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ORIGINAL ARTICLE

Evaluation of Possible Risk Factors of Lamivudine Resistance in Chronic Hepatitis B patients: A Retrospective Study in Iran

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Background and Aims: **Clinical resistance to lamivudine (LAM) can lead to exacerbation and high-level cross-resistance to all L-nucleotides in chronic hepatitis B patients, but the underlying reasons for this remain unclear. This study aimed to compare the clinicopathological features of LAM-resistant and LAM-responsive patients and tried to detect the tissue levels of Glutathione S-transferas pi (GSTpi) in the naive biopsies of both groups to find possible risk factors of LAM resistance.**

Methods: **Patients with naive biopsies and successful LAM therapy for one year were included as controls (n = 25), and patients who interrupted their LAM regimen during clinical relapse (n = 16) were considered cases. Clinicopathological characteristics of patients and GSTpi levels were compared between the two groups by an immunohistochemical analysis.**

Results: **Although no significant difference was detected between tissue levels of GSTpi in cases and controls, statistical tests showed a significant role of prior interferon-alpha (IFN-α) (P = 0.024, odds ratio [OR] = 5.25) therapy and higher HAI scores (P = 0.05, OR = 4.08) of naive biopsy samples and emphasized their roles as two relative risk factors for LAM resistance. Half of the cases showed higher HAI scores (> 6) whereas only 19% of controls showed the same pattern. A higher rate of LAM withdrawal was detected in patients with a history of IFN therapy (P = 0.024), and a history of IFN therapy was considered to be a possible risk factor of LAM withdrawal (OR = 5.21). Higher HAI scores were also detected in patients with a history of INF therapy (P = 0.041, OR = 4.8), and a history of INF therapy was considered to be a possible risk factor of tyrosine-methionine-aspartate-aspartate (YMDD) mutation (OR = 3.83).**

Conclusions: **This study has introduced two possible new predictive markers of LAM resistance in chronic hepatitis patients, which should be confirmed in future studies with larger groups to optimize the best pharmacotherapy regimens in chronic hepatitis B patients and may help physicians reduce the risk of adverse drug reactions.**

Keywords: **Chronic Hepatitis B, Lamivudine, Glutathione S-transferas pi, Cross Resistance, Interferon-Alpha**

Introduction

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide and is a major global health problem. Approximately one million people die annually because of acute and chronic HBV infection despite the availability of effective vaccines and antiviral medications [(1)] . Current treatment of chronic hepatitis B (CHB) aims to interrupt the progression and clinical outcomes of the disease by suppressing viral replication [(2, 3)]. Lamivudine (LAM) is the first

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nucleoside analogue to treat CHB by inhibiting viral DNA replication, improving liver function, and inducing histological improvement of fibrosis [4], but the major drawback of LAM monotherapy is the emergence of resistance to HBV with the mutation of the tyrosine-methionine-aspartate-aspartate (YMDD) motif at the catalytic domain (C domain) of the viral reverse transcriptase/DNA polymerase [5]. The incidence of YMDD mutants rises 15-32% in the first year to 67-69% by the fifth year of treatment [1]. This phenomenon can lead to the exacerbation of hepatitis or even hepatic failure, which needs further management [4]. Although other new agents such as entecavir are approved for LAM-resistant CHB [6], the lack of availability of these newer agents in Iran and many other developing countries have made the therapeutic regimens more complex and expensive.

Although YMDD mutations cause infected patients to be resistant to LAM therapy, many other cases have been reported in Asian countries in which patients were clinically resistant to LAM without the mentioned mutations, most likely because of heightened transaminases levels [7-9] or a rebound of the viral load at earlier times [10, 11]. It seems that LAM and many other nucleos(t)ides may not be effective in every patient because of many unknown host and viral factors, which should be investigated further in clinical studies.

