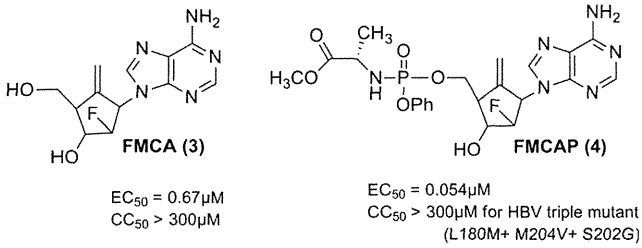


Figure 1. Structures of 2′-fluoro-6′-methylene–carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

can bypass the rate-limiting first step of monophosphorylation. Phosphoramidate prodrugs have demonstrated to enhance the nucleoside potency in cell culture as well as in patients.[10,11] This methodology greatly increases the lipophilicity of the nucleotide to increase the cell penetration as well as to target the liver cells in vivo.

In this communication, we present that a FMCA phosphoramidate prodrug is such an agent, which can potentially be used for the treatment of patients who experience viral breakthrough due to the triple mutants caused by the use of lamivudine and entecavir.

In our previous report, we have demonstrated that the novel carbocyclic adenosine analog **3** (FMCA Fig. 1) exhibits significant anti-HBV activity against wild type as well as adefovir/lamivudine resistant strains.[12] The present study describes the synthesis and antiviral evaluation of a phosphoramidate of FMCA (FMCAP), which demonstrated the significantly improved in vitro potency. Additionally, we studied its mechanism of action how FMCA-TP can effectively bind to the HBV polymerase by molecular modeling and still exerts the antiviral activity against the lamivudine–entecavir triple mutant (L180M + M204V + S202G).

FMCAP (**4**, Scheme 1)[13] was synthesized using a known method in the literature,[14,15] in which the phosphorylation of phenol with phosphorus oxychloride generates phenyl dichlorophosphate **1**, which was coupled with L-alanine methyl ester in the presence of *tri*-ethyl amine in dichloromethane to give chlorophosphoramidate reagent **2**, which, in turn, was coupled with FMCA **3** in the presence of 1-methyl imidazole in THF to furnish the phosphoramidate **4** in good yield.

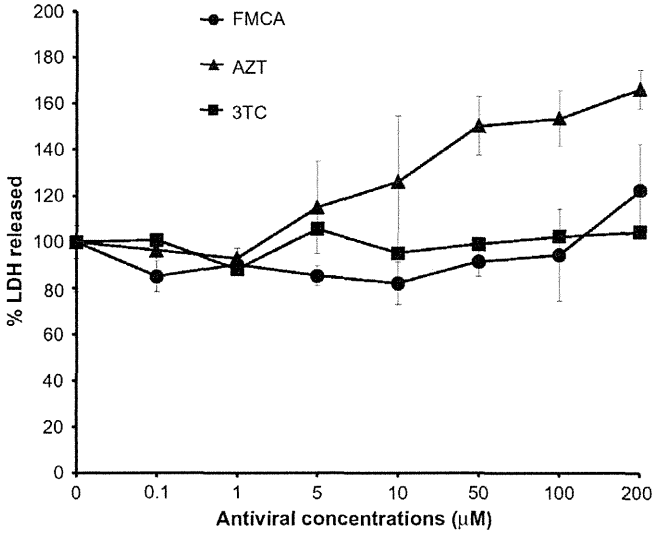
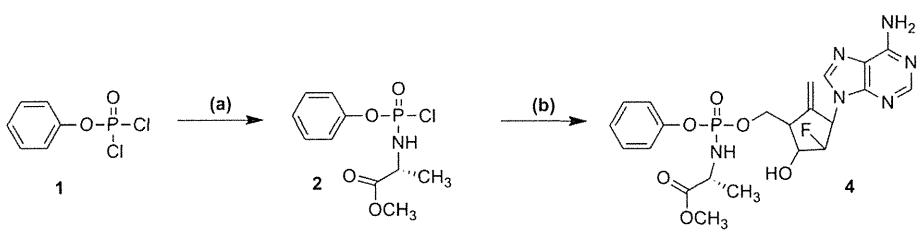


Figure 2. Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

FMCA **3** and FMCAP **4** were evaluated in vitro against the wild-type as well as the lamivudine–entecavir resistant clone (L180M + S202I + M202V). The FMCA **3** and FMCAP **4** demonstrated significant anti-HBV activity (EC_{50} 0.548 ± 0.056 & 0.062 ± 0.011 μM, respectively) against the wild-type virus, while lamivudine and entecavir also demonstrated potent anti-HBV activity (EC_{50} 0.056 ± 0.003 & 0.008 μM, respectively) (Table 1). It is noteworthy to mention that the anti-HBV potency of FMCAP (**4**) was increased to eight-fold (8×) in comparison to that of FMCA **3**, which indicates the importance of the initial phosphorylation of the nucleoside.

FMCA **3** and FMCAP **4** were further evaluated for their in-vitro antiviral potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). It was observed that the anti-HBV potency of both FMCA **3** and FMCAP **4** (EC_{50} 0.67 & 0.054 μM, respectively) were maintained against the resistant clone, and furthermore, the anti-HBV activity of FMCAP **4** was enhanced a 12-fold (12×) with respect to that of FMCA without significant enhancement of cellular toxicity. It was also noteworthy to mention that the anti-HBV potency of entecavir against the mutant



Scheme 1. Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et_3N, CH_2Cl_2; (b) FMCA (3), NMI, THF, rt overnight.

Table 1
In vitro anti-HBV activity of FMCA **3**, FMCAP **4**, lamivudine and entecavir against wild-type and entecavir drug-resistant mutant (L180M + M204V + S202G) in Huh7 cells

Compounds	HBV Strains			
	Wild-type			L180M + M204V + S202G
	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM)	EC_{50} (μM)
FMCA **3**	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP **4**	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500[17]
Entecavir	0.008	0.033	28	1.20[16]

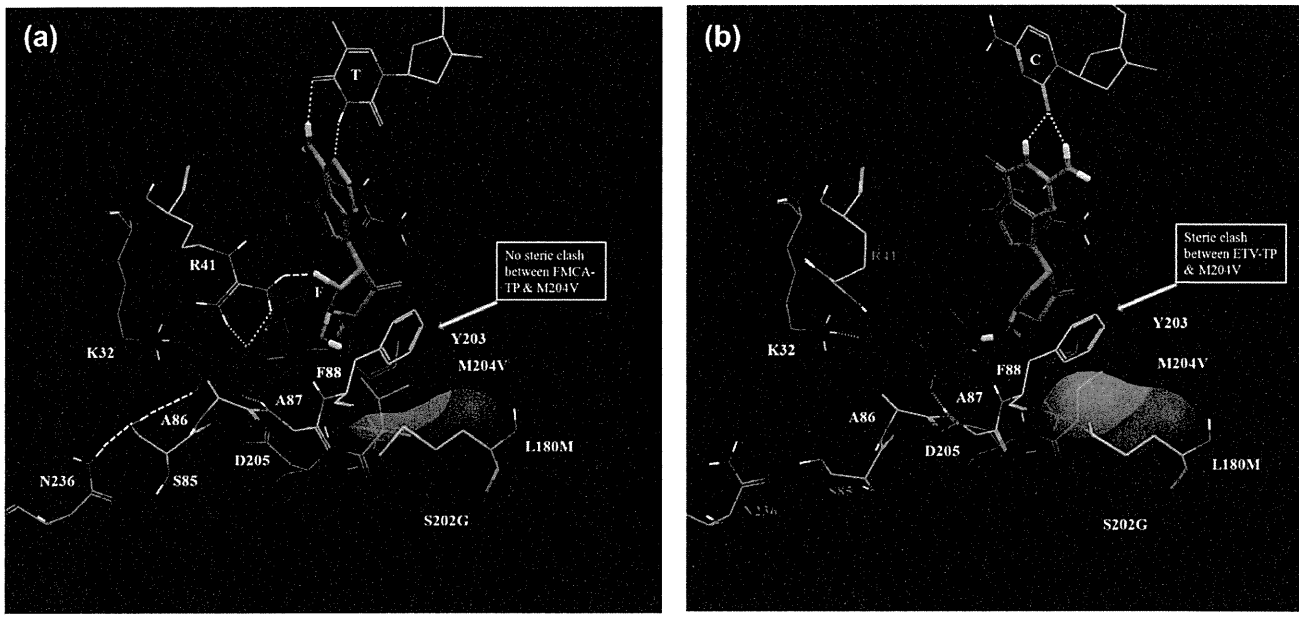


Figure 3. (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface of M204V is shown in spring green. The Van der Waals surface of S202G is colored orange. The exocyclic double bond is shown blue color.

Table 2
MBAE (multi-ligand bimolecular association with energetics) calculation of FMCA-TP and ETV-TP after Glide XP docking[21] and energy minimization[22]

Strains	Compounds	Energy difference results (ΔE, kcal/mol)		
		Total energy	VdW[a]	Electrostatic
Wild-type	FMCA-TP	−588.05	375.78	−6341.08
	ETV-TP	−597.25	350.35	−6009.65
ETVr	FMCA-TP	−591.54	359.91	−6245.68
(L180M + M204V + S202G)	ETV-TP	−320.28	248.82	−4831.12

[a] Van der Waals interaction.

was reduced by 150-fold (EC_{50} 1.2 μM) in comparison to wild type.[16]

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,[18] FMCA **3** did not exhibit any significant toxicity up to 100 μM like lamivudine (3TC), while azidothymidine (AZT) shows the increase of toxicity (Fig. 2).

