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Epidemiology of HBV S-gene mutants in the Liguria Region, Italy

Implications for surveillance and detection of new escape variants

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HBV surface antigen (HBsAg) variants may impair diagnosis or allow the virus to escape vaccine-induced immunity and their circulation in the population can represent a Public Health threat. Their prevalence, however, is not yet completely established. Evidence indicates that amino acid substitutions within HBsAg can lead to conformational changes which allow mutated HBV to escape the vaccine-induced antibodies used in the screening tests.

In such scenario, the aim of this study was to investigate the prevalence of HBV S-Gene escape mutants by sequencing the gene in a cohort of Ligurian patients monitored for viral load, genotype and drug resistance and to evaluate the risk of false negative HBsAg detection by routine screening tests.

From 2007 to 2011, in 256 consecutive samples from Ligurian HBV positive patients sequencing assay for detection of RT/S-Gene mutations using Trugene® HBV Genotyping kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) was performed. Serological HBV tests and viral load were also performed.

Analyzed sequences revealed G145R mutation in 8/256 (3.1%) examined sequences, it was alone in 5 patients and accompanied by other HBsAg mutations in 3 samples. HBsAg resulted undetectable by 3 of the 8 samples, derived from patients with multiple mutations: T126I-T131A-C139Y-E/D144G, T126I-M133L, and P120Q-T126I.

The emergence of these mutants, at least the G145R, has already been addressed as a public health concern because of its capability of escaping the immune system. In the present study we point out a second aspect connected with their existence and with similar potential negative impact on public health, that is their capability of escape punctual detection.

Introduction

Hepatitis B virus (HBV) is globally considered one of the more serious public health issues: World Health Organization (WHO) estimates that approximately two billion people have been infected with HBV[1] and chronic hepatitis B represents one of the leading causes of preventable death worldwide.[2]

Vaccination is the most effective measure to reduce the global incidence of HBV infections; since 1992, when WHO has recommended the vaccination, the number of Countries that have implemented the national program of immunization with HBV vaccine has gradually increased.[3]

The drastic decrease of prevalence of HBV infection in the post-immunization era is reported by several epidemiological surveys in high endemic regions.[4,5] In this scenario the emergence of HBV mutants represents the virus response to selective pressures, such as vaccination and antiviral therapy.

The first HBV S-Gene mutant was observed in the serum of an Italian vaccinated child with the presence of both HBs antigen and anti-HBs antibodies. The sequenced viral strain showed a substitution mutation of glycine to arginine at site 145 (G145R mutant), that involves the second loop of the immunodominant region of HBsAg ("a" determinant) causing conformational changes.[6]

G145R was later found in immunocompromised patients,[7] in infants born to HBeAg-positive mothers, both passively and actively immunized,[8] and in liver transplanted patients;[9] it represents the most frequent HBsAg mutant described in the literature.[10] Several other mutants have also been observed in the past years, including those involving the first loop of "a" determinant and the pre-S regions.[11,12]

Despite some of these mutants are able to evade vaccine-stimulated immunity, their increased prevalence has not shown a negative impact on the effectiveness of universal immunization programs so far.[13] Moreover, transmission of such mutants to vaccinated individuals has therefore possible.[14] However, they have been demonstrated to be able to avoid detection by some commercial assays, causing HBsAg false-negative results in screening

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Table 1. Patient characteristics

	n = 256
Gender, male/female	185/71
Age (yr), min-max (median)	12–88 (53)
Race, n(%) African	4 (1.6)
Asian	16 (6.2)
Caucasian	236 (92.2)
Alanine Aminotransferase levels (UI/mL), min-max (median)	12–3,486 (44.5)
HBsAg positive n(%)	253 (98.8)
HBV DNA levels (UI/mL), min-max (media)	$3x10^1$ - $6,8x10^8$ ($5.2x10^5$)
Genotype distribution, n(%)	
A	40 (15.6)
B	6 (2.3)
C	14 (5.5)
D	189 (73.8)
E	2 (0.8)
F	4 (1.6)
G	1 (0.4)

tests, as it also occurs in the case of "occult" infection.[15] This is noteworthy in relation both to blood product safety and to appropriate management of HBV infected patients. For this reason, a continuous surveillance system to monitor the possible emergence of new mutants and the spread of those already known is an important target of Public Health.

The aim of this study was to investigate the prevalence of HBV S-Gene escape mutants by sequencing the gene in a cohort of Ligurian patients monitored for viral load, genotype and drug resistance and to evaluate the risk of false negative HBsAg detection by routine screening tests.

Results

Genotype was performed in 256 patients (male/female = 185/71; age: 12–88 y, median 53; race African/Asian/Caucasian 4/16/236; HBsAg positivity 98.8%; ALT levels: 12–3,486 (median 44.5); genotype A/B/C/D/E/F/G = 40/6/14/189/2/4/1). Quantification of HBV DNA revealed levels ranging from $3x10^1$ to $6.8x10^8$ IU/mL (media $5.2x10^5$). The patients' characteristics are summarized in **Table 1**.

Eight out of 256 harbored HBsAg mutations, with a prevalence of 3.1%. G145R mutation was found alone in 5/8 examined sequences (4 D genotype and 1 C genotype), and accompanied by other mutations in the remaining 3 (2 D genotype and 1 A genotype): T126I-T131A-C139Y-E/D144G, T126I-M133L, and P120Q-T126I (**Table 2**).

Six sequences are derived from immunocompromised patients (3 neoplastic, 2 hemodialysed and 1 transplanted; this last was on lamivudine). The other two are from patients with chronic HBV infection, one of which on treatment with entecavir.

Five out of eight cases had elevated alanine aminotransferase or bilirubin levels, indicating presence of hepatitis activity.

