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The Potential Role of Interleukin 28B-gene Polymorphism and Natural Immune Enhancer Treatment Response in Chronic HCV Patients

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ABSTRACT

HCV infection is a major health problem in Egypt. Studies using safe natural products Blue green® tablet showed its antiviral effect. Polymorphisms in the interleukin-28B (IL28B) gene are associated with outcomes from infection with HCV. The rationale of the present study is to evaluate the potential role of interleukin 28B gene polymorphism (IL28B) and natural immune enhancer treatment response in chronic HCV patients. This study included 100 chronic HCV patients. Patients were classified into two groups: Group I, fifty patients with cirrhosis and advanced liver disease. Patients were unfit INF/ Ribavirin: Group II, fifty patients who refused INF/Ribavirin. Patients were treated with combination of natural products of Blue green® tablet (4 tablets daily which equivalent to 100 mg AFA/30 kg BW), vitamin D, tea spoon field paste of black seeds, olive oil and honey. Alanine Aminotransferase (AST), Aspartate Aminotransferase (ALT), complete blood count (CBC) were measured in the two groups. Detection of HCV-RNA level by quantitative PCR was done before & after 3, 6, 12 and 18 months of treatment. Genomic DNA extracted from all the 100 patient's blood samples was analyzed for the rs12979860 SNP of IL28B using a real-time polymerase chain technique incorporating SYBR Green. The results showed that, the activities of hepatic marker enzymes (AST and ALT), complete blood pictures (Hb, WBCs and Platelets), HCV RNA PCR, IL 28-B gene polymorphism were non-significantly (p>0.05) differences when compared untreated patients (Group I) and treated patients (Group II) were CC genotype before treatment was 8 (34%) & was 13 (26%) after treatment, CT genotype before treatment was 26 (52%) & was 28 (56%) after treatment, TT genotype before treatment was 7 (14%) & was 9 (18%) after treatment. Also, there were non-significant (p>0.05) differences between responder & non-responder in treated patients. In conclusion, the treatment with natural immune enhancer in chronic HCV patients showed no significance correlation with IL 28 gene polymorphism.

INTRODUCTION

Hepatitis C virus (HCV) is a global infection with an estimated 170 million people affected [1]. It appears that there are endemic strains, which have persisted in specific locations for many centuries. These can be readily identified by viral genotype. There have been iatrogenic outbreaks leading to massive spread of specific subtypes in countries such as Egypt (genotype 4A) [2,3]. HCV heterogeneity is huge based on its

capacity to develop mutations through its error-prone polymerase and its very long co-evolutionary history with man. Clinically genotype information on the virus is of major importance in defining response to conventional as well as newer therapies [4]. The standard of care for hepatitis C infection is peg-interferon/ribavirin, it gives higher sustained response rates in genotype 2/3 infected individuals [5]. But in Egypt the infection is due to genotype 4A for which is less

affected by this type of therapy, beside its high cost and numerous serious side effects [6].

The successful treatment of chronic HCV infection is determined by a reduced HCV-RNA viral load and improved liver function and histology. Many herbal and other natural compounds have now been used for the treatment of liver diseases, including HCV infection. More recently, green tea catechins have been shown to inhibit HCV attachment and transmission in human liver cells *in vitro* [7]. However, the potential benefits of herbal and other natural molecules in inhibiting the progression of HCV infection are only beginning to be understood, and more controlled studies are needed in this area. Extracts of *Spirulina platensis* (a blue green algae) and artichoke leaves have been shown to be ineffective when administered to patients with chronic hepatitis C in pilot studies [8].

Research showed that vitamin D has an important role in innate immune response against HCV [9]. In addition some studies have shown that vitamin D improves insulin sensitivity (a prediction of liver treatment response) and inhibit HCV replication [10]. Earlier findings specifically in Middle East [11] showed beneficial effect of adding supplement of vitamin D to current standard treatment of HCV as evidenced by improving both early and sustained virological responses. *Rhodiola rosea* used in the treatment of depression [12,13], has also antiviral effect against Coxsackie viruses [14]. Black seeds paste has more beneficial effect than black seed oil as during preparation of oil, there is loss of some beneficial volatile acid. Black seed has immunomodulatory effect [15]. Olive oil contains fat soluble vitamins specially vitamin D and also rich in linoleic acid that has decreasing effect on HCV replication [16]. Therefore, combination of these agents may be synergetic to treat HCV Egyptian patient cases that refused INF/Ribavirin therapy.

