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Full genome ultra-deep pyrosequencing associates G-to-A hypermutation of the hepatitis B virus genome with the natural progression of hepatitis B

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SUMMARY. Human APOBEC3 (A3) cytosine deaminases are antiviral restriction factors capable of editing the genome the hepatitis B virus (HBV). Despite the importance of the human A3 protein family for the innate immune response little is known about the clinical relevance for hepatitis B. The aim of this study was to utilize ultra-deep pyrosequencing (UDPS) data to analyse the phenomenon of G-to-A hypermutation of the complete HBV genome and to relate it to fundamental characteristics of patients with chronic hepatitis B. By analysing the viral population of 80 treatment naïve patients (47 HBeAg-positive and 33 HBeAg-negative), we identified an unequal distribution of G-to-A hypermutations across the genome. Our data indicate that G-to-A hypermutation occurs predominantly in a region between nucleotide positions 600 and 1800 a region which is usually single stranded in matured HBV particles. This implies that A3 likely edits HBV in the virion. Hypermutation rates for HBeAg-negative patients were more than 10-fold higher than those of HBeAg-positive patients. For HBeAg-negative patients higher hypermutation rates were significantly associated with the degree of fibrosis. Additionally, we found that for HBeAg-positive chronic hepatitis G-to-A hypermutation rates were significantly associated with the relative prevalence of the G1764A mutation, which is related to HBeAg seroconversion. In total, our data imply an important association of hypermutation mediated by A3 deaminases with the natural progression of chronic hepatitis B infections both in terms of HBeAg seroconversion and disease progression towards cirrhosis.

Keywords: APOBEC3G, fibrosis, HBV, hypermutation, pyrosequencing.

INTRODUCTION

Chronic hepatitis B caused by infection with the hepatitis B virus (HBV) affects about 240 million persons worldwide [1]. Even though HBV is usually not cytopathic itself it is one of the major causes of severe liver cirrhosis and liver cancer. HBV infections naturally undergo four phases that are characterized by major differences in the activity of the host's immune system and extent of viral replication. In the immune active phase, which follows the immune tolerant phase, the host's immune system recognizes HBV as a pathogen with the consequence of active liver inflammation and elevated alanine aminotransferase (ALT) levels. Spontaneous or treatment-induced seroconversion of hepatitis B e antigen (HBeAg) to anti-HBe is an important event in chronic hepatitis B. HBeAg seroconversion is either followed by the inactive carrier phase in which immunological control of the infection confers favourable prognosis or the reactive phase (HBeAg-negative chronic hepatitis) in which HBV has escaped the immune pressure and replicates without expressing HBeAg or expressing HBeAg at low levels [2–5].

In the last decade, the innate immune proteins of the APOBEC3 (A3) cytosine-deaminase family (A3A – A3H) were shown to have the capacity of inhibiting the replication of RNA- and DNA-viruses [6–11]. The enzymatic activity of A3 results in the deamination of cytosine bases in the single-stranded viral (−) DNA leads to G-to-A hypermutation on the (+) DNA strand [12,13]. The A3G protein can inhibit HBV also with less well-understood deamination-independent

Abbreviations: A3, APOBEC3; AICDA, activation-induced cytidine deaminase; ALT, alanine aminotransferase; BCP, basal core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; UDPS, ultra-deep pyrosequencing.

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mechanisms [6,14–18]. Primary human hepatocytes of healthy donors express low levels of the mRNAs of A3B – A3G, but the expression can be upregulated by interferon-α or interferon-γ stimulation [7,19–21]. Upregulation of A3 genes was also observed in HBV-associated cirrhotic liver tissue [22].

Despite the importance of A3 proteins for inhibition of HBV replication *in vitro*, little is known about their clinical relevance. G-to-A hypermutations have been detected in HBV genomes in infected patients, suggesting an enzymatic activity of A3 proteins on the HBV DNA *in vivo* [22–25]. In a recent study, G-to-A hypermutations were associated with HBeAg loss and reduction of plasma HBV DNA levels [26].

