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High circulation of hepatitis B virus (HBV) precore mutants in Tunisia, North Africa

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SUMMARY

Hepatitis B Virus (HBV) e antigen (HBeAg), HBV DNA and precore mutations affecting HBeAg expression during active replication were studied in 72 Tunisian hepatitis B surface antigen (HBsAg) positive individuals: 30 asymptomatic carriers of the virus, 37 with chronic hepatitis and 5 with acute hepatitis. HBV DNA was detected in 44 patients, but only 20% of them expressed HBeAg. Precore mutant strains, with mutations at position 1896 or at positions 1896 and 1899, were detected by PCR-hybridization in 86 and 36% of patients, respectively. Wild-type strains were detected in 54% of patients. Precore mutants were found in chronically and in acutely infected individuals, in patients with severe and asymptomatic infections, in HBeAg positive as well as HBeAg negative individuals. These results show the high frequency of HBV precore mutants in Tunisia.

INTRODUCTION

During hepatitis B virus (HBV) infection, the presence of circulating e antigen of the virus (HBeAg) generally correlates with active virus replication and often with liver disease, whereas clearance of HBeAg and the subsequent rise of the homologous antibody, anti-HBe, indicate termination of HBV replication and remission of liver disease [1, 2]. Since the early 1980s, several studies have reported HBsAg positive patients who, despite seroconversion from HBeAg to anti-HBe, still express symptoms of ongoing viral replication and/or liver inflammation [3–5]. Subsequent studies demonstrated that this profile, particularly frequent in the Far East and Mediterranean countries, was due to the emergence of viruses with mutations in the precore region of the HBV genome. These mutations block the synthesis of the precore leader peptide of the e protein and specifically prevent the expression of HBeAg during active viral replication [6–9]. Sequence analysis of these HBeAg defective viruses has led to the identification of several mutations in the precore region, of which a G–A transversion at nucleotide 1896 is the most frequent, accounting for 90–95% of all cases [7, 10–13].

Since precore mutants have been reported mainly in chronic HBV carriers with chronic liver disease [6, 7, 12, 14, 15], it was suggested that these variants are most likely escape mutants, selected during persistent infection. The lack of HBeAg expression on the cell membrane of the infected hepatocyte might help the virus to survive the anti-HBe immune response of the host [16]. Their occurrence in acute fulminant hepatitis [17–20] and their identification in both patients and their respective infective sources [17, 21] indicate that they can occasionally be transmitted to susceptible individuals.

Presently, many aspects of the infection with such mutants remain unclear. These include: host and viral

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factors that predispose to their emergence and the mechanisms by which these mutants induce severe hepatic injury. Moreover, little is known about their frequency in clinical forms of HBV infection other than in severe acute and chronic hepatitis and in regions of the world other than the Far East and western European countries.

Recently, rapid methods for detecting the most common precore mutations have been developed which do not require the time-consuming sequencing techniques [22–24]. One of these rapid molecular methods was used in the present study to detect precore mutations in Tunisian HBsAg positive people and to assess their frequency in a region of intermediate endemicity for HBV.

METHODS

Patients

Seventy-two HBsAg positive Tunisian patients were included in this study: 37 with symptomatic chronic liver disease, 30 asymptomatic HBV carriers and 5 patients with mild acute infection (serum alanine aminotransferase (ALT) 850–3770 iu/l). Liver biopsies performed in 21 patients of the first group suggested the occurrence of chronic active hepatitis (CAH) in 17 cases and chronic persistent hepatitis (CPH) in 4 patients. Among the 16 remaining patients with chronic hepatitis, 6 had liver cirrhosis suggested by clinical and echographical evidence: hepatic enlargement, clinical signs of portal hypertension, increased diameter of the portal vein and/or irregular margins of the liver parenchyma at ultrasonography.

