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ORIGINAL ARTICLE

Study of Interleukin IL-28B rs12979860 and rs8099917 Polymorphisms among HBV Infected Egyptian Patients

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ABSTRACT

Background: *HBV is a worldwide health problem as about 2 billion individuals have been infected with HBV and 350 million are the chronic carriers. The efficacy of anti-viral drugs used in treatment of HBV is high but these drugs are expensive, associated with many side effects and drug resistance was reported. Thus selection of patients with the highest probability of response is essential for clinical practice. Recent genome-wide association studies have revealed some polymorphisms near IL-28B region to be associated with HBV susceptibility, response to treatment or even spontaneous HBV clearance.* ***Objective:*** *This study aimed to investigate role of IL-28B single nucleotide polymorphisms among CHB Egyptian patients.* ***Methodology:*** *SNPs of IL-28B (rs12979860 and rs8099917) regions were genotyped using the MGB-TaqMan SNP genotyping assay in 60 HBV infected patients and 20 healthy volunteers.* ***Results:*** *IL-28B rs12979860 genotypes were expressed in similar proportions in all the studied groups and no statistical significant difference between them was found (MCP=0.272). Concerning IL-28B rs8099917 SNP; GG genotype was 90% expressed among healthy volunteers, while GT genotype was 80% expressed in naïve HBV patients, and TT genotype was expressed in 10% non- responder HBV patients only. There was statistical significant difference between the studied groups as 80% of patients in the naïve group were carrier to IL-28B rs8099917 GT allele (P=0.013).* ***Conclusion:*** *Genetic variations of IL-28B rs8099917 could be associated with HBV risk and resistance to treatment with nucleoside/ nucleotide analogues as G allele could be protective against HBV, GT genotypes could be risk factor for HBV infection while T allele may be predictive factors of treatment resistance.*

INTRODUCTION

Hepatitis B virus (HBV) is a worldwide health problem, and it is estimated that 2 billion individuals worldwide have been infected with HBV and 257 million people are chronic carriers.[1] HBV infection ranges from an asymptomatic and self-limited disease to chronic state, which progresses to chronic hepatitis (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC).[2] Approximately 60% of liver cancers are related to CHB and the subsequent LC. These HBV-related diseases impose a considerable economic burden on patients and society. [3]

CHB sufferers are treated with interferon (IFN) and/or anti-viral drugs. Alfa-interferon suppresses the viral replication and enhances T lymphocyte response, but it has many side effects and only 40 % efficacy rate in eliminating HBV. Current antiviral drugs aim at viral suppression, restoration of viral specific immune responses as well as preventing progression of liver damage to cirrhosis, hepatic decompensation, HCC and death.[4] The efficacy of anti-viral drugs used in treatment of HBV is high but these drugs are expensive, associated with many side effects and drug resistance has evolved. Thus selection of patients is essential for clinical practice. [5]

Interleukin-28B (IL-28B), also known as IFN-λ3, is a member of type III IFN family, located on 19q13 and its expression can be induced by viral infection. Recent reports have shown direct antiviral activity and immune-mediated effects of IL-28B as it can inhibit HCV replication through the JAK-STAT pathway in time-and dose-dependent manner [6,7]

Additionally, it was found that IL-28B polymorphisms are the strongest genetic predictor of outcome of hepatitis C virus infection and of response to treatment with peginterferon (PEG-IFN) alpha and/ or ribavirin (RBV), but for HBV infection, it is a matter of controversy. [4]

Several studies on the relationship between IL-28B genetic polymorphism and chronic HBV infection, HBV clearance and response to treatments have been

conducted. However, the results are inconsistent because of population heterogeneity as different races would have different causative polymorphisms.[4,7]

Therefore, this study aimed to investigate relation between IL-28B (rs12979860 C/T and rs8099917G/T) single nucleotide polymorphisms (SNPs) and response to nucleoside/ nucleotide analogues (NAs) treatment among CHB Egyptian patients.