Our aim was to utilize ultra-deep pyrosequencing (UDPS) to analyse the phenomenon of G-to-A hypermutation of HBV and to relate hypermutation to fundamental characteristics of patients with chronic hepatitis B.

MATERIAL AND METHODS

Patients

Ultra-deep pyrosequencing, as described in detail in Data S1, was performed on serum samples from 47 HBeAg-positive and 33 HBeAg-negative treatment naïve patients. Patients were enrolled in the pegylated interferon α-2a registration studies [27,28]. Patient characteristics including liver biopsy, virological and serological parameters were obtained according to the study protocols.

Hypermutation rates

The proportion of G-to-A hypermutated reads is referred to as the hypermutation rate. Several scoring schemes have been proposed to identify hypermutated reads. The G-to-A preference was defined as proportion of G-to-A mutations divided by the total number of mutations (compared with the sample consensus sequence) in the read [29]. The total number of G-to-A exchanges with reference to the sample consensus sequence were also used as indicators of hypermutation [30]. Each read was classified as normal or hypermutated based on the combination of these two statistics with individual cut-offs. Mutations were evaluated with reference to the sample consensus sequence based on the UDPS data, which included each base with prevalence of at least 10%.

RESULTS

Whole genome coverage by ultra-deep pyrosequencing

Clinical, virological and serological parameters were obtained for HBeAg-positive (n = 47) and HBeAg-negative (n = 33) treatment naïve patients. Patient characteristics are shown in Table 1. Ultra-deep pyrosequencing was performed from serum samples of all HBeAg-positive and HBeAg-negative patients applying seven overlapping amplicons (Data S1) that covered the genome of HBV. In total, 1 360 551 reads were successfully mapped onto the reference genome of HBV (GenBank accession number

Table 1 Patient characteristics

Characteristic	All patients (n = 80)	HBeAg-positive patients (n = 47)	HBeAg-negative patients (n = 33)	P-Value
Age, years [mean (range)]	37.2 (18–70)	32 (18–65)	44.6 (20–70)	<0.001*
Male sex [n (%)]	68 (85%)	42 (89.4%)	26 (78.8%)	1.0†
Ethnicity [n (%)]				
Caucasian	67 (84.8%)	35 (74.5%)	32 (97.0%)	0.01†
Other	13 (16.2%)	12 (25.5%)	1 (3.0%)	
HBV genotype [n (%)]				<0.001†
A	39 (48.8%)	33 (70.2%)	6 (18.2%)	
D	41 (51.2%)	6 (29.8%)	21 (81.8%)	
ALT, IU/L [mean (range)]	97 (14.6–300)	98.4 (29.2–300)	94.9 (14.6–300)	0.87*
HBV DNA, log copies/mL [median (range)]	9.2 (6.3–14.5)	10.1 (7.7–14.5)	8 (6.3–10.3)	<0.001*
Degree of Fibrosis				0.35†
F0	10 (12.5%)	7 (14.9%)	3 (9.0%)	
F1	27 (33.8%)	16 (34.0%)	11 (33.3%)	
F2	28 (35%)	18 (38.3%)	10 (30.3%)	
F3	7 (8.8%)	4 (8.5%)	3 (9.1%)	
F4	8 (10%)	2 (4.3%)	6 (18.2%)	

*Wilcoxon rank-sum test.
†Fisher's exact test.

AM282986) with an average sequence length of 375 ± 56 bases. The number of reads per patient showed a heterogeneous distribution and ranged from 7047 to 61 623 with a mean of 17 006 ± 7332. The median coverage (number of reads per patient sample and nucleotide position) had a mean of 1480 ± 601.

