Investigation by the Method of INNO-LiPA of Primary Resistance to Lamivudine in Patients with Chronic Hepatitis B Who Have Not Used Antiviral Therapy

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ABSTRACT

Aim: *Two billion people around the world are exposed to the hepatitis B virus (HBV) and about 350 million are infected with chronic HBV. The infection can be acquired early (neonatal) and becomes chronic in 90%; this rate reduces to 30% between ages one and five years. There is a 25% risk of chronicity in adults. Nowadays, immunomodulatory and antiviral pegylated-interferons or oral antiviral agents are used in the treatment of chronic hepatitis B. Lamivudine is an effective oral antiviral agent which inhibits the replication of HVB by blocking reverse transcriptase enzyme. The study aims to detect the resistance of HBV to lamivudine in the community and evaluate the effectiveness and suitability of early treatment with lamivudine.*

Subjects and Methods*: One hundred patients who presented to our Faculty of Medicine Hospital Infectious Diseases and Clinical Microbiology Department and had not received any antiviral treatment were recruited. The INNO-LiPA method was applied to investigate primary lamivudine resistance in patients.*

Results: *Seventy-eight patients were HBeAg-negative and 22 patients were HBeAg-positive. A statistically significant correlation was found between HBeAg positivity, alanine aminotransferase (ALT) elevation and HBV DNA (*$p < 0.05$*). The rtM204V and L180M mutation motif was found in one patient with HBeAg positivity.*

Conclusions: *Hepatitis B virus in our region is not a lamivudine-resistant strain and early treatment with lamivudine is an effective and convenient method.*

Keywords: Chronic hepatitis B, INNO-LiPA, lamivudine, primary resistance

Investigación de la Resistencia Primaria a la Lamivudina Mediante el Método de INNO-LiPA en Pacientes con Hepatitis B Crónica Que No Han Usado la Terapia Antiviral

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RESUMEN

Objetivo: *Dos billones de personas alrededor del mundo están expuestas al virus de la hepatitis B (VHB) y unos 350 millones están infectadas con el VHB crónica. La infección puede adquirirse temprano (neonatal) y se convierte en crónica en el 90% de los casos. Esta tasa se reduce al 30% entre las edades de uno a cinco años. Hay un 25% de riesgo de cronicidad en los adultos. En la actualidad, se utilizan interferones pegilados inmunomoduladores y antivirales o agentes antivirales orales en el tratamiento de la hepatitis crónica B. La lamivudina es un eficaz agente antiviral oral que inhibe la replicación del VTTB mediante el bloqueo de la enzima transcriptasa reversa. El estudio tiene como objetivo detectar la resistencia del VHB a la lamivudina en la comunidad, y evaluar la efectividad e idoneidad del tratamiento temprano con lamivudina.*

Sujetos y métodos: *Se reclutaron cien pacientes que acudieron a nuestro Departamento de Servicio de Microbiología Clínica y Enfermedades Infecciosas del Hospital de la Facultad de Medicina, que no habían recibido tratamiento antiviral. Se aplicó el método de INNO-LiPA con el propósito de investigar la resistencia primaria de la lamivudina en los pacientes.*

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Resultados: *Setenta y ocho pacientes resultaron HBeAg negativos y 22 pacientes fueron HBeAg positivos. Se encontró una correlación estadísticamente significativa entre la positividad de HBeAg, la elevación de alanina aminotransferasa (ALT), y VHB ADN (*p < *0.05). El patrón de mutación de rtM204V y L180M fue encontrado en un paciente con HBeAg positivo.*

Conclusiones: *El virus de la hepatitis B en nuestra región no es una cepa resistente a la lamivudina, y el tratamiento temprano con lamivudina es un método eficaz y conveniente.*

Palabras claves: Hepatitis B crónica, INNO-LiPA, lamivudina, resistencia primaria

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INTRODUCTION

Two billion people around the world are exposed to hepatitis B virus (HBV) and about 350 million are infected with chronic HBV (1, 2). Chronic HBV, especially in developing countries, continues to be a major public health problem. Hepatitis B virus carriage in Turkey has a rate of 3.9 to 12.5%. Turkey is located within the area of moderate endemicity (3, 4).

