Hepatitis B virus counters intracellular antiviral responses by saturating APOBEC/AID enzymes with rcDNA

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Abstract

Hepatitis B virus (HBV) causes severe liver disease by establishing persistent infection in human hepatocytes. Current medications suppress viral replication but cannot eliminate the virus from infected cells. Efforts are focused on modulating intracellular immunity to develop new therapies targeting the major genomic form of HBV, known as covalently closed circular DNA (cccDNA). One potential approach involves utilizing cytidine deaminases APOBEC/AID, which have the ability to mutate and degrade HBV cccDNA. Our study reveals a novel evasion strategy used by HBV to counteract APOBEC/AID immunity and maintain a viral reservoir within the nuclei of infected cells by saturating these enzymes with relaxed circular DNA (rcDNA), the primary target of cytidine deaminases. Reducing rcDNA levels by siRNA or lamivudine or by using transcriptionally silenced cccDNA enhances cccDNA deamination by major APOBEC/AID enzymes. We also demonstrate severe deamination of the host genome by APOBEC3A, APOBEC3B, and AID upon viral suppression by siRNA or lamivudine, indicating that APOBEC/AID cannot be regarded as safe for antiviral treatment in cells with low HBV replication levels. Finally, we show that APOBEC3C and APOBEC3H can improve anti-HBV activity of siRNA therapeutics but do not affect cccDNA deamination. Off-site deamination for these factors was not detected at selected cancer-related genes.

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Abstract

Hepatitis B virus (HBV) causes severe liver disease by establishing persistent infection in human hepatocytes. Current medications suppress viral replication but cannot eliminate the virus from infected cells. Efforts are focused on modulating intracellular immunity to develop new therapies targeting the major genomic form of HBV, known as covalently closed circular DNA (cccDNA). One potential approach involves utilizing cytidine deaminases APOBEC/AID, which have the ability to mutate and degrade HBV cccDNA. Our study reveals a novel evasion strategy used by HBV to counteract APOBEC/AID immunity and maintain a viral reservoir within the nuclei of infected cells by saturating these enzymes with relaxed circular DNA (rcDNA), the primary target of cytidine deaminases. Reducing rcDNA levels by siRNA or lamivudine or by using transcriptionally silenced cccDNA enhances cccDNA deamination by major APOBEC/AID enzymes. We also demonstrate severe deamination of the host genome by APOBEC3A, APOBEC3B, and AID upon viral suppression by siRNA or lamivudine, indicating that APOBEC/AID cannot be regarded as safe for antiviral treatment in cells with low HBV replication levels. Finally, we show that APOBEC3C and APOBEC3H can improve anti-HBV activity of siRNA therapeutics but do not affect cccDNA deamination. Off-site deamination for these factors was not detected at selected cancer-related genes.

Keywords: NGS, 3D-PCR, virus-host interactions, antivirals, chronic infection, mutations.

Introduction

Hepatitis B virus (HBV) is a virus that infects human hepatocytes and causes acute or chronic infection of the liver (chronic hepatitis B virus infection, or CHB). Continuous replication of HBV in CHB results in progressing liver damage, leading to liver cirrhosis or hepatocellular carcinoma[1]. HBV has a complicated viral life cycle, starting from infection of human hepatocytes by HBV virions containing the partially double-stranded, relaxed circular DNA (rcDNA) viral genome. This is followed by nuclear import of rcDNA with the assistance of HBV core protein[2] and its conversion into covalently closed circular DNA (cccDNA), a highly persistent genomic form that transcribes all viral RNA, including pregenomic RNA (pgRNA)[3]. HBV RNA is translated into viral proteins (X, core, surface proteins, and E-antigen), whereas pgRNA is reverse-transcribed by viral polymerase to produce rcDNA[3]. HBV cccDNA is not only very stable (persisting through the lifespan of resting hepatocytes; estimated half-life $<$ 40 days to $>$9–26 months in patients undergoing nucleot(s)ide analog therapy)[4–6], but is also replenished via intracellular amplification (re-import of generated rcDNA back to the nucleus)[7–9] and re-infection[10]. Destroying HBV cccDNA or promoting its decay in hepatocytes can lead to a cure of HBV infection[7,11]. However, no medications are approved to target HBV cccDNA. While nucleot(s)ide analogs, interferons, and Bulevirtide (inhibitor of HBV entry) suppress HBV replication and reduce the risks of CHB outcomes, they do not cure the disease. Recent findings demonstrate that combining Bulevirtide with anti-HBV antisense oligonucleotides may result in sustained silencing of HBV cccDNA transcriptional activity[12]. HBV cccDNA can also be directly cleaved and destroyed by site-specific CRISPR/Cas9 nucleases[7,13–16]. Notably, some factors of innate immunity can limit HBV replication, while others, such as APOBEC/AID cytidine deaminases, can induce HBV cccDNA mutational inactivation and/or degradation[17].

In humans, the APOBEC/AID includes 10 members: APOBEC1, APOBEC2, APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3D (A3D), APOBEC3F (A3F), APOBEC3G (A3G), APOBEC3H (A3H), and AID[18]. A3A, A3B, A3C, A3D, A3F, A3G, A3H, and AID can directly deaminate cytidines in single-stranded DNA, introducing C to T and G to A mutations on the complementary strand; while APOBEC1, A3A, and A3G deaminate RNA, forming C to U mutations[19]. Recent findings indicate that elevated (but not endogenous) levels of A3B can also edit RNA at specific hotspots, causing lethality in a model of inducible A3B expression in mice[20]. APOBEC2 is unable to edit nucleic acids.

