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Antiviral drug-associated potential vaccine-escape hepatitis B virus mutants in Turkish patients with chronic hepatitis B

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SUMMARY

Background: The hepatitis B virus (HBV) polymerase (*pol*) gene completely overlaps with the envelope (*S*) gene. Mutations in the *pol* gene of HBV, either from selection of primary or secondary resistance mutations, typically result in changes in the overlapping hepatitis B surface antigen (HBsAg). Recent studies have conferred a new acronym to these HBV *pol*/*S* gene overlap mutants: ADAPVEMs, for antiviral drug-associated potential vaccine-escape mutants. The present study aimed to assess the prevalence and pattern of ADAPVEMs in Turkish patients with chronic hepatitis B (CHB).

Methods: The investigation was conducted between March 2007 and July 2010 and involved a total of 442 patients. These patients were in the following phases of HBV infection: immune tolerant ($n = 50$), immune reactive ($n = 37$), inactive carrier ($n = 90$), HBeAg-negative CHB ($n = 217$), and HBsAg-negative ($n = 12$), or were hemodialysis patients ($n = 36$). One hundred eighty-six patients were receiving nucleos(t)ide analogue (NUC) therapy and 256 patients had treatment-naïve CHB.

Results: Seven types of ADAPVEM were detected in the total CHB patients: rtM204 V/sI195 M, rtM204I/sW196S, rtM204I/sW196L, rtV173L/sE164D, rtA181T/sW172*, rtA181T/sW172L, and rtA181 V/sL173F. The ADAPVEMs were associated with lamivudine, telbivudine, and adefovir. The prevalence of ADAPVEMs in all CHB patients was found to be 10% (46/442). The difference in the prevalence of ADAPVEMs across the different CHB clinical phases was not significant (Pearson Chi-square, $p = 0.112$). The prevalence of ADAPVEMs was 24% (44/186) in those undergoing NUC therapy and 0.7% (2/256) in the treatment-naïve group; this difference was significant (Pearson Chi-square, $p = 0.00$).

Conclusions: We determined the prevalence and pattern of ADAPVEMs in Turkish patients in the different phases of CHB. Preferred drugs in Turkey, such as lamivudine, have the potential to cause the emergence of ADAPVEMs, with the possibility that these will spread to both individuals immunized with the hepatitis B vaccine and nonimmunized individuals. ADAPVEMs should be monitored in infected and treated patients and their public health risks assessed.

1. Introduction

In many parts of the world, five hepatitis B virus (HBV)-specific nucleos(t)ide analogues (NUCs) in three subclasses targeting the viral polymerase are approved for the treatment of chronic hepatitis B (CHB): L-nucleosides (lamivudine, LAM; telbivudine, LdT), deoxyguanosine analogues (entecavir, ETV), and acyclic nucleoside phosphonates (adefovir, ADV; tenofovir, TDF).[1]

A major concern with NUC treatment is the selection of antiviral-resistant mutations. In particular, long-term therapy with NUCs is associated with an increasing risk of the development of drug resistance.[2] Mutations selected under NUCs can be split into two groups: those that cause resistance (primary), which sometimes lead to a decreased viral fitness, and compensatory (secondary) mutations, which partially or fully restore the level of viral fitness.[3] In addition, it is important to keep in mind that the HBV polymerase (*pol*) gene completely overlaps with the envelope (*S*) gene.[4] Therefore, mutations in the *pol* open reading frame, either from selection of primary or secondary resistance mutations, typically result in changes in the overlapping hepatitis B surface antigen (HBsAg). The results of changes are firstly that HBV replication may be restored by these *pol* gene mutations and result in relapse of hepatitis B infection with severe liver disease, and secondly that there may be a failure of infection prevention with vaccination or hepatitis B immunoglobulin (HBIg).[3]

LAM selected mutants – sE164D, sM198I, sI195 M, and sW196S – have shown reduced binding to hepatitis B surface antibody (anti-HBs).[3–5] Likewise, the point mutation that causes the

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rtA181T change in the *pol* also encodes a stop codon (sW172*) in HBsAg.[6] One recent report has provided evidence of involvement of HBV encoding the rtA181T/sW172* mutation in the pathogenesis of, and progression to hepatocellular carcinoma (HCC).[7] Some of the corresponding changes in HBsAg are associated with resistance mutations to ETV (rtI169T/sF161L and rtT184SL/sL176 V).[8] A recent study has conferred new acronyms to these HBV *pol*/*S* gene overlap mutants: ADAPVEMs, for antiviral drug-associated potential vaccine-escape mutants, and ADASMs, for antiviral drug-associated *S* gene mutants.[9]

