INTERLEUKIN 28B GENE POLYMORPHISMS AS A PREDICTOR OF TREATMENT RESPONSE IN EGYPTIANS WITH CHRONIC HEPATITIS C.

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Background: Egypt has the highest prevalence of HCV all over the world with 9% countrywide and up to 42% in certain rural areas. Combined PEG-IFN and ribavirin is still the only standard of care treatment in spite of its side effects, high costs and low sustained virological response rates. Hence, this provides a compelling reason for the identification of biomarker predictors of disease response to treatment.
Aim of the work: This study is designed to identify IL28B gene polymorphisms in patients with chronic hepatitis C genotype 4 who received standard of care therapy to highlight its impact on response to treatment.
Location of study: Viral Hepatitis Treatment Center at Al-Ahrar Hospital, Sharkia Governorate. and Biochemistry Departments of Faculty of Medicine, Zagazig University.
Methods: Case control study on 124 patients with chronic hepatitis C who finished their treatment with standard of care therapy (pegylated interferon and ribavirin). Sixty two patients were non responders to SOC therapy (case group) and 62 other patients were responders (control group). In addition to the chemical, laboratory data and histological parameters that were taken from their files, blood samples were taken at the time of study for detection of SNPs for rs8099917 by PCR-RFLP technique.
Results: The TT homozygous of rs8099917 genotype was detected in 54 (43.54%) of overall HCV patients, 42 of them (67.74%) achieved SVR. The GT heterozygous was detected in 48 (38.71%) of HCV patients, SVR was achieved in 9 (14.52%) of them. While, the GG genotype was found in 22 patients and 11 of them only (17.74%) were responders. Multiple regression analysis identified IL28 B SNP genotype as the single independent predictor of response to SOC therapy.
Conclusion: These data suggest that host genetics may be useful for the prediction of treatment outcomes and that IL28B SNP genotype is an important predictive biomarker for SVR in patients with HCV genotypes 4.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. There are ~170 million hepatitis C virus (HCV) carriers in the world, and 3–4 million new cases of infection are diagnosed each year hepatitis C represents the leading cause of cirrhosis and hepatocellular carcinoma, as well as the leading indication for liver transplantation [22]. The highest prevalence of infection in the world is recorded in Egypt, with an average of 13.8% [1].

In Sharkia governorate the prevalence of HCV infection is estimated to range from 4.8% among people ‹ 20 years old to 41.9 % among people › 40 years old, with an average prevalence of 25.8% [11].

In Egypt; Up to now, the standard of care (SOC) treatment consists of (pegylated) interferon-Alfa and ribavirin. However, depending on the viral genotype, treatment response rates differ significantly among infected patients. While up to 80% of the genotype 2 and 3 infected and 40–50% in genotype 1 patients can be cured, the response rate of genotype 4 in many clinical repots is showing SVR rates exceeding 60% [14] . Once achieved, an SVR is associated with long-term clearance of HCV infection, which is regarded as a virologic ‘‘cure,’’ as well as with improved morbidity and mortality [18].

Both viral [e.g. HCV genotype, amino acid substitutions in the NS5A [12] and core region [4]] and host factors [young age [17], body mass index [8] and insulin resistance [23]] influence the outcome of IFN therapy. Viral load and the stage of liver fibrosis, which vary among patients even when they have been infected by the same donor [9], have also been reported to influence the outcome of IFN therapy [31].

Data have been published on a gene polymorphism (rs12979860) upstream of IL28B that is favorably associated with treatment response to PEG-IFN and ribavirin in both African Americans (AA) and Caucasian Americans (CA) patients [13] Regardless of race, carriage of the C allele increases treatment response rates, with CC genotype patients having the highest SVR rates, CT genotype patients having intermediate rates, and TT genotype patients having the lowest rates [13]. This favorable genotype is seen more frequently in Caucasian patients and likely explains approximately half of the difference in response between AA and CA of European ancestry. The IL28B gene encodes IFN-λ3, a type III IFN induced by viral infections [16]. Although the mechanism underlying the association of IL28B genotype and HCV clearance has not been elucidated, modulation

of the innate immune response likely plays a role in controlling this viral infection. IL28B genotyping may provide useful pretreatment stratification of patients for HCV treatment in the future, but it does not completely explain response discrepancies between AA and CA patients [26].

