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PREDICTIVE FACTORS FOR RESPONSE TO LAMIVUDINE IN CHRONIC HEPATITIS B

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SUMMARY

Background: Lamivudine has been shown to be an efficient drug for chronic hepatitis B (CHB) treatment.

Aim: To investigate predictive factors of response, using a quantitative method with high sensitivity.

Methods: We carried out a prospective trial of lamivudine in 35 patients with CHB and evidence for viral replication, regardless to their HBeAg status. Lamivudine was given for 12 months at 300 mg daily and 150 mg thereafter. Response was considered when DNA was undetectable by PCR after 6 months of treatment. Viral replication was monitored by end-point dilution PCR. Mutation associated with resistance to lamivudine was detected by DNA sequencing in non-responder patients.

Results: Response was observed in 23/35 patients (65.7%) but only in 5/15 (33.3%) HBeAg positive patients. Only three pre-treatment variables were associated to low response: HBeAg ($p = 0.006$), high viral load (DNA-VHB > 3×10^6 copies/ml) ($p = 0.004$) and liver HBcAg ($p = 0.0028$). YMDD mutations were detected in 7/11 non-responder patients.

Conclusions: HBeAg positive patients with high viral load show a high risk for developing drug resistance. On the other hand, HBeAg negative patients show a good response to lamivudine even with high viremia.

KEYWORDS: Hepatitis B; HBV; Lamivudine; HBV-DNA; PCR; bDNA

INTRODUCTION

Chronic hepatitis caused by hepatitis B virus (HBV) may lead to cirrhosis, liver failure and hepatocellular carcinoma. The current treatment with interferon-α (IFN-α) remains unsatisfactory, as it is effective in fewer than 40 percent of patients[34], and previous treatment with corticosteroid is still in use to improve results[18].

Lamivudine (2', 3'-dideoxy-3'-tiacytidine) is a potent inhibitor of viral DNA polymerase and is considered one of the most promising nucleoside analog. Following entering the cells, lamivudine is phosphorylated to its active metabolite, lamivudine-5'-triphosphate, by the 2'-deoxycytidine kinase and other cellular kinase enzymes[35]. It, then, interferes with the reverse-transcriptase activity of the HBV polymerase, terminating the nascent viral DNA chain and inhibiting DNA synthesis[37].

According to a preliminary study carried out by DIENSTAG *et al.*, daily doses of 100 and 300 mg of lamivudine reduced HBV-DNA to undetectable levels in all patients during a six month therapy; but sustained undetectable levels of HBV-DNA and hepatitis-B e antigen (HBeAg) occurred in only 5-12% of the treated patients[6]. In an extended study for up to 18 months, with 23 patients who had remained HBeAg positive after six months of therapy, the same research group observed that HBV-DNA suppression was maintained during therapy in 20 (87%) of these patients. Whereas, HBeAg became negative in 39% of the patients[7] .

A recent report showed that HBV-DNA levels in serum may decrease below the detection threshold of the Polymerase Chain Reaction (PCR) assay in 46% of 51 patients, after a 24-week course of lamivudine[12] . A trend towards a deeper suppression of viral replication with a daily dose of 300 mg was also observed[12]. Such efficacy of lamivudine to impair HBV replication was also shown in HIV-infected[8] and in transplanted patients[23, 28, 29].

Daily doses of lamivudine in patients with chronic hepatitis B (CHB) has varied widely, ranging from 5 to 600 mg. Doses above 5 mg reproducibly decreased HBV-DNA levels in serum[8], but doses of 100 mg and 300 mg can reduce viremia to an undetectable level[6]. Daily doses

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of 300 mg may lead to an earlier[6] and more profound suppression of HBV DNA[12].

Viral resistance to lamivudine is associated with mutations which lead to amino acid substitutions in the highly conserved Tyr-Met-Asp-Asp (YMDD) motif, in the active site of the polymerase[22]. The appearance of such mutation (Methyonine to Isoleucine or Valine) may explain the viral "breakthrough" during lamivudine therapy[5, 7, 25, 28].

Despite the reported efficacy of lamivudine in patients not previously treated and in those who had not responded to interferon therapy[6], additional data are needed to evaluate whether there is a difference in response to lamivudine in these two groups of patients.

The level of viral nucleic acid in serum appears to be an early indicator for the effectiveness of therapy[31]. Few studies have evaluated the efficacy of lamivudine with more sensitive quantitative methods, including the end-point dilution method[27], specially in patients undergoing long-term treatment[12].

The aim of this study was to assess the efficacy and safety of lamivudine in two groups of Brazilian patients with different degrees of CHB; one who had not been previously treated with any drug (12 patients), and another after failure of IFN-α treatment (23 patients).

The present study revealed an as yet not described correlation between viral load and response to lamivudine.

