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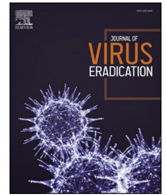

Original research

The central nervous system is a potential reservoir and possible origin of drug resistance in hepatitis B infection

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ABSTRACT

Background: The significance of hepatitis B virus (HBV) in cerebrospinal fluid (CSF) is unclear.
Methods: Synchronous serum and CSF samples were collected from 13 patients. HBV DNA, full-length genome, quasispecies, phylogenetic tree, compartmentalization and mutation of the reverse transcriptase (RT) region were performed based on PCR and sequencing methods.
Results: HBV DNA was detected in the CSF of 3 antiviral-naïve individuals and 1 individual after successful antiviral therapy. Complete full-length HBV genomes were isolated from the CSF of 5 individuals, including 2 with undetectable serum HBV DNA. Ten individuals exhibited distinct CSF-serum quasispecies, 8 harbored independent CSF-serum genetic compartmentalization and phylogenetic trees, and 5 lamivudine/entecavir-associated resistance mutations only in the CSF. The frequencies of rtL180M and rtM204I/V mutations in both serum and CSF were higher in HIV-HBV-coinfected individuals than in the HBV-monoinfected ones (serum: rtL180M: 3.9% vs. 0, $P = 0.004$; rtM204I/V: 21.3% vs. 0, $P < 0.001$; CSF: rtL180M: 7.6% vs. 0, $P = 0.026$; rtM204I/V 7.6% vs. 1.6%, $P = 0.097$).
Conclusion: CSF is a potential HBV reservoir, and HBV in CSF harbors distinct evolution and mutation characteristics from those in serum. HIV infection increases the possibility of HBV rtL180M and rtM204I/V mutations in both serum and CSF.

1. Introduction

Whether the central nervous system (CNS) is a hepatitis B virus (HBV) reservoir remains currently controversial. Some studies have demonstrated that hepatitis B surface antigen (HBsAg) and core antigen (HBcAg) are detectable in brain tissues upon autopsy,[1] which raises an interesting question: is HBV DNA present in the cerebrospinal fluid (CSF)? It is commonly believed that HBV cannot cross the blood−brain barrier (BBB).[2] However, some studies have found traces of HBV sequences and markers in individuals suffering from neurological disorders, such as fatal Creutzfeldt−Jakob disease, cryptococcal meningitis and other neurological diseases.[3–5] In addition, next-generation sequencing (NGS) analysis has revealed HBV DNA sequences in the CSF of individuals with viral encephalitis.[6] This data indicates that HBV DNA sequences could be present in the CSF in some CNS disorders. However, whether the full-length HBV genome is present in the CNS remains unknown.

Lymphocytes and neutrophils are usually poorly recruited in the normal CSF. Therefore, the CNS might be an ideal refuge for HBV because of its low immune response. If so, are HBV quasispecies in CSF

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different from those in serum? Complex HBV quasispecies in extrahepatic reservoirs might be related to HBV drug resistance and recurrence.[7–9] Of note, the currently used anti-HBV drugs, such as lamivudine (LAM or 3TC) and tenofovir disoproxil fumarate (TDF), have poor BBB penetration activity.[10,11] Presumably, low immune pressure and drug pressure in CSF may have a potential impact on HBV replication and evolution in the CNS and even on drug resistance.

In the present study, 13 HBV-infected individuals were recruited to compare the structure of the full-length genome, compartmentalization, quasispecies and reverse transcriptase (RT) region variants of HBV between serum and CSF samples with the aim of elucidating the implication of the presence in CSF of HBV in clinical practice.

2. Materials and methods

2.1. Participants

Thirteen participants (10 HIV-HBV-coinfected and 3 HBV-monoinfected) were recruited for this study: 3 had cryptococcal meningitis, 2 neurosyphilis, one with *Toxoplasma gondii* infection, and 7 with a suspected CNS disorder who underwent lumbar puncture but were not confirmed to have CNS disease. The clinical indication for lumbar puncture was determined by physicians according to the following criteria: (1) routine lumbar puncture for confirmed CNS diseases (e.g., cryptococcal meningitis and syphilis) and (2) lumbar puncture for presumed illnesses in the CNS (e.g., cerebrovascular disease or nonspecific headaches). Synchronous CSF and serum samples were obtained after participant authorization. Samples were collected between January 2014 and December 2018 and frozen at -80 °C for subsequent analysis.

2.2. Extraction of HBV DNA

Serum and CSF samples were collected on the same day as the lumbar puncture. HBV DNA was extracted from 200μL of serum/CSF samples using a QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions; the purified DNA was dissolved in 40 μL of distilled water for further use.

2.3. Quantitation of HBV DNA in serum and CSF

Quantitative PCR (qPCR) was used to quantify the HBV-DNA levels. A total of 600μL of serum or CSF was used for qPCR according to the standard protocol of the COMBAS TaqMan 96 HBV Test V2.0 Kit (Roche, IN, USA), and qPCR was performed with the Roche COBAS Ampliprep PCR system (Roche, IN, USA). The detection range was from $20\text{-}1.7\times10^{8}$ IU/mL.

2.4. Isolation of full-length HBV genomes in blood and CSF

Nested PCR was used to amplify the full-length HBV genome. The PCR primers and conditions were set according to Gunther's method.[12] After two rounds of PCR amplification, a full-length 3.2-kb or another approximately 2.0-kb PCR product of the HBV genome was obtained.

