OVERLAPPING POL/S GENE ANALYSIS IN CHRONIC HEPATITIS B PATIENTS WITH COEXISTING HBSAG AND ANTI-HBs

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ABSTRACT

Introduction*: Simultaneous HBsAg and anti-HBs positivity can be seen in patients with chronic hepatitis B (CHB) infection. The paradoxical phenomenon of ongoing viral replication despite the presence of neutralizing antibodies makes simultaneous HBsAg and anti-HBs positivity one of the most interesting atypical serological profiles. The aim of this study was to investigate the relationship between the coexistence of HBsAg and anti-HBs and S gene mutations in study and control groups with CHB infection.*

Materials and methods*: Patients included in the study group were HBsAg and anti-HBs positive; patients in the control group were HBsAg positive and anti-HBs negative. The hepatitis B virus pol gene (reverse transcriptase region, amino acids 80-250) was sequenced by the Sanger dideoxy method. HBV primary/compensatory nucleos(t)ide analog resistance mutations and pol/S gene overlap mutations were both analyzed.*

Results*: Of the 2990 patients followed for CHB, 121 (4%) exhibited simultaneous HBsAg and HBs positivity. Serologic subtype was identified as homologous ayw2 in all patients in both groups. Amino acid changes in the "a" determinant were detected in 6 of the 21 patients in the study group but none were detected in the control group. HBsAg vaccine escape, HBIg escape, and diagnostic escape mutations were detected in 6 patients in the study group. In the control group, only 1 patient carried a vaccine escape mutation.*

Conclusions*: The presence of HBsAg escape mutants observed in our study seem consistent with the variant HBsAg hypothesis. However, the absence of a typical HBsAg escape mutation in the MHR or "a" determinant in many of the patients in the study group suggests that this atypical profile may also arise via a different mechanism.*

Keywords: *Hepatitis B virus, hepatitis B surface antigen, coexistent conditions, mutation, vaccine escape, direct sequencing.*

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Introduction

Chronic hepatitis B (CHB) infection continues to be an important public health problem despite the availability of potent antiviral treatments and an effective vaccine. An estimated 275 million people worldwide are infected with hepatitis B virus (HBV[(1)]. In Turkey, the prevalence of hepatitis B surface antigen (HBsAg) seropositivity in individuals over the age of 18 was reported to be 4%[(2)]. Ten genotypes (A-J) and approximately 40 subgenotypes of HBV have been identified based on degrees of variance in whole genome sequence analysis. In addition, 9 different HBV serological subtypes (ayw, ayw2, ayw3, ayw4, ayr, adw2, adw4, adwq, adr, and adrq-) have been identified based on HBsAg antigenic determinant combinations. There is a definitive correlation between HBV serological subtypes and genotypes[(3,4)].

HBsAg is the first serological finding detected during acute HBV infection. At 6 months after acute infection, disappearance of HBsAg and appearance of hepatitis B surface antibody (anti-HBs) indicates recovery and the development of immunity against HBV, while persistent detectable HBsAg indicates chronic infection. However, contrary to this common knowledge, some patients with chronic infection may test positive for both HBsAg and anti-HBs at the same time.

The paradoxical phenomenon of ongoing viral replication despite the presence of neutralizing antibodies makes simultaneous HBsAg and anti-HBs positivity one of the most interesting atypical serological profiles[5].

The "a" determinant (aa 124-147), which is located in the major hydrophilic region (MHR) of HBsAg and contains a substantial proportion of B cell epitopes, is the region targeted by natural or vaccine-induced antibodies and serological diagnostic tests. Amino acid (aa) changes in the MHR and especially in the "a" determinant due to S gene mutations can affect HBsAg antigenicity by altering the three-dimensional structure of the epitope. This leads to the emergence of immune escape variants (vaccine escape, diagnosis escape) that cannot bind to or have decreased binding affinity for Anti-HBs[6].

To date, various possible explanations for the coexistence of HBsAg and anti-HBs have been suggested. In some studies, this atypical profile has been associated with variant HBsAg that is able to evade neutralization by anti-HBs as a result of S gene mutations[7-12]. However, other authors proposed that the lack of serological subtype specificity in anti-HBs may cause this profile[13,14]. It was also reported in a recent study that the coexistence of HBsAg and anti-HBs may be associated with host genetic variations[15].

The aim of this study was to investigate the relationship between this atypical serological profile and S gene mutations in CHB patients with and without the coexistence of HBsAg and anti-HBs.