PATIENTS AND METHODS

Patients

We carried out an open and prospective trial of lamivudine in 35 consecutive patients with CHB with evidence of viral replication. Eligible patients included 30 men and 5 women, ranging from 9 to 72 years old. All had hepatitis B surface antigen (HBsAg) in serum for at least six months, detectable HBV-DNA in serum (34 by PCR and one by bDNA) and alanine aminotransferase (ALT) levels ranging from normal levels to 10 times the upper normal limit (UNL).

The inclusion criteria were broad, allowing the enrollment of patients with low transaminase levels as well as patients with low degree of hepatitis, on one side, and patients with compensated (Child-Pugh A) and decompensated (Child-Pugh B) cirrhosis on the other side[30].

Patients were excluded if they were also infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus (HIV), had other serious medical illness, another type of liver disease or an advanced decompensated liver disease (Child-Pugh C), or were pregnant or lactating.

Patients consecutively attended from March 1996 to July 1997 were considered for treatment with lamivudine on a daily dose of 300 mg for 12 months and 150 mg thereafter. Only 4 patients with decompensated liver cirrhosis (Child-Pugh B) received 150 mg from the beginning.

Thirty five patients have been enrolled, being 30 under lamivudine treatment and most of them for longer than 6 months (long term treatment). Lamivudine was withdrawn from five patients who presented YMDD mutation after nine months of treatment (4 with YVDD and one with YIDD mutations).

Patients were divided in two groups: Group I included 12 patients who had not been previously treated ("naive") and, Group II, 23 patients who had been previously treated with interferon-α (IFN-α). Resistance to IFN-α was considered as partial in case of HBeAg clearance and persistence of HBV-DNA detected by PCR (15 patients). Complete resistance was defined as persistence of HBeAg and HBV-DNA (8 patients). None of these patients received immunosuppressive or antiviral therapy at least 6 months before lamivudine therapy.

Biopsies

Liver biopsy was performed using a Tru-Cut™ needle in 25 patients, but not in the remaining 10, due to coagulation disturbances or ascitis. In the latter cases, a clinical and ultrasonographic diagnosis of cirrhosis was performed. Liver histology was blindly evaluated by two specialized pathologists.

Immunohistochemistry

HBsAg and Hepatitis B core antigen (HBcAg) detection in liver tissue was carried out using monospecific polyclonal antibodies in the high-sensitive streptavidin-biotin-peroxidase system (LSAB, Dako, USA), after blockage of endogenous biotin and peroxidase[32].

Serology

Detection of HBsAg, HBeAg and antibodies to hepatitis B e antigen (anti-HBe) in serum samples were carried out by ELISA, using commercially available kits (Abbott Laboratories, North Chicago, IL, USA).

Detection of HBV-DNA by Polymerase Chain Reaction (PCR)

HBV-DNA was detected by a nested PCR as previously described[15, 16]. Serum samples (10 µl) were denatured with 2.5 µl of NaOH 0.5 M and incubated at 37 °C for one hour. Samples were neutralized with 2.5 µl of HCl 0.5 M. For the first round, 1 µM of each primer [1763 (5'TTG GGG CAT GGA CAT TGA CCC GTA TAA 3') and 2032R (5'CTG ACT AAT TCC CTG GAT GGG TCT 3')] were added to the mixture. Amplification was achieved in a thermocycler model 480 (Perkin Elmer, USA), in 25 cycles of 94 °C for 1 minute (min.), 42 °C for 1 min. and 72 °C for 2 min., followed by an extension step at 72 °C for 5 min. In the second round, 1:10 of the first round product was amplified by primers 1778 (5'CAT TGA CCC GTA TAA AGA ATT 3') and 2017 (5'CTG GAT GCT GGG TCT TCC AAA 3'), in the same conditions as above. Amplified products from the second round were electrophoresed in a 2% agarose gel, stained with Ethidium Bromide and visualized under ultra-violet light.

To avoid false-positive results, strict procedures proposed for nucleic acid amplification techniques were followed[19].

Detection of mutations associated with resistance to Lamivudine

HBV-DNA positive samples were also amplified with primers

corresponding to the HBV polymerase gene, targeted to the region of mutations associated with lamivudine resistance. Serum samples were denatured and neutralized as described above and submitted to nested PCR. The outer primers 5'LAM 5 (5'TGC RYY TGT ATT CCC ATC CCA TC 3') and 3'LAM 2 (5'GTT TTG TTA GGG TTT AAA TG 3') were used in the first round, while the inner primers L840 (5'ACC CCA TCT TTT TGT TTT GTT AAG 3') and L372 (5'TCG CTG GAT GTG TCT GCG GCG TTT TAT 3') were used in the second round. Amplification for the first and second round was carried out at 94 °C for 1 min., followed by 35 cycles of 94 °C for 30s, 42 °C for 30s and 72 °C for 40s and a final extension step at 72 °C for 7 min in a PTC-100 thermocycler (M J Research, Watertown, MA, USA)[1, 2].