The sequencing of the human genome, together with the development of high-throughput technologies delivering fast, affordable and accurate genomic information, represent a unique opportunity to predict treatment response. Several independent genome-wide association studies (GWAS) reported single nucleotide polymorphisms (SNPs) near the interleukin 28B (IL28B) gene (IFN-k3) locus that displayed association with treatment response, mainly in genotype 1 infected patients [17,18].

Interestingly, the association between IL28B gene polymorphism and SVR (sustained virological response) was not confirmed in other cohorts of genotypes 2 and 3 infected patients. In a cohort of 281 patients infected with HCV genotype 3, there was no association of SNP rs12979860 with SVR to PEG-IFN/ribavirin therapy [19]. Also, the association of rs12979860 with an SVR in patients with genotype2/3 HCV was present only in those who did not achieve a rapid virological response (RVR) [20].

Studies have demonstrated that the SNP rs12979860 near the IL28B gene is a strong independent predictor of treatment response in HCV monoinfected and HIV/HCV co-infected patients [21,22]. The rationale of the present study is to evaluate the potential role of IL28B gene polymorphism and natural immune enhancer treatment response in chronic HCV patients.

PATIENTS AND METHODS

Natural products materials

Blue green® tablet
Blue green® tablet was purchased from Original Natural Company, Italy. Each tablet contains (*Rodiola rosea* L.) root dry extract (entitled to 1% in salidroside); eleuterococco (*Eleutherococcus senticosus* Maxim.) root dry extract (entitled to 5% in saponin); Ginkgo (*Ginkgo biloba* L.) leaf dry extract (entitled to 24% in ginkgoflavonglycosides and 6% in lactones terpenic); Klamath microalgae (*Aphanizomenon flos aquae* 50 mg) and equiseto (*Equisetum arvense* L.) cauli sterili.

Patients
One hundred patients with chronic HCV; from April 2009 - March 2013; who refused INF/RBV therapy. Also, patients who failed to achieve sustained virological response to combined Interferon Ribavirin therapy (INF/RBV) were included in this study. HCV patients were treated with natural products of (Blue green® tablet one tablet/30 kg, vitamin, tea spoon field paste of black seeds, olive oil and honey).

Patients included in this study fulfilled the following inclusion criteria: their age 18 years or older, positive Anti-HCV antibody, detectable serum HCV-RNA by quantitative PCR. Patients were excluded if there is evidence of HCC, severe concurrent medical disease such as severe hypertension, heart failure, significant coronary heart disease, diabetes, chronic obstructive pulmonary disease and pregnant women were excluded as well. All patients gave written, informed consent before participating in the study.

Study design
Patients were classified into two groups: Group I, fifty patients with cirrhosis and advanced liver disease. They were unfit INF/ Ribavirin (support treatment of silymarin); Group II, fifty patients who refused INF, Ribavirin. They were treated with combination of natural products of Blue green® tablet (4 tablets daily which equivalent to 100 mg AFA/30 kg BW), vitamin D, tea spoon field paste of black seeds, olive oil and honey.

Biochemical parameters
Liver Function Tests (bilirubin, AST, ALT and albumin) were assayed by using the Beckman Coulter clinical Auto analyzer, USA. Estimation of Complete blood picture was assayed by using Sysmex instrument KX-21, Sysmex Inc., Japan. Viral markers (HCVAb & HBsAg) were assayed by using the third generation ELISA technique (Abbotts Laboratory, Germany). Detection of HCV-RNA level was detected by PCR quantitative measurements by using COBAS Amplicor 2.0, Roche Molecular Diagnostics, Pleasanton, CA, USA (lower limit of detection of 50 IU/mL), and it was done before treatment and after 3, 6, 12 and 18 months of treatment.

IL28B-genotyping
Genomic DNA was extracted from peripheral blood lymphocytes by using GE Healthcare illustra blood genomicPrep Mini Spin kit (GE Healthcare UK limited, Amersham Place, Little Chalfont, Buckinghamshire, HP7NA, UK) [23]. All samples were typed for the *rs12979860* SNP using a real-time polymerase chain technique incorporating SYBR Green .The primers used were as follows: 5′-GCTTATCGCATACGGCTAGGC-3′ (forward common), 5′-GCA ATTCAACCCTGGTTCG-3′ (C-allele specific reverse) and 5′-GCAATTCAACCCTG GTTCA-3′ (T-allele specific reverse). Reactions were performed on Stratagene Mx3005P

Real time PCR system (Agilent Technologies, Germany) machine using 96-well plates. Briefly, The 20 μL reaction volume contained 10 μL of 2X SYBR Green PCR Master Mix, 0.25 μL of Forward and reverse primers, 4.5 μL template (genomic DNA) and 5 μL of distilled water. The thermal cycling protocol consisted of an initial denaturation step of 95°C for 10 minutes, followed by 40 two-step amplification cycles of denaturation at 95°C for 20 seconds and annealing/fluorescence detection at 60°C for 20 seconds.

