Incidence of Genotypic Resistance to Lamivudine Long Term Therapy in Egyptian patients with Chronic Hepatitis B

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Abstract: Background: Lamivudine improves patients' outcome but is reported to be associated with increasing rates of viral resistance. The long-term benefit of lamivudine therapy and resistance rate in HBeAg negative genotype D patients is not fully known. This study **aimed** to determine the incidence of genotypic resistance to lamivudine therapy in Egyptian patients with chronic hepatitis B. **Methods:** This follow up study included 50 Egyptian patients with chronic hepatitis B who had received lamivudine 100mg daily for at least 12 months (7 females, age 32±8years). Patients were followed up for a mean period of 25±10 months. Investigations included: liver profile, hepatitis B serology and HCV Abs by ELISA, and HBV DNA by PCR. INNO-LiPA was performed in selected cases. **Results**: HBV-DNA decreased to <2000 IU/ml in 20 patients (40%), and HBV-DNA became undetectable in 30 (60 %) during the first year of treatment. The rate of relapse with either HBV-DNA reverting to positive or increasing to >2000 IU/ml after initial response was 16 patients during the second year, 3 during the third, 1 during the fourth year of follow up. Breakthrough was observed in 75% of the HBeAg positive group and only 33.3% of the HBeAg negative group. INNO-LiPA was performed for the 20 patients with relapse. Wild type was found in 14 patients; mixed type in 4 and mutant in two patients. Hence mutations were detected in 30% of the tested lamivudine-treated cases. YMDD was detected in 15%. Mean viral load was 7416.00±9232.24 IU/ml compared to 21 900 333IU/ml in the patients with the mutants. **Conclusion**: Long-term lamivudine therapy is associated with a high response rate with a rather low breakthrough rate in HBeAg negative patients and a low incidence of YMDD mutation.
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1. Introduction

Chronic hepatitis B continues to be a significant public health problem in Egypt [(1)]. Of the available treatments for hepatitis B (interferon-alpha (IFN-α), lamivudine, adefovir, entecavir and telbivudine), lamivudine remains the most frequently used drug, mostly because it is the cheapest. Lamivudine is a potent inhibitor of hepatitis B virus (HBV) replication, which was proved in several clinical trials to result in a rapid decline of serum HBV DNA levels in a majority of patients and improve patients' outcome [(2-4)]. However despite its good clinical tolerability and moderate antiviral efficacy, it is reported to be associated with rather quick development of resistance [(5-7)]. Furthermore, lamivudine mutations may confer cross-resistance to telbivudine, emtricitabine and entecavir.

The mutations related to lamivudine arise during prolonged treatment and the major mutation responsible is located in the YMDD site of the polymerase gene in which the methionine (rtM204) is replaced by either valine (rtV204) or isoleucine (rtI204) [(6,8)]. The incidence and patterns with which these mutations occur may vary among different populations. Hence, in the present study we aimed to assess the long-term response to lamivudine in Egyptian patients who have been taking lamivudine for more than one year and to determine the incidence of genotypic resistance in relapsed patients.

2. Materials and Methods:

This follow up study was conducted at the National Liver Institute, where 50 patients diagnosed with chronic HBV infection (who had been on lamivudine therapy for over one year) were recruited from the hepatology outpatient clinics and followed up during the period from the beginning of January 2009 to January 2012. Informed consent was taken from all participants in the study.

All patients were subjected to thorough history taking, abdominal examination and the following investigations: bilirubin, AST, ALT, albumin, GGT, ALP, complete blood picture, prothrombin time and concentration, viral markers; (HBsAg, HBeAg, anti-HBe, anti-HBc, anti-HDV, anti-HCV Abs by Enzyme-linked immunosorbent assay (ELISA), and HBV DNA level by polymerase chain reaction (PCR). Patients were followed up for a mean duration of 25±10 months and the relapse rate was noted. Detection of genotypic resistance was performed in the 20 patients who relapsed using INNO-LiPA HBV DR v3 (LiPA HBV GT;

Innogenetics N.V., Ghent, Belgium). The latter is a sensitive, commercially available assay, which allows rapid identification of treatment- resistant mutations in multiple patients with one assay. It also has the advantage of simultaneous detection of both wild type sequences and mutations [9] and can detect viral mutants that constitute as little as 5% of the total viral population [10, 11].