The PCR products (3.2-kb or 2.0-kb of HBV DNA if the 3.2-kb DNA was unavailable) were cloned into the vector PMD-19T with T4 DNA Ligase (Takara, Japan) and transformed into TOP10 *Escherichia coli* competent cells (Invitrogen, CA, USA). Transformants were grown on ampicillin-supplemented plates. Positive clones were identified by PCR. Approximately 1-2 randomly chosen clones per sample were used for the identification of HBV genomes.

2.5. Cloning and sequencing of the HBV RT region

The HBV RT region was amplified by nested PCR with two-round PCR primers according to the method described by Chen et al.[13] After two rounds of amplification, an approximately 1100-bp fragment encompassing the entire RT region was obtained, cloned into the PMD-19T vector (Takara, Japan) and then transformed into TOP10 *Escherichia coli* competent cells (Invitrogen, USA). The transformants were grown on ampicillin-supplemented plates. Approximately 20 randomly chosen clones per sample (one participant had only 8 clones in the CSF sample) were sequenced and analyzed.

2.6. Analysis of HBV quasispecies based on the RT region

Multiple sequence alignment was performed using CLUSTAL X (version 2.0). The HBV quasispecies were evaluated for complexity and diversity according to previously described methods.[13] The quasispecies complexity, known as Shannon entropy (Sn), was calculated using the following formula: $Sn = -\sum_i (pi\ lnpi)/lnN$ (N is the total number of clones, and pi is the frequency of each clone in the viral quasispecies population). The quasispecies complexity was calculated at the nucleotide and amino acid levels. Sn ranged from 0 to 1 (0 means that all virus sequences are identical, and 1 means that each sequence is different and unique[14]). The quasispecies diversity was calculated for each patient by the mean genetic distance (d), including the number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN). The genetic distances at the nucleotide level were calculated under the Tamura three-parameter model that incorporates transitional and transversional rates and G + C content bias, whereas genetic distances at the amino acid level were calculated under the JTT model. The dS and dN were calculated under the modified Nei–Gojobori model with Jukes–Cantor correction. Phylogenetic trees were constructed with the maximum likelihood method under the Tamura-Nei model. Mega11 software and the online tool https://itol.embl.de/were used to draw the phylogenetic trees.

2.7. HBV genetic compartmentalization tests

Compartmentalization analysis of serum and CSF samples from each patient was performed using HyPhy software (V2.5).[15] As different methods to define genetic compartmentalization (such as genetic heterogeneity and distinct lineages between two viral subpopulations) might occasionally yield discordant results in compartmentalization tests,[16] we applied genetic distance-based tests and tree-based tests for the genetic compartmentalization analysis. The distance-based tests included Fst and Snn (nearest-neighbor statistic), and Fst has several different types: 1) Hudson, Slatkin and Madison (HSM), 2) Slatkin (S), and 3) Hudson, Boos and Kaplan (HBK). We employed the HBK nonparametric test for the distance-based test and computed the K_{ST} statistic, whose values range from 0 (denoting no compartmentalization) to 1 (denoting complete compartmentalization). In addition, the Slatkin-Maddison (SM) method was used for the tree-based test. A p lower than 0.05 indicates that the elements can be judged to be compartmentalized. Compartmentalization was finally confirmed by two algorithms (K_{ST} and SM tests) when both p values were less than 0.05.

2.8. Analysis of drug resistance in the HBV RT region

Drug resistance to lamivudine (LAM, 3TC), entecavir (ETV) and tenofovir disoproxil fumarate (TDF) was defined as in previous reports[17–19]:

LAM (3TC) resistance was defined by detection of any mutation within the RT region as follows: rtL80I, rtL173M, rtL180M, and rtM204I/V.

ETV resistance was defined by detection of the combination of rtL180M and rtM204I/V mutations (rtL180M + rtM204I/V) plus any of the following mutations: rtL80I, rtI169V/M, rtA181G/C, rtT184A/F/I/L/M/S, rtS202G and rtM250V.

TDF resistance was defined by detection of four mutations, namely, rtS106, rtH126, rtD134 and rtL269.

Table 1
Basic characteristics of hepatits B virus (HBV)-infected participants.

Participant	Sex	Age	HIV	Comorbidity	CD4 count (cells/mm^3)	HBV-eAg	CSF profile					Serum HBV				CSF HBV			
							WBC (◊10^6/L)	RBC (◊10^6/L)	Protein (mg/L)	Chlorine (mmol/L)	Glucose (mmol/L)	HBV-DNA (IU/ml)	Genotype	HBV quasispecies	Full-length HBV	HBV-DNA	Genotype	HBV quasispecies	Full-length HBV
1	*M*	43	-			-	0	0	209.3	119.1	3.7	2.15×10^5	B	+		<20.0	C	+	
2	*M*	27	-				3	0	159.0	123.0	3.2	2.96×10^6	C	+	+(3.2 kb)	38.6	B	+	+(3.2 kb)
3	*M*	36	-			-	0	0	112.0	127.0	3.1	<20	B	+	ND	<20.0	B	+	+(3.2 kb)
4	*M*	51	+	syphilis	213	-	32	0	500.0	125.0	2.8	<20	B	+	ND	273.0	B	+	+(3.2 kb)
5	*M*	46	+	PCP	3	+	0	1	160.0	110.0	2.9	6.98×10^8	B	+	+(3.2 kb)	2420.0	B	+	+(2.0 kb)
6	*M*	59	+		6	+	0	0	233.0	112.0	2.9	1.01×10^7	C	+	+(3.2 kb)		C	+	ND
7	*M*	26	+	CM	21	+	25	0	684.0	112.0	2.1	3.18×10^8	C	+	+(3.2 kb)		C	+	+(3.2 kb)
8	*M*	34	+		1	-	1	5	733.0	118.0	4.0	<20	B	+	ND	<20.0	B	+	ND
9	*M*	24	+	CM	42	+	1	1	90.0	120.0	2.7	8.61×10^7	B	+			B	+	+(3.2 kb)
10	*M*	46	+	TG	15	-	10	3	792.0	114.0	2.9	8.96×10^3	C	+	ND		C	+	ND
11	*M*	32	+	CM	37	-	17	1300	641	118	3.3	<20	ND	ND	ND		C	+	ND
12	*M*	46	+		34	-	2	0	427.0	120.0	2.8	1.16×10^4	B	+		<20.0	ND	ND	ND
13	*M*	46	+	syphilis		-	0	0	450.0	122.0	3.2		ND	NA	ND	247.0	ND	ND	ND