Differently from the other 253 patients, in which HBsAg was always detected, in patients with multiple mutations, HBsAg resulted undetectable by 3 of the 5 assays used (Vitros, Monolisa Ultra and EIAgen), while it was revealed by the other two (Axsym and Architect) (**Table 2**).

Discussion

In our 256 patients, whose HBV virus was sequenced for different reasons, 8 mutants were found, harboring from one to five mutations, accounting for a prevalence of 3.1%. Although reports indicating a significant increase of the prevalence of the surface variants after the post-immunization era, it remains stable over several years even if heterogeneous in many different geographic areas. Moreover, some of these mutants, pointedly three plurimutated strains harboring both G145R and T126I, failed to be detected by three diffusely used commercial tests for the screening of HBsAg, among the five examined. The existence of these mutants, at least the G145R, has already been addressed as a public health concern because of its capability of escaping the immune system. In the present study we point out a second aspect connected with their existence and with similar potential negative impact on public health, that is their capability of escape punctual detection. In fact, the risk of failure to identify blood donors or liver patients,[16] has obvious direct consequences on blood product safety and liver patients' management and can embody the additional danger of interpersonal unrecognized mutant transmission. It is also noteworthy that surface mutations have been shown to be associated with "compensatory" mutations in the polymerase genes, affecting drug susceptibility and thus extending the problem to further aspects of HBV management.[17] It is therefore mandatory that several steps be undertaken to reduce the above mentioned risks: for instance, commercial tests for the screening of HBsAg should be updated in the light of the described findings in order to extend their efficiency to all the known mutant strains;[17] a continuous monitoring on the emergence of new mutants should be carried on by mean of international molecular-epidemiology survey programs; a periodic revision of commercial test performance should be done by specialized reference laboratories; finally, the use of at least duplicate tests with two different kits should be recommended in all blood transfusion centers and laboratories involved in the screening of patients for public health purposes and a PCR based test should be performed to solve discordant results.

Materials and Methods

From 2007 to 2011, a total of 256 serum samples were collected from HBV consecutive positive patients tested both for viral load and genotype in a Laboratory of Liguria Region, northern Italy.

Demographic, including patient age, race, gender, alanine aminotransferase levels (ALT U/L) and HBsAg serological status was obtained from medical records at the time of sample collection.

The whole blood specimens were separated by centrifugation, aliquoted and stored at -20°C until assay.

Serological tests. Commercially available immunoassay kits were used for the detection of HBsAg: MonolisaUltra (Biorad Laboratories, Marnes-la-Coquette, France) and EIAgen (Adaltis Italia, Milano, Italy). In the samples with the G145 R mutation HBsAg status was also evaluated by Vitros (Johnson and Johnson Medical S.p.A), Axsym and Architect (Abbott Laboratories, Abbott Park, IL, USA).

DNA extraction and HBV DNA detection. HBV DNA was extracted from 200 μL of serum using QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. The real-time PCR was performed for HBV DNA by Real-Time PCR kit Artus® HBV RG PCR (Qiagen). The lower detection limit was 15 copies/mL.

HBV sequencing and genotyping. RT/S-gene sequencing of HBV was obtained by Trugene® HBV Genotyping kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). Siemens' OpenGene DNA Sequencing System was used for sequencing. The obtained sequences were analyzed by GeneObjects software, which contains sequences that correspond to the surface antigen and polymerase regions for genotype A through H and a universal mutation reporting reference sequence, showing the published mutations from key scientific journal articles. The obtained FASTA sequences were also submitted into the Geno2pheno database (hbv.bioinf.mpi-inf.mpg.de/).

Disclosure of Potential Conflicts of Interest

B.B and L.S have previously participated at speaker's bureaus and advisory board meetings sponsored by GSK, Novartis, Pfizer and Sanofi Pasteur and have received research funding as principal investigators or co-investigators from CrucellBerna, Novartis, GSK, Pfizer and Sanofi Pasteur. P.C., R.C. and C.A. have no conflict of interest. No other relationships/conditions/circumstances that present a potential conflict of interest exist.

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Table 2. Demographic and clinic characteristics, treatment, serology and surface gene mutations in the 8 cases with 145R mutation

Case	Age(yr)/sex	Race	Genotype	HBV DNA level (UI/mL)	ALT (UI/mL)/bilirubin (mg/dL)*	Treatment	HBsAg detection, by immunoassay kits					HBsAg mutations
							EIAgen	AxSYM	Architect	Vitros	Monolisa Ultra	
1	88/Male	Caucasian	D	$1.7x10^7$	121/0.66	No	negative	positive	positive	negative	negative	G145R, T126I, T131A, C139Y, E/D144G
2	56/Male	Caucasian	A	$2.4x10^4$	47/1.52	Yes	negative	positive	positive	negative	negative	G145R, T126I, M133L
3	38/Female	Caucasian	D	$1.6x10^6$	562/4.8	No	positive	positive	positive	positive	positive	G145R
4	60/Female	Caucasian	D	$4.4x10^3$	11/0.27	No	positive	positive	positive	positive	positive	G145R
5	74/Male	Caucasian	D	$8.2x10^4$	84/0.65	No	positive	positive	positive	positive	positive	G145R
6	51/Male	Caucasian	D	$4.2x10^3$	324/0.48	No	negative	positive	positive	negative	negative	G145R, P120Q, T126I
7	55/Male	African	D	$6.7x10^6$	15/0.4	No	positive	positive	positive	positive	positive	G145A
8	40/Male	Caucasian	C	$3.0x10^1$	33/1.41	Yes	positive	positive	positive	positive	positive	G145R

*Alanine Aminotransferase level, reference range 0–40 U/L; total bilirubin reference range 0.4–1.2 mg/dL.

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