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Prevalence of YMDD Variants Leading to Lamivudine Resistance among Chronic HBV Patients in Gaza Strip-Palestine Running title: Lamivudine Resistance in Gaza Strip-Palestine

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Abstract

Background: Hepatitis B virus (HBV) resistance to lamivudine is reported in several areas worldwide. The prevalence of YMDD variants (YIDD and YVDD) and some risk factors leading to lamivudine resistance were investigated among chronic HBV patients in Gaza strip.

Methodology

Fifty four patients with viral load above 4500 IU/ml from two governmental hospitals were tested for Lamivudine resistance from October , 2008 to July , 2011. Real Time PCR determination for viral load and Liver function tests were also carried out .

Results: The prevalence of YMDD variants was determined and divided into five groups: YMDD (32.6%), YMDD/YIDD in (41.3%), YIDD alone (13%), YIDD/YVDD(2.2%),YMDD/YIDD/YVDD(10.9%).A statistically significant correlation was found between viral load and the preceding five groups (p=0.028). The only significant risk factor for lamivudine resistance was lamivudine intake duration (p=0.049).

Conclusion: The prevalence of YMDD variants due to lamivudine resistance among Gaza HBV patients was found to be high, therefore, re-evaluation of management protocols and policies for HBV therapy should be considered.

Keywords; Lamivudine resistance, HBV, Gaza

Introduction

Hepatitis B virus (HBV) is the most common blood borne viral infection worldwide; that is capable of causing hepatitis by attacking the liver leading to acute and chronic infection (**1**). In their 2002 report, the World Health Organization estimated that about two billion people worldwide have been infected with HBV. More than 350 millions are chronic carriers with serious long term complications like cirrhosis and hepatocellular carcinoma, and over 4 millions are acute carriers (**2**). About 1-2 millions of this population dies annually, most of them in Asia (**3,4**). Palestine is considered as

intermediate endemic area of HBV. The HBV incidence rate in Palestine was 0.5 and 1.2 per 100,000 of population in West Bank in the years 2009 and 2010 respectively **(5)** .

There are many antiviral drugs that are used to treat HBV patients or to slow its progression; in general these drugs are classified into two categories; the immunomodulatory agenst (interferon-alpha and peg-interferon) and nucleos(t)ide analogues (lamivudine, entacavir, adefovir, telbivudine) (**2,6,7).** The most common one in Gaza strip is lamivudine. Long term treatment with lamivudine results in amino acids variation in the virus and the emergence of drug resistant HBV mutants. The most common mutation in YMDD (tyrosine methionine aspartate aspartate) occurs at position 204 (**8**). This mutation (rtM204V/I/S), is a substitution of methionine by valine or isoleucine **(9,10**), and serine (**11,4)**.
There are no published reports about lamivudine resistance among chronic hepatitis B patients in Gaza strip. The aim of this study is to determine the prevalence of lamivudine resistant HBV variants YIDD and YVDD among chronic patients in Gaza strip.

MATERIALS AND METHODS

Study Population

Two governmental centers in Gaza Strip were included in this study. The first center is located at Al-Shifa hospital in Gaza city while the second is located at European Gaza hospital in Khan Yunes city. Patients were referred either to the central laboratory of the Ministry of health or Balsam Medical Laboratory at Sheikh Radwan in Gaza for viral load determination between October of 2008 to July 2011. Patients with viral load above 4500 IU/ml were selected (54 patients) for analysis for Lamivudine resistance. Liver function tests were carried out at the governmental centers laboratories (Al-Shifa hospital and European Gaza hospital).

Primers and probes

The primers and probes used for lamivudine resistance determination are listed in table (1).

