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Lamivudine Resistance in Iranian Chronic Hepatitis B Patients.

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Abstract:

Background and objectives: Lamivudine therapy for chronic hepatitis B (CHB) is associated with resistance. This study aimed to analyze the response, the incidence of LAM resistance, and different viral mutational patterns of Lamivudine therapy.
Study design: CHB patients (n=31) who had not previously received interferon or a nucleoside analogue, received Lamivudine once daily for a minimum of ≥12 months and followed. All patients were tested for presence of mutation in YMDD motif of viral polymerase gene at the end of the first year of treatment, and if indicated in rising alanine aminotransferase (ALT) or HBVDNA titer. Polymerase chain reaction along with restriction fragment length polymorphism (PCR-RFLP) method was used to detect mutations in YMDD motif.
Results: The mean age of patients was 45.2 (SD 13.5) years. The mean follow-up period of patients was 45.5 (21.9) months. Seventeen patients (54.8%) had mutations, and 45.2% of subjects were sensitive to LAM. Mean time of mutation detection after treatment was 45.5 (SD 25.3) months. The distribution of YMDD status was: 32.3 % YIDD, 3.2% YSDD, 12.9% YVDD, and 6.5% YVDD/ YIDD. The mean age, pretreatment HBeAg negativity, and high HBVDNA titer at time of mutation had significant statistical association with occurrence of YMDD mutants (PV= 0.009, 0.032, 0.049), respectively.
Conclusions: Lamivudine-resistant mutation is common in CHB patients. Regarding different mutant strains as identified in this study, is necessary for develop more useful treatment strategies, especially in patients without YMDD mutation and high HBVDNA titer, analysis for possible new mutants should be performed.

Keywords: Chronic hepatitis B ; Lamivudine; Lamivudine- resistant mutations

Background:

Hepatitis B viral (HBV) infection is a major health burden in the Asia-Pacific region.[1] Patients with elevated alanine aminotransferase (ALT) and HBV DNA levels are candidates for antiviral therapy. In patients with HBeAg-positive CHB, antiviral treatment is indicated when the serum HBV DNA level is 20 000 IU/mL and the ALT level is elevated. For HBeAg-negative patients, the threshold for initiation of therapy is lower, i.e., a serum HBV DNA level 2 000 IU/mL in association with an elevated ALT level. The presence of at least moderate necroinflammation and the presence of fibrosis on liver biopsy may be useful in supporting the decision to initiate therapy, particularly in patients with normal ALT levels.[2]

Several studies have shown that increasing HBV viral level, starting at 104 copies/mL, is a predictor of risk for the development of cirrhosis and hepatocellular carcinoma, regardless of HBV genotype, HBeAg serostatus, and baseline serum ALT level.[3,4, 5]

Licensed oral agents for antiviral therapy in patients with chronic hepatitis B virus (HBV) infection include lamivudine, adefovir, entecavir, and telbivudine. Emtricitabine, tenofovir, and the combination of tenofovir plus emtricitabine in 1 tablet, which are licensed for the treatment of human immunodeficiency virus infection, are additional off-label options for treating HBV infection. HBV antiviral drug resistance may be best prevented by using an agent or combination of agents with a high genetic barrier to resistance, and 2 potent nucleoside and nucleotide drugs with different resistance profiles may prove to be the optimal first-line treatment for chronic hepatitis B. Frequent assessment of quantitative serum HBV DNA remains the best approach to early detection of resistance, and antiviral therapy should be modified as soon as resistance is detected.[6]

Lamivudine is no longer the drug of choice because the initial enthusiasm has been tempered by the high rate of resistance development. Studies are ongoing with the newer generation of antivirals in monotherapy or in combination to determine the best strategy for achieving rapid and prolonged suppression of viral replication. These improved strategies should enhance treatment success enough to obtain clinical stabilization, to delay or prevent the need for transplantation, and to reduce the risk of hepatitis B virus recurrence on the graft.[7]

In this manuscript, the influence of lamivudine on CHB treatment, the pattern of mutations developed and the associations of Lamivudine resistance with values of viral load and alanine aminotransferase (ALT) were investigated.

