle) and refined further by visual inspection. A neighbor-joining tree was obtained with MEGA6 (www.megasoftware.net) to assess whether patients corresponded to monophyletic sequence groups, and the HBV genotype was determined using the genotyping tool available from the French HBV database (hbvdb.ibcp.fr). Sequences were visualized with MEGA6 and Jalview (www.jalview.org) and phylogenetic trees were edited with TreeDyn (www.treedyn.org).

### Mutation rate estimation by the lethal mutation method

The number of sites that can mutate to a stop codon after a single base substitution (non-sense mutation targets, NSMTs), as well as the observed premature stop codons, were extracted from sequences. For each stop codon mutation, we identified the NSMT and calculated a correction factor $C$ that accounts for the fact that there are three possible substitutions for each base ($C = 3$) and that, in some NSMTs, two of these substitutions lead to a stop codon ($C = 3/2$) [21]. These steps were performed using a custom R script. The preS1/preS2 and preC regions were excluded from the analysis because stop codons at these regions are not necessarily lethal and have been implicated in immune escape and pathogenesis [34,35]. For the other reading frames, we discarded sequences in which the stop codon was located in the 5% end of the protein, that lacked AUG initiation codon, with frameshift mutations, or with more than one stop codon. The mutation rate was calculated as the sum of stop codons, each multiplied by the correction factor $C$, divided by the total number of NSMTs.

### Overall mutation spectrum

The consensus sequence of each patient was obtained and differences relative to the consensus were counted for each sequence within this patient and for each possible substitution type, using a custom R script.

## Results

We analyzed 621 full-length molecular clone sequences obtained from seven studies [36–42], of which 451 corresponded to 22 untreated patients and 170 to 24 patients receiving long-term lamivudine/adefovir therapy (Table 1). We initially scored premature stop codons in the S, C, P, and X genes as a proxy for lethal mutations. In total, we found 23 premature stops in the S, C, P, and X genes in sequences derived from untreated patients (S1 Table). Fig 1 provides an alignment of premature stop codon-containing sequences for gene C. Mutations were not evenly distributed along the genome, since the S gene accumulated 18 of the 23 mutations

**Table 1. Dataset used and number of sequences analyzed in each patient.**

| Dataset | Treated | HBV genotype | Patient IDs (sequences)[a] | Ref. |
|---|---|---|---|---|
| I[b] | No | B | N1 (31), N2 (31), N3 (33), N4 (30), N5 (30), N6 (35), N7 (35) | [36] |
| II[c] | No | A/C/G | N8 (9), N9 (5) | [37] |
| III[d] | No | C | N10 (20) | [38] |
| IV[e] | No | C | N11 (4) | [39] |
| V[f] | No | C | N12 (3), N13 (4), N14 (4), N15 (3) | [41] |
| VI[g] | No | B | N16 (24), N17 (14), N18(44), N19 (11), N20 (26), N21 (37), N22 (18) | [40] |
| VII[h] | Yes | C | T1 (9), T2 (9), T3 (8), T4 (5), T5 (3), T6 (9), T7 (6), T8 (8), T9 (9), T10 (4), T11 (9), T12 (6), T13 (7), T14 (10), T15 (8), T16 (9), T17 (6), T18 (10), T19 (5), T20 (3), T21(7), T22 (3), T23 (8), T24(9) | [42] |

[a]Patient IDs created for this study, number of sequences retrieved for each patient shown in parentheses.

[b]Chronically infected subjects infected for >10 years; Stratagene Easy-A High Fidelity PCR DNApol was used (error rate: $1.3 \times 10^{-6}$). Accessions: GU815646-54, GU815656-70, GU815672-8, GU815548-78, GU815579, GU815581, GU815583-GU815591, GU815593-614, GU815616-45, GU815679-81, GU815684-9, GU815691-710, GU815711-3, GU815715-46, GU815747-57, GU815759-62, GU815764-83.

[c]Chronically infected man (33 years old) and woman (24 years old); Taq pol was used for PCR (error rate: $8 \times 10^{-6}$). Recombinant viral genotype. Accessions: HQ231877-85, KF425553-57.

[d]Chronically infected 31 years-old woman infected for >7 years; ex Tal pol was used for PCR (error rate: $1.8 \times 10^{-6}$). Accessions: DQ377160-5, EU306713, EU306722, EU306724-9, EU439005, EU439008, EU439010-2, EU439025.

[e]Chronically infected 48 years-old man, with two sampling dates; Taq pol was used for PCR (error rate: $8 \times 10^{-6}$). Accessions: AF182802-5.

[f]Three of four patients had occult hepatitis; nested PCR was required; LA Taq pol was used for PCR (error rate: $1.3 \times 10^{-6}$). Accessions: EU916223-5, EU916226-9, EU916215-8, EU916239-41.

[g]Chronically infected subjects through vertical transmission; Stratagene Easy-A High Fidelity PCR DNApol was used (error rate: $1.3 \times 10^{-6}$). Accessions: KP406220-43, KP406162-75, KP406176-219, KP406244-54, KP406255-80, KP406281-317, KP406318-335.