In our previous report, we described molecular modeling studies for favorable anti-HBV activity of FMCA-TP in wild-type as well as in N236T adefovir resistant (ADVr) mutant.[12] In the current studies, it was of interest to know how the FMCA and its prodrug maintain the anti-HBV activity against ETVr triple mutant (L180M + M204V + S202G) in comparison to entecavir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of FMCA-TP by using the Schrodinger Suite modules.[19] A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.[12] The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).[20]

The binding mode of FMCA-TP and ETV-TP in ETVr (L180M + M204V + S202G) HBV-RT are depicted in Figure 3a and b, respectively. Their MBAE (multi-ligand biomolecular association with energetics)[22] calculations of FMCA-TP (total energy, wt −588.05 & ETVr −591.54 kcal/mol) and ETV-TP (total energy, wt −597.25 & ETVr −320.28 kcal/mol) after glide XP (extra precision) docking[21] and energy minimization in ETVr HBV-RT are shown in Table 2. The triphosphate of FMCA-TP forms all the network of hydrogen bonds with the active site residues (Fig. 3a), K32, R41, S85 & A87 in the similar manner as in wild-type,[12] whereas ETV-TP lose the hydrogen bonding with R41 & S85. The γ-phosphate of FMCA-TP maintains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236 in ETVr HBV-RT also. However, γ-phosphate ETV-TP does not maintain this critical H-bonding with S85 and N236 (Fig. 3b).

The carbocyclic ring with an exocyclic double bond of FMCA-TP and ETV-TP makes the favorable Van der Waals interaction with F88 in ETVr HBV-RT (Fig. 3a and b). There is no steric clash in between exocyclic double bond of FMCA-TP and M204V residue, whereas ETV-TP exocyclic double bond has steric clash with M204V residue in ETVr HBV-RT. The 2′-fluorine substituent in the carbocyclic ring of FMCA-TP appears to promote an additional binding with the NH of R41 guanidino group as shown in Figure 3a, which is in agreement with the antiviral activity of FMCA-TP shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of FMCA-TP against ETVr mutant (L180M + M204V + S202G) in comparison to entecavir as shown in Table 1.

In conclusion, 2′-fluoro-6′-methylene-carbocyclic adenosine phosphoramidate prodrug (FMCAP) was synthesized, which demonstrated the significantly increased anti-HBV potency relative to the parent compound, FMCA in vitro. Molecular modeling studies delineated the mechanism of FMCA-TP and how it can effectively bind to the lamivudine–entecavir resistant triple mutant resulting

in maintaining the anti-HBV activity against the mutant. Furthermore, FMCA has been studied for the release of lactic dehydrogenase for potential mitochondrial toxicity and found that no significant increase of toxicity of FMCA compared with other commonly used anti-HIV nucleoside drugs. Very recently, a preliminary in vivo study in chimeric mice harboring the triple mutant, FMCAP was found to reduce HBV viral load while entecavir did not (data not shown). In view of these promising anti-HBV activities and non-toxicity of FMCAP as well as the interesting mechanism of antiviral activity, the chiral synthesis of FMCAP and its mitochondrial toxicity studies for preclinical investigation are warranted.

Acknowledgment

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13. *Compound* ***4***: ^{1}H NMR (500 Mz, $CDCl_3$) δ 8.35 (s, 1H), 7.86 (d, J = 3.0 Hz, 1H), 7.34–7.15 (m, 5H), 5.95 (m, 3H), 5.26 (d, J = 8.0 Hz, 1H), 5.01–4.90 (m, 1H), 4.83 (s, 1H), 4.50–4.41 (m, 2H), 4.25–4.04 (m, 3H), 3.71 (s, 3H), 3.07 (s, 1H), 1.40 (d, J = 6.5 Hz, 3 H); ^{19}F NMR (500 MHz, $CDCl_3$) δ −192.86 (m, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171, 159.0, 156.5, 152.5, 150.4, 142.9, 130.1, 121.2, 120.3, 106.7, 102.4, 72.2, 71.1, 62.3, 51.9, 46.3, 43.9, 19.1; ^{31}P NMR (202 MHz, $CDCl_3$): δ 2.67, 2.99. Anal. Calcd For $C_{22}H_{26}FN_6O_6P.0.5H_2O$: C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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Journal of Medical Virology 84:587–595 (2012)

Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

Mostafa Ragheb,[1] Abeer Elkady,[2] Yasuhito Tanaka,[2]* Shuko Murakami,[2] Fadia M. Attia,[3] Adel A. Hassan,[1] Mohamed F. Hassan,[1] Mahmoud M. Shedid,[1] Hassan B. Abdel Reheem,[1] Anis Khan,[2] and Masashi Mizokami[4]

[1]*Department of Endemic and Infectious Disease, Suez Canal University, Ismailia, Egypt*
[2]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan*
[3]*Department of Clinical Pathology Faculty of Medicine, Suez Canal University, Ismailia, Egypt*
[4]*Research Centre for Hepatitis and Immunology, International Medical Centre of Japan Konodai Hospital, Tokyo, Japan*

The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

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Mostafa Ragheb and Abeer Elkady contributed equally to this study.

*Correspondence to: Yasuhito Tanaka, MD, PhD, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan. E-mail: ytanaka@med.nagoya-cu.ac.jp

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the world is divided conceptually into zones of high, intermediate, and low HBV endemic areas [Lavanchy, 2004]. In countries where the HBV infection is endemic, most infections result from the vertical transmission from the mother to the child in the peripartum period or from the infection in the early childhood. In the low HBV endemic regions, the neonatal or the childhood HBV infection is rare or even sporadic and the transmission of HBV occurs primarily among unvaccinated adults through the sexual transmission and injecting drug use [Custer et al., 2004].

Patients with chronic hepatitis B are considered to be the major reservoirs for the transmission of HBV. High incidence of infection with HBV is observed within the household contacts of chronic HBV carriers and it is not rare to have several members of the same household who have evidence of infection with HBV [Milas et al., 2000; Thakur et al., 2002]. However, the precise mechanisms of intra-familial spread have not been established clearly.

Different prophylactic strategies for controlling the HBV infection have been used by different countries depending on the prevalence of the HBV infection in each country [Poland and Jacobson, 2004]. The widespread immunization program against hepatitis B, which was implemented in more than 100 countries, was capable of dramatic reduction in the occurrence of chronic HBV infection and HCC [Zuckerman, 1997]. In Egypt, the HBV vaccine was included in 1992 in the Expanded Program of Immunization with injection at 2, 4, and 6 months of age [El Sherbini et al., 2006]. This program resulted in a significant reduction in the rate of acute symptomatic hepatitis B among the children in the age group eligible to receive the vaccine [Zakaria et al., 2007].

At least eight HBV genotypes have been identified based on the divergence of 8% or more of the entire nucleotide sequence and most of the HBV genotypes have a distinct geographical distribution [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Accumulated evidences indicated the difference in the virological characteristics among different HBV genotypes, which is reflected by the difference in the clinical outcome of infection with hepatitis B according to the infecting genotype [Miyakawa and Mizokami, 2003; Schaefer, 2005; Ozasa et al., 2006; Sugiyama et al., 2006]. However, data regarding the specificity of the transmission routes of each genotype is still scarce globally and need to be clarified.

The prevalence of HBV ranges between 2% and 6% in Egypt with the predominance of infection with HBV genotype D [Zekri et al., 2007]. It is widely known that Egypt is one of the countries with highest prevalence rate of infection with HCV in the world [el-Zayadi et al., 1992; Arthur et al., 1993; el Gohary et al., 1995]. However, the burden of HBV related progressive liver disease including liver cirrhosis and HCC in Egypt is observable either single or in a dual infection with HCV [Abdel-Wahab et al., 2000; el-Zayadi et al., 2005].

This study aimed to evaluate the prevalence of infection with HBV within the families of chronic HBV carriers in north Eastern Egypt. In addition, the intra-familial mode of transmission of HBV genotype D was also examined in the current cohort by the molecular evolutionary analyses. The impact of the HBV immunization programme in protecting this high-risk group was also investigated.

PATIENTS AND METHODS

Patients

The present study was conducted between January 2008 and June 2008 at the Communicable Disease Research and Training Centre, in Suez city. The study protocol was approved by the ethics committees of the participating institution and an informed consent was obtained from the included subjects.

Chronic HBV carriers were defined as individuals whose serum samples tested positive for HBsAg for at least 6-months period. Patients who fulfilled the criteria of chronic HBV carriers and were first detected within their families, were defined as the index cases (n = 55). The index cases included 40 (72.7%) men and 15 (27.3%) women. Their mean age (± SD) was 41 ± 10.7 years and all the index cases were negative for HBeAg.

A total of 230 household contacts of the index cases were included in the study and defined as family members group. Data regarding their family relationship to the index cases, age, and the HBV vaccination history have been obtained.

According to the kinship of the family members to the index case group, the family members included 139 offspring, 4 parents, 46 spouses, 15 siblings, and 26 defined as other relatives who are living in the same house with the index cases.

Serological Methods

Serum samples were collected from the index cases and family members groups.

The Serum samples were examined for HBsAg, anti-HBc, anti-HBs, and HBeAg by the chemiluminescence enzyme immunoassay with the commercial assay kits (Fujirebio, Inc., Tokyo, Japan). The examination of the serum samples for anti-HCV and HIV was conducted using commercial kits (Abbott Laboratories, Abbott Park, IL).

Molecular Evolutionary Analysis

The HBV/DNA was extracted from 200 μl of serum samples positive for HBsAg using the QIAamp DNA MiniKit (QIGEN, Inc., Hilden, Germany), and re-suspended in 100 μl of a storage buffer (provided by the kit manufacturer).

The entire preS2 and S regions of the HBV genome (799 nucleotides; nucleotide positions 34–833) were amplified using the primers set and the conditions described previously [Sugauchi et al., 2001].

The amplified products were sequenced using Prism Big Dye (Pekrin–Elmer Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer according to the manufacturer's protocol. The sequences were aligned together with the CLUSAL X software programme [Thompson et al., 1994].

The phylogenetic tree was constructed using the neighbor joining method with Tamura-Nei's distance correction model using the Online Hepatitis Virus database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. The Bootstrap values were determined on 1000 database resampling tests. The sequences of other HBV isolates used for the construction of the phylogenetic tree were retrieved from the DDBJ/EMBL/GenBank sequence database and were indicated in their accession numbers. The new nucleotide sequences data that were reported in this manuscript will appear in the DDBJ/EMBL/GenBank sequence database with accession numbers AB561825-AB561856.

Statistical Analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent *t*-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL). *P*-values (two-tailed) <0.05 were considered to be significant statistically.

