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ORIGINAL RESEARCH ARTICLE

Lamivudine Monotherapy in Chronic Hepatitis B Patients from the Indian Subcontinent: Antiviral Resistance Mutations and Predictive Factors of Treatment Response

Ashrafali Mohamed Ismail · Prasanna Samuel · Jeyamani Ramachandran · Chundamannil Eapen Eapen · Rajesh Kannangai · Priya Abraham

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Abstract
Background and objective Management of chronic hepatitis B is a global public health challenge. There are several updated guidelines proposed based on treatment outcome data from the respective study populations. In this study, we aim to characterize the antiviral resistance mutations to lamivudine monotherapy in patients diagnosed with chronic hepatitis B from the Indian subcontinent.
Methods A total of 147 lamivudine-treated patients with a median treatment duration of 13 (interquartile range 8–24) months were studied. Virological response was measured by hepatitis B virus (HBV) DNA levels. Antiviral resistance mutations were identified by sequencing HBV reverse transcriptase domains. Factors associated with virological response and antiviral resistance mutations were analyzed.
Results Virological response was observed in 50 (35 %) patients while 84 (57 %) were non-responders. The virological response for the remaining 13 (9 %) patients was undetermined. Forty patients (27 %) developed lamivudine-resistant mutations. HBV genotypes, subgenotypes and hepatitis B surface antigen subtypes did not show significant association with virological response or lamivudine-resistant mutations. High HBV DNA levels and increased treatment duration were strongly associated with the development of lamivudine-resistant mutations ($p = 0.002$ and $p < 0.001$). Patients who continued to be positive for hepatitis B e antigen have an increased risk for treatment failure ($p = 0.010$). High baseline aspartate transaminase levels were significantly associated with subsequent lamivudine response ($p = 0.037$).
Conclusion Considering the limited potency and high resistance rates to lamivudine therapy, our study emphasizes the use of more potent drugs in the management of chronic hepatitis B in the Indian subcontinent.

1 Introduction

Globally, an estimated two billion people have been infected with hepatitis B virus (HBV) and around 240 million live with chronic infection [1]. Approximately 75 % of these patients reside in the Asia-Pacific region, with India harbouring the second largest pool of about 50 million chronic HBV carriers [2, 3]. About 15–40 % of HBV-infected patients develop complications leading to cirrhosis, decompensated cirrhosis and hepatocellular carcinoma, contributing to over 1 million deaths per year [4, 5]. Thus, HBV-associated liver diseases are considered to be of public health importance, emphasizing the need for the prevention and control of disease progression.

There are two formulations of antiviral drugs currently available for HBV treatment: immunomodulators and nucleos(t)ide analogues. Interferons are immunomodulatory drugs that are administered for a finite period of treatment and because of their significant adverse effects, need for subcutaneous injection and cost, nucleos(t)ide analogues

A. M. Ismail · R. Kannangai · P. Abraham (✉)
Department of Clinical Virology, Christian Medical College, Vellore 632 004, Tamil Nadu, India
e-mail: priyaabraham@cmcvellore.ac.in

P. Samuel
Department of Biostatistics, Christian Medical College, Vellore 632 004, Tamil Nadu, India

J. Ramachandran · C. E. Eapen
Department of Gastrointestinal Sciences, Christian Medical College, Vellore 632 004, Tamil Nadu, India

have come to be the drugs of choice and are widely used in the Indian subcontinent. Nucleos(t)ide analogues inhibit the enzymatic action of HBV reverse transcriptase (rt) and thus act as a chain terminator of DNA synthesis [6]. Lamivudine, an analogue of cytidine, was the first oral drug approved by the US Food and Drug Administration for the treatment of chronic hepatitis B. It remains the drug of choice because of its administration efficacy, easy intake, clinical safety, and lower cost.

The major limitation of lamivudine therapy is the development of antiviral-resistant mutations. The primary mutation conferring lamivudine resistance involves rt amino acid substitution from methionine to valine or isoleucine at codon 204 (rtM204V or rtM204I) and rtA181V/T mutation [7, 8]. Other amino acid substitutions at sites rtL80I, rtI169T, rtV173L, rtL180 M and rtQ215S occur during lamivudine therapy to restore the replication capability; these are called secondary or compensatory mutations [9]. The incidence of lamivudine resistance is in the range of 10–32 % at 1 year, 37–48 % at 2 years, 52–60 % at 3 years, 60–67 % at 4 years and 69–80 % at 5 years [10–14]. However, in the Indian subcontinent, the treatment of HBV largely depends on lamivudine despite its stated limitations and challenges. The importance of considering lamivudine monotherapy in this study population is because of its cost effectiveness.

