

Case Report

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Mutation Patterns at Codons Rt204 And Rt180 of the HBV Polymerase Gene Associated with Lamivudine Resistance in Treated and Untreated Chronic HBV Patients in Kuwait: A Case Series

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Abstract

Introduction: Lamivudine is a potent inhibitor of hepatitis B virus (HBV) replication via suppression of the RNAdependent DNA polymerase. However, patients with prolonged therapy were previously detected harboring drugresistant mutants. Such mutants though partially replication defective, confer resistance to lamivudine and can elicit exacerbation of hepatonecro-inflammation.

Cases presentation: In this case series, we examined mutations in the YMDD motif gene in five lamivudinetreated patients (60 yr male, 50 yr female, 46 yr male, 36 yr male and 42 yr male) and in four untreated patients (34 yr female, 29 yr male, 29 female and 37 yr male). Rare mutational patterns of rtM204L in conjunction with rtL108M were recognized conferring resistance to lamivudine. The rtL180M compensatory mutation was identified in conjunction with rtM204V/I/L; among which three patients had viral and biochemical breakthrough associated with serum HBV DNA levels exceeding 10⁶ copies/mL.

Conclusions: These results indicate that; (i) lamivudine resistant HBV strains are naturally occurring mutants as detected in lamivudine untreated patients and; (ii) New mutational patterns (rtM204L: YLDD and rtL180M) were identified conferring resistance to lamivudine and resulted in biochemical and virological breakthrough. Based on these findings, we propose that such mutations can be used as a marker to predict development of viral breakthrough in the HBV patients whether or not treated with lamivudine.

Keywords: YMDD mutants; YVDD; YIDD; YLDD; rtL180M

Abbreviations: ALT: Alanine Aminotransferase; Anti-HBe: The antibody to Hepatitis B e-antigen; CHB: Chronic Hepatitis B Virus; DNA: Deoxyribonucleic acid; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B Virus; IFN: Interferon; PCR: Polymerase Chain Reaction; RNA: Ribonucleic acid; ULN: Upper Limit of Normal; YIDD: Tyrosine-Isoleucine-Aspartate-Aspartate; YMDD: Tyrosine-Methionine-Aspartate-Aspartate; YMDN: Tyrosine-Methionine-Aspartate-Aspartate; YVDD: Tyrosine-Valine-Aspartate-Aspartate

Introduction

Lamivudine is an oral nucleoside analog that inhibits the replication of Hepatitis B Virus (HBV) by interfering with HBV reverse transcriptase activity. It is used to treat patients with Chronic Hepatitis B Virus (CHB) infection by decreasing the HBV-DNA viral load and Alanine Aminotransferase (ALT) levels as well as improving liver histology [1]. However, the emergence of lamivudine-resistant HBV strains in patients on long-term lamivudine-therapy has been reported at a rate of 14-32% after 1 year, increasing to 40%, 53% and 76% after 2, 3 and 4 years of continuous therapy, respectively [2]. Lamivudine-resistant mutants have also been reported in untreated asymptomatic carriers and in CHB patients [3-5]. Such resistant viruses exhibit characteristic mutations at positions rt204 (rtM204V: YVDD, rtM204I: YIDD and rtM204S: YSDD) and rt180 (rtL180M) in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase domain of the polymerase gene [6]. These mutations result in the re-elevation of HBV-DNA known as viral breakthrough and rising ALT levels known as biochemical breakthrough [7].

Given the loss of therapeutic efficacy associated with the development of resistance to lamivudine and the availability of new

alternative treatments for CHB patients, a better understanding and early detection of viral genotypic resistance could allow the clinician to consider therapy modification before viral breakthrough and biochemical relapse occur. In this study we conducted a chart review of the Kuwait Hepatitis Registry to evaluate the target patients' viral and biochemical profiles. Nine cases were found to exert lamivudineresistance in treated and untreated HBV-infected patients in Kuwait.

Case Presentation

Case 1

A 34-year-old female who presented with jaundice on first medical examination and was found to acquire HBV infection through her husband (as indicated in our questionnaire via interviewing the patient). She had HBeAg positivity, anti-HBe negativity, elevated serum ALT levels at 18 times the upper limit of normal (ULN) which is 40 iu/L in our laboratory, with elevated bilirubin (111 μ mol/L) and HBV DNA levels more than 10⁶ copies/mL. A rare pattern of YMDD mutation

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(YLDD) was detected by direct DNA sequencing of polymerase gene; the extracted HBV DNA was amplified by semi-nested PCR in a 50 µl reaction mixture. PCR products corresponding to 406 bp were analyzed on 1% agarose gel electrophoresis as described previously [8] and then was purified using QIAquick PCR purification columns (Qiagen). Both strands of purified amplicons were sequenced by using cycle DNA sequencing kit (DTCS CEQ8000, Beckman Coulter) as described in detail previously [9] except that primer POL-1 and SS were used for bidirectional sequencing as sequencing primers. Reverse complements were generated and aligned with forward sequences using ClustalW analysis. GenBank identity search using Basic Local Alignment Search Tool (BLAST) was performed for genotype identification and YMDD mutation detection. The DNA sequencing data reported in this study have been submitted to [GenBank under accession numbers: AM279420 to AM279439] [8]. Follow-up of patient revealed that she died due to liver failure.

