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Hepatitis B virus depicts a high degree of conservation during the immune-tolerant phase in familiarly transmitted chronic hepatitis B infection: deep-sequencing and phylogenetic analysis

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SUMMARY. When intrafamilial transmission of hepatitis B virus (HBV) occurs, a virus with the same characteristics interacts with diverse hosts' immune systems and may thus result in different mutations to escape immune pressure. In this study, the HBV genomic characterization was assessed longitudinally after intrafamilial transmission using nucleotide sequence data of phylogenetic and mutational analyses, including those obtained by deep-sequencing for the first time. Furthermore, HBeAg-anti-HBe profile and variability of HBV core-derived epitopes were also evaluated. Strong evidence was obtained from intrafamilial transmission of HBV genotype D1 by phylogenetic inferences. HBV isolates exhibited high degree (~99%) of genomic conservation for almost 20 years, when patients were persistently HBeAg positive with normal amino transferase levels. This identity remained high among immune-tolerant siblings. In contrast, it diminished significantly ($P = 0.02$) when the mother cleared HBeAg (immune clearance phase). By deep-sequencing, the quantitative analysis of the dynamics of basal core promoter (BCP) (A1762T, G1764A; A1766C; T1773C; 8-bp deletion; and other) and precore (G1896A) variants among HBV isolates from family members exhibited differences during the follow-up. However, only those from the mother showed amino acid variations at core protein that would impair their MHC-II binding. Hence, when intrafamilial transmission occurs, HBV was highly conserved under the immune-tolerant phase, but it exhibited mutations more frequently during the immune clearance phase. The analysis of the HBV BCP and precore mutants after intrafamilial HBV transmission contributes to a better understanding of how they evolve over time.

Keywords: deep-sequencing, hepatitis B virus, intrafamilial transmission, phylogeny.

INTRODUCTION

Chronic hepatitis B virus (CHB) infection in pregnancy is of important global health concern. Over 50% of the 350 million chronic hepatitis B carriers had acquired the infection perinatally [1]. The high carrier rate among siblings is attributed to infection in early infancy mostly from hepatitis B e antigen (HBeAg)-positive and surface antigen (HBsAg) carrier mothers. The intrafamilial transmission is a useful issue to characterize the infection evolution pattern in different hosts infected with a virus coming from a unique source.

Patients with infancy-acquired CHB become immunotolerant, with high viral load but minimal liver inflammation. Their HBV-specific immune responses are undetectable or weak and narrowly oriented [2–6]. Several mechanisms may account for this immune hyporesponsiveness. It has been proposed that HBeAg may serve as a decoy to buffer anticore protein immune response or to induce immune tolerance [5–7].

Abbreviations: ANNs, artificial neural networks; BCP, basal core promoter; CHB, chronic hepatitis B virus; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IEDB, Immune Epitope Database; MHC-II, major histocompatibility complex II; UDPS, ultra-deep pyrosequencing.

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During chronic HBV infection, two major types of core gene variants occur frequently that affect the expression of HBeAg: the precore (PC) and the basal core promoter (BCP) mutants. Considering that the BCP regulates the transcription of pregenomic RNA and precore mRNA, these variants have been related with HBV replication enhancement and evasion of the host immune response [8]. The hepatitis B core (HBcAg), HBeAg and HBsAg are important targets for antiviral immunity, but the first one seems to be the most immunogenic [9] and subjected to a wide variation of amino acid sequences [10]. In persistent viral infection, most of these amino acid changes have been shown to occur primarily in well-recognized B- and T-cell epitopes [10,11], when the virus must adapt to a new immunological environment.

At present, three previous studies have addressed the HBV mother-to-child transmission by analysing viral complete genomes [12–14], but taking into account the fulminant outcome in two of them, the long-term HBV analysis was impracticable among siblings. The aim of this study was to characterize the HBV longitudinally through full and subgenomic sequences including those obtained from massively parallel pyrosequencing. Phylogenetic analyses were performed to provide robust evidence for intrafamilial transmission. Furthermore, the impact of HBV genomic heterogeneity on HBeAg profile, level of HBV replication, and B- and T-cell epitopes was analysed.

MATERIAL AND METHODS

Patients

Four patients chronically infected with HBV, a mother and her three children were enrolled from the Gastroenterology Division of the Hospital 'Cosme Argerich', Buenos Aires, Argentina. The three siblings – presumed to have acquired HBV perinatally – and the mother tested positive for hepatitis B surface antigen (HBsAg). Mother sera were obtained from three time points: Ma (2007), Mb (2010) and Mc (2012). Her three children belonged to two different marriages. The older son (-S1- 18 years old) came from the first marriage, and the other son (S2, 11 years old) and the daughter (D, 12 years old) from the second one. At least two plasma samples were obtained in 2011 and 2012 from each one. The hepatitis B serological markers (HBsAg, HBeAg, total anti-HBc, anti-HBe, anti-HBs, tested by chemiluminescent assay, Architect i2000; ABBOTT, Chicago, IL, USA), HBV viral load (COBAS® TaqMan® HBV Test, v2.0, range: 29 to 110 000 000 HBV IU mL-1; Roche, Branchburg, NJ, USA) as well as different liver biochemical determinations were measured at several sampling times (Table 1). At the end of 2012, liver biopsy was performed for HBV-related histology in the mother and the two younger children (Metavir scores were mother, A3F4; D, A2F2; S2, A2F3). All family members tested negative for hepatitis C and D virus markers. There was no history of alcohol abuse, parenteral drug use or hepatotoxin exposure. No data were available from spouses.

