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Molecular Characterization of Drug Resistance in Hepatitis B Viruses Isolated from Patients with Chronical Infection in Turkey

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Abstract

Background: Hepatitis B virus (HBV) has a high mutation rate due to its unusual replication strategy leading to the production of a large number of virions with single and double mutations. The mutations, in turn, are associated with the development of drug resistance to nucleos(t)ide analogs (NUCs) in patients before and during NUCs therapy.
Objectives: The current study aimed at investigating the molecular characterization of HBV in Turkish patients with chronic hepatitis B (CHB) infection.
Methods: Polymerase chain reaction (PCR) amplification and direct sequencing procedures were used to analyze mutations. The detected drug resistance mutations were divided into the nucleos(t)ide analogs primary, partial, and compensatory resistance groups. The amino acid substitutions of hepatitis B surface antigen (HBsAg) were categorized into antiviral drug - associated potential vaccine - escape mutations (ADAPVEMs) and typical HBsAg amino acid substitutions, which included hepatitis B hyperimmunoglobulin (HBIg) - selected escape mutation, vaccine escape mutation, hepatitis B misdiagnosis, and immune - selected amino acid substitutions.
Results: The number of patients included in the study was 528 out of which 271 (51.3%) were treatment - naive and 351 (66.3%) were hepatitis B e antigen (HBeAg) - negative. Moreover, 325 (61.6%) were males with a mean age of 38 years (range: 18 - 69). Primary, partial, and compensatory resistance to NUCs was reported in 174 (32.9%) patients. Six different ADAPVEM motifs were determined in both treatment - naive and treatment - experienced patients, namely, sF161L/rtI169X, sE164D/rtV173L, sL172L/rtA181T, sL173F/rtA181V, sS195M/rtM204V, and sS196L/rtM204I. The prevalence of ADAPVEMs and typical HBsAg escape mutations was 5.3% (n = 28) and 34.8% (n = 184), respectively.
Conclusions: The analysis of drug resistance should constitute a fundamental part of the follow - up period of patients with CHB undergone treatment with NUCs. The surveillance of development of drug resistance mutations, while receiving treatment for hepatitis B is of paramount importance to monitor and control the emerging resistance.

Keywords: Hepatitis B Virus, Sequence Analysis, HBsAg, Antiviral Drug Resistance, Chronic Hepatitis B, HBV Polymerase

1. Background

Hepatitis B virus (HBV) is a prototype member of the family Hepadnaviridae. It consists of a partially double - stranded circular DNA genome of approximately 3200 bases with 4 overlapping open reading frames (ORFs) (1). The 4 ORFs are the core/precore, polymerase (*pol*), envelope (*env*), and X. The circular nature of the DNA and the arrangement of the ORFs cause the *env* gene to completely

overlap with the *pol* gene, and consequently result in important changes in hepatitis B surface antigen (HBsAg) and a considerable reduction in HBsAg - specific antibodies (anti - HBs) binding in vitro (2).

The high magnitude of HBV replication leads to the considerable production of virions (> 10^{12}) during each replicative cycle of the virus. As the reverse transcriptase (rt) encoded by the *pol* gene lacks a proofreading activity, HBV replication is associated with a high mutation rate of 10^{-5} substitutions/base/cycle. This results in the generation of all possible single - base changes in the genome, including single and double mutations, which in turn are responsible to develop nucleos(t)ide analogs (NUCs) resistance in patients before and during NUCs therapy (3).

The NUCs treatment strategies should be implemented as early as possible following the detection of drug - resistant HBV variants, especially before the virological and clinical breakthrough (4). Mutations may occur primary and secondary forms. Primary drug resistance mutations tend to reduce the susceptibility to an antiviral agent. However, secondary compensatory mutations repair replication defects related to primary drug resistance (5).

Turkey is one of the countries with a prevalence of 2% to 8% of intermediate endemicity for HBV infecion (6, 7). In a systematic review in Turkey, the estimated overall population with HBV prevalence was 4.57%, whereas the estimated total number of patients with chronic hepatitis B (CHB) was 3.3 million. However, the prevalence varied in different regions of the country; the prevalence was 3.47% and 6.72% in the Western and Eastern regions, respectively (8, 9).

With this background, the current study aimed at investigating the molecular characterization of HBV in Turkish patients with CHB.