Case 2

A 29-year-old male with HBV infection classified as asymptomatic carrier who acquired HBV infection through intrafamilial transmission (as indicated in our questionnaire via interviewing the patient). At that time, the patient dropped-out from the study. Direct sequencing of the PCR products identified a rare mutational pattern (YMDN) at the YMDD motif of the HBV polymerase gene. The biological activity and clinical impact of this polymorphism were not established yet. The patient returned to his original country of residence where he received lamivudine at a dose of 100 mg daily for six months following detection of mutations. During follow-up, the patient retained adequate viral and biochemical responses, viral load and biochemical parameters are shown in table 1.

Case 3

A 29-year-old female who was asymptomatic at the initial medical examination and was diagnosed with HBV infection. Our patient interview revealed that she had acquired HBV infection via unknown source. Her work-up showed HBeAg negativity and anti-HBe positivity. Serum ALT level was normal and HBV DNA viral load was less than 2000 copies/mL. She had not received lamivudine treatment or any other antiviral therapy because the viral markers were consistent with inactive infection. Direct sequencing of polymerase gene segment revealed the rtL180M+YVDD double mutation.

Case 4

A 37-year-old male with HBeAg negative (asymptomatic carrier) who acquired HBV infection via intrafamilial transmission (as indicated in our questionnaire via interviewing the patient). Serum ALT level was normal and HBV DNA viral load was less than 2000 copies/mL similar to the previous patient. YMDD locus mutation (YIDD) was detected by direct DNA sequencing.

Case No.	HBV DNA	Biochemical Profiles*			Platelets Count*
	Viral* Load (copies/ml)	ALT (iu/L)	Albumin (iu/L)	Tbili (g/L)	
Case 2	<2×10 ³	21	39	11	205,0×10 ³ /mm ³
Case 6	<2×10 ³	21	32	12	221,0×10 ³ /mm ³
Case 9	<2×10 ³	21	35	11	203,0×10 ³ /mm ³

*Lower limit of quantification for HBV DNA viral load=300 copies/mL, Upper Limit of Normal (ULN) for AL=60 iu/L, ULN for Albumin=30 g/L, ULN for Tbili=17 μ mol/L, platelets normal range (140-400) cells

Table 1: The viral load and biochemical profiles for cases 2, 6 and 9.

Case 5

A 60-year-old male with HBeAg negativity, anti-HBe positivity, serum HBV DNA more than 100×106 copies/mL and ALT level above 1.5 times ULN (baseline measurements). Our patient interview revealed that he acquired HBV infection via unknown source ((as indicated in our questionnaire via interviewing the patient). Histological assessment showed mild necroinflammation and moderate liver fibrosis. The patient received peg-IFN-a-2a at a dose of 180 µg subcutaneously for 24 weeks. Failure of therapy was reported and the patient was switched to lamivudine monotherapy with a dose of 100 mg daily. Serum HBV DNA reduction and ALT level normalization were achieved during the first few months of therapy. However, after 1 year of therapy, viral breakthrough was reported, which was characterized by an HBV DNA level more than 100×106 copies/mL and rising ALT (90 iu/L), viral breakthrough. Direct sequencing results showed emergence of rtL180M+YVDD double mutation. With these findings, adefovir at a dose of 10 mg daily was added to lamivudine, continued with same previous dosage. Recent follow-up showed a reduction in the HBV DNA viral load to 210,000 copies/mL and decreased ALT level to 56 iu/L, viral suppression.

Case 6

A 50-year-old female with HBeAg negativity, anti-HBe positivity, HBV DNA level of 100×10^6 copies/mL and normal ALT level (21 iu/L). Mild necroinflammation and liver fibrosis were demonstrated on her liver biopsy. The patient acquired HBV infection from her husband; who developed YMDD mutants (YVDD as mentioned in his file) after 2 years of therapy. Therefore, adefovir at a dose of 10 mg daily rescue therapy was initiated. Direct DNA sequencing of polymerase gene showed occurrence of rtL180M+YVDD double mutation. After 28 weeks of adefovir monotherapy, the patient was shifted to adefovir addon lamivudine therapy. Effective viral suppression and biochemical remissions were achieved and reported as shown in table 1.