It has been demonstrated in a study on HBV-seronegative chimpanzees that an in vitro generated polymerase gene mutant containing a combination of three mutations (rtV173L, rtL180 M, rtM204 V), two of which resulted in changes to the *S* gene (sE164D, sI195 M), was infectious.[10] To date, clinical evidence for the spread or transmission of ADAPVEMs has been limited.[11] No cases of infection by ADAPVEMs have yet been reported in human vaccinees, but the transmission of drug resistance-associated HBV mutants to unvaccinated individuals has been reported; however, the cases identified were infected by the LAM resistance-associated rtM204 V/sI195 M mutant. Whether or not the infecting HBV variants were naturally occurring is unknown.[12–14] Similarly, ADAPVEMs (LAM-resistant mutations) have also been found in the HBV isolated from Turkish hemodialysis patients with occult HBV infection, and these patients were not undergoing antiviral therapy.[15]

The present study aimed to assess the prevalence and types of ADAPVEMs in Turkish patients with CHB.

2. Materials and methods

2.1. Patients

The study was carried out between March 2007 and July 2010 at Kocaeli University Hospital and included patients already under treatment during this time period and those who were started on NUC therapy. The study involved all HBV DNA-positive samples detected by PCR from a total of 442 patients. These patients were in the following phases of HBV infection: immune tolerant ($n = 50$), immune reactive ($n = 37$), inactive carrier ($n = 90$), HBeAg-negative CHB ($n = 217$), and HBsAg-negative ($n = 12$), or were hemodialysis patients ($n = 36$). One hundred eighty-six patients were receiving nucleos(t)ide analogue (NUC) therapy and 256 patients had treatment-naïve CHB. The patients undergoing NUC therapy were being treated with oral LAM (Zeffix® 100 mg/day; GlaxoSmithKline, Middlesex, UK), ADV (Hepsera® 10 mg/day; Gilead Sciences, Inc., Foster City, CA, USA), ETV (Baraclude® 0.5 mg/day and 1 mg/day; Bristol-Myers Squibb Co., Princeton, NJ, USA), and TDF (Viread® 245 mg/day; Gilead Sciences, Inc.) as monotherapy or in combination therapy. All of the patients were categorized as chronic HBV carriers according to the European Association for the Study of the Liver (EASL) clinical practice guidelines.[1] The timing of serum sample collection was based on virological and/or biochemical breakthrough in those patients who were receiving NUC therapy. Blood samples were immediately separated by centrifugation, and the serum separated, aliquoted, and kept at −20 °C until testing. Serological markers of HBV (HBsAg, anti-HBs, antibodies to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to hepatitis B e antigen (anti-HBe)) were tested using commercially available microparticle enzyme immunoassay kits (Axsym, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany). However, serological data showed that all patients were HBsAg-positive, and hepatitis C virus (HCV; Axsym, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany), hepatitis D virus (HDV; Dia.Pro Diagnostic Bioprobes, Milan, Italy), and HIV (Axsym, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany) markers were negative. All of the patients had HBsAg positivity. None of the patients had received the HBV vaccination prior to their diagnosis as a chronic HBV carrier. Clinical and laboratory features of the study patients according to the EASL clinical practice guidelines are listed in Table 1.

2.2. DNA isolation and real-time PCR

HBV DNA was detected and quantified from serum samples by a commercial real-time PCR assay – COBAS Ampliprep/COBAS TaqMan HBV test (Roche Diagnostics, Mannheim, Germany).

2.3. Sequencing of the HBV pol gene region

Briefly, a pair of primers was designed (forward: 5′-TCGTG GTGGACTTCTCTCAATT-3′ and reverse: 5′-CGTTGACAGAC TTTCCAATCAAT-3′) for amplification of the HBV *pol* region. The PCR conditions were: 95 °C for 10 min, and then 35 cycles consisting of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. The final concentration of the primers was 0.3 μM. The size of the amplicon in HBV was around 742 bp, and included all the known NUC resistance mutations in HBV. Phire Hot Start DNA polymerase (Finnzymes Oy, Vantaa, Finland) was used in the sequencing protocol. All PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). Sequencing was carried out using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). The BigDye Terminator v3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), 36 cm capillary array, and