INNO-LiPA test utilizes a nitrocellular strip coated with different numerous bands of DNA probes each corresponding to specific codons for the known relative mutations. Nested biotinated primers to the polymerase gene are used to produce a biotinated product. Incubation at specific temperature with hybridisation buffers was done. In case of present exact base matching, the PCR product was found to bind to any biotinylated hybrid DNA probe. Streptavidin labeled with alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen resulted in a purple/brown precipitate. The developed strips were read manually against a chart provided with the kit so that the wild type and/or the mutations for the relative codons could be determined.

Statistical analysis:

Data was collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 19; Inc., Chicago. IL). Two types of statistics were done; *1) Descriptive statistics;* where quantitative data was shown as mean, SD, and range, and qualitative data was expressed as frequency and percent, *2) Analytical statistics;* where Student t-test was used to compare mean and SD of 2 sets of quantitative normally distributed data, while Mann Whitney test was used when this data was not normally distributed. Fisher exact test was used for 2x2 qualitative variables when more than 25% of the cells had expected count less than 5. P-value was considered statistically significant when it was less than 0.05. Cumulative bar chart was plotted.

3. Results:

Patients' Demographic and clinical characteristics:

Fifty patients with chronic hepatitis B were recruited in the current study; 43 were males and 7 were females. Their age ranged from 20 to 45 years with a mean of 32 ± 8 years. Chronicity was confirmed in all cases with positive results for HBsAg and anti-HBcAg-IgG. Twenty-five patients presented with elevated liver enzymes and only one patient was cirrhotic (Child C). Most patients did not express HBeAg; 42 (84%) were HBeAg negative and only 8(16%) positive (Table I).

Table II shows the pretreatment laboratory characteristics. Mean HBV DNA was statistically higher in the Hbe Ag positive group (3, 065 725 IU/ml) than that in the HBeAg negative group (432 675.1 IU/ml) {Z=3.87, p= <0.001}. Pretreatment ALT was significantly higher in the HBeAg positive group (Z=4.37, p=0.001) (Table III).

Treatment of the studied patients:

Forty-five patients received lamivudine monotherapy. Five patients received lamivudine followed by add-on adefovir. ALT normalized in 76% of the patients and in the other group the mean was 41.96 ± 73.79 U/L. In the patient with decompensated cirrhosis, ALT normalized, prothrombin concentration improved from 37% to 50%, serum bilirubin decreased from 1.9 to 1mg/dl, serum albumin rose from 2.6 to 2.8. Three of the HBeAg positive patients seroconverted on follow up; two of them had had elevated pretreatment ALT. HBV-DNA decreased to <2000 IU/ml in 20 patients and became undetectable in 30 patients. In the cirrhotic patient, HBV DNA became undetectable after eight months of lamivudine treatment (Table IV).

Relapse on lamivudine therapy:

Twenty patients (40%) suffered from relapse after initial response; sixteen during the second year of therapy; three during the third year and one patient during the forth year of therapy. As regards, Lamivudine resistance mutations, results of INNO-LiPA HBV DR assay detected wild type in 14 patients; mutant in 2 and mixed (wild+mutant) in 4 patients. Duration of lamivudine treatment ranged from 12-48 months in the wild group with a mean of 27 months while in the other group, the range was 12-26 months and mean 20 months (Figure 1).

Eleven mutations/variants were detected in the six patients. Three patients were HBeAg positive. The most prevalent mutations were M204V (YMDD) in three patients and L180/T181 (detected alone in three patients). Two of the patients with the YMDD were HBeAg positive. In two patients, multiple mutations were detected. In both of the patients, the M204V was accompanied by M204 I and M180/A181 with an additional I80 in one. Both pre-treatment and post-treatment viral loads were significantly higher among the mutant group (p=0.022 and 0.006 respectively) (Tables V, VI).