APOBEC/AID are famous mutators, implicated in the development of numerous cancers[21], and important factors that restrict foreign RNA and DNA, such as viral nucleic acids or intracellular DNA leaks, e.g., from nucleus or mitochondria[19,22]. APOBEC/AID suppress replication of many viruses infecting humans, like human immunodeficiency virus, herpes simplex virus 1, Epstein–Barr virus, hepatitis C virus, human papilloma virus, and HBV[19,23]. Pioneering studies demonstrated HBV-editing activity of A3G, A3C, A3H, and A3F[24–26]. A3G was also shown to inhibit HBV without cytidine deamination by suppressing pgRNA packaging into viral capsids and inhibiting reverse transcription[27]. More recently, Chen *et al* . extensively evaluated APOBEC/AID cytidine deamination activity of HBV rcDNA, demonstrating that deaminating activity decreases in the order A3B >> A3G > A3H or A3C[28], while the activity of A3A, A3D, A3H, and A3F was very low or undetectable. Several studies provided evidence that A3A, A3B, and AID can hyper-edit and destroy HBV cccDNA without off-site mutagenesis[17,29,30]. However, we recently described that transiently overexpressed A3A, A3B, and AID enzymes induce frequent mutations in cancer-related genes in the human genome in cells with low levels of HBV replication even upon transient activation, while A3G inflicts DNA double-stranded breaks [31].

Due to their potent anti-HBV activity, induction of APOBEC/AID is considered a promising antiviral strategy for potentially curing CHB patients. Intracellular expression of cytidine deaminases in the liver can be induced by different agonists, including interferon alpha (IFN-α)[17], IFN-γ[32], IFN-λ[33], lymphotoxin-β receptor agonist[17], and others. Otherwise, APOBEC/AID can be transcriptionally controlled by dCas-based molecular activators (CRISPR activation systems, CRISPRa)[19,31,34]. CRISPRa enables precise activation and tunable control of target gene expression and monogenic or simultaneous activation of several antiviral factors[35]. Typically, CRISPRa consists of a nucleolytically dead Cas9 protein (dCas) recruited to the regulatory regions of genes via single-guide RNA (sgRNA)[36,37]. Either dCas9 or sgRNA could also harbor units that mediate activation of transcription[34].

HBV's interactions with innate immunity are complex[18]; HBV is frequently regarded as a stealthy virus that evades immune recognition[38], and a resistant one as it is only moderately sensitive to the IFN-α antiviral response[39]. Some evidence indicates active countering of innate immunity by the virus (via STAT1 signaling[40], TLR2 recognition[41], etc.). In this study, we demonstrate the existence of another type of mechanism: saturation of APOBEC/AID factors with their primary target, HBV rcDNA. This "background defense" is provided by the vast overabundance of HBV rcDNA compared to 0–50 intracellular copies of cccDNA and by the preference of APOBEC/AID for single-stranded DNA. We demonstrate that transcriptionally inactivated cccDNA is more efficiently deaminated by APOBEC/AID than the transcriptionally active form. Reducing HBV rcDNA with a reverse transcriptase inhibitor or HBV-specific siRNA markedly enhances cccDNA deamination by APOBEC/AID. This positions HBV rcDNA as an important player in viral suppression mechanisms. However, our results indicate that A3A and A3B, which were not shown to deaminate the host genome in some previous studies, deaminate the host genome when rcDNA is depleted. A3C, A3D and A3H demonstrated weak rcDNA deamination and did not exhibit cccDNA-deaminating properties, but A3C and A3H increased antiviral activity of anti-HBV siRNA. At the same time, we did not detect off-site deamination by A3C, A3D, or A3H in a limited set of cancer-related genes.

Results

Transcriptionally silenced HBV cccDNA exhibits increased susceptibility to deamination by A3A, A3B, and AID.

Currently, there is still disagreement on whether HBV cccDNA is directly mutated by APOBEC/AID. Lucifora *et al* . provided extensive evidence of cccDNA deamination by A3A and A3B[42], but this effect was later questioned by Seeger and Sohn[43]and Nair and Zlotnik[44], who observed very low cccDNA mutation rates and hypothesized preferential deamination of rcDNA. Using primer extension analysis and clone sequencing, Chen *et al* . also did not detect cccDNA deamination by any APOBEC/AID enzymes overexpressed from DNA vectors[28]. Contrastingly, Faure-Dupuy *et al* . observed prolific deamination of HBV cccDNA by A3B, and first attempted to assess anti-HBV activity of A3B at transcriptionally inactive HBV ΔX genomes, which have a deleted X gene that is required for HBV transcription and rcDNA formation. The group showed robust mutation by A3B at both transcriptionally active and inactive cccDNA[30]. Our own group also showed that even very transient upregulation of A3A, A3B, or AID profoundly reduced and hyper-edited cccDNA[31]. cccDNA degradation by A3B was confirmed in another study that focused on the role of HIF-1α in suppressing A3B in CHB patients[45].

In this study, we used a well-established model of transcriptionally inactivated, hypermethylated recombinant cccDNA (meth-rcccDNA) that does not form pgRNA or rcDNA[7,14,46] to assess direct cccDNA deamination by APOBEC/AID factors with the most potent anti-HBV activity. CRISPRa of A3A, A3B, or AID similarly reduced both recombinant cccDNA (rcccDNA) and meth-rcccDNA by >50–75% (Figure 1A). Analysis of cccDNA deamination by 3D-PCR and semi-quantitative 3D-PCR, techniques enabling preferential amplification of deaminated nucleotides[17], revealed extensive deamination of cccDNA in the order A3A > A3B > AID (Figure 1B). Notably, this order of anti-cccDNA activity may be related to different target gene overexpression levels and duration by CRISPRa, as A3B is known to be the most potent DNA mutator of the APOBEC/AID family members. Contrastingly to similar reduction of HBV DNA levels, meth-cccDNA harbored >100-fold more mutations than cccDNA (Figure 1B).