Sequencing

PCR products were submitted to cycle sequencing reactions, using the second round primers described above and the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (PE Biosystems, Foster City, CA, USA). Nucleotide sequences from both strands were determined in an Automated DNA sequencer model ABI 310 FS (PE Biosystems, Foster City, CA, USA). Sequences were compared with those previously described in the literature using the Lasergene program (DNAstar, Madison, WI, USA).

Quantification of HBV DNA

HBV viremia was followed in sequential samples from the patients using an in-house end-point dilution PCR, or simply EPD method.

Samples were initially diluted in water to a 10-fold series till 10^{10}. Each dilution was submitted to the same PCR protocol described above for detection of HBV-DNA. As the sensitivity of the method was reported to be around 3 copies per 10 µl or 300 copies/ml[15, 16], quantitative results were estimated by multiplying the least positive dilution by 3×10^2 copies/ml.

Assessment of response

Patients were considered as responders if HBV-DNA was undetectable by PCR at the sixth month of treatment.

Other endpoints such as HBeAg clearance, HBeAg to anti-HBe seroconversion and disappearance of HBsAg were also investigated. HBeAg clearance was defined as the absence of HBeAg in two consecutive samples.

Statistical analysis

Univariate analysis for non-responder and responder patients were performed according to the Chi-square (χ^2) and Fisher's Exact Test, taking into consideration the following variables: sex, previous treatment with IFN-α, viremia, presence of serum HBeAg, HBcAg *in situ*, and liver cirrhosis. Age and pre-treatment ALT levels were analysed using the Student's t-test. Pre-treatment ALT levels were also analysed by the non-parametric Mann-Whitney-Wilcoxon test. This test was also used to analyse the viral load median (represented by the log of the number of genomes/ml) in the two groups. For viral load analysis, we also compared two groups with high viremia ($> 3x10^6$) and low viremia ($\leq 3x10^6$) by the Chi-square test.

The logistic regression technique was performed to estimate the probability of non-response based on variables that demonstrated statistical significance in the univariate analyses. The stepwise method was used to select the variables more associated with the probability of non-response.

The level of significance was 5% ($\alpha = 0.05$); and the SAS software (Statistical Analysis System, SAS Institute, NC, USA) was used.

All patients gave informed consent for the study, which was approved by the Local Ethics Committee.

RESULTS

Therapy efficacy

Lamivudine therapy induced a rapid decrease in serum HBV-DNA concentration in 27 out of 29 patients (93.1%) whose quantification was performed after 12 weeks of treatment: 14 (51.9%) had undetectable HBV-DNA, and 13 (48.1%) showed a decrease in viremia. The other 2 patients presented no change in the HBV-DNA concentration (Table 1 and Table 2). At the sixth month of treatment, chosen as the time point to define response in this study, HBV-DNA was undetectable in the serum

Table 1
HBV-DNA levels (log of genomes/ml) in the Responder Group

Patient	Sex	Age	Group	PCR (log genomes/ml)		
				Pre	12 weeks	24 weeks
1	M	17	I	3	NEG	NEG
3	M	29	I	4	NEG	NEG
5	M	72	I	10	3	NEG
6	M	62	I	12	4	NEG
8	F	9	I	8	NEG	NEG
9	M	59	I	9	2	NEG
10	M	47	I	4	NEG	NEG
12	M	67	I	6	3	NEG
14	M	33	II	3	NEG	NEG
15	M	27	II	4	NEG	NEG
19	M	48	II	3	ND	NEG
20	M	46	II	8	NEG	NEG
24	M	29	II	3	NEG	NEG
25	M	72	II	3	NEG	NEG
26	M	37	II	10	6	NEG
27	F	18	II	3	NEG	NEG
28	F	37	II	6	6	NEG
30	M	49	II	6	ND	NEG
31	M	43	II	3	NEG	NEG
32	M	32	II	3	NEG	NEG
33	M	24	II	8	3	NEG
34	M	50	II	4	ND	NEG
35	M	45	II	6 [(a)]	4	NEG

Sex (M = male, F = female); Age (years); Group (I = no previous treatment; II = previous treatment with Interferon-α); [(a)] = bDNA; ND = not done; NEG = negative.

Table 2
HBV-DNA levels (log of genomes/ml) in the Non-Responder Group

Patient	Sex	Age	Group	PCR (log genomes/ml)		
				Pre	12 weeks	24 weeks
2	F	22	I	10	3	4
4	M	42	I	10	4	3
7	M	51	I	12	NEG	4
11	M	11	I	10	6	4
13	M	27	II	10	10	6
16	M	30	II	8	NEG	4
17	M	65	II	12	4	7
18	M	48	II	10	4	3
21	M	44	II	10	ND	3
22	F	23	II	3	ND	4
23	M	25	II	3	ND	3
29	M	60	II	10	6	4

Sex (M = male, F = female); Age (years); Group (I = no previous treatment; II = previous treatment with Interferon-α); ND = not done; NEG = negative.

of 23 out of 35 patients (65.7%) (responder group). The relevant characteristics of the 23 responder and the 12 non-responder patients are shown in Table 3.

