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Mutational analysis of reverse transcriptase and surface proteins of patients with partial virological response during mono and combination antiviral therapies in genotype D chronic hepatitis B

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Abstract

Introduction: The mutational pattern of chronic Hepatitis B virus (HBV) is unclear in patients who show incomplete response to antiviral therapy. The aims of this study were 1) to determine the benefit of combination therapy with adefovir dipivoxil (ADV) and Lamivudine (LAM) versus ADV or LAM alone in maintaining virological, biochemical and histological responses and 2) to investigate the patterns of mutations in the reverse transcriptase and surface proteins of HBV with LAM and/or ADF-resistant in partially-responded chronic hepatitis B (CHB) patients.

Methods: The study group consisted of 186 chronic HBV carriers who were admitted to the Tehran Hepatitis Network from 2010 to 2013. We retrospectively selected 86 patients who partially responded to different nucleoside analogue regimens. After 48 weeks of therapy, five groups of patients were defined including eight Lamivudine (LAM) Group (I), 30 Adefovir (ADV) Group (II), 16 ADV add on LAM Group (III), 32 ADV+LAM Group (IV), and 100 controls (no therapy). Reverse transcriptase (RT) and surface genes were amplified and sequenced for mutational analysis.

Results: All groups showed differences between mean values for age, gender, alanine transaminase (ALT), aspartate transaminase (AST), and HBV DNA levels groups showed significant differences than other groups ($p < 0.05$). The mutation frequencies for groups were I (1.7%), II (1.39%), III (2.28%), IV (2.0%), and V (0.38%). T54N, L80I/V, I91L/V, L180M, M204I/V, Q215P/S, and F221Y/S showed the highest number of mutations in all groups with different frequencies. Four new, unreported mutations were found.

Conclusion: Those patients who failed to respond in the first 48 weeks, whether they were receiving mono or combination therapy, should be tested genotypically, for the early modification of treatment.

Keywords: nucleoside analogues (NA), partial response, drug resistant variants, hepatitis, hepatitis b virus

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1. Introduction

Hepatitis B virus (HBV) infection is one of the most important causes of death worldwide; these infections induce chronic liver disease (1), and 400 million chronic infections have been documented (2). Annually, more than a million deaths are attributed to end-stage HBV liver disease, such as decompensated liver cirrhosis and hepatocellular carcinoma (HCC). The main goal of therapy in chronic hepatitis B (CHB) patients is to drive viral replication to the lowest possible level in order to halt the progression of chronic hepatitis and prevent liver failure, which occurs due to subsequent liver cirrhosis and the emergence of HCC. Lamivudine (LAM) and adefovir dipivoxil (ADV) were among the first approved oral nucleoside analogues (NA) that suppress viral replication to be used extensively due to their convenient administration and favorable tolerability (3, 4). They enhance hepatitis B e antigen (HBeAg) seroconversion and can markedly reduce HBV DNA levels as much as possible, ideally below the lower limit of detection of molecular assays, followed by biochemical remission, decreased necroinflammatory activity, and prevention of complications (4-6). To prevent recurrence of the disease, long-term polymerase inhibitor maintenance therapy often is required (7). Despite the initial effect of NA in suppressing HBV replication and reducing alanine aminotransferase activity being promising, the emergence of drug-resistant variants conferring reduced susceptibility to the inhibitory action of these drugs leads to a gradual loss of the benefits of antiviral treatment (8). The prevalence of resistance is in the range of 14–20% annually, and it reaches as high as 70% after four years of treatment for lamivudine (9, 10). There is a lower frequency of resistance to adefovir. While the resistance is 0% in the first year of therapy, the resistance to adefovir increases to 29% after five years of therapy (11). Therefore, ADV has a favorable resistance profile and could be effective for treatment especially as a standard therapy in LAM-resistant patients (12, 13). Although switching from one nucleoside analogue to another that does not share the same pattern of antiviral resistance can reestablish clinical benefit, sequential monotherapy predisposes patients to multidrug resistance (14, 15), and LAM-resistant patients treated short-term with ADV or without overlapping LAM have shown high incidence of ADV resistance (16-19). In many Asian countries, occurrences of multidrug-resistant HBV during treatment with LAM and ADV have increased (15). While mutations related to LAM and ADV resistance are not present within the same viral genome, combining the two antiviral agents may be enough to suppress dual-resistant HBV (20). However, data are still limited concerning the long-term efficacy of ADV rescue therapy, either in combination with LAM or switching treatment in LAM-resistant CHB patients. .According to the guidelines of clinical practice, a decrease in HBV DNA of more than 1 log10 IU/ml from the baseline pre-treatment is defined as partial virological response (21). Previous studies have been conducted using different protocols for alternate cross regimen in partially-responsive patients based on new NA drugs (22-25).

