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Amino Acid Substitution in Hepatitis C Virus Core and Genetic Variation in Interleukin 28β Gene and their Correlation to Interferon Treatment Failure in Chronic HCV Egyptian Patients

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Abstract: ***Hepatitis C virus becomes a one of the top curable chronic diseases worldwide; this was referred to the remarkable efficacy of antiviral therapy; however, many cases showed resistance to interferon/Ribavirin combination therapy. Genetic polymorphism is the major cause of relapse after therapy. To determine whether Hepatitis C virus (HCV) core substitution and IL-28β (rs8099917) play a role in the response to INF/RBV antiviral therapy; 115 HCV chronically infected patients initiated treatment with Peglated INF plus ribavirin (INF/RBV) for 48 weeks were tested for baseline substitution at codon 70 of the viral core protein and for genetic polymorphism in IL-28β rs8099917 (TaqMan Probe assay, Applied Biosystems). In this study, we observed that, TT genotype in IL-28β polymorphism was the favourable genotype as 75% of this genotype achieved therapeutic success, defined as sustained viral response at 12 weeks of therapy. 23.5% of studied patients presented a mutant HCV core substitution at core position 70; those were resistant to therapy and failed to achieve a viral response. A multivariate analysis revealed three independent predictors of therapeutic success: age≤40years (P<0.001); IL-28β rs8099917 genotype (P=0.04) and absence of HCV core substitution at position 70 (P<0.001). This study concluded that IL-28β rs8099917 and HCV core mutations can be considered as predictors for therapeutic response to INF/RBV therapy; so those who are affordable should treated with direct acting antiviral drugs (DAAs) rather than INF based therapy to prevent evolution towards end-stage liver disease.***

Keywords: Hepatitis C virus, IL-28β (rs8099917), INF/RBV antiviral therapy, HCV core substitution

1. Introduction

Chronic hepatitis C infection is considered a global health problem, affecting about 3% of world's population **[1, 2].** An estimated 170 million people are infected worldwide; most of them become chronically infected and develop cirrhosis. They will be at high risk to hepatocellular carcinoma (HCC)**[3].** In Middle East and North Africa; Hepatitis C virus genotype -4 (HCV-4) is the most common variant. The highest prevalence was detected in Egypt **[4]**. A survey study was done by Egyptian's Ministry of Health at 2008 on a representative sample of 11 126 Egyptian citizens aged between 15 and 59 years, the overall prevalence of anti-HCV antibody was 14.7% and active infection (HCV RNA viremia) was found in 9.8% **[5].**The highest frequency was detected in risky medical procedures which promotes infection with HCV and maintains Egypt as the highest country in HCV infection incidence (6.6–6.9/1000 per person per year)**[6]**.It was recorded that 7.5 million individuals have chronic hepatitis C and 1.5 million are cirrhotic in Egypt. In the latter group, 75 000 (5%) will be decompensated, 30,000 (2%) will have hepatocellular carcinoma (HCC) and 60,000 (4%) will die each year **[7]**.

Pegylated interferon alfa (PEG-IFNα) and **ribavirin** (RBV) combination represents a standard treatment approach for Chronic HCV infection worldwide **[8]**. The response to therapy varies with different HCV genotypes; so sometimes an administration of triple therapy regimen including direct acting antivirals (DAAs) as telaprevir or boceprevir is required to achieve better outcome **[9]**. Several factors have been detected to be associated with the resistance to PEG-IFNα/RBV therapy and development of post-treatment relapse **[10]**. They includes; (Interleukin 28β single nucleotide polymorphisms (IL-28β SNPs), age, gender, HCV genotypes, viral load, obesity and amino acid substitution in the CORE region **[11]**.

Many **IL28β SNPs** have been identified by Genome- wide association studies. SNPs which were in proximity to the interleukin-28B (IL-28B) gene can predict sustained viral response (SVR) in chronic hepatitis C (CHC) patients who undergo therapy with PEG-IFN/RBV **[12, 13]**.The two most studied IL-28B SNPs variants were, rs12979860 and rs8099917 who showed favourable outcome and considered a strong pretreatment predictors of early HCV viral clearance with genotype 1 HCV infection **[8, 9]**. Limited data were collected regarding the role of IL28B SNPs in HCV-4 patients with respect to response to antiviral therapy and progression of fibrosis **[13, 14]**; however, the majority of studies were focused on other HCV genotypes 1, 2 and 3 **[13-15]**. The predictive power of IL28B SNPs in genotype 1 patients has been validated in many studies conducted in different geographical areas. In contrast to HCV-2 and 3 patients; the impact of IL28B polymorphisms on sustained virological response (SVR) rates are less pronounced **[14, 18]**. Nowadays, it is extremely important to understand the predictive role of IL28B SNPs in HCV-4 due to its

important clinical implication role **[17]**. The clinical significance of these studies relies on different points including; HCV-4 genotype prevalence among Middle East, North Africa **[19]**, Europe and Western countries **[20]**; difficulties to be cured among populations and rapid spread among drug addicts **[21-22]**.