Table 1
Clinical and laboratory characteristics of the patients by study group

Characteristic	Immune tolerant phase	Immune reactive phase	Inactive carrier	HBeAg-negative CHB	HBsAg-negative phase	Hemodialysis[a]
No. of patients	50	37	90	217	12	36
Sex (M/F)	32/18	21/16	47/43	142/75	9/3	24/12
Age, median (range) years	45 (10–64)	33.5 (4–58)	27 (2–78)	29.5 (4–81)	44 (28–76)	35.5 (17–80)
ALT, median (range) U/l	59.5 (18–1082)	40.5 (14–157)	17.5 (10–59)	49 (12–537)	25 (16–498)	7.5 (1–77)
HBV DNA, median (range) IU/ml	$>1.16 \times 10^6$ (10^6–$>10^8$)	1.34×10^4 (10^2–10^5)	1.15×10^2 (10^1–10^3)	5.00×10^6 (10^3–$>10^8$)	5.10×10^3 (10^2–10^4)	4.4×10^3 (10^1–10^4)
HBV genotype (%)	D (100%)	D (100%)	D (100%)	D (99.5%) A (0.5%)	D (100%)	D (100%)
HBV subgenotype, *n* (%)	D1, 47 (94%) D2, 1 (2%) D3, 2 (4%)	D1, 31 (84%) D2, 4 (11%) D3, 2 (5%)	D1, 73 (81%) D2, 9 (10%) D3, 8 (9%)	D1, 183 (84%) D2, 24 (11%) D3, 8 (4%) D4, 1 (0.5%) A2, 1 (0.5%)	D1, 12 (100%)	D1, 25 (70%) D2, 8 (22%) D3, 3 (8%)

HBeAg, hepatitis B e antigen; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; M, male; F, female; ALT, alanine aminotransferase.
[a] No HBeAg data were obtained from the hemodialysis study group; however the median duration of dialysis in these patients was 10.5 years (range 5 months–27 years).

POP-7™ polymer (Applied Biosystems Inc.) were used. Sequencing was carried out according to the manufacturer's protocol (http://www3.appliedbiosystems.com).

2.4. Determination of HBV genotype/subgenotype and pol/S gene mutations

The Genafor/Arevir–geno2pheno drug resistance tool (Center of Advanced European Studies and Research, Bonn, Germany; http://coreceptor.bioinf.mpi-inf.mpg.de/) for HBV is a database that is specifically designed for rapid computer-assisted virtual phenotyping of HBV, and accepts genome (nucleic acid) sequences as input. Geno2pheno searches for homology between input sequences and others already stored in its database, which also stores relevant clinical data for HBV genotype/subgenotype and drug resistance/*S* gene mutations. The data accumulated by direct sequencing were analyzed either manually or using the geno2pheno tool for drug resistance and *S* gene mutations. The overlapping *pol*/*S* gene segments were searched for 344/266 amino acid codons in the reverse transcriptase (rt) domain of the *pol* gene.

2.5. Statistical analysis

Age, HBV DNA load, and alanine aminotransferase (ALT) levels were treated as numeric variables; gender, clinical phase of CHB, and ADAPVEM were treated as categorical variables. The significance of the difference between two numeric variables was compared using a Levene's test for equality of variances. The significance of the difference between two proportions was measured using the Pearson Chi-square test or Fisher's exact test. *p*-Values of ≤ 0.05 were considered statistically significant. Statistical analyses were done using SPSS v.13.0.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

All of the ADAPVEMs detected in patients undergoing treatment occurred in those with background LAM therapy (LAM monotherapy or ADV in addition to LAM therapy). Two of these patients had HCC related to HBV and three patients had cirrhosis (one patient had compensated cirrhosis and two had decompensated cirrhosis). Eighty of the patients who received LAM therapy had experience with peginterferon alpha-2b therapy. Three of these patients developed virologic relapse at the end of 48 weeks of treatment, four patients developed virologic resistance during treatment, and one of the patients had no virologic response at the end of 24 weeks of treatment. Clinical and laboratory characteristics of the ADAPVEM group of patients are shown in Table 2.

Seven types of ADAPVEM were detected in the total CHB patients: rtM204 V/sI195 M, rtM204I/sW196S, rtM204I/sW196L, rtV173L/sE164D, rtA181T/sW172*, rtA181T/sW172L, and rtA181 V/sL173F (Table 3). The ADAPVEMs were associated with LAM, LdT, and ADV. The prevalence of ADAPVEMs in all CHB patients was found to be 10% (46/442). With regard to CHB clinical phase, ADAPVEM prevalence was found to be 16% (8/50) in the immune tolerant phase, 8% (3/37) in the immune reactive phase, 7% (6/90) in inactive carriers, 13% (28/217) in HBeAg-negative CHB, 8% (1/12) in HBsAg-negative phase, and 0% (0/36) in hemodialysis patients. The difference in prevalence of ADAPVEMs across the different CHB clinical phases was not significant (Pearson Chi-square, $p = 0.112$).