2. Methods

2.1. Patients

The current study was conducted from 2010 to 2015 on 762 patients chronically infected with HBV from 7 regions and 35 clinics in 25 cities across Turkey. Due to the unsuccessful DNA sequencing reactions, 110 and 124 patients in the treatment - naive and treatment - experienced categories were excluded from the study, respectively. The clinical and demographic characteristics of the patients are mentioned in Table 1. The study consisted of 528 patients, treatment - naive (n = 271, 51.3%) and those under NUCs treatment (n = 257, 48.7%). All the patients were classified as HBV chronic carriers according to the European Association for the Study of the Liver (EASL) clinical practice guidelines (10). The study was also approved by the clinical research ethics committee of Kocaeli University (Project no. KKAEK 2009/24; date: November 24, 2009; approval no. 5/16), and written informed consent was obtained from each patient before entering the study. Blood samples were obtained before starting the therapy and during the viral breakthrough of the treatment. The samples were centrifuged immediately, and the sera were separated, aliquoted, and then, stored at - 20°C until use. Serological markers of HBV were measured by enzyme - linked immunosorbent assay (ELISA) in all local clinic units.

2.2. HBV DNA Detection

HBV DNA was isolated from the serum samples obtained from the patients on the BioRobot workstation using the magnetic particle technology (QIAsymphony SP; Qiagen GmbH, Hilden, Germany). HBV DNA was detected by a commercial real - time polymerase chain reaction (PCR) assay (Artus HBV QS - RGQ test; Qiagen GmbH, Hilden, Germany) on the real - time platform (Rotor - Gene Q; Qiagen GmbH, Hilden, Germany).

2.3. HBV pol Gene Sequencing

Specific primer pairs were constructed (forward: 5' - TCGTGGTGGACTTCTCTCAATT - 3' and reverse: 5, - CGTTGACAGACTTTCCAATCAAT - 3') for the amplification of the HBV *pol* gene region (11). The PCR conditions were as follows: denaturation at 95°C for 10 minutes followed by 35 cycles consisting of an annealing step at 95°C for 45 seconds, extension at 60°C for 45 seconds, and a final step at 72°C for 45 seconds. The final concentration of the primers was 0.3 mM. The size of the derived amplicon in HBV was approximately 742 bp and included all the known NUCs resistance mutations in HBV. Phire Hot Start DNA polymerase (Finnzymes Oy, Vantaa, Finland) was utilized in the sequencing protocol. All PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany). Sequencing was performed using an ABI PRISM 3130 genetic analyzer (applied biosystems Inc., Foster City, California, United States). The BigDye Terminator version 3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, United States), 36 cm capillary array, and POP - 7™ polymer (applied biosystems Inc.) were used for sequencing. For cycle sequencing, the following thermal protocol was used: 35 cycles consisting of 95°C for 20 seconds, 50°C for 25 seconds, and finally 60°C for 2 minutes. The reverse and sequencing primers were used at a final concentration of 0.5 mM.

2.4. HBV pol/surface Gene Mutation Determination

The sequencing data were analyzed using the Genafor/Arevir - geno2pheno drug resistance tool (center of advanced European studies and research; Bonn, Germany [http://coreceptor. bioinf.mpi - inf.mpg.de]). The geno2pheno tool for HBV is a database specifically designed for the rapid computer - assisted virtual phenotyping of Hepatitis B and utilizes genome (nucleic acid) sequences as input. The program searches for homology between the input sequence and other DNA sequences already stored in its database, including relevant clinical data for drug resistance and surface gene mutations (12). The tool searches for HBV drug resistance mutations in the rt domain of the polymerase at amino acid positions 80 to 250 (13). Drug resistance mutations to the NUCs were categorized into primary, partial, and compensatory resistance groups (14).

The overlapping S - gene segment was obtained using the geno2pheno tool for amino acid substitutions at positions from 100 to 196 (15). The amino acid substitutions in HBsAg were categorized into antiviral drug - associated potential vaccine - escape mutants (ADAPVEMs) and typical HBsAg amino acid substitutions. The latter included hepatitis B hyperimmunoglobulin (HBIg) - selected escape mutation, vaccine escape mutation, hepatitis B misdiagnosis, and immune - selected amino acid substitutions (5, 14, 16-19).

Some mutations, especially ADAPVEMs, were not located in the "a" determinant of the HBsAg protein. Further, the important neutralizing domains of the HBsAg protein including the region outside the "a" determinant of the HBsAg protein for ADAPVEMs were analyzed.

2.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics software, version 21.0 for Windows (SPSS; IBM corporation, New York, United States). The chi - square and t tests were utilized in the statistical analysis. A P value < 0.05 was considered significant.

3. Results

The current study consisted of a total of 528 patients, of which 325 (61.6%) were male with a mean age of 38 years (range: 18 - 69). Based on their HBeAg status, 351 (66.3%) patients were HBeAg - negative. Seven patients were anti - HIV antibody positive, 4 were anti - HDV IgG positive, and 1 patient was anti - HCV positive (Table 1).

The number and percentage of male patients in the treatment - naive and treatment - experienced groups were 170 (62.7%) and 155 (60.3%) (P = 0.56), respectively. The HBeAg positivity in these groups was detected in 65 patients (24.0%) and 113 patients (44.0%) (P = 0.01), respectively.