RESULTS

The family member included 96 (41.7%) males and 134 females (58.3%). Their mean age (±SD) was 20.6 ± 14.6. The rate of seropositivity for HBsAg and anti-HBc was 12.2% (28/230) and 23% (53/230) of the family members group with no statistical significant difference between the males and females members.

Age Group Distribution of HBV Infection Within the Family Members Group

Figure 1 illustrates the HBsAg and anti-HBc prevalences among different age groups of the family members. The highest prevalence of HBsAg seropositive cases was observed in the age group, 21–30 years old; (10/43; 23.3%) followed by the age group, 0–10 years old; (11/68; 16.2%). No statistical significant difference was found in the HBsAg seropositive rates between these two age groups. The prevalence of HBsAg was 7.7% (5/65), 3.4% (1/29), and 4% (1/25) in the age groups; 11–20, 31–40, and ≥41 years old, respectively. The prevalence of anti-HBc seropositive cases was significantly increasing with the age and the highest rate was observed in the age group ≥41 years old. The prevalence of anti-HBc was 8.8% (6/68), 20% (13/65), 25.6% (11/43), 37.9% (11/29), and 48% (12/25) in the age groups; 0–10, 11–20, 21–30, 31–40, and ≥41 years old, respectively.

The HBsAg and anti-HBc seropositive rates were analyzed in the family members with respect to their relationship to the index cases (Fig. 2A). As overall, the HBsAg was positive in 6.5% (3/46) spouse of index cases, 10.8% (15/139) of the offspring, 25% (1/4) of the parents, and 40% (6/15) of the siblings (Fig. 2A).

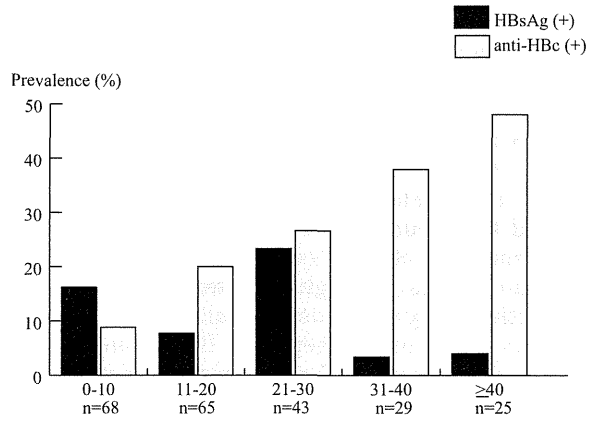

Fig. 1. Age distribution and HBV serological status among family members.

The prevalence of anti-HBc was 34.8% (16/46) in the spouse of index cases, 17.3% (24/139) in the offspring, 50% (2/4) in the parents, and 46.7% (7/15) in the siblings of the index cases (Fig. 2A).

Interestingly, the prevalence of HBsAg and anti-HBc was significantly higher in the family members of the females (19.2%, 15/78) than that of the males index cases (8.6%, 13/152; $P = 0.034$) and a trend of higher incidence of anti-HBc in the family members of the females than the males index cases (Fig. 2B). Among the offspring group, HBsAg and anti-HBc seropositive rates were significantly higher in the offspring of the females index cases (HBsAg; 23%, 11/47, anti-HBc; 29.8%, 14/47) cases than in the offspring of the males index cases (HBsAg; 4.3%, 4/92, antiHBc; 9.8%, 9/92), ($P = 0.001$, 0.003 respectively; Fig. 2C).

Further analysis was performed regarding the HBsAg seropositive rate in the offspring according to HBV infection of both one and two parents and the parent gender who is infected with HBV. Significantly higher rate of HBsAg positive (26.5%, 13/49) and anti-HBc positive (31.8%, 14/49) off spring were found in families where the mother was positive for HBsAg compared with families where the father was HBsAg positive (HBsAg; 4.7%, anti-HBc; 10.5%), ($P = 0.0006$, 0.009 respectively) (data not shown).

The seropositive rate of HBsAg was higher in the non-sexual contacts (13.6%, 25/184) of the index cases (parents, offspring, siblings, and cousins) than the sexual contacts (spouses; 6.5%, 3/46) with no statistical significant difference. Anti-HBc seropositive cases were observed more frequently in the sexual contacts (spouses) than in the non-sexual contacts (parents, offspring, siblings cousins) of the index cases. (Sexual vs. non-sexual contacts, 34.8% vs. 20.1%, $P = 0.049$) (data not shown).

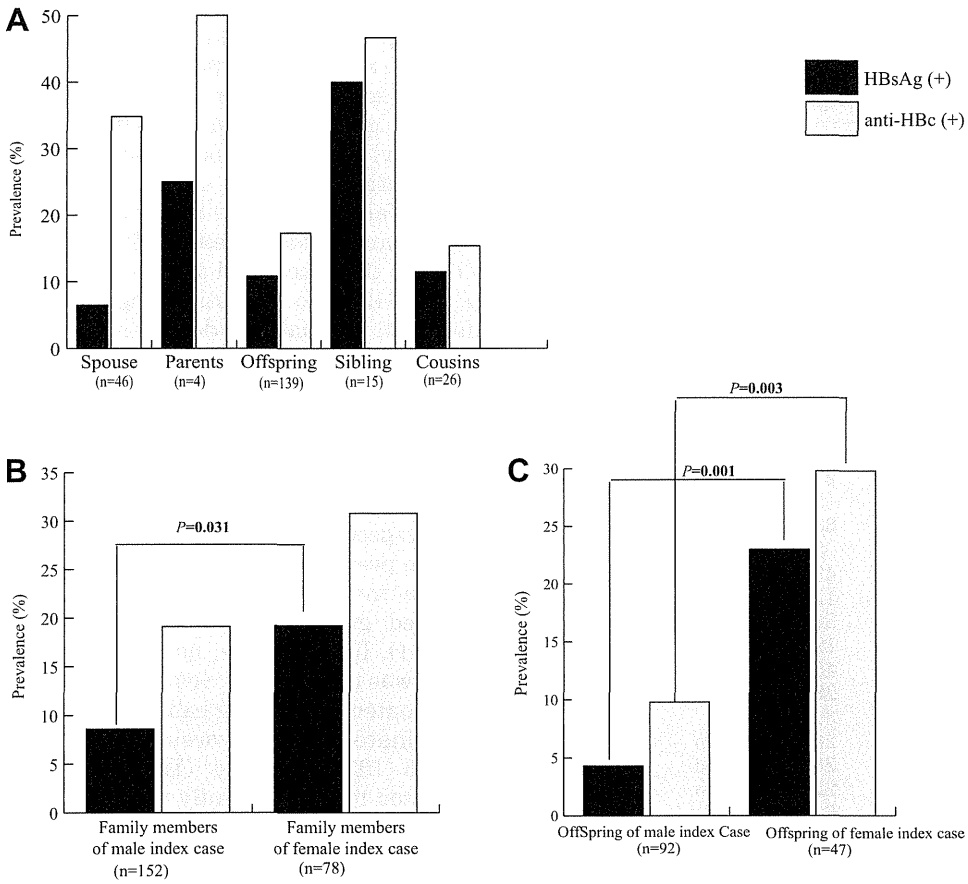

Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

Molecular Evolutionary Analysis and Transmission Pattern of Hepatitis B in the Family Members Group

Eighteen index cases out of 55 (32.7%) were found to have at least one family member positive for HBsAg. The age range of these index cases was 26–56 years and 50% (9/18) of them were male (Table I). Twenty-eight family members were found to be positive for HBsAg. The data regarding the degree of relativity of each family member infected with HBV to the index case, the age of the infected family member, and the vaccination status were indicated in Table I. The mean age (±SD) of the family members with active HBV infection was 17.8 ± 13.0 years old (Table I).

The HBV genomic region of 799-nt length and spanning PreS2 and S region was amplified in 44% (8/18) of the index cases and in 50% (14/28) of the family members infected with HBV. However, the target genomic region could be amplified and sequenced simultaneously in the index cases and their related family members in six subjects. These six subjects are defined in the present report, Table I and Figure 3 as F 3, F4, F19, F35, F37, and F 43 (Table I, Fig. 3).

To confirm the family clustering, a phylogentic tree was constructed by (1) the previous mentioned sequences (2) sequences isolated from the index cases whose family members were negative for HBsAg (3) HBV nucleotide sequences isolated from HBV chronic carriers residing in different districts in Egypt (North and South) either retrieved from the data base band or further included in the present study.

The phylogenetic analysis of the preS2 and S regions of the HBV genome revealed that the HBV isolates were of subgenotype D1 (Fig. 3). Using the phylogenetic analysis, in family 4 (F4), a high homology was detected between the HBV strains isolated from the grandmother together with her daughters and her grandchildren (Fig. 3). In the Family 35 and Family 43 (F35, and F43), the father and the child harbored very closely related HBV isolates and the phylogenetic analysis suggesting that the father may have been the source of infection for his child in Family 35 (F35) and Family 43 (F43). Similarly, very closely related HBV isolates were also detected in the

TABLE I. Descriptive Analysis of the Family Members Positive for the HbsAg

Subject	Relation (gender)	Age	HBV-vaccine[a]	PreS2 + S
F3	Index (F)	42		(+)
F3-1[b]	Daughter	13	Yes	(+)
F10	Index (F)	30		(−)
F10-1	Daughter	3	Yes	(+)
F11	Index (F)	33		(+)
F11-1	Daughter	8	Yes	(−)
F11-2	Cousin	10	Yes	(−)
F30	Index (F)	42		(−)
F30-1	Son	8	Yes	(−)
F34	Index (F)	30		(−)
F34-1	Son	7	Yes	(+)
F34-2	Son	9	Yes	(+)
F48	Index (F)	30		(−)
F48-1	Son	5	Yes	(−)
F35	Index (M)	29		(+)
F35-1[b]	Daughter	5	Yes	(+)
F39	Index (M)	33		(−)
F39-1	Daughter	5	Yes	(−)
F43	Index (M)	47		(+)
F43-1[b]	Daughter	12	Yes	(+)
F55	Index (M)	56		(+)
F55-1	Daughter	12	Yes	(−)
F37	Index (M)	45		(+)
F37-1[b]	Wife	26	Yes	(+)
F36	Index (M)	31		(−)
F36-1	Brother	26	No	(−)
F36-2	Brother	28	No	(−)
F36-3	Brother	22	No	(+)
F36-4	Mother	63	No	(+)
F4	Index (F)	54		(+)
F4-1	Daughter	35	No	(+)
F4-2	Daughter	20	No	(+)
F4-3	Grandchild	6	Yes	(+)
F4-4[b]	Grandchild	4	Yes	(+)
F19	Index (M)	29		(+)
F19-1[b]	Wife	27	No	(+)
F40	Index (M)	26		(−)
F40-1	Relative	24	No	(−)
F40-2	Relative	29	No	(−)
F41	Index (F)	53		(−)
F41-1	Daughter	23	No	(−)
F41-2	Daughter	17	No	(−)
F45	Index (M)	33		(+)
F45-1	Wife	27	No	(−)
F50	Index(F)	27		(−)
F50-1	Sister	25	No	(−)

[a]HBV vaccination history is provided for the family member.
[b]Index and family members who are positive simultaneously for the PreS2and S region.

man and his wife in Families 19 and 37 (F19 and F37) (Fig. 3). The molecular evolutionary analysis of the sequences isolated from the mother and her daughter in Family 3 (F3), yielded two separate but distinct groupings of the HBV isolates, suggesting that the presence of two different HBV viral isolates infecting the mother and her daughter (Fig. 3).