There were no significant differences between the two groups (responders and non-responders) with respect to age, gender, response to previous interferon treatment, base-line alanine aminotransferase levels and degree of liver injury, as shown by the semi-quantification of the stage or grade of inflammation in each liver compartment.

Only three pre-treatment variables were statistically associated with low response to lamivudine: HBeAg positivity (p = 0.006), high viral load (p = 0.004) and HBcAg *in situ* immune-expression (p = 0.028) (Table 3). Fourteen (93%) out of the 15 HBeAg positive serum presented high viremia at baseline (greater than 3×10^6 genomes/ml); and response to lamivudine at the sixth month was observed in 5 (36%) of these 14 patients . Baseline low viremia was observed in one HBeAg-positive patient who responded to lamivudine. Of the 20 HBeAg-negative at the baseline, 3 (15%) patients presented high and 17 (85%) low viremia, of whom, 2 and 15, respectively, responded to lamivudine after the sixth month. As a whole, a lack of parallelism was seen between HBeAg positivity and degree of viremia in 4 (11%) out of 35 patients. The presence of HBcAg was significantly related to the presence of HBeAg in sera (Fisher's Exact Test: p = 0.002).

Levels of viral load at pre-treatment was correlated with response to lamivudine in two ways: when comparing patients with high ($> 3 \times 10^6$ genomes/ml) and low viremia ($\leq 3 \times 10^6$ genomes/ml) (p = 0.004), and when analyzing the log of number of such values using a non–parametric test (p = 0.006).

The stepwise method selected two variables associated with response to lamivudine: high or low viremia (genomes/ml) and level of viremia (log genomes/ml). With the first variable, the probability of non-response to lamivudine is 11.4 times higher in patients with high viremia ($> 3 \times 10^6$ genomes/ml), with a very wide 95% confidence interval (1.938 to 66.355). For this reason, the variable log of number of genomes (*G*), as determined by EPD-PCR, was selected and the model was written as follows, where *p* is the probability of non-response to lamivudine.

$$p = \frac{\exp(-3.507 + 0.387G)}{[1 + \exp(-3.507 + 0.387G)]}$$

In this model, the variable *G* assumes continuous values from 3 to 12. The estimated odds-ratio (1.473; 95% confidence interval, 1.109 to 1.958) showed that the chance of non-response increases 1.5 times for each increase of one log in the viral load, as shown in Figure 1.

The HBV-DNA levels (log of genomes/ml) found for responders are shown in Table 1 and for non-responders in Table 2.

All but one non-responder patients at the sixth month persisted as PCR-positive at the twelfth month, regardless of their viremia level by PCR. On the other hand, all but one responder patients were PCR negative at the twelfth month.

Serum HBV markers

At the sixth month of therapy, HBeAg became negative in only 3 (20%) out of 15 patients. Among the remaining 12 HBeAg positive, 5 (42%) became negative after one year of therapy. HBeAg negativation was followed by anti-HBe seroconversion in all responder patients; in one patient, HBeAg disappeared only after 21 months of therapy. Interestingly, despite the detection of HBV-DNA and YVDD mutation, the HBeAg antigen became negative in one non-responder. Moreover, HBsAg clearance was observed in only one responder patient, after 20 months of therapy.

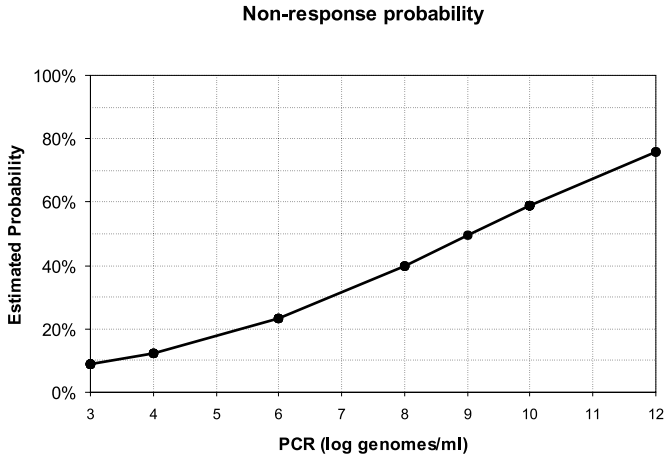

Fig. 1 - Probability of non-response according to the regression model.