In the last few years, three independent research groups have reported the results of separate genome-wide association studies (GWAS), supporting a strong association of two single nucleotide polymorphisms (change at a particular position in the gene sequence) of the IL28B gene on chromosome 9, which encodes type III interferon lambda (IFN-λ-3), rs8099917 and rs12979860, with treatment outcomes of Peg-IFN α- 2a plus RBV therapy [13, 27, 28].

These variations in the IL28B gene correlated well with natural clearance of HCV and with SVR . In the first study, performed with European-American, African-American, and Hispanic individuals, the rs12979860 SNP was most strongly associated with SVR, which is located 3 kilo bases upstream of the IL28B gene. The minor allele (T) was associated with a lower rate of SVR (26% in those with genotype TT and 79% in those with genotype CC) [13].

In the second study, carried out with 293 Australian patients, a significant association between the SNP rs8099917 and SVR was found. This was further validated by an independent cohort of 555 European individuals. From 392 patients who achieved SVR, 247 (63%) were homozygotes for the allele T, which was significantly higher than genotype GG (SVR of 3.8%)[27].

Similar findings were also reported in a Japanese study. Results of a GWAS showed a significant association between treatment response with two SNPs (rs12980275 and rs8099917), both located in the IL28B gene region, with the latter being the same SNP found by Australian researchers. In this case, for the SNP rs8099917, the G allele was associated with a significantly lower SVR (0% for genotype GG and 78% for genotype TT) [28].

Although, several groups have reported an association between several SNPs in the IL28 locus and the effect of PEG-RBV combination therapy for genotype 1 [27 , 21], but only a few studies have examined the role of these SNPs in the treatment of other genotypes especially genotype 4.

Aim:

This study was performed to study the difference in clinical, biochemical, histological parameters and genotypes of IL28B between responders and non responders to standard of care (SOC) therapy, to find out the desirable genotype for the good response (favorable group). And to study the effect of different factors that may affect the response to the interferon therapy e.g. (age, sex, BMI, co-morbid diseases, type of interferon, histological parameters, and IL28B genotypes). And to find out the most important independent predictor for good response.

Type of Study

Case control study.

PATIENTS AND METHODS

The current study was conducted on 124 chronic HCV patients from interferon clinic of Viral Hepatitis Treatment Center at Al-Ahrar Hospital, Sharkia Governorate. and Internal Medicine and Biochemistry Departments of Faculty of Medicine, Zagazig University. in the period from June 2012 to June 2013. Selected patients received combined interferon and ribavirin therapy. All patients gave written informed consent before the study.

All chronic HCV patients were genotype 4, and were all given the SOC therapy. (pegylated interferon-alfa 2a 180 mcg per week or pegylated interferon-alfa 2b 1.5 mcg per kg body weight in combination with ribavirin 600–1400 mg per day according to body weight for 48 weeks).

Patients were stratified according to response to (SOC) therapy into two groups: The first group is responders (control group) to treatment, the chronic HCV patients who had received the (SOC) therapy and have shown negative HCV RNA 6 months (24 weeks) following completion of a 48 weeks treatment course. The second group is non responders (case group) to the (SOC) therapy (no disappearance of HCV RNA at the end of the 12, 24, 48 week or relapsers). with the following inclusion criteria [14]:

- Age 18 years or older
- HCV RNA positive in serum by PCR
- Liver biopsy showing chronic hepatitis with significant fibrosis (bridging fibrosis or higher)
- Compensated liver disease:-
  - total serum bilirubin <1.5 mg/dl, direct bilirubin 0.3 mg/dl or within 20% of ULN (upper limit normal) AND indirect bilirubin 0.8 mg/dl or within 20% of ULN
  - Prothrombin time < 2 seconds above ULN, and INR <1.5,

- serum albumin >3.4 gm/dl
- and no evidence of hepatic decompensation (hepatic encephalopathy or ascites)

❖ Acceptable hematological and biochemical indices:-
- White blood cells (WBC) >3,000/mm^3
- Hemoglobin 12 gm/dl for men and 11 gm/dl for women,
- Neutrophil count >1500 /mm^3,
- Platelet count >80,000 /mm^3
- Fasting blood sugar 115 mg or within 20% of ULN
- Serum creatinine WNL (within normal limit).
- TSH WNL
- HbsAg negative
- ANA < 1:160
- If diabetic, Hb A1C < 8.5%
- Alpha- Fetoprotein <100

❖ Female patients practicing adequate contraception

❖ Male patient's wife practicing adequate contraception

❖ Willing to be treated and to adhere to treatment requirements

❖ No contraindications

And the following exclusion criteria [14]