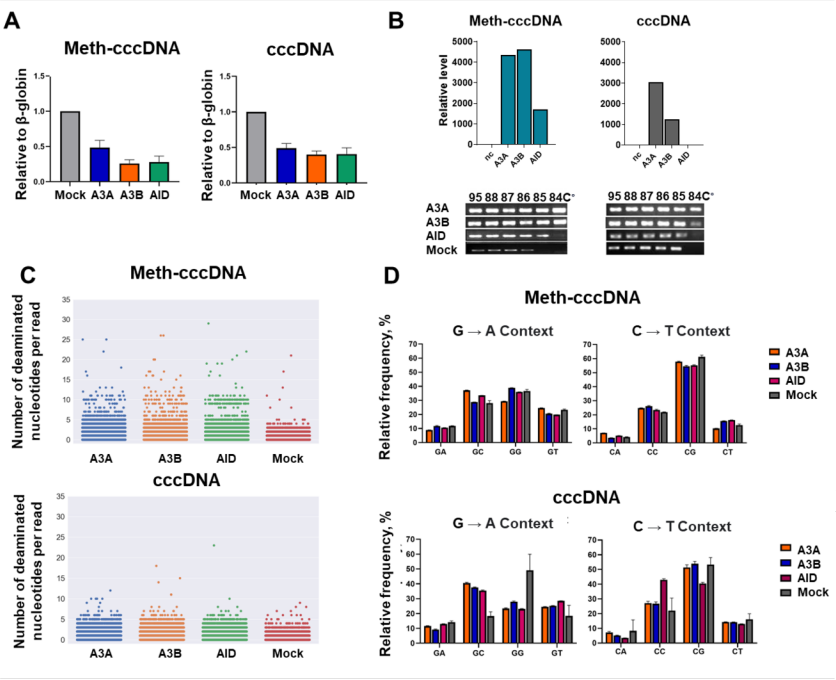

Figure 1. Effects of A3A, A3B, and AID on transcriptionally active and inactivated cccDNA. (A) Real-time PCR analysis of recombinant cccDNA (rcccDNA) or hypermethylated recombinant cccDNA (meth-rcccDNA) levels upon CRISPR activation of A3A, A3B, and AID. (B) 3D-PCR analysis of deamination in rcccDNA and methylated rcccDNA (top); semi-quantitative 3D-PCR analysis (bottom). (C) Frequency of G-A and C-T deaminated nucleotides in rcccDNA and meth-rcccDNA per read. (D) Analysis of rcccDNA and meth-rcccDNA dinucleotide G-A and C-T mutation context.

We deep-sequenced the X region of rcccDNA and meth-rcccDNA and analyzed the mutation profile caused by APOBEC/AID. Frequency of C-T/G-A mutations was markedly higher in meth-rcccDNA in all studied groups, indicating both higher frequency of C-T/G-A mutations per read and emergence of extensively edited reads (Figure 1C). Nucleotide context analysis of A3A, A3B, and AID showed similar nucleotide preference, characteristic for these factors (Figure 1D). Thus, A3A, A3B, and AID directly deaminate cccDNA and are more efficient in deaminating transcriptionally inactivated meth-cccDNA.

CG content and CpG methylation are significant factors that confer resistance to or lower activity of some APOBEC/AID enzymes[47]. Reports suggest weaker DNA deamination of methylated CpG by A3B[48] and AID[49], whereas A3A is generally not affected[50]. Thus, it was particularly surprising to observe more pronounced deamination of meth-rcccDNA than rcccDNA. The major difference between rcccDNA and meth-rcccDNA is that cccDNA launches viral replication and formation of other viral intermediates, mostly HBV rcDNA. Therefore, we endeavored to further assess the role of the most prevalent HBV intermediate – HBV rcDNA – in restricting APOBEC/AID activity.

Depleting HBV rcDNA by lamivudine or HBV-specific siRNA enhances cccDNA deamination by APOBEC/AID.

Currently, HBV rcDNA levels can be effectively reduced by a number of approaches, including the use of reverse transcriptase inhibitors such as lamivudine (LAM), which has a long history of clinical use for treating CHB patients[51], and by siRNA therapeutics, which exhibited potent antiviral activity in clinical trials[52]. Metabolite of LAM is incorporated into newly synthesized HBV DNA on the template of pgRNA, causing chain termination, and competitively inhibits HBV reverse transcriptase activity[53]; siRNA suppresses viral RNA, including pgRNA, and reduces HBV DNA and protein levels[54].

To elucidate the role of HBV rcDNA in APOBEC/AID-mediated deamination of cccDNA, we CRISPR-activated A3A, A3B, or AID in cells treated with either LAM or DMSO as vehicle (Figure 2), or co-transfected with HBV-targeting siRNA (targeting the X region of HBV) or mock control (Figure 3). Four days post transfection, HBV replication (assessed by measuring HBsAg, intracellular HBV DNA, and cccDNA levels) and cccDNA deamination were analyzed. HBsAg levels were not affected by LAM, CRISPRa, or combined treatment (Figure 2A), while siRNA potently reduced HBsAg expression (Figure 3A). CRISPRa reduced intracellular HBV DNA levels, but LAM or siRNA alone were as efficient as combined treatment (Figure 2B, 3B). Expectedly, cccDNA was not affected by LAM treatment, whereas CRISPRa of A3A, A3B, or AID diminished cccDNA by >2-fold, similar to our previous results[31] (Figure 2C). The combination of CRISPRa and LAM treatment exhibited comparable efficacy to CRISPRa monotherapy, while selectively activating A3A with LAM demonstrated significantly greater efficiency in reducing cccDNA than CRISPRa alone (Figure 2C). At the same time, the reduction in cccDNA levels in the siRNA + CRISPRa groups was significantly more prominent than in individual treatment groups. The findings suggest that both LAM and siRNA affect anti-cccDNA activity of A3A, A3B, and AID, with LAM showing limited improvement and siRNA demonstrating more pronounced effects.

Further 3D-PCR analysis revealed stark differences in the rates of APOBEC/AID-induced deamination of cccDNA and of secreted and intracellular HBV DNA between control and LAM/siRNA groups (Figure 2D-I, Figure 3D-I). It appears that, as a biochemically primary target for APOBEC/AID, the less abundant HBV rcDNA becomes much more actively deaminated. Upon LAM treatment, secreted HBV DNA was much more actively deaminated by A3A and A3B (Figure 2G), and moderately improved deamination of cccDNA (AID) (Figure 2E) and intracellular HBV DNA (A3B) (Figure 2I). Using siRNA, the results were very pronounced,

as increased deamination of intracellular and secreted HBV DNA was observed (Figure 3G, I), as well as of cccDNA by A3A and AID (Figure 3E).

Finally, NGS analysis of cccDNA and secreted rcDNA demonstrated stark differences in deamination between mock control and siRNA groups (Figure 4) consistent with 3D-PCR results (Figure 3D-G). Suppressing HBV replication with siRNA induced massive deamination of cccDNA by A3A and AID (Figure 4A). A3B-induced deamination was already prolific in the mock group, but was at the control level in siRNA group (Figure 4A). Potential explanation could be in degradation of heavily deaminated cccDNA as shown previously[30]. Nucleotide context deamination analysis demonstrated typical APOBEC/AID-induced mutations with preference of GA/GC context in secreted HBV DNA over GC/GG in cccDNA (Figure S1).