[h]Chronically infected subjects treated for over two years; LA Taq was used for PCR (error rate: $1.3 \times 10^{-6}$). Accessions: KR013835-41, KR014021, KR014023, KR013820-6, KR013995, KR013997, KR013854-7, KR014035-9, KR013770, KR013900, KR013902-3, KR013905, KR013817-19, KR013878-9, KR013881-6, KR013890, KR013777-8, KR013913, KR013915, KR013919, KR013923, KR013842-9, KR013792-6, KR013954, KR013956-8, KR013850-3, KR013799-801, KR013968-9, KR013972, KR013974-5, KR013977, KR013858, KR014046, KR014048-9, KR013771-4, KR013776, KR013908-9, KR013809-16, KR013985-6, KR013828-34, KR014019, KR013859-62, KR014055-8, KR014060, KR013872-7, KR013802-8, KR013982-4, KR013827, KR013998, KR014000, KR014006-7, KR013791, KR013943, KR013948, KR013788-90, KR013938-9, KR013941-2, KR013797-8, KR013961, KR013779-86, KR013761-9.

doi:10.1371/journal.pone.0163363.t001
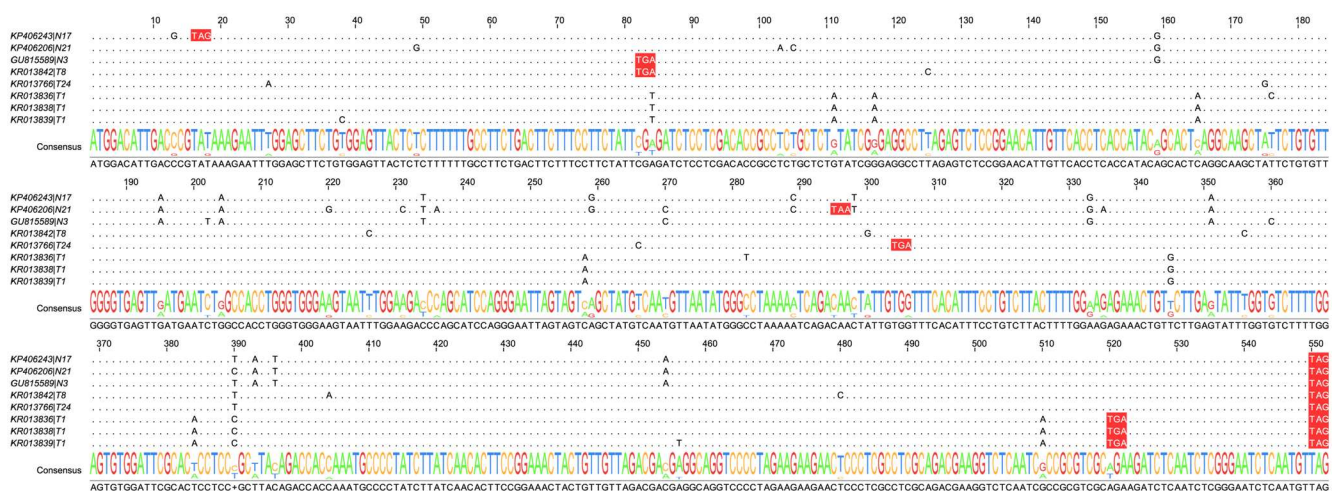


**Fig 1. Premature stop-codon mutations found in HBV gene C.** Sequence labels indicate the GenBank accession and the patient code according to Table 1. Below is shown the consensus sequence and the colored sequence logo summarizes variability at each position. Only variable sites are shown in the patient sequences, and stop codons are highlighted in red. As also indicated on Table 2, some stop codons appeared repeatedly in the same patient, or even in different patients. Similar alignments could be obtained for the other HBV genes by retrieving GenBank accession numbers from S1 Table.

doi:10.1371/journal.pone.0163363.g001

**Table 2. Summary of stop codons found in each HBV gene.**

| Gene | Treated | NSMTs | Stops (total) | Rate (total) | Stops (unique) | Rate(unique) |
|:---:|:---:|:---:|:---:|:---:|:---:|:---:|
| **S** | No | 36197 | 18 | $1.5 \times 10^{-3}$ | 9 | $7.5 \times 10^{-4}$ |
| **P** | | 110138 | 1 | $2.7 \times 10^{-5}$ | 1 | $2.7 \times 10^{-5}$ |
| **X** | | 17593 | 1 | $1.7 \times 10^{-4}$ | 1 | $1.7 \times 10^{-4}$ |
| **C** | | 27062 | 3 | $2.8 \times 10^{-4}$ | 3 | $2.8 \times 10^{-4}$ |
| **S** | Yes | 12928 | 17 | $3.8 \times 10^{-3}$ | 5 | $1.0 \times 10^{-3}$ |
| **P** | | 40179 | 11 | $8.2 \times 10^{-4}$ | 6 | $4.5 \times 10^{-4}$ |
| **X** | | 7030 | 5 | $2.1 \times 10^{-3}$ | 2 | $8.5 \times 10^{-4}$ |
| **C** | | 9718 | 5 | $1.5 \times 10^{-3}$ | 2 | $6.1 \times 10^{-4}$ |