- Major uncontrolled depressive illness
- Solid organ transplant (renal, heart or lung)
- Autoimmune hepatitis or other autoimmune condition known to be exacerbated by peginterferon and ribavirin (Ulcerative colitis, Crohn's disease, ITP, SLE, Hemolytic Anemia,Scleroderma)
- Untreated thyroid disease,
- CNS trauma which requires medications, active seizures which requires medications
- Pregnant or unwilling to comply with adequate contraception or breast feeding.
- Severe concurrent medical disease such as severe hypertension, heart failure, significant coronary heart disease, poorly controlled diabetes or chronic obstructive pulmonary disease.
- Concurrent hepatic diseases as (co-infection with HBV, hemochromatosis, alpa 1-antitrypsin deficiency, Wilson's disease, alcoholic liver diseases,
- Known hypersensitivity to drugs used to treat HCV
- Clinically significant retinal abnormalities
- Substance abuse (alcohol >80 gm/d) IV drugs and inhaled drugs.

METHOD

All patients were subjected to the following

1) Retrospective collection of patient's data (history, laboratory and histological parameters) from their files as well as filling missed data via patients interview.

2) Specific investigations including : measurement of IL28B gene polymorphism by PCR technique as follow:

A) DNA will extracted from peripheral blood using the DNA extraction kit.

B) Analysis of IL28 gene polymorphism at rs 8099917 by PCR according to **Venegas et al, [32].** 4µl PCR- product was combined with 4µl Loading dye and 1X buffer solution to form a total volume appropriate for the electrophoresis gel comb that used. Once combined, carefully load sample into each well.

Collection of samples:

Two ml of peripheral venous blood was taken from each subject under complete aseptic condition at the time of study on EDTA, Centrifuged and serum was collected and stored at -20° C until the time of assay.

Statistical analysis:

Statistical package for SPSS (statistical package for social science) program version 13 for windows and Epi info computer program was used for data analysis. Quantitative variables were summarized using Mean±SD. Student's t-test was done to compare two normally distributed variables Mann Whitney non parametric variables. Fisher's exact test and the Chi square (X2) test for categorical variables. Correlation coefficients (r) were calculated using the Pearson's correlation analysis. p value was significant at <0.05 level.

RESULTS

The characteristics of the total 124 patients with chronic HCV infection (before therapy) are shown in Table (1). The study included, 42.74 % of stage 1 fibrosis, 43.55 % of stage 2, and 13.71% of stage 3. The frequency of SNPs of IL28B showed that: for genotype rs8099917, TT was detected in 43.55%, GT in 38.71% and GG in 17.74% of overall HCV patients.

The TT genotype of rs8099917 was identified in 54 patients, 42 of them (67.7%) were achieved SVR. The unfavourable SNP genotype (GT+GG) was identified in 70 patients, 20 of them (32.26%) were achieved SVR.

The frequency of TT genotypes was associated with SVR as compared to other genotypes (p<0.001). In contrast, the frequency of (GT+GG) genotypes was associated with non responder patients (p<0.001) (Table 1).

Table (1) Comparison of the different genotypes of Interleukin 28 B between responders and non responders

		responders		Non responders		total		X^2	P	sig
		n	%	n	%	n	%			
IL 28 B	TT	42	67.7%	12	19.4%	54	43.5%	29.5	0.000	H.S.
	GG	11	17.7%	11	17.7%	22	17.7%	0.0	1.0	N.S.
	GT	9	14.5%	39	62.9%	48	38.7%	30.5	0.000	H.S.
IL 28 B	TT	42	67.74	12	19.35	54	43.55	29.5	0.000	H.S.
	TG+GG	20	32.26	50	80.65	70	56.45			

Table (2):- The relative risk of various clinical, biochemical, degree of histological parameters and different genotypes on response to interferon therapy.