METHODOLOGY

Subjects

This study was carried out on 80 subjects;60 HBV patients selected from hepatology unit, internal medicine department, Medical Research Institute, Alexandria University and 20 healthy volunteers. Subjects were divided into 4 groups; group I (naïve HBV patients) included 20 HBV infected patients (12 males vs. 8 females) who did not received any treatment, group II (responder HBV patients) included 20 CHB patients (12 males vs. 8 females) who responded after 6 months of treatment with nucleoside /nucleotide analogues (NAs), group III (non-responder HBV patients) included 20 CHB patients (10 males vs. 10 females) who did not responded to NAs treatment or had a relapse after 6 months of the treatment and group IV (control group) included 20 healthy volunteers (8 males &12 females) matched in age and sex with patients in the other groups. Patients were classified as responders by achieving any of these targets: (i) the loss of HBeAg; (ii) presence of anti-HBeAb (iii) HBV DNA level < 200 IU/ml and (iv) alanine aminotransferase normalization. Relapse was defined as the reappearance of HBV-DNA during follow-up in patients with previous end of treatment response (ETR), which is defined as undetectable serum HBV-DNA at the end of therapy.

Inclusion criteria included HBV patients untreated or receiving NAs treatment for 6 months. CHB infection was confirmed by positive serology for HBe and HBcAgs with active viral replication confirmed by the detection of HBV-DNA in the serum by PCR while ***Exclusion criteria*** included HCV infection and blood transfusion in the last three months, renal, cardiac, neoplasic or immunological disorders. All participants were asked to freely volunteer to the study and informed written consents were gathered prior to their inclusion in the study protocol, according to ethical guidelines of the Medical Research Institute, Alexandria University (Informed Written Consent for Patient Participation in a Clinical Research, 2011).

Liver functions' tests:

Serum albumin, ALT (SGPT) (RANDOX; alanine aminotransferase EC 2.6.2 IFCC kit), AST (SGOT) (RANDOX; aspartate aminotransferase EC 2.6.2 IFCC kit) were performed for all subjects enrolled in the study.

Hepatitis B markers tests:

HBs-Ag and HBe-Ag were measured in the serum samples of CHB patients, using commercial ELISA kit (AcuuDiag™ enzyme linked immunosorbant assay, Diagnostic Automation/ Cortez Diagnostics, Inc USA). Anti-HBe was measured in the serum samples, using commercial ELISA kit (EXPRESSBio®, Express Biotech International, USA). Total anti-HBc was measured in the serum samples, using (Accu-Tell ®ELISA kit, AccuBioTech Co., China**)**, according to the manufacturers' instructions.

Quantitative Detection of HBV-DNA by Real Time PCR

HBV-DNA level was measured in serum for the assessment of active viral replication, First; viral DNA extraction from patients' serum was performed using QIAamp® viral DNA mini kits, according to the manufacturers' instructions followed by PCR amplification using commercial Real time Artus ® HBV RG/TM PCR kit (Artus-Biotech, Qiagen, Hamburg, Germany**)**, according to the manufacturers' instructions..

For PCR amplification, the reaction mixture consisted of 15 µl TaqMan universal PCR master mix (Artus ® HBV RG PCR) and 10 µl of extracted DNA to make the reaction to a final volume of 25µl. For quantitation 10 µl of five external standard (RG/TM QS, Qiagen) ranging from 10^5 I.U/ml to 10^1 I.U/ml were added in separate tubes containing 15 µl of the Master Mix and their corresponding cycle threshold (CT) was used to draw a standard curve. Real time PCR was performed with the Mx3000P ™ (Stratagene) real time PCR system. The Thermal profile used was as follows: 95° C for 10 min, followed by 40 cycles of two PCR-step amplification, denaturation for 95° C for 15 sec, followed by annealing and extension at 60° C for 1 min. Results were interpreted by blotting the CT of different samples against the standard curve. The viral load was measured in IU/ml.

Assessment of IL-28 rs12979860 and rs8099917 SNP (TaqMan® SNP Genotyping Assays Protocol, Applied Biosystems**).**

Genomic DNA was extracted from EDTA whole blood samples using PureLink® Genomic DNA Kit (Invitrogen, Life Technologies) followed by assessment of DNA concentration and purity with a Nanodrop spectrophotometer.