doi:10.1371/journal.pone.0163363.t002

(Table 2). Based on these anomalously high counts and on previous findings suggesting that S proteins with stop codons can be maintained at high frequencies in patients [43–46], we removed this gene from subsequent analyses. Considering the counts for genes C, P, and X, the estimated HBV mutation rate was $8.7 \times 10^{-5}$ per nucleotide per cell infection cycle. This value is within the same order of magnitude as the rate reported for duck HBV and is also within the range of mutation rates reported for RNA viruses and retroviruses [18,19].

Although there were fewer available sequences for analysis in treated patients we found 21 premature stops in the C, P and X genes for this group, a significantly elevated frequency compared with untreated patients (S1 Table; Fisher exact test: $p < 0.001$). The estimated mutation rate for treated patients was $1.1 \times 10^{-3}$ m/n/c, i.e. 12-fold higher than in untreated patients. In the treated group, we also found that several stop codons appeared repeatedly in the same or different patients, suggesting non-lethality or the presence of mutational hotspots. Removing these cases, there were still 10 stop codons in treated patients, yielding a mutation rate estimate of $5.2 \times 10^{-4}$ m/n/c, a value still 6-fold higher than in untreated patients (Fisher exact test: $p < 0.001$).

These results suggest that lamivudine/adefovir treatment has a marked effect on the mutation rate of HBV in vivo. However, several possible confounders may have affected our conclusion. First, we found limited information about patient infection times, but this should not represent a major source of error because the lethal mutation rate estimation method is not sensitive to the number of viral generations elapsed. Second, some of the observed mutations might have been introduced during PCR amplification. However, for all patients except three untreated, high-fidelity polymerases with similar error rates were used (Table 1), making it unlikely that the observed differences were driven by PCR errors. Third, as mentioned above, stop-codon counts were not homogeneous among genes. However, the fact that we used always full-length genome sequences should make differences between treated and non-treated patients robust to among-gene variations. Fourth, all treated patients were infected with genotype C, whereas most sequences from untreated patients belonged to genotype B (Fig 2). Therefore, we cannot rule out the possibility that the observed differences in mutation rate were driven by a higher spontaneous mutation rate of HBV genotype C compared to genotype B. To address this issue, we downloaded 1481 molecular clone sequences of a region of the preC/C reading frames from a single study in which untreated patients infected with HBV genotype B or C were enrolled [47]. After genotyping the sequences and removing the preC region, we found four unique premature stops in the 724 sequences belonging to genotype B, versus one such stop in the 757 sequences belonging to genotype C, hence arguing against a higher spontaneous mutation rate in genotype C. Therefore, the conclusion that lamivudine/adefovir
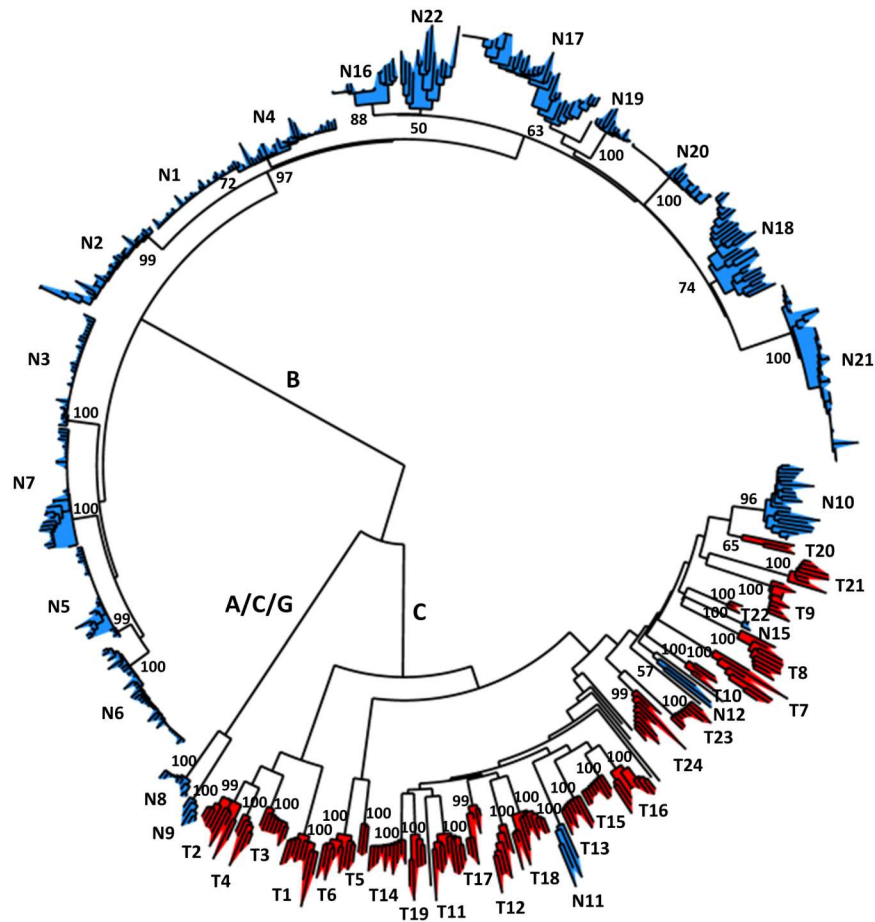
Fig 2. Phylogenetic analysis of HBV sequences used in this study. A neighbor-joining containing the 621 full-length sequences used is shown. Patient names are as in Table 1. Sequences from untreated patients are marked in blue and those from treated patients in red. For each indicated patient, numbers show the bootstrap value of the node that delimitates the sequences from this patient. Sequences from patients N16 to N22 correspond to a study in which several family members were studied and are partially intermingled (not shown). Patients N13 and N14 are not shown because their sequences did not form well-defined monophyletic groups. Letters in the center of the tree indicate the viral genotype.