In this study, we aim to characterize the antiviral-resistant mutations to lamivudine monotherapy in patients diagnosed with chronic hepatitis B from the Indian subcontinent. The analysis would thus be helpful in devising strategies for the management of chronic hepatitis B.

2 Materials and Methods

2.1 Sample Size

In an earlier study from India, the prevalence of lamivudine-resistant mutations (rtM204I/V) at 12 months was 6 % [15]. Taking this prevalence for the calculation of our sample size with the precision of 4 and a 95 % confidence interval (CI), a sample size of 136 lamivudine-treated chronic hepatitis B patients was calculated.

2.2 Patients

The patients comprised individuals attending the liver clinic of a tertiary care teaching hospital in South India. These patients were identified from their blood samples referred to the Department of Clinical Virology for HBV DNA quantification and were recruited between January 2007 and November 2011. The study was approved by the Institutional Review Board and informed written consent was obtained from all patients.

The inclusion criteria were chronic HBV infection with documented evidence of hepatitis B surface antigen (HBsAg) positivity for more than 6 months. All patients received lamivudine at a standard dosage of 100 mg/day. A total of 147 lamivudine-treated patients were recruited. Among the 147 patients, 90 had pre-treatment (baseline) and intra-treatment follow-up samples available for testing. The remaining 57 patients were started on lamivudine monotherapy prior to their visit to this hospital and their baseline samples were not available. Treatment compliance was checked by verbal questioning and by reviewing the clinical records. Only patients who reported strict adherence to the treatment schedule without any interruption were recruited. The exclusion criteria were history of previous treatment with other HBV antiviral drugs and immunomodulators (switch-off therapy); add-on or combination therapy; infection with hepatitis C virus (HCV), hepatitis D virus (HDV), or human immunodeficiency virus (HIV); history of alcohol abuse; and use of immunosuppressive drugs and chemotherapy. Blood samples (8–10 mL) were collected and the plasma was stored in aliquots at −60 °C until testing.

2.3 Serology Markers

HBsAg was tested in any one of these assays: AxSYM (Abbott, Weisbaden, Germany), ARCHITECT (Abbott) and Monolisa HBsAg ULTRA (Bio-Rad, Marnes-la-coquette, France). Hepatitis B e antigen (HBeAg) and anti-HBe testing was performed in an enzyme immunoassay (EIA) (Diasorin S.P.A., Saluggia, Italy). Anti-HCV, anti-HDV and HIV were screened in Ortho HCV 3.0 (Ortho Clinical Diagnostics, Raritan, NJ, USA), IgM anti-HD EIA (Diasorin S.P.A.) and an AxSYM or ARCHITECT HIV Ag/Ab combination (Abbott), respectively. The manufacturer's instructions were strictly followed for all the procedures.

2.4 Molecular Methods

2.4.1 HBV Quantification Polymerase Chain Reaction (PCR)

DNA was extracted from 200 µl of plasma using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Then, the DNA was eluted in 50 µl of elution buffer. The extracted DNA was used as a template for the Artus® HBV RG PCR assay (Qiagen GmbH) and the viral load was estimated in the Rotor-Gene™ 3000 or 6000 platform (Corbett Research, Mortlake, VIC, Australia). We established the lower limit of detection of the HBV RG PCR assay as 82 IU/mL (95 % detection limit) [16].

2.4.2 Amplification of rt Region of HBV Genome

A segment of the HBV polymerase gene covering the entire rt region was amplified using Platinum® *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, CA, USA) as described previously [17]. The PCR was performed for all samples that were positive in HBV quantification PCR. The amplification reactions were carried out on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) or MyCycler™ (BioRad, Hercules, CA, USA).