HCV core amino acids substitutions considered a key factor in determining resistance for both PEG-IFNα/RBV or telaprevir/ PEG-IFNα/RVB treatment **[23-24]**.Of these, substitution of arginine (Arg, R) by glutamine (Gln, Q) or histamine (His,H) at amino acid 70 (R70Q/H) in the core protein. It was reported in previous studies; that patients with IL28B rs8099917 genotype non-TT, only 12 % of those with 70Q/H exhibited SVR, while 50 % of those with 70R developed SVR **[25]**. However, the mechanisms explain this association may rely on the fact that the core region antagonizes the antiviral response induced by IFN by interacting with the IFN-activating and signalling pathways **[26, 27]**. Substitutions in in positions 70 and 91 of the core protein region can give rise to viral quasispecies resistant to interferon treatment **[28].** Which alter the outcome of CHC patients on interferon-based therapy **[29, 30].**Therefore, this study aimed to evaluate the IL28B- SNP rs8099917 and HCV core mutation as predictors for SVR in Egyptian chronic hepatitis C patients' genotype 4 treated with (PEG-IFNα) and ribavirin (RBV), Moreover, to determine whether HCV core substitutions are present and play a significant role in the outcome of interferon-based treatment in Egyptian patients, as well to inform better selection and prioritization of those patients who can still benefit from this affordable therapy.

2. Materials and Methods

A Cohort of 115 patients infected with chronic hepatitis C virus (HCV); they were eligible for treatment with peglated Interferon and ribavirin combination therapy. All patients met the following inclusion criteria: detected HCV viremia proved by HCV-RNA real time PCR analysis and previously untreated. Those who were infected with HBV or HIV co-infection were excluded. All patients gave their informed consent for the collection and storage of their serum samples for research purposes. The selected patients received a subcutaneous injections pf Peg-INF once /week in a dose of 180ug combined with oral ribavirin; dose: 1000mg/day, 1200mg/day) for those ≤75Kg and >75Kg, respectively; while Peg-INF dose was 1.5ug/Kg body weight for those weighting <65Kg. Patients were assessed initially at diagnosis (untreated), 12,24 and 48weeks after therapy. Laboratory biochemical test includes serum alanine transaminases (AST), Bilirubin, complete blood counts (CBC). Basal HCV-RNA viremia was determined by real time PCR at the same intervals as other laboratory tests to evaluate the therapeutic effect; successful response to treatment was based on a sustained virological response (SVR); defined as HCV-RNA negative six months after therapy, non virological response (NVR) was considered when HCV-RNA viral load was <2-log unit decline from pretreatment baseline levels in patient serum at 12 weeks post- therapy or detectable viremia 24 weeks after treatment.

Detection of Amino Acid Substitutions (70 aa mutation)-(Arg70) or glutamine/histidine (Gln70/His70)) in the HCV Core Regions:

5 ml blood was collected from each patient in k_2 EDTA vacutainer, Viral RNA was extracted from 140 ul serum using usingQIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA).mRNA was reversed transcribed by RT- two steps RT kit (*Qiagen, Germany*) cDNA was used in a semi-nested PCR with a specific sequence primers (Table 4); the generated amplicon were sequenced to assess the presence of substitutions at position 70 in the HCV core; sequencing was performed by (BigDye Terminator 130 Genetic analyzer, ABI prism; Applied Biosystems).

Table 1: Nested PCR Primer Sequences

	PCR	Foreword primer sequence (5'-3')	Reverse primer sequence (5'-3')
CE1/CE2	First PCR	5'GTCTGCGGAACCG GTGAG TA-3'	5'GACGTGGCGTC GTAT TGTCG-3'
CC9/CC6	Second PCR	5'ACTGCTAGCCGAG TA GTG TT-3'	5'GAGCAGTCG TTCGTGACAT-3'

Statistical analysis

Statistical analysis was performed with SPSS Statistics version 18.Non parametric tests (chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to sustained virological response. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P< 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses.

3. Results

Demographic and Clinical baseline characteristics

The demographic and clinical characteristics of the 115 studied patients who enrolled in this study are presented in Table (2). There were 96 males (83.5%) and 19 females (16.5%). All patients were of Egyptian ethnicity. Their ages range from 26 to 59 years with mean value of 46.52 ± 9.38. 62(54%) patients had a diagnosis of fibrosis before starting therapy; this was done according to Ishak liver fibrosis staging classification. The meanvalue of body mass index was 26.39 before starting treatment. At baseline, the mean±SD of alanine aminotransferase (ALT) and total bilirubin levels were 41.9 ± 3.7 and 0.81 ± 0.34, respectively. The mean leucocyte counts, platelet count, andhaemoglobin levels were $4.2x10^9$/L, $179x10^9$/L and 13.6 mg/dl, respectively. The mean viremia level (log ^{10} IU/ml) baseline,(12weeks, 24 weeks and 48 weeks) after start therapy were 2.56, 2.08, 2.77 and 7.87 respectively.