HBeAg-negative patients have higher hypermutation rates than HBeAg-positive patients

The UDPS data were scanned to identify G-to-A hypermutated HBV genomes. Hypermutation rates were computed with a combination of two hypermutation criteria (at least four G-to-A mutations and G-to-A preference of at least 70%) for nonoverlapping 100 base-pair windows along the genome (Fig. 1). Median hypermutation rates per 100 base-pair window varied between 0.0% and 0.047% for HBeAg-positive and between 0.0% and 0.69% for HBeAg-negative patients, respectively. Thus, the highest median hypermutation rate for HBeAg-negative patients was 14.6 times higher than that of HBeAg-positive patients. The data further showed that hypermutations were nonuniformly distributed across the genome. The highest hypermutation rates were observed between NT positions 600 and 1800 of the HBV genome. In this genomic area, the average hypermutation rate was 0.067 ± 0.2% for HBeAg-positive and 0.35 ± 0.6% for HBeAg-negative patients, respectively. Intermediate hypermutation rates were observed between NT position 1 and 600 with an average hypermutation rate of 0.03 ± 0.01% and 0.1 ± 0.2% for HBeAg-positive and HBeAg-negative patients, respectively. Between NT position 1800 and 3221, the observed average hypermutation rates were of 0.01 ± 0.07% and 0.05 ± 0.2% for HBeAg-positive and HBeAg-negative patients, respectively. The peak location of high hypermutation rates was independent of the applied combination of hypermutation criteria (data not shown).

Hypermutation dinucleotide context indicates A3 activity

The dinucleotide context of the G-to-A hypermutations showed preferred editing in the GA and the GG context and little editing in the GT context for amplicons 1–4 (Fig. 2a). For amplicons 5–7, the data were very sparse as the median hypermutation rates were near zero in this genomic region. In patients with significant fibrosis, (histology states F2, F3 and F4) editing patterns were not significantly different from non- or mild fibrotic (histology states F0 and F1) patients ($P = 0.21$; permutation test). The preference for the GG context was only slightly higher in fibrotic samples (37.7% GG context for fibrotic vs. 35.8% GG context for nonfibrotic). Similarly, the dinucleotide editing profile did not differ significantly between HBeAg-positive and HBeAg-negative patients ($P = 0.07$; permutation test). Previous studies analysed the dinucleotide editing profiles