The patients in the treatment - naive and treatment - experienced groups reported a mean ± standard deviation (SD) age of 39.32 ± 11.47 and 38.38 ± 12.72 years, mean HBV viral load of 8.5 + E7 (± 6.6 + E8) and 6.2 + E7 (± 6.5 + E9) IU/mL, mean alanine aminotransferase (ALT) levels of 69.99 ± 199.84 and 73.68 ± 107.92 U/L, and mean aspartate transaminase (AST) levels of 51.01 ± 110.44 and 50.18 ± 60.12 U/L, respectively. The differences in age, HBV viral load, ALT, and AST levels in the patients in the treatment - naive and treatment - experienced groups were not significant according to t test results (P = 0.43, 0.71, 0.81, and 0.92, respectively).

Among 50 (18.5%) and 112 (43.6%) patients in the treatment - naive and treatment - experienced groups, respectively, there were 191 (70.5%) and 142 (55.3%) patients in HBeAg - negative CHB phase and immune - reactive phase, respectively (P = 0.01).

The patients were administered lamivudine (LAM, 33.5%), combination therapy (27.6%), tenofovir (TDF, 14.0%), entecavir (ETV, 11.7%), PEGylated interferon (5.4%), telbivudine (Ldt) (3.9%), and adefovir (ADV, 3.9%) at the time of viral rebound in the treatment - experienced group.

Genotype D was identified in 526 patients (99.6%), and genotype H only in 2 naive patients (0.4%) (Table 1). However, subgenotypes D1, D2, D3, and D4 were determined in 461 (87.3%), 29 (5.5%), 30 (5.7%), and 6 (1.1%) patients, respectively.

In total, 174 (32.9%) Turkish patients with mutations in the HBV *pol* gene were primary, partial, or compensatory resistant to NUCs. Among them, 79 (45.4%) patients belonged to the treatment - naive group, whereas 95 (54.6%) belonged to the treatment - experienced group. The prevalence of the mutation in the gene encoding HBV polymerase in the treatment - experienced patients was statistically different from those of the patients in the treatment - naive group (P = 0.05). The most common primary resistance mutation both in the treatment - naive and treatment - experienced patients was rtM204I/V ± rtV173L ± rtL180M. The frequencies and patterns of mutations in the HBV *pol* gene are displayed in Table 2.

The total prevalence of typical amino acid substitutions in HBsAg was 34.8% (n = 184). According to the treatment status, the prevalence was 33.5% (n = 91) and 36.1% (n = 93) in the treatment - naive and treatment - experienced patients, respectively. The HBIg escape mutation, vaccine escape mutation, misdiagnosis, and immune - selected escape mutations were 8.3%, 4.1%, 3.8%, and 18.6%, respectively. There was no statistically significant difference in the prevalence of HBsAg escape amino acid substitu-

Table 1. Clinical and Demographic Characteristics of the Patients

Variables	Value
Patients, no	528
Gender, M/F, (%)	325/203 (61.6/38.4)
HBeAg positivity, n (%)	178 (33.7)
ALT, median (range) U/L	37 (9 - 2584)
HBV DNA load, median (range) IU/mL	1.3 E+4 (6 E+2 - 9.8 E+6)
Participation from Regions[a], n (%)	
Marmara (Balikesir, Bursa, Edirne, Istanbul, Kocaeli)	218 (41.3)
Central Anatolia (Ankara, Konya, Kayseri)	104 (19.7)
South - eastern Anatolia (Adiyaman, Batman, Diyarbakir, Antep, Urfa)	73 (13.8)
Black Sea (Bolu, Giresun, Sakarya, Samsun, Tokat)	55 (10.4)
Aegean (Afyon, Denizli, Izmir)	48 (9.1)
Mediterranean (Antalya, Maras, Mersin, Osmaniye)	30 (5.7)
Clinical status, n (%)	
HBeAg negative CHB phase	333 (63.1)
Immune reactive phase	162 (30.7)
Inactive HBV carrier state phase	18 (3.4)
Immune tolerant phase	15 (2.8)
HBV genotype, n (%)	D: 526 (99.6) ; H: 2 (0.4)
HBV subgenotype of genotype D, n (%)	D1: 461 (87.7); D2: 29 (5.5); D3: 30 (5.7); D4: 6 (1.1)
Co - infection status, n (%)	
Anti - HCV positive	1 (0.2)
Anti - HDV IgG positive	4 (0.8)
Anti - HIV positive	7 (1.3)
Treatment status, n (%)	
Treatment - naive	271 (51.3)
Treatment - experienced	257 (48.7)
Drug choice status[b], n(%)	
Lamivudine	86 (33.5)
Combination	71 (27.6)
Tenofovir	36 (14.0)
Entecavir	30 (11.7)
Interferon	14 (5.4)
Telbivudin	10 (3.9)
Adefovir	10 (3.9)

Abrrevations: CHB, chronic hepatitis B; F, female; M, male.
[a] Patients included 35 different clinics from 25 cities in Turkey.
[b] Therapy situation during rebound.

tions in the treatment - naive and treatment - experienced patients (P = 0.57). Typical patterns of amino acid substitutions in the HBsAg escape mutants are depicted in Table 3.