Study design:

We retrospectively, studied thirty-one consecutive chronic hepatitis B patients attending to the hepatitis clinic. Patients were provided treatment if they had elevated transaminases (i.e. serum ALT, AST) levels greater than 1.5 times the upper limit of normal for greater than 6 months and hepatitis B virus (HBV) DNA levels of > 105 copies/ml for both hepatitis B e antigen (HBeAg) positive and negative patients. Patients with decompensated liver disease, defined as clinical findings of a variceal hemorrhage, as-

cites, encephalopathy or portal hypertension confirmed by ultrasound were excluded. Also, patients who had received previous therapy with either interferon or a nucleoside analogue, or with co-infection with hepatitis C virus, or human immunodeficiency virus (HIV), were excluded. CHB patients (n=31) who had not previously received interferon or nucleoside analogue, received lamivudine 100 mg once daily for a minimum of ≥12 months lamivudine (LAM) therapy and followed. The mean time of follow-up of patients was 45.5 (21.9) months. They visited every 3-month while receiving lamivudine. ALT was measured monthly and HBVDNA every three months. All patients were tested for presence of mutation in YMDD motif of viral polymerase gene at the end of the first year of treatment, and if indicated in rising alanine aminotransferase (ALT) / or HBVDNA titer. Peripheral blood samples were collected, polymerase chain reaction along with restriction fragment length polymorphism (PCR-RFLP) method [8] was used to detect mutations in YMDD motif. The study performed with agreement of patients for that current treatment and follow-up.

DNA extraction-HBVDNA was extracted from 100 µL of patients' serum samples. 100 mL of serum sample was diluted in 150 µL of TES buffer. To this 15 mL of 10% SDS and 20 µL of proteinase K (20 mg/ mL) was added and mixture incubated at 60 degree centigrade for one hour. After a final extraction with chloroform, DNA was precipitated with ethanol then dissolved in 50 µL of buffer (10 mmol Tris-Hcl 1 mmol EDTA PH 8). Resistance was defined as an increase in viral load, with polymerase gene sequencing confirming a lamivudine resistance mutation. Viral breakthrough was defined as HBV DNA ≥ 5 log10 copies/mL on two consecutive visits in patients who, on treatment, achieved HBV DNA < 5 log10 copies/mL.

Statistical analysis- Data were analyzed with SPSS 11.5 software using Student's t test, $\chi 2$ test and Fisher's exact test.

Results:

Thirty one chronic hepatitis B patients were studied. Their mean age was 45.2 [standard deviation (SD) 13.5] years. 83.9% of them were male. Baseline HBV DNA titer of all patients was high (HBV DNA > 5 log10 copies/mL), and in only 4 patients HBeAg was positive. The mean time of follow-up of patients was 45.5 (SD 21.9) months. Time of mutation ranges from 12 to 96 months and its mean time was 45.2 (SD 25.3) months. Most patients found mutation during time period of months 13-24 (29.4%), and months 49-72(29.4%). Seventeen patients (54.8%) had mutations. Distribution of frequency (%) of CHB subjects with mutations after Lamivudine treatment according to time (month), and distribution of frequency (%) of mutations after Lamivudine treatment is depicted in figure 1, and Figure 2, respectively.

Mean ALT at time of mutation detection was 86.7 (SD 59.3) IU/L, normal range (5-35) IU/L.

45.2% of CHB subjects were sensitive to LAM. There were different viral mutational patterns: 32.3 % YIDD, 3.2% YSDD, 12.9% YVDD, and 6.5% mixed-type YMDD mutants (YVDD, YIDD).

Except two patients, all other patients at time of detection of YMDD mutation had high HBVDNA titer.In five patients without mutation, HBVDNA titer was high. The rate of mutation was 11.8, 29.4%, 17.6%, 29.4%, and 11.8% at < = 12, 13-24, 25-48, 49-72, and > 72 months, respectively. Two patients with mutation had undetectable HBVDNA (HBV DNA was below the detection threshold < 200 copies/ml): both patients used immunosuppressive drugs and corticosteroids, one of them for liver transplantation and another for arthritis rheumatoid and asthma.

Because two out of 17 patients at time of mutation had undetectable HBVDNA level, patients who fulfill criteria of Lamivudine resistance (mutation and high HBVDNA titer at time of mutation) were 15 patients (48%). When resistance was defined as an increase in viral load with polymerase gene sequencing confirming a lamivudine resistance mutation, we had 15 patients that fulfilled this criteria.

Of five patients without mutation had high HBVDNA titer, three subjects of this group had yet indetermined mutation. So, the rate of 45.2% sensitive to Lamivudine may decrease further by more investigating about these five patients. In two patients without mutation; last HBVDNA titer was not checked.

The mean age, pretreatment HBeAg negativity, and high HBVDNA titer at time of mutation had significant statistical association with occurrence of mutants (PV= 0.009, 0.032, 0.049), respectively. Sex, baseline and after three months of treatment of Lamivudine, AST and ALT value, and ALT at time of mutation detection had no significant statistical association with occurrence of mutation. Table 1 shows the demographic, biochemical, and viral load comparison of two groups with and without mutation.