Virological assays

HBsAg and HCV antibody were detected by commercial ELISA kits (Hepanostika HBsAg UniformII; Organon Teknika and Monolisa antiHCV; Sanofi Diagnostics Pasteur). All individuals included in the study were HBsAg positive and HCV antibody negative. HBeAg and anti-HBe were detected by commercial ELISA kits (Hepanostika HBeAg and Hepanostika antiHBe; Organon Teknika). HBV DNA was detected by a PCR method using primers corresponding to highly conserved sequences in the core gene (Table 1). HBV DNA was extracted from 100 μl of serum with proteinase K (10 mg/ml) in the presence of 0·6% sodium dodecyl sulphate (SDS), 6 mM ethylenedinitrilotetra-acetic acid (EDTA), 12 mM Tris hydrochloride (Tris/HCl) pH 8 and polyadenylic acid (2 mg/ml). After incubation for 2·5 h at 58 °C, the solution was extracted with phenol-chloroform and DNA was precipitated with ethanol and then dissolved in 100 μl of distilled water. The PCR reaction was conducted in a 50 μl mixture containing 10 μl of the serum DNA sample, 2·5 units of Taq polymerase (Amersham), 10 mM of each deoxynucleotides (dNTP) and 15 pM of each primer. The reaction was cycled 35 times with 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C and 2·5 min of extension at 72 °C. After electrophoresis, Southern-blot hybridization was performed to confirm the presence of the homologous sequence in the amplified product using a specific probe (Table 1). The sensitivity of this detection PCR method was estimated, by using serial dilutions of a titrated positive control, to 3 HBV DNA copies per PCR tube (100 μl of serum), which corresponds to 30 HBV DNA copies per ml of serum.

Detection of precore mutants

The detection of precore mutations at nucleotide 1896 and 1899 was carried out using a rapid method described by Li and colleagues [22]. Briefly, HBV DNA was isolated and the precore region was amplified by PCR in 50 μl of a solution containing 10 μl of the extracted DNA, 10 μM of each dNTP, 10 mM Tris pH 9, 1% Triton X-100 (Sigma), 10 pM each of primers (pX32 and pC30, Table 1). The reaction was cycled 30 times with 2 min of denaturation at 92 °C, 2 min of annealing at 66 °C and 3 min of extention at 70 °C. Amplification products were analysed by agarose gel electrophoresis and ethidium bromide staining. In the absence of amplification, a nested PCR was carried out in the same conditions, using an internal pair of primers (P13 and C126, Table 1). Products from the first or the second round of PCR were hybridized with three radiolabelled probes: probe M0 corresponding to the non-mutated sequence, probe M1 mutated at position 1896 and probe M2 mutated at both positions 1896 and 1899 (Table 1). In each assay, four positive controls were included carrying single or mixed viral strains.

RESULTS

HBeAg, anti-HBe and HBV DNA were studied in samples from all patients. The results are summarized in Table 2. HBV DNA was detected in all five patients with acute hepatitis, 33% of healthy carriers and 78%

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Table 1. *Primers and probes*

PCR amplification of the core region				
Primers	(1838–1862)	5′-TTC AAG CCT CCA AGC TGT GCC TTG G-3′		
	(2397–2377)	5′-TCT GCG ACG CGG CGA TTG AGA-3′		
Probe	(1986–2015)	5′-GTA TCG GGA GGC CTT AGA GTC TCC GGA ACA-3′		
PCR amplification of the precore region				
Primers	First PCR	pX32	(1783–1806)	CTG TAG GCA TAA ATT GGT CTG CGC
		pC30	(2171–2149)	GTT AAC ATA ACT GAC TAC TAG GTC
	Nested	P13	(1828–1847)	CAC CTC TGC CTA ATC ATC TC
		C 126	(2028–2008)	TAA GGC TTC TCG ATA CAG AGC
Probes for wild-type and mutant strain detection				
M0	(1887–1908)	TGG GTG GCT TTG GGG CAT GGA C		
M1*	(1888–1908)	GGG TGG CTT TA G GGC ATG GA C		
M2*	(1887–1908)	TGG GTG GCT TTA GGA CAT GGA C		

* The letters in bold in the sequences of M1 and M2 probes indicate point mutations at nt 1896 and 1899.