Table (I): Demographic and clinical characteristics of studied patients with Chronic Hepatitis B

Variables	Mean±SD
Age in years:	32 ± 8
Gender:	**Number (%)**
- Male	43(86%)
- Female	7 (14%)
HBeAg:	
Positive	8 (16%)
Negative	42 (84%)
Patients' Presentation:	
- Presented with Abnormal ALT	25 (50%)
- Presented with normal ALT	24(48%)
- Presented with decompensated cirrhosis	1 (2%)

Table (II): Pretreatment laboratory characteristics of the studied patients:

Parameter	Mean± SD
ALT (U/L)	90.74±224.05
Albumin (g/dl)	4.20±0.47
Bilirubin (mg/dl)	1.00±1.13
Prothrombin concentration (%)	88.72±11.30

Table (III): Comparison between HBeAg positive and HBeAg negative groups regarding some pretreatment parameters.

Parameter Group	HBeAg positive Patients	HBeAg negative Patients	Mann Whittney Z	*p*-value
Pretreatment HBV DNA (IU/ml) -Range -Mean	5800 - 20 000 000 3 065 725	2000 -10 400 000 432 675.10	3.87	<0.001
Pretreatment ALT (U/L) -Range -Mean	10-311 98.56	26-148 59.25	4.37	0.001

Table (IV): Posttreatment laboratory characteristics of the studied patients:

Variables	Number (n/Total=%)
• **ALT normalized**	19 patients (19 /25=76%)
• **HBeAg seroconversion**	3 patients (3/8=37.55%)
• **Improvement in CTP score**	1 patient (1/1=100%)
• **HBV-DNA decreased to <2000 IU/ml**	20 patients (20/50=40 %)
• **HBV-DNA became undetectable**	30 patients (30/50=60 %)
	Mean±SD
• **Posttreatment ALT (U/L)**	41.96 ± 73.79
• **Albumin (g/dL)**	4.23± 0.42
• **Bilirubin (mg/dL)**	0.8± 0.32
• **Prothrombin concentration (%)**	89± 0.097

Table (V): Profile of detected mutations in tested cases

Patient No.	Type	Type of treatment/ Duration	Hbe Ag (+ve/-ve)	Pretreatment ALT (U/L)	Pretreatment HBV DNA levels (IU/ml)	HBV DNA after emergence of resistance (IU/ml)	Type of mutation/variant
1	Wild/ Mutant	LAM 24m and add-on ADEF/2m	negative	50	10 400 000	2 800 000	L180/Thr181
2		LAM/ 12m	negative	19	56 000	10 000 000	M180/A181 M204V, M204I (YMDD)
3		LAM/ 24m	negative	29	122 000	17 800 000	L180/T181
4		LAM/ 24m	positive	39	1 360 000	50 000	L180/T181
5	Mutant	LAM/ 16m	positive	59	2 100 000	5 100 000	M204V (YMDD)
6		LAM/12m then add-on ADEF/9m	positive	49	20 000 000	19 000	I 80 M180/A181 M204V, M204 I (YMDD)

Table (VI): Comparison of Laboratory Profile of Wild and Mutant cases tested by INNO-LiPA

Variables	Group 1 Wild (n=14) No. %	Group 2 Mutant and mixed (n=6) No. %	Test of Significance	*P*- value
HBeAg:				
+ve	0 0	3 50	χ^2=0.028*	0.017
-ve	14 100	3 50		
Mean±SD of the duration of Lamivudine treatment (months)	27± 11.83	20 ±5.43	t=1.45	0.160
Pretreatment HBV DNA (IU/ml)	**Mean±SD**	**Mean±SD**	**Mann-Whitney Z**	**P- value**
	424 167.35±936828.23	6 063 666.66±7661672.96	2.29	0.022
Pretreatment ALT (U/L)	60.20±24.05	40.83±14.83	1.56	0.120
HBV DNA after treatment (IU/ml)	7416.00±9232.24	21 900 333.33±43697699.68	2.74	0.006