Serum alanine aminotransferase (ALT) activity:

Serum ALT activity was determined in 33 patients at the sixth month. Of 18 patients with increased ALT level at baseline, 12 became normal (including three non-responders). Whereas of 15 patients with normal ALT at baseline, only one non-responder showed an increased ALT level after 6 months of treatment. Therefore, lamivudine was equally effective at suppressing viral load among patients with normal and elevated ALT levels (Table 3).

The seven patients with lamivudine resistance and YMDD mutations (see below) showed a slight elevation of the ALT level (0.5 to 3.0 times the upper normal limit) with abnormal values in four of them. No biochemical flare was observed, except in one patient during an one-month association of famciclovir.

Previous treatment with Interferon-α

As shown in Table 3, no difference was found between Group I (naive of treatment) and Group II [resistant to Interferon-α (IFN-α)]. However, considering the pattern of previous response to IFN-α , we observed a trend (Fisher's Exact Test: p = 0.058) to a better response to lamivudine in patients who had shown a partial response (12 out of 15 patients) as compared to those who did not respond (3 out of 8 patients) to IFN-α, respectively.

As for HBV-DNA levels, a baseline high viremia was detected in 8 out of 12 patients naive of treatment and in 9 out of 23 interferon-resistant patients, with a response to lamivudine of 50% and 33%, respectively. On the other hand, a baseline low viremia was observed in 4 out of 12

Table 3
Characteristics of the 35 patients at baseline and comparison between responder and non-responder. Patients were considered "responders" if presented undetectable HBV-DNA by PCR at the sixth month of treatment

Variable	Responder (%) (23 patients)	Non-responder (%) (12 patients)	Total	p	Test
Male/female	20 : 3	10 : 2	35	1.00	Fisher
Age (mean; SD)	41.4 ; 17.5	37.3 ; 16.8	35	0.51	Student's
HBeAg positive	6 (40)	9 (60)	15	0.006	χ^2
HBeAg negative	17 (85)	3 (15)	20		
Non-cirrhosis	15 (65)	8 (35)	23	1.00	Fisher
Cirrhosis	8 (67)	4 (33)	12		
Previous IFN-α: Yes	15 (65)	8 (35)	23	1.00	Fisher
No	8 (76)	4 (33)	12		
Pre-treatment ALT (x UNL) (mean; SD)	2.66 ; 2.95	1.33 ; 0.86	35	0.054	Student's
Pre-treatment ALT (x UNL(*)) (median)	1.26	0.97	35	0.37	Mann-Whitney-Wilcoxon
Viral load (median of log genomes/ml)	4	10	34 (*)	0.006	Mann-Whitney-Wilcoxon
Viremia(genomes/ml):					
High (>3x10^6)	7 (41)	10 (59)	17	0.004	χ^2
Low (≤3x10^6)	15 (88)	2 (12)	17		
HBcAg *in situ*:					
Positive	4 (40)	6 (60)	10	0.028	Fisher
Negative	13 (87)	2 (13)	15		

(*) One patient was excluded because his pre-treatment viral load was determined only by bDNA. SD = Standard Deviation; UNL = upper normal limit.

naive patients and in 14 out of 23 Interferon - resistant patients, with a response frequency of 100% and 86%, respectively. No statistical significant difference was found in both cases.

Resistance to Lamivudine

Of the 12 non-responders to lamivudine, samples of 11 were sequenced for the YMDD motif of the C region, and 7 (64%) of them showed mutations associated with lamivudine resistance. Six (55%) patients showed a YVDD mutation and one (9%) of them a YIDD mutation. The other 4 (36%) patients presented the wild type virus, and in the 12th patient, sequencing was not possible as no DNA amplification of this region was obtained after several PCR attempts.

At the time samples were collected for sequencing the YMDD motif, 8 out of the 11 patients were under lamivudine treatment (period of 6 to 24 months, average 12.5 months). In the other 3 patients, treatment had been withdrawn at 2 (one patient) and 8 months (2 patients) before the mutation analysis, and all showed the wild type YMDD motif

All 7 patients with resistance mutation were HBeAg positive at baseline. A detailed analysis of YMDD mutations during and after withdrawal of lamivudine will be described elsewhere.

DISCUSSION

The recent introduction of the drug lamivudine added a new expectative to the treatment of Chronic Hepatitis B (CHB). In the present study, in agreement with previous results using Abbott Genostics liquid hybridization assay[6, 26], a gradual or a rapid decrease in HBV-DNA levels was observed. This pattern was detected by the three different quantitative methods: end-point dilution (EPD), Amplicor Monitor and branched DNA (bDNA). The EPD method with 10-fold serial dilutions was also used by YOTSUYANAGI *et al.* who described a highly sensitive detection limit (10 copies per reaction) [36].

