Viral and clinical factors associated with surface gene variants among hepatitis B virus carriers

Anne Marie Roque-Afonso[1,2,3], Marie-Pierre Férey[2], Thoai Duong Ly[4], Arielle Graube[2], Luciana Costa-Faria[1], Didier Samuel[1,3] and Elisabeth Dussaix[1,2,3]*

[1]INSERM U785, 94804 Villejuif, France
[2]AP-HP, Hôpital Paul Brousse, Virologie, 94804 Villejuif, France
[3]Université Paris-Sud, UMR-S 785, 94804 Villejuif, France
[4]Laboratoire Claude Lévy, Ivry sur Seine, 75014 Paris, France

*Corresponding author: Tel: +33 1 4559 6956; Fax: +33 1 4559 3724; E-mail: anne-marie.roque@pbr.aphp.fr

Background: Understanding the prevalence of potential antigenic variation of the hepatitis B virus (HBV) surface antigen (HBsAg) is fundamental for assay design and to future changes in vaccine formulation. In this study, the nature and frequency of HBsAg polymorphisms occurring in France in chronic carriers and in newly diagnosed patients were determined. We focused on variations in the major hydrophilic region (MHR), the central core of HBsAg known to be exposed on the surface and involved in antibody binding.

Methods: Two patient groups were identified: 51 chronic HBV carriers followed at our institution for >1 year; and 129 newly diagnosed patients (63 of whom had a first HBsAg-positive result at our hospital laboratory and 66 a first positive result in a private laboratory).

DNA sequences of HBsAg were obtained from these 180 patients and compared with consensus sequences built with 168 full-length HBV sequences imported from GenBank. Polymorphisms of the MHR of HBsAg were analysed with the Mutation Master Software. Literature review and BLOSUM scores were used to define potentially altered antigenicity.

Results: The global frequency of MHR variants was 27.8%. Occurrence of MHR variants was independent of viral load, HBeAg status and sex, but was associated with the chronic carriers' group, advancing age, the presence of antibodies to HBsAg, immunoprophylaxis administration, antiviral treatment and genotypic resistance to antivirals. In multivariate analysis, the independent variables associated with MHR variants were advancing age and the presence of genotypic resistance to nucleoside or nucleotide analogues.

Conclusion: Most MHR variants emerge with longer disease duration and upon indirect selective pressure. Variation of the MHR may serve to restore virus replication of resistant strains. Combined envelope and polymerase variants could impair diagnostic assays and limit treatment alternatives.

Introduction

Chronic hepatitis B (CHB) affects over 300 million individuals worldwide. The risk of hepatitis B virus (HBV) transmission through blood exposure or vertical contamination has dramatically reduced over the past three decades through the use of sensitive tests to screen HBV in blood donors and pregnant women and through the implementation of vaccination programs [1]. The serological diagnosis of acute and chronic HBV infections is based on the detection of the small hepatitis B surface antigen (HBsAg) encoded by the *S* gene, and most assays detect as little as 0.1 IU/ml of HBsAg [2]. Although the three-dimensional structure of HBsAg is unknown, structure–function studies indicate that its central core, extending from amino acids 99 to 169, is exposed at the surface and involved in binding to antibodies directed against HBsAg [3]. This region, referred to as the major hydrophilic region (MHR), contains eight cysteine residues that maintain the conformational structure and are highly conserved among HBV subtypes [4]. Antibodies found in vaccinated patients or patients with resolved HBV infection are directed to this region and, in particular, to a cluster of B-cell epitopes called the 'a' determinant, which comprises amino acids 124–147. HBsAg immunoassays also use antibodies that target the MHR and the 'a' determinant. Point substitutions in the *S* gene due to selection or natural variation may induce conformational changes and affect the antigenicity of HBsAg. These mutations can lead to false-negative results depending on the targeted epitope used in the HBsAg assay [5–9] and account, in part, for so-called occult HBV infections [7,10,11]. Point

© 2007 International Medical Press 1359-6535

mutations also have clinical implications such as vaccine escape. A vaccine escape mutant harbouring an arginine instead of a glycine at position 145 of the *S* gene (sG145R) was first identified in children born from HBV-positive mothers [12]. This mutant and others might not be neutralized by antibodies induced by the recombinant vaccine and cause infection in vaccinated individuals [13]. These variants also partially account for breakthrough infections in liver transplant recipients receiving passive immunoprophylaxis [14,15].

MHR variants can occur naturally in chronic carriers who have not received active or passive HBV immunization [7,10]. Their prevalence among chronic carriers is generally assumed to be low; however, the prevalence of MHR variants was 24% for chronic carriers in Japan [16], up to 28% in Taiwan [17], close to 40% in Spain [18] and close to 50% in Korea [19]. From previous studies, virological or clinical factors associated with MHR variants include age, advanced liver disease, hepatitis B virus e antigen (HBeAg)-negative status and the presence of antibodies against HBsAg (anti-HBs) [16,17,19–22]. The understanding of the prevalence, diversity and potential antigenic alteration of HBsAg variants is fundamental for assay design and to change vaccine formulations in the future. For this purpose, we determined the nature and frequency of MHR polymorphisms of HBV strains detected in different clinical settings in France. The influence of the observed polymorphisms on the overlapping reverse transcriptase reading frame was also considered.