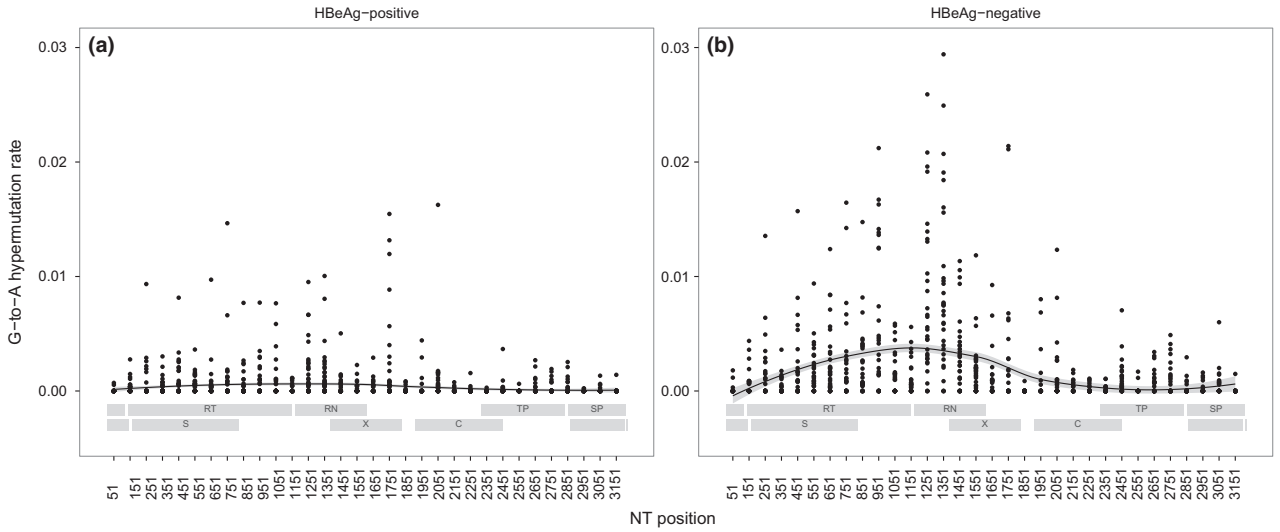

Fig. 1 Analysis of G-to-A hypermutation rates along the hepatitis B virus genome. The scatter plots show per-patient hypermutation rates across the genome for HBeAg-positive (**a**) and HBeAg-negative (**b**) patients. Each point corresponds to one patient and one 100 base-pair window. The hypermutation criteria require at least four G-to-A exchanges and a G-to-A preference of at least 70% within each 100 base-pair window. The local polynomial regression lines (LOESS) indicate that hypermutation rates for HBeAg-negative patients are on average higher than for HBeAg-positive patients. A peak location of hypermutation can be identified approximately between NT positions 600 and 1800. Positions are with respect to reference strain AM282986. The HBV genome map was added to subplots a and b. Abbreviations read as follows: RT, Reverse Transcriptase; RN, RNase; TP, Terminal Protein; SP, Spacer Domain; S, short surface gene; X, X gene, C, Core gene (not including precore).

