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ORIGINAL PAPER

Evaluation of the *pol*/S Gene Overlapping Mutations in Chronic Hepatitis B Patients in Northern Cyprus

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Abstract

Mutations associated with the *pol* and the S gene can emerge as a consequence of the high replication capacity and proofreading deficiencies of hepatitis B virus during replication. The current study was constructed to evaluate primary, partial, compensatory and the escape mutations in chronic hepatitis B patients in Northern Cyprus. The samples of HBsAg positive treatment naïve 100 patients were involved in this study. HBV *pol* gene region was sequenced, amplified and HBV *pol*/S gene mutations were determined. The samples of thirty-two patients were excluded because of their low viral load (HBV < 1000 ıu/ml). Among the sequenced 68 samples, there was a partial mutation (1.5%) and 36.7% displayed a resistance profile to lamivudine, adevofir, and telbivudine. Immune response escape, vaccine escape, HBIg and diagnosis escape mutations were determined in 24%, 10%, 6%, and 4% samples of the patients, respectively. Additionally, there were six different combined mutations. These data underscored that there is no concern for primary mutations in Northern Cyprus, however, we have identified a compensatory mutation (rtV173M) that may have primary mutation characteristics by combining with other mutation patterns. Additionally, HBsAg escape mutants demonstrated that detection of the S gene together with the *pol* gene mutations might be beneficial and important to monitor the surveillance of S variants.

K e y w o r d s: Drug resistance, mutations, hepatitis B, Northern Cyprus

Introduction

Hepatitis B virus (HBV) affects more than 2 billion people worldwide and approximately 247 million chronic individuals are known to be chronic carriers (Caligiuri et al. 2016; WHO 2018). HBV can cause severe liver infections and cirrhosis may develop in 15–40% of individuals if they are not treated (Tang et al. 2018). There are several novel treatment strategies that have been used for the treatment of chronic hepatitis B infection in order to prevent the risk of developing liver failure, cirrhosis, and cancer (Lapiński et al. 2013). Interferon alpha (IFN-α) 2a- 2b, PEGylated interferon-α-2a (*Pegasys*), PEGylated interferon-α-2b (*Pegintron*), and nucleos(t)ide analogues (NAs) are worldwide used for fighting chronic hepatitis B (CHB) infection by suppressing HBV replication (Ward et al. 2016). Lamivudine (LAM), telbivudine (LdT), entecavir (ETV), adefovir (ADV), and tenofovir (TDF) are antiviral agents that have been approved in Europe, America and many Asia-Latin America countries for the treatment of chronic HBV, however, there is a big concern of long term use of these agents as they are associated with the development of antiviral resistance mutations (Sun et al. 2016; Ozguler and Sayan 2016; Zhao et al. 2016; Ozguler and Sayan 2018). These mutations are associated with the amino acid substitutions in the reverse transcriptase (RT) domain and are classified as primary or secondary compensatory resistance mutations (Rugieri Pacheco et al. 2017). Mutations at the amino acid positions rt169, rt181, rt184, rt194, rt202, rt204, rt236, rt250 are classified as the primary resistance mutations which

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are associated with reducing the sensitivity to antivirals, whereas, the secondary compensatory resistance mutations occur at the amino acid positions rt80, rt173, rt180 and generally restore the replication capability of viral polymerase (Choi et al. 2018).

HBV is characterized by its high replication capacity ($>10^{12}$ virion/day) and a lack of proofreading activity during replication (10^{-5} substitution/base/cycle). This means that each nucleotide on the HBV genome can be changed within a day and cause antiviral resistance-related mutations before treatment (Tezcan et al. 2015). Also, the S gene is completely overlapped by the *pol* gene. Because of this overlap between the S and the *pol* gene, changes that are related to antiviral resistance in the *pol* gene may also occur in the S gene as well (Sayan et al. 2012). This overlap leads to amino acids structure, found in surface antigen (HBsAg) of HBV to change and thus form naturally occurring antiviral resistance mutations. The vaccine escape, diagnosis escape, hepatitis B immunoglobulin (HBIg) escape, and immune escape variants against oral antivirals that are used in chronic HBV treatment may emerge. Another reason for the emerging of these mutations is the occurrence of the mutant viruses deriving from antiviral-treated patients (Zhao et al. 2016). These changes cause reactivation of HBV in vaccinated people and concerns during diagnosis and/or HBIg vaccination failure (Wang et al. 2017; Asan et al. 2018).