2. Material and Methods

2.1. Design and setting

The study group consisted of 186 chronic HBV carriers who were admitted to the Tehran Hepatitis Network.

2.2. Sampling

We retrospectively selected 86 patients who partially responded to a regimen of different nucleoside analogues after 48 weeks of therapy; we had 100 controls (no therapy). Eighty-six adult patients with chronic hepatitis B with HBV DNA levels above 10^6 copies/mL and high serum aminotransferase levels were eligible for enrollment, irrespective of their hepatitis B e antigen (HBeAg) status. Inclusion criteria were as follows: persistence of serum hepatitis B surface antigen for more than six months prior to antiviral therapy and experienced partial viral breakthrough, which was defined as the reappearance of serum HBV-DNA using real time PCR-based assays on two or more occasions after its initial disappearance. We excluded patients who had any evidence of liver cirrhosis. In this study, the exclusion criteria were patients with autoimmune hepatitis, alcoholic liver disease, hepatitis C virus, hepatitis D virus or HIV markers, decompensated liver disease, organ transplantation, immunosuppressive therapy, and active use of alcoholic beverages.

2.3. Patient Groups

Group I consisted of eight patients who had received LAM mono therapy for an average of 48 months. Group II was composed of 30 patients who had been receiving ADF mono therapy for 48 months. Group III consisted of 16 patients who previously had received LAM, and, subsequently after resistance, received add-on therapy with ADF for > 48 months. Group IV consisted of 32 carriers who had received LAM and ADV in combination for > 48 months. Group V was composed of 100 patients who had not received any antiviral treatments (control group).The dosage for lamivudine and adefovir dipivoxil were 100 and 10 mg per day orally, respectively. All patients were followed up every 12 weeks during their treatment with LAM or ADV, according to European Society for the Study of Liver (ESAL) guidelines (21). The decrease in HBV DNA of more than 1 log10 IU/ml was defined as partial

virological response. All patients provided written, informed consent for enrollment in the study, which was approved by the institutional review board of the local ethics committee.

2.4. DNA extraction, Polymerase Chain Reaction, and DNA Sequencing

HBV DNA was extracted from 200 µl of sera using a Qiagen Mini Blood Kit (Purelink 96 Viral RNA/DNA Qiagen, Hilden, Germany) by automated extractor following the manufacturer's instructions. DNA was eluted using 100 µl of elution buffer and stored at -20 °C. For the amplification of RT and surgafe genes, nested polymerase chain reactions were conducted in 100 and 50 µl, respectively, of a mixture that contained 5 µl of the extracted DNA using appropriate primers as described previously (26, 27). Direct sequencing of Polymerase Chain Reaction (PCR) products was conducted in a DNA sequencer (Perkin Elmer ABI-3130XL DNA Sequencer, Foster City, CA, USA) using 0.5 µl of internal specific primers (22). The electropherograms were checked and edited by Chromas software.

2.5. Mutational analysis

Genotyping was done for all sequences according to amino acid variants specifying HBV genotypes A to H within overlapped surface proteins. Amino acid variations within polymerase protein were compared with reference sequences obtained from different HBV genotype and sequences from Iranian isolates obtained from Gen Bank and NCBI. Comparing to the former, any amino acid changes were defined as "variants." Regarding the Iranian database sequences, amino acid differences were defined as "mutations." The sequences were analyzed using Bio Edit Software, version 7.0.5.3. The amino acid mutation frequencies were obtained by drug-resistance mutation found in individual RT domains (F, A, B, C, D and E) divided by the number of amino acid residues in that particular domain.

3. Results

3.1. Basic characteristics of patients

The demographic and baseline characteristics and the mean changes of serum ALT, AST, and HBV loads are shown in Table 1. All patients completed 48 weeks of therapy. In the groups of patients, 64 were males (74.4%), and 22 (25.6%) were females. In the control group, 70 (70%) were males, and 30 (30%) were females. The mean age of the patients in the patient groups was 34.6. As shown in Table 1, the group-I patients were older than the patients in the other groups. Significant associations were found between HBV DNA and AST levels among all of the groups ($p < 0.05$). Groups I and IV had the highest and lowest HBV viral load levels, respectively ($p < 0.05$). The mean level of ALT was higher in group I than in the other groups (however, $p > 0.05$). Group II had the lowest levels of ALT/AST.