Other studies reported higher rates of HBV-DNA suppression after lamivudine therapy at week 24 or 52[7,11,20]. However, their quantification methodology was based on the solution hybridization assay (Abbott), with a detection limit of 1.6 to 2.0 pg/ml, corresponding to $4.5x10^5$ to $5.6x10^5$ genomes/ml[4,6,20,31]. Concentrations calculated by the Abbott assay were approximately 40 to 100 fold and 35 fold lower than those obtained when the same sample was tested by the Quantiplex and Hybrid Capture System HBV-DNA assays, respectively[4,17,31]. On the other hand, like us, LAU *et al.* observed a lower rate of response when using a quantitative PCR method[21].

The HBV-DNA levels were found to be low or undetectable at the sixth month of therapy in our patients. However, in patients with low HBV-DNA levels at base line, only the quantitative PCR-based methodology was able to show variation in viremia. Therefore, some patients with low levels at baseline would not have been treated if selection had been based only on bDNA determinations. As emphasized by HADZIYANNIS[10], quantification of HBV-DNA in sera by techniques with a detection limit of at least 1,000 copies/ml is recommendable for both the inclusion of low-viremia patients in treatment trials as well as for evaluating therapy efficacy.

Supporting data for the hypothesis that persistent low degree of viremia (detected only by PCR quantitative methods) is clinically relevant were shown by the evolution of our non-responder patients at the sixth month. All but one of the 12 non-responder patients persisted with positive PCR and a viral load from 10^3 to 10^7 genomes/ml and out of these 11, seven patients showed a progressive increase of viremia and a resistance mutation in the YMDD motif. Conversely, all but one of the 23 responder patients (PCR negative at the sixth month) persisted as such after 12 months of therapy. These results suggest that highly sensitive PCR assays will be useful for evaluating new drugs and therapy regimens[31] and that a negative PCR at the sixth month is an useful criterion for determining response to lamivudine.

Our results also showed that non-response to lamivudine is mainly observed in serum of patients with high viremia and, most of them, positive for HBeAg. This correlation was so significant that a proportion between probability of non-response and increase in the logarithm of genome copies per ml could be established (Figure 1). MUTIMER *et al.*[24] showed that high titers of hepatitis B virus in serum of pre-treated patients predicted failure of lamivudine prophylaxis and graft re-infection after liver transplantation.

Mutations in the YMDD motif were observed only in non-responder patients with high viremia and positive HBeAg. If we take into consideration only the first 12 months of treatment, 33% of our patients (5/15) presented such mutation - which is comparable to the frequency obtained by HONKOOP *et al.* (39%, in 14 patients)[13], but higher than the one reported by LAI *et al.* (14%, in 335 patients)[20] - both within the same therapy period. Furthermore, if we consider the full length of our treatment (24 months), the frequency of mutation carriers was even higher: 47% (7 out of 15 patients).

In a later paper, HONKOOP *et al.* calculated an actuarial cumulative incidence of YMDD mutation carriers of 32% and 53% after 52 and 78 weeks, respectively, of lamivudine therapy in 15 patients treated for more than 12 months[14].

According to LAU *et al.*, lamivudine resistance tends to appear specially in patients whose serum levels of HBV-DNA persist above 1,000 Eq/ml[21].

The response frequency found in HBeAg-negative patients was 85%; higher than that reported by TASSOUPOULOS *et al.* patients with the same serological profile (63%), probably because we included patients with low viremia, not detected by the bDNA method employed by these workers[33]. Interestingly, 27% of patients in the Tassoupoulos' study showed YMDD variant at week 52. This mutation was not observed by us in HBeAg negative patients.

It is worth mentioning that one patient became HBeAg negative, despite the detection of a YVDD mutation to lamivudine resistance. This fact was also observed by GARRETT *et al.*[9].

Another important point is that the response to lamivudine did not depend on the presence or absence of liver cirrhosis. Furthermore, the tolerance to lamivudine was equally good in both situations, even in patients with ascitis (Child Pugh B).

Summing up, two important conclusions arose from our observations: first, patients positive for HBeAg and with a high viral load are less prone to respond to lamivudine as monotherapy due to the high risk of drug resistance and selection of mutation in the YMDD motif; second, patients negative for HBeAg generally showed good response to lamivudine, even in case of liver cirrhosis or of high viremia. A longer follow-up for these and other patients with YMDD mutation will be described elsewhere.

RESUMO

Fatores preditivos para resposta da lamivudine na hepatite crônica B

Introdução: A Lamivudina tem-se mostrado útil no tratamento da hepatite crônica pelo vírus B (HC-VHB).

Objetivo: Investigar os fatores preditivos da resposta à Lamivudina na HC-VHB.

Material e Métodos: Estudo prospectivo com Lamivudina em 35 pacientes com HC-VHB e evidência de multiplicação viral, independentemente do resultado do AgHBe. Administrou-se a Lamivudina na dose diária de 300 mg por 12 meses, seguida de 150 mg diários. Critério de resposta: DNA-VHB negativo (por técnica de PCR) aos 6 meses de tratamento. Nos pacientes não respondedores, pesquisaram-se mutações associadas com resistência à Lamivudina, através do sequenciamento do DNA viral.

