Original Article

Reduction in Intrahepatic cccDNA and Integration of HBV in Chronic Hepatitis B Patients with a Functional Cure

Weiqiang Gan[1,2#] (iD), Na Gao[1#] (iD), Lin Gu[1], Zhishuo Mo[1], Xiuqing Pang[1], Ziying Lei[1*] (iD) and Zhiliang Gao[1,2,3*] (iD)

[1]Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China; [2]Guangdong Key Laboratory of Liver Disease Research, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China; [3]Key Laboratory of Tropical Disease Control (Sun Yat-sen University), Ministry of Education, Guangzhou, Guangdong, China

Abstract

Background and Aims: Functional cure (FC) is characterized by the clearance of the hepatitis B surface antigen from the serum of patients with chronic hepatitis B (CHB). However, the level of intrahepatic covalently closed circular DNA (cccDNA) and hepatitis B virus (HBV) integration remains unclear. We conducted this study to determine them and reveal their value in the treatment of CHB. ***Methods:*** There were two sessions to elucidate the changes in intrahepatic cccDNA and HBV integration after antiviral therapy. In the first session, 116 patients were enrolled and divided into FC, non-functional cure (NFC), and CHB groups, including 48 patients with functionally cured CHB, 27 with CHB without functional cure after antiviral treatment, and 41 with treatment-naïve CHB. Patients were tested for both intrahepatic cccDNA and other viral markers. All patients in the FC group were followed up for at least 24 weeks to observe relapse. In the second session, another ten patients were included for in-depth whole-genome sequencing to analyze HBV integration. ***Results:*** Thirteen patients in the FC group were negative for intrahepatic cccDNA. Intrahepatic cccDNA was much higher in the CHB group compared with the FC group. Seven patients had HBsAg seroreversion, including two with virological relapse. Integration of HBV was detected in one (33.3%) functionally cured patients and in seven (100%) with CHB. 28.0% of the HBV breakpoints were assigned in the 1,500 nt to 1,900 nt range of the HBV genome. ***Conclusions:*** After achieving an FC, the rate of intrahepatic cccDNA and HBV integration was significantly reduced in patients with CHB. For those patients who cleared intrahepatic cccDNA, the chances of developing virological relapse were even lower.

Citation of this article: Gan W, Gao N, Gu L, Mo Z, Pang X, Lei Z, *et al*. Reduction in Intrahepatic cccDNA and Integration of HBV in Chronic Hepatitis B Patients with a Functional Cure. J Clin Transl Hepatol 2022. doi: 10.14218/JCTH.2022.00177.

Introduction

Chronic hepatitis B (CHB) is a significant threat to human health. More than 240 million people are currently chronically infected with the hepatitis B virus.[1] A functional cure (FC) is defined as a sustained virological response and the disappearance of the hepatitis B surface antigen (HBsAg) with normalization of alanine aminotransferase (ALT) and improvement of liver histology after the completion of a finite course of treatment.[2] Through the efforts of hepatologists worldwide, the use of nucleot(s)ide analogues (NAs) and interferon alpha (IFNα) or different combinations of pegylated interferon alpha (Peg-IFNα), FC rates have greatly improved. However, some patients still experience virological relapse after discontinuation of antiviral therapies, which may be the result of the persistence of covalently closed circular DNA (cccDNA) capable of transcribing different mRNAs of the hepatitis B virus (HBV) genome, leading to relapse.[3] The level of intrahepatic cccDNA and its importance in virological relapse have not been previously reported. It is necessary for clinicians to know the level of intrahepatic cccDNA after an FC.

Complete cure of CHB is defined as the removal of cccDNA based on an FC. A sterile cure is defined as the disappearance of both cccDNA and integrated HBV DNA.[2,4] Whether that can be achieved with current treatment strategies has never been reported. Over the last 30 years, HBV DNA has been reported to integrate into the human genome during different stages of CHB infection, and HBV integration can accumulate during disease progression.[5] In hepatocellular carcinoma (HCC) associated with latent HBV infection, HBV DNA integration is considered an attractive oncogenic risk.[6] When FC is achieved, cccDNA and HBV integration remains. However, viral DNA integration is considered as a replicative dead end for HBV, and the presence and potential clinical implica-

tions of HBV integration in patients with HBsAg loss remain unclear. Therefore, we conducted this study to better understand intrahepatic cccDNA after obtaining an FC, and we also performed deep sequencing of the liver genome to explore HBV integration in patients with HBsAg seroclearance.

Methods

Patients

There were 48 HBsAg-negative FC patients and 27 HBsAg-positive NFC patients in the clinic of our department. Three patients in the FC group were treated with NAs alone, and all the other patients were treated with Peg-IFN, switching from NAs with undetectable HBV DNA and HBsAg below 1,500 IU/mL. All patients in the FC group discontinued Peg-IFN after HBsAg disappeared. The patients in the NFC group were switched to NA monotherapy, and liver biopsies were performed on all patients. The liver biopsies were performed between 12 January 2017, and 11 August 2020. The patients were followed by regular visits. Forty-one positive control samples were originally stored in the liver tissue specimen bank of our department, which was retained before antiviral therapy in treatment-naïve CHB-group patients, and synchronous serum was collected. Specimens were stored in a refrigerator at −80°C. Liver biopsies were performed between 21 August 2010, and 26 May 2014. Ten patients outside the above cohorts were included for deep whole-genome sequencing of liver tissue, including four hepatitis B e antigen (HBeAg)-positive patients, three HBeAg-negative patients with treatment-naïve CHB, and three with an FC after antiviral treatment. The study protocol was consistent with the International Conference on Harmonization Guidelines, applicable regulations, and the ethical guidelines of the Declaration of Helsinki. The protocol and consent forms were approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University, China (2016, 2-129). Patients with antiviral therapy were obtained from a real-world study registered at clinicaltrails.gov (NCT02745704). All authors had access to the study data and reviewed and approved the final manuscript.

Serological and virological parameters

qHBsAg was measured with Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN, USA). Serum HBV DNA was assayed with a Roche COBAS AmpliPrep/COBAS TaqMan HBV Test v2.0 (Roche Molecular Diagnostics, Branchburg, NJ, USA). All procedures were performed following the manufacturer's instructions. Serum hepatitis B core related antigen (HBcrAg) levels were measured with an automated chemiluminescent enzyme immunoassay (CLEIA) immunoassay analyzer and Lumipulse G HBcrAg kits (both from Fujirebio, Inc., Tokyo, Japan). HBcrAg values were expressed as log U/mL, and the measurement range was 3.0–7.0 U/mL. Serum samples were diluted with normal human serum and retested if the HBcrAg levels exceeded the upper limit.