Cyprus is an island located in the Mediterranean Sea and has been divided into two communities (Northern and Southern Cyprus). Northern Cyprus, officially the Turkish Republic of Northern Cyprus, is a multinational society due to universities, casinos, luxury hotels, beaches, and other entertainment centers; thus, there are many people coming from foreign countries for education, business and/or tourism purposes. For HBV infection, the prevalence rate ranges from 1.2% (160/13 892) (Arikan et al. 2016) to 1.35% (339/25 442) (Guler et al. 2018). For Southern Cyprus, this rate was given as 0.77% and 1.01% (Altindis et al. 2016). Although there are few studies regarding the prevalence, up to now, there has been no publication on drug resistance in patients infected with HBV in Northern Cyprus (Altindis et al. 2006; Suer et al. 2014). Therefore, we aimed to show the presence of resistance mutations and their clinical significance in untreated chronic hepatitis B patients in Northern Cyprus.

Experimental

Materials and Methods

In our study, the samples of 100 patients with HBsAg positive who had never been treated with NAs or IFN, were involved retrospectively. The study group consisted of 13 892 people who applied to the Near East University Microbiology Laboratory for assessment of hepatitis markers during three years. Of these individuals, 100 samples with positive HBsAg were included in the study for drug resistance analysis. The levels of viral markers [HBsAg, anti-hepatitis B core antigen (anti-HBc), anti-hepatitis B e antigen (anti-HBe), hepatitis B e antigen (HBeAg), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)] were determined by using the chemiluminescent enzyme immunoassay kits according to the manufacturer instructions [(Architect i200, Abbott, USA), (Roche, Cobas E411),

Table I
Demographic and clinical characteristics of the study group.

Variable		Study Group
Patient, n		100
Gender, F/M, n (%)		13 (13%)/87 (87%)
Age, median year (range)		35 (18–65)
Nationality, region/country, n (%)		
	Asia	**68 (68)**
	Turkey	43 (63)
	Northern Cyprus	14 (21)
	Pakistan	3 (4)
	China	3 (4)
	Turkmenistan	3 (4)
	Syria	1 (2)
	Georgia	1 (2)
	Africa	**29 (29)**
	Nigeria	28 (97)
	Benguela	1 (3)
	North America	**1 (1)**
	Mexican	1 (100)
	Europe	**2 (3)**
	Azerbaijan	1 (50)
	Bulgaria	1 (50)
Anti HBc IgG positivity, n (%)		96 (96)
HBeAg positivity, n (%)		5 (5)
ALT (average ± SD) (U/L)		23 ± 19
AST (average ± SD) (U/L)		28 ± 19
HBV DNA median IU/ml (range)		1.0 + E7 (1.9 + E1 – 2.8 + E8)
Genotype/subgenotype of HBV, n (%)		
Genotype D		**53 (78.0)**
D1		48 (70.6)
D2		4 (5.9)
D3		1 (1.5)
Genotype A		**7 (10.2)**
A1		5 (7.3)
A2		2 (2.9)
Genotype E		**8 (11.8)**
Treatment status, n (%)		
Naïve		100 (100%)
Under treatment		–

F – female, M – male, ALT – alanine aminotransferase, AST – aspartate aminotransferase, SD – standard deviation

(Olympus AU680, Beckmann Coulter IFCC)]. HBsAg positive sera were stored at –80°C until use. The ethical approval of the study was taken from Near East University Scientific Researches Evaluation Ethics Committee (YDUBADEK, 20/06/2013 date and NEU/2013/16-88 decision number).