Informed consents were obtained from the adult and from the parents of the three infants, and the study protocol was approved by the Hospital 'Cosme Argerich' Ethics Committee.

HBV-DNA extraction, PCR and complete genome sequencing

Briefly, DNA was extracted from a 200-μL serum sample using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Six subgenomic nested PCRs were performed to obtain the complete genome of the isolates (protocol available upon request). Complete HBV genomes were sequenced directly for each sample as reported previously (ABI Prism 310 genetic analyzer; Applied Biosystems, Foster City, CA, USA) [15].

Cloning of BCP-PC, and spacer domain of the polymerase gene

Isolates of the first sample time obtained from each child and the three isolates from the mother were studied in depth by cloning the nested PCR products from both BCP-PC (nt 1740 to 2180) and spacer (nt 2370 to 2950) partial regions (pGEM-T Easy vector, Promega, Wisconsin, MN, USA) following manufacturer's instructions. After sequencing, both partial nucleotide sequences obtained from the same cloned amplicon were manually concatenated.

Ultra-deep pyrosequencing

The viral isolates to be sequenced by ultra-deep pyrosequencing (UDPS) were the same than previously selected for cloning. PCR amplicons were sequenced on the 454 Life Science platform (GS-FLX, Roche Applied Science). A total of 24 296 partial HBV precore–core gene sequence reads were returned from the UDPS; the number of reads per sample ranged between 2834 and 5296. To analyse the UDPS data, the GS Amplicon Variant Analyzer (454 Life Sciences, Roche) was used. To distinguish authentic minority variants from technical artefacts, we used an estimation threshold of 1% [16].

Sequence analyses and phylogenetic relatedness

For each data set, an appropriate model of nucleotide substitution was selected according to Akaike's information criterion (jModelTest software) [17]. Phylogenetic trees were constructed with the maximum likelihood methodology [18] using the PhyML v3.0 software, and the branch support was assessed by nonparametric bootstrapping (100 pseudoreplicates). HBV sequences were aligned and visualized using

Table 1 Laboratory HBV serology, liver biochemical tests and HBV viral load

ID*	Date	AST (U/L)	ALT (U/L)	FAL (U/L)	GGT (U/L)	BT/BD (mg/dL)	HBsAg	Total anti-HBc	HBeAg	Anti-HBe	Anti-HBs	CV (UI/mL)
Mother												
(Ma)	May-07	128	233	186	80	0.20/0.09	POS	POS	POS	NEG	NEG	>110 000 000
	June-2009	18	15	155	15	0.4/0.09	ND	ND	ND	ND	ND	ND
(Mb)	November-10	24	16	56	17	0.65/0.17	POS	POS	NEG	POS	NEG	8000
	Apr-11	20	22	66	20	0.31/0.09	POS	POS	NEG	POS	NEG	ND
(Mc)	March-12	22	31	NR	29	0.32/0.11	POS	POS	NEG	POS	NEG	1060
Son 1												
	September-10	23	25	89	15	0.47/0.15	POS	POS	POS	NEG	NEG	ND
(S1a)	April-11	19	21	84	15	0.91/0.26	POS	POS	POS	NEG	NEG	>110 000 000
(S1b)	March-12	20	20	ND	18	0.73/0.23	POS	POS	POS	NEG	NEG	>110 000 000
	August-12	22	26	77	18	0.68/0.18	ND	ND	ND	ND	ND	ND
Son 2												
(S2a)	August-11	35	21	417	15	0.40/0.15	POS	NEG	POS	NEG	NEG	>110 000 000
(S2b)	March-12	23	20	ND	23	0.38/0.12	POS	NEG	POS	NEG	NEG	>110 000 000
Daughter												
(Da)	August-11	56	23	271	7	0.23/0.20	POS	NEG	POS	NEG	NEG	>110 000 000
	October-11	22	20	333	11	0.26/0.09	POS	NEG	POS	NEG	NEG	ND
(Db)	March-12	48	37	ND	13	0.41/0.14	POS	NEG	POS	NEG	NEG	>110 000 000
	August-12	19	19	228	12	0.36/0.10	ND	ND	ND	ND	ND	ND

*Those samples available for HBV genomic characterization appear between parentheses. Normal values for liver biochemical test: ALT: 0–41; AST: 0–38; Alkaline phosphatase (FAL): 50–250; gammaglutamyl transferase (GGT):7–50; bilirubin: 0–0.3 mg/dL.

Mafft online application and the program BioEdit v7.0.5.3 (T. Hall. Ibis Biosciences, Carlsbad, CA, USA), respectively.

The HBV genotype was determined by phylogenetic analysis of full-length genomes by comparing 23 reference sequences retrieved from the NCBI Viral Genotyping Tool [http://www.ncbi.nlm.nih.gov/projects/genotyping]. The selected nucleotide substitution model was GTR+I+G.