CSF: cerebrospinal fluid; HIV: human immunodeficiency virus; HBV: hepatitis B virus; WBC: white blood cell; RBC: red blood cell; M: male; PCP: Pneumocystis jiroveci pneumonia; CM: cryptococcal meningitis; TG: *Toxoplasma gondii;* ND: not detectable; -:negative; +: positive; TDF: tenofovir disoproxil; ETV: entecavir.
Participants 4 and 8 were on anti-HIV regimen of TDF+3TC+efavirenz ≥ 2 months; participant 3 was on ETV treatment > 1-year.

An online tool (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) was used to analyze the mutations in the RT region and HBV genotypes. A graphical representation of the amino acid sequence alignment of the HBV RT region was drawn using an online tool (https://weblogo.threeplusone.com). Each logo consisted of stacks of symbols, with one stack for each position in the sequence. The overall height of the stack indicated the sequence conservation at that position, whereas the height of symbols within the stack indicateed the relative frequency of each amino at that position.

2.9. *Statistical analysis*

Continuous normally distributed variables are presented as the means ± standard deviations, and continuous nonnormally distributed variables are presented as medians (interquartile ranges, IQRs). Categorical variables are presented as the numbers of cases (percentages). Continuous variables were compared by Student's *t*-test or the Mann–Whitney *U* test, whereas categorical variables were compared using the χ2 test or Fisher's exact test. A P value <0.05 (two-tailed) was considered to indicate significance. The data analyses were performed using SPSS version 24.0 (IBM, NY, USA).

2.10. *Ethics*

Written consent was obtained from all the patients. The study was in accordance with the 1975 Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 2014-335). All patient data were anonymously analyzed.

3. Results

3.1. *Basic demographic characteristics of participants*

The participant demographic details, morbidities and initial CSF profiles are listed in Table 1. The average age was 39.7 ± 10.7 years. Three were on anti-HBV treatment (HBV/HIV-coinfected participants 4 and 8 with ≥2-months of anti-HIV regimen of TDF+3 TC + efavirenz, and HBV-monoinfected participant 3 on 1-year entecavir treatment) at enrollment. Among 11 participants with sufficient serum samples and 8 with sufficient CSF samples, serum HBV DNA was detectable in 8 individuals with a mean level of 6.5 ± 1.9 (lg) IU/mL, and CSF HBV DNA was detectable in 4 individuals with a level of 2.4 ± 0.7 (lg) IU/mL. There were 7 individuals with HBV genotype B and 4 with genotype C in serum, whereas there were 6 individuals with genotype B and 5 with genotype C in CSF. Notably, 2 individuals (participants 1 and 2) exhibited discordant genotypes between serum and CSF.

3.2. *Full-length HBV genomes were detectable in CSF samples*

The full-length HBV genome was successfully isolated from serum samples of 4 participants and from CSF samples of 5 participants. In addition, a 2.0-kb HBV genome fragment was isolated from the CSF of participant 5. Among these, HBV genomes were isolated from both serum and CSF samples of 3 participants (2, 5 and 7) (Table 1). Notably, the full-length HBV genome was obtained in the CSF of participants 3 and 4, although their serum HBV DNA level was <20 IU/mL.

A map of the HBV genome structure was drawn according to the full-length sequence of HBV. PreS1 and S2 region deletion mutations in the CSF HBV genome were found in 3 participants (3, 4 and 9). Participant 5 had the PreS1 and S2 region deletion mutations in serum but only the PreS2 deletions in CSF, whereas participant 6 had a partial gene deletion in Pre S2 in serum (Fig. 1).