Quantification of serum HBV pgRNA

Serum HBV pre-genomic RNA (pgRNA) levels were measured with a HBV pgRNA quantification kit (Supbio, Inc., Guangzhou, China) as previously described.[7,8] HBV pgRNA in serum was isolated, treated with DNase I, and then reverse-transcribed with a commercial kit according to the manufacturer's instructions. Finally, qPCR was performed without the use of reverse transcriptase. The lower limit of detection was 25 copies/mL.

Quantification of intrahepatic cccDNA

Liver biopsies were performed with ultrasound-guided techniques, and the tissue samples were preserved in liquid nitrogen. HBV cccDNA was purified from liver tissue using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Enzyme treatment, and PCR amplification were performed with the HBV cccDNA quantitative detection kits (Supbio, Inc., Guangzhou, China). In brief, 10 µL of extracted DNA was removed, 1 µL plasmid-safe ATP-dependent DNase (PSAD) enzyme and 39 µL of buffer were added, with incubation at 37°C for 30 m, and immediate transfer to a 70°C incubator for 30 m followed by centrifugation at 13,000 rpm for 1 minute, and storage at −20±5°C. qPCR reactions were performed with an Applied Biosystems 7500 real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The lower limit of detection was 600 copies/10^6 cells, and the quantitative range was 1×10^3 to 5×10^7 copies/10^6 cells.

Clinical samples and DNA extraction for deep sequencing of the liver genome

DNA was extracted from liver tissue using TIANamp Genomic DNA Kits (Tiangen Biotech, Beijing, China), and 1 µg genomic DNA was sheared using Covaris M220 (Covaris, Inc., Woburn, MA, USA). Sequencing was performed on the Illumina Novaseq 6000 platform (Illumina Inc., San Diego, CA, USA) with 150 bp paired-end reads. Deep sequencing was prepared for HBV integration analysis and HBV genotyping.

HBV integration sites

Clean reads obtained were mapped to the human reference genome (hg38) and the HBV genome. We used the paired-end nature of the reads to search for HBV integration. If a paired-end read uniquely mapped to hg38 at one end and the HBV genome at the other end, it was divided into discordant paired reads and chimeric reads. Chimeric reads were considered as integration sites. The position of the breakpoint was defined as the junction between the human and HBV sequences in the reads assembled at the end of the pairs (chimeric reads). The reads in each category were clustered according to their coordinates if the minimum distance between the edges of the two reads was <50 bp (HBV genome) or 100 bp (human genome) in both paired-end reads.[9] Our goal was to sequence 2 billion read pairs. Approximately 2.6 billion paired-end 150 bp reads were obtained for each sample. Humans were partially annotated in HBV integrations from the National Center for Biotechnology Information (NCBI), the University of California, Santa Cruz (UCSC) genome browser home page, Ensemble, HumCFS database,[10] and the DriverDBv2 database.[11] Reads only mapped to HBV genome were used to analyze HBV genotype.

Statistical analysis

SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used to establish the database and perform the statistical analysis.

Table 1. Demographic and virological features of patients in the study groups

Parameter	FC (N=48)	NFC (N=27)	CHB (N=41)	*p*-value
Age, years, mean (SD)	37.2 (8.2)	39.7 (9.3)	38.2 (8.0)	0.507
Sex (F/M)	7/41	4/23	8/33	0.796
ALT, median (IQR)	25.5 (19.3, 34.5)	29.5 (21.0, 35.0)	60.0 (30.0, 148)	<0.001
Serum HBV DNA ($\log_{10}$ IU/mL)	ND	ND	5.8 (1.5)	NA
qHBsAg ($\log_{10}$ IU/mL)	ND	1.4 (1.1)	3.4 (0.6)	<0.001
Serum pgRNA (ND/Positive)	48/0	18/3 (*n*=21)	1/38 (*n*=39)	NA
pgRNA ($\log_{10}$ copies/mL)	ND	3.0 (1.0) (*n*=3)	4.2 (1.6)	0.193
HBcrAg ($\log_{10}$ IU/mL)	3.6 (0.5)	3.3 (1.0) (*n*=11)	6.1 (1.7)	<0.001
Intrahepatic cccDNA (ND/Positive)	13/35	3/24	0/41	0.105
Intrahepatic cccDNA ($\log_{10}$ copies/cell)	−1.3 (0.9) (*n*=35)	−0.9 (0.6) (*n*=24)	0.3 (0.9)	<0.001

ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; FC, functional cure; HBcrAg, hepatitis B core related antigen; HBV, hepatitis B virus; NA, not available; ND, not detected; NFC, non-functional cure; pgRNA, pre-genomic RNA; qHBsAg, quantitative hepatitis B surface antigen.

Normally distributed variables were reported as means±SD. Independent samples were compared using *t*-tests or one-way analysis of variance. Non-normally distributed measurements were reported as medians and compared using the rank-sum test. Statistical analysis of HBV integration was performed with R3.6.0. Pathway enrichment analysis of integrated genes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) was performed with the R package ClusterProfiler. Chi-square tests were used to compare the ratios. Differences were considered statistically significant if *p* was <0.05.

Results

Intrahepatic cccDNA and serum viral markers in different groups

There were no differences in age and sex among the three groups. The levels of ALT were significantly higher in the CHB group than in the other two groups (*p*<0.05). Serum HBV DNA was not detected in the FC and NFC groups, compared with 5.8 $\log_{10}$ IU/mL in the CHB group. HBsAg was detected in both the NFC and CHB groups, with the latter being significantly higher (1.4 vs. 3.4 $\log_{10}$ IU/mL, *p*<0.05). The FC group was negative for pgRNA. Three of the 21 patients in the NFC group and 38 of the 39 patients in the CHB group were positive, with mean levels of 3.0 and 4.2 $\log_{10}$ copies/mL respectively. Serum HBcrAg levels were significantly lower in the FC and NFC groups than in the CHB group (*p*<0.05). Intrahepatic cccDNA was negative in 13 patients in the FC group and three in the NFC group. None of the patients in the CHB group had undetectable intrahepatic cccDNA. Intrahepatic cccDNA levels were significantly higher in the CHB group than in the other two groups, but the difference in the levels of the FC and NFC groups was not significant (Table 1).