Table 2: Baseline Characteristics of the studied HCV-4 Patients

Patients characteristics		Overall (n=115)
Age		
	Range	26-59
	years (mean ±SD)	46.52 ± 9.38
Gender		
Male	(n)%	96(83.5%)
Females	(n)%	19(16.5%)
Laboratory		
Total Bilirubin (mg/l)	(Mean ± SD)	0.81 ± 0.34
Serum alanine aminotransferase (IU/L)	(Mean ± SD)	41.9 ± 3.7
HCV RNA (log ^{10} IU/ml)		
Basal	(Mean ± SD)	2.56 ± 6.95
At 12 weeks	(Mean ± SD)	2.08 ± 1.09
At 24 weeks	(Mean ± SD)	2.77 ± 1.28
At 48 weeks	(Mean ± SD)	7.87 ± 4.18
Hematological		
Total Leucocyte count(x10^9/L)	(Mean ± SD)	4.2 ± 2.56
Haemoglobin (grm /dl)	(Mean ± SD)	13.57 ± 2.04
Platelets count (x 10^9)	(Mean ± SD)	179 ± 5.2
Response to PegINF/RBV therapy		
Non virological response (NVR)	(n)%	36(31.3%)
Sustained virological response (SVR)	(n)%	79(68.7%)
IL28β rs8099917 GTgenotype		
Homozygous GG	(n)%	5(4.3%)
Homozygous TT	(n)%	72(62.6%)
Heterozygous GT	(n)%	38(33%)
HCV core-70 amino-acid substitution		
Wild	(n)%	88(76.5%)
Mutant	(n)%	27(23.5%)

IL-28β Single nucleotide polymorphism genotypes

The genotypes of studied IL-28β SNPs (rs8099917) were measured for each patient. The results revealed that among 115 studied patients who completed the 48 weeks of INF/RBV therapy and 48 weeks follow up by HCV-RNA viremia level, the favourable genotype (TT) for rs8099917 was identified in 72 patients (62.6%); GT in 38(33%) , GG in 5(4.3%).

Prevalence of substitution of amino acid 70 at HCV infected patients:

In the studied HCV infected patients, only 27(23.5%) of patients had glutamine/histidine at position 70(R70G/H);this was found in contrast to 88(76.5) who had wild type of HCV core – defined as presence of arginine at core 70.

Impact of IL28β polymorphism and HCV core 70aa substitution on treatment outcomes:

The patients were divided into two groups according to the virological response; 79 (68.7%) of patients achieved SVR in contrast to 36 (31.3%) with NVR. By stratifying patients on the basis of their IL28β genotype polymorphism; it was found that in rs8099917; the favorable genotype (TT) achieved significantly higher SVR rates 75% compared with GT, GG genotypes (63, 20)% respectively (P<0.05) Table (3).Analysis of the correlation between the HCV core 70 aa mutation and viral response to INF/RBV combined therapy; it was found that the presence of substitution at position 70of the HCV core was associated with higher rates of NVR 23(85%). However; patients had wild type HCV core achieved higher rates of SVR 75(85%). High significant difference was found between wild versus mutant types and virological response to INF/RBV therapy (p=0.000). No significant differences related to demographic characteristics or virological parameters (HCV viremia, ALT, liver fibrosis) were detected between wild/mutant types was found between the two groups (p≥0.05) Table (3).

Predictive factors for INF/RBV treatment Response:

By Univariate analysis, age≤40 years old, IL28β (rs8099917) TT, absence of core 70 aa mutations and achievement of SVR were significantly correlated with INF/RBV therapeutic success; defined as SVR (Table 3).Logistic regression analysis was performed to evaluate the impact of patient's characteristics, viral load on the likelihood of achieving SVR; all statistical significant predictors in univariate analysis were included in the model. Multivariate analysis revealed that young age (≤40 years) old, IL28β (rs8099917) TT genotype and absence of HCV core70 aa mutation were independent predictors associated with achieving SVR to INF/RBV combined therapy (Table 4).