The comparisons of HBV at both inter and intrahost levels during the follow-up included the construction of identity matrixes. To this end, a matrix aimed to compare full-length nucleotide sequences was constructed and other four for the amino acid identities according to each HBV ORF.

Once the HBV genotype was assigned, the monophyletic origin of viral sequences was additionally supported by two phylogenetic analyses using the concatenated HBV sequences (length = 1210 bp). At first, these sequences were analysed with the best 29 not redundant matches of BLASTN, which represented the most closely related sequences to the data set analysed. The selected model for this analysis was TPMuf+I+G. Secondly, the sequences were analysed with genotype D references following the model TVM+I+G.

Comparative analysis of the major histocompatibility complex II peptide binding

As the major histocompatibility complex II (MHC-II) peptide binding is a necessary requirement for its recognition by a T cell, mutations affecting anchor positions could alter their capability to bind MHC molecules. Taking into account the discordance of anti-HBc reactivity among the family members (Table 1) and considering the plausible different amino acid constitution in the core-derived epitopes, the impact on the MHC-II peptide binding was predicted by the algorithm based on matrix method included in the Immune Epitope Database (IEDB) [http://www.immuneepitope.com]. Two different tools were used to predict the MHC-II binding: first, using the IEDB analysis resource Consensus tool [19,20]; second, the NN-Align tool, which uses artificial neural networks (ANNs) and weighs matrices, was used [21]. The MHC-II HBV core T-lymphocyte epitope sequences were defined according to Chisari & Ferrari [2]. The main B-cell HBc-derived epitopes according Tordjeman *et al.* [22] (from amino acid 74 to 84, 107 to 118, 117 to 125, and 120 to 138) were also analysed.

Nucleotide sequence accession numbers

The authors are awaiting for accession numbers assignment from GenBank.

Statistical analysis

Data were analysed for mean and standard deviation at 0.95 confidence interval, and differences between means were compared by one-way ANOVA at 0.05 level of significance.

RESULTS

Laboratory HBV serology, liver biochemical tests and HBV viral load during the follow-up

During the longitudinal evaluation of each family member, the only liver biochemical abnormal pattern was observed at the first sampling time in the mother. Elevated transaminases and enzymatic measures of cholestasis (alkaline phosphatase, gammaglutamyl transferase) observed in contrast with other parameters (bilirubin, albumin, coagulation factors, prothrombin time) were normal. Subsequently, these tests were normalized in the mother moving towards an inactive chronic infection (defined as loss of HBeAg, serum ALT $\leq$40 IU/mL, and HBV DNA <6 log IU/mL, according to [23]). Her children exhibited almost constant normal values in these liver biochemical tests, except for transient elevation of AST in the daughter.

Hepatitis B virus serological markers showed continuous presence of HBsAg in all family members. Anti-HBc total antibodies were detected in the mother and S1, but not in S2 and D. HBeAg seroconversion occurred only in the mother after the first sampling time. The three siblings remained HBeAg reactive throughout the study. The viral load was >8 logs IU/mL in the mother at first followed by a marked decline (3.9 log IU/mL). In contrast, her children exhibited a steady high level of viral DNA (Table 1).

HBV complete genome sequences and phylogenetic relatedness

Nine full-length HBV genome sequences were obtained from the family members during the follow-up. By phylogenetic relatedness, they were ascribed to genotype D1 in a separate cluster, showing a monophyletic origin statistically supported by a high bootstrap value (Fig. 1). In comparison with the other 29 complete sequences of genotype D1, 13 distinctive specific point mutations were observed in the nine complete genomes from the family as follows (amino acid changes at Polymerase): A544C (N474H), T996C, A1361T (Y746F), A1634G, G2648A, T2859C (S185P), C2928T (R208C), A2991G (S229G), A3000C (I232L), G3039A (G245L), C3083A (S259R), G3120A (K272E), and 3171A (H289N). Eight of 13 of these variations were at the polymerase spacer domain (from aa 183 to 349). Only the C3083A caused a nonsynonymous mutation T/A79E at preS-S region, whereas the others were synonymous substitutions in this ORF.

The mean similarity rate of complete nucleotide sequences obtained at each sampling time from the mother and her offspring was 98.96%, indicating a remarkable consistency and homology from mother to children.