Table 1. Classification, demographic, and clinical features of the patients

Groups	Description	Male/Female	Mean Age (±SD)	Mean HBV (Copy/mL)	Mean ALT (U/ml)	Mean AST (U/ml)
I	LAM only	5/3	49±16.7	155,757,791	94.5	60.2
II	ADF only	23/7	38±12	31,502,263	46.6	33.9
III	ADF add on LAM	12/4	35±13	97,787,744	63.8	39.6
IV	LAM & ADF	24/8	37.5±12	21,157,044	61.2	35.2
V	Control	70/30	34.6±12	15,000	84	41
p-value	-	$p > 0.05$	$p > 0.05$	$p < 0.05$	$p > 0.05$	$p < 0.05$

3.2. Distribution of Mutation in RT Domains

The distribution of amino acid substitutions within the main functional domain of RT (including A to E) showed significant differences between the five groups ($p < 0.05$) (Figure 1). In domains A, B, C, and E, the numbers of amino acid changes were higher in group III (ADV add on) than in the other groups (Figure 1). Group IV had the highest number of amino acid substitutions in domain D. In all domains, the control group (V) showed the lowest percentage of amino acid mutations. According to the results (Figure 1), in brief, the respective domains of RT in terms of weight of amino acid changes were as follows: Group I (LAM only): C > A > B > E > D; group II (ADV only): C > A > E > B > D; group III (ADV add on): C > A > B > E > D; group IV (ADV+LAM): C > A > D > B > E; and group V (control): A > C > B > D > E (p value for all domains between groups ranged from < 0.001 to <0.01, results not shown). Therefore, domains C and A were the most common domains that harbored the highest number of mutations between all groups, including the control group.

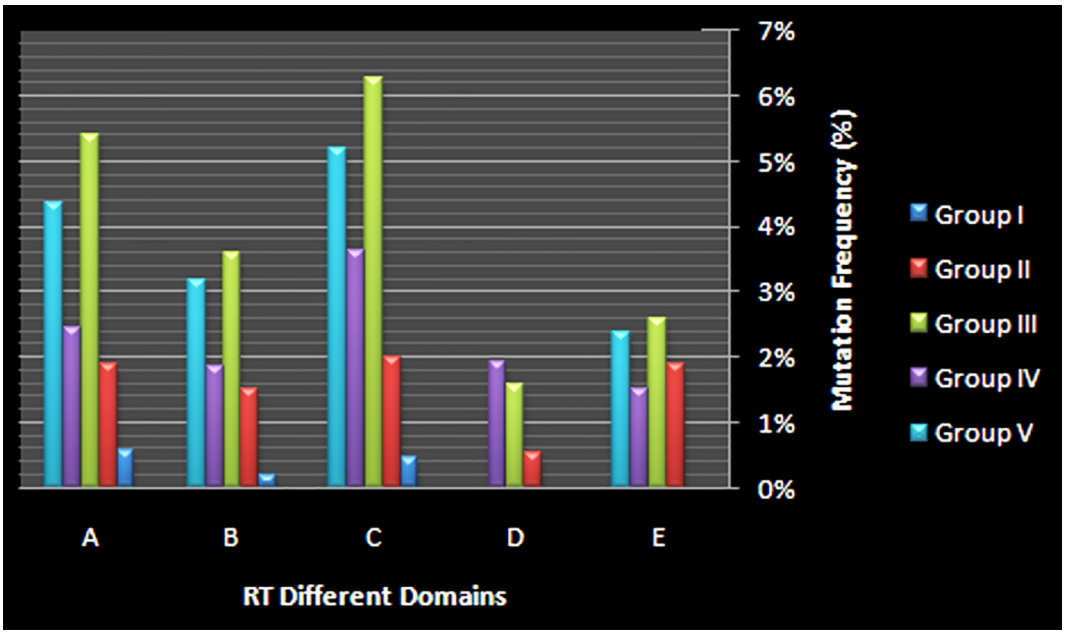

Figure 1. Frequency of mutations patient groups within different RT domains (A to E)