Materials and methods

Patients

We defined two groups of patients (Table 1). A first group (group A) included 51 patients followed at our institution for >1 year. All had a viral load >1,500 copies/ml. HBV sequencing was routinely performed to identify genotypic resistance to nucleoside or nucleotide analogues in 44 patients; among them, 36 were on lamivudine monotherapy of whom two had previously escaped from hepatitis B immunoglobulin (HBIG) prophylaxis, one was on adefovir monotherapy and had previously escaped from HBIG prophylaxis, one had lamivudine therapy combined with adefovir, one had lamivudine combined with HBIG and five had no treatment but had previously received lamivudine. Among these 44 patients, four were co-infected with human immunodeficiency virus (HIV) and had received lamivudine as part of their antiretroviral therapy. Mutations associated with nucleotide or nucleoside analogue resistance were found in 20/44 patients. HBV sequencing was performed to identify HBsAg mutations responsible for HBIG escape in four patients who had HBV recurrence within 3 years of liver transplantation; two of them were transplanted for HBV cirrhosis and two had received an anti-HBc-positive liver graft. All four were receiving HBIG monoprophylaxis and presented mutations associated with HBIG escape at position 144 and/or 145 of the *S* gene. In addition, HBV sequencing was performed in three patients with chronic hepatitis B who tested HBsAg-positive with the immunoassay used in our laboratory, but HBsAg-negative with other assays. None of these three patients was treated with nucleoside or nucleotide analogues at the time of sequencing.

A second group (group B), included 129 patients with newly diagnosed hepatitis B. Sixty three of these were selected from 143 consecutive HBsAg-positive patients who visited our centre in 2004 for the first time. Among these 143 newly diagnosed patients, 42 (29.4%) had an undetectable HBV viral load and 63 patients among those with detectable viraemia had viral loads >1,500 copies/ml and were selected for sequencing. Of these, two had acute hepatitis. The remaining 61 patients had chronic hepatitis and only

Table 1. Characteristics of the 180 HBsAg-positive patients with viral loads >1,500 copies/ml

	Group A (*n*=51)	Group B (*n*=129)	*P*-value
Age, years ±SD	48.1 ±13.8	40 ±16	0.001
Male, *n* (%)	41/51 (80.4)	88/129 (68.2)	0.14
Viral load, log copies/ml	7.03	7.46	0.06
D Genotype, *n* (%)	20/51 (39.2)	39/129 (30.2)	0.32
HBIG exposure, *n* (%)	8	0	<0.001
Nucleoside or nucleotide analogue exposure, *n* (%)	44 (86.3)	5 (3.9)	<0.001
Genotypic resistance to antivirals, *n* (%)	20 (39.2)	4 (3.1)	<0.001
Anti-HBe positivity, *n* (%)	35/51 (68.6)	60/86 (69.8)	0.95
Anti-HBs positivity, *n* (%)	7/32 (21.8)	1/57 (1.7)	0.005
HIV-positive status, *n* (%)	4/51 (7.8)	8/63 (12.7)	0.59

Anti-HBe, antibody against hepatitis B virus e antigen; anti-HBs, antibody against hepatitis B virus surface antigen; HBIG, hepatitis B immunoglobulin; HBe, hepatitis B virus e antigen; HBs, hepatitis B virus surface antigen.

© 2007 International Medical Press

five HIV-coinfected patients were receiving an anti-HIV treatment that was also active against HBV: either lamivudine alone (4/5) or lamivudine and tenofovir (1/5). A further 66 patients were selected from among 166 consecutive HBsAg-positive patients having a first positive HBsAg result at the Laboratoire Claude Levy (LCL) between 1st May and 30th June 2005. Of these 166 newly diagnosed patients, 40 (24.1%) had an undetectable HBV viral load and 66 among those with detectable HBV DNA had viral loads >1,500 copies/ml and were selected for sequencing. No clinical information was available for these 66 patients except that none was receiving any antiviral therapy.

Serological assays

HBsAg was detected with the EtiMak4 ELISA assay (DiaSorin, Salluggia, Italy) in patients from groups A and in hospital-diagnosed patients from group B. The remaining patients from group B were tested with the AxSYM HBsAg V2 (Abbott Diagnostics, Delkenheim, Germany). When enough serum was available, HBe status was determined with the Vidas HBe assay (Biomérieux, Marcy l'Etoile, France) and anti-HBs status was determined with the Monolisa anti-HBs Plus assay (Biorad, Marnes la Coquette, France).

HBV DNA quantification

Viral load was quantified in all sera by using the Cobas HBV Monitor assay (Roche Diagnostics, Meylan, France).

HBV sequencing

HBV sequencing was performed with the TRUGENE HBV assay (Bayer Diagnostics, Puteaux, France), as recommended by the manufacturer. Briefly, 200 µl of serum was extracted using the QIAmp DNA blood Mini Kit (QIAgen, Les Ulis, France). A 1,200 bp fragment of the HBV genome was amplified from 5 µl of extracted DNA and added to a set of four CLIP sequencing reaction tubes. This chemistry produces bidirectional sequences using two fluorescent primers. The 520 bp reaction products are submitted to electrophoresis on the sequencer. Forward and reverse sequences are combined and automatically aligned with reference sequences. Sequencing allows the detection of minor variants when they constitute >20% of the viral quasispecies. When a mixed population was present at a given position, only the variant form was taken into account for subsequent analysis.