IL-28 polymorphisms; rs12979860 and rs8099917 were analyzed using 5' nuclease assay with a TaqMan MGB (minor groove binder) probe using *StepOne*™ Real-Time PCR System (Applied Biosystems, Life Technologies). IL-28rs12979860 SNP and IL-28B.rs8099917 SNP primers and TaqMan MGB probes were provided by the assay on-demand ™ service by Applied Biosystems, Life Technologies. Each PCR tube contained 2 µl genomic DNA, 7 µl DNase-free water, 10 µl (2X) TaqMan Universal PCR Master Mix and 1 µl

working stock of SNP genotyping assay (20X). The assay contained sequence-specific forward and reverse primers to amplify the sequence of interest, two TaqMan® MGB probes (one VIC®-labeled probe to detect Allele 1 sequence and one FAM™-labelled probe to detect Allele 2 sequence). Negative controls were also included in each run. Thermal cycling conditions were adjusted to 10 min. at 95ºC followed by 40 PCR cycles of 2 step PCR each consisted of 15 sec at 92ºC and 1 min. at 60ºC. When probes that have hybridized to their complementary sequences they were cleaved leading to an increase in fluorescence signal. Thus, the fluorescence signal generated by PCR amplification indicates which alleles were present in the sample (table 1).

Table 1: Fluorescence signals and sequences in a sample.

A substantial increase in...	Indicates...
VIC-dye fluorescence only	Homozygosity for Allele 1
FAM-dye fluorescence only	Homozygosity for Allele 2
Both VIC- and FAM-dye fluorescence	Allele 1-Allele 2 heterozygosity

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described by number and percentage, while Quantitative data were described using mean, standard deviation and median. Comparison between different groups was done using Chi-square test. When more than 20% of the cells have expected to count less than 5, correction for chi-square was conducted by using Monte Carlo correction. For normally distributed data, comparison between different groups was analyzed using F-test (ANOVA) and correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, comparison between different groups were done using Kruskal Wallis test. Significance of the obtained results was judged at the 5% level of significance. [8]

RESULTS

Liver functions & viral markers:

Results of liver functions (Albumin, ALT, AST) were markedly deteriorated in HBV patients compared to the control group. There was a statistical significant differences between responder group compared to other groups in albumin levels (P=0.005), in addition, there were statistical significant increase in ALT and AST values among naive and non-responder groups compared to responder and control groups (P=0.003 and 0.002, respectively) (table 2).

Regarding viral markers, there were statistical significant differences between HBV responder patients and other groups with respect to HBe-Ag (all responders showed negative HBe-Ag, P=0.005), HBe-Ab (85% were HBe-Ab positive, P=0.03) and HBc-Ab (90% were HBc-Ab positive, P=0.005) (table 3).

Table 2: Comparison between the studied groups as regard liver functions.

liver functions	*Group*				*F (P)*
	Naïve (N=20)	**Responder (N=20)**	**Non responder (N=20)**	**Controls (N=20)**	
Albumin (mg/dl)					4.4
▪ Mean ± SD	3.7± 0.4	3.9± 0.3	4.2 ± 0.4	3.3± 0.5	(0.005)*
▪ Median	3.8	4	4.1	3.5	
ALT (U/L)					5.6
▪ Mean ± SD	38.5±9.4	27.4±8.1	35.4±11.3	20.5±1.7	(0.003)*
▪ Median	37.3	27.0	36.7	19.1	
AST (U/L)					6.2
▪ Mean ± SD	36.7±6.6	25±6.0	35±12.3	21.2±5	(0.002)*
▪ Median	38.5	23.3	35.6	21.0	

F: One way ANOVA, * P < 0.05 (significant)

Table 3: Different viral markers among the studied HBV infected groups.

Viral profile	*Group*						MCP
	Naive		Responder		Non responder		
	No	%	No	%	No	%	
HBS-Ag							-
▪negative	0	0.0	0	0.0	0	0.0	
▪positive	20	100.0	20	100.0	20	100.0	
HBe-Ag							0.005*
▪negative	12	60.0	20	100.0	6	30.0	
▪positive	8	40.0	0	0.0	14	70.0	
HBe-Ab							0.03*
▪negative	16	80.0	3	15.0	10	50.0	
▪positive	4	20.0	17	85.0	10	50.0	
HBc-Ab							0.005*
▪negative	17	85.0	2	10.0	5	30.0	
▪positive	3	15.0	18	90.0	15	70.0	

MCP: Monte Carlo test of association

Viral load "HBV- DNA":

HBV-DNA was expressed as IU/ml and their values were summarized in table 4. There was a statistical significant increase in HBV-DNA level among naïve HBV patients compared to other responder and non responder groups (P=0.002) (table 4).