Table (1): Primers and probes sequences used for lamivudine resistance detection

Primers/probe ID	Description	Sequence (5' to 3')	specificity	Position
COMfor	Common forward primer	CACCTGTATTCCCATCCCAT	HBV genome	595-615
YMDD	M-specific reverse primer	CCCCCAATACCACATCATCC	rt M204 wild type	740-760
YIDD	I-specific reverse primer	CCCCCAATACCACATCATCR[§]	rt M204I (ATT)	740-760
YVDD	V specific reverse primer	CCCCAATACCACATCATCCGC	rt M204V (GTG)	738-759
TaqMan	Common probe	(5′-FAM TGTTGACAARAATCCTCACCATACCRCAGA-MGB-3′,		

[§] *the letter R indicates a degenerate nucleotide (G or A).*

Viral DNA Extraction

The viral DNA was extracted using materials provided with the COBAS Amplicor HBV Monitor test (Roche Diagnostics, Germany).

Real Time PCR determination for viral load

Quantitation of the viral DNA was performed using the COBAS Amplicor HBV Monitor test (Roche, Germany) according to the manufacturer recommendation; or using an in-house designed Real-time standard curve method. Amplification was performed in a 50-µl reaction mixtures containing 1X TaqMan Universal Master Mix (Applied Biosystems, USA), 0.72 µM of a specific 6-FAM labeled probe and 2 µM of each of the forward primer and the reverse primer and 5 µl of isolated DNA.

Amplification and detection were performed with the ABI StepOne real-time PCR system (Applied Biosystems, USA). After incubation for 2 min at 50°C, which enables uracil N9-glycosylase present in the Universal MasterMix to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N9-glycosylase. The PCR cycling program consisted of 40 two-step cycles of 15 seconds at 95°C and one minute at 60°C.

Real Time PCR genotyping

The reactions were carried out using TaqMan universal master mix (Applied Biosystems, USA) on the StepOne Real-Time PCR System (Applied Biosystems, USA). The amplification was performed in a 15-µl reaction mixture in three parallel tubes each containing 2 pmoles of each

primer and the probe in the case of YMDD and YVDD; and 1 pmole of each primer and probe in the case of YIDD. The mixture also contained 1X of the provided Taq Man universal master mixture and 5 µl of extracted DNA. The reaction cycling conditions were the same for those of viral load determination.

RESULTS

Patients Description

The study included 54 patients receiving 100 mg lamivudine who have viral load above 4500 (IU/ml) during the period from October of 2008 and July 2011. The patients age ranged from 4 to 71 years and the mean age was 32.4 years**.** The 54 patients (41 males and 13 females) represent three regions of Gaza strip (8 patients from the Northern governorate, 6 patients from Khan Yunes governorate, and 19 patients from Rafah governorate). The patients were grouped according to lamivudine intake duration into two categories**:** less than one year (18 patients) and more than one year (26 patients).

The prevalence of lamivudine resistance

HBV genotypes were divided in this study into five categories; the first category is the wild type (YMDD) which was found alone in 15 (32.6%) patients , the second genotype is YMDD mixed with YIDD and was found in 21 patients (41.3%), the third was YIDD alone and found in 6 patients (13%), the fourth is YIDD/ YVDD and found in one patient (2.2%), the fifth was YMDD/ YIDD/ YVDD and found in 5 patients (10.9%). Eight samples were found to be non-typable, probably, as a result of the presence of other mutations in the PCR primers' binding location.

Factors associated with lamivudine resistance

No statistically significant relationship was found between YMDD (Wild type), YIDD, YIDD/YVDD,YIDD/ YMDD, and (YMDD /YIDD/ YVDD) and age, sex, lamivudine intake duration, and liver function tests (ALT & AST). But a significant relation with these five groups among viral load was found (Table 2).

For YMDD (Wild type) and other genotypes (resistant type) versus age, sex, viral load, and liver function tests (ALT & AST); the study showed that there is no significant relationship. Whereas a significant relation was present between wild type and resistant type versus lamivudine intake duration (Table 3).