Hepatitis B virus is a small enveloped DNA virus, which is replicated by reverse transcriptase (RT) enzyme codes and RNA agent (5, 6). Today, in the treatment of chronic hepatitis B, immunomodulatory and antiviral effective pegylated-interferons or oral antiviral agents are used. Lamivudine (beta-L-2', 3'-dideoxy thiacitidine) is the first nucleoside analogue used orally in the treatment of chronic hepatitis B. It is well tolerated by patients and there are no serious and common side effects restricting the use. Lamivudine prevents replication of the virus by blocking the RT enzyme providing the replication of HBV. The most important disadvantage is that the duration of treatment is not exact and that it causes the formation of resistant strains (7).

Hepatitis B virus polymerase/RT gene is an important target in the treatment of chronic hepatitis B. Therefore, antiviral resistance in such patients is one of the major problems. Lamivudine is a nucleoside analogue with 2'-3 'dideoxy 3'-tiyasitidin negative enantiomer. It enters into the DNA chain through the active triphosphate added to it enzymatically in the cell and leads to premature chain termination. Thus, it prevents HBV-DNA synthesis (8). It has high oral bioavailability and a long plasma half-life. Hepatitis B e antigen (HBeAg) seroconversion rate increases in direct proportion to the duration of treatment and rises to 50% at five years (9).

Mutant types begin to appear within six months from the start of lamivudine therapy and as treatment duration increases, mutation rates increase. Studies conducted so far indicate that lamivudine resistance rate is up to 12–15% in the first year, 35–45% in the second year, 45–50% in the third year, 50–60% in the fourth year and 60–70% in the fifth year, and that the resistance rates have increased in direct proportion to the duration of illness (10, 11).

Drug resistance should be investigated in cases of increase of 1.0 log10 IU/mL in viral load, increase in serum alanine aminotransferase (ALT) level and clinical worsening manifested by two sera taken within an interval of two months in a patient regularly receiving the treatment and who showed response to the treatment (12).

Mutation observed with lamivudine therapy is an L-nucleoside pathway (rtM204V/I) mutation and occurs in C of viral polymerase. rtM204V/I mutation is a tyrosine-methionine-aspartate-aspartate (YMDD) mutation and rtM204V/I mutant strains are selected with lamivudine, embtrisitabin, telbivudine and klevudin treatment. While rtL180M, located in the upper side B areas, leads to partial resistance, the second mutation causes a higher resistance with rtM204V. Entecavir resistance may also be observed in patients who received lamivudine (12).

Studies have shown that patients with chronic hepatitis B who have not received prior lamivudine therapy have viral genome YMDD mutation (13, 14). New infection can develop from these mutant strains. The study aims to detect the resistance of HBV to lamivudine in the community and evaluate the effectiveness and suitability of early treatment with lamivudine.

SUBJECTS AND METHODS

One hundred patients with chronic hepatitis B disease who had not received any antiviral treatment were recruited from the Faculty of Medicine Hospital Infectious Diseases and Clinical Microbiology Department between June 2010 and May 2011. This study was approved by the Faculty of Medicine Ethics Review Commission. The project was supported by Gilead Sciences, Pharmaceutical Trading Co, Ltd Sti. The study was conducted in Istanbul Düzen Laboratories.

Five millititres of blood was taken for biochemistry from the patients who were applicable for operation and centrifuged for five minutes at 3000 rpm. Sera were stored at -80 ˚C until the day of use.

Hepatitis B virus DNA levels were studied with Roche Cobas Taqman (COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0, Roche Diagnostics, Basel, Switzerland). Serum HBV-DNA levels and mutation patterns causing lamivudine resistance were analysed by INNO-LiPA HBV DR v2 Auto-LiPA 48 (Innogenetics, Ghent, Belgium).