doi:10.1371/journal.pone.0163363.g002

treatment increases the mutation rate of HBV appears to be robust to several sources of error and bias.

To test whether treatment modified the type of mutations produced by HBV, we scored all intra-patient single-nucleotide polymorphisms (SNPs) in the 621 full-length molecular clone sequences used above for mutation rate estimation by comparing each sequence with the consensus sequence of the patient. In total, we found 3342 intra-patient SNPs along the HBV genome, and we calculated the percentage contribution of each type of nucleotide substitution to the total number of SNPs found in each patient (mutational spectrum). The most frequent type of SNP was T→C, followed by the other base transitions A→G, C→T, and G→A, whereas base transversions were less frequent. However, the mutational spectrum differed between untreated and treated patients (Fig 3). Lamivudine/adefovir treatment was associated to a greater relative abundance of A→G SNPs (Mann-Whitney test: $p < 0.001$) and the complementary T→C changes ($p = 0.001$). All other types of nucleotide substitutions were not significantly modified in treated patients, or showed lower relative abundance.
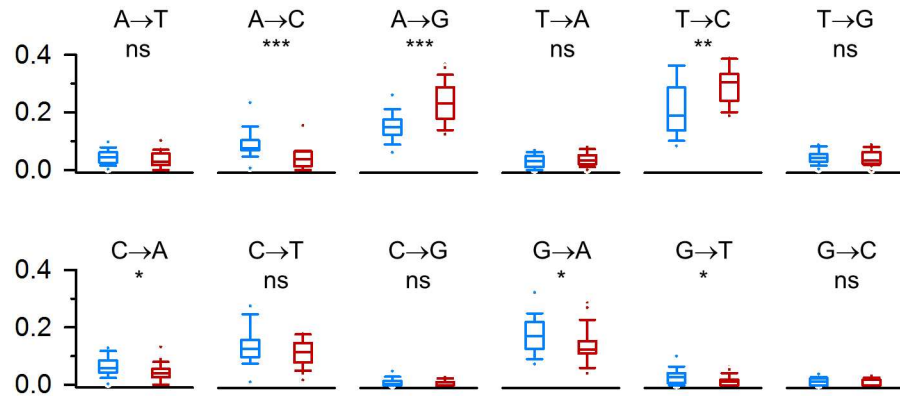
**Fig 3. HBV mutational spectrum in untreated patients and in patients undergoing lamivudine/adefovir treatment.** The mutational spectrum was obtained in sequences derived from untreated (blue) or treated (red) patients by scoring all intra-patient SNPs along the HBV genome. Box plots indicate the relative contribution of each substitution type to the total SNPs found. Lower and upper box limits indicate percentiles 25th and 75th, respectively, and the middle line shows the median. Whiskers show the 10th and 90th percentiles, and outlying points are plotted individually. Differences between treated and untreated patients in the frequency of each substitution type were assessed by a Mann-Whitney non-parametric test (***: $p < 0.001$; **: $0.001 < p < 0.01$; *: $0.01 < p < 0.05$; ns: non-significant).