Table (2): Comparison between the mean viral load (IU/ml) and Lamivudine resistance genotypes

Genotypes	No.	Mean	Std. Deviation	P-value
YMDD	15	1.07×10^8	2.855×10^8	0.028
YIDD	6	9.10×10^7	1.208×10^8	
YIDD / YVDD	1	7.28×10^8	.	
YMDD /YIDD	19	2.44×10^8	3.295×10^8	
YMDD /YIDD / YVDD	5	8.02×10^8	1.035×10^9	
Total	46	2.51×10^8	4.633×10^8	

Table (3): Comparison between the mean of lamivudine intake (in months) duration for YMDD and all different Lamivudine resistant genotypes.

Genotypes	No.	Mean	Std. Deviation	Sig.
YMDD	11	27.36	14.362	0.049
Other	28	18.43	11.507	
Total	39	20.95	12.843	

DISCUSSION

Hepatitis B virus (HBV) remains one of the most common viruses in the world. This is the first study in Gaza strip of Palestine that deals with resistance of HBV to Lamivudine. Among the successfully genotyped samples, 31 patients (76.4%) have a lamivudine resistance genotype either alone or in combination with the wild type or another resistance genotype. This high prevalence rate of lamivudine resistance recorded in our study is comparable to other populations like Brazilians patients, (67%) (**3),** and Netherland (59%) **(12).** On the other hand, it is slightly higher than that of other populations such as, Jordan (31%) (**13),** and Japan (31%) **(14). Tuncbilek *et al.*,** reported that one tenth of chronic hepatitis B virus patients have lamivudine resistance in Turkey (**15**).

In this research YIDD mutation was the most prevalent mutation (45.7%). This is in agreement with the studies in Jordan (YIDD: 25%, YVDD: 6.25%) **(13)**, Canada (YIDD: 30%) **(16)**, Netherland (YIDD: 61.5%, YIDD/YVDD: 38.46%) **(12)**, and Korea (YIDD: 34%, YVDD: 21.25%) **(17)** . On the other hand this result mismatch with other investigations, where the prevalence of mutation YVDD was the most dominant mutation. For example in Germany (YVDD: 62%, YIDD: 38%) **(8),** Italy (YVDD: 54.2%, YIDD: 29.1%) **(18),** and Turkey (YVDD: 5.19%, YIDD: 2.59%) **(15) .**

The variant types of mutations may suggest that the patients were infected by a virus that is probably from lamivudine treated cases or less likely from a spontaneous mutation due to HBV reverse transcriptase lacking proofreading activity. It is possible that mutations could naturally and accidentally occur, even at YMDD motif location **(19).**

By comparing the different genotypes with each others, or comparing the wild type with other resistant genotypes collectively, there was no statistically significant difference with respect to the patient age. This finding is in agreement with studies from Turkey, Germany and China **(4,8,15).** Also the five combinations of genotypes obtained in the study did not show a statistically significant distribution according to the patient gender. Such a result was also seen in other populations, such as in Turkey, Germany and China **(4,8,15).**

There is no significant relationship between YMDD, YIDD, YIDD /YVDD, YIDD/YMDD, (YMDD/YIDD/ YVDD) with respect to lamivudine intake duration (P-value =0.696). While there is a significant relation between the wild type and resistant genotypes versus lamivudine intake duration (p value =0.049). As in Germany, there was significant correlation between lamivudine resistant patients and the duration time for treatment **(8).**

In this study, the difference in the mean viral load was statistically significant among the five genotype groups (P-value =0.028). This coincides with study in Germany **(8).** With a common sense, one might expect the resistance genotypes to be associated with higher viral load because patients become less respondent to therapy, or simply because the occurrence of mutation will change the antigenicity of the virus and help it to evade the host immune response. No such significance was obtained by comparing the mean viral load of the YMDD wild type with the other 4 resistance genotypes collectively. This is in agreement with studies conducted in Brazil **(3**), and Turkey **(15).**

In conclusion, lamivudine resistant genotypes among Gaza HBV patients were found to be high and a re-evaluation of the treatment policy should be considered. Further studies to determine the non-typable genotypes are recommended.