Materials and methods

Patient Group

The study group comprised 21 patients who were simultaneously HBsAg and anti-HBs positive, anti-HBe positive, HBeAg negative, and had HBV DNA level ≤103 IU/mL and normal ALT levels. The control group included 21 patients who were HBsAg positive and anti-HBs negative, anti-HBe positive, HBeAg negative, and had HBV DNA level ≤103 IU/mL and normal ALT levels.

The study and control groups had similar age and gender distributions. Patients in both groups were examined for co-infection with hepatitis C, hepatitis D, and HIV, and history of previous antiviral therapy or immunoprophylaxis was determined. Patients who had co-infection, history of antiviral therapy, immunosuppression, and those presenting with acute exacerbation of chronic disease were not included in the study. In both groups, the patients' immune phases were identified as "inactive carrier" according to the old terminology and "phase 3" according to the new terminology[16].

Serological markers of hepatitis B

Electrochemiluminescent assays were done using a Roche Modular Analytics E-602 device (Roche Diagnostics, USA). HBsAg titers > 0.05 IU/ml and anti-HBs titers ≥ 10 mIU/ml were regarded for positivity.

HBV DNA quantitation

HBV DNA quantification was performed using commercial real-time PCR assay (Roche/Cobas Ampliprep® TaqMan® System).

HBV sequencing

Primer pairs (F;5'-tcgtggtggacttctctcaatt-3' and R;5'-cgttgacagactttccaatcaat-3') were designed against HBV pol gene region. PCR run protocol was as follows: denaturation step; 95°C for 10 min. than followed by 35 cycles consisting of an annealing step; 95°C for 45 sec., and extension step; 60°C for 45 sec., a final step at 72°C for 45 sec. The PCR primers concentration was 0.3 mM. HBV product size was 742 bp and included overlapping pol/S gene region for HBsAg protein (111 - 227 codons). Hot Start polymerase was used for cycle sequencing. All PCR products were purified using the Highly Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced with ABI PRISM 310 Genetic Analyzer equipment using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequencing reaction was as follows: 35 cycles consisting of 95°C for 20 sec., 50°C for 25 sec., and finally 60°C for 2 min. The sequencing primers concentration was 0.5 mM. The sequences obtained with an electropherogram which were assembled using Vector NTI v.5.1 (InforMax, Invitrogen, Life Science Software, Frederick, MD).

HBV pol/S gene nucleotides were analyzed by the geno2pheno tool (Center of Advanced European Studies and Research; Bonn, Germany, http://coreceptor.bioinf.mpi - inf.mpg.de). The geno2pheno tool for HBV is designed as a database for the virtual phenotyping of hepatitis B and used nucleic acid sequences as input. The tool searches HBV mutations at the positions of amino acids for 80 to 250 in the rt domain in pol gene (13). We analyzed aa codons 80., 84., 85., 91., 169., 173., 180., 181., 184., 191., 194., 202., 204., 214., 215., 233., 236 - 238. for pol gene

mutations and aa codons 121., 135., 137., 139.-149., 151-153., 155-157., 161., 164., 172., 173., 175., 176., 182., 193 - 196. for HBsAg mutations. HBsAg mutations were categorized into four categories; vaccine escape, HBIg escape, diagnostic escape and immune escape[4,6,17].

Statistical analysis

Statistical analyses were performed using the MedCalc Statistics version 17.4 software package (MedCalc Software bvba, Ostend, Belgium) and SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were expressed as as percentage, minimum-maximum, mean, and standard deviation. The Kolmogorov-Smirnov test was used to determine whether variables conformed to normal distribution. Student's t-test and Mann-Whitney U test were used to compare continuous variables, and chi-square and Fisher's Exact tests were used to compare continuous variables. Level of significance was accepted as p=0.05.

The study was approved by the Ethics Committee of our Hospital (Date, 29 September 2011; Approval no., 11/2). Informed consents were obtained from each patient.

Results

Of the 2990 patients followed for CHB between May 2016 and May 2017, simultaneous HBsAg and anti-HBs positivity was detected in 121 (4%). The mean age of these 121 patients was 52 ± 14 years and 81% were aged 40 years or older. The mean age of the patients included in the study (study + control group) was 50 ± 13 years; 20 (47.6%) were male and 22 (52.4%) were female. HBsAg level was lower in the study group compared to the control group (p=0.006).