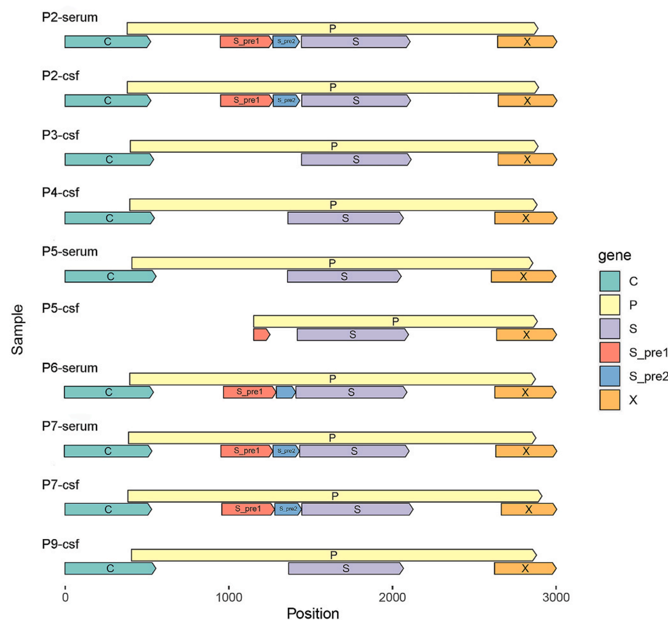

Fig. 1. Map of the hepatitis B virus (HBV) genome structure. Pre-S1 and S2 region deletion mutations in the CSF HBV genome were found in participants 3, 4 and 9. Pre S1 and S2 region deletion mutations were detected in the serum of participant 5, but only Pre S2 deletion mutations were found in CSF. P1-P10: participant 1- participant 10.

3.3. CSF HBV quasispecies were divergently distinct from serum HBV quasispecies

Serum HBV sequences of the RT region from 11 of 13 participants and CSF HBV sequences of the RT region from 11 of 13 participants were obtained, respectively. Among these, simultaneous CSF and serum HBV sequences of the RT region were isolated from 10 participants. Notably, HBV RT region sequences were also isolated from CSF samples of participants 3 and 11, although their serum HBV DNA levels were less than 20 IU/ml (Table 1).

The HBV quasispecies complexity ranged from 0.944 to 1.000 in serum and 0.943 to 1.000 in CSF at the nucleotide level and from 0.913 to 1.000 in serum and 0.865 to 1.000 in CSF at the amino acid level, indicating that all HBV sequences in CSF were highly complex and unique compared with those in serum. Furthermore, the composition and distribution of HBV quasispecies in CSF were also obviously divergent from the quasispecies in serum (**supplement 1** and Fig. 2).

The quasispecies diversity, including d, dS and dN, was compared between serum and CSF samples. We found that participants 1, 2, 4 and 5 had higher d, dS and dN values for the HBV RT region in serum than in CSF ($P < 0.005$). Moreover, participant 3 had lower d, dS and dN values in serum than in CSF ($P < 0.005$), participants 6 and 9 had lower d and dS values ($P < 0.05$) in serum but a similar dN value in CSF ($P = 0.150$ and $P = 0.801$, respectively), and participant 7 had lower d and dN values in serum ($P < 0.005$) but a similar dS value in CSF ($P = 1.000$) (Fig. 3).

3.4. HBV sequences in CSF are highly compartmentalized

The genetic compartmentalization of CSF and serum HBV sequences in 10 participants was analyzed by distance-based tests and tree-based tests. We found that both distance and tree tests agreed for 8/10 individuals (including 3/3 HBV-monoinfected and 5/7 HIV-HBV-coinfected ones) but not in 2 (5 and 8). For participant 5, the distance-based test demonstrated compartmentalization, but the tree-based test did not. Neither tree-based nor distance-based tests revealed blood-CSF HBV genetic compartmentalization in participant 8 (**supplement 2**).