Case 7

A 46-year-old male who underwent kidney transplantation in 1989. The patient had HBeAg negativity, anti-HBe positivity; slight elevation in serum ALT level (66 iu/L) and HBV DNA level of 270×10^6 copies/mL (baseline measurements). Evidence of mild necroinflammation and moderate liver fibrosis were seen on histopathology. In 2001, lamivudine monotherapy with an oral dose of 100 mg once daily was initiated. After 3 years of therapy; viral and biochemical breakthrough were reported; characterized by serum HBV DNA level more than 100×10^6 copies/mL and rising ALT of 84 iu/L (viral breakthrough). Results of direct DNA sequencing of polymerase gene segment showed the emergence of rtL180M+YIDD double mutation. Follow-up of this patient revealed that he had been switched to Entacavir 1 mg orally once a day (in 2007) which resulted in a reduction in the HBV DNA viral load to 3×10^3 copies/mL, an ALT level of 35 iu/L, viral suppression.

Case 8

A 36-year-old male with HBeAg positivity, anti-HBe negativity, HBV DNA viral load of 340×10^6 copies/mL and ALT level 4 times more than the ULN. The patient acquired HBV infection through intrafamilial transmission (vertical as revealed from our questionnaire via interviewing the patient). Liver histology showed moderate necroinflammation and mild liver fibrosis. The patient received subcutaneous standard IFN- α -2a (5 MU) daily for 1 year. HBeAg clearance rate was suboptimal. The patient was considered a nonresponder and switched to lamivudine monotherapy at a dose of 100 mg daily. During therapy, sustained adequate viral and biochemical responses were achieved, characterized by the suppression of HBV DNA and normalization of serum ALT levels. After 2 years of treatment, an increase in HBV DNA level accompanied by rising ALT flare were reported. DNA sequencing analysis showed the occurrence of YIDD single mutation. Therefore, it was recommended to start adefovir add-on lamivudine combination therapy. Unfortunately, the patient could not afford to buy adefovir. Therefore, he was switched to daily peg-IFN- α -2a at a dose of 180 µg plus daily lamivudine at a dose of 100 mg combination therapy. After 1-year follow-up; the patient's work up showed failure of therapy manifested by viral rebound of HBV DNA levels of 5×10⁶ copies/mL.

Case 9

A 42-year-old male who presented with abdominal pain on first medical examination and acquired HBV infection through intrafamilial transmission (as indicated in our questionnaire via interviewing the patient). He had HBeAg negativity, anti-HBe positivity, serum HBV DNA level more than 5200×106 copies/mL and ALT level above 1.3 times the ULN. Histological assessment showed moderate necroinflammation as well as moderate liver fibrosis. The patient was treated with standard IFN-a-2a monotherapy at a dose of 5 MU daily. During his 16 weeks follow-up visit, failure of therapy was observed and the patient was switched to lamivudine monotherapy at a dose of 100 mg daily. Serum HBV DNA reduction and ALT level normalization were achieved. After 3 years of therapy, viral and biochemical breakthroughs occurred. YMDD motif analysis showed the emergence of rtL180M+YLDD mutations. Therefore, therapy was switched to adefovir monotherapy at a dose of 10 mg daily and the patient is responding well to the treatment as shown in table 1.

Discussion

Lamivudine was the first nucleoside analogue licensed for the treatment of chronic HBV infection. Lamivudine is a cytosine analogue that inhibits Reverse Transcriptase (RT) by competing for incorporation into growing DNA chains causing chain termination. Lamivudine can be taken orally in a dosage of 100 mg daily, is generally well tolerated, and has an excellent safety profile [10].

The major drawback of lamivudine, which significantly limits its use as first-line therapy for chronic HBV infection, is the high rate of occurrence of viral resistance. Resistance to lamivudine may emerge after 9-10 months of therapy, with an incidence of 38% and 67% after two and four years of therapy, respectively. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartateaspartate (YMDD) motif of the RT which is part of the catalytic site of the HBV polymerase [7,11,12]. Three types of mutations are observed in the polymerase gene. The consensus RT domain numbering system described by Stuyver et al. [13] has been used to describe these variants. These include M204V (YVDD) mutation associated with L180M (gp I); the M204I mutation alone (gp II); and the M204I (YIDD) mutation associated with L180M (gpIII) [14]. The L180M and M204V mutations act synergistically to increase resistance to lamivudine [7]. While the L180M mutation is reported to enhance M204V resistance, its relevance is probably more related to being a compensatory mutation resulting in an increase of viral fitness. In addition, all cases were genotyped to be D while only case 3 was infected with mixed genotypes (A+D) and case 9 was infected with recombinant genotypes (A/D) as revealed by sequencing [8].