Serological Markers of HBV Infection in the Vaccinated and Unvaccinated Family Members

The family members group was subdivided into two subgroups according to the history of full regimen schedule of HBV vaccination as shown in Table II; (1) A group of vaccinated family members which includes a total of 142 subjects, who received the complete HBV vaccine regimen. (2) A group of unvaccinated family members, which included 88 subjects with no previous history or incomplete regimen of HBV vaccination.

The family members in the unvaccinated group were significantly older (mean ± SD; 32.5 ± 12.5 years old) than in the vaccinated group (mean ± SD; 13.3 ± 10.4, $P = 0.012$). No statistical significant difference was found in the male gender distribution between the two groups. The anti-HBs seropositive rate was significantly higher in the vaccinated group than the unvaccinated group [69.8% (99/142) vs. 33% (29/88), respectively, $P < 0.0001$] (Table II). The mean anti-HBs titre was significantly higher in the vaccinated than unvaccinated family members (70.1 ± 129.7 vs. 21.6 ± 51.7 mIU/ml, respectively $P < 0.0001$).

The prevalence of anti-HBc was significantly higher in the unvaccinated family members compared to vaccinated groups (37.5% vs. 14.1% respectively, $P < 0.0001$). Interestingly, no statistical significant difference was detected between the vaccinated and the unvaccinated groups regarding the prevalence of HBsAg [vaccinated vs. unvaccinated; 10.6% (15/142) vs. 14.8% (13/88), $P = 0.4$] (Table II). The HBV DNA was detected in 50% of family members positive for HBsAg with no statistical significant difference between the vaccinated (53%, 8/142) and unvaccinated groups (46.2%, 6/88) (Table II).

Mutations in the "a" determinant region. The available nucleotide sequences spanning the S gene of HBV isolated from the nine vaccinated and five unvaccinated members were translated into amino acid and aligned in correspondence to the reference sequences. The amino acid substitutions in the "a" determinant region that was reported to be associated with vaccine escape mutation were not detected. However, an amino acid substitution at the second loop of "a" determinant region (T143L) was clustered in the family subject F37 (F37 and F37-1) and found in one unvaccinated family member (F4-1). Another substitution was detected in the second loop of "a" determinant region (T140I) in an unvaccinated member (F36-1). P127A substitution in first loop of the "a" determinant region was clustered in the family 43(F43 and F43-1; Fig. 4).

DISCUSSION

The investigation of the intra-familial transmission in a particular region usually reveals valuable information about the routes of HBV spread in general and may help in exploring the HBV spread problem and local peculiarities. This study is the first one in Egypt done to explore the intra-familial spread of HBV infection and inclusively HBV genotype D transmission routes in Egypt. An evaluation of the impact of the universal HBV vaccination on the intra-familial transmission of HBV was also done.

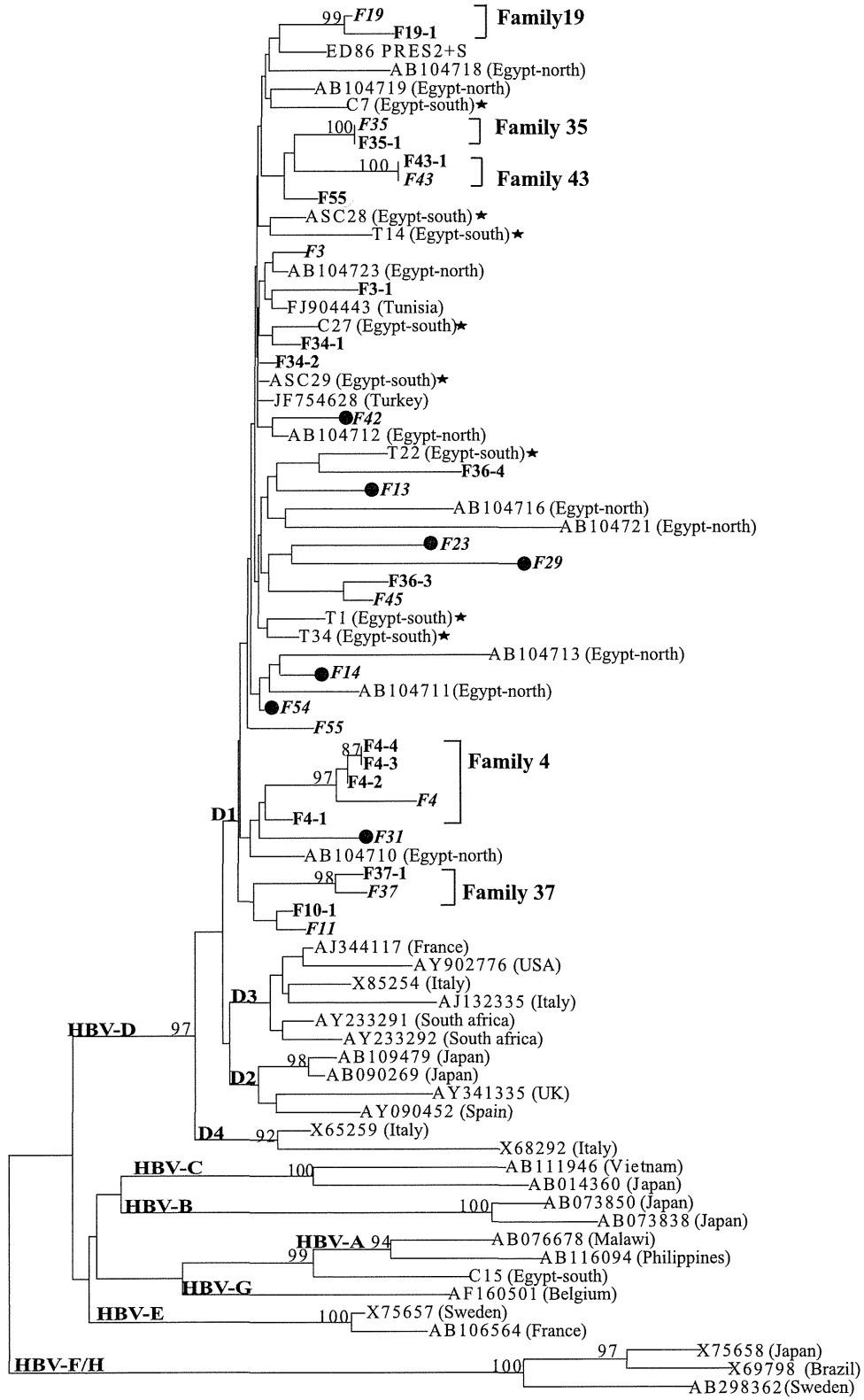

Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family members are indicated in italic bold and bold fonts respectively. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted in their accession numbers. Solid black rounds indicate sequences from index cases with family members negative for HBsAg. (★) Strains isolated from chronic hepatitis B carriers residing in Egypt south. The country origin of the reference sequences are indicated in brackets. HBV genotypes A–H are indicated in the cluster roots.

TABLE II. Comparison of Hepatitis B Serological Markers in Vaccinated Versus Unvaccinated Family Members Group

	Total (N = 230)	Vaccinated group (N = 142)	Unvaccinated group (N = 88)	*P*-value
Age[a]	20.6 ± 14.6	13.3 ± 10.4	32.5 ± 51.7	<0.0001
Gender (Male)[b]	96(41.7)	64 (45.1)	32 (36.4)	NS
Anti-HBc (+)[b]	53 (23)	20 (14.1)	33 (37.5)	<0.0001
HBsAg (+)[b]	28 (12.2)	15 (10.6)	13 (14.8)	NS
Anti-HBs (+)[b]	128 (55.7)	99 (69.8)	29(33)	<0.0001
HBV-DNA (+)[b]	14 (50)	8 (53.3)	6 (46.2)	NS

[a]Mean ± SD.
[b]N (%).

In the present study, 12.1% of the family members were infected with HBV. This incidence was much higher than that detected among the blood donors (1.4%) resident in the same area in Egypt (data not shown). Clustering of the HBV infection within the families has been described in nearby countries located within the same zone of the HBV endemicity but with different incidences; 30% in Turkey, 15.8% in Greece, and 11.9% in Iran [Alizadeh et al., 2005; Zervou et al., 2005; Ucmak et al., 2007]. An important risk factor was found to be implicated in acquiring the infection among the family was the presence of female infected with HBV. Furthermore, the higher incidence of HBsAg positive rate among the offspring of the females' index cases than that of males index cases illustrates clearly the role of the mother in the transmission of HBV. Similarly, Salkic et al. [2007] reported the same observation in his study from Bosnia [Salkic et al., 2007]. However, in Taiwan no significant difference was found in the HBsAg positivity among the offspring of the two groups, suggesting the importance of the paternal as well as the maternal transmission for the HBV intra-familial spread in Taiwan [Lin et al., 2005].