All patients in both the study group and control group were found to have HBV genotype D. Subgenotype distributions were 20 D1 and 1 D2 in the control group, and 15 D1, 3 D4, 2 D2, and 1 D3 in the study group. In terms of serological subtype, all patients in both groups exhibited ayw2 homology. The results are shown in Table 1 and Figure 1.

When aa changes related to serological subtype were excluded, one or more aa changes were detected in the MHR (aa 100-169) in 10 patients (47.6%) in the study group and 5 patients (23.8%) in the control group (p=0.107). The rate of aa change in the MHR was 1.76% in the study group and 0.4% in the control group (p<0.0001). Aa changes in the "a" determinant (aa 124-147) were detected in 6 (28.5%) of the 21 patients in the study group but none were detected in the control group. The aa changes in the "a" determinant were located in the first loop (aa 124-137) in 4 patients, the second loop (aa 138-147) in 1 patient, and both loops in 1 patient. Aa changes in the HBsAg C-terminal region (aa 170-226) were detected in 19 (90.4%) of the patients in the study group and 10 (23.8%) of the patients in the control group (p=0.002).

Characteristic	Study group, (n=21)	Control group, (n=21)	P value
Age, (mean±SD)	51±12	48±14	0.396
Gender, male, n (%)	9 (45)	11(55)	0.758
HBV genotype, n (%)			
D1	15 (42.9)	20 (57.1)	
D2	2 (66.7)	1 (33.3)	0.168
D3	1 (100)	0	
D4	3 (100)	0	
HBV DNA load, (mean±SD, IU/mL)	408±297	527±288	0.195
HBsAg titer, (mean±SD, IU/mL)	2107±1646	3545±1549	0.006

Table 1: Demographic characteristics and laboratory findigs of study and control groups.

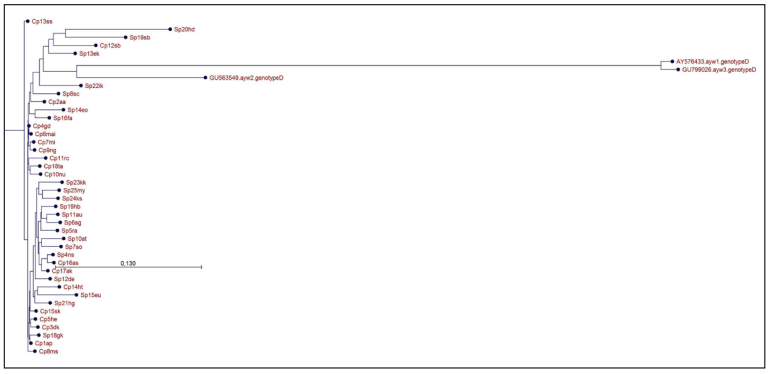

Figure 1: Phylogenetic tree of HBV strains in the study (Sp) and control patients (Cp). The phylogenetic analysis has been made according to Neighbor-Joining construction method and Jukes-Cantor protein distance measure bye polymerase/S gene overlapping region codons; reverse transcriptase (codon; 43-344) and S gene (codon; 34-277) using CLC Sequence Viewer v8.0 (Qiagen Aarhus A/S, www.qiagenbioinformatics.com) software. Bootstrap analysis was performed by 500 replicates. HBV genotype D reference sequences; AY576433.ayw1, GU563549.ayw2, GU799026.ayw3 obtained from GenBank. (HBV DNA sequence analysis was performed in 26 patient samples, but 4 patients were excluded from the study due to a history of exacerbation and a patient's history of antiviral drug use)

The rate of a substitution in the C-terminal was 5.17% in the study group and 1.33% in the control group (p<0.0001). The most common C-terminal aa changes were in aa 204 (6 patients), 206 (11 patients), 207 (10 patients), and 220 (6 patients). The results are shown in Table 2.

Amino acid status in HBsAg	Study group,(n=21)	Control group, (n=21)	P value
Patients with aa change in MHR, n (%)	10 (47.6)	5 (23.8)	0.107
*Rate of aa change in MHR, (%)	1.76	0.4	<0.0001
Patients with aa change in "a" determinant, n (%)	6 (28.5)	0	-
Rate of aa change in "a" determinant, (%)	2.97	0	-
Patients with aa change in C-terminal, n (%)	19 (90.4)	10 (47.8)	0.002
Rate of aa change in C-terminal, (%)	5.17	1.33	<0.0001

Table 2: Distribution of amino acid changes in the HBsAg in study and control group patients.