Literature supports the role of Glutathione *S*-transferas pi (GSTpi) as an important tumor marker of drug resistance in hepatocellular carcinoma (HCC) [12-14]. It has also been reported that HBV infection may induce the maintenance of GSTpi in hepatocytes, which could affect hepatocarcinogenesis [15], but its expression in CHB patients as well as its predictive role in LAM-R remain unclear. This study tried to understand the clinical roles of GSTpi and other clinicopathological factors as possible risk factors of LAM resistance, which could be used as affordable, easy, and reliable clinical tools for predicting possible resistance to LAM therapy before starting a drug regimen.

Materials and Methods

Patients

All patients had CHB, had already received naïve liver biopsies, and had been administered LAM monotherapy for one year (January 2006 to January 2007). The patients were seen and followed-up in Prof. Daryani's Gastroenterology Clinic or the Iranian Charity for Hepatic Patients and were enrolled in the present study. All patients had tested positive for hepatitis B surface antigen (HBsAg) and negative for hepatitis B e antigen (HBeAg) for at least 6 months and had undergone a liver biopsy to evaluate the degree of necroinflammation in the liver before starting LAM regimens. Patients were excluded if they were habitual heavy drinkers or IV drug abusers, showed any evidence of hepatic cirrhosis, or were coinfected with hepatitis C virus, hepatitis D virus, or HIV.

Demographic information

Demographic information of the CHB patients was collected with a questionnaire designed for this study. The information included age at diagnosis, sex, marital status, occupation, disease initiation date, histories of background disease (e.g., diabetes, cardiovascular diseases, talassemia, history of other infectious diseases, AIDS), drug or alcohol addiction, tattoos, and blood transfusion. In addition, the starting date, dose, and duration of LAM treatment were noted for each patient. In order to confirm LAM resistance in the present study, sequencing information of all patients with detectable HBV DNA at the end of one year or when they discontinued their LAM regimens from clinical resistance signs was collected.

Sample collection

Liver biopsies of patients before entering drug therapy were selected. If they continued their regimen for at least one year with acceptable clinical improvement, they were considered to be part of the control group. Patients who interrupted their LAM regimen during breakthrough infection or had a clinical relapse at any time during their drug regimen were considered to be LAM-resistant cases. LAM-resistant cases were those who developed a breakthrough infection during treatment and were defined by a rebound of viral load (HBV DNA > 10^5 copies/Ml) or the presence of an LAM-resistant mutant polymerase chain reaction /restriction fragment lengths polymorphism (PCR/RFLP) or elevated serum transaminases (> upper limit of normal /ULN). This information was determined by reading their clinical data and confirming the information via correspondence with collaborative laboratories and clinicians. Accessible parrifinized blocks of the abovementioned groups were found in 9 different hospitals: Dr. Shariati, Baqiyatallah, Amir-Alam, Imam Khomeini, Mehr, Atiyeh, Toos, Sevome Shaban, Arad and Shahryar. Five micrometer tissue samples were requested and obtained from the abovementioned hospitals for pathological studies (HAI scores and stage) and evaluation of GSTpi expression.

The following information was recorded for each sample: date of biopsy, HBV DNA level at the time of sampling, method used for viral-load quantification, and the sequencing result at the time of sampling. Optionally, alanine aminotransferase (ALT) levels and the presence of HBeAg and anti-HBeAb were recorded if there existed in the patient's history. As the presence of HBeAg was not accessible for all of cases, we therefore focused the study on HBeAg-negative cases and excluded the few HBeAg-positive ones. For ethical reasons, all cases without naïve liver biopsy at the beginning of the study or the RFLP report at the end of our one-year experiment were excluded from the present research, too.

Pathological studies

Liver-biopsy samples were scored on a hepatitis activity index (the sum of the component scores for piecemeal necrosis, lobular inflammation, and portal inflammation) (grading score/HAI) and were classified into pathological stages by a pathologist who was blinded to the treatment outcomes according to Knodell *et al.*'s criteria [16].

