Prevalence of nucleotide 1858 variants of Hepatitis B Virus in HBsAg-positive Malaysians

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Abstract

Hepatitis B DNA (HBV-DNA) was determined in 265 HBsAg positive individuals using polymerase chain reaction (PCR). Out of this, 35.8% (95/265) were positive for the viral DNA. Precore variants were analyzed and T-1858 variant (CCT as codon 15) predominate significantly over C-1858 variant (82.1% versus 14.8%; $p < 0.0001$). No individual was infected exclusively by the TAG mutant (TGG →TAG at codon 28) but three individuals were co-infected by a mixture of wild type and the TAG mutant. Relation between T-1858/C-1858 variants and HBeAg/anti-HBe status showed that 55.1% of T-1858 infected individuals were anti-HBe positive, 39.1% were HBeAg positive and 5.1% were negative for both HBeAg and anti-HBe. This is not significant ($p > 0.05$). Individuals infected by the C-1858 variant had more HBeAg positivity (64.3% versus 28.6%; $p < 0.005$). When the prevalence of T-1858 variant was compared in the asymptomatic individuals, individuals with chronic hepatitis B and individuals with hepatocellular carcinoma, it was observed that many more of the asymptomatic and HCC individuals were infected by this variant than the individuals with chronic hepatitis ($p < 0.001$). In conclusion it was found that Malaysians were infected mainly by the T-1858 variant compared with those in the Western European countries where C-1858 predominates. Low TAG mutation was seen in our population.

Key words: T-1858, C-1858 variant, TAG mutant

Introduction

Hepatitis Be (HBe) seroconversion is frequently accompanied by the emergence of a G→A mutation at nt 1896 in the precore region. This introduces a premature stop codon, TAG, at codon 28 (Carmen *et al.*, 1989). This will prevent the synthesis of HBe antigen (HBeAg). HBeAg expression, although not required for viral replication or infectivity (Brown *et al.*, 1992) may represent a target at the surface of infected cells for the anti-HBe immune response (Schlicht *et al.*,1989; Schlicht *et al.*, 1991).

The emergence of this mutation depends on the nucleotide at position 1858 (Li *et al.*,1993; Lok *et al.*, 1994; Lindh *et al.*, 1995a,b) which forms a base pair with nt 1896 in the pregenomic RNA loop (Pollack *et al.*, 1993; Tong *et al.*,1993). Nt 1858 may either be a thymine (T-1858) as in CCT or a cytosine (C-1858) as in CCC for proline in precorecodon 15 (nt 1856- 1858). U-1858 (i.e. T-1858 in DNA) can base pair with G-1896 and A-1896. This allows a G→A mutation at nt 1896. In C-1858 variant this mutation apparently does not develop because it destabilizes the loop and thus impairs its function (Li *et al.*, 1993). Apparently this restriction of the development of precore is likely to be of pathogenic significance, since it was reported that carriers infected with C-1858 variant showed more severe liver damage than those infected with T-1858 strains (Lindh *et al.*, 1996). The low prevalence of the TAG mutation in carriers of Northern European origin has been attributed to the presence of cytosine at nt 1858 in hepatitis B virus (HBV).

In view of the fact that HBV-DNA prevalence in hepatitis B antigen (HBsAg) carriers in Malaysia is 22.5 % (Ton *et al.*, 1995) this study was undertaken to analyze the prevalence of nt 1858 variants i.e. T-1858 or C-1858 in Malaysian HBsAg carriers and to correlate them with the TAG mutation at codon 28 (TGG→TAG) and HBeAg/anti HBe status (antigen and antibody to the e antigen).

Materials & Methods

Patients

HBV-DNA from sera of 95 HBsAg positive individuals

were analyzed for nt 1858 CCT/CCC, (nt 1856-1858) and nt 1896 TGG/TAG, (nt 1895-1898). The patients were classified as asymptomatic, chronic hepatitis individuals or hepatocellular carcinoma patients (HCC). The 69 patients with chronic hepatitis B included 25 Malays (16 males, 9 females), 39 Chinese (14 males, 25 females), and 5 Indians (2 males, 3 females). The 11 patients with hepatocellular carcinoma included 8 Malays (5 males, 3 females) and 3 Chinese (1 male, 2 females), while the 15 asymptomatic carriers were 8 Malays (5 males, 3 females), and 7 Chinese (6 males, 1 female).

