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Molecular characterization of multiple putative novel hepatitis B virus spliced DNA from a chronic hepatitis B patient

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Spliced hepatitis B virus (HBV) RNA has been reported to affect the viral life cycle and progression of liver disease. As much as 30% of HBV RNA are spliced in infected cells, and some can be reverse transcribed to spliced DNA. To date, only a small number of spliced DNA have been identified and the majority require further investigation. This study aimed to identify the types of spliced DNA from the sera of a chronic hepatitis B patient. HBV DNA was isolated from the serum, and both full-length and spliced DNA were amplified by PCR. The amplified PCR products were then subcloned and sequenced using a Sanger sequence analysis. As a result, 19 types of spliced DNA were identified, 11 of which were assumed to be putative novel spliced DNA. These spliced DNA were doubly spliced with varied splice donor sites and splice acceptor sites. In addition to the frequently detected HBV SP1, SP2, and SP4, we also identified 11 putative novel spliced DNA in the serum. These findings indicate the complexity of HBV RNA splicing during viral life cycle.

KEYWORDS

hepatitis B virus, chronic hepatitis B, spliced DNA, splice protein, hepatocellular carcinoma

Abbreviations: ccc, covalently closed circular; HBV, hepatitis B virus; HBSP, HBV splice-generated protein; HCC, hepatocellular carcinoma; nt, nucleotide; pSD, putative spliced DNA; rc, relaxed circular; SP, spliced protein.

Introduction

It has been estimated that approximately 7.18% of the population of China are Hepatitis B virus (HBV) carriers, and are thus at risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) (Chen et al., 2006; Chen et al., 2013; Wang et al., 2014; Rapti and Hadziyannis, 2015). Thus, the HBV infection represents a public health problem in China.

HBV is a hepatotropic DNA virus with a partially double-stranded genome, also known as relaxed circular (rc) DNA (Delius et al., 1983). Upon entry into hepatocytes, the rcDNA is transported to the nucleus to form covalently closed circular (ccc) DNA by completing the positive strand and ligating gaps in the positive and negative DNA strands, respectively (Tuttleman et al., 1986; Hu and Seeger, 2015). The cccDNA functions as a template for all HBV viral RNA, including pregenomic (pg) RNA, precore RNA, preS1/preS2/S mRNA and X mRNA. Among these RNAs, pgRNA encodes the core protein and HBV reverse transcriptase, and also serves as the template for HBV DNA synthesis (Summers and Mason, 1982).

Unlike human immunodeficiency virus-1 and human papilloma virus, splicing of HBV RNA does not appear to be required in the HBV life cycle (Su et al., 1989a; Karn and Stoltzfus, 2012; Graham and Faizo, 2017; Kremsdorf et al., 2021). However, spliced HBV RNA has frequently been detected in chronic hepatitis B patient's liver tissue (Su et al., 1989b; Bayliss et al., 2013; Chen et al., 2015). Some spliced RNAs have been shown to be further reverse transcribed into HBV DNA and subsequently secreted as defective HBV particles (Chen et al., 2015; Kremsdorf et al., 2021). This phenomenon has also been observed in an *in vitro* cell culture system (Abraham et al., 2008; Chen et al., 2015). Moreover, several studies have reported that the expression of isoforms and the amount of HBV splice variants, including spliced RNA and DNA vary in patients, transfected cell types, and HBV genotypes (Abraham et al., 2008; Chen et al., 2015; Kremsdorf et al., 2021; Lim et al., 2021). Moreover, these variants have been reported to modulate HBV replication and hepatocyte apoptosis and are associated with a poor response to interferon treatment and development of HCC (Chen et al., 2015; Betz-Stablein et al., 2016). However, the precise clinical and virological relevance of HBV splice variants remains poorly understood.

Currently, there are at least 20 pgRNA-derived HBV splice variants, including spliced RNA and DNA that have been detected in HBV infected liver tissue, serum, and transfected cells (Kremsdorf et al., 2021). However, the high proportion of HBV RNA splice variants among total HBV RNA production coupled with abundant HBV daily production (approximately 10^{11} copies/day) suggests currently reported HBV splice variants may only account for a small portion of the total variants (Lau and Wright, 1993; Nowak et al., 1996).

Thus, the aim of the study is to investigate the molecular characteristics of HBV spliced DNA in clinical serum sample. Here, we report 11 putative novel HBV spliced DNAs from the sera of a chronic hepatitis B patient.