High Pure PCR Template Preparation Kit was used for HBV DNA extraction (Roche, Germany).

Polymerase chain reaction (PCR)

Gene Amp 9700 PCR instrument was used for denaturation. After PCR products were executed in acrylamide gel electrophoresis, the bands were examined in ultraviolet transilluminators and bands were observed as 867 bp. Polymerase chain reaction products were stored until reverse hybridization process at -20 ˚C. The reverse hybridization process was performed with Auto-Lipa Device (Innogenetics, Ghent, Belgium). Codons numbered 80, 173, 180/181, 204, 236 of HBV polymerase gene were scanned and the mutations of HBV were evaluated.

A total of 237 chronic hepatitis B patients who were admitted to the Infectious Diseases and Clinical Microbiology Department of Fırat University Hospital between January 2009 and May 2012 were screened in order to determine secondary resistance ratios. Lamivudine treatment was started in 161 of these patients and HBV DNA and liver function tests were studied at six-month intervals. Hepatitis B virus DNA was tested on follow-up in 37 patients who developed liver enzyme elevation and HBV DNA was found to be elevated. YMDD mutation was examined with INNO-LiPA method in order to determine lamivudine resistance.

RESULTS

Mean age of patients was 40 ± 13 years. Forty-one patients were female and 59 were male. Alanine transaminase levels were normal in 63 of the patients included in the study but high in 37 patients. Average ALT level was 69 ± 10 IU/mL.

There was AntiHBe positivity in 78 of the patients included in the study. In the remaining 22 patients, there was HBeAg positivity. Hepatitis B virus DNA level was 10^4 copies/mL in 50 patients, 10^5 copies/mL in 25 patients, 10^6 copies/mL in 13 patients, 10^7 copies/mL in 2 patients, 10^8 copies/mL in 8 patients and 10^{11} copies/mL in 2 patients.

Percutaneous liver biopsy was performed with Hepafix Braun Luer lock 17G/1.4 mm needle in 25 patients. The average histological activity index (HAI) of patients, according to ISHAK score, was detected as 8 ± 2 and the phase was 2 ± 1. The lowest HAI was 5 and the highest HAI, 12. The lowest phase was phase 2 and the highest phase was phase 3.

By taking the serum samples of patients included in the study, the points of resistance mutations were investigated by INNO-LiPA method. All patients were infected with genotype D. Only one patient had primary lamivudine resistance. Test strip image of the positive patient is seen in the Figure. The patient was HBeAg-positive and also had rtM204V and rtL180M mutation.

In our study, YMDD mutation was detected in a total of 28 patients who had received lamivudine treatment. Resistance was detected in the third year in 16 patients, in the second year in 11 and in the first year in one. Resistance rates were found as 43% in the third year, 29% in the second year and 2.7% in the first year. Mean secondary resistance rate was 57%.

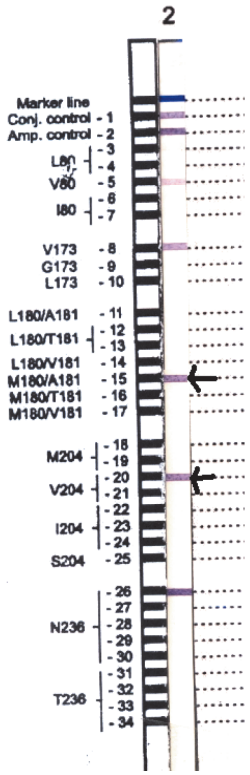

Figure: Appearance of lamivudine resistance on INNO-LiPA test strips.

DISCUSSION

Approximately 300 million patients around the world have chronic hepatitis B infection and 10% of these patients die due to HBV-related chronic disease. Lamivudine is a nucleoside analogue which had Food and Drug Administration (FDA) approval for treatment of chronic hepatitis B in 1998. Lamivudine, a cytosine analogue (dideoxy-2', 3'-thiacitidine), causes premature chain termination by entering into the growing DNA (10–12, 15).