*** Yates corrected Chi-square**

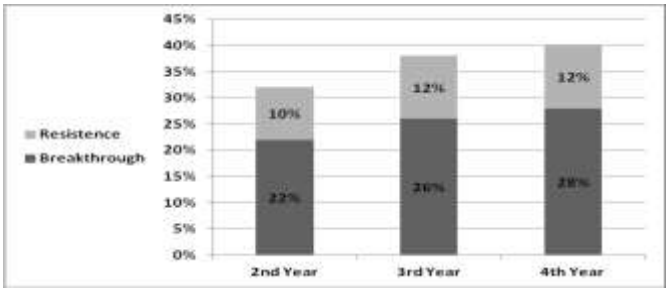

Fig. (1): Lamivudine breakthrough and resistance in patients on LAM > 1 year

4. Discussion:

In the current study, 86% of the studied chronic hepatitis B patients were HBeAg negative, and only (14%) positive. This is in accordance with *El-Zayadi et al., 2009*, who reported that 90-95% of patients in Egypt were HBeAg negative with low viremia before starting therapy. [(1)] Actually reports from other Middle Eastern countries state that the patients are predominantly HBeAg negative [(12, 13).]

Lamivudine showed good clinical tolerability in the studied patients as well as moderate antiviral efficacy. [(14)] This is in accordance with the randomized multicentre study by *Chan, 2007*, and that by *Da Silva, 2000*, who stated that lamivudine therapy is of potent efficacy in hepatitis B e antigen (HBeAg)-negative chronic hepatitis B. [(15,16).] HBe Ag seroconverison was observed in three (25%) of the HBeAg positive patients, two of whom had elevated pretreatment ALT. This agrees with the report of *Dienstag et al., 2003*, stating that the higher the patient's ALT level was before treatment, the better the HBeAg seroconversion rate.[(17)] Also they reported that HBeAg seroconversion rate was found to rise during treatment. In their study, the seroconversion rates at 1, 2, 3, 4, and 5 years of treatment were 16%, 17%, 23%, 28%, and 35%, respectively. In the current study, the mean follow up was only 28 months and the rate of HBeAg seroconversion might have increased with longer follow up periods.

Although the benefit of lamivudine is compromised by the development of resistance, [(14,18)] breakthrough rate (40%) was considerably low in the current study. In other studies, rates of lamivudine resistance reach near 70% by year four of continuous therapy. [(19 , 20)] Breakthrough occurred in 16 patients in the second year, 3 in the third and 1 in the fourth year of therapy respectively. Breakthrough was observed in 75% of the HBeAg positive group and only 33% of the HBeAg negative group. In accordance, *Tong, 2005*, reported that the number of HBV DNA breakthrough cases was significantly different between pretreatment HBeAg-negative and positive individuals (6.3% and 41.7% respectively).[(21)] Since the majority of our studied patients were HBeAg negative, this may explain the considerably low breakthrough rate. There is also evidence that the rate of resistance to lamivudine is lower in patients infected with genotype D. [(22)] Although genotypic testing was not performed in the current study, the epidemiological studies of HBV genotypes arising from the Middle-Eastern region, including Egypt, suggest that genotype D is the most prevalent. [(23-26)]

Genotypic resistance testing of the twenty cases revealed detection of wild type in 14 cases, mutant type in two patients and both mutant and mixed types in four patients. The prevalence of mutations in the tested group was thus 30%. It is difficult to compare this prevalence to other studies; first because of our small sample size and second because our cases had received variable durations of lamivudine treatment. However, this prevalence seems broadly similar to those in other reports showing that there is a 20% yearly chance for resistance. It is worth mentioning, though, that a major disadvantage of these assays is that they can detect only known mutations, and existing assays may not detect all mutations that have been shown to be associated with resistance to HBV antiviral drugs.