Table 4: Comparison between HBV patients as regard viral DNA

HBV_DNA IU/ml * 10^3	*Group*			^{H} ***P***
	Naïve (N=20)	**Responder (N=20)**	**Non responder (N=20)**	
Minimum	480	0.12	11.6	18.4 (0.002)*
Maximum	63500	170	920	
Median	3260	1.8	26.5	

H: Kruskal-Wallis test, * P < 0.05 (significant).

IL-28B rs12979860 and rs8099917variants among different groups:

The expression of IL-28B rs12979860 and rs8099917 SNP genotypes is summarized in (figure 1, table 5). As regards to rs12979860 genotypes (TT, CT and CC) no statistical significant difference was found between the studied groups (MCP=0.27).

Concerning IL-28B rs8099917 SNP; there was a statistically significant difference between the studied groups in the expression of TT, TG and GG genotypes as 80% of naïve group carried IL-28B rs8099917 GT allele (P=0.013).

Table 5: IL-28 B genotypes among different groups

IL-28 B		*HBV infected patients*								MCP
		Naïve (N=20)		Responders (N=20)		Non responders (N=20)		Control (N=20)		
		No	%	No	%	No	%	No	%	
rs12979860	TT	2	10	4	20	4	20	8	40	0.27
	CT	14	70	8	40	8	40	12	60	
	CC	4	20	8	40	8	40	0	0	
rs8099917	TT	0	0	0	0	2	10	0	0	0.013
	TG	16	80	8	40	6	30	2	10	
	GG	4	20	12	60	12	60	18	90	

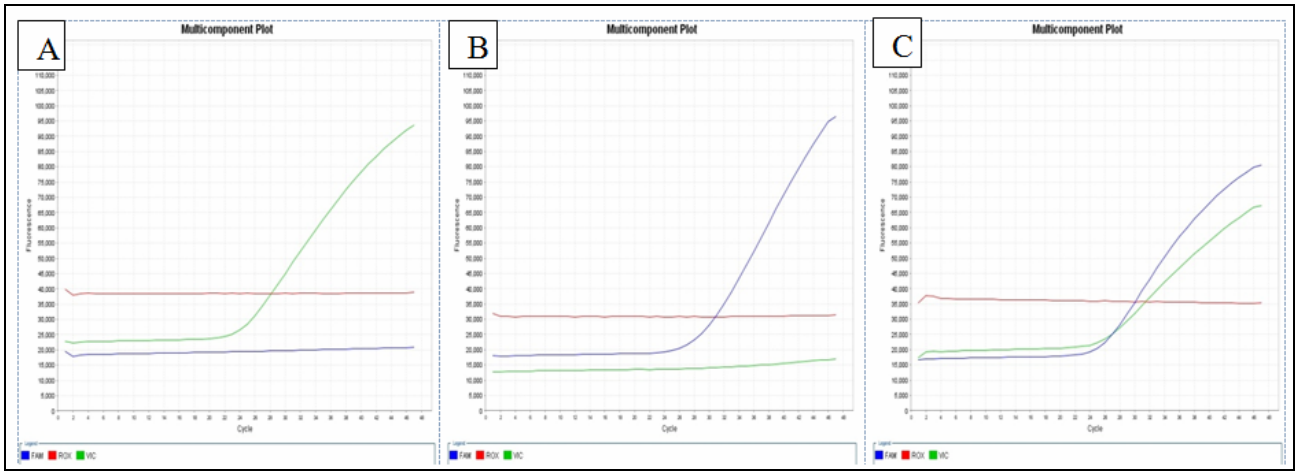

Fig. 1: Real-time PCR picture displaying **A)** homogenous expression of IL-28B rs12979860 CC or rs8099917 TT (VIC® dye is associated with the C allele of IL-28B rs12979860 or T allele of IL-28B rs8099917, **B)** homogenous expression of IL-28B rs12979860 TT or rs8099917 GG (FAM™ dye is associated with the T allele of IL-28B rs12979860 or G allele of IL-28B rs8099917), **C)** heterozygous expression of both alleles "rs12979860 CT or rs8099917 TG".