Asymptomatic carriers in this study were blood donors from the Tengku Ampuan Rahimah Hospital, Klang. They were diagnosed as carriers through routine screening and were found to be HBsAg-positive for more than 6 months on repeated testing. Patients with chronic HBV infection/hepatitis and HCC were seen in the Gastroenterology Clinic, Department of Medicine, Medical Faculty, Universiti Kebangsaan Malaysia. Diagnosis of HCC patients was made histologically. Sera from the patients were collected and stored at –20°C until required for analysis.

Primers and codon analysis

Detection of nt 1858 (codon 15 of precore region) and nt 1896 (codon 28 of precore region) was done by amplification-created restriction site (ACRS) and an allele-specific PCR to detect nt 1858 (Lindh, *et al.*, 1995a; 1995b; Lindh *et al.*, 1996).

The primers and enzymes used are as in Table 1. The mismatches in the primers may create restriction sites in combination with the adjacent downstream nucleotides in the template. The mismatch in P4 creates a Bsu361 site (CCTNAGG) if the TAG mutation (codon 28) is present in the template; the mismatch in P7 creates an EcoN1 site (CCTN5AGG) if codon 15 is CCT. All samples were analyzed using primers P4 and P8 for the detection of TAG at codon 28 and using primer P2 for the detection of CCT at codon 15.

Analysis of nt 1896 (codon 28 of precore region)

10 µL of the extracted HBV-DNA was used for PCR in a 50 µL reaction volume containing 1.5 mM MgCl2, 50 mM KCl, 10 pmol of primers P4 and P8, and 1 U of TAG polymerase (Perkin Elmer). After an initial denaturation of 94 °C for 3 min, 40 cycles of amplification (including 45 seconds of denaturation at 94°C, 60 seconds of annealing at 58°C and 90 seconds of extension at 72°C, which was extended by 3 seconds per cycle) were followed by 72°C final extension for 7 minutes. 10 uL of the PCR product was then mixed with 1.5 µL of 10X buffer, 3 µL of water and 7 U of Bsu361 and incubated overnight at 37°C. The mixture was then run on a composite gel containing 2% NuSieve agarose (FMC Bioproducts, Rockland, ME) and 1% standard agarose. 10 µL of the PCR products in a mixture with 1.5 µL of 10X buffer and 3.5 µL of water, but without Bsu361 was run in a parallel lane. The DNA was visualized by ethidium bromide staining. The presence of the TAG mutant or wild-type. TGG was detected by comparing the migration of the DNA bands in the two lanes.

Analysis of nt 1858 (codon 15 of precore region)

PCR was performed as described above but using 10 pmol of primers P2 and P7 and an annealing temperature of 55°C. The PCR products were incubated with 7.5 U of EcoN1. CCT band appeared as an altered migration of a single band and a mixed CCC/CCT infection could be detected as a double band.

Nested PCR was done when no band was obtained by a single PCR. It was carried out using primers P1 and P9 and 30 cycles of amplification was carried out. However the annealing temperature used was 60°C. 5 µL of the PCR product was then subjected to 35 cycles of amplification using the primers as for codon 28 or codon 15.

Table 1. Primer sequences and restriction enzyme sites (Lindh *et al.*, 1996)

Primer sequences	Function	Restriction Enzyme/Restriction Site Sequence
P1 5'GTTGCATGGAGACCACCGTGAAC (nt 1603-1625)		
P2 5'ATGTCGACAACCGACCTTGA (nt 1680-1699)		
P4 5'CAAGCCTCCAAGCTGTGCCTTGGGTGGCCTT (nt 1865-1895)	Identifies TAG at codon 28	Bsu361 (CCTNAGG)
P7 5'CCCAAGGCACAGCTTGGAGGCTCCTACAGT (nt 1888-1859)	Identifies CCTat codon 15	EcoN1 ($CCTN_5AGG$)
P8 5'GTATGGTGAGGTGAACAATG (nt 2058-2039)		
P9 5'ATGGGATCCCTGGATGCTGGGTCTTCCAAA (nt 2145-2118)		

Serum HCV, HDV and HBV Markers

HBsAg and HBeAg/anti-HBe were determined by radioimmunoassay using commercially available kits (Austrai II for HBsAg and HBeAg/anti-HBe (rDNA) for the e antigen and antibody, Abbott Laboratories, North Chicago Ill). Hepatitis C antibodies (anti-HCV) were determined by Murex anti-HCV while antibody to hepatitis D virus (anti-HDV) was determined by using EIA (Organon Teknika Hepanostika.). Alanine aminotransaminase (ALT) levels were done by the Biocbemistry Laboratory, Tengku Ampuan Rahimah Hospital and the Chemical Pathology Unit, Medical Faculty UKM. Levels above 50 IU/L were considered raised.