Clearance of intrahepatic cccDNA in the FC group and its possible influencing factors

Of the 48 FC patients, cccDNA was undetectable in 13, a clearance rate of 27%. There were still 73% of patients with positive intrahepatic cccDNA at levels in the logarithmic range of 0.00365 to 1.54 copies/cell, or −2.44 to 0.19 $\log_{10}$ copies/cell, significantly lower than in the CHB groups (*p*<0.001).

Patients were further divided into cccDNA-negative and cccDNA-positive groups according to the cccDNA status. Sex, age, family history, duration of hepatitis B disease, and antiviral treatment in the two groups were compared. There were no differences between the two groups in sex, age, duration of hepatitis B disease, history of NAs treatment, type of interferon used, or the time to HBsAg clearance. More patients in the cccDNA-positive group had a family history of hepatitis B than in the negative group, but the difference was not statistically significant (45.7% vs. 23.1%, *p*=0.145). Before interferon treatment, more patients in the cccDNA-negative group had lower levels of HBsAg (≤200 IU/mL) than those in the positive group, but the difference was not statistically significant (*p*=0.153, Table 2).

Relationships between virological markers, pathological changes, and cccDNA clearance

To understand whether virological markers and pathological changes were associated with cccDNA clearance, we compared those parameters in the cccDNA-positive and cccDNA-negative groups. The results showed that the patients in the cccDNA-negative group had more advanced liver fibrosis (*p*<0.05). However, there were no differences in virological markers or other pathological changes, including serum HBcrAg, hepatitis B surface antibody (HBsAb) levels, liver inflammation, and HBsAg/hepatitis B core antigen (HBcAg) immunohistochemistry (*p*>0.05; Supplementary Table 1).

Association between viral markers and HBsAg relapse

The 48 patients with clearance of HBsAg were followed for more than 24 weeks after discontinuation of the drug (Fig. 1). A total of seven patients showed HBsAg seroreversion (i.e. >0.05 IU/mL). qHBsAg increased to 0.05–1 IU/mL in one case and to 1–20 IU/mL in four cases, with HBV DNA remaining negative (Fig. 2). qHBsAg increased to 1.99 IU/mL in one case with 0.0216 copies/cell of intrahepatic cccDNA, 12 weeks after discontinuation of the drug, and HBV DNA remained undetectable. However, qHBsAg increased to 130 IU/mL, and HBV DNA rebounded to 1.09e8 IU/mL with normal ALT at week 24 after discontinuation of the drug. Another patient with 0.0487 copies of intrahepatic cccDNA/cell had a relapse of HBsAg at week 12 after drug discontinuation, with a qHBsAg of 0.419 IU/mL, while HBV DNA remained undetectable. qHBsAg increased to 18.96 IU/mL

Table 2. Comparison of demographic features in cccDNA-negative and positive groups

	cccDNA-negative (N=13)	cccDNA-positive (N=35)	*p*-value
Age (years) Mean (SD)	37.5 (6.5)	37.1 (8.8)	0.875
Sex (F/M)	2/11	5/30	1.000
Family history of hepatitis B*, n (%)	3 (23.1%)	16 (45.7%)	0.197
NAs duration (years) median (IQR)	6.0 (3.0, 8.0)	3.0 (2.0, 8.0)	0.197
Type of interferon (N=45)			0.721
PEG α-2a	4 (30.8%)	8 (25.0%)	
PEG α-2b	9 (69.2%)	24 (75.0%)	
HBsAg prior to IFN (N=44)			0.153
>1,000, ≤1,500	2 (15.4%)	2 (6.3%)	
>200, ≤1,000	2 (15.4%)	15 (46.9%)	
≤200	9 (69.2%)	15 (46.9%)	
Maximal ALT level on treatment, U/L, mean (SD)	108.5 (72.2)	108.4 (65.7)	0.998
Minimal WBC ×10^9/L, mean (SD)	2.9 (0.8)	3.0 (0.7)	0.813
Minimal PLT ×10^9/L, mean (SD)	98.0 (37.0)	101.1 (33.6)	0.792
Time of HBsAg clearance			0.751
≤24 weeks	6 (46.2%)	13 (40.6%)	
>24 weeks	7 (53.8%)	19 (59.4%)	
IFN duration, weeks, median (IQR)	37 (30, 54)	44 (33, 48)	0.870

*Parents or siblings; ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; HBsAg, hepatitis B surface antigen; IFN, interferon; IQR, interquartile range; NAs, nucleot(s)ide analogues; PEG, pegylated; PLT, platelet; SD, standard deviation; WBC, white blood cell.
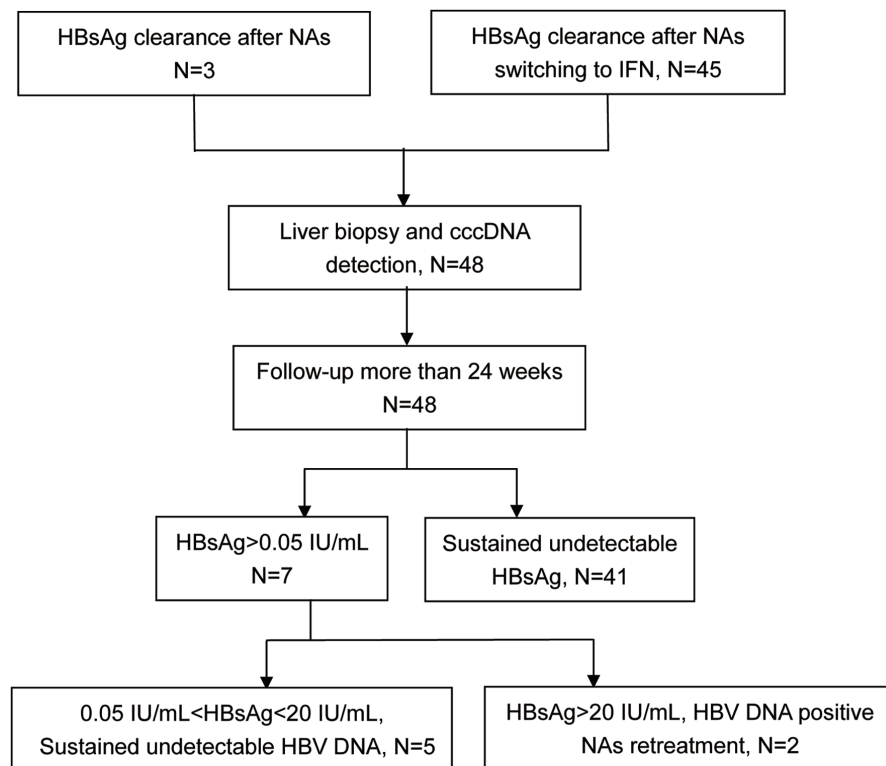
Fig. 1. Roadmap of follow-up in chronic hepatitis B patients with a functional cure. cccDNA, covalently closed circular DNA; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; NAs, nucleot(s)ide analogues.
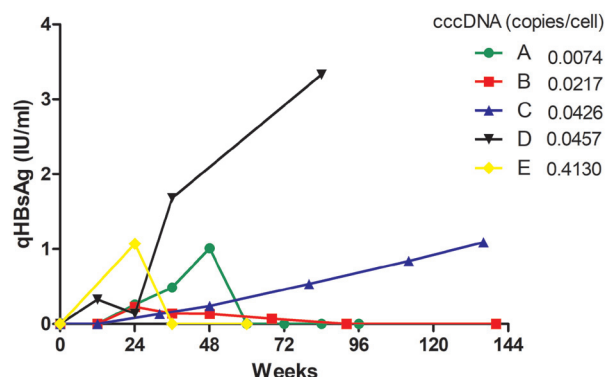
Fig. 2. Dynamics of qHBsAg in five HBsAg seroreversion patients without virological relapse. Patient C and D remained positive until the observational date while patient A, B, E dropped to normal at around week 60 during follow-up. cccDNA, covalently closed circular DNA; qHBsAg, quantitative hepatitis B surface antigen.