Statistical analysis

All statistical analyses were performed using SPSS (statistical package for social science) program version 17 for windows (SPSS INC., Chicago, IL, USA). Comparisons between two groups were performed using Fisher exact test for categorical data. P values of < 0.05 were considered to indicate statistical significance. Results were expressed as mean ± S.D.

RESULTS

Data represented in Table 1 shows the results (total 100 chronic HCV patients) of hepatic marker enzymes (Bilirubin T, AST and ALT) activities as well as CBC and HCV RNA PCR values of untreated patients (Group I) and treated patients (Group II). Activities of hepatic marker enzymes (Bilirubin T, AST and ALT) in serum were non-significantly (p>0.05) differences when compared untreated patients (Group I) with treated patients (Group II). Where, Total bilirubin level before treatment was (0.8±0.2) & was (1.0 ± 0.4) after treatment; mean AST level before treatment was (48.1±40.4) & was (49.1 ± 32.0) after treatment and mean ALT level before treatment was (50.5±31.2) & was (49.9 ± 28.1) after treatment. Also the Table shows the results of Hb, WBCs, Platelets and HCV RNA PCR values.

Values of Hb, WBCs, Platelets and HCV RNA PCR in serum were non-significantly (p>0.05) differences when compared untreated patients (Group I) with treated patients (Group II).Where, mean Hb before treatment was (13.3±1.4) & was (12.7 ± 4.67) after treatment; mean WBCs before treatment was (6156±1984) & was (8744 ±1302) after treatment and mean Platelets before treatment was (215.64 ±55.42) & was (209.61±54.30) after treatment, mean HCV PCR value before treatment was (578692.7±883846.4) & was (390916.1±749228.8) after treatment.

Table 2 shows the results of IL 28-B gene polymorphism of untreated patients (Group I) and treated patients (Group II). The results of IL 28-B gene polymorphism were non-significantly (p>0.05) differences when compared untreated patients (Group I) with treated patients (Group II). Where CC genotype before treatment was 8 (34%) & was 13 (26%) after treatment, CT genotype before treatment was 26 (52%) & was 28 (56%) after treatment, TT genotype before treatment was 7 (14%) & was 9 (18%) after treatment. The results of IL28 gene Polymorphism according to the response to treatment (responder & non-responder) in treated patients (Group II) were illustrated in Table 3, **Fig. 1 and Fig. 2**. There were non-significant (p>0.05) differences between responder & non-responder in treated patients.

Table 1. Comparison of laboratory variables (Bilirubin T, AST, ALT & HCV RNA PCR) between Group I & Group II.

laboratory variables	Group I n=50		Group II n=50		P-value
	M	SD	M	SD	
Age (years)	39.3	11.1	51.2	7.3	
Bilirubin (mg/dL)	0.8	0.2	1.0	0.4	
Aspartate Aminotransferase AST (U/L)	48.1	40.4	49.1	32.0	> 0.05
Alanine transaminase ALT (U/L)	50.5	31.2	49.9	28.1	
Hemoglobin (Hb) (g/dL)	13.3	1.4	12.7	4.6	
White Blood Cells (WBCs) μL	6156	1984	8744	13027	
Platelets μL	215.64	55.42	209.61	54.30	
HCV RNA PCR (IU/ml)	578692.7	883846.4	390916.1	749228.8	

Group I: Untreated patients (32 men and 18 women). Group II: treated patients (29 men and 21 women). n: number of patients. P: is considered non significant when > 0.05

Table (2): Comparison of IL 28-B gene polymorphism between group I & group II.

IL28B Polymorphism	Group I (n=50)		Group II (n=50)		P-value
	N	%	N	%	
CC	13	26	17	34	
CT	28	56	26	52	> 0.05
TT	9	18	7	14	

Group I: Untreated patients. Group II: treated patients. IL28B: interleukin 28B gene Polymorphism. CC, CT & TT: IL28B genotypes. n: number of patients. N: Number of IL28B genotype patients. %: Percentage of IL28B genotype patients. P: is considered non significant when > 0.05.