2.4.3 Nucleotide Sequencing and Sequence Analysis

The amplified HBVrt products were sequenced using the ABI Prism BigDye® terminator v3.1 cycle sequencing ready reaction reagents (Applied Biosystems) as described in our previous study [18]. The nucleotide sequences of good read lengths of at least 550 bp were taken for analysis. The sequencing reaction and purification steps were repeated for sequences with low signal and poor read lengths. Obtained bidirectional sequences were analyzed using BioEdit v7.0.9 and the consensus sequence was generated. The generated sequences were submitted to the HBVSeq program for HBV mutation analysis in the Stanford database developed by Stanford University (Stanford, CA, USA; http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) [19].

Nucleotide sequences generated from this analysis have been deposited in the GenBank database under accession numbers JQ514380–JQ514499.

2.4.4 Determination of HBV Genotypes, Subgenotypes and HBsAg Subtypes

HBV genotypes were determined by HBVrt sequence analysis in the Stanford database. HBV subgenotypes were determined by phylogenetic analysis in MEGA4 using the neighbour joining method with a bootstrap test of 1,000 replicates and a maximum composite likelihood algorithm. HBsAg subtypes were predicted using the HBVrt overlapping surface gene amino acids as deduced by Purdy et al. [20].

2.5 Biochemical Tests

Serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels were obtained from the patients clinical records.

2.6 Statistical Analysis

A univariate analysis of study variables was performed using non-parametric tests; Mann–Whitney *U* test, Kruskal–Wallis test or a Chi-square test as appropriate. A p value of <0.05 was considered statistically significant. All variables that showed significant association in the univariate analysis were further analysed using a multivariate analysis. A multivariable logistic regression analysis was performed to assess the predictive factors of treatment response and antiviral-resistant mutations. All analyses were performed using STATA 12 (StataCorp, College Station, TX, USA).

3 Results

A total of 147 lamivudine-treated patients with a median treatment duration of 13 (interquartile range [IQR] 8–24) months were studied. Among these patients, 119 (81 %) were male and 28 (19 %) were female; their median age was 39 (IQR 24–50) years.

3.1 Virological Response

All 147 patients continued to be positive for HBsAg. Ninety-three patients were HBeAg positive (63 %) while the remaining 54 (37 %) were HBeAg negative. Seven (7.5 %) HBeAg-positive patients and 47 (87 %) HBeAg-negative patients were positive for the anti-HBe antibody. The median HBV DNA level was 3.6 (IQR 2.63–5.69) $\log_{10}$ IU/mL. Among the 147 patients, 50 (34 %) were classified as responders who showed ≥ 1 $\log_{10}$ IU/mL of HBV DNA reduction with a median treatment duration of 6 months ($n = 12$) or undetectable HBV DNA (<82 IU/mL) with a median treatment duration of 12 months ($n = 38$). Eighty-four (57 %) were non-responders showing <1 $\log_{10}$ IU/mL reduction of HBV DNA with a median treatment duration of 6 months ($n = 5$) or continued to be positive for HBV DNA with a median treatment duration of 12 months ($n = 79$). The remaining 13 (9 %) had only one timepoint of sampling and HBV DNA continued to be positive with ≤ 9 months of lamivudine treatment. For the purposes of this study, the virological responses for these 13 patients could not be studied and were excluded for the analysis including measurement of virological response. The HBVrt sequences generated from these 13 patients were included for the antiviral-resistant mutation analysis.

3.1.1 Intra-Therapy Factors Associated with Virological Response

To identify intra-therapy factors associated with virological response, a univariate analysis for age, gender, ALT, AST, HBeAg and anti-HBe was performed. On analysis, male gender, elevated ALT and AST levels, HBeAg-positive

status and anti-HBe-negative status were significantly associated with lamivudine non-response.

All variables in the univariate analysis significantly associated with virological response ($p < 0.05$) were entered into the multivariate model. Gender (male vs. female), ALT levels (≤2 ULN vs. >2 ULN; ≤70 and >70 U/L), AST levels (≤2 ULN vs. >2 ULN; ≤80 and >80 U/L) and anti-HBe status (positive vs. negative) did not differ significantly with virological response but HBeAg status (positive vs. negative) showed a significant association with virological response. Compared with intra-therapy HBeAg-negative serostatus, intra-therapy HBeAg-positive status was associated with a decreased response to lamivudine [odds ratio [OR] 0.2, 95 % CI (0.06–0.68); $p = 0.01$] (Table 1).