at week 24, and HBV DNA was negative. However, at week 48, qHBsAg reached 37.36 IU/mL, HBV DNA rebounded to 347 IU/mL, and ALT was normal. Two patients with virological relapse were retreated with NAs and became undetectable for HBV DNA at 24 weeks.

Levels of intrahepatic cccDNA, serum HBcrAg, HBsAb, and HBsAg

Immunohistochemistry results in patients with and without HBsAg relapse are shown in Supplementary Table 2. Seven of 35 patients (20%) with positive intrahepatic cccDNA experienced HBsAg relapse, while no relapse occurred in patients with negative intrahepatic cccDNA. No relapses occurred in those with HBsAb above 100 IU/mL, otherwise, the relapse rate was 24.1% (7/29, $p<0.05$). There was no statistical effect of HBcrAg and HBsAg immunohistochemistry on relapse.

Characteristics of patients with deep sequencing

We performed deep whole genome sequencing (WGS) to search for HBV integration sites across the human genome. A total of 10 patients were included, including three CHB pa-

tients with an FC, four HBeAg-positive, and three HBeAg-negative treatment-naïve CHB patients (Supplementary Tables 3 and 4). Patient F808 received 38 months of entecavir (ETV) monotherapy, switching to a combination of Peg-IFN and ETV therapy for 12 weeks, followed by 36 weeks of Peg-IFN monotherapy. HBsAg disappeared at week 12 of Peg-IFN monotherapy. Patient R228 was treated with 13 months of tenofovir disoproxil fumarate (TDF) and was switched to a combination of Peg-IFN and TDF for 72 weeks. HBsAg disappeared at week 44 of combination therapy. Patient F807 experienced acute on chronic liver failure and was treated with ETV for 22 months. HBsAg disappeared in the eighth month. Seventy percent of the patients had at least one family member with HBV-associated disease. Based on WGS with HBV reads, we examined the HBV genotypes of all patients. Excluding F807, which lacked HBV reads, we found five cases with genotype B and four cases with genotype C, consistent with the predominant genotypes in South China.[12]

Integration of viruses into the human genome

There were 439 viral integration sites in 10 liver samples (Supplementary Table 5). The numbers of integration sites in the HBeAg-positive CHB group, the HBeAg-negative CHB group, and the FC group were 132, 304, and 3, respectively. There was a different conclusion about whether HBV integration was distributed throughout the genome. To answer that question, we compared the observed chromosome distribution with the expected group, which was a random selection of 444 sites from the entire human genome after removing assembly gap sites from the genome. Integration sites were preferentially located on chromosomes four and 11, and fewer integration sites were located on chromosome X than expected (Fig. 3). Overall, 45.79% of the integration sites were located in protein-coding genes. The promoters were defined as 5 kb upstream of the transcription start sites. HBV integration was not preferentially located in the promoters, exons, introns, and 3'UTR, affecting gene expression.