Table (3): Comparison of IL28 gene Polymorphism in treated patients according to the response to treatment.

IL28B Polymorphism	Responder		Non responder		P-value
	N	%	N	%	
CC	8	35	9	33	
CT	12	52	14	52	> 0.05
TT	3	13	4	15	

IL28B: interleukin 28B gene Polymorphism. CC, CT & TT: IL28B genotypes. N: Number of IL28B genotype patients. %: Percentage of IL28B genotype patients. P: is considered non significant when > 0.05.
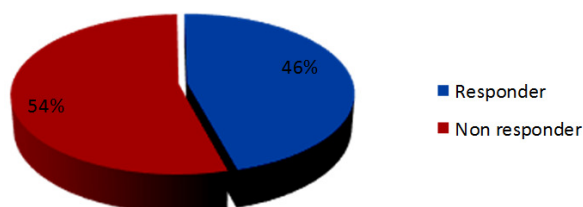

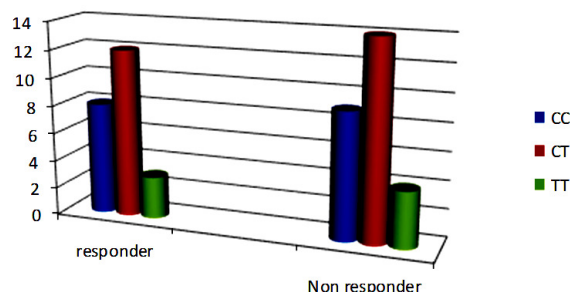**Fig.** 1. Virological response & non-response after treatment (18 months).

Fig. 2. Prevalence of the IL28B gene polymorphism in responder and non-responder treated patients.

DISCUSSION

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide, the available treatment nowadays for HCV is the combination of pegylated interferon and ribavirin, many side effects are observed with this combination [24]. IL28B single nucleotide polymorphisms (SNPs) play an important role in the management of hepatitis C virus infections and are strongly associated with spontaneous and treatment-induced HCV clearance (REF) .In the near future, treatment of HCV will include the addition of direct-acting antivirals (DAAs) with a protease inhibitor to PEG-IFN plus ribavirin [25]. Further studies will be needed to demonstrate whether genotype 4-infected patients with good predictors of response, including IL28B CC, may benefit from shorten therapy. Therefore, in genotype 4 patients, IL28B polymorphism may be important as a companion diagnostic for guiding treatment strategies.

The current standard treatment is expensive and also has numerous serious side effects and is only effective in approximately 50% of patients. In addition, relapse may occur [26]. Drugs target specific viral proteins; especially NS3-4A serine protease and NS5B polymerase are promising. Different peptidomimetic inhibitors, nucleoside analogs, and non-nucleoside analogs are at various stages of development and show high potency against HCV. However, these drugs show a high susceptibility for resistance emergence as detected by in vitro studies. In most cases, one single point mutation is sufficient to achieve tolerance against the drug or worsen cross mutations against another one. These observations indicate that drug resistance is likely to remain a problem. The lack of vaccine for HCV infections, the high cost of the drugs specially in low-income countries with a high prevalence of HCV, ineffective therapy and the rapid emergence of new drug-resistant viruses have urged a growing need for developing new, more effective chemotherapeutic agents with less side effects for successful HCV treatment. *Takeshita* et al. at the university of Miyazaki in Japan believed that since HCV is localized in the liver and can take 20 years or more to developed into disease, dietary supplement might help slow or stop disease progression [27].

Consumption of *Aphanizomenon flos aqua* has rapid effects on the circulation, function of immune cells in humans, and have antiviral effect. Jensen et al. [28] conducted a randomized, placebo-controlled study to examine the short-term effects of consumption of 1.5 grams of the blue green algae (AFA), on the immune system. They found that consumption of an AFA results in rapid changes in immune cell trafficking i.e. generalized mobilization of lymphocytes and monocytes, but not polymorph nucleated cells. This included increases in CD3+,

CD4+, and CD8+ T cell subsets and CD19+ B cells with reduction in relative proportions and absolute numbers of Natural Killer (NK) cells. The polymorph nucleated cells showed a mild but significant reduction in phagocytic activity. In vitro, AFA did not induce a direct activation of lymphocytes, as evaluated by tyrosine phosphorylation and proliferative activity. They concluded that AFA increases the immune surveillance without directly stimulating the immune system [29].