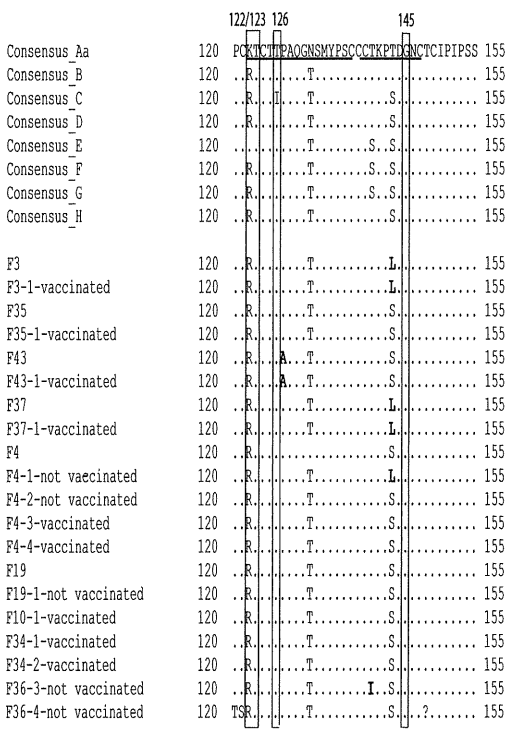

Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank database. Dots in alignment indicate identity of amino acids to the consensus sequence of genotype Aa/A1. First and second loop positions are underlined in the consensus sequence of the genotype Aa/A1 and positions of previously reported vaccine escape mutants are indicated in numbers and included in boxes.

Despite being a tedious and labor-intensive method, sequencing of the viral genomes isolated from different individuals, with the subsequent homology comparison and the phylogenetic analysis remains the golden approach for demonstrating the HBV transmission in a given population [Dumpis et al., 2001; Zampino et al., 2002; Tajiri et al., 2007].

The full length HBV sequence analysis is the gold standard for this purpose but remains a cost approach [Datta et al., 2007]. Highly variable HBV genomic region is recommended by some investigators to study the transmission event. Variability of the genomic region is affected by several factors one of which is the clinical characteristics of the studied cohort [Wu et al., 2005]. PreC/C region exhibit high variability in the cases of acute or fulminant hepatitis and thus analysis of this region is preferable for investigating the chain of recent/nosocomial fulminant cases [Bracho et al., 2006; Ozasa et al., 2006]. However, a high S gene variability is documented among the chronic hepatitis B carriers and their families, thus investigating the genotype, subgenotype, subtypes, and mutations by the sequence analysis of the S gene with further analysis by testing the constructed phylogenetic tree with the bootstrap resampling maximum-likelihood test, may provide enough confidence to prove the transmission event in the case of chronic HBV carriers [Thakur et al., 2003]. Hence, in the present study, the phylogenetic analysis of the HBV nucleotide sequences spanning the entire preS2 and S HBV genomic regions and isolated from chronic hepatitis B carriers which include index cases and their family members revealed the infection with HBV genotype D which coincides with the previous

data regarding the predominance of infection with HBV genotype D in Egypt [Saudy et al., 2003]. In addition, the phylogenetic analysis documented the presence of three different patterns of HBV genotype D transmission within the families in Egypt; maternal transmission (from mother to child as in the family 4), paternal transmission (from father to child as in family 35 and family 43) and spousal transmission (between spouses as in family 19 and family 37). This was different from the transmission pattern characteristics of genotype D in Uzbekistan where the horizontal transmission was the predominant route of infection with HBV genotype D within a family [Avazova et al., 2008].

The Data regarding the difference of transmission routes of HBV infection between different genotypes are controversial and scarce. Based on the findings that the patients infected with HBV genotype C may exhibit delayed HBeAg seroconversion decades later than the patients infected with other genotypes, Livingston et al. [2007] speculated that genotype C is the most responsible for the perinatal transmission and that the other genotypes (A, B, D, and F) are mainly transmitted horizontally [Livingston et al., 2007]. A recent study has shown a different data through exploring that both genotypes B and C can be transmitted by maternal and horizontal routes [Wen et al., 2011]. Whether different HBV genotypes have different transmission routes remains a question, which needs further global studies to clarify this interesting and important issue.

In an attempt to evaluate the influence of the universal vaccination on the intra-familial HBV infection, it was surprising to find a high prevalence rate of HBsAg among the vaccinated members with no significant difference when compared to the unvaccinated group. In an agreement with the present data, El Sherbini et al. [2006] reported the unchangeable prevalence of HBsAg among the vaccinated school children across a decade despite the significant decrease of the anti-HBc rate [El Sherbini et al., 2006]. The possible explanation for this vaccine failure is the acquiring of the HBV infection in the lag period between the birth and the time of receiving the first HBV vaccine dose at the age of 2 months. Supporting our explanation is the recent data coming from Taiwan where a different HBV infection prophylactic strategy is applied by administrating the first dose of the HBV vaccine at birth with the administration of the hepatitis B immunoglobulin to the infants born to the HBeAg positive mother within 24 hr after birth. The recent study has clearly demonstrated that the current HBV prophylactic strategy in Taiwan was capable of reducing the intra-familial HBV transmission and reducing the overall HBsAg positive rate among the infants [Mu et al., 2011]. In Japan, the extension of the active and passive immunization to the babies born to HBeAg negative mother had greatly reduced the HBsAg prevalence to 0.2% of blood donors younger than 19 years old [Noto et al., 2003; Matsuura et al., 2009]. The present study recommends the changing of the current HBV prophylactic policy in Egypt. It would be needed to provide the first dose of the HBV vaccine at birth together with screening for HBV infection markers prenatally and administration of the HBIG to the infants born from HBeAg-positive mothers. The documented role of the HBV spousal transmission in the present study by the phylogenetic analysis (Family 19 and Family 37), coincides with the recent data conducted in Egypt that the first sexual contact with an infected spouse was a significant risk factor for infection with HBV among females and may further emphasize the importance of the premarital screening for HBV in Egypt [Paez Jimenez et al., 2009]. Investigating the " a" determinant region of viral isolates retrieved from the vaccinated members infected with HBV provides no evidence of breakthrough infection by previously reported vaccine escape mutant virus [Carman et al., 1990].

In conclusion, the present study has clearly explored the role of the HBV intra-familial transmission and spread in north Eastern Egypt. Three patterns of HBV transmission were determined in the current cohort infected with HBV genotype D; maternal, paternal, and spousal. The present study recommends the change of the current prophylactic policy against the HBV infection in Egypt by including the first dose of HBV vaccine at birth, screening of pregnant women for HBsAg and the administration of HBIG to the infants born from HBeAg positive mothers within 24 hr after birth. Further studies are needed globally to determine the transmission patterns of different HBV genotypes and locally in different districts in Egypt to explore the impact of familial transmission in HBV infection in Egypt.

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Novel Evidence of HBV Recombination in Family Cluster Infections in Western China

Bin Zhou[1], Zhanhui Wang[1], Jie Yang[1], Jian Sun[1], Hua Li[2], Yasuhito Tanaka[3], Masashi Mizokami[4], Jinlin Hou[1]*

1 Institute of Hepatology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China, **2** Qinghai Provincial Infectious Diseases Hospital, Xining, Qinghai, China, **3** Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan, **4** The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Kounodai, Ichikawa, Japan

Abstract

Two hepatitis B virus (HBV) C/D recombinants were isolated from western China. No direct evidence indicates that these new viruses arose as a result of recombination between genotype C and D or a result of convergence. In this study, we search for evidence of intra-individual recombination in the family cluster cases with co-circulation of genotype C, D and C/D recombinants. We studied 68 individuals from 15 families with HBV infections in 2006, identified individuals with mixed HBV genotype co-infections by restriction fragment length polymorphism and proceeded with cloning and DNA sequencing. Recombination signals were detected by RDP3 software and confirmed by split phylogenetic trees. Families with mixed HBV genotype co-infections were resampled in 2007. Three of 15 families had individuals with different HBV genotype co-infections in 2006. One individual (Y2) had a triple infection of HBV genotype C, D and C/D recombinant in 2006, but only genotype D in 2007. Further clonal analysis of this patient indicated that the C/D recombinant was not identical to previously isolated CD1 or CD2, but many novel recombinants with C2, D1 and CD1 were simultaneously found. All parental strains could recombine with each other to form new recombinant in this patient. This indicates that the detectable mixed infection and recombination have a limited time window. Also, as the recombinant nature of HBV precludes the possibility of a simple phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

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* E-mail: jlhousmu@yahoo.com.cn

Introduction

Not all viruses are equally prone to recombination. Recombination has not been detected in several viruses despite repeated searches [1]. Whether recombination does or does not exist is important for understanding the evolution and replication mechanism of a specific kind of virus. Hepatitis B virus (HBV), a major human pathogen, has been classified into 10 genotypes and several sub-genotypes [2,3]. Many sub-genotypes were identified by polygenetic analysis as recombinants. But there is no direct evidence to indicate that these subgenotypes arose as a result of recombination or perhaps a result of convergence.

Coinfection with different HBV genotype strains is a prerequisite for recombination. As more than one genotype is predominant in most of the geographic regions, coinfection between the predominating HBV genotypes is not a rare finding, especially for B and C, or A and D. The prevalence of mixed HBV genotype infections has been reported using varied genotyping methods [4,5,6].

Our previous study found two kinds of HBV C/D recombinants in northwest China [7]. In a further study of ethnic groups of five provinces, we confirmed the geographic and ethnic distribution of the HBV C/D recombinant in northwest China [8], and found that family-cluster HBV infections were common in these endemic areas. We hypothesize that infected members of HBV family clusters would gain exposure to various genotypes through marriage, while at the same time; competent strains would be selected through vertical transmission. It would be useful to observe the mixed infection in family-cluster cases, especially in patients infected with C/D recombinants.

The aim of this study was to evaluate the possibility of recombination between two HBV genotypes within an individual by finding cluster-infected families in which individual members were infected with different HBV genotypes. We would then look for individuals within these families with multiple-genotypes that were likely to have been obtained from other family members as a result of vertical or horizontal transmission. Novel viral genomes within an individual with a multiple genotype infection that were mosaics of the known viral genotypes in the family, but not present in any of the other family members, would be consistent with the hypothesis that they arose within the individual with multiple genotype infections.