doi:10.1371/journal.pone.0163363.g003

## Discussion

Our spontaneous mutation rate estimate of $8.7 \times 10^{-5}$ m/n/c should be taken as an upper-limit for several reasons. First, a fraction of the observed premature stop codons may be artefacts introduced during PCR amplification, cloning, or sequencing. Second, hypothetical read-through of some stop codons may prevent their lethality. Third, the lethal effects of some stop codons may be rescued by trans-complementation in cells co-infected with other, functional variants of the virus, leading to higher-than-expected frequencies of premature stop codons. Trans-complementation may explain the large differences among genes in the frequency of stop codons, which were >20-fold higher in the S gene than in the polymerase gene P in untreated patients (Table 2). Since reading frames are strongly overlapped in HBV, we do not interpret these variations as real changes in mutation rate along the HBV genome. We suggest that the lethality of some stop codons is more likely to be rescued by trans-complementation in the surface envelope than in the RT because HBV-infected cells produce a large excess of surface proteins, which are released to the serum in the form of empty, non-infectious sub-viral particles [48,49]. In contrast, the viral polymerase is produced in smaller amounts and should be more tightly associated with the viral genome, making trans-complementation less likely. Considering this, the mutation rate obtained for the P gene of $2.7 \times 10^{-5}$ m/n/c might be more accurate than the value obtained for other genes. Interestingly, among-gene stop-codon frequency variation was smaller in treated patients than in untreated patients (Table 2), potentially indicating a lower effect of trans-complementation. The co-infection probability increases with viral load and, hence, should tend to be higher in untreated patients than in treated patients.

Although it is commonly accepted that the main source of spontaneous mutations in HBV is the low replication fidelity of the viral polymerase, the HBV genome is subject in vivo to genome editing by cellular enzymes of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (A3) family, which produce in G→A substitutions in the viral genome [50,51]. Of the five premature stop codons found in X, C, and P sequences derived from untreated patients, one involved a G→A substitution (TGG→TGA), two involved C→T

substitutions (unlikely to be driven by A3), and two involved base transversions (clearly not driven by A3). This suggests that A3 is not the main source of spontaneous mutations in HBV. In recent work with HIV-1, we have shown that A3 activity contributes approximately half of the spontaneous mutations found in plasma, but 98% of those found in peripheral blood mononuclear cells [33]. Here, the vast majority of sequences analyzed were obtained from plasma samples. Analysis of sequences from liver tissue may contribute to better elucidate whether A3 activity contributes significantly to the total rate of spontaneous mutation of HBV.

Lamivudine/adefovir treatment increased significantly the frequency of premature stop codons. This effect was strongest in the P gene (>15-fold) and mildest in the S gene (approximately twofold). We argue that the actual mutagenic effect of lamivudine/adefovir is best reflected by the P gene because estimation bias is probably less pronounced than in the other genes, particularly S. Our analysis of the full set of SNPs revealed a specific enrichment in A/U→G/C substitutions in HBV sequences derived from treated patients. Recent work in mice chronically exposed to lamivudine has shown the same mutagenic pattern, as determined by sequence analysis of the highly variable D-loop region of mitochondrial DNA isolated from cortical neurons [52]. The fact that the mutational spectrum observed in treated patients was similar to that found in mouse mitochondrial DNA suggests a direct mutagenic effect of lamivudine/adefovir, as opposed to an alternative scenario in which HBV polymerase variants with modified mutational properties would have evolved in response to treatment. Since lamivudine is a cytosine analogue, it is expected to be incorporated into DNA by base-pairing with guanosine, but it is unclear how this should lead to A/U→G/C substitutions. Adefovir is an adenosine analogue and may cause A/U→G/C substitutions after being incorporated into DNA by base-pairing with thymidine, followed by incorporation of cytosine by base-pairing with adefovir, or through other pathways. Further work will be required to elucidate the mechanistic basis of the observed mutagenic effects.

Our results clearly show that lamivudine/adefovir treatment increases the HBV mutation rate above $10^{-4}$ m/n/c in each viral gene (Table 2). For a per-site mutation rate of $5.2 \times 10^{-4}$ m/n/c, approximately 1.5 new mutations should be produced per genome after a single cell infection cycle. This suggests that, in treated patients, HBV produces mutations faster than many RNA viruses. For comparison, the spontaneous mutation rate of hepatitis C virus (HCV) *in vivo* estimated by the lethal mutation method is on the order of $0.3–1.2 \times 10^{-4}$ m/n/c, and ribavirin/interferon treatment increases this rate between two- and three-fold [21,32]. It is noteworthy that the effect of ribavirin/interferon treatment on the HCV mutation rate appears to be less marked than that of lamivudine/adefovir treatment for HBV. Previous work has suggested that modest increases in the mutation rate of RNA viruses can situate them close to the maximum tolerable mutation rate, or error threshold [53]. In the presence of treatments, the estimated mutation rates of HBV and HCV are similar and, therefore, we speculate that treatment may also situate HBV close to the error threshold, particularly if we consider the high degree of gene overlap shown by this virus, in which many single-point mutations modify the sequence of two proteins simultaneously. However, this extremely high mutation rate may also boost viral evolvability and promote the rapid emergence of drug-resistance and immune escape mutants. Previous work has demonstrated that, in HBV and RNA viruses, high fidelity variants can evolve in response to nucleoside analogue mutagenesis [13,54–58]. If a similar process occurs in vivo for HBV, it seems not to be sufficient to compensate for the mutagenic effect of lamivudine/adefovir treatment. Future work may further elucidate how HBV evolves in response to treatment-induced mutagenesis. Ideally, this should be addressed in longitudinal studies that include samples before and after the onset of treatment, or using HBV genotype-matched patients in the case of cross-section studies. Similarly, sequencing at both the acute

and chronic diseases stages for patients with known infection times would allow testing whether the HBV mutation rate evolves throughout the course of infection.