Figure 1- Distribution of frequency (%) of CHB subjects with mutations after Lamivudine treatment according to time (month)

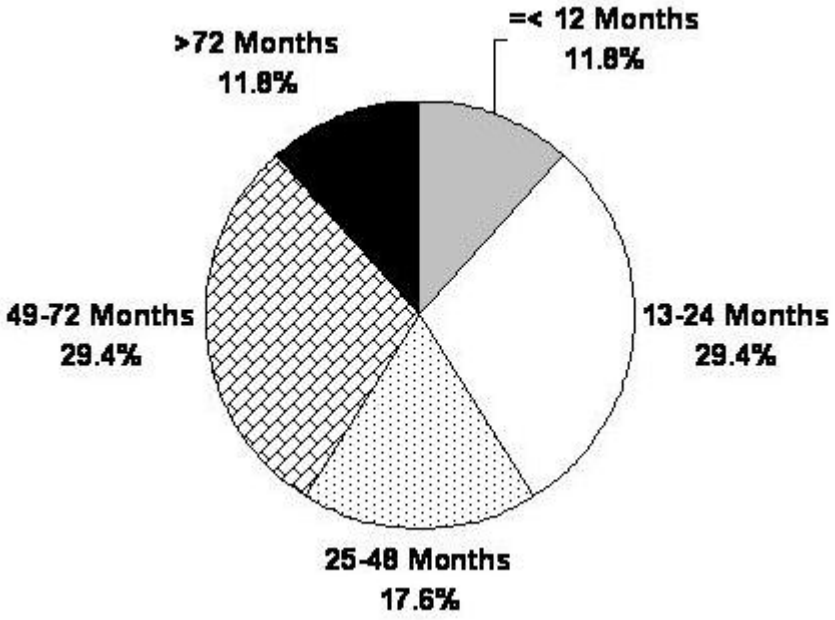

Figure 2 - Distribution of frequencies of mutations after Lamivudine treatment

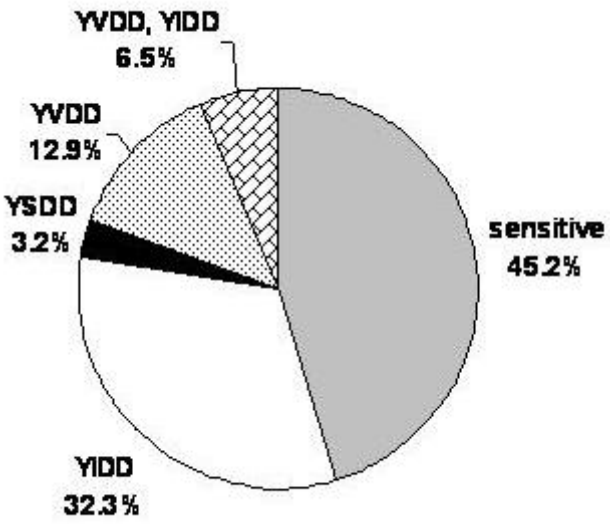

Table 1- The demographic, biochemical, and viral load comparison of group with mutation and those without mutation after Lamivudine treatment

Variable	mutation		PV
	yes	no	
Mean age (SD)	50.8 (12.3)	38.5 (12.1)	0.009
Male (%)	82.4	85.7	1.000
Baseline AST	60.9 (37.8)	54.8 (36.1)	0.652
Baseline ALT	86.5 (68.6)	55.0 (30.7)	0.103
% positive baseline HBeAg	0	28.6	0.032
AST after third months of LAM treatment	71.2 (49.9)	60.2 (69.2)	0.629
ALT after third months of LAM treatment	72.9 (67.1)	59.6 (53.4)	0.573
ALT at the time of mutation	86.7 (59.3)	62.5 (29.8)	0.444
The last high HBV DNA at the time of mutation	88.2%	53.8%	0.049

Discussion:

The early emergence of lamivudine (3TC)-resistant tyrosine-methionine-aspartate-aspartate (YMDD) mutants has been reported during 3TC therapy in patients with chronic hepatitis B (CHB) in hepatitis B virus (HBV)-endemic areas. According to a study, YMDD mutation at 3 months after Lamivudine treatment was significantly related to viral breakthrough within 24 months.[9] In our study, most patients found mutation during time period of months 13-24 (29.4%), and months 49-72(29.4%). Detection for HBV mutations at months 13-24 and 49-72 may be useful to predict the long-term outcomes of 3TC therapy in CHB patients.