Sequence analysis

From GenBank, 168 sequences of the complete HBV genome [23] were imported and aligned with CLUSTAL X. A protein consensus sequence of the MHR, amino acids 102–169, was built with these 168 sequences by selecting the most frequent amino acid at each position. In addition, a consensus sequence was built for each genotype. Clinical sequences were imported from the TRUGENE software and aligned. HBV genotype was determined by phylogenetic analysis using MEGA software version 2.1 [24].

Each amino acid position of clinical sequences was compared to the consensus sequence with the Mutation Master software (http://tandem.bu.edu/cgi-bin/mutationmaster/mutmast.exe). This program allows the position and frequency of specific changes to be visualized and enables variable and conserved positions to be identified starting from the multiple alignment data file of the 180 clinical sequences. Mutation Master distinguishes positions with many different amino acids from those with a high frequency of only one or two substitutions. A position with only one or two naturally occurring substitutions may be subjected to more constraints than positions with a higher variability. Mutation Master also allows the graphical presentation of BLOSUM scores (Blocks Matrix Substitution), reflecting changes in the chemical and structural properties of the amino acids [25].

The same sequence analysis was conducted for the overlapping reverse transcriptase sequence encompassing amino acids rt111–rt235, according to the consensus numbering system for HBV reverse transcriptase [26].

Statistical analysis

Age, sex, HBeAg and anti-HBs status, viral loads and genotype distribution were compared between the three groups. The frequency of MHR variants was analysed according to age, sex, HBe and anti-HBs status, viral load, HBV genotype, antiviral treatment and the presence of genotypic resistance to antivirals on the overlapping reverse transcriptase open reading frame. The χ^2 or Student's *t* tests were used. Multivariate analysis was conducted with StatView software version 5.0.

Results

Demographic and viral characteristics of the two groups

Patients' characteristics are presented in Table 1. Patients from group A, followed at our centre mostly for chronic hepatitis, were older than the newly diagnosed patients of group B. They were more frequently exposed to antiviral agents or HBIG and more often had resistant strains. Anti-HBs positivity was more prevalent in this group and HBV viral load tended to be lower than in group B (7.03 log UI/ml versus 7.46 log UI/ml; *P*=0.06). The groups were similar in terms of sex ratio, HBV genotype distribution, HBeAg status and frequency of HIV infection. Genotype D was detected in >30% of the patients in each group and genotype A was detected in ~20% of cases in each group. The

Figure 1. Nature of MHR polymorphisms and frequency of single-character variation within the MHR (amino acids 102–168)

Position	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169
WT	G	M	L	P	V	C	P	L	I	P	G	S	S	T	T	S	T	G	P	C	K	T	C	T	T	P	A	Q	G	T	S	M	F	P	S	C	C	C	T	K	P	S	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	A	K	Y	L	W	E	W	A	S	V	R
A													T																	N												T																										
B																																										T																										
C									L			T													I																																		R	F								
D																					R												Y																											G		F							A	
E																					R					L													S																			G		F							A	
F									L				T																																												L	G									A	
G																														N			Y																																				
Ref		I5		H				Q	T2	L	R					I	v3	E	S	R				M5	S3	T13	v3	R	N	K		T							I	T	T	M	E2										P			T		V3	S		P		G	G2	V			
								P	M	T						G	A3								A3																	L	V													P												
																																																								G												
%		3.0		0.6				1.2	1.8	1.2	0.6					1.2	3.6	0.6	0.6	0.6				3.0	3.6	7.7	1.8	0.6	0.6	0.6		0.6							0.6	0.6	0.6	1.2	1.8										0.6			1.8		1.8	0.6		0.6		0.6	1.2	0.6			
B		R						Q			A	A	A		A		V3		S2			N2		M7	N2	T15	V5	H3	N4			T3	N3	R					I			L	G2	R2						L							S3	V		H			D2		G2			
								M				G	P				A2		L2			S			A	I2		R2	R			L	I	H					L				V	A2																			G					
																	M		T							A			E			I											E	K																									
																			Q																																																		
A	S	I					L	I	R		R	A				I	K	R	T2			N	R	M5	A	T7		N2	N		F	L2	V2	A	Y						S	M2	G2	R5					T				L		G			E	N				V2		G			H
							H	V									A	E	S									K				I3	H2								L	L	E4	A																			G2					
							I												Q													T	N										A																									
																																	S																																				
																																	L																																				
%	0.6	1.1					1.7	2.2	0.6		1.1	1.7	1.1		0.6	0.6	4.4	1.1	5.5			1.7	0.6	6.7	2.2	13.9	2.8	4.4	3.9		0.6	6.1	6.1	1.7	0.6				1.1		1.1	2.2	6.1	6.1					0.6	0.6			0.6		0.6		1.7	1.1	0.6	0.6			3.9		1.7			0.6

The upper part of the figure represents the consensus amino acid sequence of the major hydrophilic region (MHR) and variation found in different genotypes (A-G). The lower part represents the substitutions observed in the different reference strains and clinical sequences from group B and group A. Substitutions highlighted with grey squares correspond to substitutions associated with diagnostic and/or vaccine and/or immunoprophylaxis escape or to substitutions with BLOSUM scores ≤-3, or to both. Numbers associated with amino acid symbols correspond to the number of occurrences of a given amino acid when >1. The frequency of variation is given as a percentage. The positions with highest degrees of polymorphism were 103, 125 and 127 for the 168 reference strains and 118, 120, 125, 127, 129, 130, 133, 134, 144 and 145 for the 180 clinical sequences.

prevalence of genotype E was <10% in patients from group A, but reached 25% in group B.