Table 2. *Expression of HBV serological markers in HBsAg positive patients according to their clinical status*

Clinical status (*n*)	HBeAg positive *n* (%)	Anti-HBe positive *n* (%)	HBV DNA positive *n* (%)
Acute infection (5)	2 (40)	3 (60)	5 (100)
Healthy carriers (30)	1 (3)	29 (97)	10 (33)
Chronic liver disease (37)	6 (16)	30 (81)	29 (78)
Total (72)	9	62	44

Table 3. *Detection of precore mutants in HBV DNA positive patients: correlation with the expression of HBeAg*

			Hybridization patterns						
	No PCR amplification	No hybridization	M0	M0 + M1	M0 + M2	M0 + M1 + M2	M1	M1 + M2	M2
HBeAg positive (9)	0	0	2	6	0	1	0	0	0
HBeAg negative (35)	2	1	1	12	0	2	4	13	0
Total (44)	2	1	3	18	0	3	4	13	0

of patients with chronic liver disease, while only 40, 3 and 16%, respectively, expressed HBeAg.

All 44 HBV DNA positive patients were assessed for HBV precore mutations: 9 were HBeAg positive, 34 anti-HBe positive and 1 patient was negative for both markers. PCR amplification of the precore region was obtained for 42 patients: 21 after the first round of PCR and 21 after the second round; 2 sera failed to amplify. Amplification products were hybridized with probes M0, M1 and M2 (Table 1). Twenty-four samples (54%) hybridized with M0, 38 with M1 (86%) and 16 with M2 (36%) (Table 3). One sample failed to hybridize with any of the three probes. Figure 1 shows the hybridization patterns with M0, M1 and M2, obtained for 22 serum samples.

The correlation between the detection of precore mutated viruses and the expression of HBeAg or anti-HBe is shown in Table 3: all nine HBeAg positive sera carried non-mutated viruses, alone in 22% of cases (2/9) and together with mutated viruses in 78% of cases (7/9). In contrast, the majority of HBeAg negative sera carried mutated viruses: 48% (17/35)

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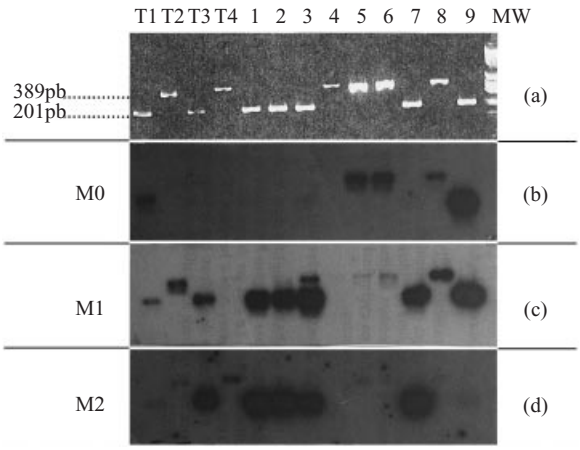

Fig. 1. PCR amplification and hybridization patterns with the wild-type probe (M0) and the two mutated probes (M1 and M2). Part (*a*) shows the PCR products on agarose gel obtained after the first amplification (389pb) or after the nested PCR (201pb). Parts (*b*), (*c*) and (*d*) show the hybridization results with M0, M1 and M2 respectively. T1, T2, T3 and T4 are serum samples containing sequenced viruses, T1 should hybridize with M0, M1 and M2, T2, T3 and T4 should hybridize with M1 and M2. Samples 1, 2, 3 and 7 hybridized with M1 and M2. Sample 4 did not hybridize with any of the three probes. Samples 5 and 9 hybridized with the three probes. Samples 6 and 8 reacted with M0 and M1. Sample 7 reacted with M1 and M2.

carried mutated strains exclusively and 43% (15/35) a mixture of mutated and unmutated viruses. Only one serum (3%) carried the unmutated virus exclusively.

The correlation between the presence of precore mutations and the clinical status of patients is shown in Table 4. In patients with acute HBV infection, 40% (2/5) had a co-infection with wild type and a 1896 mutated strain. In asymptomatic carriers of the virus, precore mutants were found in all HBV DNA positive patients which corresponds to 33% (10/30) of total healthy carriers studied. In patients with chronic liver disease, mutant viruses were detected in 24 patients which corresponds to 65% (24/37) of the chronic hepatitis study group.