Immunohistochemical studies

As previously described [17] dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH = 6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections in 0.3% hydrogen peroxide in methanol for 30 minutes and 3% BSA for 60 minutes, respectively. Sections were then incubated for 30 minutes at room temperature with GST pi mouse monoclonal antibody (353-10) to GST3/GSTpi and prediluted (ab856, Abcam company) to recognize the nuclear and cytoplasmic expression of human GSTpi in hepatocytes. The results were visualized using the streptavidine-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation-Denmark) and DAB chromogen (Dakocytomation- Denmark) based on the manufacturer's instructions with some necessary modifications. Sections were also counterstained with Meyer's haematoxyline. In each series, a section in which incubation with the primary antibody was omitted was used as a negative control. The ideal staining conditions were established in our preliminary experiments. Staining was considered negative only after careful examination of the entire tissue section. Semiquantization of the intensity and number of positive hepatic cells was performed by independent pathologists blinded to the clinical outcome. In cases in which the observers disagreed, the immunohistochemical scoring was repeated ensure agreement by both observers. Biopsy samples were then classified into four categories based on the expressions of markers. Based on quantization of the intensity and number of positive cytoplasms, hepatocells were scored as 3+ if they had strong staining (> 50%), 2+ if they had moderate staining (25-50%), 1+ if they had mild staining (5-25%), and 0 if staining was < 5% or nonexistent.

Ethical considerations

As with hepatitis C, a biopsy sample is recommended for initial assessment of the severity of the disease in CHB patients. Biopsy is generally not regarded as essential for determining whether treatment is needed, even though it is preferable to have a baseline histological assessment [15]. Due to ethical considerations, we selected our case and control groups from patients who had biopsy samples before starting LAM monotherapy. The ethical viewpoint of the present study was approved by the ethics committee of the Pharmaceutical Sciences Research Center.

Statistical analysis

Values were expressed as percent per population or as the mean ± standard deviation (SD). To assess the association between expressions of markers and clinicopathological data, nonparametric chi-square tests were used. A Fisher exact test was used for frequencies less than five. Relative risks and odds ratios were calculated by the Cochran-Mantel-Haenszel statistic using SPSS 17 [18]. Probability values < 0.05 and odds ratios > 1 were considered significant.

Results

Clinicopathological characteristics of patients

IFN-α therapy: Out of 80 CHB patients who were in accordance with the abovementioned inclusion criteria, the biopsy samples of 41 patients (16 cases and 25 controls) were accessible. The clinicopathological features of the patients are summarized in Table 1. Although no significant difference were observed between the age, sex, smoking patterns, level of alcohol consumption, and the pathological stages of both groups, history of prior treatment with IFN-α was significantly higher in cases (P = 0.024, odds ratio [OR]= 5.25, 95%, confidence interval [CI] = 1.23-22.3) than controls. That means half of the LAM-resistant patients (8/16) showed a history of treatment with IFN-α, whereas only 19% (4/21) of controls had the same

Table 1. Clinicopathological features of cases and controls

Variables	Cases	Controls	P-value	Odds Ratio 95% Confidence Interval
Age (years)				
Mean+SD (range)	34+16.43(11-65)	33.09+15.07(13-68)	0.863	1.41 (0.38-5.2)
Sex				
Males	9(56.25%)	14%(56%)	0.621	0.99(0.27-3.5)
Females	7(43.75%)	11 (44%)		
History of INF alpha regimens (before starting LAM)				
Positive	8(50%)	4(19%)	0.024	5.25 (1.23-22.3)
Negative	8(50%)	21(81%)		
YMDD mutation (RFLP)				
Positive	9(56.2%)	2(8%)	0.001*	14.78(2.56-85.1)
Negative	7(43.75%)	23(92%)		
Rise of transaminases levels				
Positive	9(56.2%)	4(19%)	0.009	6.75(1.57-28.93)
Negative	7(43.75%)	21(81%)		
Rebound Viral Load				
Positive	13(81.25%)	5(20%)	0.001*	17.3(3.5-85.2)
Negative	3(18.75%)	20(80%)		
Smoking				
Positive	2(25%)	4(19%)	0.374	1.75(0.369-8.30)
Negative	12(75%)	21(81%)		
Alcohol Consumption				
Positive	3 (18.75%)	23	0.291	2.65(0.39- 17.99)
Negative	13 (81.25%)	2		
Pathological Stage (1-6)*				
<3	11	15	0.606	1.09(0.23- 5.02)
>3	4	5		
HAI Score(1-13)				
<6	8(50%)	21(81%)	0.05	4.08(0.952-17.5)
>6	8(50%)	4 (19%)		