Table 2. Mutation residues and mutation frequencies at positions of HBV Reverse Transcriptase (rt) for common antiviral drug-associated resistance variants. Note: The order of amino acid positions is arranged according to the pattern of related drug resistance to NAs mono- or combination therapy

Group Description		I (LAM only)	II (ADF only)	III (ADF add on LAM)	IV (LAM & ADF)	V (Control)
Afefovir-related Resistance	rtT54N (sT45N)	0%	3.3% (6.6%)	0%	21.80%	0% (3%)
	rtV84M (sM75I/R)	0%	0%	12.50% (12.5%)	0%	0%
	rtS85A (sC76S/Y)	0%	3.3%	0% (12.5%)	0%	0% (3%)
	rtM129L (sP120A/T/S)	0% (12.5%)	3.3%	0% (6.25%)	0% (6.4%)	5% (1%)
	rtS213T (sS204R/N)	0%	6.6% (6.6%)	0%	0% (3.2%)	5% (13%)
	rtV214E/A (sL205P/M)	0%	0%	6.25%	0%	1% (3%)
	rtL217R (sI208T/S)	0% (37.5%)	3.3% (9.9%)	3.3% (12.5%)	0% (22.4%)	0% (4%)
	rtF221Y/S (sF212L)	25%	6.6%	6.25%	19.2% (3.2%)	4% (1%)
	rtN236T	0%	0%	0%	3.2%	0%
	rtP237H	0%	16.5%	0%	0%	0%
	rtN238H/T/Y	0%	3.3%	18.75%	3.2%	0%
Lamivudin-related Resistance	rtR153Q (sD144)	0%	3.3%	0%	0%	2%
	rtI169T (sK160S)	0%	3.3% (3.3%)	0%	0%	0%
	rtA200V (sW191)	0%	3.3%	0%	0%	0%
	rtS202G (sS193A/L)	0% (12.5%)	9.9% (6.6%)	0%	0% (3.2%)	0% (4%)
	rtC256G/S	25%	0%	12.5%	6.40%	0%
	rtS53N (sG44E)	0%	3.3%	0% (6.25%)	0% (3.2%)	0% (1%)
	rtL80I/V (sG71)	40%	13.2%	25%	16%	2%
	rtI191L/V (sW182G)	60%	13.2%	12.5%	16% (3.2%)	8%
	rtT128A/D (sG119R)	12.50%	6.6%	12.5%	6.4%	3% (1%)
	rtN139D/T (sT131P)	0%	6.6% (6.6%)	6.25% (6.25%)	0%	0%
Afefovir & lumivudin-related Resistance	rtQ215P/S (sY206C/H)	25% (12.5%)	0%	6.25% (6.25%)	9.6%	8% (4%)
	rtM204I/V (sI195M) (sW196L/stop)	50% (12.5%) (37.5%)	23.10% (6.6%) (16.5%)	68.75% (12.5%) (56.25%)	22.4% (9.6%) (12.6%)	4% (1%) (3%)
	rtA181T/P (sW172C/stop)	0%	16.50% (6.6%)	6.25%	6.4% (6.4%)	0%
	rtL180M (sS171Y)	25%	9.90%	43.75%	16.%	3%
	rtV173L/M (sE164D)	0%	3.30%	6.25% (6.25%)	3.2%	1% (4%)

3.3. Analysis of the Mutation Frequencies

The mutation frequencies between all groups were as follows: I (1.7%), II (1.39%), III (2.28%), IV (2%), and V (0.38%). Table 2 shows the mutation frequency for individual mutated residues of different groups according to previously-published common HBV antiviral resistant variants. Group I contained mutation frequencies between 10 and 60% for eight amino acids that were common for both LAM and ADV resistance. In group II, for 13 amino acids, the frequencies of substitutions were 3 to 22%. All of the mutations were involved in both LAM and ADV resistance (with a higher number of mutations for the latter). Group III contained 3 to 60% of mutation frequencies for 10 amino acids. For group IV, the mutation frequencies were 3 to 22% for 10 amino acids, and group V contained 1 to 8% mutation frequencies for 13 amino acid substitutions.