Six different ADAPVEM motifs were located both in the treatment - naive and treatment - experienced patients.

Table 2. Characteristics of Genotypic Resistance Mutations to the Nucleos(t)ide Analogues

Mutation Characteristic, No. (%)	Mutation Pattern	Nucleos(t)ide Analogue	Patient No. (%)		P Value
			Naive	Experienced	
Primary Resistance Mutation n = 84 (15.9)	rtA181G/S/T/V	LAM, LdT, L - FMAU, ADV, TDF[a]	1 (0.18)	8 (1.5)	0.01
	rtT184A/I/L	ETV[a]	0	9 (1.7)	
	rtM204I/V ± rtV173L ± rtL180M	LAM, LdT, L - FMAU, FTC	9 (1.8)	50 (9.4)	
	rtM204V + rtT184S	LAM, LdT, ETV	0	1 (0.18)	
	rtI233V	ADV	3 (0.5)	1 (0.18)	
	rtN236T	ADV, TDF	1 (0.18)	1 (0.18)	
		Total	14 (2.6)	70 (13.2)	
Partial Resistance Mutation n = 20 (3.7)	rtT184L	ETV	0	1 (0.18)	0.06
	rtA194S/T/X	TDF	4 (0.7)	6 (1.1)	
	rtS202G	ETV	1 (0.18)	6 (1.1)	
	rtM250V/R	ETV	1 (0.18)	1 (0.18)	
		Total	6 (1.1)	14 (2.6)	
Compensatory Mutation n = 143 (27.0)	rtL91I	LdT	23 (4.3)	23 (4.3)	0.09
	rtQ149K	ADV	19 (3.5)	15 (2.8)	
	rtV191I	TDF	0	1 (0.18)	
	rtV214A	LAM, L - FMAU, FTC, TDF	2 (0.3)	3 (0.5)	
	rtQ215H/P/S	LAM, L - FMAU, FTC, TDF	37 (7)	18 (3.4)	
	rtN238D	ADV	1 (0.18)	1 (0.18)	
		Total	82 (15.5)	61 (11.5)	

[a] Propable resistance

They were sF161L/rtI169X, sE164D/rtV173L, sL172L/rtA181T, sL173F/rtA181V, sS195M/rtM204V, and sS196L/rtM204I. The total prevalence of ADAPVEMs was 2.9% (n = 8) and 7.7% (n = 20) in the treatment - naive and treatment - experienced groups, respectively. The prevalence of ADAPVEMs in the treatment - experienced patients was statistically different from that of the treatment - naive patients (P = 0.03). The ADAPVEM patterns were related to LAM, LdT, and ADV drugs. The ADAPVEMs motifs in the treatment - naive and treatment - experienced patients are displayed in Table 4.

4. Discussion

The current study focused on analyzing the prevalence of mutations in the Turkish people chronically infected with HBV. Six different primary resistance mutation patterns were detected in 84 (48.2%) patients. Fourteen (8%) of them belonged to the treatment - naive, whereas 70 (40.2%) of them belonged to the treatment - experienced group. However, the most common primary resistance mutation was reported rtM204I/V ± rtV173L ± rtL180M. The prevalence of primary resistance mutations in the treatment - experienced patients was statistically different from that of the treatment - naive patients (P = 0.01), indicating that primary resistance mutations occurred mainly in patients with the use of NUCs during treatment. The viral rebounds during antiviral treatment should be analyzed in terms of drug resistance. Antiviral resistance can be detected prior to treatment; therefore, screening to detect resistance prior to the commencement of treatment may be regarded as a rational approach. The primary resistance mutations associated with amino acid positions 181, 204, 233, and 236 were detected in the treatment - naive patients. Sayan et al., reported 2 resistance mutations (rtI233V and rtN236T) associated with acyclic phosphonates (ADV); however, the group could not detect YIMM or YMDD mutations in patients with naive - CHB in Turkey (11). The current study findings suggested a possible accumulation of primary resistance mutations for NUCs, which may be attributed to their widespread use in the last 5 years in Turkey.