In a study, of 260 consecutive CHB patients treated with lamivudine for >12 months, 231 patients were tested for A (1896) variant of HBV using direct sequencing. In multivariate analysis, the absence of A (1896) variant and high serum HBVDNA level were independent

factors for viral breakthrough following lamivudine therapy. The stop codon mutation at the precore region of HBV in addition to low serum HBV-DNA level may be associated with low breakthrough rate following lamivudine therapy.[10] Different mutational patterns were observed in the lamivudine-treated patients with and without exacerbation. There was an association of the basic core promoter and stop codon mutations with lamivudine resistance in patients with disease exacerbation.[11]

Patients who develop YMDD mutant during lamivudine therapy for HBV infection exhibit various clinical courses. Some patients show normal ALT levels, whereas others develop severe hepatitis exacerbations (SHEs) due to YMDD mutants. Negativity for HBeAg at commencement of therapy or before emergence of YMDD mutant was an important factor among non-elevated group. Patients with SHEs had more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of exacerbation than those without SHE, although no specific substitutions were noted. More substitutions in the rt region and the other proteins may be related to the emergence of severe hepatitis caused by lamivudine-resistant virus.[12] In our study, ALT level at time of mutation was 86.7 (SD 59.3) IU/L, and not statistically different from those without mutation. This finding shows also YMDD mutant is accompanied with biochemical relapse and breakthrough hepatitis, but it may not supposed as sole sign of appearance of mutants. Negativity for HBeAg at commencement of therapy or before emergence of YMDD mutant was an important factor among our patients. Regular measurement of HBVDNA titer is more important than ALT measurement.

In a study of 234 chronically HBV-infected Japanese patients who were treated with lamivudine for more than 12 months, comprised patients with HBV genotype A (n = 8), genotype B (n = 21), genotype C (n = 203) and other HBV genotypes (n = 2). Multivariate analysis also identified high HBV DNA level and HBeAg positivity as factors associated with emergence of resistance.[13] In our study, five patients without YMDD mutation had high HBVDNA titer. Hepatitis B virus DNA sequences should be analyzed for pre-S, surface, polymerase, core promoter, precore and core regions in the undetermined or suspicious samples.

In a total of 183 CHB patients, the cumulative rates of viral breakthrough were 9.6%, 39.0%, 55.8% at 12, 24, and 36 months, respectively. Serum HBV DNA level of 6 months of lamivudine therapy and presence of HBeAg were independent predictors for viral breakthrough. An alternate therapy should be considered when serum viral load is high at 6 months of lamivudine therapy.[14] Of 79 patients who had received lamivudine therapy for 9-57 months, 34 were HBeAg-positive and 45 were HBeAg-negative; 24 developed virologic breakthrough (VBT) and 55 did not. By logistic regression, the most important predictor of virologic breakthrough was the baseline HBV DNA ($r(2) = 0.12$, $P < 0.05$). Lamivudine may remain an effective first line therapy for those HBeAg-positive patients with a baseline HBV DNA < 6.6 log (10) copies/mL.[15]

In mentioned study, it is not clear why used persistent viremia (at 6 months) as a criteria for Lamivudine resistance. We believe it is better to lower the viral load as soon as possible.

Lamivudine is associated with the risk of developing viral mutants and, after therapy discontinuation, to high rate of relapse. In relapsing patients severe acute recurrence of hepatitis B may occur. Decisions about lamivudine monotherapy should take into account the limited long-term efficacy, effects of relapse, costs and predictive factors for response.[16]

Patients with precore variant hepatitis B virus are likely to develop lamivudine resistance early and should be considered for alternate first-line monotherapy. In the future, combination antiviral therapy may limit the development of resistance.[17]

Recent clinical observations reported the occurrence of amino acid substitutions at position 181 of the HBV polymerase, associated with a viral breakthrough under lamivudine or adefovir therapy. In a study, the main variants harboring the rtA181T/V mutation isolated from 10 consecutive patients who developed lamivudine and/or adefovir resistance was charachterized. The observations suggest that a single amino acid change at position rt181 may induce cross-resistance to lamivudine and adefovir. These data emphasize the clinical relevance of genotypic and phenotypic analysis in the management of antiviral drug resistance.[18]

Virologic breakthrough (VBTH) during long-term lamivudine therapy is believed to be due to the emergence of rtYM204I/VDD mutants. Both core promoter and YMDD motif mutation(s) are associated with VBTH in patients on long-term lamivudine therapy. Whether or not these promoter mutations in the absence of YMDD mutations confer drug resistance needs to be studied in an in vitro cell culture system, as they could create novel and stronger binding sites for hepatocyte nuclear factors.[19]