Table 3: Distribution of IL28β rs8099917 genotypes and HCV core-70aa substitution according to virological response to (INF/RBV) therapy

Virological response to (INF/RBV)	IL28β rs8099917 genotype			HCV core-70 amino-acid substitution	
	GG (n= 5)	GT (n=38)	TT (n=72)	Wild (n=88)	Mutant (n=27)
NVR	4 (80%)	14 (36%)	18 (25%)	12 (14.8)	23 (85%)
SVR	1 (20%)	24 (63%)	54 (75%)	75 (85.2)%	4 (15%)
Pearson Chi-Square	7.38			1.154	
P value	0.025			0.000*	

*=high statistical significance; p≤0.01

Table 4: Multivariate analysis on independent predictors factors for SVR to INF/RBV therapy

Independent Variable	Adjusted OR	95% CI	*P* value
Age (≤40 years)	0.92	0.82 – 0.97	<0.001
IL28β (rs8099917) TT genotype	4.6	2.1 – 10.4	0.04
Absence of HCV core 70aa mutations	5.75	2.3 – 14.2	<0.001

SVR: sustained viral response; INF/RBV: pegylated- interferon/ribavirin combination therapy; OR: Odds ratio; CI: confidence interval.

4. Discussion

IL28β gene polymorphism and its impact response to antiviral therapy have been implicated in previous studies **[31-32].** It was suggested as a putative biomarkers for predicting response to therapy **[33-34].**However, the majority studies were done on HCV genotype 1, 2 and 3; few studies concentrate on HCV genotype 4. To our knowledge this study is designed to evaluate the role of (IL28β rs8099917 genotypes and HCV core 70 codon mutation) as a predictors of SVR to PegINF/RBV in Egyptian patients mono-infected with HCV-4 genotype.

In this study, we observed that the absence of substitution in core position 70; together with IL28β polymorphism are a good predictors for achieving SVR with Peg-INF/RBV therapy in Egyptian patients. SVR rates were significantly higher in TT patients compared with GT/GG in rs8099917 (75%) versus (63%, 20%) respectively. Our findings are in agreement with **Sticchiet al. [35],** who observed similar findings in northern Italy, the distribution of rs8099917 genotypes was: TT in 55%, TG in 40%, and GG in 5% of the study participants.

In the current study, we confirmed that TT genotype was the most favorable genotype in HCV-4 patients who showed a trend toward higher SVR rates (75%) compared to 25% who relapsed after therapy. On the other hand; NVR was observed in 80%, 36% of GG, GT genotypes respectively. A similar finding was reported by **Dzeova-Vidimliski et al. [36]** who observed a significant higher SVR in rs8099917 TT genotype (71.4%) than in patients with non-TT genotypes (43.4%). Studies analyzed IL28β polymorphism revealed that IL-28 polymorphism genotypes variants are associated with endogenous activation of innate immunity **[37]**, thus inducing upregulation in INF stimulating genes, activation of INF signaling pathways that render patients resistant to INF based antiviral therapy **[38,39].** Unfavorable IL-28β genotypes are not only the reason behind low viral response to INF, a serious of others factors should be taken in consideration; presence of adverse events that could interrupt treatment, preexisting mutations in other genomic regions **[37].** These findings can explain the different findings regarding IL28 polymorphism obtained by several studies.

Our study detected higher rates of core protein substitution at position 70 (23.4%) in Egyptian patients infected with HCV-4 subtype; 85% of them were unresponsive to treatment. Higher rates 88% of mutated HCV-core 70 aa substitution was observed by **Sultana et al. [37]**, but he studied core protein substations at position 70 and 91; he reported that these mutations were linked with a significantly decreased probability of achieving SVR to INF/RBV therapy. Only two studies with limited studied patients have suggested an association between 70 aa substation and an increased response to therapy **[40, 41].** No significant association with viral load. Similar findings were observed in Caucasian patients infected with HCV subtype 1b **[30, 40, and 42].Funaoka et al. [41]** confirmed these results in vitro; he evaluated the effect of INF on HCV core mutations in terms of viral replication, he found a higher rate of INF resistance in mutant compared to wild type; this was associated with downregulation of INF-stimulating genes. These findings can be explained by the theory based on that the INF resistance of core mutants that was related to the inhibition of INF-signaling pathway, involving SOCS3 (suppressor of cytokine signaling).IL-6 is upregulated in cells transfected with a core mutant and it was known that it stimulates SOCS3 proteins. In vivo, this theory can explain increased levels of inflammatory cytokines such as IL-6 and TNF-α in chronic HCV patients **[43].**Moreover, structural changes at mini-core proteins isotypes of normal core protein can alter viral sensitivity to INF **[28].**

In conclusion, this study reports that absence of core genomic mutations associated with IL28β rs8099917 TT genotypes are good predictors for SVR to Peg-INF/RBV antiviral therapy as well as favorable outcome in HCV-4 chronic infected Egyptian patients. Another potentially beneficial gain from this study is to select patient subclass who are responsive to interferon based therapy according to genomic study, thus reduce the cost-effectiveness and achieve higher rates of HCV patients outcome.

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