The available literature reports an overall prevalence of

Table 3. Typical HBsAG Escape Amino Acid Substitutions of the Study Patients

HBsAg Amino Acid Substitution Category	Mutation Pattern	Patient No. (%)		Combined Pattern	Number of Patients	P Value
		Naive	Experienced			
HBIg escape	sT118A, sP120T, sT123A,sQ129R, sM133L, sY134N,sD144E, sG145K	23 (8.4)	21 (8.2)	sT123A + sG145K	2[a]	0.89
Vaccine escape	sP120S, sM133L, sS143L,sD144E, sG145R, sS193L	10 (3.7)	12 (4.6)			0.57
Hepatitis B misdiagnosis	sP120S/T, sR122K, sT131I, sM133T, sS143L	11 (4)	9 (3.5)			0.73
Immune - selected amino acid substitution	sQ101H/R, sI110L, sG119I/R, sP120T,T123A/N, sP127T, sG130K/R, sT131N, sT140I, sS143T, sD144E, sG145R	47 (17.3)	51 (19.8)	sS143T + sD144E + sG145R	1[a]	0.46
				sI110L + sP120T	2[a]	
				sQ101H + sI110L	1[a]	
				sQ101H + sP127T	1[a]	
				sQ101H + sI110L + P120T	1[a]	
	Total	91(33.5)	93 (36.1)			

[a] Number of combined pattern patients included to the mutation pattern.

Table 4. ADAPVEM according to nucleos(t)ide analogues in treatment naive and experienced patients

Mutation Characteristic	Mutation Pattern	Nucleos(t)ide Analogue	Patient, N (%)		P Value
			Treatment - Naive	Treatment - Experienced	
ADAPVEM N = 28[a] (5.3)	sF161L/rtI169X	ETV	1	-	0.03
	sE164D/rtV173L + sS195M/rtM204V	LAM, LdT	-	3	
	rtA181T/sL172L	ADV	-	1	
	rtA181V/sL173F	ADV	-	3	
	sS195M/rtM204V	LAM, LdT	4	7	
	sS196L/rtM204I	LAM, LdT	3	6	
Total			8 (2.9)	20 (7.7)	

Abbreviation: ADAPVEM, antiviral drug - associated potential vaccine - escape mutant; ADV, adefovir; LAM, lamivudine; LdT, telbivudine.
[a] Some of the patients had multiple mutations, however the percentage of mutation was calculated for 28 patients.

primary resistance mutations among treatment - naive patients to range from 1% to 30%. Such variability is likely to occur due to the differences in the methods applied to determine the mutation in the HBV *pol* gene, overall study design, and study population (20-29). However, Sayan et al., mentioned that the direct sequencing approach could limit the detection of primary resistance mutations (11). In the current study, the major primary resistance mutation, namely rtM204I/V, was usually found in combination with other mutation patterns both in the treatment - naive and treatment - experienced patients (30). The combination status regarding the rtM204V mutation may serve as a useful tool when selecting the study methodology.

The current study detected and characterized 4 different partial resistance mutations. They were mainly associated with ETV (rtT184L, rtS202G, and rtM250R/V). How-

ever, in half of the patients, the mutations in ADV gene (rtA194S/T/X) were detected. Patients carrying these mutations and undergoing a long - term therapy may require frequent monitoring for primary drug resistance against ETV and TDF (31, 32). The national insurance policy covered only LAM, Ldt, and interferons for first - line treatment (patients with HBV DNA < 2 E + 6 IU/mL). Until July 2015, the choice of treatment for CHB was limited; therefore, the current study did not discuss the type and duration of the therapy.

The most common compensatory mutations in the current study were rtQ215H/P/S, rtL91I, and rtQ149K in either single or combined profiles both in treatment - naive and treatment - experienced patients. The rtQ215H substitution was detected in patients receiving LAM or ADV therapy; however, its virological and clinical importance remained unclear (33). The primary function of compensatory mutations is to repair replication defects in viral polymerase activity related to the generation of primary drug resistance (11, 30, 34). Therefore, compensatory mutations can be detected during viral rebound, but without primary resistance during the NUCs therapy in patients with chronic hepatitis B. These, in turn, may assist to discriminate from primary drug resistance. Compensatory mutations without any primary or partial resistance mutations were detected in 27 patients in the treatment - naive and in 59 patients in the treatment - experienced groups. The substitution mutation, rtQ215, occurred even without exogenous selection pressures (35).

In the current study, six different types of ADAPVEMs were determined in Turkish patients with CHB predominantly associated with L - nucleosides (LAM and LdT) and ADV. However, ADAPVEMs were mainly observed in patients undergoing NUCs therapy (Table 4). The data gathered in the current study coincided with the findings of other studies (11, 36, 37), particularly, the frequency of rtM204I/V + sI195 M/sW196S/L mutations in LAM - resistant cases demonstrated a predominant presence (34). Thus, the ADAPVEMs can adversely affect the local or global immunization programs to control HBV with a potential to spread to individuals vaccinated against HBV infection (18, 19, 38, 39).