A study aimed at determining any differences in the antiviral response and risk of YMDD mutations between lamivudine-treated patients with HBV genotype B and genotype C. In conclusion, there was no difference in the antiviral response and the rate of development of YMDD mutations in Chinese patients with genotype B and C after 1 year of lamivudine. Determination of HBV genotypes before lamivudine therapy was probably not an important pretreatment investigation to predict antiviral responses in Chinese patients.[20] In a study, the significance of various viral factors and changes of viral population with lamivudine treatment was defined. There was no difference in treatment response between patients with genotype B and C. Achieving HBV DNA levels <1,000 copies/ml at 24 week is the best target for short- and long-term treatment efficacy. Core promoter and precore mutations were associated with better treatment outcome, and rt256C polymorphism in the polymerase gene with poor response.[21]

In a study, multivariate analysis showed that HBV genotype and pretreatment ALT levels was independently associated with YMDD mutational patterns. The results showed that the YMDD mutational patterns, precore mutation and serum HBV DNA levels differ between lamivudine-resistant HBV genotypes B and C in vivo.

It recommended that it is valuable for treatment of lamivudine-resistant HBV in clinic.[22]

Pre-therapy viral factors including viral load, genotype, precore (PC) stop codon status, basal core promoter status and pre-S deletion were determined to correlate with therapeutic endpoints. For lamivudine-treated HBeAg-positive CHB patients with pre-therapy ALT levels >5xULN, the PC stop codon mutation could predict a higher HBeAg seroclearance rate at the end of 12-18 months of therapy.[23]

Sequential on-treatment monitoring of hepatitis B virus (HBV) DNA levels, known as the roadmap concept, might predict the efficacy of oral therapy with nucleoside/nucleotide analogues among patients naive to this treatment.[24]

Serological markers are key elements in diagnosing acute hepatitis B virus (HBV) infection and determining its possible evolution towards chronicity. Advances in the molecular diagnosis of drug resistance using highly sensitive methodologies such as DNA hybridization assays can further pinpoint the type of mutation responsible and, more importantly, detect upcoming viral resistance at an early stage when the variant represents only a minor fraction of the total viral population.[25]

HBV shows high rates of turnover in the absence of proof-reading ability. As a result, large amounts of quasispecies are produced naturally or antiviral-associated. HBV consists of four open reading frames, namely preS/S gene, precore/core gene, polymerase gene, and X gene. Mutations on polymerase genes are often induced by antiviral therapy.[26]

In conclusion, virological and biochemical markers (HBeAg, anti-HBe antibodies, serum HBVDNA, and serum ALT) are used in the diagnosis and monitoring of HBV disease. A combined end point of biochemical response (ALT normalization) and virologic (serum HBV-DNA suppression) response is used frequently. In this study, most of patients were HBeAg negative CHB on admission. We did not study other markers include liver covalently closed circular DNA (cccDNA) and quantitative hepatitis B surface antigen (HBsAg), HBV genotype, and genotypic resistance markers. Also, baseline and after mutation detection of necrioinflammation and stage of liver fibrosis was not investifgated. Also, lack of define of a target serum HBVDNA level, and different assays for a patient make the interpretation difficult. We calculated the amount of mutations according to rise of serum HBVDNA titer or ALT level and showed it in certain divided times. But, in many clinical trials resistance is calculated by regarding the cumulative probability of HBV polymerase mutations.

In clinical trials, the reporting of resistance has varied tremendously from one trial to another.

The incidence of resistance, which now is assessed by sequencing, should be reported as a cumulative probability of occurrence. In all clinical trials, the cumulative probability of the onset of (1) resistance, (2) resistance with virologic (HBV DNA) breakthrough, and (3) resistance with virologic (HBVDNA) and biochemical (ALT) breakthroughs, should be reported every year according to the duration of follow-up evaluation (EB). Calculation

should be made by means of the following formula:

$P=1-(1-n1/N1)(1-n2/N2)\ldots(1-nx/Nx)$

where P is the cumulative probability that the event will occur, nx is the number of cases at

year x, and Nx is the number of patients still followed up at year x.[27,28]

In present study, most chronic hepatitis B patients on Lamivudine treatment developed mutations in mean 45.2 (SD 25.3) months. In CHB patients without YMDD mutation and high HBVDNA titer, analysis for possible new mutants should be performed. High HBVDNA titer may be supposed as a clue to drug resistant, regardless of ALT level or mutation report.

Conflicts of interest:

Commercial or other associations did not pose a conflict of interest to prepare of this review. Also there was not any financial support or grant for this manuscript.

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