Methods

Subjects

We enrolled 68 patients with a chronic HBV infection from 15 families. All the families were from a district located at the boundary of Gansu and Qinghai provinces, where the prevalence of genotype C2, D1 and C/D recombinant HBV were known to be high [8]. The families were initially identified with cluster HBV infection in an epidemiological survey in 2002. Sixty-eight individuals were sampled in June 2006 and December 2007 for the purpose of assigning HBV genotypes to chronically infected individuals and finding individuals with multiple HBV genotype co-infections. None of the patients received anti-viral therapy or immunosuppressant drugs. A written, informed consent was obtained from each family, and the study protocol was approved by the Southern Medical University Ethics Committee.

HBV DNA Extraction and HBV Genotyping

HBV DNA was extracted from 400 μL of serum by QIAamp UltraSens Virus Kit (Qiagen GmbH, Germany), then re-suspended in 50 μL water and stored at −20°C until analysis. HBV genotypes, including C/D recombinant, were initially assigned using the PCR based restriction fragment length polymorphism (RFLP) methods described previously [9], [8].

Cloning of Mixed Infection Samples

For samples with mixed genotype infections, PCR cover HBV S gene (nt136-1110) was performed using the primers and thermocycling conditions descirbed by Sugauchi et al [10]. For samples needing further recombination analysis, PCR was performed using the primers and thermocycling conditions described by Günther to obtain full-length HBV genome [11]. Alternatively, a nested PCR was used to produce two overlapping fragments in subjects with low HBV DNA levels as described by Sugauchi et al [12]. The spanning of fragment A cover nucleotides 2813 to 1824, and fragment B included nucleotides 1821 to 237. LA-Taq (TAKARA, Japan) and high-fidelity polymerase COD-FX (TOYOBO, Japan) were used to produce amplimers for cloning and direct sequencing respectively. Finally, Fragment C (HBV nt56-nt1824) was obtained from a PCR amplification of Y2 HBV-DNA to which an aliquot of genotype B HBV-DNA had been added. The purpose of this experiment with in-tube control of genotype B was to determine if the recombinant clones were being generated during the PCR amplification. PCR products were gel-purified and cloned into the PMD19-T vector (TAKARA, Japan) according to the manufacturer's instructions, and used to transform JM109 competent cells (TAKARA, Japan). A minimum of 15 clones were sequenced from subjects with a mixed-strain infection and three clones were sequenced from family members with a single-strain infection. All sequencing of clones and PCR products was performed by Invitrogen Ltd. (Shanghai, China).

Phylogenetic and Recombination Analysis

Genotypes of clones were determined by phylogenetic tree analysis and recombination analysis. The sequences were assembled using SeqMan II software (DNAStar Inc.). Sequence alignments were performed using ClustalW and confirmed by visual inspection. Phylogenetic trees were constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987). To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. A phylogenetic tree analysis of HBV strains isolated from the mixed infection family was compared with reference strains from GenBank. Accession numbers are indicated on the tree. Bootstrap values are shown along each main branch. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site. The regions included in the analysis were the same with fragment A, B and C or a little shorter. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011).

Recombination signals were initially detected by RDP3.β.4 software [13,14]. Bootscan, Geneconv and Siscan were used. The highest acceptable P-value was 0.05. Bootscan and Siscan window sizes were 300 bp, step size was 30, replicates for 100 times. A genotype F sequence (GenBank accession numbers is X75658 and X75663) was used as external reference. The precise map of recombination was determined by split phylogenetic tree and alignment. Split phylogenetic trees were constructed by the method same as above. In alignment, each clone was compared to reference C2, D1 and CD1 consensus sequences. We then inspected the alignments to determine the identical crossover sequences around the breakpoint within which the recombination occurred.

Accession Number of the Sequences

GenBank accession number of reference sequences of HBV genotype C2, D1, CD1 and CD2 are indicated in phylogenetic tree. Accession Numbers of Y2 clones are JX036326-JX036359.

Results

Mixed-genotype Infections in HBV Cluster Families

Different HBV genotypes were found in three families among 15 families. The flow of participants in the study and family trees of families with mixed genotypes/subgenotypes of HBV infection are shown (Figure 1).

Family V had infected members across two generations and two genotypes: In 2006, the mother (V1W) and daughter (V2F) were infected with subgenotype D1 while the son (V2M) had a CD1 recombinant. In 2007, the daughter (V2F) had subgenotype D1 while other family members had HBV DNA levels below the detection limit of the nested PCR assay.

Family Q had infected members across three generations and two genotypes/subgenotypes. In 2006, the grandmother (Q1W) and grandson (Q3M) were infected with CD1 recombinant while father (Q2) and granddaughter (Q3F) had mixed infections of genotype C2 and CD1 recombinants. In 2007, the same genotypes were detected in all family members except that the granddaughter (Q3F) had an HBV DNA level below the detection limit of the nested PCR assay.

Family Y had affected members across three generations and three genotypes/subgenotypes. In 2006, the grandfather of family Y (Y1) was infected with genotype C2 while grandmother (Y1W) had mixed infections of CD1 and C2. Mother (Y2W) and granddaughter (Y3F) were infected with the CD1 recombinant. Father (Y2) had triplicate infections of genotype C2, D1 and CD recombinant. Grandson's (Y3M) serum was unavailable. In 2007, the grandfather (Y1) and mother (Y2W) had HBV DNA levels below the detection limit while the grandmother (Y1W) and granddaughter (Y3F) had genotype CD1. Father (Y2) and grandson (Y3M) had genotype D1.

Phylogenetic Analysis of Family Y, Family Q and Family V

A phylogenetic tree constructed from HBV nt 36-1110 from the clones of family Y is given (Figure 2A). The clones (dotted) of family Y exhibits three clusters on genotype C2, D1 and CD1.

The phylogenetic tree construct from HBV nt136-1110 from the clones of families Q and V is given (Figure 2B). The clones of

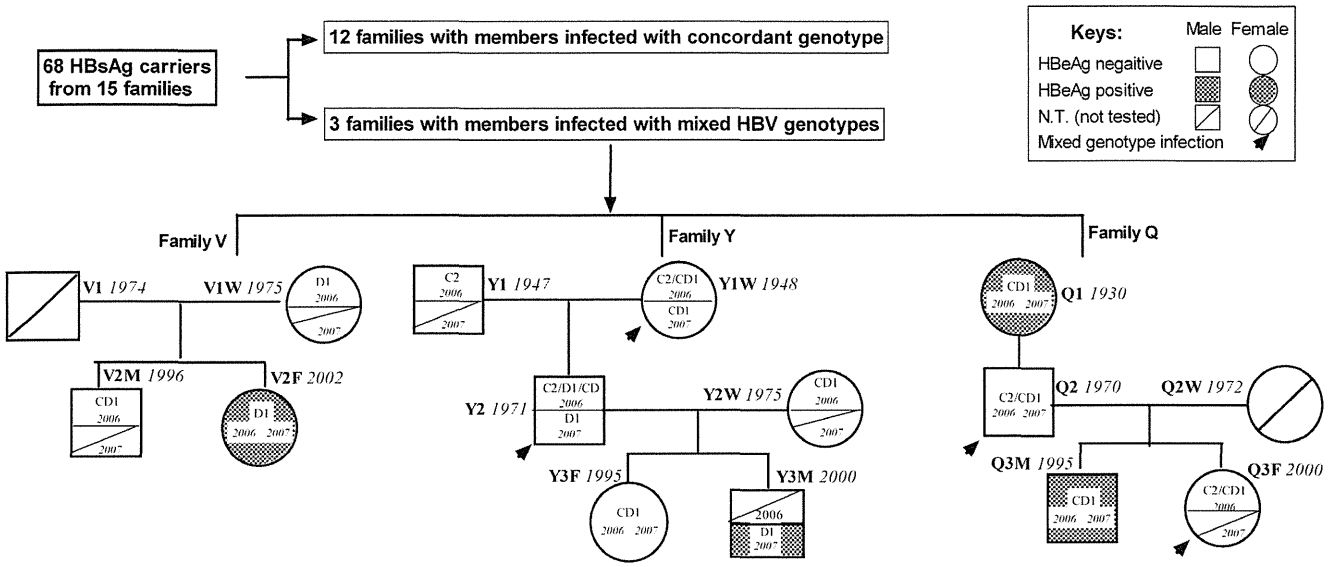

Figure 1. Flow of participants in the study and family trees of family with mixed genotypes/subgenotypes HBV infection. Circles and rectangles correspond to female and male individuals, respectively. Family name and birth date of the patients are indicated beside the circles and rectangles. Subgenotype and the year of blood sampling are indicated inside the circles and rectangles. Family V with affected members across two generations and two genotypes/subgenotypes. Family Q with affected members across three generations and two genotypes/subgenotypes. Family Y with affected members across three generations and three genotypes/subgenotypes. Specially, father (Y2) of family Y with triplicate infection of genotype C, D and CD recombinant in 2006. N.T: Not tested for HBV DNA level below the detection limit of the nested PCR assay or no serum was available.
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family Q (indicated by black dots) exhibit two clusters of subgenotypes C2, and CD1. The clones (indicated by black triangles) from family V exhibit two clusters of subgenotypes D1 and CD1.

A phylogenetic tree constructed from HBV nt 36-1110 of novel recombinants clones of Y2 is given in Figure 2C. The dotted clones are from Y2. The topology of phylogenetic tree with recombinants is totally different from typical trees. Recombinant sequences blurred the typical branch,in other words, blurred the typical genotype.

Recombination and Crossover Analysis of Quasi-species of Y2

Results of recombination analysis of Y2 clones are as bellow: Three kinds of analytical methods certificated the same recombination map. The initial pictures of the three methods were all provided as supplemental figures. Recombination events detected by RDP software are shown in Figure S1, S2, and S3. Split phylogenetic trees constructed by MEGA software are shown in Figure S4, S5, and S6, (clone number and fragment used to construct tree are indicated beside each tree). Sequence alignments are shown in Figure S7, S8, and S9.