These results indicate that reduced levels of HBV rcDNA are more prone to deamination, while reduced HBV rcDNA levels also increase availability of cccDNA to APOBEC/AID enzymes. The lack of complete uniformity in deamination results between APOBEC/AID groups and LAM/siRNA groups may be explained by the complex dynamics of HBV DNA/cccDNA decline and deamination.

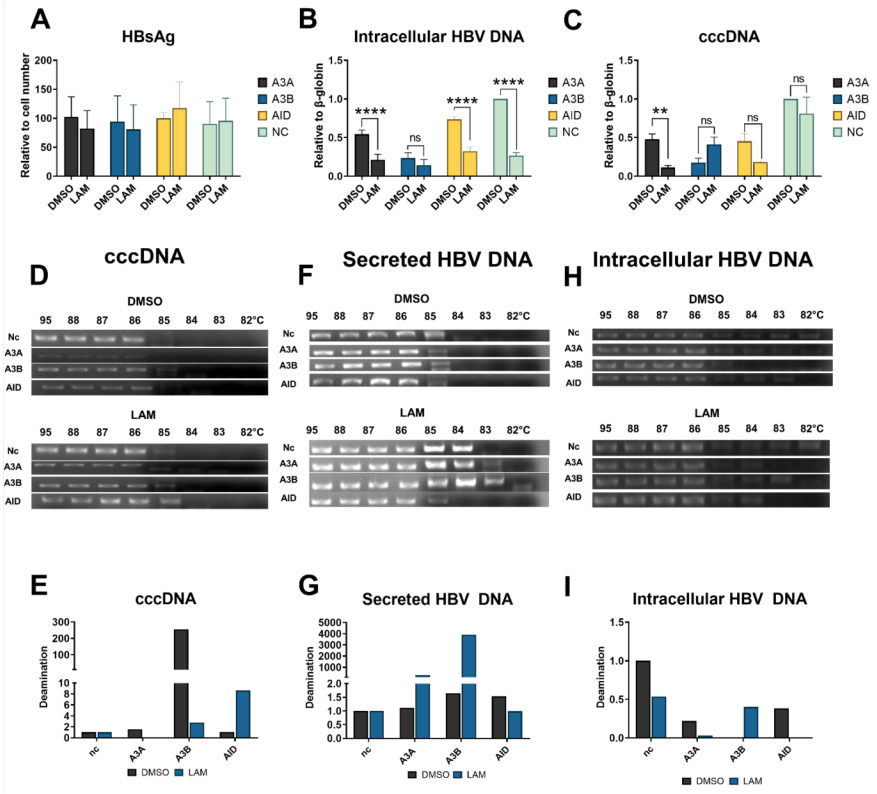

Figure 2. Antiviral and deaminating activity of APOBEC/AID combined with LAM treatment. **(A)** Analysis of secreted HBsAg. Effects of LAM treatment on **(B)** intracellular HBV DNA and **(C)**cccDNA levels with or without CRISPR activation of A3A, A3B, or AID.**(D)** 3D-PCR analysis of cccDNA deamination in a control (DMSO-treated) and experimental (LAM-treated) groups, and **(E)**semi-quantitative evaluation of cccDNA deamination levels. **(F)**3D-PCR analysis and **(G)** semi-quantitative evaluation of secreted HBV DNA deamination. **(H)** 3D-PCR analysis and**(I)** semi-quantitative analysis of intracellular HBV DNA. NC, non-targeting sgRNA. **p < 0.01; ****p < 0.0001.

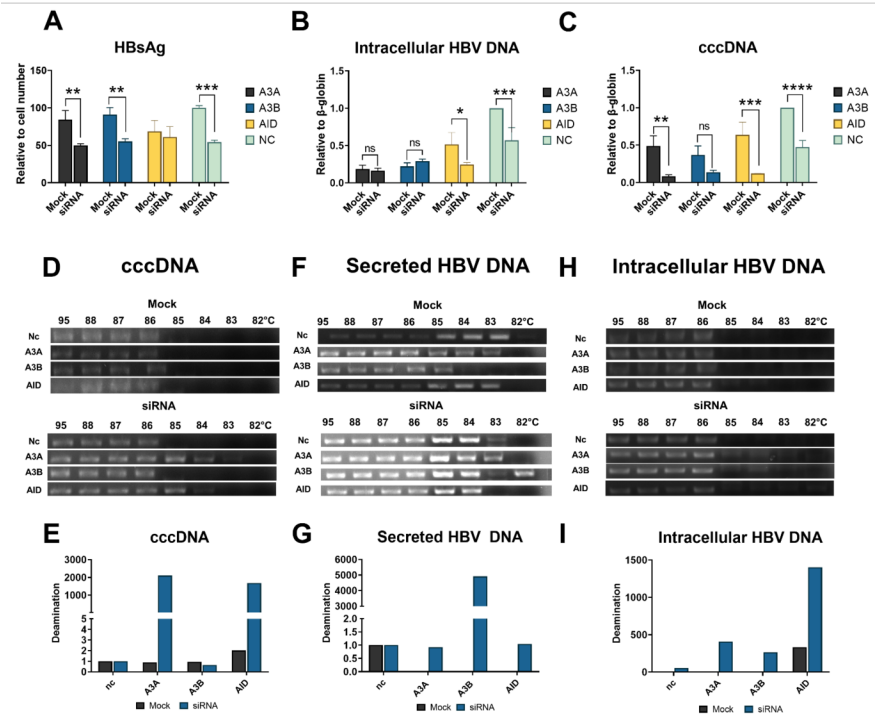

Figure 3. Effects of anti-HBV siRNA on APOBEC/AID-mediated antiviral and deaminating activity. Antiviral activity of CRISPR-activated APOBEC/AID with or without anti-HBV siRNA measured by determining levels of **(A)** HBsAg, **(B)** intracellular HBV DNA, and **(C)** cccDNA. HBV cccDNA deamination was analyzed by**(D)** 3D-PCR analysis. **(E)** Semi-quantitative analysis of cccDNA deamination. **(F)** 3D-PCR analysis of secreted HBV DNA deamination. **(G)** Semi-quantitative analysis of secreted HBV DNA deamination. **(H)** 3D-PCR results and **(I)**semi-quantitative analysis on intracellular HBV DNA. Mock, scrambled siRNA. NC, non-targeting sgRNA. *p $<$ 0.05; **p $<$ 0.01; ***p $<$ 0.001; ****p $<$ 0.0001.