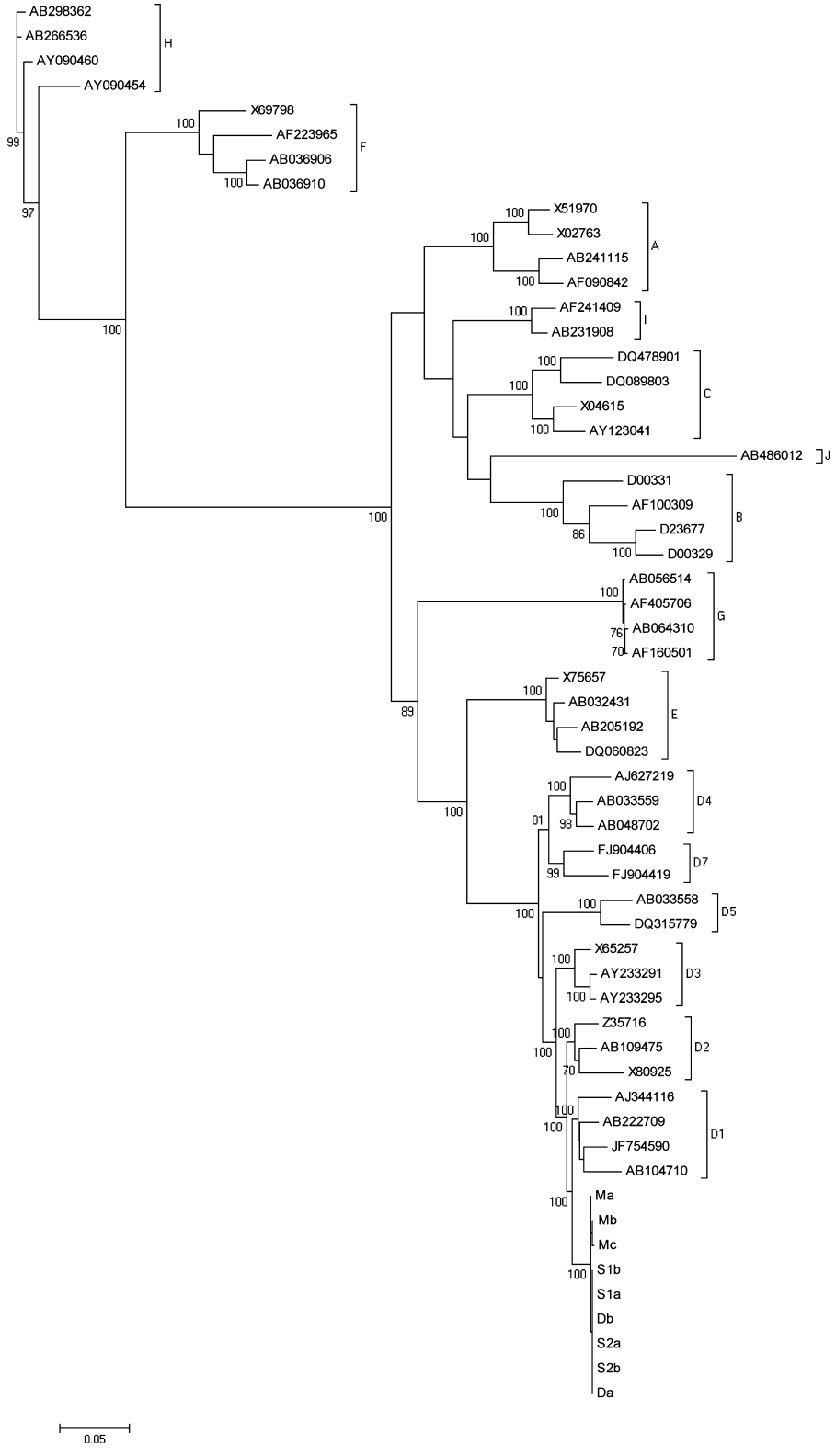

Fig. 1 Maximum likelihood (ML) phylogenetic tree based on complete genome sequences of HBV strains isolated from family members at different sampling times along with 48 reference strains (indicated by GenBank accession numbers) with well-known HBV genotypes (indicated with brackets). The number in the tree indicates the bootstrap reliability. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site. M (mother), S1 (son 1), S2 (son 2), D (daughter). a, b and c, are consecutive sampling times.

However, when comparing the mean (±SD) similarity rate of complete amino acid sequences prior to and after the mother HBeAg seroconversion, the identity was significantly lower (99.5 ± 0.8% *vs* 97.2 ± 2.4%, respectively; $P = 0.02$), suggesting an increase in sequence divergence after HBeAg seroconversion. The lowest identity was observed in core amino acid sequences. In contrast, the siblings showed high nucleotide and amino acid similarity rates among them (99.8 ± 0.00%, and 99.4 ± 0.75%) while all of them remained HBeAg-detectable and normal amino transferases (Table S1).

The monophyletic origin of HBV isolates from the family that upholds the hypothesis of intrafamilial transmission was further confirmed by two analyses implying the concatenated sequences. By both analyses was observed a distinctive 'family' group exhibiting a branching order statistically supported by high bootstrap values. Nucleotide sequences from the family members appeared intermingled among themselves; only the mother showed two distinguishable viral lineages after the HBe seroconversion (Fig. 2).

Analysis of mutations in the BCP-PC region after molecular cloning and deep-sequencing, HBeAg profile and HBV viral load

The cross-analysis of UDPS data obtained from the BCP-PC amplicon showed the presence of several HBV variants with different relative abundance among family members. Several nucleotide variations at PC (G1896A) and BCP (T1753C+A1762T+G1764A; A1766C; T1773C) were detected as well as an 8-bp (nt 1763-1770) deletion. Some of these variants were present in all members but showing a distinctive relative abundance. Other viral variants, however, were only found in some members (Fig. 3).