## Supporting Information

**S1 Table. List of premature stop codons indicating the patient, gene, genome position, type of stop and accession number, in Excel format.**
(XLSX)

## Acknowledgments

We thank Álvaro Fajardo and Martín Soñora for help with preliminary analysis of the sequence data.

## Author Contributions

**Conceptualization:** RS.

**Formal analysis:** MPG JVB IA.

**Funding acquisition:** RS.

**Investigation:** MPG.

**Methodology:** RS.

**Software:** IA JVB.

**Supervision:** RS.

**Visualization:** RS.

**Writing – original draft:** RS.

**Writing – review & editing:** RS JVB IA.

## References

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet. 2015; 386:1546–55. doi: 10.1016/S0140-6736(15)61412-X PMID: 26231459

2. Beck J, Nassal M. Hepatitis B virus replication. World J Gastroenterol. 2007; 13:48–64. PMID: 17206754

3. Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. Virology. 2015; 479–480:672–86. doi: 10.1016/j.virol.2015.02.031 PMID: 25759099

4. Halegoua-De MD, Hann HW. Then and now: the progress in hepatitis B treatment over the past 20 years. World J Gastroenterol. 2014; 20:401–13. doi: 10.3748/wjg.v20.i2.401 PMID: 24574709

5. Tawada A, Kanda T, Yokosuka O. Current and future directions for treating hepatitis B virus infection. World J Hepatol. 2015; 7:1541–52. doi: 10.4254/wjh.v7.i11.1541 PMID: 26085913

6. Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut. 2015; 64:1972–84. doi: 10.1136/gutjnl-2015-309809 PMID: 26048673

7. Menéndez-Arias L, Alvarez M, Pacheco B. Nucleoside/nucleotide analog inhibitors of hepatitis B virus polymerase: mechanism of action and resistance. Curr Opin Virol. 2014; 8:1–9. doi: 10.1016/j.coviro.2014.04.005 PMID: 24814823

8. Harrison A, Lemey P, Hurles M, Moyes C, Horn S, Pryor J, et al. Genomic analysis of hepatitis B virus reveals antigen state and genotype as sources of evolutionary rate variation. Viruses. 2011; 3:83–101. doi: 10.3390/v3020083 PMID: 21765983

9. Zhou Y, Holmes EC. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. J Mol Evol. 2007; 65:197–205. PMID: 17684696

10. Okamoto H, Imai M, Kametani M, Nakamura T, Mayumi M. Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through materno-fetal transmission. Jpn J Exp Med. 1987; 57:231–6. PMID: 3430800

11. Osiowy C, Giles E, Tanaka Y, Mizokami M, Minuk GY. Molecular evolution of hepatitis B virus over 25 years. J Virol. 2006; 80:10307–14. PMID: 17041211

12. Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet. 2008; 9:267–76. doi: 10.1038/nrg2323 PMID: 18319742

13. Hong YB, Choi Y, Jung G. Increased DNA polymerase fidelity of the Lamivudine resistant variants of human hepatitis B virus DNA polymerase. J Biochem Mol Biol. 2004; 37:167–76. PMID: 15469692

14. Caligiuri P, Cerruti R, Icardi G, Bruzzone B. Overview of hepatitis B virus mutations and their implications in the management of infection. World J Gastroenterol. 2016; 22:145–54. doi: 10.3748/wjg.v22.i1.145 PMID: 26755866

15. Gao S, Duan ZP, Coffin CS. Clinical relevance of hepatitis B virus variants. World J Hepatol. 2015; 7:1086–96. doi: 10.4254/wjh.v7.i8.1086 PMID: 26052397

16. Zhang ZH, Wu CC, Chen XW, Li X, Li J, Lu MJ. Genetic variation of hepatitis B virus and its significance for pathogenesis. World J Gastroenterol. 2016; 22:126–44. doi: 10.3748/wjg.v22.i1.126 PMID: 26755865

17. Sheldon J, Rodes B, Zoulim F, Bartholomeusz A, Soriano V. Mutations affecting the replication capacity of the hepatitis B virus. J Viral Hepat. 2006; 13:427–34. PMID: 16792535

18. Pult I, Abbott N, Zhang YY, Summers J. Frequency of spontaneous mutations in an avian hepdnavirus infection. J Virol. 2001; 75:9623–32. PMID: 11559794

19. Sanjuán R, Nebot MR, Chirico N, Mansky LM, Belshaw R. Viral mutation rates. J Virol. 2010; 84:9733–48. doi: 10.1128/JVI.00694-10 PMID: 20660197

20. Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci USA. 2001; 98:6895–900. PMID: 11371613