The viral loads of positive samples were determined by using real-time polymerase chain reaction (PCR) technique according to the manufacturer instructions (artus HBV QS RGQ Qiagen, Hilden, Germany). The HBV *pol* gene (RT region between 80.–250. aa) was sequenced (between 254.–995. nucleotides), amplified and analyzed for *pol*/S gene mutations (Sayan et al. 2010a). Hepatitis B virus DNA (HBV DNA) was extracted from positive serum samples (Anatolia Geneworks, Bosphore® Viral DNA Extraction Spin Kit ve Magnesia® 16 Magnetic Bead Extraction System, Istanbul, Turkey). HBV polymerase region was amplified by using a pair of primers (forward: 5'-TCGTGGTGGACTTCTCTCAATT-3'and reverse: 5'-CGTTGACAGACTTTCCAATCAAT-3') (Sayan et al. 2010a; 2010b). PCR conditions were 95°C for 10 min, followed by 35 cycles consisting of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s (Sayan et al. 2010a). High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to purify all PCR products. The sequencing was performed on ABI PRISM 3130 (Applied Biosystems Inc. Foster City, USA) platform by using Phire Hot Start DNA polymerase (FinnzymesOy) enzyme and BigDye Terminator v3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc.), 36 cm capillary and POP-7 TM polymer (Applied Biosystems Inc.). The obtained data were analyzed by using Geno2pheno Drug Resistance program (the Center of Advanced European Studies and Research, Bonn, Germany) which searches for HBV drug resistance mutations at amino acid positions 80., 84., 85., 91., 169., 173., 180., 181., 184., 191., 194., 202., 204., 214., 215., 233., 236.–238., and 250. in the RT domain of the polymerase (Shaw et al. 2006). At the same time, the overlapping S gene segments at amino acid positions 121., 135., 137., 139.–149., 151.–153., 155.–157., 161., 164., 172., 173., 175., 176., 182., 193.–196. were also analyzed and monitored for HBsAg vaccine escape, diagnosis escape, HBIg escape, and immune escape mutations (Avellon and Echevarria 2006).

Results

The current study consisted of HBsAg positive-treatment naïve 100 CHB patients, of whom 13 were female and 87 were male, whose ages varied between 18–65 ages (with a median of 35 years). The origins of the patients were from Asia (68%), Africa (29%), North America (1%) and Europe (2%), and none of them have been taken antiviral therapy when their serum samples were collected. Ninety-six percent and 5% of the study group were positive for anti-HBc IgG and HBeAg, respectively. The serum HBV DNA level was calculated as a median of 1.0 + E7 (range: 1.9 + E1 – 2.8 + E8). ALT and AST levels were reported as 23 ± 19 and 28 ± 19, respectively. We sequenced only the samples of 68 patients in this study because HBV DNA was determined below 1000 IU/ml in 32 (32%) patients' samples. The distribution of genotypes of the sequenced patients (68/100) was determined as D1 (n:48, 70.6%), D2 (n:4, 5.9%), D3 (n:1, 1.5%), A1(n:5, 7.3%), A2 (n:2, 2.9%), and E (n:8, 11.8%) (Arikan et al. 2016). Demographic and laboratory findings of the study are displayed in Table II.

In the *pol* region, there was no primary drug resistance mutation detected in this study. There was only one treatment-naïve patient (1.5%) who had the partial resistance mutation at rt173M amino acid substitution that may be related with LAM and LdT resistance. Twenty-five patients (36.7%) had secondary/compensatory mutations and the mutations patterns (rtL91I, rtQ149K, rtQ215H/P/S and rtN238D) were depicted in Table II.

In the *S* region, 30 (44%) patients had typical HBsAg escape mutations. Vaccine escape, diagnosis escape,

Table II
Mutation characteristics of the HBV *pol*/S gene mutation patterns to the nucleos(t)ide analogues in treatment naïve HBV carriers.