The region where recombination breakpoints had the highest probabilities was recognized as crossover region, which is a region that one parental genotype switches to another. Upstream sequence of crossover region will have specific mutation of one genotype but with no specific mutation of another, downstream just opposite. At the same time, these two genotypes should share same sequence at crossover region. We indicated the crossover region in direct alignment by black bars in Figure S3 initially and marked it in recombination map by colorful bars in Figure 3A and black bars in Figure 3B. The clonal sequences of 2006 showed 17 unique crossover regions in fragments A, B and C. We could not identify any common motif within these sequences that might suggest a common mechanism for crossovers in the HBV. The size of switch region share the same sequence are different in different strains, from 6–174 bp (6 bp for Y2M-2 clone in Figure S7 and 174 bp for Y2M-29 clone in Figure S8).

To illustrate the recombination map in a simple way. An abbreviated alignment of fragment A, B and C are shown in Figure 3B. Green and pink bars indicated the genotype C2 and D1 respectively. Black bars showed the crossover region. The aligned sequences provide a snapshot of the recombinant HBV strains. Genotype C2, D1 and CD1 recombinant clones of Y2 were all used as parental sequences to recombine with each other to form new recombinants. A series of novel recombinants were found in three fragments.

In 15 clones of fragment A, there were five genotype C (Y2-6,9,13,14,15,); two genotype D (Y2-11,12); one CD1 (Y2-10) and seven novel different C/D recombination (Y2-1,2,4,7,8,3,5).

In 16 clones of fragment B, there were four genotype C (Y2-23,71,78,75); seven genotype D (Y2-25, 27,79,76,72,22,210); one CD1 (Y2-29) and four novel C/D recombinants (Y2-212,21 73,77).

Of the 56 clones of fragment C(in which genotype B HBVDNA were added as an in-tube control to exclude the recombination by PCR procedure), there were 32 pure genotype B clones; nine genotype C clones(Y2-B10,B5,B8,B9,B13,B16.B17.B18,B24); five genotype D clones(Y2-B22,B3,B4,B21,B23), two CD1 clones (Y2-B1,B11) and eight novel C/D recombinants (Y2-B6,B7,B14,B15,B19,B2,B12,B20). No recombinants of genotype B were found.

Discussion

Recombination is one of the major mechanisms contributing to the evolution of retroviruses [15]. Since the HBV has a reverse transcription step in its life cycle, it is conceivable that recombination also contributes to diversity in HBV genomes. Although just four cases were observed with mixed genotype

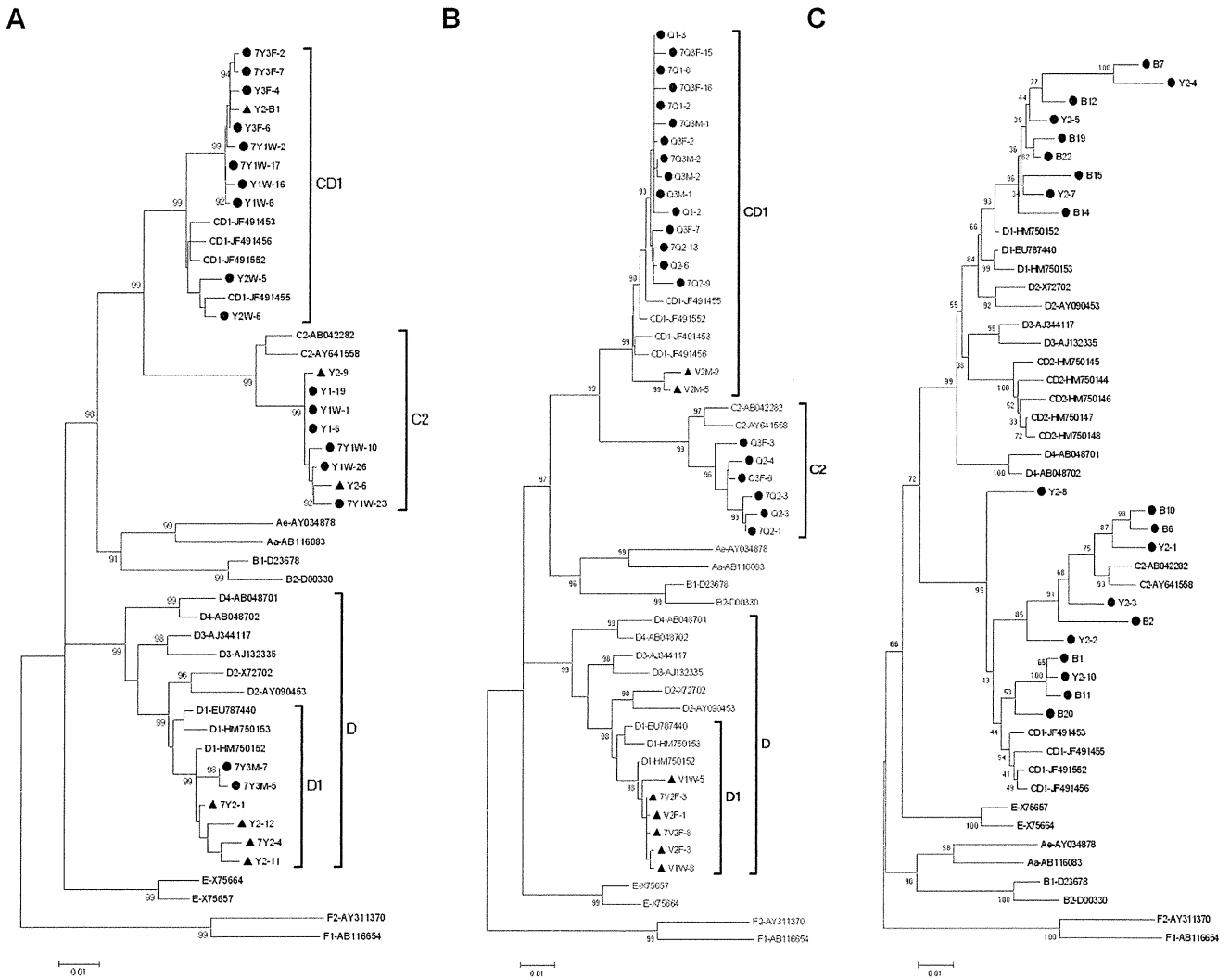

Figure 2. Phylogenetic tree construct by HBV nt 136-1110. (**A**) clones of family Y. Solid dots indicate the clones from Y1,Y1W,Y2W,Y3F and Y3M; Solid triangles indicate the clones from Y2. Family names starting with number 7 means the samples collected in 2007 otherwise in 2006. Novel recombinants of Y2 were excluded from the phylogenetic tree. (**B**) clones of family Q and family V. Solid dots indicate the clones from family Q; Solid triangles indicate the clones from family V. A family name starting with number 7 means the samples collected in 2007, otherwise, in 2006. (**C**) Novel recombinant clones of Y2. Solid dots indicate the clones from Y2.
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infections, we obtained a snapshot of naturally occurring HBV recombinants generated in the absence of selection and after selection. Our result showed direct evidence of HBV recombination, with new information of recombining crossovers compared with similar studies [16,17,18,19].

The recombination analysis of Y2 quasi-species showed variable types of recombinant between genotype C2, D1 and CD1 in 2006. Some studies show that hotspots of recombination most on the boundary of ORFs [12,20]. Our results showed that two or more strains of HBV can recombine with each other at any region along the genome. Crossover regions can be hundreds or just several base pairs, The length of crossover region is depends on the location of it on HBV genome. If it is located in a conserved HBV region, for another word, where many different genotypes share the same sequence, the length of crossover region may be long. If it is located in a non-conserved region, it may be very short. At the same time, we found that the crossover region distributed totally at random on HBV genome. Consistent with our results, *in vitro* evidence showed the initial recombination events in a laboratory system of MHV were almost entirely randomly distributed along the sequence [21]. It was only after passage through cell culture, with the opportunity for selection to remove less fit variants, that crossover sites became "localized" to just a small area of the region examined. Crucially, they also suggested initial products of recombination may go undetected because of the action of strong purifying selection which will remove new, deleterious combinations of mutations. The conclusion is therefore an interpretation for the genotype change of Y2. The Y2 presented multiple strain infections of C2/D1/CD1 and many new recombinants with no obvious dominant genotype strain in 2006. After 18 months, however, all the type C2 and CD recombinant strains disappeared while the D strain became dominant. A similar case of mixed HBV genotype infection in which one genotype was lost and another prevailed was previously described in patients with HBeAg seroconversion [4,22].

Epidemiologically, HBV genotype CD1 and C2 are the most common strains in ethnic minorities of northwest China with CD2 and D1 as minor strains. Precise mapping of recombination