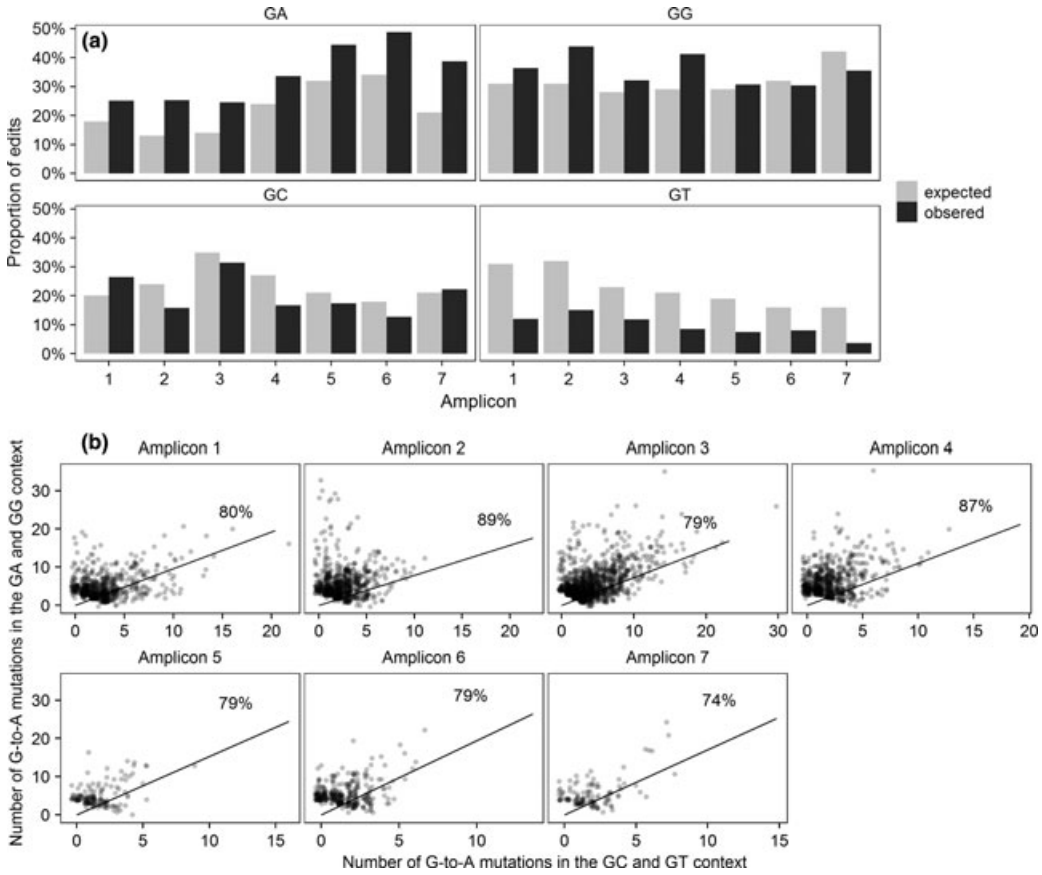

Fig. 2 Dinucleotide editing profile. Hypermutated sequences were analysed with respect to the dinucleotide context in which the G-to-A mutations were found. Hypermutation was defined by at least four G-to-A mutations per read and a G-to-A preference of at least 70%. (**a**) The actual number of mutations (black) that occurred in the dinucleotide contexts GA, GG, GC and GT was compared with the relative frequency of the respective dinucleotides in the reference strain AM282986 (grey). Most of the editing was observed in amplicons 1–4 with a strong preference for the GG, and the GA indicating that mutations are due to human APOBEC3 deaminases. (**b**) Each dot in the scatterplot refers to one hypermutated sequence derived from ultra-deep pyrosequencing. The *x*-axis denotes the number of G-to-A mutations in the GC and GT dinucleotide context per read and the *y*-axis displays the number of G-to-A mutations in the GA and GG context. The diagonal lines extending from the origin account for the frequencies of the dinucleotides in the reference strain AM282986 of the respective genomic region (amplicons 1–7) and separate the scatterplot into an upper left region in which mutations in the GA and GG context are more prevalent than expected and a lower right regions in which mutations in the GC and GT context are more prevalent than expected. The percentage number gives the fraction of sequence reads in each genomic region that express higher mutation rates in the GA and GG context. For all of the seven amplicons (1–7), at least 74% (up to 89%) of the hypermutated reads show higher number of G-to-A mutations in the GA and GG context than expected. Thus, editing is likely due to human APOBEC3 deaminases.

of several A3 deaminases and activation-induced cytidine deaminase (AICDA) *in vitro* and suggested to use the frequency of G-to-A exchanges in the GG and the GA context as a hallmark of editing mediated by A3 deaminases [22,23,31–33]. A3 proteins prefer to cause mutations in the GG and the GA dinucleotide context. Clonal analysis of the hypermutated sequences revealed that depending on the genomic region (amplicons 1–7) 74% to 89% of the hypermutated sequences mapped to the editing profiles of typical A3 proteins (Fig. 2b). Thus, after accounting for dinucleotide frequencies in the reference sequence editing was more frequently observed in the GG and the GA context compared with the GC and GT context.

Hypermutation rates correlate with fibrosis in HBeAg-negative patients

Next, we asked whether the hypermutation rates correlate with any patient characteristics. The results of the statistical tests are summarized in Table 2. Hypermutation rates were computed using sequence data from amplicons 3 (NT positions 1093–1660), which overlaps

with the peak location of hypermutation. We found that G-to-A hypermutation rates are significantly correlated with the degree of fibrosis for HBeAg-negative patients ($P = 0.004$). This did not hold for HBeAg-positive patients ($P = 0.09$). Figure 3 shows increasing hypermutation rates for both HBeAg-positive and HBeAg-negative patients with increasing degree of fibrosis. Additionally, we found that G-to-A hypermutation rates are correlated with the patients' age for HBeAg-negative patients ($P = 0.04$). Nonetheless, in a multivariate analysis, including degree of fibrosis, age and hypermutation rates, age was no longer significantly associated with hypermutation rates ($P = 0.07$). Other patient characteristics including sex, ethnicity, HBV genotype, ALT and viral load did not reveal significant associations.

Correlation of hypermutation rates to precore/core, BCP and HBsAg mutations

Several studies suggested that G-to-A hypermutation might be one of the driving forces of HBeAg seroconversion due to mediation of escape mutations [18,22,26]. We found that the relative prevalence of the G1764A ($P = 0.0002$) mutation was significantly associated with G-to-A hypermutation rates (amplicon 3) in HBeAg-positive patients. No association was found for the mutations G1896A

Table 2 Hypermutation rate associated with patient characteristics

	HBeAg-positive		HBeAg-negative	
Patient characteristic	*P*-value (HMR1*)	*P*-value (HMR2†)	*P*-value (HMR1*)	*P*-value (HMR2†)
Age	0.4422	0.7009	**0.0429**	0.1610
Sex	0.7937	0.8443	0.3650	0.4475
Ethnicity	0.6132	0.1612	0.4948	0.18922
HBV genotype	0.7869	0.8757	1.0000	0.7088
ALT	0.1499	0.0929	0.8811	0.8903
HBV DNA	0.4245	0.4809	0.9739	0.8708
Degree of Fibrosis	0.0868	0.1063	**0.0044**	**0.0161**

*HMR1: hypermutation defined by at least four G-to-A mutations and G-to-A preference of at least 70% (amplicon 3 reads only).
†HMR2: hypermutation defined by at least ten G-to-A mutations and G-to-A preference of at least 70% (amplicon 3 reads only).
P-values less than 0.05 were considered significant (bold).