Two of the patients detected with ADAPVEMs were treatment-naïve: patient 1 had primary LAM, LdT, and intermediate ETV drug resistance (rtL80I + rtM204I + rtQ215S/sW196L) and patient 2 had primary LAM, LdT, and ETV drug resistance (rtL180 M + rtS202C + rtM204 V + rtV214P/sI195 M). The prevalence of ADAPVEMs was 24% (44/186) in those undergoing NUC therapy and 0.7% (2/256) in the treatment-naïve group; this difference was significant (Pearson Chi-square, $p = 0.00$).

rtA181T/sW172* was found in 2% (1/46) of patients in whom ADAPVEMs were detected and in 11% (1/9) of acyclic phosphonate (ADV)-resistant cases (Table 3). However, none of the patients with HCC related to HBV had rtA181T/sW172* mutation combination.

The treatment-naïve patients had a median HBV viral load of 5.22×10^4 (range 10^1–>10^8) compared to 5.75×10^5 (range 10^1–>10^8) in patients undergoing treatment; treatment-naïve patients had a median ALT value of 17 (1–515) U/l compared to 180 (12–1082) U/l in patients undergoing treatment. According to Levene's test for equality of variances, the difference in the median HBV viral load value and ALT levels between treatment-naïve patients and those undergoing treatment were significant ($p = 0.001$ and $p = 0.012$). However, these values were not found to be closely related to ADAPVEMs. In addition, median age and gender ratios (male/female) were 55 (2–80) years and 39 (4–81) years, and 154/102 and 121/65, in treatment-naïve patients and patients undergoing treatment, respectively. The difference in median age was significant, but the difference in gender variation was not significant between the two groups (Levene's test for equality of

Table 2
Laboratory data and clinical features of ADAPVEM group patients

Characteristic	ADAPVEM
No. of patients	46
Sex (M/F)	30/16
Age, median (range) years	50.5 (24–77)
ALT, median (range) U/l	31.5 (13–537)
HBV DNA, median (range) IU/ml	5.02×10^5 (10^2–>10^8)
HBV genotype (%)	D (100%)
HBV subgenotype, *n* (%)	D1; 38 (83%)
	D2; 3 (6%)
	D3; 4 (9%)
	D4; 1 (2%)
Clinical status	Immune tolerant phase, *n* = 8 patients
	Immune reactive phase, *n* = 3 patients
	Inactive carrier, *n* = 6 patients
	HBeAg-negative CHB, *n* = 28 patients
	HBsAg-negative phase, *n* = 1 patient
Treatment status	Treatment-naïve, *n* = 2 patients
	Undergoing treatment, *n* = 44 patients

ADAPVEM, antiviral drug-associated potential vaccine-escape mutant; M, male; F, female; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen.

Table 3
Emergence of ADAPVEM according to the nucleos(t)ide analogues used to treat the patients

Nucleos(t)ide analogue	*pol* gene mutation	*S* gene mutation	*n* (%)[a]
L-nucleosides	rtV173L	sE164D	5 (11)
(LAM, LdT)	rtM204V	sI195M	11 (24)
	rtM204I	sW196S/L	25 (54)
Acyclic phosphonates	rtA181T	sW172*	1 (2)
(ADV, TDF)	rtA181T	sW172L	2 (4)
	rtA181V	sL173F	6 (13)
	rtV191I	sW182*	ND
Deoxyguanosine analogues	rtI169T	sF161H/L	ND
(ETV)	rtT184C	sL175F + sL176V	ND
	rtT184L/S	sL175F	ND
	rtT184G/M	sL176V/*	ND
	rtS202C/G	sS193F/L	ND
	rtS202I	sS194F/S	ND

ADAPVEM, antiviral drug-associated potential vaccine-escape mutant; LAM, lamivudine; LdT, telbivudine; ADV, adefovir; TDF, tenofovir; ETV, entecavir; ND, not detected.

[a] Some patients had multiple mutations, however the percentage of the mutation was calculated for 46 patients.

variances: $p = 0.014$ and $p = 0.091$, respectively). However, neither age nor gender was correlated with the presence of detected ADAPVEMs.

Direct sequencing results revealed HBV of genotype D in all patients, except one patient with genotype A (A2, 0.2% (1/442)) who was an HBeAg-negative CHB patient in the treatment-naïve group. According to the results of the HBV subgenotyping analysis, D1 was predominant, found in 84% (371/442) of all study patients, and subgenotypes D2, D3, and D4 were found in 10% (46/442), 5% (23/442), and 0.2% (1/442), respectively (Table 1).