		Non responders	responders	total	Relative risk
		N	N		
Fibrosis	F3	12	5	17	1.51
	F1+F2	50	57	107	
Activity	A3	10	3	13	1.61
	A1+A2	52	59	111	
PCR ***IU/ml***	≥600,000	26	25	51	1.03
	< 600,000	36	37	73	
ALT ***(IU/L)***	≥ 40	38	46	84	0.75
	< 40	24	16	40	
AST ***(IU/L)***	≥ 40	33	36	69	0.91
	< 40	29	26	55	
BMI ***kg/m²***	≥ 25	48	41	89	1.35
	< 25	14	21	35	
Hyperglycaemia Mg/dl	YES (FBS>100)	6	3	9	1.37
	NO(FBS<100)	56	59	115	
Hypertension	YES	5	3	8	1.23
	NO	59	57	116	
AGE Years	>40	31	30	61	1.03
	<40	31	32	63	
Sex	MALE	37	47	84	0.70
	FEMALE	25	15	40	
Type of Interferon	2a	36	40	76	0.87
	2b	26	22	48	
IL 28 B	TG+GG	50	20	70	3.21
	TT	12	42	54	

Table (3):- Logistic Regression multivariate analysis for the most predictable factors for response to treatment by standard of care therapy

Variables in the Equation

		B	*S.E.*	*Wald*	*df*	95.0% C.I.for EXP(B)		Exp(B)	*sig*	
						Lower	Upper			
Step1 (a)	**IL28B gene TT**	*2.449*	*.515*	*22.664*	*1*	*4.225*	*31.751*	*11.582*	*.000*	*HS*
	Activity ↓	*1.160*	*1.058*	*1.200*	*1*	*.401*	*25.380*	*3.189*	*.273*	*NS*
	Fibrosis ↓	*.189*	*.937*	*.041*	*1*	*.192*	*7.586*	*1.208*	*.840*	*NS*
	Sex ♂	*-.646*	*.510*	*1.606*	*1*	*.193*	*1.424*	*.524*	*.205*	*NS*
	BMI <30	*-.088*	*.579*	*.023*	*1*	*.295*	*2.848*	*.916*	*.880*	*NS*
	AST<40	*-.766*	*.530*	*2.087*	*1*	*.165*	*1.314*	*.465*	*.149*	*NS*
	PCR <600	*-.028*	*.441*	*.004*	*1*	*.433*	*2.442*	*1.029*	*.949*	*NS*
	DM FBS <100	*-.225*	*.855*	*.069*	*1*	*.149*	*4.266*	*.798*	*.792*	*NS*
	Age <40	*-.904*	*.497*	*3.308*	*1*	*.153*	*1.073*	*.405*	*.069*	*NS*
	ALT <40	*-.100*	*.537*	*.035*	*1*	*.316*	*2.590*	*.905*	*.853*	*NS*
	Constant	*-1.062*	*2.675*	*.158*	*1*			*.346*	*.691*	*NS*

a Variable(s) entered on step 1: IL28_NEW, ACTIV, FIROS, SEX, BMI30, AST40, PCR600, DIABETIS, AGE40, ALT40.

DISCUSSION

Egypt has the highest prevalence of HCV worldwide with 9% countrywide and up to 50% in certain rural areas [15] and the highest prevalence of HCV-4, (previously called the Egyptian genotype) which is responsible for almost 90% of infections and is considered a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and liver transplantation in the country [2].

The current study was designed to clarify, the effect of rs8099917, SNP located nearest to interleukin 28B (IL28B), the gene that encodes for IFN lambda-3 on HCV-4 outcome after combined IFN/RVN therapy.

According to the demographic and the clinical description of our study; our patients mean age was about (39.25 ± 9.86) yrs, 67.74 % of them were males, 32.26% of them were females with male to female ratio 84/ 40. With mean BMI was about (27.70 ± 4.59).

In the current study, TT genotype of rs8099917 was identified in 43.55 % of overall HCV patients; the GT heterozygous was detected in 38.71% and the GG 17.74 %. **Olfat et al, 2011** on the other hand, found that, the frequencies of the IL-28B genotypes were as follows: TT, 46%; GT, 42%; and GG, 12%. **Sharafi et al, 2012 (a)** [24] recorded that TT, GT and GG genotypes were 59.6%, 35.6% and 4.8%, respectively. **Sharafi et al, 2012 (b)**[25] reported that the frequency of IL28B rs8099917 TT, GT, and GG genotypes in chronic hepatitis C patients was 58.3%, 37.1%, and 4.6% and in healthy individuals was 64.1%, 32.4% and 3.5%.

SVR were achieved in 62 (50 %) among overall 124 HCV patients (responders). And the remaining 62 (50%) of patients failed to respond (non responders). The TT genotype of rs8099917 who achieved SVR was 67.74%, which is significantly higher compared to GT (14.52%) and GG (17.74%) genotypes. **Olfat et al ., 2011** [19]revealed that, SVR was detected in 80.4% of patients who harbour homozygous TT of rs8099917, in 21.4% of heterozygous GT and in 16.7% of the GG genotype. **Antaki et al., 2013** [5] found that SVR was achieved in 26% of *rs8099917* TG and GG carriers compared with 60% of TT carriers ($P < 0.0001$).

