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Evaluation of Lamivudine Resistance Mutations in HBV/HIV Co-infected Patients

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ABSTRACT

Background and Aim: The drug resistance mutations are key elements in the failure of long-term treatment of Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infections. The mutation in the YMDD motif in the *P* gene of HBV is the most critical factor in antiviral drug (especially lamivudine) resistance. This study aimed to assess the YMDD motif and other polymerase gene mutations in individuals with HBV/HIV coinfection.

Materials and Methods: All enrolled patients were under lamivudine treatment. Blood samples were collected from 37 HBV/HIV-positive patients, and DNA was extracted. The *P* gene was amplified by the PCR method with appropriate primers. The PCR products for detecting mutations in the *P* gene were sent to the Macrogen. To investigate the *P* gene mutations, the obtained sequences were compared with the polymerase gene of the HBV standard sequence in the GeneBank (accession number AB033559).

Results: The mean age of the patients was 34.1±5.7 years, of which 59.5% were male, and 40.5% were female. Of all patients, 56% were drug abusers, 35% had risky sexual behavior, 56% had prison history, and 33% had addicted wives. The 37 extracted samples were sequenced successfully. Among the studied samples (n =37), 28 patients had simultaneous mutations of YIDD and FLMAQ, 1 patient had YINN and FLIPH and 1 patient had YIDD and FSLAQ.

Conclusion: In summary, drug-resistant variants were detectable in most coinfected patients with chronic Hepatitis B (CHB) and HIV. As a result of mutations, therapeutic strategies sometimes are not effective. Therefore, recognition and monitoring of drug resistance mutations are critical.

Keywords: Coinfection, HBV, HIV, Lamivudine, Mutation, *P* gene

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1. Introduction

Hepatitis B virus (HBV) is known as a blood-borne virus and a member of the Hepadnaviridae family, which with approximately 400 million carriers, it is one of the major concerns for public health **(1)**. HBV and human immunodeficiency virus (HIV) have a common transmission route and therefore, coinfection is common in both infections. Almost 10% of patients worldwide infected with HIV are chronically coinfected with HBV **(2)**. Some studies have reported that HBV/HIV coinfection occurred in the course of HBV infection can increase HBV DNA levels in patients and eventually can lead to cirrhosis and Hepatocellular carcinoma **(1, 2)**. After HBV infection in HIV-infected people, the development of chronic hepatitis is 6

times more common than HIV-negative people **(3)**. This is more common in men with HIV infection and individuals with decreased CD4. In addition, in people with HIV infection development, protective anti-HBs responses may be defective **(4)**. The genetic characteristics of the HBV are very effective in the progression of the disease and the development of cirrhosis and even Hepatocellular carcinoma **(5)**.

By harboring four overlapping open reading frames (ORFs), including S, C, P, and X, HBV has a unique genomic structure **(6)**. The HBV *P* ORF is the longest ORF and encodes a 90-kDa protein with polymerase activity **(7)**. The YMDD motif in the catalytic domain of the reverse transcriptase region of the HBV *P* gene consists of tyrosine, methionine, and aspartate **(8, 9)**. It is also a common motif in RNA-dependent DNA polymerase in retroviruses, hepadnaviruses, retrotransposons, group II intron, and the catalytic subunit of telomerase and is involved in nucleotide binding in the polymerase **(10, 11)**. The mutation in the YMDD motif is the most important factor in antiviral drug (especially lamivudine (LAM)) resistance **(12)**. These mutations may reduce the efficiency of the drug against mutated strains **(13)**. In addition, the FLLAQ motif (glutamine, alanine, leucine, and phenylalanine) is also located in the reverse transcriptase region of the polymerase gene and, together with YMDD, can cause resistance to lamivudine **(14, 15)**.

For this reason, the management and treatment of HBV/HIV coinfection is a significant concern. Although anti-HIV and anti-HBV treatments can relieve symptoms and possibly minimize the level of HIV RNA and HBV DNA, Long-term LAM therapy may induce YMDD mutations and drug resistance **(16)**. Therefore, the study of the YMDD motif in patients with hepatitis B can provide beneficial information for physicians to adopt appropriate therapies. In this regard, the present research was undertaken to evaluate the HBV polymerase gene mutations in individuals with HBV/HIV coinfection under lamivudine therapy and determine other mutations associated with antiviral drugs.