Breakpoints and integrative links in the HBV genome

HBV reads were detected in liver samples from one (33.3%) FC patients and seven (100%) CHB patients. The number of HBV reads per patient was much lower in the FC group
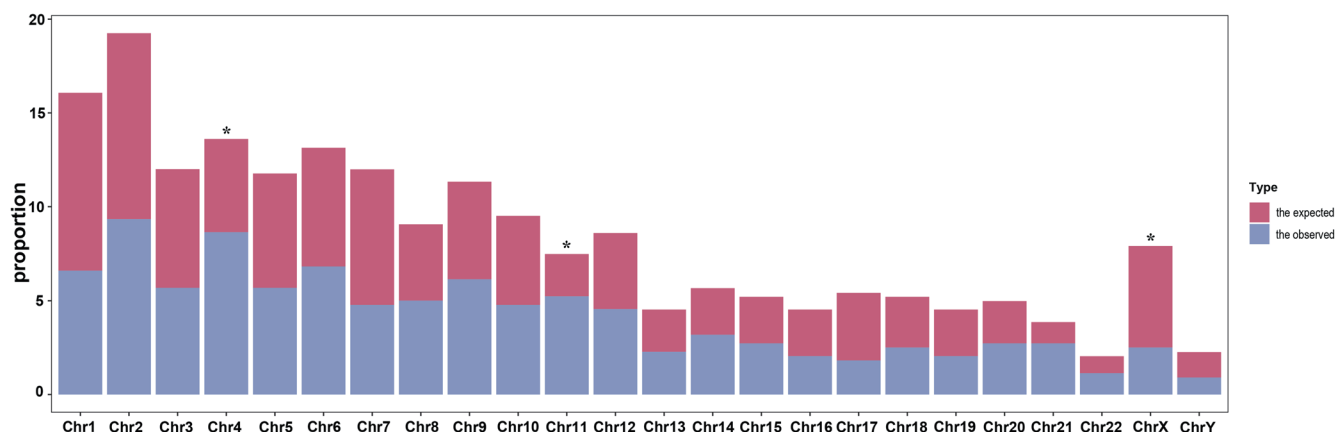
Fig. 3. Chromosome distribution of HBV integration in the human genome. Whole-chromosome represents the expected (assuming uniform, random distribution, dark red) and the observed (actual numbers, violet) ratio of HBV integration at a particular chromosome in human genome. *P*-values were calculated by chi-square tests. HBV, hepatitis B virus; Chr, chromosome.
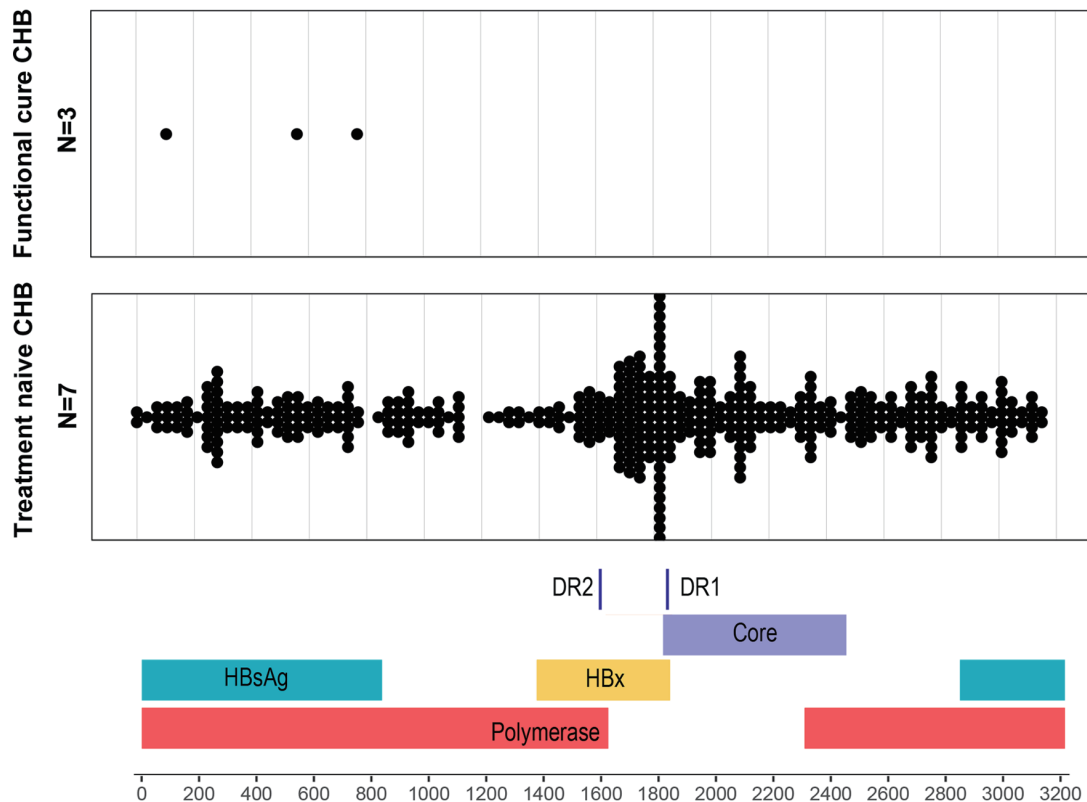
Fig. 4. Distribution of HBV break points across the HBV genome. The figure includes sites from genotype B and C of HBV. Viral junctions determined from chimeric reads. The locations of the genes encoding HBV polymerase (red), core protein (violet), HBsAg (blue) and HBx (yellow) are shown. HBV, hepatitis B virus; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBx, hepatitis B X protein.

than in the HBeAg-positive CHB group and the HBeAg-negative CHB group (Fig. 4). For HBV integration, 28.0% of HBV breakpoints were mapped within the 1,500 nt to 1,900 nt range of the HBV genome, which contains DR1 and DR2, which play a crucial role in integrating HBV DNA into the host cell genome.[13] The viral genome had a clear preference for breakpoints near the 3' end of HBx and the 5' end of the Precore/Core gene, which may lead to HBV-human fusion reads. However, all three breakpoints were located in the S/P region in the FC group. It is thought that double-strand break repair plays a role in HBV integration, which assumes that linearized double viral DNA ends are joined to the human genome via non-homologous end joining and microhomology-mediated end joining using microhomologous sequences during the alignment of the break ends prior to joining.[14] Based on the hypothesis, we searched for microhomologous sequences between human sequences and inserted HBV DNA at integration sites in the chimeric reads, and compared them with the expected group. We found that 39.64% of HBV fragments were linked to the human genome by microhomologous sequences greater than or equal to 2 bp.
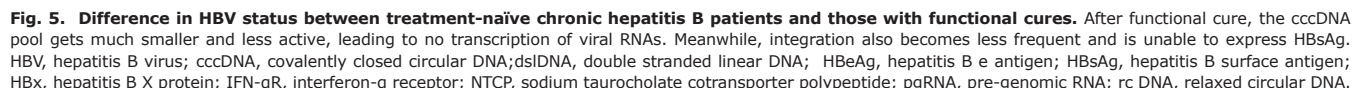
HBV integration in the driver gene and functional annotation of the HBV integration-targeted gene

Four integration sites in the HBeAg-positive CHB group, 23 in the HBeAg-negative CHB group, and one in the FC group were located in the liver cancer driver gene. The liver cancer driver gene was identified in the DriverDBv2 database.[11] In one patient who achieved an FC, HBV DNA remained integrated in the driver gene, which may indicate that HBV integration might be a potential risk for liver carcinogenesis despite the patient achieving the ultimate goal of HBV infection.[15] KEGG analysis of integrated genes showed enrichment in cancer-related pathways, such as small-cell lung cancer and choline metabolism in cancer (we selected only the top 30 based on *p*-values, Supplementary Fig. 1). The cancer pathways imply that influencing the expression or function of integrated genes associated with oncogenic pathways may be an oncogenic factor for HBV integration.