3.2 Antiviral-Resistant Mutations

Among the 147 lamivudine-treated patients, 38 were HBV DNA negative in real-time PCR and HBVrt sequencing was performed in the remaining 109 samples. In the lamivudine-treated patients, 40 (27 %) developed lamivudine-resistant mutations with a median treatment duration of 13 (IQR 8–24) months.

The primary rtM204V/I and rtA181V/T mutations were exclusively detected in 11 (27.5 %) patients. The rtL180M and rtM204V combination was the predominantly identified mutation, $n = 11$ (28 %), followed by the rtL80I and rtM204I combination, $n = 10$ (25 %). The rtM204V/I mutation was also detected along with rtA181V and rtM250L mutations and other compensatory mutations including rtL80V, rtV173L or rtL180M (Table 2). The rtV173L mutation was identified in two study patients with longer treatment durations of 72 months. Additionally, rtI169L antiviral-resistant mutation and rtA181G atypical mutation with unusual amino acid substitutions at crucial HBVrt sites were detected exclusively in one subject each. The cumulative rates of lamivudine-resistant mutations at the median treatment duration of 6 (IQR 6–8), 12 (IQR 12–16), 24 (IQR 24–27) and 41 (IQR 36–60) months are shown in Fig. 1.

3.2.1 *Intra-Therapy Factors Associated with Antiviral Resistant Mutations*

The frequency of antiviral-resistant mutations did not differ significantly with age, gender or ALT and AST levels, but was significantly lower in patients with low HBV DNA levels [2.84 (IQR 0–5) $\log_{10}$ IU/mL] than in those with higher virus loads [5.95 (IQR 4.75–7.15) $\log_{10}$ IU/mL]; was significantly lower in those with a shorter treatment duration [12 (IQR 6–18) months] when compared with a longer treatment duration [24 (IQR 15–34) months] and in HBeAg-negative and anti-HBe-positive patients.

A multivariate analysis, including HBV DNA levels (≤4 $\log_{10}$ IU/mL and >4 $\log_{10}$ IU/mL), treatment duration (median treatment duration of 6, 13, 24 and 41 months), HBeAg status (positive vs. negative) and anti-HBe status (positive vs. negative), was performed. The risk of antiviral resistance was associated with HBV DNA levels [OR 5.9, 95 % CI (1.94–17.7); $p = 0.002$] and treatment duration [OR 2.8, 95 % CI (1.71–4.57); $p < 0.001$] (Table 3).

Table 1 Factors associated with lamivudine response

	Univariate analysis			Multivariate analysis		
	Response ($n = 50$)[a]	Non-response ($n = 84$)[a]	p value	Odds ratio	95 % CI	p value
Age, years[b]	44 (28–52)	39 (26–49)	0.269			
Gender, male[c]	35 (70)	69 (86)	0.024	1.94	0.71–5.3	0.194
ALT, U/L[b]	28 (20–44)	36 (27–59)	0.006	1.58	0.36–6.89	0.539
AST, U/L[b]	34 (22–44)	40 (32–57)	0.008	0.21	0.04–1.2	0.080
HBeAg positive[c]	17 (34)	67 (80)	<0.0001	0.20	0.06–0.68	0.010
HBeAg negative[c]	33 (66)	17 (20)				
Anti-HBe positive[c]	31 (62)	19 (23)	<0.0001	1.89	0.56–6.36	0.306
Anti-HBe negative[c]	19 (38)	65 (77)				

Factors significantly associated ($p < 0.05$) in the univariate analysis were entered into the multivariate analysis, which included gender (male vs. female), ALT (≤70 and >70 U/L), AST (≤80 and >80 U/L), HBeAg and anti-HBe status

ALT alanine transaminase, *AST* aspartate transaminase, *CI* confidence interval, *HBeAg* hepatitis B e antigen

[a] Among 147 lamivudine-treated patients, the virological response for 13 subjects could not be categorised and were not included for analysis

Values are [b]median (interquartile range) or [c]number (%)

Table 2 Profile of lamivudine-resistant HBVrt mutations with varying treatment duration