Abbrevations: aa; amino acid, MHR; major hydrophilic region
**Rate of aa change: number of amino acid substituted ÷ total number of amino acid x 100*

HBsAg vaccine escape (P120S, Q129R, G130N, T131N, S143L, G145R, S193L), HBIg escape (C121S, Q129R, Y134N, G145R), and diagnosis escape (P120S, G130N, M133T, Y134N, S143L) mutations were detected in 6 patients in the study group. In contrast, only 1 patient in the control group was found to carry a vaccine escape mutation (S193L). Immune escape mutations (I110L, S207N, T189I, Y134F) were detected in 8 patients in the study group and 4 patients in the control group. Primary drug resistance mutation in the RT region was not detected in any of the patients in either group. However, compensatory mutations were noted in 10 patients in the study group. Compensatory mutations associated with lamivudine and adefovir (Q215H, V214A) were detected in 7 patients, with telbivudine (L91I) in 2 patients, and with adefovir (Q149K, N238D) in 3 patients. One patient in the control group had a lamivudine- and adefovir-associated compensatory mutation (Q215H). The results are shown in Table 3.

HBsAg mutation position	Mutation category	Study group,(n=21) (%)	Control group, (n=21) (%)
P120S	Vaccine and diagnostic escape	1 (4.7)	-
C121S	HBIg escape	1 (4.7)	-
Q129R	Vaccine escape	1 (4.7)	-
G130N	Vaccine and diagnostic escape	1 (4.7)	-
T131N	Vaccine escape	1 (4.7)	-
M133T	Vaccine and diagnostic escape	1 (4.7)	-
Y134N	Diagnostic and HBIg escape	2 (9.5)	-
S143L	Vaccine and diagnostic escape	2 (9.5)	-
G145R	Vaccine and HBIg escape	1 (4.7)	-
S193L	Vaccine escape	3 (14.2)	1 (4.7)
M197T, S204N, Y206C/H, F220L	Low level HBsAg and HBV DNA-related	13 (61.9)	2 (9.5)
I110L, G130E, S207N, T189I, Y134F	Immun escape	8 (38)	4 (19)
Q215H, V214A	Lamivudine and Adefovir-related compensatory mutation	7 (33.3)	1 (4.7)
L91I	Telbivudine-related compensatory mutation	2 (9.5)	-
Q149K, N238D	Adefovir-related compensatory mutation	3 (14.2)	-

Table 3: The mutations-identified in the study and control group patients-which determined clinical significance were determined.

Discussion

The prevalence of the coexisted status in CHB has ranged between 2.6% and 8.9% in studies conducted in different countries[7-13]. It is more common among patients who experience exacerbations during immunosuppressive therapy or during the course of CHB (5). In this study, the prevalence of the coexisting of HBsAg and anti-HBs among patients with CHB was 4%. This rate is reported as 3.6% in another study from Turkey examining atypical serological profiles[18]. In a community-based cohort study conducted in China, this profile was more common with the D genotype of HBV and 84% of patients were over the age of 40; these findings were attributed to mutant accumulation that occurs with aging and the greater genetic variability of the D genotype[12].

The fact that 81% of these patients were aged 40 years or older in our study may support this conclusion.

Different views regarding the underlying mechanisms of HBsAg and anti-HBs coexistence have been put forth in previous studies. In two studies it was suggested that this atypical profile may emerge as a result of insufficient antigen-antibody binding due to a lack of serological subtype specificity in anti-HBs, which is expected to neutralize HBsAg[13,14]).

Most studies on simultaneous HBsAg and anti-HBs positivity have associated this profile with S gene mutations causing aa substitutions in the MHR and the "a" determinant. Thus emergenced HBsAg variants may be able to evade neutralization of anti-HBS and leading to the coexistence of HBsAg and anti-HBs status[7-12].

The high rate of aa changes in the "a" determinant and the detection of previously identified immune escape mutants in our study group seems consistent with this hypothesis. However, the fact that we did not detect a typical HBsAg escape mutation in the MHR or "a" determinant of 11 patients in the study group suggests that this profile may also emerge via a different mechanism. In these patients may required in-vitro investigation to explain of the HBsAg antigenicity affecting. Monoclonal antibody studies have demonstrated that not all aa substitutions in the S protein lead to loss of antigenicity, and that antigenicity can even be regained through another mutation accompanying the mutation known to cause antigenicity loss[19,20]. Therefore, elucidating the effect of each aa substitution on HBsAg antigenicity rather than focusing on aa variation rates in

HBsAg may help explain this atypical profile.