The source of CSF HBV sequences was further analyzed based on RT

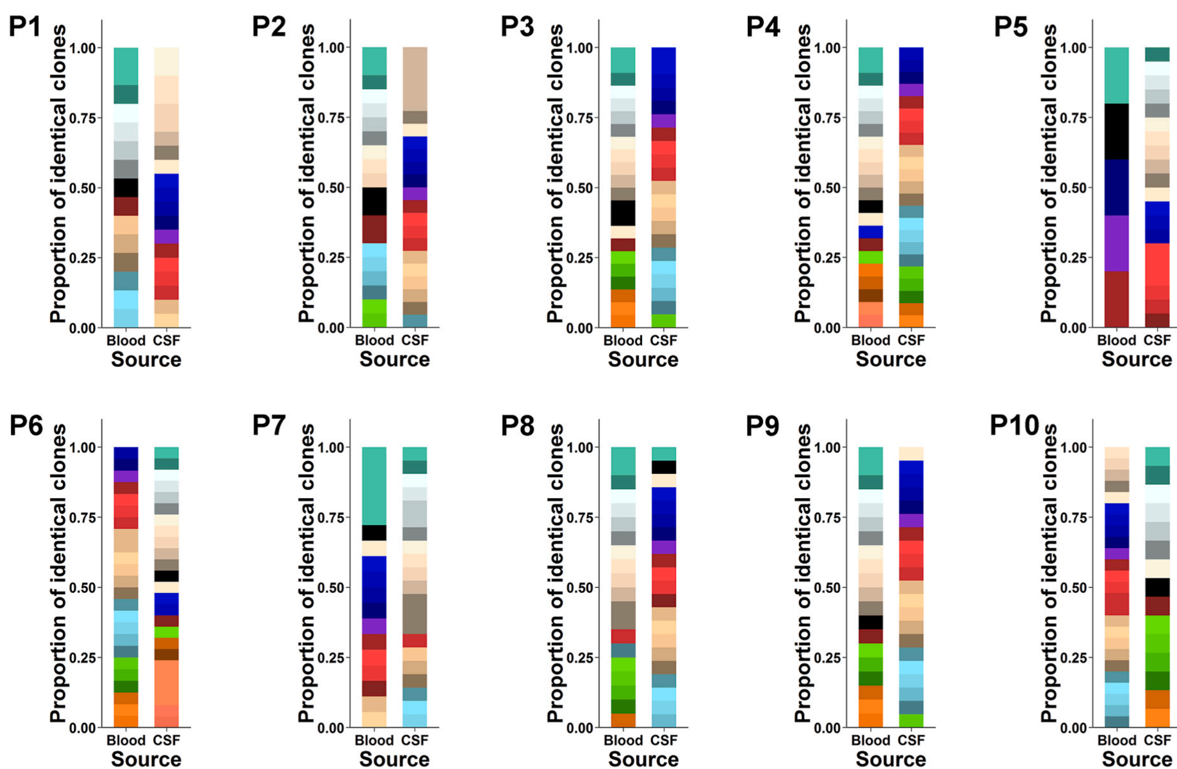

Fig. 2. Composition and distribution of quasispecies in serum and CSF samples. Each color represents one kind of quasispecies. The vertical bars indicate the number and proportion of viral quasispecies within each sample. Complex and unique hepatitis B virus quasispecies were found independently in serum and CSF, respectively. P1-P10: participant 1- participant 10.

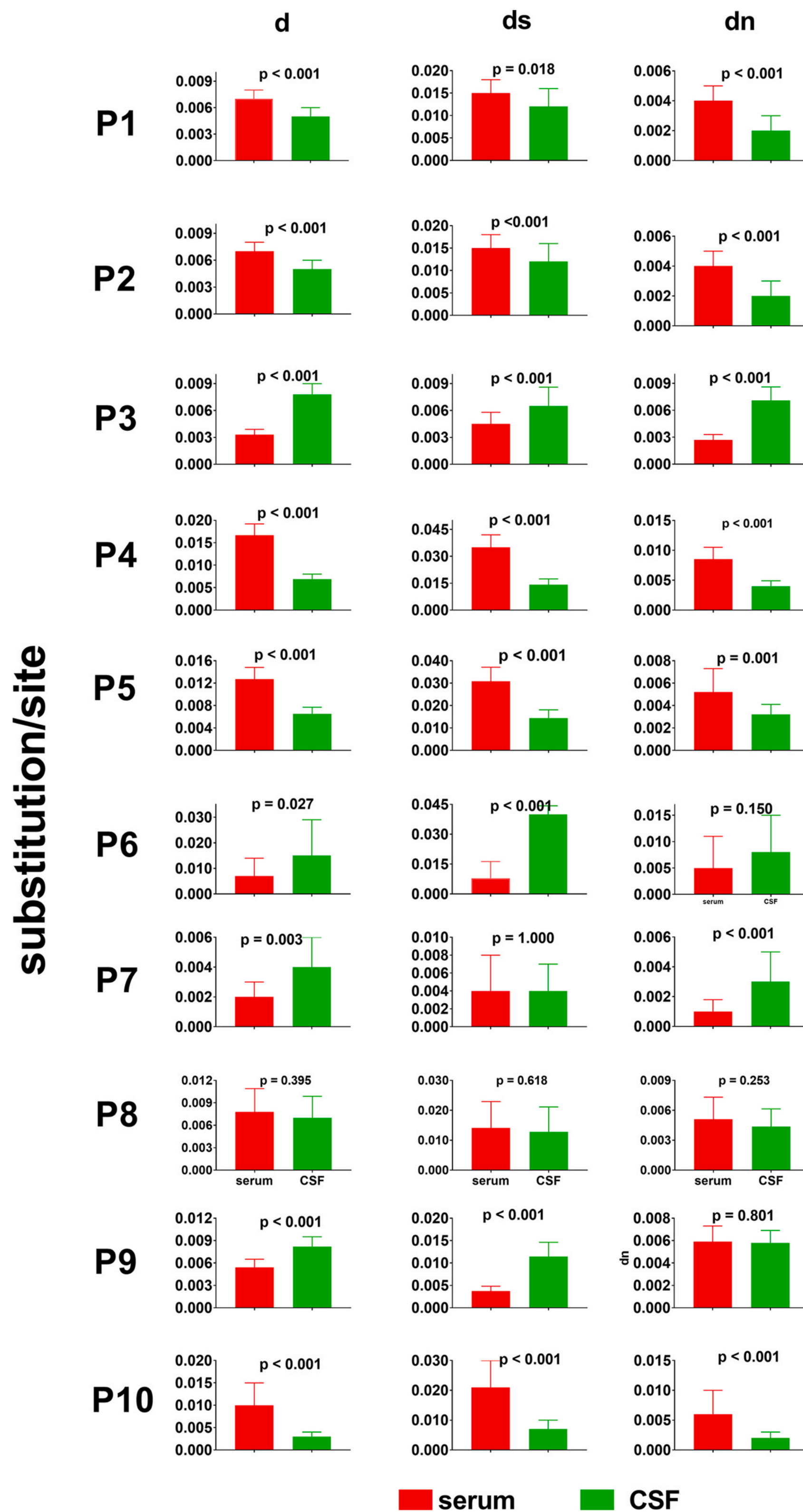

Fig. 3. Hepatitis B virus quasispecies diversity in serum and CSF samples. The viral genetic distance (d), synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) were compared between serum and CSF (Unit:substitution/site). P1-P10: participant 1- participant 10.

region sequences. We found that 5 of 23 CSF sequences were amplified from the same clone in participant 2, and one CSF sequence and 5 serum sequences were from one clone in participant 7. More importantly, no clonally amplified sequence was found in the serum or CSF compartmentalized viral population among other participants.

3.5. Phylogenetic analysis of HBV DNA sequences in paired serum and CSF samples

The phylogenetic trees were constructed under the maximum likelihood algorithm for paired serum and CSF samples in 10 participants.