21. Cuevas JM, González-Candelas F, Moya A, Sanjuán R. The effect of ribavirin on the mutation rate and spectrum of Hepatitis C virus in vivo. J Virol. 2009; 83:5760–4. doi: 10.1128/JVI.00201-09 PMID: 19321623

22. Graci JD, Cameron CE. Mechanisms of action of ribavirin against distinct viruses. Rev Med Virol. 2006; 16:37–48. PMID: 16287208

23. Airaksinen A, Pariente N, Menéndez-Arias L, Domingo E. Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis. Virology. 2003; 311:339–49. PMID: 12842623

24. Day CW, Smee DF, Julander JG, Yamshchikov VF, Sidwell RW, Morrey JD. Error-prone replication of West Nile virus caused by ribavirin. Antiviral Res. 2005; 67:38–45. PMID: 15919121

25. Severson WE, Schmaljohn CS, Javadian A, Jonsson CB. Ribavirin causes error catastrophe during Hantaan virus replication. J Virol. 2003; 77:481–8. PMID: 12477853

26. Mansky LM, Pearl DK, Gajary LC. Combination of drugs and drug-resistant reverse transcriptase results in a multiplicative increase of human immunodeficiency virus type 1 mutant frequencies. J Virol. 2002; 76:9253–9. PMID: 12186909

27. Menéndez-Arias L. Mutation rates and intrinsic fidelity of retroviral reverse transcriptases. Viruses. 2009; 1:1137–65. doi: 10.3390/v1031137 PMID: 21994586

28. Dapp MJ, Heineman RH, Mansky LM. Interrelationship between HIV-1 fitness and mutation rate. J Mol Biol. 2013; 425:41–53. doi: 10.1016/j.jmb.2012.10.009 PMID: 23084856

29. Guimaraes NN, de Andrade HH, Lehmann M, Dihl RR, Cunha KS. The genetic toxicity effects of lamivudine and stavudine antiretroviral agents. Expert Opin Drug Saf. 2010; 9:771–81. doi: 10.1517/14740331003702384 PMID: 20377473

30. Torres SM, Walker DM, Carter MM, Cook DL Jr., McCash CL, Cordova EM, et al. Mutagenicity of zidovudine, lamivudine, and abacavir following in vitro exposure of human lymphoblastoid cells or in utero exposure of CD-1 mice to single agents or drug combinations. Environ Mol Mutagen. 2007; 48:224–38. PMID: 17358033

31. Gago S, Elena SF, Flores R, Sanjuán R. Extremely high mutation rate of a hammerhead viroid. Science. 2009; 323:1308. doi: 10.1126/science.1169202 PMID: 19265013

32. Ribeiro RM, Li H, Wang S, Stoddard MB, Learn GH, Korber BT, et al. Quantifying the Diversification of hepatitis C virus (HCV) during primary infection: estimates of the in vivo mutation rate. PLoS Pathog. 2012; 8:e1002881. doi: 10.1371/journal.ppat.1002881 PMID: 22927817

33. Cuevas JM, Geller R, Garijo R, López-Aldeguer J, Sanjuán R. Extremely high mutation rate of HIV-1 in vivo. PLoS Biol. 2015; 13:e1002251. doi: 10.1371/journal.pbio.1002251 PMID: 26375597

34. Jammeh S, Tavner F, Watson R, Thomas HC, Karayiannis P. Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. J Gen Virol. 2008; 89:901–9. doi: 10.1099/vir.0.83468-0 PMID: 18343830

35. Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, et al. Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. Proc Natl Acad Sci USA. 1991; 88:4186–90. PMID: 2034663

36. Wang HY, Chien MH, Huang HP, Chang HC, Wu CC, Chen PJ, et al. Distinct hepatitis B virus dynamics in the immunotolerant and early immunoclearance phases. J Virol. 2010; 84:3454–63. doi: 10.1128/JVI.02164-09 PMID: 20089644

37. Su H, Liu Y, Xu Z, Cheng S, Ye H, Xu Q, et al. A novel complex A/C/G intergenotypic recombinant of hepatitis B virus isolated in southern China. PLoS One. 2014; 9:e84005. doi: 10.1371/journal.pone.0084005 PMID: 24475029

38. Shen T, Yan XM, Zhang JP, Wang JL, Zuo RX, Li L, et al. Evolution of Hepatitis B Virus in a Chronic HBV-Infected Patient over 2 Years. Hepat Res Treat. 2011; 2011:939148. doi: 10.1155/2011/939148 PMID: 21785721

39. Lin X, Qian GS, Lu PX, Wu L, Wen YM. Full-length genomic analysis of hepatitis B virus isolates in a patient progressing from hepatitis to hepatocellular carcinoma. J Med Virol. 2001; 64:299–304.