Discussion

Our study showed that cccDNA was undetectable in 27% (13 of 48) of CHB patients after achieving an FC. For patients with positive intrahepatic cccDNA, the levels were significantly reduced compared with treatment-naïve CHB patients. Many studies have shown that NAs and IFNs significantly reduce intrahepatic cccDNA levels.[16,17] Our data confirm that result, and more importantly, all patients who achieved cccDNA clearance did not experience virological relapse, which implies that cccDNA clearance can be achieved with current treatment and is essential to predict whether CHB patients will relapse after drug discontinuation in short-term follow-up. Although HBsAg seroreversion was achieved in seven patients, only two experienced a virological relapse. All HBsAg relapses occurred in the cccDNA-positive group, which is not surprising because the original template is required for transcription and replication. Our study shows that CHB patients have a very low risk of relapse after FC. We hypothesized that might be because the

Fig. 5. Difference in HBV status between treatment-naïve chronic hepatitis B patients and those with functional cures. After functional cure, the cccDNA pool gets much smaller and less active, leading to no transcription of viral RNAs. Meanwhile, integration also becomes less frequent and is unable to express HBsAg. HBV, hepatitis B virus; cccDNA, covalently closed circular DNA;dslDNA, double stranded linear DNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBx, hepatitis B X protein; IFN-αR, interferon-α receptor; NTCP, sodium taurocholate cotransporter polypeptide; pgRNA, pre-genomic RNA; rc DNA, relaxed circular DNA.

cccDNA pool became smaller and inactive (Fig. 5). Since some patients in our study were followed up for only 24 weeks, their dynamics still need to be observed over time.

To better illustrate the levels of serum or intrahepatic viral markers, the methodologies should be appropriate and accurate. Currently, there are various methods to detect serum pgRNA determined by targets used by the assays. Shi Liu *et al*.[18] reported that methodologies targeting both the 5'and 3'ends of the HBV pgRNA, the S region, and the X-region had similar performance in predicting clinical relapse and HBeAg loss. Targeting polyA tail of HBV RNA was not satisfactory. In this study, the assay targeted the X-region and performed well in both treatment-naïve and experienced CHB patients.[7,8] However, there is no acknowledged standard to detect HBV pgRNA. Our method had a detection limit of 25 copies/mL, and methodologies having lower detection limits are required. Likewise, multiple strategies to detect intrahepatic cccDNA have been reported. Among them, droplet digital PCR (ddPCR) had higher sensitivity and specificity than qPCR.[19] However, its high cost, complicated procedure, and unstable upper limit, its availability hindered its application. The method we used in this study was relative quantification using primers that crossed the gap of relaxed circular DNA (rcDNA). We also added PSAD to degrade rcDNA to exclude the amplification of rcDNA. Although it would not completely exclude the possibility of interference by rcDNA, we still aimed to minimize its influence and present a relatively reliable and cost-effective result.

Several serum viral parameters have been reported to be associated with cccDNA levels.[20,21] We also analyzed the relationship between cccDNA and serum viral markers. Our results showed that none of the serum viral markers were

associated with cccDNA levels. Most published results were obtained from treatment-naïve CHB patients or those experienced patients with high levels of viral markers and intrahepatic cccDNA who did not obtain an FC. Therefore, we believe that this contradiction is due to the low levels of viral markers and intrahepatic cccDNA as well as the small sample size.

Understanding the mechanisms of cccDNA clearance and finding the parameters that show cccDNA clearance is crucial for hepatologists. We analyzed a variety of parameters, including family history, type of treatment, duration of treatment, serum viral markers, and histological parameters. Unfortunately, the results showed that none of the above parameters was associated with cccDNA clearance. Therefore, more effort should be made to expand the sample size or explore other factors, such as immune indices.

To further understand HBV integration after an FC and whether a sterilizing cure can be achieved, we performed deep sequencing of the liver genome. Our data show that HBV integration occurred more frequently in HBeAg-negative CHB-infected patients than in other infected patients, consistent with previous reports.[22] As HBV-associated liver disease progresses, the percentage of HBV double stranded linear DNA in the serum is presumed to be a precursor to HBV integration, which gradually increases.[23] In addition, it has been proposed that the frequency of integrated DNA increases with HBV infection duration.[24,25] Although integrated HBV DNA has lost its ability to produce viral progeny, it can still produce HBsAg, particularly in patients with negative HBeAg.[26] Although Summers and Mason[25] reported a marked decrease in viral cccDNA, but the integrated viral DNA in a woodchuck hepatitis model did not show a significant decrease during NAs treatment.[25] In contrast, Ning

et al.[27] found that HBV DNA integration was significantly reduced after NAs treatment, which may explain the decreased risk of HCC after NAs treatment.[27] Although HBsAg was not detectable in patient R228, three integration sites were still present in the liver after therapeutic stress. It is well documented that integrated DNA is stable *in vitro* after at least 17 cell divisions, unlike cccDNA, which can be diluted by cell division.[28] The most noteworthy finding was that the number of HBV integrations was significantly lower in patients with FCs than in treatment-naïve CHB patients.

Unlike a previous study by Yang *et al*.[29] showing that HBV integration was more common on chromosomes 1 and 2 in non-tumor tissue with HBV infection, our data show that integration sites were preferentially located on chromosomes four and 11. As the depth of sequencing increases, more integrations are detected.[30] Alu repeats are usually separated by >3,000 bp, leading to scarce detection by Alu PCR, especially in low clonal samples that can produce more Alu-Alu products.[31,32] However, deeper WGS with genome-wide coverage provides an objective and comprehensive view of HBV integration. Discrepancies associated with different experimental approaches to detect HBV integration may lead to different conclusions regarding the distribution of integration sites throughout the genome.

Chimeric reads showed breakpoints for HBV integration enriched in the HBV genome between 1,500 bp and 1,900 bp, consistent with previous studies.[33–35] The breakpoints of functionally cured were within the S/P open reading frame, different from the type in CHB patients, which revealed a preferential pattern within the 3' end of the X gene, and the C-terminal truncated X protein (Ct-HBx) was shown to initiate hepatocarcinogenesis.[36] In addition, breakpoints in S region would lead to the failure to express HBsAg by integrated HBV fragments. Whether patients with an FC share the same patterns of integration needs to be validated. We are planning to enroll more FC and NFC patients after antiviral treatment to better understand their differences.