REFRANCES:

1. Shepard C, Simard E, Finelli L, Fiore A, Bell B: **Hepatitis B Virus Infection: Epidemiology and Vaccination**. Epidemiol Rev 2006, **28**:112–125.
2. World Health Organization. **Hepatitis B**. Geneva, 2002. http://www.who/cds/csr/lyo/2002.hepatitis_a.html. (Accessed on 15/5/2012).
3. Niesters H, Pas S, Man R: **Detection of hepatitis B virus genotypes and mutants: current status.** Clin Virol 2005, **34**(1): S4-8.
4. Li S, Qin L, Zhang L, et al.: **Molecular epidemical characteristics of Lamivudine resistance mutations of HBV in southern China**. Med Sci Monit 2011, **17**(10): 75-80.
5. Health Status in Palestine: Ministry of Health Annual Report 2010, State of Palestine Ministry of Health (Palestinian health information center), Nablus, Palestine.
6. Sablon E, Shapiro F: **Advances in molecular diagnosis of HBV infection and drug resistance.** Int J Med Sci 2005, **2**(1): 8-16.
7. Block T, Guo H, Guo J: **Molecular virology of hepatitis B virus for clinicians**. Clin Liver Dis 2007, **11**(4): 685-706.
8. Zollner B, Peterson J, Puchhammer E, et al.: **Viral features of lamivudine Resistant Hepatitis B genotype A and D.** Hepatol 2004, **39**(1):42-50.
9. Bozdayi A, Eyigun C, Turkyilmaz A, et al.: **A novel pattern (sW195a) in surface gene of HBV DNA due to YSDD (L180M plus M204S) mutation selected during lamivudine therapy and successful treatment with adefovirdipivoxil**. J Clinl Virol 2004, **31**(1): 76-7.
10. Chien R, Liaw Y: **Nucleos(t)ide analogues for hepatitis B virus: strategies for long-term success.** Best Prac and Res Clin Gastroenterol 2008, **22**(6):1081-92.
11. Lupo J, Larrat S, Hilleret M, et al.: **Assessment of selective real-time PCR for quantitation of lamivudine and adefovir hepatitis B virus-resistant strains and comparison with direct sequencing and line probe assays**. J Virol Methods 2009, **156** (1-2) 52-8.

12. Pas S, Man R, Fries E, Osterhaus A, Niesters H: **The dynamics of mutations in the YMDD motif of the hepatitis B virus polymerase gene during and after lamivudine treatment as determined by reverse hybridisation** . J Clin Virol 2002, **25**(1): 63–71.
13. Masaadeh H, Hayajneh W, Alqudah E: **Hepatitis B virus genotypes and lamivudine resistance mutations in Jordan**. World J Gastroenterol 2008, **14**(47) 7231–7234.
14. Kumashiro R, Kuwahara R, Ide T, et al.: **Subclones of drug-resistant hepatitis B virus mutants and the outcome of breakthrough hepatitis in patients treated with lamivudine.** Intervirology 2003**, 46** (6): 350-4**.**
15. Tunçbilek S, Köse S, Elaldi A, Akman S: **Lamivudine resistance in untreated chronic hepatitis B patients in Turkey**. Turk J Gastroenterol 2008, **19** (2): 99-103.
16. Allen M, Deslauriers M, Andrews C, et al.: **Identification and characterization of mutations in hepatitis B virus resistant to lamivudine.** Hepatol 1998**, 27**(6): 1670-7.
17. Jang H, Cho M, Heo J, et al.: **Oligonucleotide Chip for Detection of Lamivudine-Resistant Hepatitis B Virus**. Clin Microbiol 2004, **42** (9): 4181-8.
18. Vincenti D, Solmone M, Garbuglia A, Iacomi F, Capobianchi M: **A sensitive direct sequencing assay based on nested PCR for the detection of HBV polymerase and surface glycoprotein mutations**. J Virol Methods 2009, **159**: 53-57.
19. Tsubota A: **How do naturally occurring YMDD-motif mutants influence the clinical course of lamivudine-naïve patients with chronic hepatitis B virus infection?.** J Gastroenterol Hepatol 2006, **21** (12): 1783-8.