Other studies **Tanaka, et al, 2009** [28], **Thomas, et al, 2009** [29] **and Rauch et al, 2010** [21] demonstrated that two copies of the *T* allele (*TT* genotype) for the *rs8099917* SNP were strongly associated with natural HCV clearance and SVR. Similar to the *rs12979860* pattern, the *rs8099917 TG* or *GG* genotype was less responsive to treatment.

In our study there was no significant difference between responders and non responders regarding age (P value = 0.857), sex (P value = 0.055), associated disease as HTN (P value = 0.71), DM (P value = 0.299), type of interferon 2a and 2b (P value = 0.461), fibrosis (P value =0.068), viral load (P value =0.855), ALT (P value =0.124), AST(P value =0.588), or BMI (P value =0.378)**.** However there was significant difference between responders and non responders as regarding disease activity (P=0.04)

In addition, we have found that rs8099917 TT genotype is associated with lower serum ALT than the remaining genotypes, a finding that is in agreement with data of **(Abe et al., 2010)** although these authors did not provide the level of significance for this association. These findings are contradictory with those reported by **Thompson *et al 2010*.** obtained from a GWAS study that disclosed that only the rs12979860 SNP was significantly associated with baseline ALT levels. Serum ALT is considered as a marker of necroinflammatory activity in the liver as confirms the analysis of our data that shows a linear increase of ALT levels related to METAVIR activity score.

The relation between *IL28B* polymorphism and the stage of fibrosis is controversial. **Abe et al, 2010 [3],** found higher fibrosis METAVIR scores among carriers of the rs8099917TT genotype. However, **Di Marco et al, 2011 [10],** in a group of 131 patients with thalassemia major and chronic HCV infection who underwent a liver biopsy reported that older age and the carrier state of the minor alleles at rs8099917 sites were associated with more severe liver fibrosis ($p<0.005$).

If the patient is classified to favorable (TT) and unfavorable (TG+ GG) groups there was no significant difference between the two groups regarding DM (P value = 0.18), fibrosis (P value =0.07), disease activity (P= 0.11), viral load (P value =0.94), ALT (P value =0.08), or BMI (P value =0.25) **.** However there was highly significant difference between the two groups regarding age (P=0.002). In accordance with our results , **Olfat et al, 2011 [19]** revealed that, there was no significant difference between the two groups regarding gender, albumin, Hb%, viral load. But it gives significant difference between the two groups regarding ALT and AST (P<0.05)

Our study revealed that there was significant difference between responders and non responders as regard activity (P value =0.04) and highly significant difference as regard interleukin 28 B genotype (P value<0.005). In accordance with our results , **Olfat et al ., 2011** [19] revealed that, there was highly significant difference as regard interleukin 28 b genotype (P value<0.005) [7] **.** linking the rs8099917G allele, that is associated with poor response to therapy, with lower necroinflammatory activity (p = 0.04) and milder fibrosis (p = 0.02) in patients infected with non-1 HCV genotypes.

Suppiah et al, (2009)[27] reported an association to SVR within the gene region encoding interleukin 28B (rs8099917). IL28B contributes to viral resistance and is known to be upregulated by interferons and by RNA virus infection. These data suggest that host genetics may be useful for the prediction of drug response, and they also support the investigation of the role of IL28B in the treatment of HCV and in other diseases treated with IFN-α.

In a study by **Aparicio et al, (2010**) [6], patients carrying rs8099917 G alleles had high rates of treatment failure. The rate of treatment failure in patients infected with HCV genotype 3 was not affected by rs8099917 genotype.

The high prevalence of the rs8099917 G allele in HCV genotype 1- or 4-infected patients shows that the rs8099917 TT genotype may have a protective effect in terms of preventing the persistence of these two HCV genotypes. Since the rs8099917 G allele has been correlated with lower expression levels of IL28 genes [28].

In conclusion: This study identified a polymorphism 3 kb upstream of IL28B (namely; rs8099917) that is significantly associated with response to PEG-IFN and RBV for patients with chronic genotype 4 HCV infection. It seems likely that the gene product is involved in the innate control of HCV. These findings, and further study of the functional mechanism underlying the IL28B response association, may help to identify patients for whom therapy is likely to be successful.

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