2. Materials and Methods

Sample Collection

In this study, 37 HBV/HIV coinfected patients under lamivudine therapy were selected. Blood samples from patients were collected and separated plasma stored in -70°C. All patients were tested for the HBV serological markers (HBsAg and HBeAg) and HIV antibodies using an ELISA kit (Acontech, California, USA). Patients vaccinated for HBV, individuals with a history of immunoglobulin therapy, and antibodies against hepatitis C and D viruses were excluded. The study was approved by the Ethics Committee of Golestan University of Medical Sciences with the code number 773A2032980.

DNA Extraction and PCR Assay

The extraction of the viral genome from plasma was performed using the High Pure Viral Nucleic Acid Kit (Roche, Hamburg, Germany). The extraction of HBV DNA was performed according to the manufacturer's protocol, and then the extracted DNA was stored at -20°C for PCR assay. Totally, the DNA extraction was successful in 37 samples. The *P* gene of HBV was amplified by PCR method with appropriate primers. The length of the DNA sequence was 645 bp, and PCRs were performed with the sets of forward primer (5' GAT GTG TCT GCG GCG TTT TA '3') and reverse primer ('5' CAG CAA AGC CCA AAA GAC CCA C 3'), corresponding to the nucleotide positions 376-395 and 1021-1000 for forward and reverse primers, respectively **(15)**. For PCR, 5 µL of extracted DNA was added to an amplification mixture containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM/L dNTP mixture, 2.5U of Taq DNA polymerase (QIAGEN, Hamburg, Germany) and 1 pmol of each primer in a total volume of 50 µL. The amplification thermal conditions were as: an initial incubation at 94°C for 3 min and 30 cycles comprised of denaturation for 30 sec at 94°C, annealing for 30 sec at 55.9°C and extension for 50 sec at 72°C, with a final extension at 72°C for 2 min. The PCR products were electrophoresed, as shown in Figure 1. The PCR products for detecting mutations in *P* gene were sent to the Macrogen (South Korea).

DNA Sequencing and Mutation Analysis

To identify the location of the mutation, we compared our sequences with the reference sequence attained from GeneBank (accession number AB033559). In addition, the sequenced *P* gene was compared with the HBV genome sequence in Iran, which was registered in the GeneBank by Tehran University of Medical Sciences and Digestive Disease Research Institute. To determine the mutations, the resultant sequences were blasted with the reference sequences for HBV genotypes. Gene Runner software was also utilized to determine mutations in amino acid levels.

Statistical Analysis

Statistical analysis was performed using the SPSS version 22 (SPSS Inc., Chicago, IL, USA). Descriptive data were presented as mean± SD. T-Test was used to compare means. P-values less than 0.05 were considered statistically significant.

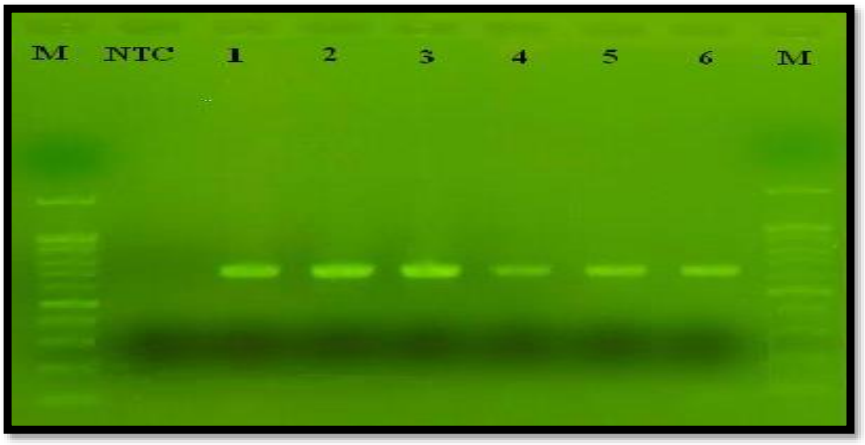

Figure 1. PCR products for samples 1-6. NTC: No template control

3. Results

Mutations in Nucleotide Sequences

In this study, the mean age of patients was 34.1±5.7 years, of which 59.5% were male and 40.5% were female. All HIV-positive patients were HBsAg positive, and 6 (10%) of patients were HBeAg positive. Of all patients, 56% were drug abusers, 35% had risky sexual behavior, 56% had prison history, and 33% had addicted wives. Demographic characteristics of enrolled patients are listed in Table 1.