SD: standard deviation; LAM: lamivudine; YMMD: tyrosine-methionine-aspartate-aspartate; RFLP: restriction fragment lengths polymorphism.

* Pathological stages were not recorded for a few patients.

history.

YMDD mutation: RFLP showed a higher prevalence of YMDD mutations in cases than controls (P = 0.001, OR = 14.78, 95% CI = 2.56-85.1), but mutation in the YMDD motif was not recorded in any LAM-resistant patients after one year of LAM therapy or during withdrawal from LAM monotherapy at any time after starting the therapy.

Rebound viral load: Although other possible mutations in the RT region of HBV polymerase have not been analyzed, we realize that some explanations for withdrawal may be mutations such as rtL80V/I, rtI169T, rtV173L, rtA181T, rtT184S,ALT elevation or rebound viral load (10^5 copies/Ml). Incidence of rebound viral load was significantly higher in cases than controls (P = 0.001, OR = 17.3, 95% CI = 3.5-

85.2).

Elevation of transaminases: ALT elevation was significantly higher in cases than controls as well, without recording YMDD mutation (P = 0.009, OR = 6.75, 95% CI = 1.57-28.93). Although a few cases from the control group showed a rebound viral load (5/25) or elevated transaminases levels (4/25) during LAM therapy, these cases did not show YMDD mutation or any clinical signs of recurrence and continued their LAM regimens during this study. Their clinical improvement was also considered acceptable by the physicians.

Relative risk factors for LAM resistance

The Cochran-Mantel-Haenszel statistic showed several relative risk factors of LAM resistance and few factors related to responsiveness to LAM. Of the evaluated factors, prior regimens of IFN-α (OR = 5.25), higher HAI scores (OR = 8.5), mutation in the YMDD motif (OR = 14.78), transaminases elevation (OR = 6.75), smoking patterns (OR = 1.75), level of alcohol consumption (OR = 2.65), and rebound viral load were considered to be relative risk factors of LAM resistance after LAM therapy, which may help the prediction of this phenomenon and LAM withdrawal after at least one year of therapy (Table 1). A Fisher exact test showed significant differences between cases and controls in HAI scores, history of IFN therapy, rebound viral load, and transaminases levels. Interestingly, LAM-resistant cases showed higher HAI scores in their initial biopsy samples when compared with the control group (P = 0.05). Half of LAM-resistant cases (8/16) showed high HAI scores (> 6), whereas 15% of controls (4/25) showed the same pattern.

Clinicopathological characteristics of patients with and without a history of IFN therapy

Regarding the sampling limitations, we could not obtain an equal number of patients with and without a history of LAM therapy, and we could not clearly conclude that prior interferon therapy would play a role in future LAM-resistant phenotypes. Therefore, we decided to divide the patients into two new case and control groups. This time the control group was made up of patients without IFN therapy and the case group was made up of patients with IFN therapy. Both groups were treated by LAM for at least one year. Table 2 compares all of the demographic, clinical, and pathological data for both groups.

Although no significant differences were discovered between the two groups regarding their age, sex, smoking patterns, level of alcohol consumption, ALT elevation and tissue pathological stages but this new modeling helped us to discover some other significant differences between two groups clearly. Cases have withdrawn LAM therapy after 21.58 ± 10.94 months of therapy whereas controls were continued their treatment even after 39.76 ± 18.34 months of therapy (during running the present study). In fact higher rate of LAM withdrawal was detected in cases (P=0.024) when compared with the control group and a history of Interferon therapy was considered as possible risk factor of LAM withdrawal (OR = 5.25, 95% CI = 1.23-22.3). Other important parameters in this comparison were YMDD mutatiom (OR = 3.83), ALT elevation (OR = 1.87), rebound viral load (OR=3.8), smoking patterns (OR = 3.12), level of alcohol consumption (OR = 4.5) and HAI score (OR = 3.8) (Table 2).