HBV virion	Mutation characteristic	Mutation pattern	Nucleos(t)ide analogues	n (%)
Pol gene	Primary resistance mutation	ND	ND	–
	Partial resistance mutation	rtV173M	LAM-LdT related	1 (1.5)
	Compensatory mutation	rtL91I	LdT related	25 (36.8)
		rTQ149K	ADV related	
		rtQ215H/P/S	LAM-ADV related	
		rtN238D	LAM-ADV related	

ND – not determined, LAM – lamivudine, ADV – adevofir, LdT – telbivudine

Table III
Typical HBsAg escape and combined mutations of the study patients (n = 68).

Typical HBsAg escape mutation	Mutation patterns	n (%)	Combined mutations	Mutation patterns	n (%)
Immune escape	sY100C, sI110L, sP120L/R, sT123N, sT127L, sP127T, sA128V, sT131N, sS132P, sY134F/H, sT140I/S, sS143T, sD144E, sS144T, sP210S	16 (24)	Immune response -vaccine	sI110L + sS193L	1 (1.5)
Vaccine escape	sT126I, sD144A/E, sG145A/R, S193L, sP210T	7 (10)	Vaccine-HBIg	sP120L + sT123N + sT126I + sA128V + sY134H + sD144E + sG145A	1 (1.5)
HBIg* escape	sT118A, sP120T, sD144A/E, sG145A/E/R	4 (6)	Immune response -HBIg	sT118A + sP127T	1 (1.5)
Diagnostic escape	sT118A, sT131I, sP120T, sC121Y, sD144A, sG145R	3 (4)	Immune response-vaccine-HBIg Immune response-diagnosis	sI110L + sP120T + sD144A + sG145R sP120R + sC121Y, sT131I + sS132P	1 (1.5) 2 (3)
Total		30 (44)			6 (9)

* HBIg – Hepatitis B immunoglobulin
** Patients may have more than one mutation pattern

HBIg, immune escape mutations were determined in 7/68 (10%), 3/68 (4%), 4/68 (6%), 16/68 (24%) samples of the patients, respectively and these mutations are presented in Table III.

There were also six different combined mutations. The mutation patterns were sI110L + sS193L, sP120L + + sT123N + sT126I + sA128V + sY134H + sD144E + + sG145A, sT118A + sP127T, sI110L + sP120T + sD144A+ + sG145R and sP120R + sC121Y + sT131I + sS132P. The resistance mutation patterns are given in Table III.

Discussion

Even in treatment naïve patients because of the high replication capacity and the overlapping reading frames, antiviral resistance mutations may occur throughout the HBV genome (Yano et al. 2015). To our knowledge, this is the first study that focused on the resistance mutations in the HBV *pol* and S gene regions in treatment naïve CHB patients in Northern Cyprus. This information has confirmed that there was no primary drug resistance mutation in untreated patients. So, even if patients undergo long-term therapy with NA, they may not require periodic monitoring for the primary drug resistance. The previous studies indicated that primary resistance mutations could occur not only in patients undergoing an NA therapy but also might be detectable in patients receiving any treatment (Bottecchia et al. 2016; Zhao et al. 2016; Asan et al. 2018). The mutations that occur mainly at the amino acid positions 181, 204, 233 and 236 have been shown to be related with the primary resistance mutations in the treatment naïve patients (Asan et al. 2018). However, the mutations at the amino acid positions rt184, rt194, rt202, rt250 are mainly classified as partial resistance mutations and often act as compensatory mutations. The main functions of primary and partial resistance mutations are to reduce drug susceptibility and restore the viral replication defects, respectively (Asan et al. 2018). Thereof, the drug resistance should be analyzed during and/or before initiating the treatment to reduce the risk of liver damage and consequently progression of hepatocellular carcinoma. Sayan and his colleagues from Turkey have shown in many studies that acyclic phosphonate related mutations could occur in CHB patients (Sayan 2010b; Asan et al. 2018; Ozguler and Sayan 2018). Similarly, resistance mutations have been identified from other studies conducted in China and Pakistan (Mahmood and Anwar 2017; Zhao et al. 2016) which supports that primary resistance mutations can occur all over the world.