Table 1. Demographic and other characteristics of enrolled patients

Patient No.	Gender	Age	Intravenous drug abuse	Risky Sexual Behavior	Prison	Wife addicted	YMDD Mutation	FLLAQ Mutation
1	MALE	48	yes	yes	no	no	Negative	Negative
2	FEMALE	39	no	no	no	yes	Negative	Negative
3	FEMALE	25	no	no	no	yes	Negative	Negative
4	FEMALE	54	no	no	no	yes	Negative	Negative
5	MALE	35	yes	yes	yes	no	Negative	Negative
6	FEMALE	17	no	no	no	no	positive	positive
7	MALE	54	yes	no	yes	no	positive	positive
8	FEMALE	35	no	no	no	yes	positive	positive
9	FEMALE	28	no	no	no	yes	positive	positive
10	FEMALE	32	yes	no	yes	yes	positive	positive
11	FEMALE	26	no	no	no	yes	positive	positive
12	MALE	45	yes	yes	yes	no	positive	positive
13	MALE	24	yes	no	yes	no	positive	positive
14	MALE	25	yes	no	yes	no	positive	positive
15	FEMALE	30	no	yes	yes	no	positive	positive
16	MALE	30	yes	no	yes	no	positive	positive
17	MALE	26	yes	yes	yes	no	positive	positive
18	FEMALE	17	no	yes	no	yes	positive	positive
19	FEMALE	45	no	no	no	yes	positive	positive

Patient No.	Gender	Age	Intravenous drug abuse	Risky Sexual Behavior	Prison	Wife addicted	YMDD Mutation	FLLAQ Mutation
20	MALE	40	yes	yes	yes	no	positive	positive
21	MALE	38	yes	no	yes	no	positive	positive
22	MALE	35	yes	yes	yes	no	positive	positive
23	MALE	45	yes	yes	yes	no	positive	positive
24	MALE	45	yes	yes	no	no	positive	positive
25	MALE	24	yes	no	yes	no	positive	positive
26	FEMALE	25	no	no	no	yes	positive	positive
27	MALE	52	yes	no	yes	no	positive	positive
28	MALE	44	yes	yes	no	no	positive	positive
29	MALE	40	yes	no	yes	no	positive	positive
30	MALE	26	yes	no	yes	no	positive	positive
31	MALE	23	no	yes	yes	no	positive	positive
32	FEMALE	24	no	yes	no	yes	positive	positive
33	FEMALE	40	no	no	no	yes	positive	positive
34	MALE	42	yes	no	yes	no	positive	positive
35	MALE	43	yes	no	yes	no	positive	positive
36	FEMALE	17	no	no	no	no	Negative	Negative
37	MALE	24	yes	no	yes	no	Negative	Negative
Total	M:22(59.5%) F:15(40.5%)	34.1±5.7	21 (56%)	13 (35%)	21 (56%)	12 (33%)	30 (81%)	30 (81%)

After sequencing, all sequences were aligned. According to our previous study, all isolates corresponded to Genotype D **(16)**. Generally, 7 deletions at position 419 and 13 deletions at position 1009 were found. In addition, 5 mutations at positions 420-421 and 20 mutations at positions 1013-1014 were insertion mutations, and the other mutations were translocation as listed in Table 2.

Table 2. Frequency of mutations in nucleic acid level

Position	Type	Nucleotide	Frequency
419	Deletion	C	7
420-421	Insertion	G	5
420	translocation	T-G	8
427	translocation	C-G	7
472	translocation	T-G	34
493	translocation	A-T	35
514	translocation	C-A	36
533	translocation	C-A	36
561	translocation	C-A	36
574	translocation	A-C	36
592	translocation	C-T	34
667	translocation	T-C	7

Position	Type	Nucleotide	Frequency
667	translocation	T-A	27
741	translocation	G-T	29
861	translocation	T-G	35
864	translocation	T-C	36
868	translocation	C-T	36
871	translocation	A-C	36
899	translocation	G-A	35
900	translocation	G-T	36
903	translocation	A-T	36
916	translocation	A-G	35
930	translocation	C-G	35
957	translocation	C-T	36
964	translocation	C-A	36
966	translocation	T-C	36
987	translocation	G-C	35
996	translocation	A-T	34
1005	translocation	C-T	32
1009	Deletion	C	13
1009	translocation	C-T	21
1010	translocation	T-G	31
1013	translocation	G-C	14
1014	translocation	T-C	11
1013-1014	Insertion	C	20