HBVrt mutations	Treatment duration in months [median (IQR)]			
	6 (6–8)	12 (12–16)	24 (24–27)	41 (36–60)
L80I + M204I	1	3	5	1
L180M + M204I	1	–	–	1
L80V + L180M + M204V	1	–	–	–
[a]L180I + A181C	1	–	–	–
A181T	1	–	–	–
[a]A181G	1	–	–	–
M204I	–	1	3	3
L80V + M204I	–	1	–	1
L80V + M204V	–	1	–	–
I169L	–	1	–	–
L180M + A181V + M204V	–	1	–	–
M204I + M250L	–	1	–	–
L180M + M204V	–	5	5	1
M204V	–	–	1	1
L80V + L180H + M204V	–	–	1	–
L80I	–	–	–	1
A181V	–	–	–	1
V173L + L180M + M204V	–	–	–	1
V173L + L180M + M204I + M250L	–	–	–	1
L80I + L180M + M204V	–	–	–	–

IQR interquartile range, *HBVrt* hepatitis B virus reverse transcriptase

[a] Atypical mutations with unusual amino acid substitutions in HBVrt positions that are crucial for antiviral action

3.3 HBV Genotypes, Subgenotypes and HBsAg Subtypes

3.3.1 Lamivudine Response and Resistance Mutations According to HBV Genotypes

HBV genotypes A, C and D were identified in 29 (19.7 %), 33 (22.4 %) and 85 (57.8 %) patients, respectively. In the samples analysed, 9 (35 %), 9 (31 %) and 32 (41 %) patients infected with genotypes A, C and D, respectively were associated with lamivudine response. On analysis of antiviral-resistant mutations, 10 (34 %), 7 (21 %) and 23 (27 %) study patients of genotypes A, C and D developed resistance. There was no significant difference in study patients who showed lamivudine response ($p = 0.633$) or lamivudine-resistant mutations ($p = 0.503$) and the genotypes tested.

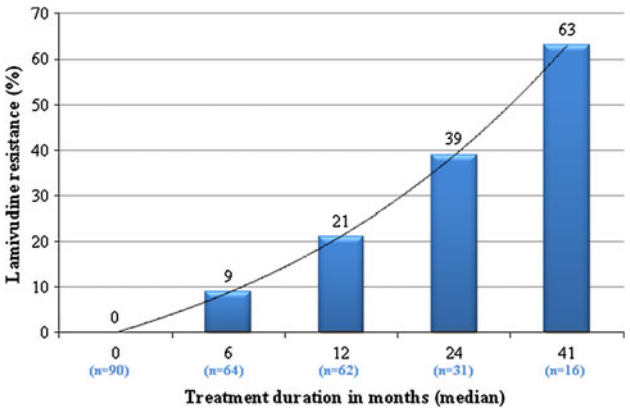

Fig. 1 Percentage frequency of lamivudine-resistant mutations over the course of therapy

3.3.2 Lamivudine Response and Resistance Mutations According to HBV Subgenotypes

All HBV genotypes A and C sequences were identified as subgenotype A1 and C1, respectively. HBV subgenotypes D1, D2, D3 and D5 were identified in 7 (8.2 %), 55 (64.7 %), 7 (8.2 %) and 15 (17.6 %) patients, respectively. The subgenotype for one genotype D could not be assigned. The virological responses for subgenotypes D1, D2, D3 and D5 were 4 (57 %), 20 (40 %), 4 (57 %) and 4 (29 %) patients, respectively ($p = 0.489$). Antiviral resistance mutations in subgenotypes D1, D2, D3 and D5 were identified in 3 (43 %), 15 (27 %), 1 (14 %) and 4 (27 %) patients, respectively ($p = 0.694$).