The results were analyzed using Chi square and was considered significant when p was ≤ 0.05.

Results

HDV and anti-HCV were determined in 265 HBsAg positive individuals (37 asymptomatic carriers, 199 patients with chronic hepatitis and 27 with hepatocellular carcinoma). It was found that all were negative for antibodies to HDV (anti-HDV) while anti-HCV was detected in 5 (1.9%) of the HBsAg positive individuals and they were all from HCC patients. Sera from the 265 individuals were subjected to PCR and 35.8 % (95/265)

Table 2. HBV variants in HBsAg positive individuals

	HBV-DNA +	Codon 15 CCT, T-1858 variant	Codon 15 CCC, C-1858 variant	Mixture of T-1858/C-1858 variant	Codon 28 TAG	Mixture of TGG/TAG
Total HBsAg individuals	35.8 % (95/265)	82.1 % (78/95)	14.7 % (14/95)	3.2 % (93/95)	-	3.5 % (3/95)
Asymptomatic carriers	38.5% (15/39)	93 % (14/15)	7 % (1/15)	-	-	-
Chronic hepatitis individuals	34.7 % (69/199)	78.3 % (54/69)	17.4 % (12/69)	4.6 % (3/69)	-	2.9 % (2/69)
Hepatocellular carcinoma individuals	40.4 % (11/27)	90.9 % (10/11)	9.1 % (1/11)	-	-	9.1 % (1/11)

CCT/CCC = co-infection by 2 variants i.e. T-1858 and C-1858

Table 3. Relation between variants T-1858/C-1858 (Codon 15 -CCT or CCC) and HBeAg/ Anti-HBe status in HBsAg positive individuals

Group	Codon 15						
	CCT			CCC			CCT/CCC
	e +	anti-e +	e/anti-e -	e +	anti-e +	e/anti-e +	e +
Total HbsAg Individual (n = 95)	39.7% (31/78)	55.1% (43/78)	5.1% (4/78)	64.3% (9/14)	28.6% (4/14)	7.1% (1/14)	100% (3/3)
Chronic Hepatitis (n = 69)	48.1% (26/54)	59.9% (27/54)	1.9% (1/54)	66.7% (8/12)	25.0% (3/12)	8.3% (1/12)	100% (3/3)
HCC (n = 11)		70% (7/10)	30% (3/10)		100% (1/1)		
Asymptomatic Carrier (n = 15)	35.7% (5/14)	64.3% (9/14)		100% (1/1)			

CCT/CCC= co-infection by 2 variants T-1858 and C-1858

were positive for the virus (Table 2).

When the HBsAg positive individuals were divided into the three groups namely asymptomatic carriers, individuals with chronic hepatitis and HCC patients, no difference was seen in the prevalence of HBV-DNA between the groups ($p > 0.05$) (Table 2).

Nucleotides 1858 (for codon 15, CCT/CCC-T-1858, C-1858) and 1896 (for codon 28, TGG/TAG) were analyzed using a combination of PCR and restriction enzyme action as described by Lindh *et al.* (1995; 1996). Of the 95 PCR-positive patient samples, 78 (82.1%) had T (thymine) as nt 1858 i.e. codon 15 of the precore region is CCT, 14 (14.7%) had C (cytosine) as nt 1858 i.e. CCC as codon 15 while 3 (3.2%) were infected with a mixture of T-1858 and C-1858 This difference is significant ($p < 0.0001$). When the prevalence of the T-1858 variant was compared in the three groups, it was observed that a lot more of the asymptomatic and the HCC groups were infected by the T-1858 variant than the group with chronic hepatitis B. This observed difference was significant ($p< 0.001$) (Table 2). Co-infection by a mixture of variants was seen only in individuals with chronic hepatitis B.

None of the HBsAg positive individuals were infected exclusively by the TAG mutant i.e. stop codon, TAG, at codon 28. However, 3.5 % of the population was infected by a mixture of wild type and the TAG mutant (Tables 2 & 4). All the three individuals were infected by the T-1858 variant.

The relationship between T-1858 and C-1858 variants and HBeAg/antiHBe status is shown in Table 3. As seen, 55.1% of the individuals infected by the T-1858 variant were anti-HBe positive, but this is not significant ($p > 0.05$), while most of the individuals infected by the C-1858 variant were HBeAg positive ($p < 0.005$). Again comparison between the three groups showed that the majority of the individuals infected by T-1858 variant were anti-HBe positive especially in the HCC patients.

