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HBV genotypes and antiviral-resistant variants in HBV infected subjects in Northern Italy

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SUMMARY

HBV genotypes were investigated in sera/plasma from 97 HBV positive subjects. Genotype D was revealed in 80.4% followed by E in 6.2%. Genotypes A, B, and C were also found, as well as for the first time a new combination of HBV D and G genotypes. In a cohort of subjects of this population, the relationship with lamivudine and/or famciclovir-resistant HBV mutants was also investigated. Among 12 untreated subjects, 25% carried HBV drug-resistant strains suggesting that drug-resistant variants naturally exist in untreated Italian HBV chronically infected subjects.

KEY WORDS: HBV, genotype, antiviral-resistant variants

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The major seven genotypes (A-G) of hepatitis B virus (HBV) have distinct geographic distributions (Kramvis *et al.*, 2005). Co-infection with HBV of distinct genotypes is of growing interest from the virological and clinical points of view. One of the main reported co-infections involves A and G genotypes (Kato *et al.*, 2003; Kato *et al.*, 2004). Genomic sequence heterogeneity is one of the major features of HBV due to the lack of proof-reading activity of viral polymerase (Steinhauer and Holland, 1986). Point mutations related to lamivudine and/or famciclovir resistance occur within the P open reading frame (ORF) of the HBV genome, and affect domains B and C of the HBV reverse transcriptase (RT), leading to various aminoacid substitutions.

The substitution of wild-type strains by drug-resistant mutants appears to be more frequent when specific HBV genotypes are involved in the chronic infection (Ben-Ari *et al.*, 2003; Zöllner *et al.*, 2001). Nonetheless, cases of early emergence of mutants have been described, suggesting that mutations conferring lamivudine resistance were already present in these patients before starting therapy (Paik *et al.*, 2001). This would mean that these mutant strains circulate among the population, so that a certain proportion of HBV carriers from a given geographical area might have any of them.

Studies aimed at investigating naturally occurring variants among untreated infected subjects have been reported from few European countries, such as France (Thibault *et al.*, 2002) and Spain (León *et al.*, 2004). Data from Japan suggest that the prevalence of lamivudine-resistant strains among naïve HBV carriers in that country could be as high as 28% (Kobayashi *et al.*, 2001).

The aim of this study was to obtain data on the prevalence of HBV genotypes in Northern Italy. A further aim was to investigate the relationship between HBV drug resistance and genotype among a cohort of untreated and treated HBV chronically infected subjects.

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A total of 97 chronically infected and HBV-DNA positive subjects (56 males and 41 females, all inpatients except 5 outpatients) were included in the study. These subjects were selected for the availability of their sera among the 223 HBsAg-positive subjects submitted to the detection and/or quantification of HBV-DNA in sera/plasma for diagnostic purposes at the Virology Unit of the Microbiology Section of the University Hospital in Parma (Italy) from 1 April 2000 to 30 April 2004. Chronic HBV infection was defined as a persistent seropositivity for HBsAg for at least 6 months before enrollment. The median age of 95 subjects, whose date of birth was known, was 55 years 2 months, ranging from 12 years 6 months to 93 years 3 months. Of 16 and 76 subjects tested as part of routine diagnostic investigations for human immunodeficiency virus (HIV) and hepatitis C virus (HCV), respectively, one presented co-infection with HIV and 2 with HCV. Information about treatment was not accessible for 83 of the examined subjects. On the other hand, treatment with lamivudine or famciclovir had never been administered in 12 subjects, while antiviral treatment was started a long time before sampling in 2 subjects. In one of the 12 untreated subjects two samples were taken over a period of three months. Unfortunately no accurate data were available on when the infection had started and on the probable route of exposure for any subject.