In the HBV genome, the HBV polymerase gene and envelope gene completely overlap (40); hence, the mutations in the *pol* ORF can cause alterations in the overlapping HBsAg. Typical HBsAg escape mutations were detected in the 4 categories of the patients. The immune - selected amino acid substitution category was the most common HBsAg amino acid substitution. However, some categories, such as HBIg escape and immune - selected mutations demonstrated certain patterns. Some typical HBsAg escape mutations, commonly detected in the patients with CHB, are sP120T, sM133I, sS143L, SD144A/E, sG145R, and sE164D (13, 14, 41). Typical HBsAg escape mutations may result as a consequence of a failure to control infection with vaccination or HBIg and misdiagnosis in the HBsAg testing stage (2). Patients with CHB undergoing NUCs treatments should be surveyed for typical HBsAg escape mutations for the benefit of public health (30, 41).

The determination of viral genotypes assists to analyze the progression of the disease, thereby aid to develop a suitable anti - viral therapy. Genotype D is widespread in Turkey and other Mediterranean countries (41, 42). D1 was more frequent with the presence of D1 - D4 subgenotypes in Turkey (43). The determination of genotypes and subgenotypes of HBV may contribute to accurate data generation associated with their circulation and transmissibility.

The coinfection data were very limited in Turkey; the coinfection of HBV/HIV, HBV/HDV, and HBV/HDV was 1.3%, 0.8%, and 0.2%, respectively. In a single large - scale study, Sayan et al., analyzed 1306 HIV - positive patients and coinfection of HIV/HBV was 2.7%. Sexual contact was reported as the acquisition route in 98.6% of the patients, whereas the use of injection drug was only 0.3% (44). Amiri et al., reported the coinfection of HBV/HIV as 1.8% and concluded that the use of injection drug severely affected the degree of coinfection (45). Both of the references 44 and 45 are regional (from Turkey and Iran).

In conclusion, the findings on drug resistance mutations in the treatment group require rational approaches such as prevention of unnecessary drug modifications due to compensatory mutations. The increasing incidence of drug resistance mutations warrants an analysis of drug resistance as an integral part to manage patients with CHB using NUCs. In addition, the surveillance of drug resistance mutations when treating HBV should be regarded as an area of supreme importance both for regional and global control of CHB.

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References

1. Miller RH, Kaneko S, Chung CT, Girones R, Purcell RH. Compact organization of the hepatitis B virus genome. *Hepatology*. 1989;**9**(2):322–7. [PubMed: 2643549].
2. Torresi J, Earnest-Silveira L, Deliyannis G, Edgtton K, Zhuang H, Locarnini SA, et al. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. *Virology*. 2002;**293**(2):305–13. doi: 10.1006/viro.2001.1246. [PubMed: 11886250].

3. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology*. 2006;**44**(6):1656–65. doi: 10.1002/hep.21422. [PubMed: 17133475].
4. Zoulim F, Perrillo R. Hepatitis B: reflections on the current approach to antiviral therapy. *J Hepatol*. 2008;**48 Suppl 1**:S2–19. doi: 10.1016/j.jhep.2008.01.011. [PubMed: 18304680].
5. Locarnini S. Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a consequence of treatment failure. *Hepatol Int*. 2008;**2**(2):147–51. doi: 10.1007/s12072-008-9048-3. [PubMed: 19669299].
6. Mistik R. *Viral hepatit*. In: Tabak, F , Balik, İ , Tekeli, E , editors. **1**. Istanbul: VHSD; 2007. Turkiye de viral hepatit epidemiyolojisi yayinlarin irdelenmesi, Yayinların irdelenmesi; p. 10–50.
7. Gurol E, Saban C, Oral O, Cigdem A, Armagan A. Trends in hepatitis B and hepatitis C virus among blood donors over 16 years in Turkey. *Eur J Epidemiol*. 2006;**21**(4):299–305. doi: 10.1007/s10654-006-0001-2. [PubMed: 16685581].
8. Tozun N, Ozdogan O, Cakaloglu Y, Idilman R, Karasu Z, Akarca U, et al. Seroprevalence of hepatitis B and C virus infections and risk factors in Turkey: a fieldwork TURHEP study. *Clin Microbiol Infect*. 2015;**21**(11):1020–6. doi: 10.1016/j.cmi.2015.06.028. [PubMed: 26163105].
9. Toy M, Onder FO, Wormann T, Bozdayi AM, Schalm SW, Borsboom GJ, et al. Age- and region-specific hepatitis B prevalence in Turkey estimated using generalized linear mixed models: a systematic review. *BMC Infect Dis*. 2011;**11**:337. doi: 10.1186/1471-2334-11-337. [PubMed: 22151620].
10. European Association For The Study Of The L. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol*. 2012;**57**(1):167–85. doi: 10.1016/j.jhep.2012.02.010. [PubMed: 22436845].
11. Sayan M, Akhan SC, Meric M. Naturally occurring amino-acid substitutions to nucleos(t)ide analogues in treatment naive Turkish patients with chronic hepatitis B. *J Viral Hepat*. 2010;**17**(1):23–7. doi: 10.1111/j.1365-2893.2009.01149.x. [PubMed: 19566788].
12. Papatheodoridis GV, Deutsch M. Resistance issues in treating chronic hepatitis B. *Future Microbiol*. 2008;**3**(5):525–38. doi: 10.2217/17460913.3.5.525. [PubMed: 18811237].
13. Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol*. 2006;**44**(3):593–606. doi: 10.1016/j.jhep.2006.01.001. [PubMed: 16455151].
14. Sheldon J, Rodes B, Zoulim F, Bartholomeusz A, Soriano V. Mutations affecting the replication capacity of the hepatitis B virus. *J Viral Hepat*. 2006;**13**(7):427–34. doi: 10.1111/j.1365-2893.2005.00713.x. [PubMed: 16792535].
15. Avellon A, Echevarria JM. Frequency of hepatitis B virus 'a' determinant variants in unselected Spanish chronic carriers. *J Med Virol*. 2006;**78**(1):24–36. doi: 10.1002/jmv.20516. [PubMed: 16299725].
16. Locarnini S, Bowden S. Drug resistance in antiviral therapy. *Clin Liver Dis*. 2010;**14**(3):439–59. doi: 10.1016/j.cld.2010.05.004. [PubMed: 20638024].
17. Sheldon J, Soriano V. Hepatitis B virus escape mutants induced by antiviral therapy. *J Antimicrob Chemother*. 2008;**61**(4):766–8. doi: 10.1093/jac/dkn014. [PubMed: 18218641].
18. Teo CG, Locarnini SA. Potential threat of drug-resistant and vaccine-escape HBV mutants to public health. *Antivir Ther*. 2010;**15**(3 Pt B):445–9. doi: 10.3851/IMP1556. [PubMed: 20516564].
19. Locarnini SA, Yuen L. Molecular genesis of drug-resistant and vaccine-escape HBV mutants. *Antivir Ther*. 2010;**15**(3 Pt B):451–61. doi: 10.3851/IMP1499. [PubMed: 20516565].
20. Sayan M, Cavdar C, Dogan C. Naturally occurring polymerase and surface gene variants of hepatitis B virus in Turkish hemodialysis patients with chronic hepatitis B. *Jpn J Infect Dis*. 2012;**65**(6):495–501. [PubMed: 23183201].
21. Vutien P, Trinh HN, Garcia RT, Nguyen HA, Levitt BS, Nguyen K, et al. Mutations in HBV DNA polymerase associated with nucleos(t)ide resistance are rare in treatment-naive patients. *Clin Gastroenterol Hepatol*. 2014;**12**(8):1363–70. doi: 10.1016/j.cgh.2013.11.036. [PubMed: 24342744].
22. Han Y, Huang LH, Liu CM, Yang S, Li J, Lin ZM, et al. Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naive patients. *J Gastroenterol Hepatol*. 2009;**24**(8):1417–23. doi: 10.1111/j.1440-1746.2009.05864.x. [PubMed: 19486254].
23. Jardi R, Rodriguez-Frias F, Buti M, Schaper M, Esteban R, Guardia J. Mutations at HBV-polymerase gene associated with entecavir drug resistance in patients not undergoing entecavir therapy. *Hepatol*. 2006;**44**:547A.
24. Lampertico P, Viganò M, Facchetti F, Puoti M, Minola E, Suter F, et al. Effectiveness of entecavir for the treatment of NUC-naive chronic hepatitis B patients: a large multicenter cohort study in clinical practice. *Hepatol*. 2008;**48**(Suppl 1):707A–8A.
25. Ludwig AD, Goebel T, Adams O, Baumann N, Hauck K, Fey H, et al. Primary resistance mutations against nucleos (t) ide analogues in treatment name patients with hbv-infection. *Hepatol*. 2008;**48**(4):701A.
26. Mirandola S, Campagnolo D, Bortoletto G, Franceschini L, Marcolongo M, Alberti A. Large-scale survey of naturally occurring HBV polymerase mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B. *J Viral Hepat*. 2011;**18**(7):212–6. doi: 10.1111/j.1365-2893.2011.01435.x. [PubMed: 21692935].
27. Salpini R, Svicher V, Cento V, Gori C, Bertoli A, Scopelliti F, et al. Characterization of drug-resistance mutations in HBV D-genotype chronically infected patients, naive to antiviral drugs. *Antiviral Res*. 2011;**92**(2):382–5. doi: 10.1016/j.antiviral.2011.08.013. [PubMed: 21920388].
28. Ergunay K, Kahramanoglu Aksoy E, Simsek H, Alp A, Sener B, Tatar G, et al. [Investigation of baseline antiviral resistance in treatment-naive chronic hepatitis B cases]. *Mikrobiyol Bul*. 2013;**47**(4):628–35. [PubMed: 24237431].
29. Bartholomeusz A, Locarnini SA. Antiviral drug resistance: clinical consequences and molecular aspects. *Semin Liver Dis*. 2006;**26**(2):162–70. doi: 10.1055/s-2006-939758. [PubMed: 16673294].
30. Sayan M, Akhan SC, Senturk O. Frequency and mutation patterns of resistance in patients with chronic hepatitis B infection treated with nucleos(t)ide analogs in add-on and switch strategies. *Hepat Mon*. 2011;**11**(10):835–42. doi: 10.5812/kowsar.1735143X.775. [PubMed: 22224083].
31. Liu Y, Wang CM, Cheng J, Liang ZL, Zhong YW, Ren XQ, et al. Hepatitis B virus in tenofovir-naive Chinese patients with chronic hepatitis B contains no mutation of rtA194T conferring a reduced tenofovir susceptibility. *Chin Med J (Engl)*. 2009;**122**(13):1585–6. [PubMed: 19719953].
32. Xu Y, Zhang YG, Wang X, Qi WQ, Qin SY, Liu ZH, et al. Long-term antiviral efficacy of entecavir and liver histology improvement in Chinese patients with hepatitis B virus-related cirrhosis. *World J Gastroenterol*. 2015;**21**(25):7869–76. doi: 10.3748/wjg.v21.i25.7869. [PubMed: 26167087].
33. Osiowy C, Villeneuve JP, Heathcote EJ, Giles E, Borlang J. Detection of rtN236T and rtA181V/T mutations associated with resistance to adefovir dipivoxil in samples from patients with chronic hepatitis B virus infection by the INNO-LiPA HBV DR line probe assay (version 2). *J Clin Microbiol*. 2006;**44**(6):1994–7. doi: 10.1128/JCM.02477-05. [PubMed: 16757589].
34. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, et al. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatol*. 2007;**46**(1):254–65. doi: 10.1002/hep.21698. [PubMed: 17596850].
35. Amini-Bavil-Olyaee S, Herbers U, Mohebbi SR, Sabahi F, Zali MR, Luedde T, et al. Prevalence, viral replication efficiency and antiviral drug susceptibility of rtQ215 polymerase mutations within the hepatitis B virus genome. *J Hepatol*. 2009;**51**(4):647–54. doi: 10.1016/j.jhep.2009.04.022. [PubMed: 19586679].
36. Sayan M, Akhan SC. Antiviral drug-associated potential vaccine-