HBeAg and anti-HBe were tested in all group A patients and in 86 patients from group B. Anti-HBe-positive patients were significantly older than anti-HBe-negative patients (48 ±13 years versus 35.5 ±16 years; *P*<0.0001). Anti-HBs was tested in 32 patients from group A and in 57 patients from group B. Anti-HBs positivity was found in 8/89 tested patients.

Polymorphism of the major hydrophilic region

The amino acid sequence of the MHR was deduced from the nucleotide sequence (GenBank accession numbers DQ855663 to DQ855842). Mixed populations were observed at the amino acid level in 11/51 sequences from group A (21.6%) and in 34/129 (26.3%) sequences from group B. Only the variant amino acid was taken into account. The nature and frequency of MHR polymorphisms of reference and clinical sequences are shown in Figure 1. Fifteen invariant positions among the 68 studied positions (22%) are common to the 348 sequences. Genotype-dependent polymorphisms were not regarded as mutants. In addition, considering the high frequency of T125M and P/L127T substitutions, they were not taken into account for analysis. Literature review and BLOSUM scores were used to define substitutions associated with potentially altered antigenicity of HBsAg. This includes substitutions previously reported to be associated with diagnostic or immune escape [7,10,15,27–29] and substitutions with BLOSUM scores ≤-3. This cut-off was chosen because -3 is the BLOSUM score of the sG145R substitution known to significantly alter HBsAg antigenicity [15]. Such positions are highlighted by grey squares in Figure 1. The most frequent substitutions of this type in reference sequences were T/I126A (1.8%) and D144E/V (1.8%); in clinical sequences, they were M133I/T/L, D144E/G/A/V and G145R/A/K (6.1%), followed by P120S/T/Q/L (5.5%), Q129R/H/N and E164G/D/V (3.8%), F/Y134N/S/L (3.3%), S/T143M/L (2.2%), T123N (1.6%), and T/I126N/A and F/L158S (1.6%). The extent of MHR variability would be much higher if all observed substitutions were taken into account.

Nature and frequency of MHR variants with potentially altered antigenicity

Variants harbouring substitutions associated with potentially altered antigenicity are listed in Table 2. Their frequency was 7% (12/168) in reference sequences and 27.8% (50/180) in clinical sequences, including 44 strains with mutations within the ‘a’ determinant, amino acids 121–147 (24.4%).

© 2007 International Medical Press

Table 2. Nature of MHR substitutions, HBV genotype, antiviral treatment exposure and genotypic resistance to antivirals of 50 variant strains

HBV genotype	MHR mutations	Antiviral treatment	Previous treatment	Genotypic resistance (RT mutations)	Group*
A	M133L	LAM		L180M, M204V	A
A	D144E, G145R	LAM		L180M, M204V	A
A	G145A, E164D	LAM		V173L, L180M, M204V	B
B	M133I, S132F	LAM		M204I	A
B	M133L	LAM		L180M, M204V	A
C	Q129N	LAM		L180M, M204V	A
C	Q129N	LAM		L180M, M204V	A
C	I110R, S117I, G119R, T123N, C124R	ADV	HBIG	A181T	A
D	P108L, E164V	LAM		A181T	A
D	M133I, F/Y134H, S/T143M	LAM		M204I	A
D	P120Q, F/Y134V, D144G, A166G	LAM	HBIG	L180M, M204V	A
D	Q129H, G130E, M133T	LAM		L180M, M204V	B
E	G145A	HBIG/LAM		L180M, M204V	A
G	Q129H, E164D	LAM		V173L, L180M, M204V	B
A	L109V, F/Y134L	LAM			A
D	T118K, M133T, S136Y, S/T143M	LAM			A
D	M103I, G112R, L109I, S113A, P120T, F/Y134S		LAM		A
D	T118R, G145R	HBIG/LAM	HBIG		A
E	F/Y134V, D144E	LAM			A
G	P108H, S154L	LAM			A
A	P120S, D144E, G145R	HBIG			A
A	D144A, A/G159E	HBIG			A
A	P142S, G145R	HBIG			A
D	F/Y134H, P142L, D144E, G145R	HBIG			A
A	T118V, P120Q, T126A, D144G, G145R				B
A	Q129R, G130N				B
B	T126A, M133I, W156G, E164V				A
C	T126N				B
D	P120T, S132F, F/Y134N, P135A, D144G, I150T				A
D	P120L, T123N, D144G				B
D	F/Y134N				B
D	F/Y134N				B
D	S143L				B
D	G145R				B
D	E164G				B
D	G112A, M133T, T140I, Y/F161H				B
E	A129V, G130R				B
E	D144E				B
A	P120L				B
A	A/G159V				B
B	M133L				B
C	G145A				B
D	L109Q, S113A, S114A, T116A, P120S, T123N, F/Y134I P135R, P150L, P151L				B
D	L109M, M133T, F/Y134N				B
D	Q129R, F158S				B
D	G130N, M133I				B
E	P120T				B
E	Q129H				B
E	D144V, G145K				B
F	S143L				A

*Study group. ADV, adefovir; HBIG, hepatitis B immunoglobulin; LAM, lamivudine; MHR, major hydrophilic region; RT, reverse transcriptase.