YMDD mutation (M204 V and M204 I) was detected in three of the tested patients. In the other three, L180/T181 was detected. This mutation was not previously reported. Previously lamivudine resistance was attributed solely to M204V or M204I. However an uncommon mutation (M204S) was reported by Hubert (2002) in which methionine was replaced by serine [(27)] and they concluded that it was possible that more variants would be found to arise during treatment with lamivudine. In fact, *Lok, 2008*, stated that as an increasing number of patients with divergent HBV sequences are exposed to nucleos(t)ide analogue therapies, additional mutations may be identified. However caution must be exercised in attributing differences in HBV sequences as the cause of antiviral drug resistance, particularly when baseline sequences are not available for comparison. [(28)] In two of the patients with L180/T181, viral load was very high which implies that the mutation is responsible for the resistance in our studied patients. One of these patients was receiving add-on adefovir for two months. Three of the patients having mutations were HBeAg positive.

Compensatory mutations (rtV173L, rtL180M) are reported to be identified in domain B [(29).] By phenotypic analysis, the rtM204V and rtL180M combined mutations induce a 1000-fold decrease of susceptibility to lamivudine *in vitro* by comparison with wild-type (wt) HBV. The main effect of the compensatory mutations is to restore replication fitness of the drug-associated HBV mutant. Thus, HBV DNA level usually increases with continuous therapy after the emergence of the primary mutation. [(29, 30)] In agreement, in the current study, the viral load in patients with wild type was significantly lower than mutants.

The emergence of YMDD mutants were reported to precede biochemical flare by 19 weeks in lamivudine-treated chronic hepatitis B patients, an opportunity for therapy reevaluation. [(31)] In the current study, three of the mutants had normal enzymes. Hence genotypic testing is important as it will permit early initiation of new therapies effective against lamivudine- resistant HBV. Indeed, the LiPA assay is becoming of great importance. The role of the different mutation patterns had little clinical relevance when lamivudine and adefovir were the only therapeutic options. However, this relevance of the mutation pattern might change with the availability of telbivudine and entecavir. Resistance to these two drugs is associated with ‘‘opposite’’ changes in the YMDD motif: rtM204V is associated with entecavir resistance, and rtM204I is associated with telbivudine resistance.[(32-34)] Up to 40% of lamivudine-resistant patients develop full entecavir resistance after 3 years of treatment. [(35)] Futhermore the LiPA analysis is important to monitor treatment compliance. The forteen patients with detected wild viral load (i.e. viral breakthrough in the absence of mutant virus) might be indicative of non adherence to the treatment protocol.

Regardless of the emergence of YMDD motif mutant, continuation of lamivudine resulted in improvement especially those who received additional treatment. In the current study there was a marked drop in the viral load in the patient with YMDD mutant who was added adefovir. This is in accordance with *Akuta et al., 2005*, who reported that 80% of patients who received additional treatment for breakthrough hepatitis, regardless of continuation of lamivudine, were ALT normal level on follow up. [(36)] Also *Gaia et al., 2004*, reported that the clinical outcome with YMDD mutants was benign despite severe post virological breakthrough hepatitic flares in about 12% of cases.[(37)]

In conclusion, this is the first report from Egypt that investigates lamivudine resistance mutations. Mutations were detected in 30% of the tested lamivudine-treated cases (YMDD in 15%). We also found a relatively low prevalence of HBeAg expression among examined cases (14%). Awareness of these serologic, genotypic and resistance patterns might help in the formulation of management plans and in predicting clinical outcomes since LiPA assay will detect mutant virus populations before viral breakthrough permitting timely initiation of new therapies effective against lamivudine- resistant HBV. Although lamivudine is considered obsolete according to guidelines from several clinical practice regions, [(38, 39)] lamivudine remains today’s mostly used drug against HBV infection in Egypt. Our data show that long-term lamivudine is associated with a high response rate with a rather low breakthrough rate and a low incidence of YMDD mutation. Further larger scale studies are needed to confirm our results and to examine possible associations among clinical, serologic, and genetic patterns of HBV infections in Egypt.

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