3.4. Individual Mutation analysis

Of the total of 39 amino acid changes, residues 54, 80, 91, 180, 204, 215, and 221 showed the highest number of mutations in all groups (Table 2). I54V (ADV-related variant) was found in group IV with the highest frequency (22%). L80I/V was found at the frequencies of 40 and 25% in groups I and III, respectively. I191L/V was found in 60% of group I. These two latter LAM-associated variants showed different frequency in all groups, including the control group. L180M mutation (common to both LAM and ADV drug resistant variants) was found to range from 9.9% of the patients in group II to 43.75% of the patients in group III. As was expected, M204I/V showed the highest frequency in all of the groups that were studied (the highest and the lowest values were 22.4 and 68.7% in groups IV and III, respectively). In the control group, 4% contained this variation. Q215P/S was found in all groups except group II with frequencies of 6.25 to 25%. F221Y/S was highest in groups I and IV, at 25 and 19.2%, respectively.

3.5. Comparison of amino acid substitution between groups

1) *Group I:* In addition to the nine amino acid substitutions that related to LAM resistance, F221Y/S, which is related to ADV resistance, also was found in the patients in this group, who only received LAM (Table 2).
2) *Group II:* Six amino acid variations related to ADV therapy were found in this group. Moreover, 3 and 4 LAM and LAM+ADV-related mutations were found, respectively. This group showed the smallest percentage of amino acid changes among the groups that received the treatment. Interestingly, residues W153Q, I169T, A200V, and S202G, which are related to LAM resistance, were found only in this group with a frequency between 3.3 and 9.9% (Table 2). No ADV-related variant N236T was found in this group.
3) *Group III:* This group had different features of mutations that previously were related to LAM, ADV, and LAM+ADV. They contained the highest frequency of substitutions for residues L180M (43.7%) and M204I/V (68.7%) among all the groups. Again, N236T, which is related to ADV therapy, was absent in this group (Table 2).
4) *Group IV:* Despite the fact that this group contained critical residues for variations related to LAM, ADV, and LAM+ADV, the frequency of mutations was less than that of the patients in group III, who had ADV add-on therapy (Table 2). N236T and compensatory mutation (A181T/P) were found to be between 3.2 and 6.4%, respectively (Table 2).
5) *Group V:* This group showed an uneven distribution of mutations in different residues between 0 to 13% (Table 2). Altogether, groups III and IV showed the highest and lowest numbers of amino acid changes over the RT, respectively.

3.6. New, unreported mutation

Besides the known LAM- and ADV-resistant mutations, all of the groups but group V had additional amino acid changes in the RT domain at residues that have not been reported before at different rates (Table 3). The highest number occurred in group I, which was followed by group III. Group IV had the lowest percentage of new mutations. None of patients in the control group had these mutations. L72I was found in groups II (5%) and III (10%). I187L was distributed in groups I (25%), II (< 5%), and III (< 5%). D263E was found in groups II (3%) and III (13%). I266R/M was found in all of the groups with a frequency > 20% (Table 3).

Table 3. Unpublished mutation residues and their frequencies found between different groups

Groups	L72I	I187L	D263E	I266R/M	No (%)
I (LAM only)	0%	25%	0%	25%	47.5%
II (ADF only)	6%	3%	3%	20%	23.1%
III (ADF add on LAM)	12%	0%	13%	25%	37.5%
IV (LAM & ADF)	0%	3%	0%	24%	18.75%
V (Control)	0%	0%	0%	0%	0%