A female patient who survived acute on chronic liver failure was not found to have any cccDNA or HBV in the liver after entecavir treatment, implying a potential sterilizing cure. That may indicate a different trend in those patients who lost HBsAg through different immune statuses at the start of treatment had a distinguishable outcome of HBV DNA integration. Patient R228 achieved an FC and had fewer HBV integration sites after combined therapy with Peg-IFN and tenofovir. However, noteworthy HBV integration occurred at ATF7IP, a cancer driver gene, which could potentially contribute to the development of HCC and needs to be validated in future studies. Functional annotation analysis of host genes involved in HBV integration sites showed that some functional terms belong to cancer-related pathways. Therefore, the oncogenicity of HBV integration may be determined by targeting hepatocellular carcinoma-associated host genes.

HBV infection can exist for decades, during which time DNA damage is enhanced by liver inflammation and hepatocyte regeneration, which may provide more available DNA ends in host genomic DNA and promote HBV integration. Thus, there may be a trend toward increased HBV integration during long-term HBV infection. Our data show that patients had more integration sites after HBeAg seroconversion. It is generally accepted that liver inflammation and fibrosis may improve to some extent when patients achieve an FC; however, some integration remains in hepatocytes, and some of these integration sites play a potential role in hepatocellular carcinoma. This will require long-term cohort studies to observe whether HBV integration is progressively reduced over the course of treatment.

The study has some limitations. First, the number of functionally cured patients was not large enough for HBV integration analysis and may have produced selection bias. The data need to be validated in a larger population. Second, although deep sequences were used, some HBV integrations may have been missed across the human genome. Efforts are underway to combine deep WGS and RNA sequences to mitigate these deficiencies.

Conclusions

In conclusion, intrahepatic cccDNA levels were significantly reduced in CHB patients with FCs. cccDNA clearance was achieved in 27% of patients. The risk of virological relapse was very low in FC patients. Although the rate of integration of HBV in patients with FCs was substantially lower than in patients with CHB naïve treatment, they are nonetheless at potential risk of developing HCC.

Funding

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Analysis and writing of the manuscript (WG), HBV integration (NG), storage of the specimens and analysis of the data related to cccDNA (LG), follow-up of the patients (ZM), collection of the data and part of the statistical analysis (XP), conduct of the liver biopsies and analysis of the data (ZL), and overall supervision of the study and guidance of the co-authors (ZG).

Ethical statement

The study protocol was consistent with the International Conference on Harmonization Guidelines, applicable regulations, and the ethical guidelines of the Declaration of Helsinki. The protocol and consent forms were approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University, China (2016, 2-129). Patients with antiviral therapy were obtained from a real-world study registered at clinicaltrails.gov (NCT02745704).

Data sharing statement

The datasets used in the study are available from the corresponding author on reasonable request.

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(10003):1546–1555. doi:10.1016/S0140-6736(15)61412-X, PMID:26231459.

[2] Terrault NA, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, *et al*. Update on Prevention, Diagnosis, and Treatment of Chronic Hepatitis B: AASLD 2018 Hepatitis B Guidance. Clin Liver Dis (Hoboken) 2018;12(1):33–34. doi:10.1002/cld.728, PMID:30988907.

[3]   Xu WX, Peng L. Response to: Comment on "48-Week Outcome after Cessation of Nucleos(t)ide Analogue Treatment in Chronic Hepatitis B Patient and the Associated Factors with Relapse". Can J Gastroenterol Hepatol 2019;2019:2970510. doi:10.1155/2019/2970510, PMID:31187027.

[4]   Testoni B, Levrero M, Zoulim F. Challenges to a Cure for HBV Infection. Semin Liver Dis 2017;37(3):231–242. doi:10.1055/s-0037-1606212, PMID:28847034.

[5]   Yang L, Ye S, Zhao X, Ji L, Zhang Y, Zhou P, *et al*. Molecular Characterization of HBV DNA Integration in Patients with Hepatitis and Hepatocellular Carcinoma. J Cancer 2018;9(18):3225–3235. doi:10.7150/jca.26052, PMID:30271481.

[6]   Saitta C, Tripodi G, Barbera A, Bertuccio A, Smedile A, Ciancio A, *et al*. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. Liver Int 2015;35(10):2311–2317. doi:10.1111/liv.12807, PMID:25677098.

[7]   Gu Y, Chen L, Lian Y, Gu L, Chen Y, Bi Y, *et al*. Serum HBV pregenomic RNA is correlated with Th1/Th2 immunity in treatment-naïve chronic hepatitis B patients. J Med Virol 2020;92(3):317–328. doi:10.1002/jmv.25612, PMID:31642539.

[8]   Wang X, Chi X, Wu R, Xu H, Gao X, Yu L, *et al*. Serum HBV RNA correlated with intrahepatic cccDNA more strongly than other HBV markers during peg-interferon treatment. Virol J 2021;18(1):4. doi:10.1186/s12985-020-01471-2, PMID:33407619.

[9]   Hama N, Totoki Y, Miura F, Tatsuno K, Saito-Adachi M, Nakamura H, *et al*. Epigenetic landscape influences the liver cancer genome architecture. Nat Commun 2018;9(1):1643. doi:10.1038/s41467-018-03999-y, PMID:29691395.

[10] Kumar R, Nagpal G, Kumar V, Usmani SS, Agrawal P, Raghava GPS. HumCFS: a database of fragile sites in human chromosomes. BMC Genomics 2019;19(Suppl 9):985. doi:10.1186/s12864-018-5330-5, PMID:30999860.

[11] Chung IF, Chen CY, Su SC, Li CY, Wu KJ, Wang HW, *et al*. DriverDBv2: a database for human cancer driver gene research. Nucleic Acids Res 2016;44(D1):D975–D979. doi:10.1093/nar/gkv1314, PMID:26635391.

[12] Hepatitis B virus infection. Nat Rev Dis Primers 2018;4:18036. doi:10.1038/nrdp.2018.36, PMID:29877317.

[13] Hino O, Ohtake K, Rogler CE. Features of two hepatitis B virus (HBV) DNA integrations suggest mechanisms of HBV integration. J Virol 1989;63(6):2638–2643. doi:10.1128/JVI.63.6.2638-2643.1989, PMID:2542576.

[14] McVey M, Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends Genet 2008;24(11):529–538. doi:10.1016/j.tig.2008.08.007, PMID:18809224.

[15] Jang JW, Kim JS, Kim HS, Tak KY, Nam H, Sung PS, *et al*. Persistence of intrahepatic hepatitis B virus DNA integration in patients developing hepatocellular carcinoma after hepatitis B surface antigen seroclearance. Clin Mol Hepatol 2021;27(1):207–218. doi:10.3350/cmh.2020.0115, PMID:33317255.