Materials and methods

Isolation and amplification of serum HBV DNA

HBV DNA was isolated from 200 µL of serum of a 36-year-old male chronic hepatitis B patient, who had not been received antiviral treatment yet and was negative for hepatitis C virus, using a QIAamp MinElute Virus Spin Kit (Qiagen, German) in accordance with the manufacturer's instructions. The full-length HBV genome was amplified using forward primer P1and reverse primer P2 with KOD DNA polymerase (TOYOBO, Japan), which possesses proof-reading activity, to minimize nucleotide mismatch. The sampling procedures were approved by the Ethics Committee of the Hainan Medical University, Haikou, Hainan province, China.

Clonal analysis

PCR products including approximately 3.2kb-long DNA (full-length HBV DNA) band and near 2kb-long DNA (short fragment HBV DNA) were recovered from a 1% agarose gel, ligated with the pLB-vector (TIANGEN, China), and subsequently transformed into DH5α *Escherichia coli* according to standard protocol. Positive colonies detected with conventional colony PCR with P1/P2 primers were further cultured in Luria Broth medium containing ampicillin (100 mg/mL) at 37°C for 16 h. Plasmids were isolated by TIANprep mini plasmid kit (TIANGEN, China) and subjected to HBV genome sequencing using ABI 3730xl DNA analyzer (Sangon Biotech, China). Primers for the sequencing of full-length (primer T7, P4, P5, P6, and P7) and short fragment (primer T7, RT-R, and pLB-R) HBV DNA are listed in Table 1 and the primer binding site is described in Figure 1 (Gunther et al., 1998).

Sequence analysis

The alignment of the obtained HBV sequences was performed using the ClustalW method and the HBV genotype was determined with a neighbor-joining phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA) software, version X. Reference sequences obtained from GenBank were used to compare the HBV full-genome sequences from the present study. Accession

TABLE 1 List of primer pairs.

	Primers	Sequence (5'→3')	Nucleotide position
PCR	P1	5′- TTTTTCACCTCTGCCTAATCA -3′	nt 1,821–1,841
	P2	5′- AAAAAGTTGCATGGTGCTGG -3′	nt 1,825–1,806
Sequencing analysis			
Full length	T7	5′-CTAGAGAACCCACTGCTTAC-3′	
	P4	5′-CCTTGGACACATAAGGTGGG-3′	nt 2,457–2,476
	P5	5′-GTGGAGCCCTCAGGCTCAGG-3′	nt 3,075–3,094
	P6	5′-GCTGCTATGCCTCATCTTC-3′	nt 415–433
	P7	5′-CCAACTTACAAGGCCTTTC-3′	nt 1,102–1,120
Short fragment	T7	5′-TAATACGACTCACTATAGGG-3′	
	RT-R	5′-TTGCCGGGCAACGGGGTAAAG-3′	nt 1,141–1,161
	pLB-R	5′-AAGAACATCGATTTTCCATGGCAG-3′	

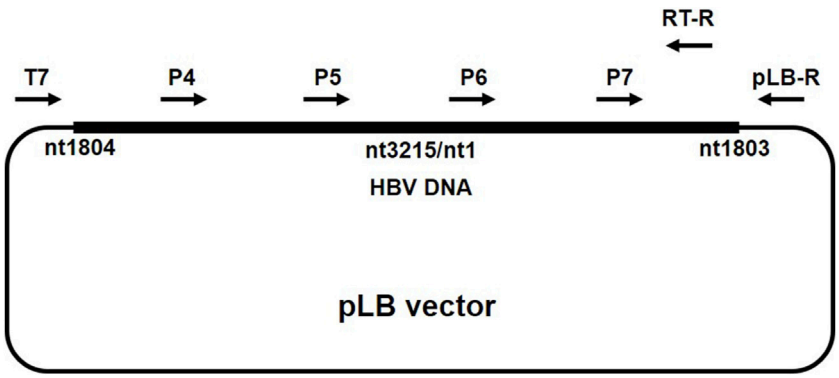

FIGURE 1
Primer binding site. The position of primers used in the sequencing of full-length HBV DNA and short fragment HBV DNA.

numbers: AB697490, GQ358158, DQ069801, HM011493, EU410080, EU670263, GU721029, AP011106, AP011109, AB540583, AB554019, and AB554025.

Results

Amplification of full-length and short fragment of HBV genome

To study the types of secreted HBV spliced DNA, HBV genome isolated from the serum was amplified by PCR using primer pairs P1/P2. In addition to full length HBV genome, a large amount of approximately 2 kb-long short fragments were detected in the sera of a chronic hepatitis B patient (Figure 2, lane 2). The short fragment was assumed to be HBV spliced DNA due to the similar molecular size of a previously reported HBV splice variant (Kremsdorf et al., 2021).

Identification of HBV splice variants

To confirm the assumption and identify the position of the genetic deletion in the short fragment, 50 colonies of subcloned short fragments were subjected to a sequencing analysis.