3.3.3 Lamivudine Response and Resistance Mutations According to the Predicted HBsAg Subtypes

HBsAg subtypes *adw2*, *adr*, *ayw1, ayw2* and *ayw3* were predicted in 31 (21.1 %), 32 (21.8 %), 1 (0.7 %), 15

Table 3 Factors associated with lamivudine resistance

	Univariate analysis			Multivariate analysis		
	No antiviral resistance ($n = 107$)	Antiviral resistance ($n = 40$)	p value	Odds ratio	95 % CI	p value
Age, years[a]	37 (26–49)	46 (26–54)	0.123			
Gender, male[b]	85 (80)	31 (84)	0.630			
Treatment duration, months[a]	12 (6–18)	24 (15–34)	<0.0001	2.8	1.71–4.57	<0.001
ALT, U/L[a]	33 (24–49)	36 (23–65)	0.472			
AST, U/L[a]	39 (30–54)	41 (31–64)	0.395			
HBV DNA, $\log_{10}$ IU/mL[a]	2.84 (0–5)	5.95 (4.75–7.15)	<0.0001	5.9	1.94–17.7	0.002
HBeAg positive[b]	59 (55)	34 (85)	0.001	1.2	0.22–7.14	0.794
HBeAg negative[b]	48 (45)	6 (15)				
Anti-HBe positive[b]	47 (44)	7 (18)	0.003	0.64	0.13–3.29	0.595
Anti-HBe negative[b]	60 (56)	33 (82)				

Factors significantly associated ($p < 0.05$) in the univariate analysis were entered into the multivariate analysis, which included treatment duration [median 6 (IQR 6–8), 12 (IQR 12–16), 24 (IQR 24–27) and 41 (IQR 36–60) months]; HBV DNA (≤4 and >4 log IU/mL); HBeAg and anti-HBe status

ALT alanine transaminase, *AST* aspartate transaminase, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *IQR* interquartile range

Values are [a]median (IQR) or [b]number (%)

(10.2 %) and 64 (43.5 %) patients, respectively. The subtype for 4 (2.7 %) patients could not be identified. The virological response for the predicted subtypes *adw2*, *adr*, *ayw2* and *ayw3* were 9 (32 %), 9 (32 %), 8 (53 %) and 23 (39 %), respectively ($p = 0.505$). Antiviral-resistant mutations in the predicted subtypes *adw2*, *adr*, *ayw2* and *ayw3* were identified in 12 (39 %), 6 (19 %), 4 (27 %) and 16 (25 %) patients, respectively ($p = 0.333$).

3.4 Baseline Factors for Prediction of Lamivudine Response and Resistance Mutations

Among the 147 lamivudine-treated patients, 90 had baseline samples available. Baseline variables were used to identify factors that could predict subsequent virological response and development of antiviral-resistant mutations.

Among the 90 baseline samples analysed, 48 (53 %) responded to lamivudine treatment subsequently and 42 (47 %) were non-responders. To identify baseline factors that would predict the lamivudine response, a univariate analysis for age, gender, ALT, AST, HBV DNA, HBeAg and anti-HBe was performed. In the factors analysed, AST is the only factor that showed significant association for the prediction of virological response ($p = 0.037$) (Fig. 2).

There was also no significant difference between the study patients who developed lamivudine-resistant mutations ($n = 16$) and those who lacked lamivudine-resistant mutations ($n = 74$) as shown in Table 4.

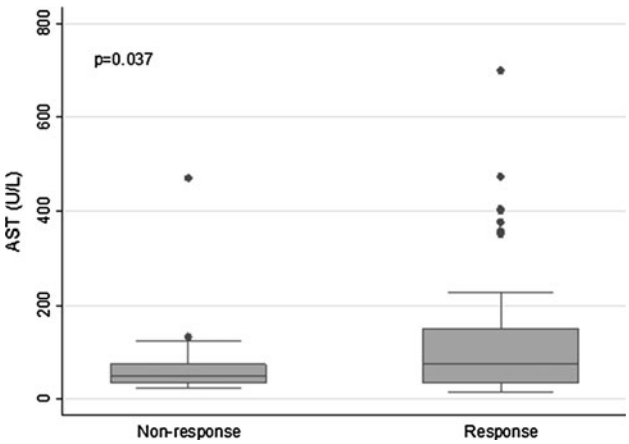

Fig. 2 Box and Whisker plot showing the baseline serum aspartate transaminase (AST) levels in lamivudine responders ($n = 48$) and non-responders ($n = 42$). The *box* represents the median and interquartile range. The *areas covered by the whiskers* indicate 1–99 percentile and the *dots* are outliers

4 Discussion

Virological response and antiviral resistance development were the two major outcomes determined in this study. In the samples analysed, intra-therapy variables were used to identify factors associated with lamivudine response and antiviral-resistant mutations; baseline variables were used to identify the predictive factors of lamivudine response and antiviral-resistant mutations.