Status of GSTpi expression in cases and controls

No significant difference was recorded between the case and control groups regarding GSTPi expression (4/16 *vs.* 3/25) according to Fisher's exact test (P = 0.254).

Clinicopathological significance of GSTpi

In the LAM-resistant group, a history of IFN-α regimens (before starting LAM) showed a higher risk of GSTpi expression (OR = 2.33, 95% CI = 0.167-32.58). That means GSTpi was rarely detectable in naïve CHB biopsy samples of patients who did not receive INF-α before starting LAM therapy. A higher risk of GSTpi expression was also detected in LAM-resistant patients with higher HAI scores (OR = 4.2), higher pathological stages (OR = 10), and higher rates of YMDD mutation (OR = 2.14) (Table 3).

Discussion

More recently, hepatitis B has become a treatable disease with five approved agents including IFN-α (standard and pegylated) and three oral antiviral agents (LAM, adefovir dipivoxil, and entecavir). All five licensed agents are effective in suppressing HBV DNA levels and improving serum ALT levels and hepatic histology, but it is still unclear who should be treated, with which agent (or combination of agents), for how long, with what level of monitoring, and with the use of which endpoints to measure the success or failure of treatment [6]. Therefore, much research is needed to optimize treatment for individual patients and to prevent the risk of treatment failures [19].

The present study has suggested the importance of prior IFN-α therapy as a possible risk factor of LAM-resistance in CHB (OR = 5.25, 95% CI =

Table 2. Comparison of the clinicopathological features of patients with and without a history of Interferon Therapy

Variables	History of Interferon Therapy(+)	History of Interferon Therapy(-)	P-value	Odds Ratio 95% Confidence Interval
Age (years)				
Mean+SD (range)	26.44+8.77	34.92+16.26	0.147	0.693(0.153-3.13)
Sex				
Males	9	14	0.110	0.31(0.068-1.4)
Females	3	15		
LAM Withdrawal				
Positive	8	8	0.024	5.25(1.23-22.3)
Negative	4	21		
Months of LAM therapy				
Mean+SD (range)	21.58 + 10.94	39.76 + 18.34	0.003*	
YMDD mutation (RFLP)				
Positive	6	6	0.069	3.83(0.9-16.26)
Negative	6	23		
Rise of transaminases levels				
Positive	6	8	0.3	1.87(0.45-7.69)
Negative	6	21		
Rebound Viral Load				
Positive	8	10	0.061	3.8(0.915-15.77)
Negative	4	19		
Smoking				
Positive	8	4	0.158	3.125(0.63-15.45)
Negative	4	25		
Alcohol Consumption				
Positive	9	2	0.139	4.5(0.646-31.36)
Negative	3	27		
Pathological Stage (1-6)*				
<3	9	17	0.639	0.944(0.19-4.69)
>3	3	6		
HAI Score (1-13)				
<6	6	24	0.081	3.8*(0.84-17.03)
>6	6	5		

SD: standard deviation; LAM: lamivudine; YMMD: tyrosine-methionine-aspartate-aspartate; RFLP: restriction fragment lengths polymorphism.

* Pathological stages were not recorded for a few patients.