Resultados: Observou-se resposta em 23/35 pacientes (65,7%). Dos 15 pacientes com AgHBe positivo antes do tratamento, apenas 5 (33,3%) responderam. As variáveis prévias ao tratamento que puderam prever uma má resposta foram: AgHBe positivo (p = 0,006), carga viral elevada (> 3 x 10^6 genomas/ml) (p = 0,004) e AgHBc no tecido positivo (p = 0,0028). Mutações na região YMDD foram detectadas em 7/11 pacientes não respondedores.

Conclusões: Pacientes com AgHBe positivo e com alta carga viral apresentam um alto risco de desenvolver resistência à Lamivudina. Por outro lado, pacientes com AgHBe negativo, mesmo com alta carga viral, mostraram uma boa resposta à Lamivudina.

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REFERENCES

1. AYE, T.T.; BARTHOLOMEUSZ, A; SHAW, T. *et al.* - Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. **J. Hepat.**, **26**: 1148-1153, 1997.

2. BARTHOLOMEW, M.M.; JANSEN, R.W.; JEFFERS, L.J. *et al.* - Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. **Lancet, 349**: 20-22, 1997.

3. BENHAMOU, Y.; KATLAMA, C.; LUNEL, F. *et al.* - Effects of lamivudine on replication of hepatitis B virus in HIV-infected men. **Ann. intern. Med., 125:** 705-712, 1996.

4. BUTTERWORTH, L.-A.; PRIOR, S.L.; BUDA, P.J. *et al.* - Comparison of four methods for quantitative measurement of hepatitis B viral DNA. **J. Hepat., 24**: 686-691, 1996.

5. CHAYAMA, K.; SUZUKI, Y.; KOBAYASHI, M. *et al.* - Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. **Hepatology, 27**: 1711-1716, 1998.

6. DIENSTAG, J.L.; PERRILLO, R.P.; SCHIFF, E.R. *et al.* - A preliminary trial of lamivudine for chronic hepatitis B infection. **New Engl. J. Med., 333**: 1657-1661, 1995.

7. DIENSTAG, J.L.; SCHIFF, E.R.; MITCHELL, M. *et al.* - Extended lamivudine retreatment for chronic hepatitis B. **Hepatology, 24**: 188A, 1996 [Abstract].

8. DUSHEIKO, G.M. - New treatments for chronic viral hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODÉS, J., ed. **Therapy in liver diseases: the pathophysiological basis of therapy**. Barcelona, Masson, 1997. p. 317-330.

9. GARRETT, L.; DIENSTAG, J.L.; GAUTHIER, J. *et al*. - Hepatitis B e-antigen (HBeAg) seroconversion in two patients with evidence of genotypic resistance following extended lamivudine treatment. **Hepatology, 26**: 431A, 1997 [Abstract].

10. HADZIYANNIS, S.J. - Natural course and therapy of anti-HBe-positive chronic hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODÉS, J., ed. **Therapy in liver diseases. The pathophysiological basis of therapy.** Barcelona, Masson, 1997. p. 301-308.

11. HONKOOP, P.; DE MAN R.A.; ZONDERVAN, P.E. *et al.* - Histological improvement in patients with chronic hepatitis B virus infection treated with lamivudine. **Liver, 17:** 103-106, 1997.

12. HONKOOP, P.; DE MAN R.A.; NIESTERS, H.G.M. *et al*. - Quantitative hepatitis B virus DNA assessment by the limiting-dilution polymerase chain reaction in chronic hepatitis B patients: evidence of continuing viral suppression with longer duration and higher dose of lamivudine therapy. **J. viral Hepatitis, 5:** 307-312, 1998.

13. HONKOOP, P.; NIESTERS, H.G.; DE MAN R.A. *et al*. - Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. **J. Hepat., 26**: 1393-1395, 1997.

14. HONKOOP, P.; DE MAN, R.A.; NIESTERS, H.G.M. *et al.* - Incidence, characteristics and clinical impact of lamivudine resistance in chronic hepatitis B**. J. Hepat., 28**(suppl.1): 48, 1998 [Abstract].

15. KANEKO, S.; FEINSTONE, S.M. & MILLER, R.H. - Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique**. J. clin. Microbiol., 27**: 1930-1933, 1989.

16. KANEKO, S.; MILLER, R.H.; DI BISCEGLIE, A.M. *et al.* - Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. **Gastroenterology, 99**: 799-804, 1990.

17. KAPKE, G.E.; WATSON, G.; SHEFFLER, S. *et al*. - Comparison of the Chiron Quantiplex branched DNA (bDNA) assay and the Abbott Genostics solution hybridization assay for quantification of hepatitis B viral DNA**. J. viral Hepatitis, 4**: 67-75, 1997.