The only disadvantage in comparison with other antiviral agents used for the treatment of chronic hepatitis B is drug resistance seen mostly during lamivudine therapy. Lamivudine increases cross-resistance to nucleoside analogues for the other L nucleosides, and limits the sensitivity of other antiviral agents. For this reason, it limits treatment options (10–12, 15).

Studies conducted so far indicate that lamivudine resistance rate is up to 12–15% in the first year, 35–45% is the second year, 45–50% in the third year, 50–60% in the fourth year and 60–70% in the fifth year, and resistance rates have increased in direct proportion to the duration of illness (10–12, 15).

In the present study, YMDD mutation was detected in a total of 28 patients who had been receiving lamivudine treatment.

While secondary resistance rates were found lower at the end of year 1 and 2, it was found similar to the literature in the third year. The reason for this may be that the patients did not come for review regularly, were admitted to different clinics and the difference of HBV in our region. Mean resistance rates were found similar to the literature.

Use of a genotyping method with high sensitivity and determination has an important place in analysis of viral gene pool for the treatment of HBV virus infection and various methods have been used (13, 14, 16–19).

In a study by Malmström *et al* (20), 180th and 204th codons of viral polymerase gene were screened by Taqman PCR method in 27 serum samples of five patients known to have lamivudine resistance. This study compared PCR with restriction fragment length polymorphism (RFLP) and sequence analysis. Taqman PCR method is fast, cost-effective and convenient.

Restriction fragment length polymorphism and 5'-nuclease-activated Taq DNA polymerase studies are the other two new mutation screening methods. These tests can detect < 1000 copies in HBV DNA genome. Both methods have been designed for scanning single nucleotide changes in HBV DNA polymerase gene. The study by Allen *et al* (16) indicates that these two new tests compared favourably with the sequence analysis and that YMDD mutants can be sorted with wild-type virus even at low concentrations.

Research in various parts of the world has shown that there are chronic hepatitis B carriers infected with lamivudine-resistant strains in the community. Some researchers have found that there are strains with YMDD motif mutation in the serum of patients with chronic hepatitis B infection having HBeAg positivity or HBeAg negativity who never used lamivudine (13, 21–31).

In our study, lamivudine-resistant strains were found in only one in a series of 100 patients. Compared to the work done throughout the world, primary lamivudine resistance rate in our study is lower. Some of the reasons suggested are a) genotypic differences or mixed genotype may exist in countries where the studies were done; b) combination therapy with interferon and lamivudine (long-term) in our region; c) in recent years, drugs such as tenofovir and entecavir, which are more potent antivirals, may have been used more; d) the high rates of HIV observed in the world may be higher in countries with YMDD mutation and therefore, lamivudine was used more intensively in these countries in the past.

Compared with other studies conducted throughout the country, the result of our study was lower. The reasons for this may be that geographic areas are different where data are collected, and the antiviral preferences used in the treatment are different.

In our region, there has not been such a study before. This result confirms that HBV virus in circulation and observed in our region is mainly the wild strain, and that especially starting with lamivudine as treatment is an effective and convenient choice for patients that do not have higher viral load.

As a result, we conclude that lamivudine may be used as the first choice of antiviral in a group of patients with the appropriate conditions. However, it should be taken into consideration that one patient in this study had primary resistance to lamivudine, and other studies have indicated that lamivudine-resistant virus may be dominant in the community and may also cause problems in treatment. According to the results of work done throughout the world, it is recommended that each patient be evaluated for the presence of YMDD mutation prior to treatment. However, there is no applicability for this proposal in the scale of our country and it is not considered cost-effective. Due to the low level of resistance to lamivudine detected in our region, we think that starting with lamivudine as the treatment is suitable for patients with low viral load.

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