DISCUSSION

HBV is one of the most prevalent viral infections worldwide. It is well known that elimination of HBV is attributed to coordinated innate, humoral and cell-mediated immune response. During this immune process, cytokines play a crucial role in modulating almost all phases of the host immune response. Genetic polymorphisms of the cytokines and factors regulating cytokines that may influence the expression of cytokines, thus will determine disease progression, various clinical outcomes of HBV infection and treatment response. [9,10]

Several genome-wide association studies (GWAS) identified SNP near the IL-28B gene that was associated with liver diseases and treatment-induced viral clearance in chronic HCV infection. [11-13] In contrast, for HBV several retrospective studies yielded conflicting results on the association between IL-28B polymorphism and treatment response. This may be due of population heterogeneity; different races would have different causative polymorphisms.[14-16]

In the present study, we determined the associations of variations at IL-28B rs12979860 and rs8099917 gene among Egyptian HBV patients. The laboratory finding of all subjects under the study showed significant increase in albumin levels among responders group while ALT and AST were significantly elevated among naïve and non-responder HBV infected patients. Viral load showed the highest levels among naïve group. There was no association between IL-28B rs12979860 genotypes and HBV susceptibility or response to nucleoside /nucleotide analogues (direct anti-viral) treatment. Concerning IL-28B rs8099917 SNP; there was statistically significant association between GT genotype and HBV carrier state (P=0.013) and TT genotype was expressed only in 10% of non- responder HBV patients but without any statistical significance. Thus IL-28B rs8099917 TT allele is a susceptibility marker for HBV infection and carriers of this allele may show higher resistance to anti-viral treatment. This needs to be confirmed by more extensive studies as the small sample size is a major obstacle in the present study.

In accordance with our results, there are many other studies that did not support the role of IL-28B rs12979860 SNP in HBV infection, persistence, outcome or response to treatment [7,17,18] Holmes et al., [17] have documented that IL-28B genotyping is likely to have limited clinical utility for predicting peg-IFN treatment outcome for CHB patients in the Asia-Pacific region. Also,Tang et al.[7] have reported that no association was found between IL-28B rs12979860 C/T polymorphism and HBV infection.

Conde et al.[19] have investigated the prevalence of the IL-28B polymorphisms rs12979860 and rs8099917 in CHB patients in Eastern Amazonia. Results demonstrated that the distribution of IL-28B rs12979860 C/T and rs8099917 G/T polymorphisms were similar between the infected and control groups, indicating that they were not associated with susceptibility and the clinical evolution of HBV in the examined population. Additionally, Jiao et al., [15] and Komatsu et al., [10] have observed that IL-28B rs8099917 SNP had no correlation with the outcome of HBV infection or HBeAg sero-conversion.

On the other hand; Boglione et al., [14] have investigated the effect of IL28-B polymorphisms in the treatment with pegylated-interferon (PEG-IFN) of patients with CHB. They found that IL-28B rs12979860 CC genotype was significantly associated with serological and virological response ($p<0.001$); whereas in rs8099917 TT genotype was mostly related to virological response.

In contrary to our results, Ren et al., [20] have confirmed that HBV-infected individuals with the rs12979860 CC genotype have higher IL28 mRNA and IL-28B protein levels than those carrying the T-alleles. They suggested that IL-28B expression tend to have an effect on immune defence against HBV and so response to treatment.

Yu et al., [21] have assessed the association between IL-28B rs8099917 SNP and the response to lamivudine treatment in naïve of Chinese rural patients. They found that IL-28 genotypes were independently associated with responses at 1 year post-treatment with lamivudine in CHB patients. IL-28B rs8099917 GT genotype appeared to be associated with a higher probability of response to treatment.

Thus results of different researches in distinct populations are still conflicting so the relationship between IL-28B polymorphisms and HBV has not been confirmed.

Conclusions & Recommendations

According to our study findings, we can conclude that genetic variations of IL-28B rs8099917 could be associated with HBV risk and response to treatment with nucleoside/ nucleotide analogues; as GG allele could be protective against HBV infection, GT genotypes could be risk factor for HBV infection and TT allele may be predictive factors of treatment resistance.These results need to be confirmed by more extensive studies on a larger number of patients.

Conflict of interest

There is no conflict of interest or financial ties to include.

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