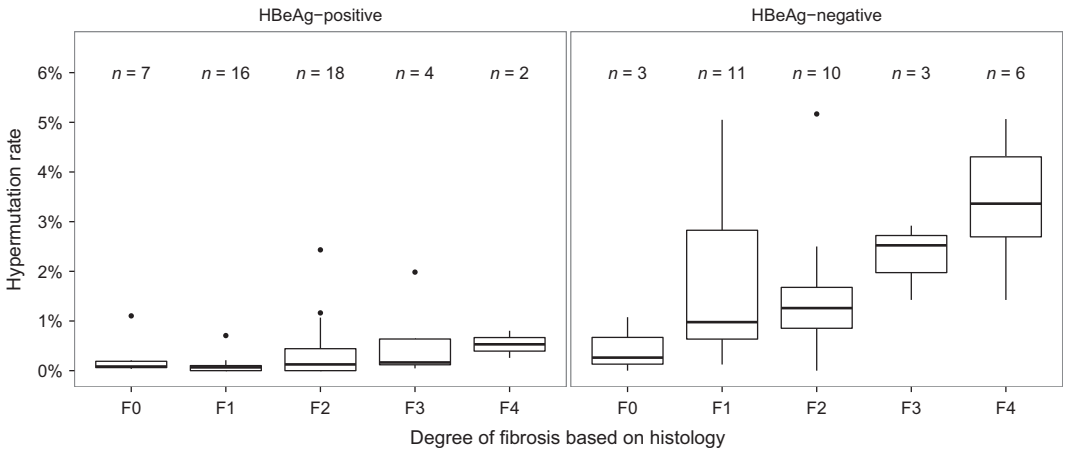

Fig. 3 Hypermutation rates by HBeAg status and degree of fibrosis. Hypermutation rates increase with higher degrees of fibrosis for both HBeAg-positive and HBeAg-negative patients. Hypermutated reads were defined by at least four G-to-A mutations and a G-to-A preference of at least 70% (amplicon 3 sequences only). Hypermutation rates increase with more severe fibrosis indicating higher activity of APOBEC3s in fibrotic tissue. The association of hypermutation rates with the degree of fibrosis is significant for HBeAg-negative patients ($P = 0.004$) but not significant for HBeAg-positive patients ($P = 0.09$). Boxplots show the median and interquartile ranges of perpatient hypermutation rates. The points indicate outliers, that is, patients with a hypermutation rate which lies beyond the end of the upper whiskers.

($P = 0.8$) and A1762G ($P = 0.4$). G-to-A hypermutation was also suspected to be relevant for the development of immune escape in the surface open reading frame of HBV for example mutations G145R or G145E (both mediated by a G-to-A transitions) [18,22]. We found that for HBeAg-positive patients the relative prevalence of these mutations strongly correlated with G-to-A hypermutation rates ($P < 0.0001$). For HBeAg-negative patients weaker correlations for G145R ($P = 0.9$) and G145E ($P = 0.01$) were observed.

DISCUSSION

By analysing ultra-deep pyrosequencing data of the complete HBV genome of 47 HBeAg-positive and 33 HBeAg-negative treatment naïve patients, we identified a nonuniform distribution of G-to-A hypermutations across the genome and significantly different hypermutation rates for HBeAg-positive and HBeAg-negative patients. We also found that hypermutation rates were significantly correlated with the degree of fibrosis in HBeAg-negative hepatitis.