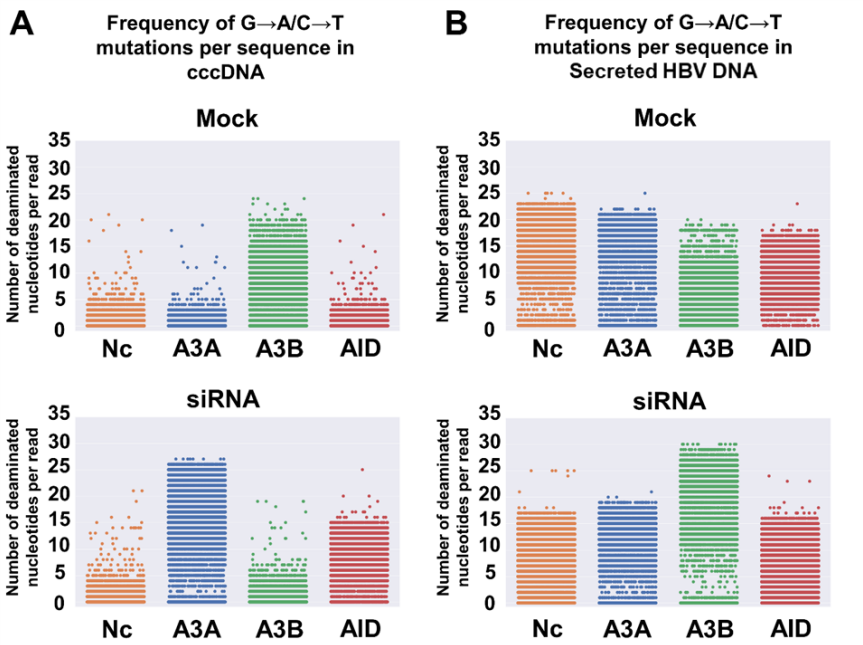

Figure 4. Analysis of G-A/C-T mutations per sequence in HBV cccDNA and rcDNA. Number of deaminated nucleotides per read in (A) cccDNA and (B) secreted HBV DNA upon CRISPR-activation of A3A, A3B or AID with anti-HBV siRNA or Mock control.

Suppression of HBV replication by LAM/siRNA promotes host genome deamination by A3A and A3B.

Lucifora *et al* .[17] and Qiao *et al* .[55] demonstrated that A3A, A3B, and AID can edit cccDNA but do not affect the host genome in HBV-infected cells due to association with HBc protein. Faure-Dupuy *et al* . did not detect A3B deamination of the host genome by NGS analysis as well[30]. However, our recent study observed a trend towards APOBEC/AID-mediated deamination of the host genome in cells with lower rates of HBV replication[31], suggesting that the use of APOBEC/AID therapeutic strategies may be disadvantageous in patients receiving antivirals or in patients with low viral loads.

Thus, we assessed potential impact of LAM and siRNA treatments on off-site genomic deamination of genes implicated in numerous types of human cancers (*TP53* , *AIRD2* , and*PAX5*)[56] by A3A, A3B, and AID. To study the impact of LAM/siRNA, a model of very active rcccDNA-driven HBV replication, which does appear susceptible to APOBEC/AID-driven mutations, was used[31]. Indeed, CRISPRa of A3A, A3B, and AID did not lead to deamination in any of the studied genes (Figure 5). On the other hand, LAM treatment resulted in deamination of*TP53* by A3A. Prolific deamination of *TP53* by A3A and A3B was also detected upon siRNA treatment (Figure 5A). Under these experimental conditions, no mutations were found in *ARID2* and*PAX5* genes (Figure 5B, C).

Together, these results indicate that therapeutic suppression of HBV replication makes the host genome prone to A3A and A3B pro-mutagenic activity.

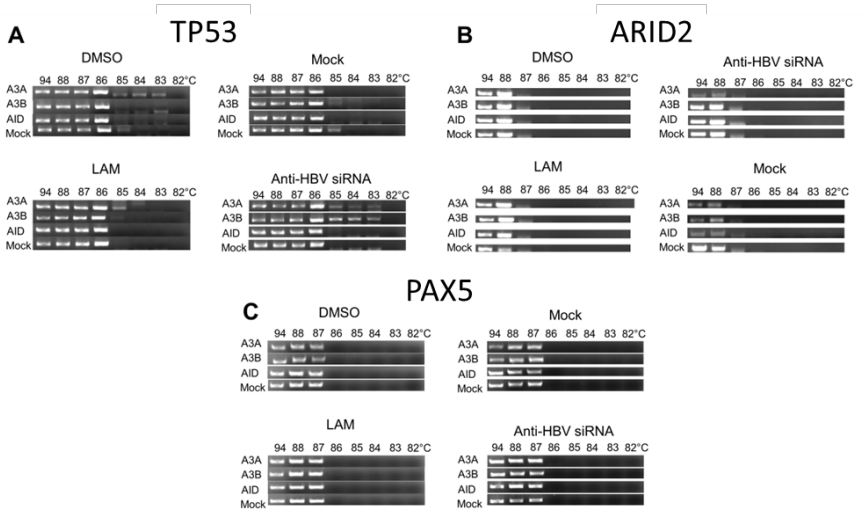

Figure 5. Off-site genome deamination by A3A, A3B, and AID upon HBV rcDNA depletion. Off-site mutations were measured by 3D-PCR in CpG-rich regions of **(A)** *TP53* , **(B)** *ARID2* , and **(C)** *PAX5* genes. Mock, non-targeting siRNA control; LAM, treatment with lamivudine; DMSO, treatment with DMSO as a control for LAM treatment. Generation of amplicons at lower melting temperatures indicates accrual of deamination-associated mutations.