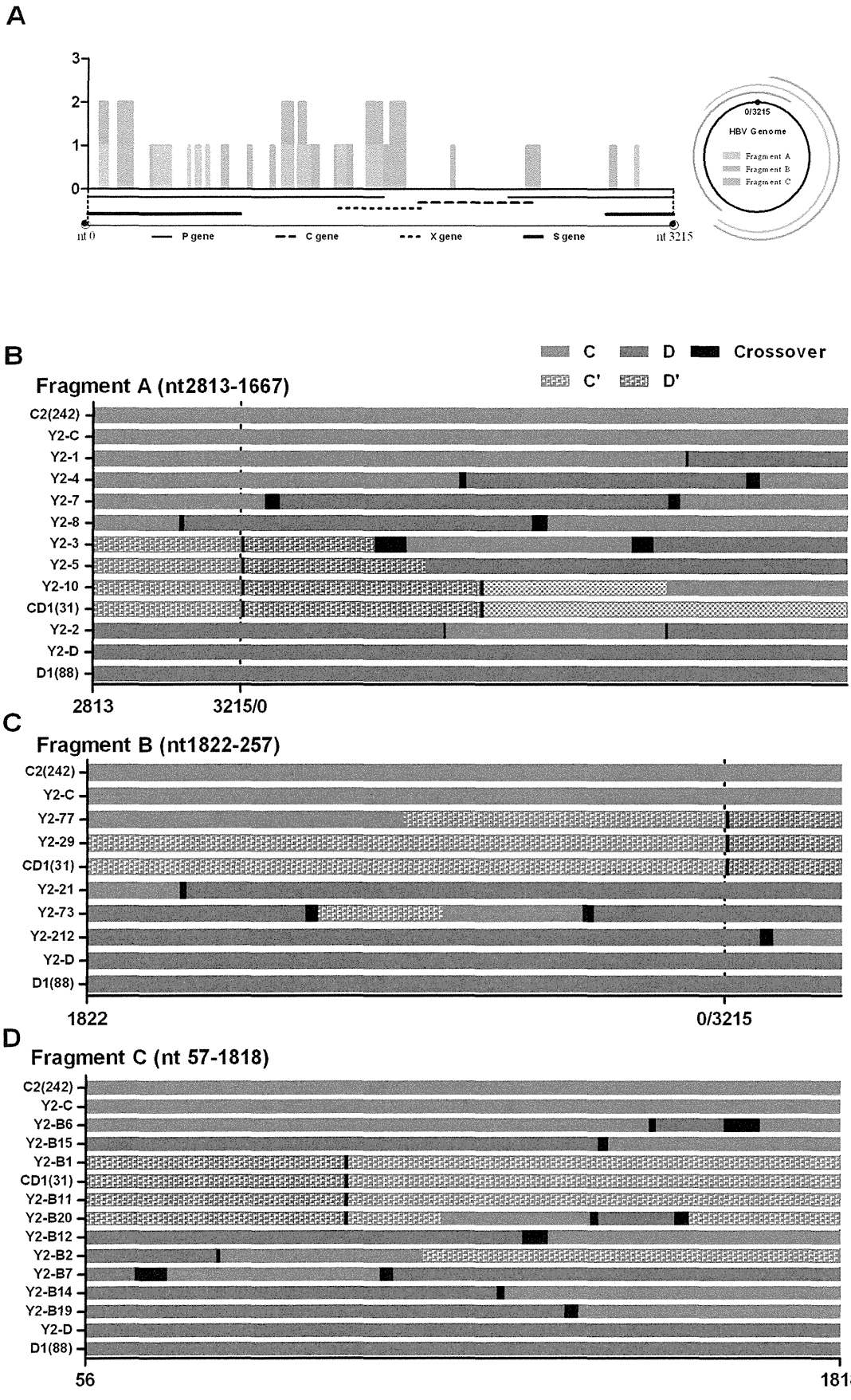
A
3
2
1
0
nt 0
P gene
C gene
X gene
S gene
nt 3215
0/3215
HBV Genome
Fragment A
Fragment B
Fragment C
B
C
D
Crossover
C'
D'
Fragment A (nt2813-1667)
C2(242)
Y2-C
Y2-1
Y2-4
Y2-7
Y2-8
Y2-3
Y2-5
Y2-10
CD1(31)
Y2-2
Y2-D
D1(88)
2813
3215/0
Fragment B (nt1822-257)
C2(242)
Y2-C
Y2-77
Y2-29
CD1(31)
Y2-21
Y2-73
Y2-212
Y2-D
D1(88)
1822
0/3215
Fragment C (nt 57-1818)
C2(242)
Y2-C
Y2-B6
Y2-B15
Y2-B1
CD1(31)
Y2-B11
Y2-B20
Y2-B12
Y2-B2
Y2-B7
Y2-B14
Y2-B19
Y2-D
D1(88)
56
1818

Figure 3. Alignment and recombination crossover regions found in Y2 clones. (**A**) Frequency and distribution of the recombination crossover regions found in Y2 clones along the HBV genome. The bars indicate the number of clones (y axis) showing recombination crossover regions at each site. The 1-3215 of x axis was consistent with the nt1-3215 of HBV genome. Different colors represent the sites find from clones of different PCR region: pink bars for fragment A, grey bars for fragment B and green bars for fragment C. (**B**) Alignment of fragment A (HBV nt 2813-0-1667). Y2-1'12: clones from fragment A of Y2 patients. (**C**) Alignment of fragment B (HBV nt 1822-0-257). Y2-21'212: clones from fragment B of Y2 patients. (**D**) Alignment of fragment C (HBV nt 57-1818) of Y2 clones. Y2-B1'B22: clones from fragment C of Y2 patients. The number on the x axis was consistent with the site of nucleotides of HBV genome. Solid green lines are genotype C2, solid pink lines are genotype D1, speckled green lines are the C2 component of genotype recombinant CD1 and speckled pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242) is the consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88) is the consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33) is the consensus sequence formed by CD1 recombinant sequences from GenBank.
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suggests C2 and D1 are parental sequences of CD1 and CD2 recombinants. Virological differences among HBV genotypes were demonstrated in vitroand in CHiM mice, with genotype C having a higher replication capacity than D [23]. Why does the replication-deficient genotype D virus predominate over replication-competent genotype C? As mixed HBV infections together with recombination are rare, we have little knowledge about i this situation. On the one hand, we know little about host impact on different genotypes and recombinants. On the other hand, we know little about interference and competition in the quasi-species of mixed infection. In vitro results showed the replication capacity of individual clone, exclude the influence of host and other strains of quasi-species. An example from a ChiM mice study showed that monoinfection of HBV/G in ChiM mice display a very slow replication while coinfection with HBV/A remarkably enhanced the replication of HBV/G. The replication of HBV/G is heavily dependent on coinfection with other genotypes. When HBV/G superinfected on other genotypes, a rapidly takes over of HBV/G from original genotype were observed, though they are indispensable [24]. This study confirms that in a mixed infection system of different genotypes, the replication capacity of a genotype may be different from that of monoinfection. At the same time, replication capacity is not the only factor to influence which strain will become dominant. Variable recombinants found in our study may be mechanistically capable of genetic exchange, but strong selection guaranteed the elimination of hybrid genomes. The mechanism of selection in mixed infection also needs more investigation.

We found mixed HBV genotypes infection with many novel recombinants at one point in time, but just one genotype was found 18 months later. This may indicate that the detectable mixed infection and recombination has a limited time window due to the sensitivity of detection or strong selection power of the host. That's why in most studies, we can identify a major genotype in one patient. Even so, evolutionarily visible and invisible recombination of HBV could occur and play an important role by generating genetic variation or reducing mutational load. However, this study had limitation, because recombination signals were detected by RDP3 software and confirmed by split phylogenetic tree and alignment, indicating the recombinant or recombinant-like form should depend on the software. If we use another software, the results might be different.

Studies of HBV in endemic areas throughout the world have resulted in large numbers of full genome sequences available for phylogenetic analysis enabling the identification of novel, mosaic HBV genomes that appear to be the result of recombination between previously known sequences [7,25,26]. One of the most comprehensive analyses of putative HBV inter-genotype recombinants showed the existence of 24 phylogenetically independent HBV genomes involving all known human genotypes [27]. Some of these recombinants are unique to individual subjects, but some undergo expansion in specific populations and become recognized as new genotypes or subgenotypes [12,28,29,30]. Four stages in the process of generating popular HBV recombinant genomes should be recognized. The first stage is the co-circulation of different HBV strains or genotypes in the same geographic area. The second is the existence of individuals who have been infected with more than one strain of HBV. The third is the generation of a novel recombinant strain(s) within an individual. The fourth is the selection of a recombined strain with the ability to replicate and be transmitted. Our data show the natural process of the formation and selection of recombination though the recombinant strains of Y2 that appeared in 2006 that were all removed from samples in 2007.

By using phylogenetic trees and homology calculations, HBV variants infecting humans are currently classified into ten genotypes that differ from each other in nucleotide sequence by 7.5 to 13% [2,3]. There are some characteristic length differences between the genotypes that facilitates their detection and discrimination. However, as shown in Figure 2, existence of a recombinant makes the topology of the phylogenetic tree totally different from one with no recombinant. Recombinant strains obscured the definition of genotypes. Based on the algorithm creating a phylogenetic tree, sequences with high homologues cluster together. With the same logic, recombinants always clustered with the backbone parental sequence, in other words, with which they have high similarity with the larger proportion of the recombination region. Therefore, recombinants always seem to be a subgenotype of their backbone parental sequence. Similar to Y2-8 clone in Figure 2C, for recombinants with similar proportion of both parental genotypes, the sequence shows a divergent trend different from both parental genotypes.

Based on phylogenetic topology changes of different regions of HBV, it was hypothesized that some of the genotypes that are conventionally regarded as "pure," actually were recombinant. Genotype E strains show evidence of recombination with genotype D at 1950–2500. new reported genotype "I" actually belongs to genotype C. Furthermore, Subgenotype Ba possesses the recombination with genotype C at 1740 to 2485 [31,32,33]. Recombinants comprising regions with different histories have important implications for the way we think about HBV evolution. It means that there is no single phylogenetic tree that can describe the evolutionary relationships between genotypes.

In conclusion, mixed HBV genotypes infection with many novel recombinants at one point in time ended up with just one genotype 18 months later in this study. This may indicate that the detectable mixed infection and recombination have a limited time window due to the sensitivity of detection or strong selection power of the host. Also, as the recombinant or recombinant-like nature of HBV precludes the possibility of a "true" phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

Supporting Information

Figure S1 Recombination map of fragment A created by RDP software.
(TIF)

Figure S2 Recombination map of fragment B created by RDP software.
(TIF)

Figure S3 Recombination map of fragment C created by RDP software.
(TIF)

Figure S4 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S5 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S6 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S7 Alignment of fragment A(HBV nt 2813-0-1667)of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1 and light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-1′12: clones from fragment A of Y2 patients.
(DOC)

Figure S8 Alignment of fragment B(HBV nt 1822-0-257) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-21′212: clones from fragment B of Y2 patients.
(DOC)

Figure S9 Alignment of fragment C(HBV nt 57-1818) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. B1B22: clones from fragment C of Y2 patients.
(DOC)

Author Contributions

Conceived and designed the experiments: ZW MM JH. Performed the experiments: BZ ZW. Analyzed the data: BZ JY JS. Contributed reagents/materials/analysis tools: HL YT. Wrote the paper: BZ YT.

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