4. Discussion

In a chronically infected individual, the extent of HBV replication is considerable, reaching $>10^{12}$ virions per day.[16] As *pol* is an rt that lacks proof-reading capacity, HBV replication is also associated with a high mutational rate of 10^{-5} substitutions/base/cycle.[17] Thus, all possible single-base changes in the HBV genome are generated daily, thereby accounting for the observation that mutations associated with NUC resistance and/or HBsAg escape exist in patients prior to therapy.[13]

Successful therapy should be aimed at suppressing all existing viral variants, thus preventing the selection of minority species and their subsequent evolution.[18] The genomic changes associated with drug resistance in HBV variants can be stable and these resistant viruses can be transmitted to another individual.[9,12] In populations where LAM has been used widely to treat patients continuously for periods of several years, as in Turkey (LAM is relatively inexpensive and an obligatory drug at the start of treatment in Turkey), viruses with alterations in the *S* gene are likely to occur relatively frequently, and some will be ADAPVEMs. The current results reveal ADAPVEMs associated with LAM (rtV173L, rtM204I/V, rtA181T/V), LdT (rtM204I/V), and ADV (rtA181T/V), rather than ETV (rtI169T, rtT184C/L/G/M/S, rtS202C/G/I) and TDF (rtV191I). Consequently, monitoring of pre-existing ADAPVEMs in treated patients is of the utmost importance for the selection of drugs and in order to prevent the emergence of ADAPVEMs.

The emergence of ADAPVEMs may present a risk to the local and/or global hepatitis B immunization program. Recently published reviews have reported that ADAPVEMs have the opportunity to spread to hepatitis B vaccinated individuals.[8,9,11,13] ADAPVEMs were detected in all clinical phases of CHB in this present study. However, ADAPVEMs were predominant in patients undergoing NUC therapy and were scarcer in treatment-naïve patients. Thus, there may be evidence of the emergence of ADAPVEMs in individuals undergoing treatment for CHB.

The primary and secondary drug resistance mutations associated with LAM and LdT (rtM204 V/sI195 M, rtM204I/sW196S, rtM204I/sW196L, rtV173L/sE164D) have been shown to result in reductions in the reactivity of the altered HBsAg with vaccine induced antibody against HBsAg.[3–5] In the present study, ADAPVEMs associated with LAM and LdT were found to be predominant in Turkish patients with CHB. The frequency of rtM204I/V + sI195 M/sW196S/L mutations in LAM-resistant cases in the SeqHepB database have also been shown to be predominant.[19] However, ADAPVEMs associated with ADV (rtA181T/sW172*, rtA181T/sW172L, rtA181 V/sL173F), which are also resistant to LAM and LdT, were detected in fewer cases (Table 3). One of the drug-resistant mutants, rtA181T/sW172*, has been shown to have oncogenic potential in transgenic mice.[7] The rtA181T/sW172* variant was detected in the SeqHepB database in 1% and 11% of LAM- and ADV-resistant cases, respectively.[19] We also detected the rtA181T/sW172* variant at nearly the same rate: 2% and 11% of LAM- (predominant in ADAPVEM detected cases) and ADV-resistant cases, respectively.

In Turkey, about 6500 individuals per year are newly infected with HBV, showing intermediate (2–7%) endemicity.[20] However, little is known about HBsAg escape mutations. Recently, the first HBV vaccine escape mutation (sT143*) was identified in a child with CHB in Turkey.[21] A study with sequencing of the amplified surface gene region has suggested sM125T and sT127P mutations for HBsAg escape in Turkish patients with CHB and their family members.[20] A recently published study reported a diagnostic escape HBsAg mutation (sS143L) causing chronic HBV infection in a previously vaccinated treatment-naïve Turkish patient.[22] However, recently we have seen the monitoring of the prevalence and pattern of the typical mutations for HBsAg escape and concomitant drug resistance mutations in patients undergoing NUC therapy and in treatment-naïve Turkish patients with CHB.[23] In this study, ADAPVEMs in Turkish patients with CHB were discussed for the first time. However, because of high perinatal transmission of HBV infection,[24] ADAPVEMs should be monitored in southeastern Turkey among treated women of childbearing age, especially in those undergoing LAM therapy. Neither HBIg nor active immunization would prevent infection with ADAPVEM transmitted from mother to child.[9]

In conclusion, we have revealed ADAPVEMs across the different phases of CHB in Turkish patients. Preferred drugs in Turkey, such as lamivudine, have the potential to cause the emergence of ADAPVEMs, with the possibility that these will spread to both individuals immunized with the hepatitis B vaccine and non-immunized individuals. ADAPVEMs should be monitored in infected and treated patients and their public health risks assessed.

Conflict of interest: This study was not funded by any organization. No conflict of interest to declare.

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