The obtained sequences were aligned with the full-length HBV DNA sequence (accession number: MA12345), which was determined to be HBV genotype C5 by a neighbor-joining phylogenetic tree analysis from the same PCR reaction. As a result, we detected 19 types of spliced DNA, including 8 previously reported types and 11 that have not yet been reported (Figure 3). Among these types, SP1 (42%, 21/50) was the most abundant HBV spliced DNA, followed by SP4 (10%, 5/50), SP2 (8%, 4/50) and SP5 (4%, 2/50) (Figure 3). There were 11 putative spliced DNA (pSD) that have not been previously reported, which were denoted pSD1, pSD2 . . ., pSD11 (Figure 3).

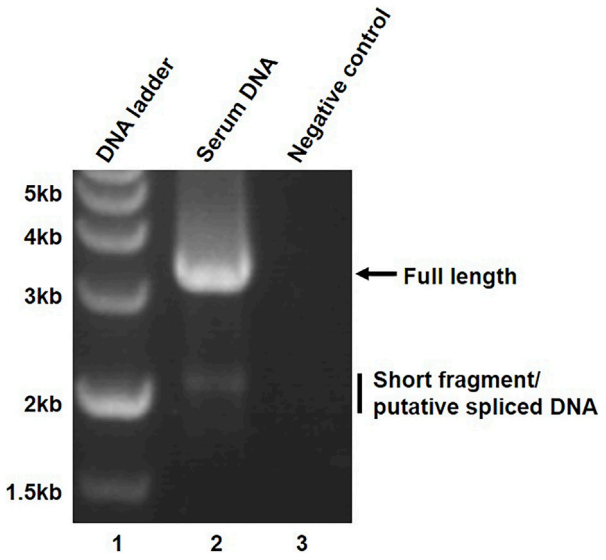

FIGURE 2
Amplification of full-length and short fragment of HBV genome. The amplified serum HBV DNA was resolved on a 1% agarose gel and the PCR products were visualized on UV light.

All spliced DNAs, except SP10, had a common splice donor at nucleotide (nt) 489 and splice acceptor at nt 2,447 or nt 2,471, however, various non-canonical splicing sites were also detected in pSDs (Table 2).

Discussion

Splicing of HBV RNA, especially pgRNA splicing, frequently occurs during HBV replication, and the spliced pgRNA can be packaged into capsid to be transcribed into HBV DNA (Kremsdorf et al., 2021). According to previous studies, the proportion of intracellular HBV spliced RNA could comprise up to 30% of the total HBV RNA in cells transfected with HBV expressing plasmid construct *in vitro*, whereas the spliced DNA only accounted for approximately 1% of the total secreted HBV DNA in the sera (Chen et al., 2015; Lim et al., 2021). This discrepancy may be related to the lower efficiency of the assembly or secretion of viral particles with defective HBV DNA. Due to the small amount of secretion, spliced DNA is not easily detected during whole HBV genome amplification using conventional PCR method. However, during our routine study