Patients who develop YMDD mutations during lamivudine therapy

for HBV infection exhibit various clinical courses. The emergence of lamivudine-resistant mutants is usually associated with an increase in serum HBV DNA level and ALT, and selection of YMDD variants has been associated with worsening of liver histology [15-17]. Among the problems associated with lamivudine is the relapse of hepatitis when such treatment is discontinued. This results in the release of HBV replication suppressed by lamivudine, and the emergence of resistant viruses during the treatment. The latter factor may cause a relapse of hepatitis, necessitating the concomitant use of other antiviral agents. Prediction of the emergence of lamivudine-resistant viruses before treatment with lamivudine would provide clinically useful information.

Early detection of antiviral resistance is important for the success of any antiviral therapy. Mechanisms resulting in early emergence of antiviral resistance are not always clear [18]. It has now become increasingly evident that YMDD HBV mutants are not always induced by lamivudine therapy, but do occur naturally and exist in HBV carriers who are not treated with lamivudine [4,12,19]. This implies that drug-resistant HBV strains with pre-existing mutations survive during therapy and contribute towards relapse and treatment failure. The purpose of this study was to investigate the occurrence of lamivudine-resistant HBV strains in lamivudine-treated and untreated HBV infected patients in Kuwait.

In the current study, YMDD mutants were detected in treated and untreated CHB patients in accordance with earlier reports [4,5,20]. Accurate ascertainment of HBV infection history via defining the source of HBV acquisition of infection helped us in refining the transmission route in case of untreated (therapy-naïve) HBV patients which was very crucial. This indicated that lamivudine resistant mutants can occur naturally (patients 1-4); a conclusion that concurs with previous reports [4,12,19,20].

The clinical impact of viral resistance was evaluated through the follow-up of patients after the emergence of lamivudine resistant mutants for a minimum duration of 12 months. Study reported that virological breakthrough was followed by a biochemical breakthrough after a median duration of 4 months in HBV infected patients with YMDD mutants. However, in our study most (7 of 9; 77.7%) patients with YMDD mutants developed virological and biochemical breakthrough after a median duration of 6-12 months. The emergence of the compensatory L180M mutation was reported earlier to be associated with breakthrough hepatitis [21]. In our study, breakthrough hepatitis occurred in 5 out of 9 cases with the L180M mutation, while only one patient with mutation L180M developed significant biochemical changes. The prediction of the occurrence of breakthrough hepatitis is very important in lamivudine treated and untreated HBV patients in the clinic setting because some patients may become severely ill and even die [22].

A typical YVDD associated with L180M variant (gp I) was detected in three patients: in two treated patients and only one patient before administration of lamivudine. Thus, viral DNA breakthrough, biochemical breakthrough and deterioration of liver histology were observed in patients treated with lamivudine while, none were observed with the lamivudine-untreated patient. A typical YIDD single mutation (gp II) was observed in two patients untreated and treated, respectively. HBV DNA level and serum ALT level were normalized in patient 4 (lamivudine-untreated) despite the emergence of YIDD single mutation. Thus, patient 8 experienced rising HBV DNA and ALT levels, which resulted in failure of therapy. A typical YIDD associated with L180M variant (gp III) was detected in only one patient on prolonged lamivudine treatment. The patient showed elevated HBV DNA levels and rising ALT levels upon emergence of the variant.

Rare mutational patterns were also detected in 3 cases: YLDD single variant, YLDD associated with L180M mutation and YMDN single variant. YMDN rare mutation was detected in only one lamivudineuntreated patient. No significant clinical association was observed. Few clinical reports have described the emergence of YLDD mutation but without an association to lamivudine resistance [21]. However, our results showed that YLDD single mutation (case 1) and YLDD associated with L180M mutations (case 9) showed disease progression or conferred resistance to lamivudine (patients 1 and 9; respectively) which was accompanied with liver failure in case 1 and breakthrough hepatitis after 36 months of therapy in case 9.

Conclusion

In conclusion, in order to avoid ALT flares that result in breakthrough hepatitis, we recommend early detection of viral breakthrough via HBV DNA monitoring every 3 months as well as early characterization of viral quasispecies at baseline before initiating rescue therapy.

Consent

Written informed consent forms were obtained from all patients participated in this study for publication of this case series. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing Interests

The authors declare that they have no competing interests.

Authors' contributions

MMA: Maisa Mahmoud Ali

FH: Fuad Hasan

WAN: Widad Al-Nakib

SA: Suhail Ahmad

SAM: Siham Al-Mufti

HA: Haifa Askar

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MMA, FH, SA and WAN designed the study. FH, HA, SF and SAM collected the specimens and provided the clinical profile and signed consent forms. MMA carried out the DNA Extraction, Direct DNA Sequencing, sequence alignments and analysis. SA participated in the sequence alignment analysis. All authors contributed in manuscript writing, read and approved the final manuscript.

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