1.23-22.3). We showed that the incidence of LAM resistance was significantly higher in patients with a history of INF (P = 0.024), which means that patients with a history of INF therapy withdrew from LAM therapy after 21.58 ± 10.94 months of therapy, whereas other patients continued their treatment even after 39.76 ± 18.34 months of therapy. Although one study has been concerned with the expression of drug-resistant-related genes in three human hepatoma cell lines and demonstrated the modulatory role of IFN-α in resistance to Cis platin (CDDP) in liver cancer [20], to the best of our

Table 3. GST pi expression levels in LAM-Resistant patients (n = 16)

Variables	GST pi (-)	GST pi (+)	P-value	Odds Ratio 95% Confidence Interval
Age (years)				
Mean+SD (range)	33.18+ 17	43.66+ 12.9	NS	
Sex				
Males	6	3	NS	NS
Females	6	1		
LAM Withdrawal				
Positive	1	0	NS	NS
Negative	11	4		
YMDD mutation (RFLP)				
Positive	7	3	NS	2.14
Negative	5	1		
Rise of transaminases levels				
Positive	8	1	NS	NS
Negative	4	3		
Rebound Viral Load				
Positive	10	3	NS	NS
Negative	2	1		
Smoking				
Positive	4	0	NS	NS
Negative	8	4		
Alcohol Consumption				
Positive	2	1	NS	NS
Negative	10	3		
Pathological Stage (1-6)*				
<3	10	1	NS	10(0.58-171.2)
>3	2	2		
HAI Score (1-13)				
<6	7	1	NS	4.2(0.33-53.1)
>6	5	3		

SD: standard deviation; LAM: lamivudine; YMMD: tyrosine-methionine-aspartate-aspartate; RFLP: restriction fragment lengths polymorphism; NS: not significant.

* Pathological stages were not recorded for a few patients.

knowledge this is the first study that suggests the clinical role of IFN-α in inducing LAM resistance and possibly inducing GSTpi expression in hepatocytes of HBeAg negative patients. Although one study [21] has reported that LAM for 52 weeks is not as effective in IFN nonresponders as in previously reported treatment-naive patients, most of the CHB patients in the present study were HBeAg positive. In fact, the present study shows a different pattern in HBeAg-negative cases. Further study is necessary to compare the LAM resistance between HBeAg-positive and HBeAg-positive INF-resistant groups, which may clarify the possible role of e-antigen status in this very important clinical phenomenon.

Although resistance to LAM therapy in CHB patients occurs by mutation in the YMDD motif [5], a breakthrough phenomenon has also been recorded

in cases without YMDD mutation [7-11]. We compared the clinicopathological features of resistant and responsive cases without focusing on YMDD mutation in the present study and tried to compare the clinicopathological features and GSTpi expression of both groups. Although the present results showed a negative association between GSTpi expression and other relative risk factors of drug resistance, the present data support the value of naive biopsy samples for predicting the risk of LAM resistance before starting the abovementioned drug regimen. A significant difference between baseline HAI scores of cases and controls (P = 0.05) was shown, which increased the risk of LAM-resistance (OR = 4.08) in CHB patients.

Although difficulty to access the biopsy samples of naïve patients and incomplete clinical records of patients have limited this preliminary study, this research showed for the first time some new clues (*e.g.*, prior drug regimen, HAI score and GSTpi expression) which may be utilized for the prediction of drug resistance by matching with other risk factors in future studies if ethical laws permit the physicians to obtain biopsy samples before starting the drug therapy. In fact, the HAI score could be used as a practical, economic, and reliable tool for predicting the risk of LAM resistance if its importance is confirmed in larger groups of patients and if ethical rules allow physicians to obtain tissue samples before starting drug-therapy regimens. To the best of our knowledge, biopsy is generally not regarded as essential for determining whether treatment is needed, even though it is preferable to have a baseline histological assessment [15], and this study has emphasized the importance of this factor as well.

In conclusion, the present findings indicate the importance of INF-α pretreatment as one of the major causes of LAM resistance. The importance of the HAI score as a possible effective strategy to identify LAM-resistant cases by studying their naive biopsy samples seems to be suggested from the present study. This study brings new insight regarding the value of prior drug-therapy regimens and the pathological features of biopsy samples in LAM-resistance prediction, but further prospective studies are required to confirm this preliminary result, to compare the clinical outcomes between HBeAg-positive and HBeAg-negative cases, and to clarify the clinical role of other drug- resistance markers in this phenomenon.

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