40. Lin YY, Liu C, Chien WH, Wu LL, Tao Y, Wu D, et al. New insights into the evolutionary rate of hepatitis B virus at different biological scales. J Virol. 2015; 89:3512–22. doi: 10.1128/JVI.03131-14 PMID: 25589664

41. Fang Y, Teng X, Xu WZ, Li D, Zhao HW, Fu LJ, et al. Molecular characterization and functional analysis of occult hepatitis B virus infection in Chinese patients infected with genotype C. J Med Virol. 2009; 81:826–35. doi: 10.1002/jmv.21463 PMID: 19319940

42. Hao R, Xiang K, Peng Y, Hou J, Sun J, Li Y, et al. Naturally occurring deletion/insertion mutations within HBV whole genome sequences in HBeAg-positive chronic hepatitis B patients are correlated with baseline serum HBsAg and HBeAg levels and might predict a shorter interval to HBeAg loss and seroconversion during antiviral treatment. Infect Genet Evol. 2015; 33:261–8. doi: 10.1016/j.meegid.2015.05.013 PMID: 25976382

43. Huang SF, Chen YT, Lee WC, Chang IC, Chiu YT, Chang Y, et al. Identification of transforming hepatitis B virus S gene nonsense mutations derived from freely replicative viruses in hepatocellular carcinoma. PLoS One. 2014; 9:e89753. doi: 10.1371/journal.pone.0089753 PMID: 24587012

44. Lai MW, Yeh CT. The oncogenic potential of hepatitis B virus rtA181T/ surface truncation mutant. Antivir Ther. 2008; 13:875–9. PMID: 19043921

45. Suppiah J, Mohd ZR, Bahari N, Haji NS, Saat Z. S gene mutants occurrence among hepatitis B carriers in malaysia. Hepat Mon. 2014; 14:e22565. doi: 10.5812/hepatmon.22565 PMID: 25737728

46. Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. Hepatology. 2008; 48:88–98. doi: 10.1002/hep.22295 PMID: 18537180

47. Li Z, Xie Z, Ni H, Zhang Q, Lu W, Yin J, et al. Mother-to-child transmission of hepatitis B virus: evolution of hepatocellular carcinoma-related viral mutations in the post-immunization era. J Clin Virol. 2014; 61:47–54. doi: 10.1016/j.jcv.2014.06.010 PMID: 24973814

48. Chai N, Chang HE, Nicolas E, Han Z, Jarnik M, Taylor J. Properties of subviral particles of hepatitis B virus. J Virol. 2008; 82:7812–7. doi: 10.1128/JVI.00561-08 PMID: 18524834

49. Patient R, Hourioux C, Roingeard P. Morphogenesis of hepatitis B virus and its subviral envelope particles. Cell Microbiol. 2009; 11:1561–70. doi: 10.1111/j.1462-5822.2009.01363.x PMID: 19673892

50. Gunther S, Sommer G, Plikat U, Iwanska A, Wain-Hobson S, Will H, et al. Naturally occurring hepatitis B virus genomes bearing the hallmarks of retroviral G→A hypermutation. Virology. 1997; 235:104–8. PMID: 9300041

51. Suspène R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. Proc Natl Acad Sci USA. 2005; 102:8321–6. PMID: 15919829

52. Zhang Y, Wang B, Liang Q, Qiao L, Xu B, Zhang H, et al. Mitochondrial DNA D-loop AG/TC transition mutation in cortical neurons of mice after long-term exposure to nucleoside analogues. J Neurovirol. 2015; 21:500–7. doi: 10.1007/s13365-015-0347-x PMID: 26015313

53. Perales C, Domingo E. Antiviral strategies based on lethal mutagenesis and error threshold. Curr Top Microbiol Immunol. 2016; 392:323–39. doi: 10.1007/82_2015_459 PMID: 26294225

54. Cheung PP, Watson SJ, Choy KT, Fun SS, Wong DD, Poon LL, et al. Generation and characterization of influenza A viruses with altered polymerase fidelity. Nat Commun. 2014; 5:4794. doi: 10.1038/ncomms5794 PMID: 25183443

55. Coffey LL, Beeharry Y, Bordería AV, Blanc H, Vignuzzi M. Arbovirus high fidelity variant loses fitness in mosquitoes and mice. Proc Natl Acad Sci USA. 2011; 108:16038–43. doi: 10.1073/pnas.1111650108 PMID: 21896755

56. Levi LI, Gnadig NF, Beaucourt S, McPherson MJ, Baron B, Arnold JJ, et al. Fidelity variants of RNA dependent RNA polymerases uncover an indirect, mutagenic activity of amiloride compounds. PLoS Pathog. 2010; 6:e1001163. doi: 10.1371/journal.ppat.1001163 PMID: 21060812

57. Meng T, Kwang J. Attenuation of human enterovirus 71 high-replication-fidelity variants in AG129 mice. J Virol. 2014; 88:5803–15. doi: 10.1128/JVI.00289-14 PMID: 24623423

58. Pfeiffer JK, Kirkegaard K. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. Proc Natl Acad Sci USA. 2003; 100:7289–94. PMID: 12754380