In the current study, we detected rtV173M partial resistance mutation in one patient according to Geno2pheno Drug Resistance Program, however, the rtV173L substitution has been reported more commonly in the literature (Lin et al. 2012; Gürsoy et al. 2019). Both amino acid substitutions are regarded as compensatory mutations and enhance viral fitness (Asan et al. 2018). The single compensatory mutation profiles are generally associated with low-level resistance; however, they may cause high-level resistance when they combine with other patterns (Lazarevic 2014). Thus, screening to detect resistance may be

regarded to be a benefit in the control of CHB. Sayan has shown in one of his studies that rtV173L mutation combined with sD144E produced HBV vaccine escape + HBIG escape (Sayan and Bugdaci 2013). LAM-associated resistance triple mutation pattern (rtV173L + rtL180M + rtM204V) has also been shown to enhance viral replication compared with rtL180M + rtM204V (Sheldon and Soriano 2008).

In the current study, among 68 patients, there were 25 (37%) secondary/compensatory resistance mutations (rtL91I, rtQ149K, rtQ215H/P/S, rtN238D) which are related to LdT, LAM, and ADV resistance (Saran et al. 2017; Asan et al. 2018). It may be a result of naturally emerging mutations due to the biology of HBV. The most common compensatory mutations have been reported to be rtQ149K, rtQ215H/P/S and rtL91I (Asan et al. 2018). These compensatory mutations are important as they assist viral replication and fitness, and hence are associated with drug resistance (Ahn 2015). In our study, we determined one of the most commonly detectable compensatory mutation rtQ215/H/P/S, which is associated with LAM and ADV resistance (Shaw et al. 2006; Altindis 2016; Asan et al. 2018).

Mutations in the *pol* gene, due to the structure of HBV, also occur in the overlapping HBsAg (Zaaijer et al. 2007; Simon et al. 2013) and some typical HBsAg escape mutations emerge. The immune response escape, HBV vaccine escape, HBIg escape, and HBsAg diagnosis escape were detected in 44 patients in this study. sP120T, Sm133I, Ss143L, sD144A/E, sD145R and Se164D are the most frequently detected typical HBsAg escape mutations in CHB patients (Sayan et al. 2010b). Misdiagnosis in the HBsAg, failure to prevent with vaccination and HBIg, reactivation of hepatitis B, and re-infection in HBV infected recipients and/or orthotropic liver transplantations may be a consequence of the typical escape mutations (Asan et al. 2018). All patients that receive or not receive antiviral treatment should be monitored for these mutations.

In conclusion, these findings on drug resistance mutations show that treatment-naïve CHB patients have low HBV polymerase resistance mutation (1.5%) rate. Hence, primary drug resistance analysis may not be necessary to be performed before the initiation of antintiviral therapy; however, we have discovered a novel compensatory mutation, rt173M that may act as a primary mutation together with other patterns. To draw attention, we preferred to define this mutation as a partial resistance mutation rather than compensatory resistance mutation in this study. Additionally, HBsAg escape mutants in patients living in Northern Cyprus demonstrated us that early detection of possible S gene mutations may be beneficial and important to monitor the surveillance of S variants. Even though national compulsory HBV vaccination program, these mutants may develop in hepatitis B vaccinated and/or HBIG vaccinated individuals with CHB, in chronic carriers and/or patients undergoing antiviral treatment and may pose danger for public health. In addition, due to the arrival of people from many countries to North Cyprus for the reasons we have stated, drug resistance mutations should be analyzed as *pol* and S gene together for monitoring CHB.

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Ethical Approval

Near East University Scientific Researches Evaluation Ethics Committee (YDUBADEK, 20/06/2013 date and YDÜ/2013/16-88 decision number.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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