Table 3. Differences between patients with variant or wild-type MHR

	Variant *n*=50	Wild type *n*=130	*P*-value
Membership of group A, *n*	24/50	27/130	0.0005
Mean age, years ±SD	49.1 ±13.7	40.1 ±15	0.0002
Male, *n*	35/50	94/130	0.90
Viral load, log copies/ml	7.2	7.4	0.28
Genotype D, *n*	20/50	39/130	0.27
Anti-HBe-positive, *n*	30/42	65/95	0.88
Anti-HBs-positive, *n*	8/33	0/56	0.0004
HIV-positive status, *n*	6/39	6/75	0.36
Previous or actual nucleoside analogues, *n*	20/50	29/130	0.027
Previous or actual HBIG, *n*	8/50	0/130	0.00002
Mutations associated with antiviral resistance, *n*	14/50	10/130	0.0008

Anti-HBe, antibody against hepatitis B virus e antigen; anti-HBs, antibody against hepatitis B virus surface antigen; HBIG, hepatitis B immunoglobulin; HBe, hepatitis B virus e antigen; HBs, hepatitis B virus surface antigen; MHR, major hydrophilic region.

No significant relationship was found between genotype distribution and potential antigenic alteration: the frequency of MHR variants was 17.5% (for genotype E), 21.7% (for genotype C), 23.8% (for genotype A), 32% (for genotype D) and 42.9% (for genotype C). Potentially altered antigenicity was independent of viral load, HBeAg status, sex and HIV status, but was associated with membership of group A, advancing age, anti-HBs-positive status, previous or actual HBIG administration, previous or actual treatment by nucleoside or nucleotide analogues and genotypic resistance to nucleoside or nucleotide analogues (Table 3). In multivariate analysis (logistic regression), the independent variables associated with MHR variants were advancing age (r=1.039, 95% confidence interval [CI] 1.014–1.034; *P*=0.0019) and genotypic resistance to antivirals (r=3.454, 95% CI 1.361–8.764; *P*=0.0091).

The frequency of mutations between positions 139 and 147, the epitope cluster that regularly undergoes mutations after vaccination or anti-HBs administration, was 12.2% (22/180). This type of mutation represented 44% of MHR variants. Eight of these 22 patients were anti-HBs-positive, of whom six were receiving HBIG prophylaxis, but mutations of this epitope were also found in 11 anti-HBs-negative patients.

Among the 49 patients with nucleoside or nucleotide analogue exposure, 24 had developed genotypic resistance. MHR variants were found in 20/49 (40.8%) treated patients and in 14/24 (58.3%) patients with treatment-induced mutations of the *P* gene. If the 17 patients with only L180M/M204V/I and M204I are looked at, the same proportion of MHR variants is found: 10/17 (58.8%).

Clinical data were available for 39 patients with potential antigenic alteration. Hepatocarcinoma was found in 11 cases (28.2%), immunosuppression (HIV coinfection, renal transplantation, dialysis or cancer chemotherapy) in nine cases (23.1%), and liver transplantation with immunoprophylaxis escape in seven cases (17.9%).