escape hepatitis B virus mutants in Turkish patients with chronic hepatitis B. *Int J Infect Dis*. 2011;**15**(10):722–6. doi: 10.1016/j.ijid.2011.05.019. [PubMed: 21784687].
37. Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. *Hepatol*. 2008;**48**(1):88–98. doi: 10.1002/hep.22295. [PubMed: 18537180].
38. Clements CJ, Coghlan B, Creati M, Locarnini S, Tedder RS, Torresi J. Global control of hepatitis B virus: does treatment-induced antigenic change affect immunization?. *Bull World Health Organ*. 2010;**88**(1):66–73. doi: 10.2471/BLT.08.065722. [PubMed: 20428355].
39. Locarnini S. Transmission of antiviral drug resistant hepatitis B virus: implications for public health and patient management. *J Gastroenterol Hepatol*. 2010;**25**(4):649–51. doi: 10.1111/j.1440-1746.2010.06255.x. [PubMed: 20492319].
40. Torresi J. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol*. 2002;**25**(2):97–106. [PubMed: 12367644].
41. Sayan M, Senturk O, Akhan SC, Hulagu S, Cekmen MB. Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analog resistance mutations in Turkish patients with chronic hepatitis B. *Int J Infect Dis*. 2010;**14 Suppl 3**:136–41. doi: 10.1016/j.ijid.2009.11.039. [PubMed: 20382061].
42. Hadziyannis SJ. Natural history of chronic hepatitis B in Euro-Mediterranean and African countries. *J Hepatol*. 2011;**55**(1):183–91. doi: 10.1016/j.jhep.2010.12.030. [PubMed: 21238520].
43. Sunbul M. Hepatitis B virus genotypes: global distribution and clinical importance. *World J Gastroenterol*. 2014;**20**(18):5427–34. doi: 10.3748/wjg.v20.i18.5427. [PubMed: 24833873].
44. Sayan M, Sargin F, Inan D, Sevgi DY, Celikbas AK, Yasar K, et al. HIV-1 Transmitted Drug Resistance Mutations in Newly Diagnosed Antiretroviral-Naive Patients in Turkey. *AIDS Res Hum Retroviruses*. 2016;**32**(1):26–31. doi: 10.1089/AID.2015.0110. [PubMed: 26414663].
45. Bagheri Amiri F, Mostafavi E, Mirzazadeh A. HIV, HBV and HCV Coinfection Prevalence in Iran–A Systematic Review and Meta-Analysis. *PLoS One*. 2016;**11**(3):151946. doi: 10.1371/journal.pone.0151946. [PubMed: 27031352].