The representative sequences for each participant were used to draw a phylogenetic tree after sequences identified as identical were discarded. We found that 3 participants (1, 2 and 10) harbored highly clustered clades of CSF HBV sequences that were completely separate from clades of serum HBV sequences, and that 5 participants (3, 4, 6, 7 and 9) harbored relatively independent clustered clades of CSF HBV sequences separate from serum HBV sequences. Clades of CSF sequences were mixed with clades of serum sequences in 2 participants (5 and 8) (Fig. 4).

3.6. Drug resistance mutations within the RT region in serum and CSF samples

Drug resistance mutations of LAM (3TC), entecavir (ETV) and tenofovir disoproxil (TDF) within the RT region were analyzed in paired serum-CSF samples.

There were 8.3% (1/12) clones from serum with the rtS202R mutation and 5.0% (1/20) clones from CSF with the rtM204V mutation in participant 1. Sequencing showed 35.0% (7/20) serum clones with rtV84I, 10.0% (2/20) with rtH126D and 75.0% (15/20) with rtD134G/E/S mutations in participant 2. There were 4.5% (1/22) serum clones bearing the rtS202G mutation in participant 3. For participant 4, 4.8% (1/21) of serum clones bore rtV84A, 8.7% (2/23) of CSF clones N236S/D, and 4.3% (1/23) of CSF clones rtD134E. The rtD134G mutation [10.5%, 2/19] and rtI169T/M one [10.5%, 2/19] were observed in 19 CSF clones from participant 5. For participant 6, triple mutations of ETV resistance in serum clones [95.8% (23/24) rtM204I, 62.5% (15/24) rtM180L and 100% (24/24) rtL80I] and quadruple mutations of ETV resistance in CSF clones [28.0% (7/25) rtM204I, 32.0% (8/25) rtM108L, 28.0% (7/25) rtL80I, 4.0% (1/25) rtM250T] were detected. In addition, participant 6 displayed 4% rtS106F and 4% rtH126R mutations in CSF clones. The rtQ215R mutation (28.0%, 7/25) in serum clones and the rtI169V mutation (4.8%, 1/21) in CSF clones were found in participant 7. Regarding participant 8, rtS106C (47.4%, 9/19), rtD134N6 (8.4%, 13/19) and L269I (100%, 19/19) mutations in serum clones were associated with TDF resistance. Strikingly, quadruple mutations of TDF resistance [rtS106C; 33.3% (7/21), rtH126R: 4.8% (1/21), D134N: 57.1% (12/21), 100% L269I] were found in CSF sequences. Furthermore, the rtT184A and rtS202G mutations were present only in 4.8% (1/21) and 4.8% (1/21) of CSF clones, respectively. Participant 9 had three TDF-related resistance mutations (5.0% rtS106P, 5.0% rtD134N and 100% rtL269I) and one ETV-related mutation (rtI169M, 5%) in 20 serum clones. In contrast, 4.8% rtD134N, 100% rtL269I, 4.8% rtV173M and 4.8% rtS202G were found in 21 CSF clones. The rtS202G mutation was found in 4.3% (1/23) of serum clones from participant 10 (Fig. 5). In addition, the rtL269I mutation was found in all serum sequences and all CSF sequences of participants 3, 4 and 5. Overall, LAM/ETV resistance-associated mutations in only CSF clones were found in 5 participants (participant 1 with rtM204I, participant 6 with rtM250T, participant 7 with rt169V, participant 8 with rtT184A and rtS202G, and participant 9 with rtV173M and rtS202G).

3.7. HIV infection increased the likelihood of rtL180M and rtM204V/I mutations in both serum and CSF clones

High frequency mutation sites of rtL180M and rtM204V/I were compared between HIV-HBV-coinfected and HBV-monoinfected participants. After participants 5 and 8 were excluded for possible blood contamination of CSF, the rtL180M mutation was found in 13.9% (15/108) of serum clones from HIV-HBV-coinfected individuals, but serum clones from HBV-monoinfected individuals did not harbor this mutation ($P = 0.004$); in addition, the rtL180M mutation was found in 7.6% (8/105) of the CSF clones from HIV-HBV-coinfected participants, and the CSF clones from the HBV-monoinfected ones did not have this mutation ($P = 0.026$). Similarly, the rtM204I/V mutation was found in 21.3% (23/108) of serum clones from HIV-HBV coinfected individuals and in none from HBV-monoinfected ones ($P < 0.001$); the rtM204I/V mutation was detected in 7.6% (8/105) of the CSF clones from HIV-HBV-coinfected participants and 1.6% (1/62) from the HBV-monoinfected ones ($P = 0.097$).

4. Discussion

Currently, few studies have investigated the evolution and mutations of HBV in the CNS. Our research suggests that 1) full-length HBV genomes could be detected in the CSF even in those with undetectable serum HBV DNA; 2) HBV quasispecies in CSF were completely different from those in serum in terms of both complexity and diversity; 3) the milieu of the CNS fosters unique HBV compartmentalization and phylogenetic trees, indicating an independent evolution model of HBV; and 4) the development of nucleos(t)ide analog resistance mutations in the CNS is probably associated with the presence of drug resistance in serum.