In the intra-therapy samples analysed, the univariate analysis showed male gender, high ALT levels, high AST

Table 4 Baseline factors for prediction of lamivudine response and resistance mutations

	Responders ($n = 48$)	Non-responders ($n = 42$)	p value	No antiviral resistance ($n = 74$)	Antiviral resistance ($n = 16$)	p value
Age, years[a]	44 (29–52)	38 (24–49)	0.308	40 (29–49)	43 (15–55)	0.903
Gender, male[b]	35 (73)	37 (88)	0.073	57 (77)	15 (94)	0.129
ALT, U/L[a]	54 (32–151)	46 (28–68)	0.092	47 (30–102)	50 (33–74)	0.788
AST, U/L[a]	77 (35–150)	51 (35–74)	0.037	66 (35–102)	52 (38–73)	0.339
HBV DNA, $\log_{10}$ IU/mL[a]	5.3 (3.8–6.8)	5.7 (4.3–7.6)	0.168	5.39 (4.3–6.95)	6.09 (4.35–7.6)	0.243
HBeAg positive[b]	25 (52)	30 (71)	0.060	44 (59)	11 (69)	0.489
HBeAg negative[b]	23 (48)	12 (29)		30 (41)	5 (31)	
Anti-HBe positive[b]	19 (40)	11 (26)	0.179	25 (34)	5 (31)	0.845
Anti-HBe negative[b]	29 (60)	31 (74)		49 (66)	11 (69)	

ALT alanine transaminase, *AST* aspartate transaminase, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *IQR* interquartile range
Values are [a]median (IQR) or [b]number (%)

levels, HBeAg-positive and intra-therapy anti-HBe-negative status to be significantly associated with non-response. When included into a multivariate model, only HBeAg showed significant association with lamivudine response. In our study, 20 % of HBeAg-positive patients responded to lamivudine when compared with 66 % in HBeAg-negative patients ($p < 0.0001$). These findings are consistent with those of previous reports, which showed that loss of HBV DNA is less likely in HBeAg-positive patients than in HBeAg-negative patients.

Likewise, on analysing the intra-therapy factors associated with lamivudine resistance, the frequency of antiviral-resistant mutations did not differ significantly with age, gender, ALT and AST levels, but was significantly lower in patients with low HBV DNA levels compared with those with high HBV DNA levels, in those with a shorter treatment duration compared with a longer treatment duration and in HBeAg-negative and/or anti-HBe-positive patients than in HBeAg-positive and anti-HBe negative patients. Further, the multivariate analysis showed that antiviral resistance was associated with increased HBV DNA levels and treatment duration. Study patients with HBV DNA levels >4 $\log_{10}$ IU/mL had a 5.9-fold increased chance of developing lamivudine resistance than patients with HBV DNA levels ≤ 4 $\log_{10}$ IU/mL. Therefore, we show that high HBV DNA levels and increased treatment duration are strongly associated with the development of lamivudine-resistant mutations [10, 14, 21, 22].

In our analysis, there was no significant difference between lamivudine response and HBV genotypes. There was also no association between the number of patients who developed lamivudine resistance and the genotypes tested. In addition, subgenotypes of genotype D did not show any association with lamivudine response and resistance mutations. Adding evidence to an earlier meta-analysis, our finding also revealed a lack of association between HBV genotypes and treatment response [23].

There are few reports that showed the association of antiviral response and resistance development with certain HBsAg subtypes. The study by Zollner et al. [24] showed subtype *adw* to have a 20-fold increased risk of lamivudine resistance compared with subtype *ayw*. Subsequently, subtype *adw* was shown to be associated with a better response to lamivudine than subtype *ayw* [25]. Our results indicated that there was no significant difference in lamivudine response or resistance mutations for the subtypes tested.