The presence of HBsAg, HBeAg, and/or anti-HBe was detected by using microparticle enzyme capture immunoassays (AxSym; Abbott Diagnostics Division). The presence of anti-HCV antibodies was assessed by enzyme immunoassay (*Vitros*™ anti-HCV assay, Ortho Clinical Diagnostics) and confirmed by recombinant immunoblot analysis (Chiron RIBA HCV v.3.0 Strip Immunoblot Assay, Chiron Corporation). HCV viremia was detected by Cobas Amplicor Hepatitis C Virus Test v.2.0 (Roche Molecular Systems, Inc.). Detection of anti-HIV antibodies was carried out by ELFA technique using VIDAS® HIV DUO Quick (bioMérieux) and by western blot assay using HIV BLOT 2.2 (Genelabs Diagnostics). For the qualitative amplification of HBV DNA, DNA extracted by EXTRAgen Kit (Amplimedical) was used in a nested PCR assay using primer targeting the pre-core/core HBV gene (HBV Oligomix, Amplimedical). HBV DNA levels were determined with Cobas Amplicor HBV Monitor Test (Roche Molecular Systems).

The sera/plasma submitted to qualitative and/or quantitative amplification of HBV DNA were stored at -20°C until tested for the detection of HBV genotypes and genetic variants.

HBV genotypes (A-G) and the genetic variants (L180M, M204V, M204I and VLM207I) were determined by the line probe assays INNO-LiPA HBV Genotyping and INNO-LiPA HBV DR (Innogenetics), respectively, following the manufacturer's recommended procedures. For comparison of the medians the Mann-Whitney U test was used. A p value of less than 0.01 was considered significant.

The prevalence of HBV genotypes in HBV chronically infected subjects in relation to ethnicity is shown in Table 1. Out of 97 subjects, 5 (5.2%) proved to be infected with HBV genotype A, 1 (1%) with genotype B, 1 (1%) with genotype C, 78 (80.4%) with genotype D, and 6 (6.2%) with genotype E. Moreover, in 2 subjects (2.1%) the genotype was unclassifiable by LiPA. Mixed D and G genotypes were found in the remaining 4 subjects (4.1%). The 74 Italian subjects were infected almost exclusively with genotype D (87.8%: 65 of 74), with the remainder being infected with genotype D/G (5.4%: 4 of 74), A (2.7%: 2 of 74), unclassified (2.7%: 2 of 74), and E (1.4%: 1 of 74). All subjects from Eastern Europe, Albania and Croatia, and North Africa were infected with genotype D. Conversely, Western and Central African subjects were infected exclusively with genotype E (62.5%: 5 of 8) or A (37.5%: 3 of 8). Asian individuals were infected with genotypes B, C, or D.

Among the 2 treated and 12 untreated HBV chronically infected subjects, the relationship between genotype and drug-resistant variant was investigated. All 2 treated subjects were infected with genotype D drug-resistant HBV strains (Table 2). Out of the 12 subjects with no record of lamivudine and/or famciclovir treatment, 3 (25%, median age 59 years) carried genotype A (2 cases) or D (1 case) resistant HBV strains and 9 (75%, median age 49 years) carried genotype D (6 cases), E (2 cases) or D/G (1 case) wild-type HBV strains. None of these subjects was co-infected with HCV or had evidence of HIV co-infection. In this subgroup, median values of viral loads of drug resistant and wild-type HBV strains

were 1.18 x 10^5 copies/ml and 1.19 x 10^6 copies/ml (for 6 subjects whose sera/plasma were available for viral DNA quantification), respectively.

Overall, we observed that the M204V mutation was found in the 5 subjects carrying HBV variants, all in combination with other point mutations (2 with L180M, 2 with L180M and M204I, and 1 with M204I and VLM207I). Wild-type strains were associated with mutant strains in all subjects except one, who showed single L180M in combination with M204V mutant. A mixture of wild-type and VLM207I mutant was found in the first sample of a patient whose follow-up sample showed the appearance of the mixture of mutants M204V and M204I. The detection of the mutants did not coincide with any significant variation in viral load.

In conclusion, the most frequent genotype was D, according to previous studies performed in the Mediterranean (Hadziyannis, 2002; Lampertico *et al.*, 2003; Westland *et al.*, 2003), followed by genotypes E and A. The correlations between race and genotype observed here are consistent with the classic geographic distributions observed in Asia, Eastern and Southern Europe, and Africa (Kramvis *et al.*, 2005). The apparent change in the geographic distribution in Northern Italy most likely is attributable to patterns of migration.