CRISPR activation of A3C, A3D, and A3H suppresses HBV replication but does not affect cccDNA or the host genome.

As we showed, short-term activation of A3A and A3B effectively suppresses HBV replication but causes off-target deamination of the host genome. Therefore, we decided to analyze the antiviral efficacy of other members of the APOBEC3/AID family with previously reported anti-HBV activity (A3C, A3D, and A3H). We designed sgRNAs targeting promoters of the selected genes (Figure 6A) and analyzed the effects of CRISPRa of these genes in the HepG2 rcccDNA co-transfection model. Transcription of*A3C* , *A3D* , and *A3H* was increased 2–20-fold (Figure 6B). HBV life cycle analysis revealed a 50% reduction in both pgRNA and cccDNA levels following CRISPRa, while HBsAg level remained unchanged (Figure 6C).

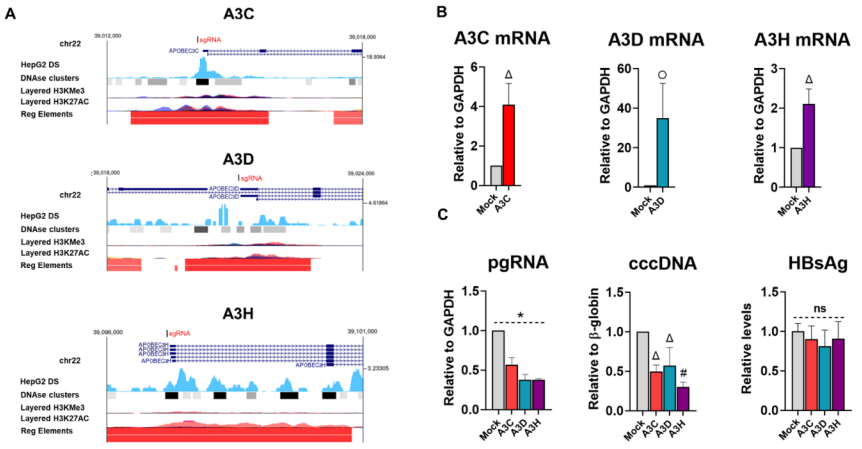

Figure 6. Activation of A3C, A3D, and A3H by CRISPR and its effects on HBV replication and cccDNA deamination. (A) Design of gene-targeting CRISPRa sgRNA. **(B)** Activation of target

genes measured by qRT-PCR. **(C)** Anti-HBV activity of CRISPR-activated genes determined by measuring pgRNA, cccDNA, and secreted HBsAg levels.

To further assess antiviral and deamination activity of A3C, A3D, and A3H under HBV rcDNA-depleted conditions, we performed experiments with anti-HBV siRNA (Figure 7). Combined treatment with siRNA, A3C, and A3H enhanced reduction in HBV rcDNA levels and cccDNA levels. However, HBV cccDNA deamination and secreted HBV DNA were not detected in either group (Figure 7E, I). Similar to the results with A3A, A3B, and AID, siRNA increased deamination of secreted HBV DNA with most factors (Figure 7I).

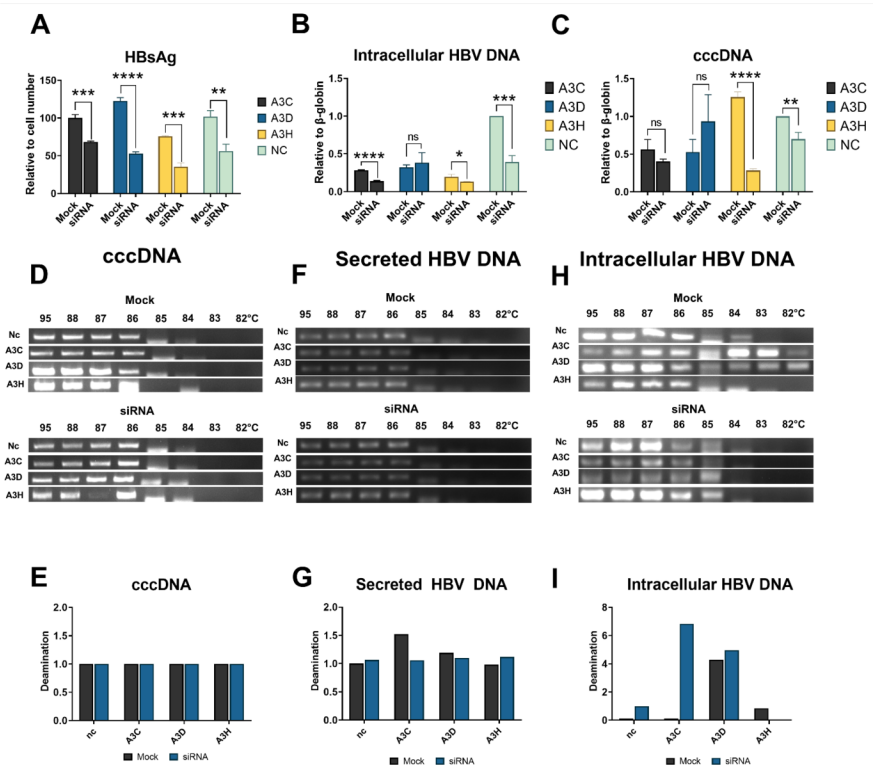

Figure 7. Effects of HBV DNA depletion on anti-HBV activity of A3C, A3D, and A3H. Analysis of **(A)** HBsAg, **(B)**intracellular HBV DNA, and **(C)** cccDNA levels. **(D-I)**3D-PCR analysis and semi-quantitative analysis of deamination of cccDNA, intracellular HBV DNA, and secreted HBV DNA.

At the same time, 3D-PCR analysis of the host genome did not detect deamination in selected regions of genes in either experimental group (Figure 8). This might be due either to lower ability of A3C, A3D, and A3H to affect the host genome, or a different set of targets mutated by these cytidine deaminases.