In our analysis of baseline variables, AST is the only factor that showed significant association for lamivudine response ($p = 0.037$). The baseline AST level was significantly higher in patients who subsequently responded to lamivudine than in non-responders. In the natural course of HBV infection, the immune clearance phase is characterised by elevated serum aminotransferase levels. To eradicate the virus, the immune system acts on the target hepatocytes and the presence of elevated liver enzymes in serum indicates lysis of infected cells. Therefore, the measure of serum aminotransferases is an indirect measure of hepatocellular damage, much of which is mediated by the immune system. The higher baseline AST levels in patients who subsequently responded to lamivudine indicated a heightened immune response. Therefore, high immune responses together with the antiviral action of lamivudine might have led to the better clinical outcome. This was also postulated in a study with similar findings [26]. Though ALT levels were not significantly different ($p = 0.092$), the baseline ALT levels were significantly higher in responders as compared with non-responders (Table 4).

High serum ALT levels and low peripheral blood HBV DNA levels at baseline were previously reported to predict

lamivudine response. In our study, we did not observe a significant association between baseline HBV DNA levels and subsequent lamivudine response. Previously, Yuen et al. [26] have also showed a similar observation. Their study suggested that lamivudine response is not solely dependent on the baseline HBV DNA levels as patients with high baseline HBV DNA levels still showed good response to lamivudine subsequently. Another study by Perrillo et al. [27] showed poor evidence for baseline HBV DNA ($p = 0.07$) being a predictive factor of HBeAg loss while on lamivudine treatment when compared with ALT ($p < 0.001$) and histologic activity index score ($p < 0.001$). These studies corroborate our findings.

Earlier studies have reported male gender, older age, lower baseline ALT levels, HBeAg positivity and higher baseline HBV DNA to be the predictors of lamivudine resistance [13, 21, 22, 28, 29]. In our analysis, none of these baseline parameters showed significant association with lamivudine resistance. One explanation for the contradictory findings could be the limited numbers studied, especially in those patients identified with lamivudine-resistant mutations ($n = 16$).

Antiviral drugs help to reduce the progression of liver disease by the suppression of HBV DNA. Additionally, in drugs with a high selection pressure, there is a complete suppression or very low levels of viral replication that limits the chance of mutant selection and resistance development [30]. The American Association for the Study of Liver Diseases and the European Association for the Study of Liver Diseases now recommend the use of more potent drugs in terms of viral DNA suppression with a high barrier to resistance, such as entecavir or tenofovir, as a first-line therapy in the management of chronic hepatitis B [31, 32]. With the availability of more potent drugs, there is a need to reconsider alternative therapeutic options for the better management of chronic hepatitis B in the Indian subcontinent.

There are several reports from other Asian countries on the antiviral efficacy of lamivudine therapy and associated antiviral-resistance mutations. The ethnic population of India, however, varies widely from other Asian groups. Unlike interferon, there are no studies on oral nucleot(s)ide analogues showing differences in antiviral efficacy rates among different ethnic groups. Moreover, India is the second largest country with chronic hepatitis B carriers. Therefore, in addition to the published literature, our data would have a significant impact on the management of chronic hepatitis B.

Recently, Nimer et al. [33] showed that vitamin D supplements influence response to anti-HCV therapy. The role of nutrition status or vitamin supplements on the antiviral efficacy of HBV drugs is largely unknown. Moreover, there are no well established studies showing the influence of antiviral therapy in patients with a family history of HBV. Therefore, we recognise the need for studying other covariates including nutritional status and a family history of HBV infection and their impact on antiviral efficacy in hepatitis B patients.

To the best of our knowledge, this is the largest report from the Indian subcontinent to bring such collective information in a sufficiently good number of lamivudine-treated patients. In this study, the sequential follow-up of patients receiving lamivudine therapy was dependent on scheduled visits to the hospital for HBV DNA quantification. The sequential follow-up at each timepoint of measurement recommended for therapeutic monitoring was not available for testing. This is considered the major limitation of this study.

In summary, virological response was observed only in 35 % of lamivudine-treated patients. It is very evident from our findings that HBV genotypes, subgenotypes and HBsAg subtypes do not influence treatment outcome or the development of antiviral resistance. High HBV DNA levels and increased treatment duration were strongly associated with lamivudine resistance. Further, patients who continued to be positive for HBeAg have an increased risk for lamivudine failure. We also show that elevated baseline AST levels are significantly associated with subsequent lamivudine response. Considering the limited potency and high-resistance rates experienced with lamivudine, our study emphasises the use of more potent drugs in the management of chronic hepatitis B.

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