Longitudinally analysed, the mother (Ma) showed a high predominance (83%) of an 8-bp deletion (1763–1770) at BCP region in the first sampling time. The remainder viral population was composed of similar amounts of wild type (8.7%) and the G1896A mutation at PC (8.3%). At this time, the patient was HBeAg positive and exhibited high viral load (>8 log UI/mL). Three years later (Mb), the HBV DNA level spontaneously dropped (3.0 log UI/mL) and HBeAg to anti-HBe seroconversion occurred. At this time, the viral population heterogeneity was increased. The BCP deleted variant was the most abundant one but appearing alone (60.3%), or combined with G1896A (4.8%), or A1762T (17.9%) mutations. Concomitantly, the G1896A was detected alone (5.3%) and combined with T1773C (3.2%). Two years later (Mc), when viral load was steadily low (3.9 log UI/mL), anti-HBe positive, the HBV population still exhibited the BCP deleted variant predominance (77.1%). The remaining variants changed slightly by adding the contribution of the variant exhibiting the BCP T1753C+A1762T+G1764A triple mutation (5.8%).

The three children were analysed in a single time considering that paired samples were separated from each other by a short period of time (6-to-11 months). All of them were persistently HBeAg positive accompanied by a high viral replication level (>8 log UI/mL). Qualitatively, the HBV variants characterized among the siblings (S1, S2, and D) showed the coexistence of wild-type (63.7%, 55.3%, 20.5%, respectively) and G1896A (16.5%, 40.1%, 73.2%, respectively) variants. Simultaneously, other 'individualized' variants such as the A1766C (both present only in S1) and the T1773C (detected only in D) were found. When comparing the results obtained by molecular cloning followed by Sanger-based sequencing against those from UDPS, the type of viral variants found did not differ, but differences in their relative abundance were observed (data not shown). Only in patient D, the presence of the wild-type variant was not detected by cloning-based characterization. This finding could be explained considering the relative abundance found by UDPS and the small number of clones analysed (from 14 to 20).

The cross-sectionally analysis of the core-coding sequences (from nucleotide position 1901 to 2450) revealed that mutations were found only among viral isolates from the mother. Interestingly, these variations appeared as mutational complexes with the above mentioned BCP-PC variations in well-defined combinations. There were 4 different complexes as follows: (i) BCP deletion+A2094C+C2102G+C2108G, (ii) BCP deletion+T19 61C +A2049G, (iii) T1773C+G1896A+T1963C+A2094C +C210 2G+A2141G+A2179G and (iv) G1764A+A2077 G+G220 7A. Most of these core variations were nonsynonymous and had an impact on MHC class-II core-derived epitopes (see below).

Comparative HBV-nucleocapsid-derived-peptides MHC-II binding predictions

The impact of viral peptides variation derived from the HBV nucleocapsid on MHC class-II molecules was analysed. Amino acid substitutions affected all regions of the core protein. Such substitutions were not necessarily confined to known B- and T-cell epitopes (data not shown). The sequence comparison of nucleocapsid-derived epitopes among HBV isolates from the family members showed differences that were no circumscribed to members with discrepant detection of anti-HBc (M, S1 *vs* S2, D). Nevertheless, those epitopes derived from two peptides (residues 28–47 and 50–69) that verified variations between mother and her children were analysed for their MHC-II binding prediction. Such *in silico* analysis showed that siblings depicted higher affinity in their MHC-II peptide binding than the mother in a vast majority of predictions (Table 2). However, it is important to recognize the inherent limitations in the use of epitope prediction programs. No epitope prediction program is perfectly accurate, and