[16] Chuaypen N, Sriprapun M, Praianantathavorn K, Payungporn S, Wisedopas N, Poovorawan Y, *et al*. Kinetics of serum HBsAg and intrahepatic cccDNA during pegylated interferon therapy in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B. J Med Virol 2017;89(1):130–138. doi:10.1002/jmv.24601, PMID:27307409.

[17] Lai CL, Wong D, Ip P, Kopaniszen M, Seto WK, Fung J, *et al*. Reduction of covalently closed circular DNA with long-term nucleos(t)ide analogue treatment in chronic hepatitis B. J Hepatol 2017;66(2):275–281. doi:10.1016/j.jhep.2016.08.022, PMID:27639844.

[18] Liu S, Wu Y, Deng R, Shen S, Fan R, Peng J, *et al*. Methodology-dependent performance of serum HBV RNA in predicting treatment outcomes in chronic hepatitis B patients. Antiviral Res 2021;189:105037. doi:10.1016/j.antiviral.2021.105037, PMID:33711337.

[19] Caviglia GP, Abate ML, Tandoi F, Ciancio A, Amoroso A, Salizzoni M, *et al*. Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection. J Hepatol 2018;69(2):301–307. doi:10.1016/j.jhep.2018.03.021, PMID:29621551.

[20] Chuaypen N, Posuwan N, Chittmittraprap S, Hirankarn N, Treeprasertsuk

S, Tanaka Y, *et al*. Predictive role of serum HBsAg and HBcrAg kinetics in patients with HBeAg-negative chronic hepatitis B receiving pegylated interferon-based therapy. Clin Microbiol Infect 2018;24(3):306.e7–306.e13. doi:10.1016/j.cmi.2017.07.016, PMID:28750917.

[21] Wang J, Shen T, Huang X, Kumar GR, Chen X, Zeng Z, *et al*. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J Hepatol 2016;65(4):700–710. doi:10.1016/j.jhep.2016.05.029, PMID:27245431.

[22] Fowler MJ, Greenfield C, Chu CM, Karayiannis P, Dunk A, Lok AS, *et al*. Integration of HBV-DNA may not be a prerequisite for the maintenance of the state of malignant transformation. An analysis of 110 liver biopsies. J Hepatol 1986;2(2):218–229. doi:10.1016/s0168-8278(86)80080-0, PMID:3958473.

[23] Zhao XL, Yang JR, Lin SZ, Ma H, Guo F, Yang RF, *et al*. Serum viral duplex-linear DNA proportion increases with the progression of liver disease in patients infected with HBV. Gut 2016;65(3):502–511. doi:10.1136/gutjnl-2014-308989, PMID:26045139.

[24] Yang L, Ye S, Zhao X, Ji L, Zhang Y, Zhou P, *et al*. Molecular Characterization of HBV DNA Integration in Patients with Hepatitis and Hepatocellular Carcinoma. J Cancer 2018;9(18):3225–3235. doi:10.7150/jca.26052, PMID:30271481.

[25] Summers J, Mason WS. Residual integrated viral DNA after hepadnavirus clearance by nucleoside analog therapy. Proc Natl Acad Sci U S A 2004;101(2):638–640. doi:10.1073/pnas.0307422100, PMID:14699050.

[26] Wooddell CI, Yuen MF, Chan HL, Gish RG, Locarnini SA, Chavez D, *et al*. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. Sci Transl Med 2017;9(409):eaan0241. doi:10.1126/scitranslmed.aan0241, PMID:28954926.

[27] Long term nucleos(t)ide analogue therapy reduced the extent of HBVDNA integration in chronic hepatitis B patients. Hepatology 2020;72(S1):19A. doi:10.1002/hep.31578.

[28] Bill CA, Summers J. Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. Proc Natl Acad Sci U S A 2004;101(30):11135–11140. doi:10.1073/pnas.0403925101, PMID:15258290.

[29] Yang X, Wu L, Lin J, Wang A, Wan X, Wu Y, *et al*. Distinct hepatitis B virus integration patterns in hepatocellular carcinoma and adjacent normal liver tissue. Int J Cancer 2017;140(6):1324–1330. doi:10.1002/ijc.30547, PMID:27943263.

[30] Jiang Z, Jhunjhunwala S, Liu J, Haverty PM, Kennemer MI, Guan Y, *et al*. The effects of hepatitis B virus integration into the genomes of hepatocellular carcinoma patients. Genome Res 2012;22(4):593–601. doi:10.1101/gr.133926.111, PMID:22267523.

[31] Budzinska MA, Shackel NA, Urban S, Tu T. Cellular Genomic Sites of Hepatitis B Virus DNA Integration. Genes (Basel) 2018;9(7):E365. doi:10.3390/genes9070365, PMID:30037029.

[32] Larsson SB, Tripodi G, Raimondo G, Saitta C, Norkrans G, Pollicino T, *et al*. Integration of hepatitis B virus DNA in chronically infected patients assessed by Alu-PCR. J Med Virol 2018;90(10):1568–1575. doi:10.1002/jmv.25227, PMID:29797342.

[33] Sung WK, Zheng H, Li S, Chen R, Liu X, Li Y, *et al*. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet 2012;44(7):765–769. doi:10.1038/ng.2295, PMID:22634754.

[34] Zhao LH, Liu X, Yan HX, Li WY, Zeng X, Yang Y, *et al*. Genomic and oncogenic preference of HBV integration in hepatocellular carcinoma. Nat Commun 2016;7:12992. doi:10.1038/ncomms12992, PMID:27703150.

[35] Tatsuno K, Midorikawa Y, Takayama T, Yamamoto S, Nagae G, Moriyama M, *et al*. Impact of AAV2 and Hepatitis B Virus Integration Into Genome on Development of Hepatocellular Carcinoma in Patients with Prior Hepatitis B Virus Infection. Clin Cancer Res 2019;25(20):6217–6227. doi:10.1158/1078-0432.CCR-19-0434, PMID:31320595.

[36] Zhang Y, Yan Q, Gong L, Xu H, Liu B, Fang X, *et al*. C-terminal truncated HBx initiates hepatocarcinogenesis by downregulating TXNIP and reprogramming glucose metabolism. Oncogene 2021;40(6):1147–1161. doi:10.1038/s41388-020-01593-5, PMID:33323975.