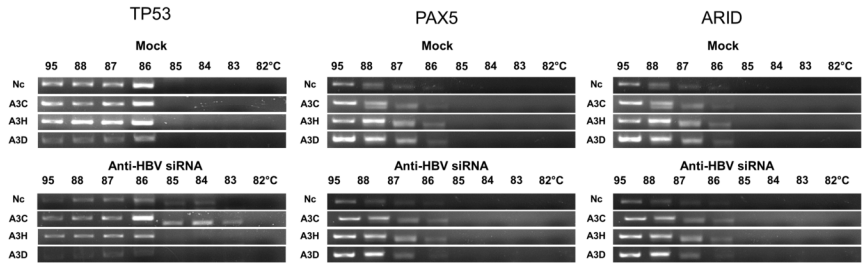

Figure 8. Off-target deamination of genomic HepG2 DNA by A3C, A3D, and A3H in combined treatments .

Discussion

In this work, for the first time, we assessed how HBV rcDNA affects antiviral and, specifically, cccDNA-targeting activity of major APOBEC/AID enzymes. Experiments with transcriptionally silenced cccDNA revealed markedly increased deamination of cccDNA by A3A, A3B, and AID (Figure 1). This prompted us to perform experiments with modern antiviral therapeutics that block or terminate HBV rcDNA production, namely lamivudine and siRNA targeting X gene of HBV. These experiments revealed increased deamination of the remaining rcDNA and, more importantly, increased cccDNA deamination (Figure 2, 3) with massive deamination demonstrated by NGS analysis (Figure 4). These observations suggest a new, background defense strategy utilized by the virus to counter innate immunity and to preserve cccDNA integrity by saturating antiviral enzymes with its genome intermediate, HBV rcDNA. This unique mechanism of immune evasion is somewhat similar to what HBV does to dampen adaptive immunity in CHB patients, when HBV produces excessive amounts of HBsAg compared to virions to establish antigen overload that exhausts antigen-presenting cells and leads to immune tolerance[57,58]. In this particular "hide-abide" strategy, HBV produces abundant HBV rcDNA, the single-stranded regions of which are the primary targets for APOBEC/AID cytidine deaminases. Overloading and saturating APOBEC/AID with ssDNA targets minimizes the effect of these enzymes on cccDNA, contributing to viral persistence. To the best of our knowledge, this is a newly characterized mechanism of HBV immune evasion that has not been reported previously for HBV or other viruses, adding to the already complex interaction of viruses with the host immune system.

Our results also discourage from further use of APOBEC/AID as potential anti-HBV therapeutics. APOBEC/AID enzymes have long been known to be important drivers of human cancers. Widespread APOBEC/AID-mediated mutations in genomic DNA of cancer patients have been detected[56]. More recent evidence adds knowledge about RNA-targeting properties of some APOBEC/AID enzymes dysregulating the host cell functioning[59] and inducing death in animals with, specifically, A3B overexpression[20]. The promise of APOBEC/AID as safe molecular tools came from the pioneering study by Lucifora *et al* .[42] who demonstrated that A3A and A3B do not deaminate the host genome due to binding HBc protein of HBV. However, we recently observed that A3A, A3B, and AID exhibit no off-site deamination only in human cells with very active HBV replication, whereas in cells with low viral loads, deamination of the host genome occurs[31]. In this study, we imitated the real-life therapeutic situation of suppressing HBV replication by antivirals, thereby reducing HBV viral loads, and then activated APOBEC/AID expression. In this scenario, A3A and A3B per se were not mutagenic, but the use of siRNA or LAM provoked host mutagenesis of the cancer-related *TP53* gene by A3A and A3B. Thus, using CRISPR deaminases able to directly interact with cccDNA[60] together with improved versions of cytidine deaminases lacking RNA-targeting properties may represent a more rational therapeutic approach[61].

At the same time, CRISPR activation and CRISPR interference approaches demonstrate high efficacy in highly specific and transient activation of antiviral genes for suppressing viral replication[19,31,62,63]. The search for the critical antiviral factors for CRISPR activation is necessary to further its use as an antiviral therapeutic[18].

To conclude, this study provides evidence of a new mechanism of immune evasion unique to HBV that works by saturating APOBEC/AID immunity with the rcDNA genomic form to preserve cccDNA pool integrity. Analysis of host genome deamination indicates severely hazardous activity of APOBEC/AID on host DNA when HBV viral loads are reduced, compromising its use as a potential therapy.

Materials and methods

Cell culture and transfection

HepG2 cells were cultured in DMEM high-glucose medium containing 10% fetal bovine serum (HyClone, Cytiva), 2 μM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. HepG2 cells were transfected with a plasmid encoding dCas9-p300 (pcDNA-dCas9-p300 Core), along with U6-PCR product encoding sgRNA and HBV rcccDNA produced using minicircle technology. After 48 h, cell culture medium was removed, and the cells were washed twice with PBS before being cultured in fresh complete medium for the next 72 h. All results were replicated in at least 3 independent studies.

Design and synthesis of anti-HBV siRNA

SiRNA targeting X-gene of HBV was selected from a study by Wooddell et al. (siHBV74)[64]. PCR product encoding siRNA under T7 promoter was synthesized using Q5 polymerase and following primers: fw_AAGCTAATACGACTCACTATAGGGACCAATTTATGCCTACAGCCttcaagagaGGCTGT, rev_AGACATAAAAAACAAAAAAAGACCAATTTATGCCTACAGCCtctctt. Mock siRNA was synthesized with primers fw_AAGCTAATACGACTCACTATAGGTTCTCCGAAC and rev_AGACATAAAAAACAAAAAAAAAACGTGACACGTTCGGAGAAC. T7 containing PCR product was used as a template for in vitro transcription (IVT) reaction using the HiScribe Quick T7 High Yield RNA synthesis kit (NEB) according to the manufacturer's protocol. The IVT reaction was incubated overnight and then treated with DNAse I (NEB) for 15 min at 37°C followed by isopropanol purification. In brief, isopropanol and 0.5 M NaCl were added to the reaction mixture and centrifuged for 30 min. Next, the pellet was consecutively washed twice with 70% and 95% ethanol. The air-dried pellet was resuspended in RNase-free water and stored at -80°C.

LAM treatment

LAM was dissolved in DMSO and stored as stock solution at -80°C until use. On the day of the experiment, an aliquot of stock solution was added to complete cell culture medium at a final concentration of 2 μM. Fresh LAM-containing medium was added to cells every 2 days.