Although the precise mechanism of simultaneous infection of distinct HBV genotypes is unknown, a second HBV strain may superinfect an individual already infected with the first HBV strain by escaping from the host's immune response (Kato *et al.*, 2003). The occurrence of coexisting distinct genotypes of HBV could also be explained by a phenomenon of genomic recombination between HBV strains of different genotypes. Even if different types of recombination have been demonstrated (Hannoun *et al.*, 2000; Owiredu *et al.*, 2001; Sugauchi *et al.*, 2002; Cui *et al.*, 2002; Kato *et al.*, 2002a; Kato *et al.*, 2002b), this is the first report demonstrating coinfection between genotypes D and G in a prevalently genotype D infected population. The coexistence of D and G genotypes was confirmed in 1 case in two consecutive sera, as well as in all cases by testing the same specimens twice.

What is noteworthy is that, in the cohort of this population without evidence of antiviral treatment, HBV variants were found in 25%. This finding supports the data obtained by Kobayashi *et al*. in Japanese subjects, and suggests that HBV variants naturally exist in Italian HBV chronically infected subjects without the administration of lamivudine and/or famciclovir. Even if in a limited number of naïve subjects, no statistically significant differences were observed in the age and viral load between subjects with HBV variants and those with wild-type strains, supporting the results obtained in a previous *in vivo* study which demonstrated that HBV carrying L180M and M204V/I mutations replicated to wild-type levels (Zöllner *et al.*, 2000).

Overall, the distribution of mutants observed in untreated and treated subjects is in agreement with other reports (Gutfreund *et al.*, 2000; Ono *et al,* 2001), i.e. that L180M + M204V is commonly found in subjects with HBV variants. It

TABLE 1 - *Ethnic origins and genotypes in 97 HBV DNA-positive subjects*

	Ethnic origin	*No.*	*Genotype*						
			A	*B*	*C*	*D*	*E*	*D/G*	*unc*[a]
No. of subjects:		97	5	1	1	78	6	4	2
	Eastern Europe	2	0	0	0	2	0	0	0
	Mediterranean area								
	Italy	74	2	0	0	65	1	4	2
	Albania, Croatia	6	0	0	0	6	0	0	0
	North Africa	4	0	0	0	4	0	0	0
	Western and Central Africa	8	3	0	0	0	5	0	0
	South East Asia	3	0	1	1	1	0	0	0

[a]unc: unclassified genotype by the line probe assay.

TABLE 2 - *Detection of HBV genotypes and variants in treated and untreated subjects*

Subject No.	*Age*	*Sampling date (month/year)*	*HBV DNA (copies/ml)*	*HBV genotype*	*LiPA HBV DR (remarker)*
Treated subjects					
1	62	Jul/03	n.t.	D	Wt+L180M+M204V+M204I
2	62	Mar/04	$3.06x10^3$	D	L180M+M204V
Untreated subjects					
1	39	Mar/03	$1.18x10^5$	D	Wt+L180M+M204V
2	59	Dec/02	$3.64x10^4$	A	Wt+L180M+M204V+M204I
3	73	Apr/00	$3.88x10^6$	A	Wt+VLM207I
		Jul/00	$2.53x10^6$		Wt+M204V+M204I+VLM207I
4	15	Aug/03	$5.77x10^7$	E	Wt
5	16	Jan/04	$2.39x10^9$	D	Wt
6	18	Sep/03	n.t.	E	Wt
7	33	Mar/01	n.t.	D	Wt
8	49	Mar/04	$6.47x10^2$	D	Wt
9	55	Jun/02	n.t.	D	Wt
10	62	Jun/03	$1.01x10^5$	D	Wt
11	80	Jun/03	$2.28x10^6$	D/G	Wt
12	89	Apr/04	$< 3x10^2$	D	Wt

Wt: wild-type
n.t.: not tested

was interesting to note that, according to previous reports stating the association of HBV genotype A with an increased risk of lamivudine-resistant mutants (Zöllner *et al.*, 2001), we found genotype A exclusively associated with HBV resistant variants.

The data presented here confirm and extend previous reports which strongly correlate HBV genotypes with race and suggest that HBV drug-resistant mutants circulate among untreated HBV chronically infected Italian subjects. It would be worthwhile to determine how co-infection D/G occurs and whether HBV genotype G needs a replication competent HBV genotype to persistently infect hosts.

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