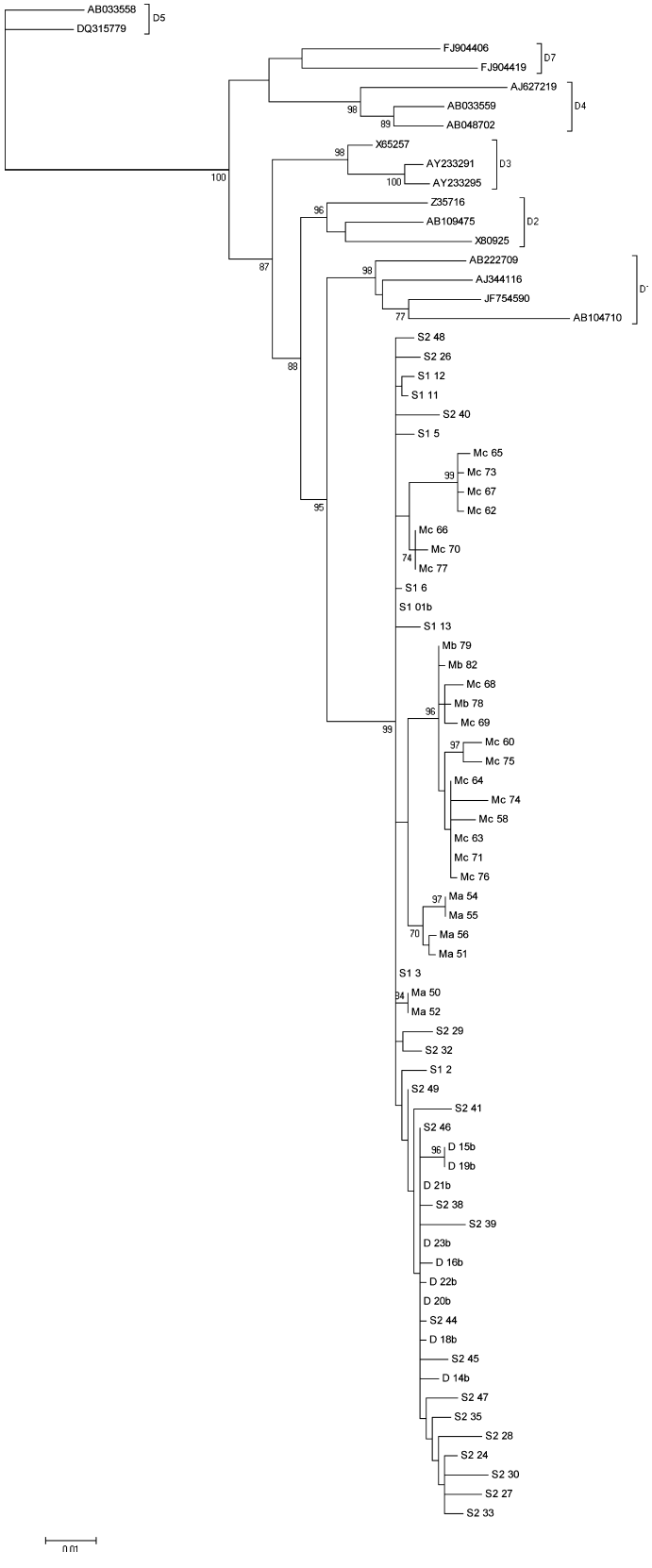

Fig. 2 Maximum likelihood (ML) phylogenetic tree based on 1210 bp alignment of 62 cloned and concatened (BCP-PC and polymerase spacer domain) nucleotide sequences of HBV strains isolated from family members at different sampling times along with 17 genotype D (with their subgenotypes indicated by brackets) reference strains. The number in the tree indicates the bootstrap reliability. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site. M (mother), S1 (son 1), S2 (son 2), D (daughter). a, b and c (only for mother samples) are consecutive sampling times. Each viral molecular clone from family members is designated by a correlative number.

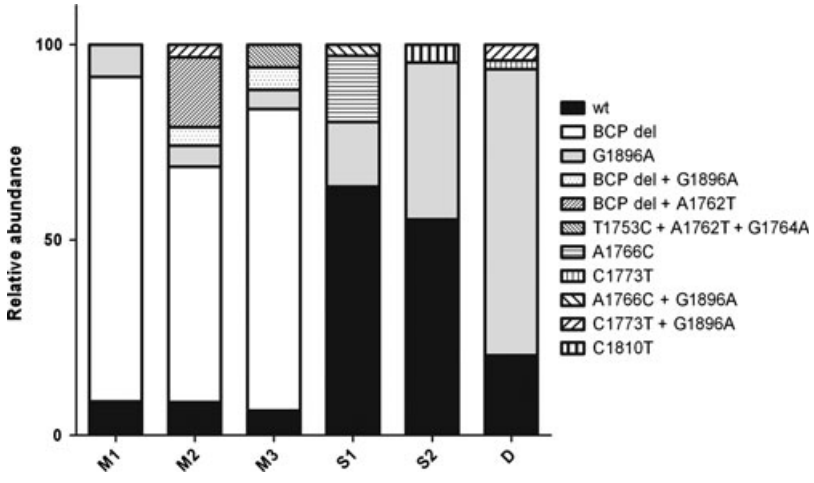

Fig. 3 Relative abundance (%) of different HBV basal core promoter (BCP) and precore (PC) variants present into the viral population for each family member. The nucleotide sequence data were obtained by ultra-deep pyrosequencing.

there is substantial disagreement among epitopes predicted by each program [19,24]. The main B-cell epitopes visually analysed (aa 74–84, 107–118, 117–125, and 120–138) showed only one with a punctual difference between mother and children viral strains located at amino acid 78 (N78D), but the remaining epitopes showed no mutations leading to amino acid changes.

DISCUSSION

Infection with hepatitis B virus in early infancy frequently results in persistent infection, and clustering of the chronic infection within a family is common. Transmission of the virus is thought to have occurred by intrafamilial contact. In this study, we aimed to document the HBV intrafamilial transmission by phylogenetic inference analysis using full-length and partial genomes. The molecular characterization of HBV variants implied both molecular cloning and deep-sequencing.

Unfortunately, samples at the birth of each children and the mother were not available making difficult to confirm vertical transmission. The family members grouped together on a separate branch of the tree ascribed to genotype D1, thus indicating the close genetic relatedness of HBV isolates and its plausible common source of infection. Our findings also suggest, like previous studies [25,26], that HBV genome is stable among HBe-positive patients over long periods of time. Such sequence constrained is supported by both the repertoire of the host immune response and by HBV genomic organization with overlapping open reading frames. However, despite this viral genomic stability, it was necessary to explain the variation in HBV genomic sequences after intrafamilial transmission occurred. In this sense, it is necessary to consider that HBV isolates from genotype D1 appear to be prone to mutations in BCP-PC regions [27]. For that reason, two genomic regions were targeted for amplification, sequencing and phylogenetic inferences. First, the BCP-PC also appears to be more appropriate for phylogenetic analysis than the well-conserved surface gene and the least overlapped gene of HBV [28]. Second, the spacer domain of the polymerase gene for its genomic plasticity was amplified to offer a host signature for each viral variant. Nucleotide sequences were obtained by means of classical cloning followed by Sanger-based sequencing, as well as by deep-sequencing. Those nucleotide sequences obtained by cloning allowed phylogenetic inferences among patients by analysing two concatenated genomic regions obtained from the same HBV isolate and comprising a long genomic fragment (1.2 kb). The deep-sequencing technique allows revealing the massive genetic heterogeneity of HBV within the host by parallel amplification and detection of sequences of a huge number of individual molecules with smaller size (0.33 kb). To the best of our knowledge, this is the first work which performs a hepatitis B virus genomic analysis involving next generation sequencing aimed to provide convincing evidence of intrafamilial transmission.