Analysis of overlapping reverse transcriptase reading frame

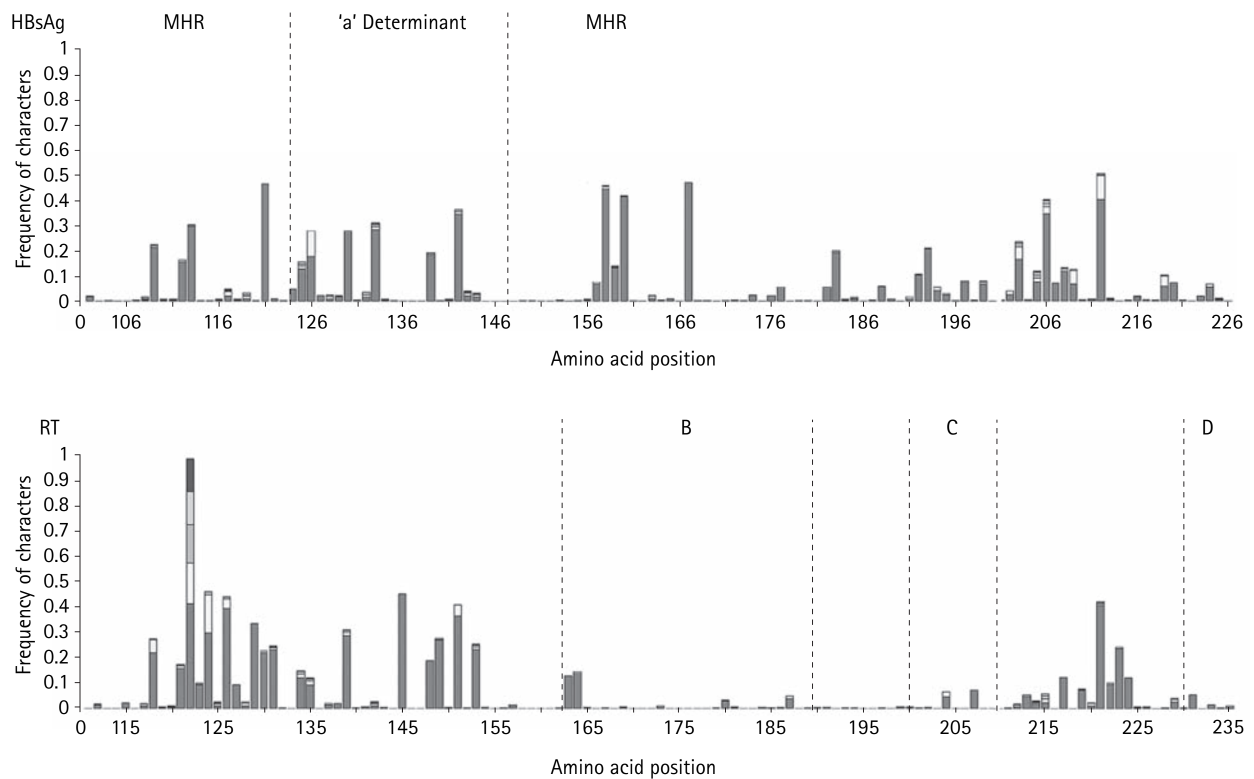
The amino acid sequence of the viral reverse transcriptase (rt111–rt235) was deduced from DNA sequences and includes B and C domains of the reverse transcriptase. This sequence encompasses amino acids 102–226 of HBsAg. The 180 clinical sequences and the 168 reference sequences were plotted on the same graph to identify polymorphic and conserved domains (Figure 2). B and C domains of the reverse transcriptase present very few polymorphisms, despite amino acid variability of the overlapping HBsAg frame. By contrast, the region between domains A and B (up to amino acid 164) is highly polymorphic and corresponds to the MHR of HBsAg. A second polymorphic zone is evidenced between the C and D domains. Thus, reverse transcriptase functional domains appear constrained with only substitutions corresponding to antiviral resistance.

Discussion

The present study addresses the question of the frequency and the factors associated with polymorphisms of the MHR of HBsAg in a large population of 180 HBsAg-positive French patients. The global frequency of such variants was 27.8%. This frequency is similar to frequencies observed in Japan [16] or Taiwan [17], but less than the frequencies (~40%) reported in Spain [18] or Korea [19]. Advancing age and genotypic resistance to antivirals were the main and independent factors associated with MHR variants in this series, suggesting that most variants emerge with longer disease duration and upon indirect selective pressure.

© 2007 International Medical Press

Figure 2. Single-character variation of HBsAg and overlapping reverse transcriptase sequence for the 180 clinical isolates



The 180 clinical sequences and the 168 reference sequences were plotted on the same graph to identify polymorphic and conserved domains. The major hydrophilic region (MHR) and 'a' determinant are represented for hepatitis B surface antigen (HBsAg); B and C domains are represented for the reverse transcriptase (RT). The height of the bars indicates the fraction of the population with a given substitution.

Previous studies have shown that patients with advancing age and end-stage liver disease exhibited higher *S* gene variability than patients with acute self-limited hepatitis [19–21]. *S* gene mutations accumulate during the natural history of chronic hepatitis B, particularly after development of hepatitis or loss of HBeAg [30]. In the present study, most patients were HBeAg-negative and this status was associated with advancing age, but not with MHR variants, suggesting that immune selection pressure is not the main factor for MHR variation in this series. However, the role of immune selection in the emergence of *S* variants has been previously noted in specific settings. This is the case with vaccine failure in neonates born from HBsAg-positive mothers [17,31] or in breakthrough infection after liver transplantation in patient receiving passive immunoprophylaxis [14,15]. Several reports have described HBsAg variation in chronic carriers where HBsAg was associated with anti-HBs antibodies [16,22,32,33]. We indeed found anti-HBs positivity associated with MHR variants in univariate analysis. All patients with anti-HBs antibodies presented mutations within the 139–147 epitope that undergoes mutations after vaccination or anti-HBs administration. Six of these eight patients had received anti-HBs immunoprophylaxis and were studied at the time of viral breakthrough. Alterations of this epitope were also found in eight anti-HBs-negative patients of whom only one had a previous immunoprophylaxis. Nevertheless, this type of variant represents only 44% of all variants, suggesting that MHR variation is not mainly due to neutralization escape. Our finding is consistent with the results of Zhang *et al.* [34], indicating that there was no significant difference in the occurrence of HBsAg variation in patients with or without anti-HBs.