Raised ALT levels were observed in 75% (8/12) of patients with chronic hepatitis infected with the C-1858 variant while 70.4 % (38/45) of the chronic patients infected with the T-1858 variant also had raised ALT level. 60% (6/10) of HCC patients infected with the T-1858 variant had raised ALT levels.

Discussion

In the present study 35.8% of the PCR samples were positive for HBV-DNA. This is different from our previous report of 22.1%. This difference could be due to the fact that the hybridization technique (dot blot) used in the previous study was less sensitive than PCR (Ton *et al.*, 1995). The majority of the infected individuals were infected by the T-1858 variant while none of the individual was infected exclusively by the TAG mutant (TGG → TAG). Only 3.5% (3/95) of the TAG mutants were detected in the present study samples and these were individuals co-infected with the wild type. The low prevalence of the TAG mutants in this study is in agreement with a report by Feray *et al.* (1993). It was suggested that the precore stop codon mutation at codon 28 is not a general condition, but might be due to epidemiological and geographical bias, contrary to other reports. Another possible reason for the low prevalence of TAG mutation in our patients could be that many of the patients had not yet seroconverted since 45.3% were HBeAg positive. As the precore mutations are more commonly seen to emerge during seroconversion or in

Table 4. Relation between codon 28 and HBeAg/anti-HBe status in HBsAg individuals

Group	Codon 28						
	TGG				TGG/TAG		
	e +	anti-e +	e/anti-e +	e/anti-e -	e +	anti-e +	e/anti-e +
Total HbsAg Individuals (n = 95)	45.3% (43/95)	47.4% (45/95)	2.1% (2/95)	3.2% (3/95)	1.1% (1/95)		1.1% (1/95)
Chronic Hepatitis (n = 69)	53.6% (37/69)	40.6% (28/69)	2.9% (2/69)		1.4% (1/69)		1.4% (1/69)
HCC (n = 11)		72.9% (8/11)	18.2% (2/11)			9.4% (1/11)	
Asymptomatic Carriers (n = 15)	40% (6/15)	60% (9/15)					

TGG/TAG = co-infection with variants with TGG and TAG as codon 28

interferon therapy it could account for the low TAG mutations in our study (Lindh *et al.*, 1995b; Lindh *et al.*, 1998; Chen & Oon, 2000; Grandjacques *et al.*, 2000).

Low prevalence of TAG mutants was also observed in the 55.1% of the anti-HBe positive individuals. Precore mutations are not always detected in HBV isolates from carriers who have undetectable levels of HBeAg or who are positive for anti-HBe in the serum. This strongly points to the existence of mutations in genomic areas other than the precore region that would interfere with the proper synthesis and secretion of HBeAg (Okamoto *et al.*, 1994).

In the present study, no relation was observed between HBeAg-negative individuals infected by the T-1858 variant and the TAG variant in patients with chronic hepatitis. This differs from a report by Lindh *et al.* (1996) who demonstrated that TAG mutation was found in 68/71 (96%) of HBeAg-negative chronic carriers infected with the T-1858 variant. The C-1858 variant was reported to be associated with more inflammation as indicated by raised ALT levels compared with T-1858 variant. Our study differs from the above as 75 % of patients with chronic hepatitis infected with the C-1858 variant and 70.4 % of those infected with the T-1858 variant had raised ALT levels.

None of the HCC patients was infected exclusively by the TAG mutant. Only one (9.1%) patient was infected by a mixture of TGG/TAG mutants. This is in contrast to a report by Park *et al.* (1997) who showed that 50/58 (86%) of HCC patients had TAG mutations. Out of this 21/50 (42%) were co-infected with the wild type.

The HBeAg-negativity in our HCC patients could be due to mutations elsewhere. Mutations could be at nt 1762/1764 in the X protein open reading frame where the core promoter is located (Okamoto *et al.*, 1994) and not in the precore region. Mutation at a non-AGG motif at position 1762 to 1764 was shown by Kidd-Ljunggren *et al.* (1997) to be significantly linked to the HBeAg negative phenotype and increased liver damage. Lindh *et al.* (1998) also demonstrated that in HBeAg negative chronic carriers, T-1762 mutants were found in 71% of patients. Thus it appears that our patients who were anti-HBe positive but do not have the G → A mutation may be infected by variants with mutations in the core-promoter region or in other region within the viral genome.

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