The BCP-PC/spacer-based analysis of HBV population for each host was different in a defined sampling time. The presence of wild-type sequences as well as those with the PC G1896A mutation was observed in all members with dissimilar contribution. In contrast, other BCP variations such as the triple C1753T+A1762T+G1764A mutation or the 8-bp deletion were detected in some members even with dissimilar relative contribution. During follow-up, a constant high proportion of the deleted BCP variant was observed in the mother, irrespectively of the HBeAg status. Such deletion affects the sequence between TA2 and TA3 as well as sites for transcriptional factors such as the liver-enriched factor that could promote an increased level of pregenomic mRNA accompanied by a reduced production of pre-C mRNA [29,30]. The dominance of such BCP deleted variant prior to seroconversion does not appear to be related with a replication advantage considering that viral load levels decreased after seroconversion, when the dominant variant was the same. Nevertheless, during the

Table 2 MHC class-II binding prediction scores for HBV core protein-derived epitopes targeted HLA molecules encoded in the DR locus

		DRB alleles-Percentile rank[†]												
M:Mother S:Siblings	Peptide sequence[‡]	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0701	DRB1 *0801	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1301	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
A.														
Ma-b-c	**RD**LLD**T**ASALYREAL	21.56	3.55	12.57	26.01	25.83	22.9	19.36	0.73	19.99	24.12	**7.76**	33.46	4.4
S1-S2-D	QN–N- - - - - - - - - -	**12.6**	**3.53**	**4.16**	**4.86**	**14.64**	**2.12**	**7.1**	**0.04**	**6.41**	**10.1**	29.45	**21.38**	**0.39**
Ma-b-c	**D**LLD**T**ASALYREALK	26.42	8.26	12.57	26.01	25.83	32.08	19.36	2.25	19.99	24.88	**7.76**	33.34	7.33
S1-S2-D	N- -N - - - - - - - - - -	**17.14**	**7.71**	**6.64**	**4.86**	**14.64**	**17.83**	**7.1**	**0.3**	**6.41**	**15.86**	38.65	**23.06**	**0.69**
Ma-b-c	LLD**T**ASALYREALKS	37.19	17.5	12.57	26.01	25.83	40.48	19.36	4.24	24.12	27.34	56.16	33.81	8.84
S1-S2-D	- -N- - - - - - - - - - - -	**27.4**	**9.61**	**7.54**	**4.86**	**14.64**	**26.97**	**7.1**	**0.57**	**10.1**	**21.5**	**48.06**	**27.85**	**1.13**
Ma-b-c	L**D**TASALYREALKSP	41.9	23.05	24.13	26.01	23.56	40.91	19.36	34.145	14.94	32.36	75.22	33.26	8.92
S1-S2-D	-N- - - - - - - - - - - -	**33.39**	**9.61**	**21.04**	26.01	**21.21**	**33.44**	**7.1**	**26.08**	**6.41**	**27.81**	75.22	**30.51**	**1.87**
B.														
Ma-b-c	PHHTALRQAILCWG**D**	35.68	66.88	41.78	**29.27**	3.86	37.87	7.35	45.29	2.4	**33.86**	52.96	3.73	**43.12**
S1-S2-D	- - - - - - - - - - - - - -E	**35.62**	**66.88**	**41.53**	29.34	3.86	**36.78**	**7.34**	**44.93**	2.4	34.55	52.96	3.73	43.33
Ma	LRQAILCWG**D**LM**N**LG	61.75	19.66	38.94	70.52	3.86	**41.08**	50.27	56.57	2.4	31.89	33.56	32.97	59.76
Mb	- - - - - - - - - - - - - T- -	55.71	17.66	31.57	64.95	3.86	44.88	47.19	56.08	2.4	29.74	28.2	31.96	56.46
Mc	- - - - - - - - - - - - - T-A	55.22	**12.49**	30.87	61.41	3.86	42.15	46.91	53.84	2.4	**28.7**	**20.55**	**31.47**	55.86
S1-S2-D	- - - - - - - - - E - - T-A	**48.26**	17.65	**28.21**	**57.05**	3.86	42.86	**35.78**	**53.2**	2.4	31.39	29.53	35.49	**48.17**
Ma-b-c	HTALRQAILCWG**D**LM	45.55	47.33	46.12	**48.87**	3.86	47.97	23.12	53.11	2.4	**29.49**	43.02	**5.11**	48.86
S1-S2-D	- - - - - - - - - - - - - E - -	**43.11**	**42.99**	**45.04**	49.87	3.86	**44.17**	**20.8**	**52.9**	2.4	29.63	**42.19**	5.36	**45.99**
Ma	ALRQAILCWG**D**LM**N**L	61.63	22.38	37.31	65.96	3.86	**40.54**	48.16	55.51	2.4	33.85	38.95	31.48	57.11
Mb-Mc	- - - - - - - - - - - - - T-	55.71	19.04	30.43	57.81	3.86	42.75	45.62	54.21	2.4	30.81	33.7	**30.25**	53.64
S1-S2-D	- - - - - - - - - - - E - -T-	**49.59**	**17.93**	**27.76**	**52.24**	3.86	43.73	**35.06**	**52.00**	2.4	**28.72**	**31.33**	33.8	**47.99**