Investigation Mutations in Amino Acid Level

Gene runner software was used to investigate mutations at amino acid levels. Also, these sequences were double-checked with ClustalW. In the term of YMDD motif, 29 cases showed substitution of methionine to isoleucine (YIDD), and in one patient, aspartic was also converted to asparagine (YINN). In terms of the FLLAQ motif, 28 patients showed the conversion of leucine to methionine (FLMAQ). In a patient, leucine was converted to serine (FSLAQ). Also, in a patient, leucine was converted to isoleucine, alanine was converted to proline, and glutamine was converted to histidine (FLIPH). Among the studied samples (n=37), 28 patients had simultaneous mutations of YIDD and FLMAQ, 1 patient had YINN and FLIPH and 1 patient had YIDD and FSLAQ. However, seven patients indicated no mutation in YMDD and FLLAQ motifs. The frequency of mutations based on positions is listed in Table 3.

Table 3. Frequency of mutations in amino acid level

Frequency	Mutation type	Position
5	A to V, C, S, G	97
5	H to D	100
30	L to V, F	115
32	I to F	122
32	L to M, I	129
32	S to Y	135

Frequency	Mutation type	Position
32	K to Q	149
29	L to M	180
30	M to I	204
37	N to H	248
37	W to Y	257
36	N to D	263
36	H to Q	267
36	H to N	279
4	L to F- R to L	293
13	L to W, G	294
23	G to A, P, L- V to L	295
12	A to C, S	297
16	A to G, W	298
10	P to N, K, F- L to F	299

Drug Resistance Mutations

The data from Table 4 depicts that mutation at amino acid position 204 in the region C of the sequenced *P* gene caused the substitution of the methionine with isoleucine and caused resistance to lamivudine and telbivudine. Also, mutation at amino acid position 180 in region B changed the leucine to methionine and resulted in resistance to lamivudine. The L180M mutation alone had little effect on lamivudine resistance, while its combination with M204V and sometimes M204I increased resistance to both emtricitabine and lamivudine compared to M204V and M204I mutations individually.

Table 4. Drug resistance mutations

Agent	Mutations	Patients No.	Cross-resistant to	Sensitive to
Lamivudine	M204I	30	Other nucleoside Analogs (Telbivudine, Emtericitabine, Adefovir)	Adefovir Tenofovir Entecavir±MPA
	L180A	28		
Telbivudine	M204I	30		
Emtericitabine	L180A±M204I	28	Lamivudine	

4.Discussion

The HBV polymerase is the main target of therapeutic agents. Long-term LAM therapy may induce YMDD mutations and drug resistance, which would limit the effects of LAM treatment **(15)**. LAM therapy in HBV/HIV coinfection inhibits both HIV and HBV reverse transcriptase and may result in undetectable HBV DNA levels, relieving liver damages and causing HBeAg seroconversion **(17)**. In previous studies, the rate of YMDD mutations after one year of LAM therapy was 14–70% **(8, 11)**. In contrast, in the present study, the rate of YMDD mutation was 81% among patients with HBV. The YMDD-specific cytotoxic T lymphocytes may have an incomplete cross-reactivity with the YIDD and YVDD motifs, which often results in drug resistance **(18, 19)**.

One of the factors related to a mutation in lamivudine resistance is the simultaneous presence of two mutations, YIDD and YVDD **(20, 21)**. Our findings demonstrated the simultaneous mutation of YIDD and FLMAQ in 28 patients, YINN and FLIPH in one patient, and YIDD and FSLAQ in another patient. Kobayashi *et al*.'s study has reported that the liver's defense mechanism occurs in association with a single mutation, and none of the patients had two concurrent mutations in YIDD and YVDD, which is inconsistent with our findings **(22)**. Another factor related to lamivudine resistance is mutations in YMDD and FLLAQ motifs **(23)**. This observation is in agreement with a previous study conducted at Golestan Province of Iran **(24)**.