DISCUSSION

Eighty-four per cent of chronic HBsAg carriers were HBeAg negative. A large proportion of these had chronic active or persistent hepatitis, the outcome of active viral replication. HBV precore mutants were detected in 65% Tunisian HBsAg positive patients with chronic HBV infection. Our results confirm previous reports suggesting that these mutants are particularly frequent in Mediterranean countries, a conclusion which was based mainly on studies conducted in European Mediterranean countries, particularly Italy [25] and France [26]. The southern rim of the Mediterranean Sea is a region with intermediate endemicity for HBV with 5–10% of the population being chronic HBV carriers [27, 28].

Conflicting data have been reported concerning the pathogenicity of HBV precore mutants: several studies reported their likely association with severe clinical presentations, i.e. fulminant hepatitis and chronic active hepatitis [6, 7, 12, 14, 15, 17, 18]. Others failed to link them with severe HBV disease, as they were equally present in a wide range of liver diseases: asymptomatic chronic carriage, typical mild acute infection [10, 29–31] and even in HBsAg negative individuals with serologically undetectectable HBV infection [32]. It has been suggested that this discrepancy could be ascribed to geographical differences, as the former reports came from Mediterranean countries while the latter came from the Far East and the Middle East [31]. In our study, we found precore mutations in patients with symptomatic chronic infection, asymptomatic carriers as well as patients with mild acute hepatitis. These results underline the differences between countries in the Mediterranean region.

The pathogenicity of precore mutants is still incompletely understood. Although it has been suggested that these variants are generally generated during persistent infection as escape mutants, their identification in patients with acute fulminant hepatitis suggests that they are fully infectious [17, 18, 21]. However, the impact of these variants in vertical transmission and in horizontal transmission within family setting is still unclear. It has been demonstrated that HBeAg positive mothers are more likely to transmit HBV to their neonates than those with anti-HBe. Thus, precore mutated strains could have a lower infectivity, at least in the neonatal period, than wild strains. The high prevalence of mutated strains found in this study and the low rate of vertical transmission previously reported in Tunisia [28] could favour this hypothesis. However, more detailed analysis is necessary to determine the role of precore mutants either in vertical and in other patterns of HBV transmission.

In this study, we used a rapid method for the identification of two types of precore mutants: one with a G–A substitution at nt 1896 and another with this mutation and a G–A substitution at nt 1899. This method, based on a PCR amplification of the precore region followed by hybridization with specific mutated

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Table 4. *Detection of precore mutants in HBV DNA positive patients: correlation with clinical status*

			Hybridization patterns						
	No PCR amplification	No hybridization	M0	M0 + M1	M0 + M2	M0 + M1 + M2	M1	M1 + M2	M2
Acute infection (5)	0	0	3	2	0	0	0	0	0
Healthy carriers (10)	0	0	0	1	0	1	2	6	0
Chronic liver disease (29)	2	1	2	8	2	3	2	9	0
Total 44	2	1	5	11	2	4	4	15	0

or unmutated probes, has some disadvantages. First, unlike DNA sequencing, the most widely used technique for such studies, it does not enable identification of all precore mutations (although these two mutations are the most frequent) [6–8, 10, 17, 21, 29]. Second, some strains cannot be characterized because they fail to amplify with the primers for precore mutant detection or fail to hybridize with any of the three probes. In our study group, the lack of amplification occurred in 4·5% [2/44] of patients with detectable HBV DNA. This could be due to the fact that, contrary to the first detection PCR method, the primers used for the amplification of the precore region are not derived from sequences in the most highly conserved region of the genome. Hybridization failure occurred in 2% of HBV DNA positive samples in our study. This could be due to point mutations, other than those at nt 1896 and 1899 [22]. Despite these shortcomings, this method enabled the detection of mixed viral populations and, unlike sequencing, is simple to perform and can be used for routine diagnosis. As precore mutants may be selected during interferon therapy [33], their identification may be helpful before starting the patient's treatment so as to enable an appropriate treatment protocol to be implemented.

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