[†]For each peptide, a percentile rank for each of the three methods (combinatorial library, SMM_align and Sturniolo) is generated by comparing the peptide's score against the scores of five million random 15 mers selected from SWISSPROT database. A small numbered percentile rank indicates high affinity (indicated by bolded values). The median percentile rank of the three methods was then used to generate the rank for consensus method. [‡]The peptide sequences correspond to core antigen (at the top: amino acid 28–47; at the bottom: amino acid 50–69). Those residues that varied are bolded. Dashes represent identical amino acids.

follow-up, the presence of the deleted variant coexisted with the wild-type virus that would compensate for the loss of function caused by the deletion [31]. The dynamics of these variants, as seen in the mother serial sampling, suggest that they have developed during the course of infection and could be correlated with the delayed HBe seroconversion only experienced by the mother [32]. Such long persistence of maternal HBeAg could contribute to the absence of spontaneous HBeAg seroconversion in her children with chronic HBV infection [33]. After HBe seroconversion, the viral population heterogeneity augmented suggesting that it could be related to escape mutantions resulting from stronger selection pressure. An increment in the input of the A1762T mutation alone or as part of the C1753T/A1762A/G1764A triple mutation was observed with a concomitant decrease in wild-type virus input. The emergence of such BCP variants after HBeAg seroconversion is associated with incomplete viral suppression, persistent hepatitis [34] and low viremia levels [35].

The common features among children (HBV high replication ability, persistent HBeAg expression and low levels of transaminases) suggest tolerance or minimal immune activity and, in turn, imply small or nonselective pressure from the host. In this scenario, their viral population was composed mainly by wild-type and G1896A variants. Whether the viral population heterogeneity was inherited from the mother and gradually decreased during the immune tolerance phase because of viral competition remains to be determined.

The two younger siblings showed the uncommon absence of the hepatitis B core antibody (anti-HBc). Hepatitis B core antigen (HBcAg) is one of the targets for immune clearance where several epitopes have been identified. Among them, two regions play a particularly important immunodominant role: the sequence from amino acid 50–69, which immunostimulates CD4+ T-helper lymphocytes [36], and the sequence from amino acid 74–84, which stimulates B lymphocytes [11,37]. Changes in the HBV core gene sequence, level of HBcAg expression and/or structure of HBcAg epitopes may affect immune clearance and activity of liver disease [5]. It is known that pairs of genetically related individuals within the same family may share none; one or two haplotypes at the MHC loci, but here unfortunately the MHC haplotypes could not be defined because cells were not available. The three siblings were HBeAg positive and had persistently normal aminotransferase levels (immune-tolerant phase), and their HBV core-derived epitopes restricted by both MHC-I and II depicted identical sequences allowing inferring that they may be sharing several MHC haplotypes. Similarly, the three children also shared the main core-derived B-cell epitopes. In contrast, HBV variations were more frequent in the mother serial analysis with elevated amino transferase levels when she cleared the HBeAg (immune clearance phase). Thus, amino acid variation between isolates from different family members may be explained in terms of the variation in MHC class-II haplotypes, and this variation does not contradict the hypothesis that the virus is transmitted within the family. However, this fact did not explain the absence of anti-HBc antibodies in two children with different genetic background that merits to be explored to elucidate its plausible contribution to this particular serological pattern. Perinatally, infected HBsAg carrier infants without anti-HBc have been reported but are unusual [38–40]. The absence of detectable anti-HBc antibodies in some chronically infected patients may occur due to altered core–protein sequences [41,42], but here among family members with discrepant anti-HBc pattern (M, S1 *vs* S2, D), the sequences exhibited an identity that rose up to 100%. Nevertheless, if the amount of transplacental maternal HBeAg is sufficient and given the susceptibility of the neonatal immune system [43], an immune tolerance could be induced to the extent that even the formation of anti-HBc is precluded. This implies that according to a variation in the magnitude of the immune tolerance during each gestation, the HBcAg-specific B cells of the offspring could be either tolerant or defective.

In summary, intrafamilial transmission of HBV genotype D1 infection was characterized by phylogenetic inferences. HBV appeared as highly conserved while family members were HBe positive. The dominant HBV precore and BCP mutants were different in the mother and children at both interhost and intrahost level, which may be explained by their dissimilar capability to evade immune pressure according to each host immune environments. This study offers a better understanding of how hepatitis B virus and its BCP-PC mutants evolve over time once intrafamilial transmission occurs.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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

Table S1. Percentages of nucleotide and predicted amino acid identities in entire HBV sequences among family members, at different sampling times.