In addition to direct selective pressures on *S* protein, indirect selective pressures can be exerted due to HBV genome organization into overlapping reading frames. As a consequence, treatment of chronic hepatitis B carriers by nucleoside analogues results in the selection of reverse transcriptase mutations that produce changes in the overlapping *S* gene. Substitutions within the HBsAg protein downstream of the 'a' determinant arising from lamivudine-selected mutations may result

in changing antigenicity [29]. The association that we found between MHR variants and genotypic resistance to lamivudine is a different phenomenon. We found a high variability of the reverse transcriptase reading frame, particularly between amino acids 118 and 153. The *S* gene mutations P120T and G145R that produce the reverse transcriptase changes rtT128N and rtW153Q have been found to restore *in vitro* the replication phenotype of lamivudine-resistant strains [35]. Other mutations in the *S* gene could thus also be compensatory mutations of the reverse transcriptase protein. In another setting, older age and previous resistance to lamivudine is associated with a higher probability of the emergence of adefovir resistance [36]. Both situations may reflect the ability of the virus to accumulate mutations and restore virus fitness over time.

Some authors have shown the accumulation of mutations due to high level replication without selection pressure. Long-term immunosuppression has been associated with a number of alterations of the HBV genome, including deletions in the *C* gene in the pre-S1/2 and the X regions and several point mutations at different positions [37,38] that are probably related to high replication rates. HBsAg mutations have also been reported in immunodepressed clinical settings such as haemodialysis patient populations [39] and in patients reactivating from resolved HBV status after immunosuppression [40,41]. Among patients with MHR variants in the present study, about 25% were immunosuppressed due to HIV coinfection, renal transplantation, dialysis or cancer chemotherapy.

The localization of polymorphic and invariant positions found within the MHR is in accordance with topological models that predict exposed and buried regions. Exposed fragments show high diversity while supposedly buried regions (107–111, 136–139 and 147–157) [3] are highly conserved. This conservation may be due to either the lack of any positive selection pressure (no elicitation of antibodies) or a strong structural constraint. The prediction of structural and biochemical effects from amino acid substitutions is always difficult, especially when the three-dimensional structure of the protein is still unknown. The BLOSUM score provides information about the conservative or non-conservative nature of each substitution in reference to the consensus amino acid. The highest score is that of the consensus amino acid, and the lowest score is that of a very rare substitution that is likely to alter the structure/function of the protein. Some of the samples containing HBV mutants with low BLOSUM scores have been tested with different commercial assays [8]. Of these, a sample with the substitutions I110R, S117I, G119R, T123N and C124R was detected by only one of the six tested assays.

In conclusion, we have shown that the prevalence of MHR variants is high in HBsAg carriers in France. Most of these polymorphisms are not associated with immune selection but with disease duration, as we found a clear association with age. The relation with antiviral resistance suggests that specific mutations within the MHR may serve to restore replication fitness of resistant strains. With the widespread use of lamivudine and other nucleoside analogues for HBV treatment, combined envelope and reverse transcriptase variants could become a public health problem, impairing diagnostic assays and limiting treatment alternatives.

References

1. Lavanchy D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *J Clin Virol* 2005; **34 Suppl 1:**S1–3.
2. Scheiblauer H, Soboll H, Nick S. Evaluation of 17 CE-marked HBsAg assays with respect to clinical sensitivity, analytical sensitivity, and hepatitis B virus mutant detection. *J Med Virol* 2006; **78 Suppl 1:**S66–70.
3. Chen YC, Delbrook K, Dealwis C, Mimms L, Mushahwar IK, Mandecki W. Discontinuous epitopes of hepatitis B surface antigen derived from a filamentous phage peptide library. *Proc Natl Acad Sci U S A* 1996; **93:**1997–2001.
4. Howard CR. The structure of hepatitis B envelope and molecular variants of hepatitis B virus. *J Viral Hepat* 1995; **2:**165–170.
5. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet* 1995; **345:**1406–1407.
6. Moerman B, Moons V, Sommer H, Schmitt Y, Stetter M. Evaluation of sensitivity for wild type and mutant forms of hepatitis B surface antigen by four commercial HBsAg assays. *Clin Lab* 2004; **50:**159–162.
7. Carman WF, Van Deursen FJ, Mimms LT, *et al.* The prevalence of surface antigen variants of hepatitis B virus in Papua New Guinea, South Africa, and Sardinia. *Hepatology* 1997; **26:**1658–1666.
8. Ly TD, Servant-Delmas A, Bagot S, *et al.* Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. *J Clin Microbiol* 2006; **44:**2321–2326.
9. Louisirirotchanakul S, Kanoksinsombat C, O'Charoen R, *et al.* HBsAg diagnostic kits in the detection of hepatitis B virus mutation within 'a' determinant. *Viral Immunol* 2006; **19:**108–114.
10. Hou J, Wang Z, Cheng J, *et al.* Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology* 2001; **34:**1027–1034.
11. Weinberger KM, Bauer T, Bohm S, Jilg W. High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. *J Gen Virol* 2000; **81:**1165–1174.
12. Carman WF, Zanetti AR, Karayiannis P, *et al.* Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336:**325–329.
13. Chakravarty R, Neogi M, Roychowdhury S, Panda CK. Presence of hepatitis B surface antigen mutant G145R DNA in the peripheral blood leukocytes of the family members of an asymptomatic carrier and evidence of its horizontal transmission. *Virus Res* 2002; **90:**133–141.