18. KROGSGAARD, K.; MARCELLIN, P.; TREPO, C. *et al*. - Prednisolone withdrawal therapy enhances the effect of human lymphoblastoid interferon in chronic hepatitis B. **J. Hepat., 25**: 803-813, 1996.

19. KWOK, S. & HIGUCHI, R. - Avoiding false positives with PCR. **Nature (Lond.), 339**: 237-238, 1989.

20. LAI, C.-L.; CHIEN, R.-N.; LEUNG, N.W.Y. *et al.* - A one-year trial of lamivudine for chronic hepatitis B. **New Engl. J. Med., 339**: 61-68, 1998.

21. LAU, D.T.-Y.; DOO, E.; GHANY, M.G. *et al.* - Lamivudine for chronic hepatitis B with typical and atypical serology. **Hepatology, 26:** 429A, 1997 [Abstract].

22. LING, R.; MUTIMER, D.; AHMED, M. *et al*. - Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. **Hepatology, 24**: 711-713, 1996.

23. MARKOWITZ, J.; PAKRASI, A.; HOLLIS, P. *et al*. - Efficacy of lamivudine for prophylaxis and treatment of hepatitis B in liver transplant patients. **Hepatology, 24**: 182A, 1996 [Abstract].

24. MUTIMER, D.; PILLAY, D.; DRAGON, E. *et al*. - High pre-treatment serum hepatitis B virus titre predicts failure of lamivudine prophylaxis and graft re-infection after liver transplantation. **J. Hepat., 30:** 715-721, 1999.

25. NAOUMOV, N.V.; CHOKSHI, S.; SMITH, H.M. *et al.* - Emergence and characterization of lamivudine-resistant hepatitis B virus variant. **Hepatology, 24:** 282A, 1996 [Abstract].

26. NEVENS, F.; MAIN, J.; HONKOOP, P. *et al*. - Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. **Gastroenterology, 113:** 1258-1263, 1977.

27. NIITSUMA, H.; ISHII, M.; MIURA, M. *et al*. - Low level hepatitis B viremia detected by polymerase chain reaction accompanies the absence of HBe antigenemia and hepatitis in hepatitis B virus carriers. **Amer. J. Gastroent., 92:** 119-123, 1997.

28. PERRILLO, R.; RAKELA, J.; MARTIN, P. *et al.* - Lamivudine for hepatitis B after liver transplantation. **Hepatology, 24:** 182A, 1996 [Abstract].

29. PERRILLO, R.P. - Antiviral therapy of transplant patients with recurrent hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODÉS, J., ed. **Therapy in liver diseases: the pathophysiological basis of therapy**. Barcelona, Masson, 1997. p. 347-350.

30. PUGH, R.N.; MURRAY-LYON, I.M.; DAWSON, J.L. *et al.* - Transection of the oesophagus for bleeding oesophageal varices. **Brit. J. Surg., 60**: 646-649, 1973.

31. ROSENSTRAUS, M.; GUTEKUNST, K. & DALE, B. - Utility of hepatitis virus nucleic acid assays in therapeutic drugs trials. In: SCHINAZI, R.F.; SOMMADOSSI, J.-P. & THOMAS, H.C., ed. **Therapies for viral Hepatitis.** London, International Medical Press, 1998. p 115-127.

32. SANTOS, R.T.M.; ALVES, V.A.F.; WAKAMATSU, A. *et al.* - Chronic hepatitis B immunohistochemistry – comparison of the amplification systems PAP and ABC for the detection of HBsAg and HBcAg. **Arch. argent. Enferm. Apar. dig., 10**: 10, 1996.

33. TASSOUPOULOS, N.C.; VOLPES, R.; PASTORE, G. *et al*. - Efficacy of lamivudine in patients with hepatitis B e antigen – negative / hepatitis B virus DNA – positive (precore mutant) chronic hepatitis B. **Hepatology, 29:** 889-896, 1999.

34. WONG, D.K.H.; CHEUNG, A.M.; O'ROURKE, K. *et al*. - Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B: a meta-analysis. **Ann. intern. Med., 119**: 312-323, 1993.

35. WONG, W.W.S. & TYRRELL, D.L.J. - Treatment of hepatitis B virus with lamivudine. In: SCHINAZI, R.F.; SOMMADOSSI, J.-P. & THOMAS, H.C., ed. **Therapies for viral Hepatitis.** London, International Medical Press, 1998. p. 353-363.

36. YOTSUYANAGI, H.; YASUDA, K.; IINO, S. *et al.* - Persistent viremia after recovery from self-limited acute hepatitis B. **Hepatology, 27:** 1377-1382, 1998.

37. ZEUZEM, S.; DE MAN, R.A.; HONKOOP, P. *et al.* - Dynamics of hepatitis B virus infection *in vivo*. **J. Hepat., 27:** 431-436, 1997.

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