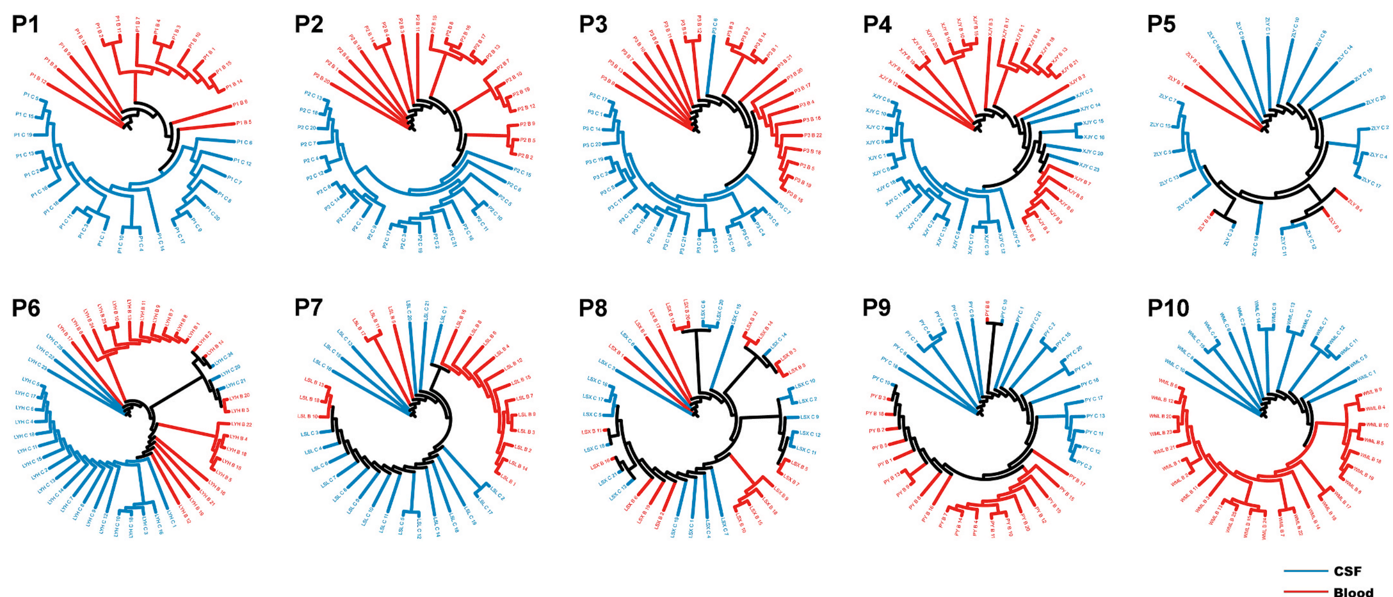

Fig. 4. Phylogenetic trees of hepatitis B virus (HBV) DNA sequences in paired serum and CSF samples. The completely or almost clustered clades obtained for the CSF were separate from the clades obtained for serum samples of participants 1-4, 6, 7, 9 and 10. P1-P10: participant 1- participant 10.

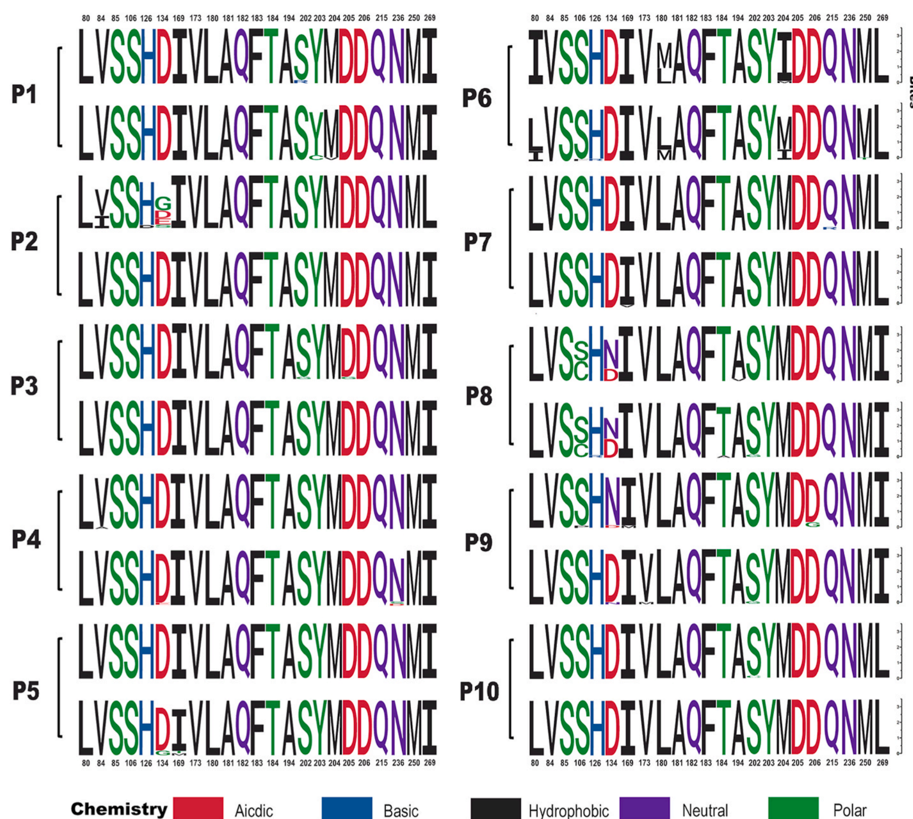

Fig. 5. Comparison of major mutations in the hepatitis B virus (HBV) RT region between serum and CSF. Serum amino acids are in the upper row, and CSF amino acids are in the lower row. Each logo represents an amino acid sequence. The height of symbols within the stack indicates the relative frequency of each amino at that position.