4. Discussion

This is the first report on the mutational profile of viral reverse transcriptase and overlapped surface proteins from CHB patients with incomplete response who were on different treatment regimens and, subsequently, acquired viral breakthrough. In this study, in addition to LAM- and ADV-resistant polymerase gene mutations, a proportion of patients had new amino acid changes in the RT domains. Whereas there was no consistent pattern in their distribution of unknown amino acid changes, the significance of these at the emergence of LAM and ADV resistance is unclear. Moreover, mutations related to a specific NA monotherapy were found in multi-drug groups (III and IV) and vice versa. In the present study, multi-drug-resistant mutations were detected in patients on monotherapy with either LAM or ADV. Multi-drug-resistant HBV has been reported in patients who received sequential treatment with nucleoside/tide analogue monotherapies (14, 28, 29). It is interesting to note that mutants L180M and M204I that were detected during LAM and ADV breakthrough showed a high prevalence in the LAM-only group (25 and 50%, respectively), followed by a low frequency in the ADV-only group (9.9 and23%, respectively), whereas they showed a very high frequency in group III in which the patients received ADV after LAM breakthrough (43.75 and 68.75%, respectively). However, group IV, in which the patients who were started with both drugs, showed an intermediate frequency of such mutations (16 and 22.5%, respectively). Collectively, these results suggest that prolonged LAM and/or ADV monotherapy may be associated with cross-resistance to combination of LAM and ADV (14, 29, 30), and individual resistant mutations to lamivudine and adefovir monotherapy have a marked reduction in sensitivity to the combination of lamivudine and adefovir (28, 29). These results also showed that pre-existing LAM-related variants (groups I and III) confer higher mutation profiles compared with ADV-only (group II) or adding ADV at the beginning of combination therapy (group IV), highlighting the previous finding that combination therapy may have heightened antiviral effects and that combining agents that do not share cross resistance has the potential to prevent resistance (29, 31). The distribution of mutations in groups I to IV showed that the RT domain C contained the highest number of primary amino acid substitutions, followed by domain A. Studies showed that the failure of therapy due to M204I/V could be related to the upstream substitutions in domains A and B [14]. This hypothesis might be emphasized to a greater extent in the treatment-naïve group in which domain A showed the highest number of variations, followed by domain C. In this study, the mean HBV DNA and ALT levels were higher in the LAM-only group (group I) than in the ADV-only group (group II) or the combination therapy group (group IV) (Table 1). Interestingly, the mutation frequency patterns in these groups were comparable, i.e., 10 and 60% (group I) compared with 3 to 22% (groups II and IV). Given the high rate of resistance in the former and the low antiviral potency of the latter, which was similar to the findings in other studies, this study showed that neither lamivudine nor adefovir should be used as monotherapy because of the low barrier of resistance (4, 6, 11, 12, 32, 33). Concerning the results obtained from the patients in group V, who did not receive any treatment and due to the quasispecies nature of HBV, the resistant viral populations could have pre-existed in a subset of the HBV-infected patients, which could have occurred randomly as a result of sustaining viral replication (26). Similarly, some substitutions could have occurred during the few months of therapy in those patients who partially responded to the therapy after reduction in previously-high HBV replication levels. Therefore, we could hypothesize that, for those individuals who did not respond to therapy, there would have been a probability of the emergence of more random substitutions getting higher if prolonged therapy had been continued; the more viral replication sustains the higher number of mutations could be emerged. This observation could be supported by the view that the mere detection of resistance–associated substitutions during therapy does not mean that a clinically relevant viral breakthrough will occur (34). Such detection indicates that a complete breakthrough might subsequently occur in partially-responded patients with moderate to high levels of viral load. However, because we did not use sequential samples in the current study to investigate either the complete response or non-responsiveness of the patients, further research should be conducted to determine whether treatment should be altered in situations in which partial response occurs in order to prevent viral breakthrough. Previous studies have shown that various mutational patterns were associated with drug resistance in relation to HBV genotypes (35-38). Versatile, unusual mutational patterns were found in this study (like finding of LAM-related mutations in group II and new, yet unreported mutations) that might be related to the nature of pure genotype D among the Iranian population. Moreover, the patients in both groups III and IV, who received LAM and ADV in combination, showed the highest rate of mutation frequency between all groups studied (2.28 and 2%, respectively). This may highlight the significance of viral genotypes in the evolution of chronic HBV. Iranian population is the only ethnic group that harbored 100% of genotype D (26). Therefore, the impact of such a pure genotypic pattern on the evolution of specific mutational patterns cannot be ruled out. Drug-resistance variants have been found to be present along with the surface protein substitutions (as compensatory mutations, in the distal part) with other nucleos(t)ide analogue antiviral agents (39-42). This study contained several limitations. It was a retrospective, observational study of a relatively heterogeneous patient population, representing the mutational outcomes of

patients who responded partially to the sequential administration of LAM and ADV, LAM and ADV in combination, and monotherapy treatment regimens. However, since we did not use sequential samples, the effect of such mutations on the relative viral breakthrough and the level of drug resistancy between different groups were not explored.

5. Conclusions

The results of amino acid distribution in the present study clearly showed that LAM+ADV combination therapy was superior to either LAM/ADV monotherapy or ADV add-on in preventing drug resistance. In addition, those patients who failed to respond in the first 48 weeks, irrespective of whether they were undergoing mono or combination therapy, should be tested genotypically for partial response, and, if confirmed, early modification of their treatment should be considered, such as using highly potent drugs with high genetic barriers of resistance.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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