Hypermutated sequence reads were analysed at the clonal level with respect to the dinucleotide context of the observed G-to-A exchanges. Depending on the genomic region, we observed that 74% to 89% of the hypermutated reads showed more GG-to-AG and GA-to-AA mutations compared with the GC-to-AC and GT-to-AT exchanges than one would expect based on the dinucleotide frequencies of the HBV genome. This indicates that the majority of hypermutations are due to human APOBEC3 deaminases, which display the relevant editing profiles [22,23,31–33].

A peak location of hypermutated reads was observed between NT positions 600 and 1800, which does not result from uneven coverage during sequencing but correlates with the replication cycle of HBV as proteins of the A3 family prefer to deaminate single-stranded DNA. In fact, mature HBV particles contain a genome in which NT positions between approximately 138 and 947 are partially single stranded, and NT positions from 947 to the plus-strand synthesis primer site are almost exclusively single stranded [34–37]. The predominant plus-strand primer site of HBV is the direct repeat element DR2 (NT positions 1824–1834). Thus, the detected hypermutation rates (Fig. 1) are in line with the prevalence of single-stranded DNA *in vivo*. High hypermutation rates were found where HBV DNA is almost exclusively single stranded (between NT positions 947 and DR2) and intermediate hypermutation rates are observed where HBV DNA is partly single stranded (between NT positions 138 and 947). Hypermutation with low frequency were also located between NT positions 1800–3221. This might hint to A3 activity on the HBV genome in mature virions before the synthesis of the plus strand, thus before cell exit. This situation is in contrast to, for example, editing of the human immunodeficiency virus which can only be targeted after cell entry and reverse transcription.

A key finding of our study is that the median G-to-A hypermutation rates in HBeAg-negative patients were more than 10-fold higher than those of HBeAg-positive patients. This finding is likely due to the fact that HBeAg-negative patients have undergone HBeAg seroconversion implying previous high immune response. Alternatively, suppression of the immune modulator HBeAg may have resulted in increased immune pressure. Our finding contradicts a study based on cloning and conventional sequencing of ten chronically infected patients where hypermutated genomes were only found in HBeAg-positive but not in HBeAg-negative patients [25]. Increasing hypermutation rates in HBeAg-positive chronic hepatitis were previously associated with HBeAg loss [26]. Interestingly, we found a significant correlation of the hypermutation rates with the relative prevalence of the G1764A mutant but not with the G1896A precore stop codon mutation. The G1764A mutation which is known to be an early indicator of HBeAg loss is located within the genomic region of high hypermutation rates but the G1896A precore stop codon mutation is not [38,39].

Several cytidine deaminases including A3B, A3C, A3G, A3H and AICDA are upregulated in HBV associated cirrhotic liver tissue [22]. Our data indicate that upregulation of A3 genes results in a significantly increased number of mutated genomes in sera of HBeAg-negative patients. This association was not significant for HBeAg-positive patients but a similar trend was observed (Fig. 3). No significant differences in the dinucleotide editing profile were identified between samples with significant fibrosis (F2, F3 and F4) and absent or mild fibrosis (F0 and F1). Editing dinucleotide profiles were not significantly different for HBeAg-positive and HBeAg-negative patients. This indicates that the proportion of reads being edited by A3G (which is known to have a strong bias towards the GG context) does not notable depend on the patients' HBeAg and fibrosis status. To some extent, our data contradict a previous study which analysed viral DNA from cirrhotic liver tissue and concluded according to the editing profiles that A3G plays a major role in editing HBV in cirrhotic tissue [22].

Our data provide new insights into G-to-A hypermutation of the HBV genome. Hypermutation significantly depends on the genomic region, the patients' HBeAg and fibrosis status. The observation that hypermutation rates are 10-fold higher in HBeAg-negative patients and that the relative prevalence of the core promoter mutation G1764A correlates with hypermutation rates for HBeAg-positive patients indicates an important association of hypermutation mediated by APOBEC3 deaminases in the natural progression of chronic hepatitis B infections both in term of HBeAg seroconversion and disease progression towards cirrhosis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1: Viral DNA isolation, amplification, and 454 pyrosequencing.