© 2007 International Medical Press

14. Terrault NA, Zhou S, McCory RW, *et al.* Incidence and clinical consequences of surface and polymerase gene mutations in liver transplant recipients on hepatitis B immunoglobulin. *Hepatology* 1998; **28:**555–561.
15. Protzer-Knolle U, Naumann U, Bartenschlager R, *et al.* Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* 1998; **27:**254–263.
16. Ogura Y, Kurosaki M, Asahina Y, Enomoto N, Marumo F, Sato C. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. *J Infect Dis* 1999; **180:**1444–1451.
17. Hsu HY, Chang MH, Ni YH, Chen HL. Survey of hepatitis B surface variant infection in children 15 years after a nationwide vaccination programme in Taiwan. *Gut* 2004; **53:**1499–1503.
18. Avellon A, Echevarria JM. Frequency of hepatitis B virus 'a' determinant variants in unselected Spanish chronic carriers. *J Med Virol* 2006; **78:**24–36.
19. Song BC, Kim SH, Kim H, *et al.* Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. *J Med Virol* 2005; **76:**194–202.
20. Rodriguez-Frias F, Buti M, Jardi R, *et al.* Genetic alterations in the S gene of hepatitis B virus in patients with acute hepatitis B, chronic hepatitis B and hepatitis B liver cirrhosis before and after liver transplantation. *Liver* 1999; **19:**177–182.
21. Liu CJ, Kao JH, Shau WY, Chen PJ, Lai MY, Chen DS. Naturally occurring hepatitis B surface gene variants in chronic hepatitis B virus infection: correlation with viral serotypes and clinical stages of liver disease. *J Med Virol* 2002; **68:**50–59.
22. Lada O, Benhamou Y, Poynard T, Thibault V. Coexistence of hepatitis B surface antigen (HBs Ag) and anti-HBs antibodies in chronic hepatitis B virus carriers: influence of 'a' determinant variants. *J Virol* 2006; **80:**2968–2975.
23. Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO and Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 2005; **77:**47–52.
24. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; **17:**1244–1245.
25. Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. *Proc Natl Acad Sci U S A* 1992; **89:**10915–10919.
26. Stuyver LJ, Locarnini SA, Lok A, *et al.* Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. *Hepatology* 2001; **33:**751–757.
27. Seddigh-Tonekaboni S, Lim WL, Young B, *et al.* Hepatitis B surface antigen variants in vaccinees, blood donors and an interferon-treated patient. *J Viral Hepat* 2001; **8:**154–158.
28. Wallace LA, Echevarria JE, Echevarria JM, Carman WF. Molecular characterization of envelope antigenic variants of hepatitis B virus from Spain. *J Infect Dis* 1994; **170:**1300–1303.
29. Torresi J, Earnest-Silveira L, Deliyannis G, *et al.* Reduced antigenicity of the hepatitis B virus HBsAg protein arizing as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. *Virology* 2002; **293:**305–313.
30. Hannoun C, Horal P and Lindh M. Long-term mutation rates in the hepatitis B virus genome. *J Gen Virol* 2000; **81:**75–83.
31. Hsu HY, Chang MH, Liaw SH, Ni YH, Chen HL. Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in Taiwan. *Hepatology* 1999; **30:**1312–1317
32. Yamamoto K, Horikita M, Tsuda F, *et al.* Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol* 1994; **68:**2671–2676.
33. Kohno H, Inoue T, Tsuda F, Okamoto H, Akahane Y. Mutations in the envelope gene of hepatitis B virus variants co-occurring with antibody to surface antigen in sera from patients with chronic hepatitis B. *J Gen Virol* 1996; **77 (Pt 8):**1825–1831.
34. Zhang JM, Xu Y, Wang XY, *et al.* Coexistence of hepatitis B surface antigen (HBsAg) and heterologous subtype-specific antibodies to HBsAg among patients with chronic hepatitis B virus infection. *Clin Infect Dis* 2007; **44:**1161–1169.
35. Torresi J, Earnest-Silveira L, Civitico G, *et al.* Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the 'fingers' subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. *Virology* 2002; **299:**88–99.
36. Fung SK, Chae HB, Fontana RJ, *et al.* Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol* 2006; **44:**283–290.
37. Preikschat P, Meisel H, Will H, Gunther S. Hepatitis B virus genomes from long-term immunosuppressed virus carriers are modified by specific mutations in several regions. *J Gen Virol* 1999; **80 (Pt 10):**2685–2691.
38. Gunther S, Fischer L, Pult I, Sterneck M, Will H. Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999; **52:**25–137.
39. Minuk GY, Sun DF, Greenberg R, *et al.* Occult hepatitis B virus infection in a North American adult hemodialysis patient population. *Hepatology* 2004; **40:**1072–1077.
40. Westhoff TH, Jochimsen F, Schmittel A, *et al.* Fatal hepatitis B virus reactivation by an escape mutant following rituximab therapy. *Blood* 2003; **102:**1930.
41. Alexopoulou A, Dourakis SP, Pandelidaki H, Archimandritis AJ, Karayiannis P. Detection of a hepatitis B surface antigen variant emerging in a patient with chronic lymphocytic leukaemia treated with fludarabine. *J Med Virol* 2006; **78:**1043–1046.

Accepted for publication 14 August 2007