A previous study has indicated that 69.2% (18/26) of HIV-HBV-coinfected adolescents with detectable plasma HBV DNA also have CSF HBV DNA.[20] Another study has shown that CSF HBV DNA sequences were detected in two HBV monoinfected individuals with CNS disease.[5] The full-length HBV genome was found in the CSF of an individual with acute HBV and transverse myelitis.[21] In contrast to these studies, our data has revealed that HBV DNA and full-length HBV genomes could be detected in the CSF of adult individuals, even those on successful antiviral therapy. This data suggests that the CNS is a reservoir for HBV. Nucleos(t)ide analogs are widely used for HIV and HBV treatment. However, most nucleos(t)ide analogs have poor penetration activity through the BBB,[10,22] and this feature may lead to insufficient drug concentrations for HBV clearance in the CNS, which might be related to its persistence in the CNS. Thus, drugs with higher penetration through the BBB should be considered as a new strategy for HBV clearance in the CNS.

Moreover, PreS1 and PreS2 deletions in the CSF HBV genome were detected in our study. It has been reported that the prevalence of PreS deletions in HBV-HIV-coinfected individuals is higher than that in HBV-monoinfected ones,[23] and a high prevalence of PreS deletion/mutation is associated with immune evasion, advanced liver disease and hepatocellular carcinoma.[24,25] The incidence of PreS1/S2 deletion in CSF samples might reflect the low immune pressure in CSF.

Our data supports the notion that the HBV evolutionary profile in CSF is completely different from that in serum based on the following results: 1) HBV quasispecies were completely different and unique in terms of complexity and diversity in serum and CSF of most participants; 2) the composition and distribution of HBV quasispecies in CSF were also divergent from those in serum; and 3) the clades of the phylogenetic trees obtained from the CSF were clearly separate from those found in serum, and this finding was observed for most particpants. The distinct HBV population between CSF and serum raises an interesting question: where does the HBV population in CSF originate from? Studies on HIV-1 in the CNS have described three different states of the HIV-1 population in CSF compared to that in the blood: 1) equilibrated (when viruses in the blood and CSF are very similar), 2) compartmentalized (when blood and CSF viral populations are distinct, indicating separately evolving populations in these compartments) and 3) clonal amplification (when a single variant is greatly expanded within a compartment).[26,27] Although the presence of red blood cells in the CSF samples obtained from participants 5, 8, 9 and 10 indicated the possibility of blood contamination during lumbar puncture, our study illustrates the fact that CSF HBV populations are highly compartmentalized variants from the serum HBV population in most except in participants 5 and 8. Indeed, the phylogenetic analysis also showed that CSF clades were independent from the serum ones of most participants. Notably, we also found that one HBV sequence in CSF and five HBV sequences in serum from participant 7 were identical clones, suggesting possible transfer of HBV between the CSF and serum compartments.

Our data suggests that HBV RT region mutations in the CNS might be a source of HBV drug resistance in peripheral blood. We found that some mutations were only detected in CSF (such as rtM204V in patient 1, rtN236S/D and rtD134E in participant 4, D134G and rtI169T/M in participant 5, and rtS106F, rtH126R and rtM250T in participant 6). Combined mutations conferring ETV resistance in the CSF sequences of participant 6 and quadruple mutations conferring TDF resistance in the CSF sequences of participant 8 were also found in our study (although HBV sequences in CSF were not compartmentalized). Furthermore, we found that HIV-HBV-coinfected individuals had higher frequencies of rtL180M and rtM204I/V mutations in both serum and CSF than HBV-monoinfected ones, indicating that low immune pressure is associated with a high frequency of mutations of the HBV RT region in both serum and CSF. More importantly, most anti-HBV regimens have a poor penetration through the BBB, which relates to insufficient drug

concentrations for suppressing HBV replication. Consequently, HBV strains harboring resistance mutations in CSF might transfer into peripheral blood and eventually become the dominant HBV strain. For instance, although HBV DNA was undetectable by qPCR in peripheral blood, the full-length HBV genome and HBV quasispecies carrying LAM resistance mutations were found in CSF samples of HBV-monoinfected participant 3. Due to pre-existing LAM-resistance mutations in HBV-RT region from serum and CSF, our data supports the recommendation that a single anti-HBV medicine containing antiretroviral treatment (ART) is not suitable for HIV-HBV-coinfected patients.[28] .Furthermore, our study also provides an explanation for why some HBV-infected patients with good virological suppression still develop drugs-resistant under long-term antiviral treatment eventually.

Our study has some limitations. First, it has been reported that HBV particles can be found in CSF by electron microscopy.[29] However, this study did not confirm the existence of HBV viral particles in CSF. Second, dynamic changes in HBV DNA levels and quasispecies after antiviral treatment were not observed longitudinally. Third, our study is based on a small-scale population, and some statistics and analyses, such as differences in quasispecies between HIV-HBV coinfected and HBV-monoinfected participants, were not performed.Forth, some studieshave indicated that HBV in CNS might be associated with neurological diseases and symptoms.[5,6,21] However, we did not describe the HBV-related neurological manifestation in our participants.

In summary, our study indicates that the CNS is a potential extrahepatic HBV reservoir, which may contribute to the occurrence of HBV drug resistance due to low concentrations of nucleos(t)ide analogs in CSF. Selecting a drug or drug combination with high penetration through the BBB should be considered a strategy for more effective HBV treatment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jve.2023.100348.

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