Synthesis of recombinant HBV cccDNA

Recombinant HBV cccDNA was generated utilizing minicircle technology offered by System Biosciences. In short, the genetic material of HBV genotype D was inserted into a minicircle-producing plasmid equipped with attB and attP recombination sites, known as mini-HBV. The engineered mini-HBV construct was introduced into *E. coli* strain ZYCY10P3S2T (System Biosciences) and specific clones were selected, followed by a 4 h incubation at 37°C in kanamycin-supplemented Luria broth (LB). The resulting cell suspension was transferred into 200 mL of TB media and incubated overnight. This culture was then combined with 200 mL of induction media (composed of 1 N NaOH and 0.2% L-arabinose in LB), and incubated for 3 h at 30°C, followed by an additional 1 h incubation at 37°C. To isolate HBV rcccDNA, the resulting bacterial pellet was processed using the QIAGEN Plasmid Maxi Kit (Qiagen). Methylation of rcccDNA was performed using M.SssI CpG methyltransferase (SibEnzyme) as described previously [7,14,23].

CRISPR/Cas9 constructs

sgRNAs targeting promoters of genes of interest (*A3A* , *A3B* , *AID* , *A3C* , *A3D* , *A3H*) were designed using the open-access web tool Chop-Chop[65]. PCR products containing the U6 promoter and sgRNA specific for every promoter region were synthesized by two-step mutagenic PCR using Q5 polymerase and purified using a Qiagen gel extraction kit. The following plasmids were used: pcDNA-dCas9-p300 Core (AddGene plasmid #61357) and pcDNA-dCas9-p300 Core (D1399Y), gifts from Dr. Charles Gersbach; pLX-sgRNA (AddGene plasmid #50662), a gift from Dr. Eric Lander and Dr. David Sabatini; dCas9-p300 Core was cloned into Lenti-Cas9-2A-Blast plasmid (AddGene plasmid #73310; a gift from Dr. Jason Moffat) instead of *Streptococcus pyogenes* Cas9. dSaCas9-p300 was generated from pJEP303-pAAV-CMV-dSaCas9-VP64-pA (AddGene plasmid #113678; a gift from Dr. Jonathan Ploski) by cloning dSaCas9 into pcDNA-dCas9-p300

Core plasmid. dSaCas9 sgRNAs were produced from a pSaGuide (Addgene plasmid #64710; a gift from Dr. Kiran Musunuru). All primers are listed in *Table S1.*

In vitro sgRNA transcription and purification

PCR products containing sgRNA sequence under the control of a T7 promoter were synthesized using Q5 polymerase (NEB). These PCR products, which contained the T7 promoter, were then utilized for an in vitro transcription (IVT) reaction utilizing the HiScribe Quick T7 High Yield RNA synthesis kit (NEB), following the manufacturer's protocol. Following an overnight incubation, the IVT reaction was treated with DNAse I (NEB) for 15 min at 37°C, followed by purification using isopropanol. The resulting pellets were air-dried and then resuspended in RNase-free water before being stored at -80°C

Isolation of nucleic acids

Upon harvest, the cell culture medium was discarded, and the cells were washed twice with PBS. Subsequently, the cells were lysed using AmpliSens Riboprep lysis buffer (AmpliSense Biotechnologies). Nucleic acids were then extracted using the AmpliSense Riboprep kit (AmpliSense Biotechnologies) following the manufacturer's instructions. To isolate RNAs, the nucleic acids were treated with RNase-free DNase I (NEB) at 37°C for 30 min, purified using the AmpliSense Riboprep kit (AmpliSense Biotechnologies), and reverse-transcribed using AmpliSens Reverta-FL (AmpliSense Biotechnologies). For the isolation of HBV cccDNA, the Hirt procedure, described by Cai*et al* .[66], was used. The isolated cccDNA was then treated with plasmid-safe ATP-dependent DNase (Epicentre) at 37°C for 12 h, followed by enzyme inactivation at 72°C for 15 min.

PCR analysis

The expression levels of cellular mRNAs, HBV pgRNA, and S-mRNA were standardized using GAPDH mRNA as a reference. Total intracellular HBV DNA and cccDNA levels were normalized against genomic β-globin levels. Real-time PCR was conducted using specific primer and probe sets (listed in Table S2) on Rotor-Gene 6000 (Corbett Research) and CFX96 cycler (BioRad). Relative expression levels were determined using the ΔΔCt method.

3D-PCR and NGS

CpG-rich regions of HBV cccDNA or secreted rcDNA were amplified using a specific primer pair and TaqF polymerase. The resulting amplicons were purified from the gel and extracted using the Qiagen gel extraction kit. Equal amounts of the purified PCR products were then subjected to nested PCR using TaqF polymerase at progressively decreasing temperatures (95–82°C). The resulting PCR products were analyzed by gel electrophoresis and subjected to next-generation sequencing (NGS). Alternatively, for semi-quantitative analysis, 3D-PCR was performed with SYBRGreen dye. Briefly, 3D-PCR amplicons generated at 87°C, 84°C, or 82°C were purified from the gel using the Qiagen gel extraction kit, quantified using a Qubit 2.0 Fluorometer (Life Technologies), and pooled in equimolar ratios. Adapters for Illumina sequencing were ligated, and the libraries were sequenced using the MiSeq instrument (Illumina) with 250 paired-end reads. To analyze the sequences, FASTQC software and Geneious software were employed for quality assessment, reference alignment, removal of low-quality reads and nucleotides, and calculation of indels. Custom Python codes (available upon request) were used for mutation analysis and mutation context analysis. 3D-PCR primers are listed in Table S3.

HBsAg and analysis

Media that had been conditioned by cells were collected and filtered through 0.2-μm filters to eliminate any cellular debris. HBsAg was quantitated using the DS-IFAHBsAg-0.01 colorimetric ELISA, following the manufacturer's instructions (Diagnostic Systems, Russia).

Statistical analysis

Values were expressed as the mean ± SD of triplicate experiments using SPSS software (SPSS 21.0.0.0). One-way ANOVA and Student's t-tests with Tukey's HSD post hoc tests were used to compare variables and calculate p values to determine statistically significant differences in means.

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