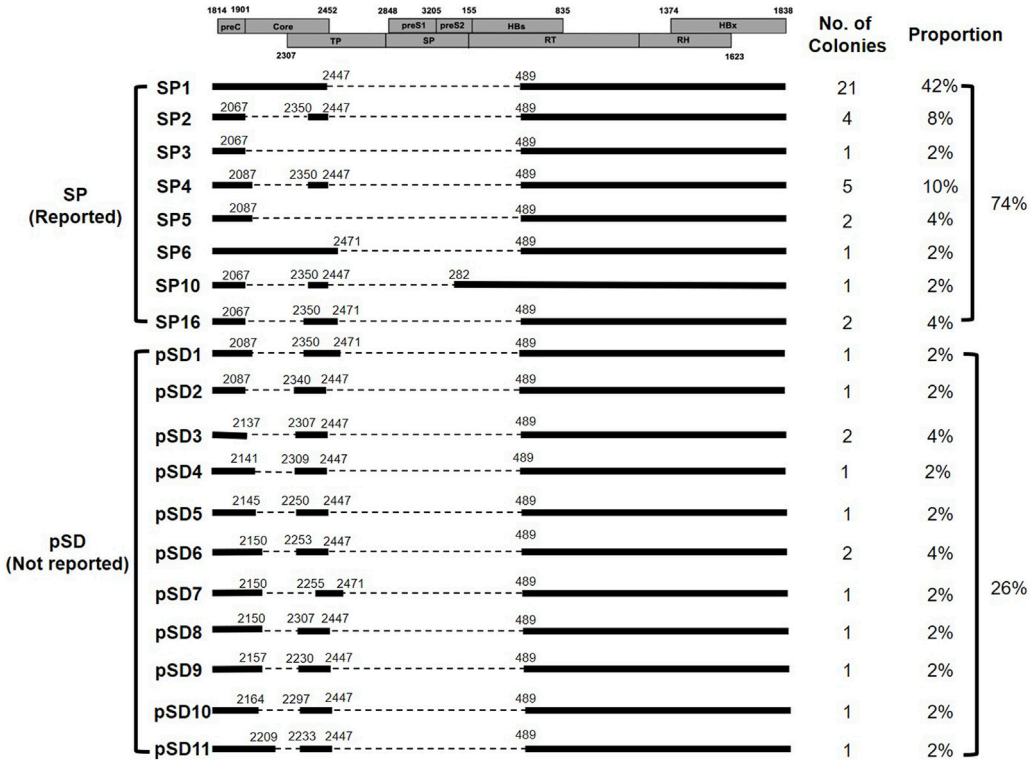

FIGURE 3
HBV spliced DNAs identified in the serum. Schematic diagram of the ORF of HBV genome (boxes), types of spliced DNAs including previously reported spliced DNA, denoted SP and putative novel spliced DNA, denoted pSD, and the proportion of the individual spliced DNA.

TABLE 2 Genetic variability of splice donor and splice acceptor.

	Position	Nucleotide
Splice donor	2,067	GC
	2,087	GT
	2,137	GC
	2,141	CC
	2,145	GG
	2,150	TA
	2,157	CA
	2,164	GT
	2,209	CA
	2,447	GT
	2,471	GT
Splice acceptor	282	AG
	489	AG
	2,230	TT
	2,233	GG
	2,250	TG
	2,253	GT
	2,255	AT
	2,297	AT
	2,306	CA
	2,307	AA
	2,309	AT
	2,340	AG
	2,350	AG

of HBV genome amplification, we detected a large amount of approximately 2kb-long PCR products that were assumed to be spliced DNA from the serum of a chronic hepatitis B patient. This finding provides us with an opportunity to study the molecular characteristics of spliced DNA.

In the present study, 19 types of HBV splice variants were detected, including 8 that have previously been reported and 11 putative novel spliced DNA (Su et al., 1989b; Chen et al., 1989; Abraham et al., 2008; Ma et al., 2009; Lim et al., 2021). Consistent with previous studies, SP1 was found to be the most abundant spliced DNA, accounting for 42% of the total variants (Liaw, 2013; Chen et al., 2015; Lim et al., 2021). In addition, we also detected SP2 (8%), SP3 (2%), SP4 (10%), SP5 (4%), SP6 (2%), SP10 (2%) and SP16 (4%) in various proportions.

All pSDs had common sties of splice donor and splice acceptor in the second region of deletion (from nt 2,447/2,471 to nt 489), however, they had varied splicing sites that were closely located to the canonical splice donor and splice acceptor sites at the first region of deletion (from nt 2,067 to nt 2,350). Moreover, unlike the previously reported consensus sequences of the splice sites (GT/GC for splice donor and AG for splice acceptor), TA/AT/TG was frequently detected in the splice donor site and the splice acceptor site of pSD (Kremsdorf et al., 2021). These findings suggest that nucleotide sequences near the canonical splice site may also contribute to HBV pgRNA splicing.

It has been previously reported that both HBV RNA and DNA variants are associated with the progression of liver disease and have a biological impact on the viral life cycle. SP1 is the most commonly studied HBV splice variant that encodes the HBV splice-generated protein (HBSP). The HBSP has been shown to inhibit HBV nucleocapsid formation, suppress host apoptosis, and impair the response to interferon treatment (Chen et al., 2015; Wang et al., 2015; Wu et al., 2018). In addition to SP1, the SP7, SP14, and SP19 have been reported to translate truncated HBV protein, which was assumed to affect the HBV viral life cycle (Huang et al., 2000; Park et al., 2008; Chen et al., 2010; Lin et al., 2012; Lin and Chen, 2017). In the present study, SP1 was the most abundantly detected HBV variant, whereas SP7, SP14, and SP19 were not detected.

Similar to SP2, SP4, SP10 and SP16, all pSDs were doubly spliced DNA, however, there are limited studies on the biological properties of doubly spliced variants. It has been reported that SP10 can suppress HBV transcription (Tsai et al., 2015). Considering that pSDs exhibited a similar pattern of splicing to that of SP10, they may have an impact on the HBV viral life cycle and pathogenesis, however, the precise mechanism requires further study.

Taken together, we identified multiple pSDs with varied splice donor and splice acceptor sites from the serum of chronic hepatitis B patient. These findings imply the complexity of HBV RNA splicing in the viral life cycle. A limitation of this study is the lack of liver tissue from the same patient for a comparison of the splice variants profile between the serum and liver tissue.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Hainan Medical University, Haikou, Hainan province, China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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