A survey on HBsAg-positive patients has reported that all patients with lamivudine resistance have the YMDD mutation; however, no FLLAQ mutation was observed in any of the patients **(22)**. Mutations affect the YMDD motif in the reverse transcriptase catalytic domain of the HBV polymerase gene, rendering the conversion of methionine to valine or isoleucine in codon 741 **(19)**. Mutation in the amino acid position 204 in the C region of the *P* gene has been shown to change the methionine to valine, leucine, and serine. In addition, a mutation in the amino acid position 180 (in the B region) changes the leucine to methionine **(25)**. Inoue *et al*. (2011) detected the mutation of codon 552 from methionine to valine (rtM204V) and methionine to isoleucine (rtM204I) in 42.9% and 28.6% of the patients, respectively. They also found a mutation in codon 528 from leucine to methionine (rtL180M) in 28.6% of the patients **(26)**. The YSDD mutation, which is a substitution of methionine to serine in codon 204 in the c-terminal polymerase region, was first reported by Bozdayi *et al*. in 2001 **(27)**.

In the present study, amino acid variations (change in methionine to isoleucine amino acids or change in leucine to methionine amino acids) resulted in resistance to lamivudine, adefovir, tenofovir, and entecavir. The viral resistance to adefovir is lower than lamivudine and occurs in patients with lamivudine resistance. In most cases, the rtM204I mutation in the YMDD motif disappears after treatment with adefovir. Delaney *et al*.'s (2011) study on lamivudine-resistant and adefovir-treated patients showed a link between the rtV173L mutation and both M204 and L180 mutations **(28)**. In addition, the rtV173L mutation is involved in resistance to lamivudine and famciclovir **(29)**. Co-occurrence of HBV/HIV infection and combination therapy with lamivudine and adefovir can be the reasons for this mutation **(28)**. Long-term treatment with nucleoside/nucleotide analogs can cause HBV drug resistance in patients with chronic hepatitis B. Arrese *et al*. have shown limited responses in patients with monotherapy and stronger responses with combination therapy **(30)**. Unlike their study, in which they detected rtM204I and rtM204V + rtL180M mutations, we did not find such mutations. Lacking observation of the other mutations in our study could be due to the research methods used above **(31)**.

The rate of YMDD mutations varies among different populations, and this variation may be related to the genotype of the infected population **(24)**. Relation between the types of mutations and the virus genotype in the population is one of the main factors in lamivudine resistance mutations **(32)**. Wang *et al*.'s study in Southern China disclosed that although genotype B is predominant in the region, the highest drug resistance was among individuals with genotype C **(33)**. Horgan *et al*. reported a connection between the genotype C and YMDD mutation and drug resistance **(34)**. Zhang *et al*. (2003) reported that 55.9% and 44.1% of patients are infected with genotypes B and C, respectively. They also recorded 22 mutations in 54.7% (238/435) of patients, in which the frequency of drug resistance mutations in genotype C was much higher than B (63.0% vs. 48.1%, *P*=0.003). The positions 180, 181, 191, 200, 202, 221, 229, and 224 were common mutation sites in the genotype C, and mutation in position 236 was more common in genotype B **(18)**. In patients with virological breakthrough, 11 (169, 202, 250, 173, 180, 200, 207, 214, 237, 242, and 245) and 9 (191, 207, 213, 218, 221, 224, 229, 238 and 242) mutations sites coexisted with M204I or M204V. In our study, all the infected individuals had a D genotype, and the YMDD mutation was found in this genotype. It must be mentioned that one of the genotypes that have YMDD mutation is genotype D.

The present survey detected M204I and L180M mutations in 30 and 28 patients, respectively; however, both M204V + L180M and M204I + M204V mutations were observed in all the patients. In this regard, Liaw *et al.*'s study, which was performed on patients with HBV, reported 12 patients with M204V + L180M mutation and 20 patients with M204I mutation **(11)**. Our results showed that the L180M mutation alone provides little resistance to lamivudine. Still, its combination with M204V and M204I gives rise to resistance due to the increased reverse transcriptase and transcription activity. In contrast, previous investigations in HBV/ HIV coinfected patients have displayed that these mutations alone can induce resistance to lamivudine, though the M204I mutation was more resistant to this agent **(35, 36)**.

4. Conclusion

In summary, drug-resistant variants were detectable in the majority of coinfected patients with chronic Hepatitis B and HIV. Today, different drug agents are active toward both HIV and HBV and different treatment algorithms. Therefore, we suggest that physicians draw a separate algorithm for HIV and HBV before starting treatment, and additionally, drug resistance mutations in both HBV and HIV are examined.

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Author Contribution

Conceive and design of the experiments: A.M; data analysis: A.M, E.B; writing of the paper: A.M, E.B, M.N, I.S; performance of the experiments: F.S.

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Conflict of Interest

None declared.

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