Since 1983, the blue green algae *Aphanizomenon flos-aquae* (AFA) has been harvested from Upper Klamath Lake (UKL) in Southern Oregan and marketed as a dietary supplement. It is consumed for its putative beneficial effects including detoxification, increasing energy, elevating mood and loss of weight [30].There have been many scientifically-controlled studies analyzing the immune enhancing properties of (AFA) from Klamath Lake, Oregon. On the basis of the Provisionally Tolerable Daily Intake (PTDI) I value established by the WHO, a guidance value of 1 mg microcystin-LR/g AFA algae was issued for AFA algae in the USA and the daily intake of AFA algae is 2 g/person weighted 60 kg [31].

Baicus and Tanasescu [32] studied the effect of one month treatment of Spirulina (extract of blue green algae) on serum aminotransferases and general state, compared to placebo, in 24 patients with chronic HCV. They found no effect on the level of aminotransferases with improvement in the general status. However, these results may be due to the very short duration of treatment. Several compounds were extracted from blue green algae including a protein called Cyanovirin-N (CV-N) which appears to irreversibly inactivate diverse strains of HIV virus [33].

CV-N has antiviral activity against HCV as it inhibits HCV entry into host cells at low nanomolar concentrations [34]. Also, blue green algae compounds selectively inhibit the penetrations of enveloped viruses (Herpes simplex, human cytomegalovirus, measles virus, mumps virus, influenza virus and HIV virus) into host cells thereby preventing replications [35,36]. Also, consumption of AFA has rapid effect on the circulation of immune cells in humans [37].

In Chronic HCV infection, early normalization of the ALT levels is predictive of the response to interferon, Silybum marianum and Lactoferrin have been associated with a decrease in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels [38,39]. Serum ALT and AST levels are recognized as markers reflecting the degree of the histological liver damage and has served as parameters for starting therapy or judging response to antiviral treatment in chronic hepatitis C [40].

Freedman et al. [41] analysed the baseline characteristics of 766 patients with chronic hepatitis C and found that higher coffee consumption was associated with a lower AST/ALT ratio, less steatosis and lower levels of alpha-fetoprotein. After 4 years of follow-up, the authors showed that patients who drank three or more cups of coffee per day had a 53% lower risk of liver disease progression than those who took less than three cups. Low ALT levels In the present study showed no statistical with those who taken treatment, This is in disagreeing with Serfaty et al. [42] that showed that pretreatment ALT level tend to be lower in responder [42]. This is in agreement to Carreño et al. showed that silymarin was safe and well tolerated, but it had no effect on ALT or serum HCV-RNA levels [43]. In other studies elevated ALT levels (three

fold higher than the upper limit of normal) is associated with a good response to treatment [44].

In the last few years, IL28B gene polymorphisms have been extensively studied in HCV genotype 1-infected patients because of their predictive role in the treatment outcome and possible association with disease progression [45]. When studied in different ethnic populations, it has been shown that allele distributions of IL28B SNPs are different between races and ethnic backgrounds [46].

In the present study IL28B gene Polymorphism (CC, CT, TT), Seventeen patients (34%) were of genotype C/C, 26 of patients (52%) were of genotype C/T and 7 patients (14%) were of genotype T/T. This is in accordance with Clark et al. who observed that different ethnic groups have marked differential distribution of IL28B gene polymorphisms. The favorable CC allele of rs12979860 is least frequent in African-Americans and most frequent in Asians. Allele frequencies differ between ethnic groups, largely explaining the observed differences in response rates between Caucasians, African Americans and Asians [46].

De Nicola et al. studied the prevalence of IL28B in 103 HCV-4 patients at two liver centers at the Maggiore Hospital Milan (Italy) and found that 23% were genotype CC, 63% CT and 14% TT [47]. In the present study that was no statistically significant difference between the IL28B as regard the response to treatment; 17 CC genotype achieved SVR (34%), 26 CT genotype (52%) while only 14% of TT genotype. In another study of *Abdo* et al. , study IL28B effect in 129 treatment- naïve patients showed that patients carrying CT and CC genotypes achieved SVR equally (47%) in comparison to TT genotype (5.6%) [48]. El-Awady et al. showed that IL28B typing in end stage liver disease (ESLD) was CT in 80% of patients and TT in 20% of patients, while CC was not detected in any patient with ESLD [49]. By comparing group I(untreated patients) with group II (treated patients) , there was no statistical difference in IL28B polymorphism expression.

CONCLUSIONS

Treatment with natural immune enhancer in chronic HCV patients could have not a significance correlation with IL 28 gene polymorphism.

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