In contrast to these views, it was proposed in a recent study that the coexistence of HBsAg and anti-HBs may be associated with host genetic factors, independent of viral dynamics. The authors of that study reported that OAS3 (oligoadenylate synthetase 3) gene variants were more common among patients with this atypical profile compared to the control group, and suggested that OAS3 polymorphism may lead to HBsAg and anti-HBs coexistence by altering expression of the antiviral enzyme OAS (oligoadenylate synthetase)[15].

Our findings of genotype D and subgenotype D1 predominance, serological ayw2 homology, and similar demographic and laboratory values in both the patient and control groups (Table 1, Figure 1) may also suggest that HBsAg and anti-HBs coexisted may be independent of viral dynamics.

Evidence indicating that simultaneous positivity of HBsAg and anti-HBs in CHB patients may stem from host genetic factors would potentially change our perspective on the diagnosis and treatment of CHB.

During reverse transcription step in HBV replication, lack of proofreading capacity of RT enzyme dominate to creates HBV variants[3,4]. However, it has been shown that there are fewer HBV variants in patients in the immunotolerant phase, which shows that the genetic diversity of the virus is not independent of host immune response[21]. For this reason, unlike in other studies, we also matched immune phases of the patients in the study and control groups in the present study in order to investigate the relationship between HBsAg and anti-HBs coexistence and S gene mutations.

S gene mutants can become dominant due to naturally selection by the immune system during the course of chronic infection, or through selective suppression by external factors such as vaccination and HBIg or antiviral therapy[3]. It has been reported that naturally occurring "a" determinant mutations are more commonly found in the first loop and those associated with external factors are found in the second loop[22]. Considering that the results of our study reflect naturally occurring S gene mutations, the fact that aa changes in the "a" determinant were found in the first loop seems consistent with these literature data.

A noteworthy finding of our study was the high aa substitution rate in the C-terminal region of HBsAg among patients in the study group. Similar to our results, two studies on HBsAg and anti-HBs coexistence showed that the rate of aa substitution in the C-terminal region was high[11-13], while no significant difference was observed in the others[7-9]. Our current general knowledge is that the C-terminal region contains CTL epitopes pertaining to cellular immunity rather than humoral immune epitopes. It is argued that the immunoreactive region of HBsAg may extend to aa 207 and that the mutations in T cell epitopes located outside the MHR can also affect the three-dimensional structure of immunogenic determinants[23-26]. On the other hand, certain HBsAg aa alterations that are not escape mutations (M197T, S204N, Y206C/H, F220L) have been associated with low HBV DNA and HBsAg levels[27].

In our study, at least one of these 5 specific mutations was found in 13 patients in the study group but in only 2 patients in the control group. The significantly lower HBsAg level in the study group ($p=0.006$) might be related to the presence of these specific mutations. Similar to our results, several other studies reported low HBsAg levels in patients with HBsAg and anti-HBs coexisted status[9,11,13]. Although this may be attributable simply to partial neutralization of HBsAg or its reduced binding affinity for the antibody used to measurement it, other authors have suggested the role of intracellular accumulation and decreased extracellular secretion of HBsAg due to S gene mutations[6,28].

There are also differing opinions regarding the clinical significance of HBsAg and anti-HBs coexistence. In addition to studies reporting that this profile is associated with poor prognosis such as advanced fibrosis and cirrhosis, there are also studies that show no significant clinical difference and even that the majority of these patients are asymptomatic carriers[11,29,30]. Longitudinal cohort studies including histopathological analysis in well-matched patient and control groups may provide more information on the clinical significance of this atypical profile.

Due to the overlapping points in the HBV S and P genes, mutations in the S gene can lead to primary or compensatory antiviral resistance mutations in the P gene (encoding the RT enzyme), which in turn can potentially result in antiviral resistance[3]. In our study, we detected no mutations associated with primary drug resistance, but the rate of lamivudine, adefovir, and telbivudine-related compensatory drug resistance mutations was high in the study group in parallel to the high prevalence of S gene mutation.

In conclusion, the findings of this study suggest that coexisting of HBsAg and anti-HBs in patients with CHB may be partially attributable to typical HBsAg escape mutants; however, investigation of

different mechanisms such as host-related dynamics may further elucidate the possible causes of this profile and lead to a better understanding of the balance and interaction between the virus and host immune response in HBV infection.

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The study has been approved by the Clinical Research Ethics Committee of Kocaeli University (project no., KKAEK 2011/104; Date, 29 September 2011;Approval no., 11/2) and a written informed consent was obtained from each patient.

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