Inducible Nitric Oxide Synthase Polymorphism In Hepatitis C Patients Treated With Interferon Based-Treatment As A Predictor of Early Virological Response

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HCV infection represented a foremost communal health trouble and Egypt has the largest epidemic of HCV in the world with prevalence of 14.7% for HCV antibody and 9.8% HCV-RNA. Nitric oxide (NO) is a signaling molecule participated in inhibiting of microbial diseases. Pro-inflammatory stimuli can trigger resting cells to produce inducible nitric oxide synthase ((iNOS) also referred to as (NOS2), which is very crucial for host response to contagious agents. NOS2A gene haplotypes has been associated with a number of diseases. This study aimed to assess the relation between NOS2A gene haplotypes and HCV treatment response in pegylated interferon alpha /ribavirin (PEG-IFN /RBV) in chronic HCV patients (CHC) in an attempt to find a predictor biomarker to detect poor responders to therapy. DNA was extracted from blood samples and subjected to detection of NOS2A gene haplotypes using real time PCR. Non-responder patients showed statistically significant higher percentages of unclassified haplotypes than responder patients (85.7% versus 58.6%, respectively) (*P*<0.0001) and of haplotypes 4 and 5 (GTT and ATC) than non-responder patients (25.7% and 14.3% versus 0% and 0%, respectively) (*P*<0.0001). The NOS2A gene haplotypes were not associated with response to PEG-IFN /RBV at 12th week Early Virological Response (EVR). In conclusion, NOS2A gene haplotypes are not considered predictors of response to PEG-IFN /RBV treatment. Further studies are required to elucidate predictor markers.

HCV is a challenging disease affecting nearly 3% of the world population, only 20–30% of individuals exposed to it recovered spontaneously while the remaining 70–80% will develop CHC [1]. Outright treatment of hepatitis C stays tricky and in a vast majority of the patients did not attain sustained virological response (SVR) [2]. Egypt has by far the largest HCV prevalence in the [3], in the age group varied from 15 to 59 years the estimated percentage who are positive for HCV antibody was 14.7% and over 80% of HCV infected Egyptian population is among individuals aged 30 years and above [4]. NO is one of the signaling molecules participated in inhibiting the microbial diseases that formed by nitric oxide synthase (NOS) that oxdatively delaminates L-arginine to L-citrilline and had three isoforms, neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and NOS2 [5]. *in vitro*, NO had a potent antimicrobial effects due to its capability to stop the growth of many infectious organisms6. NOS2 is the most potent in the three NOS forms, which is formed rapidly, efficiently and speedily articulated in reply to pro-inflammatory stimuli such as cytokines, including interferon. When present, NOS2 is capable of synthesizing 100–1000 times more NO than the other forms over a prolonged period of time, which made it an important part of

the host response to infectious agents [6]. NOS2A gene haplotypes has been associated with a number of diseases and polymorphism in promoter of NOS2A influences the gene adjustment and accordingly, the consequence of illness [7]. This study was aiming to assess the relation between NOS2A gene haplotypes and HCV treatment response in an attempt to find a reliable measurable predictor biomarker to detect none and poor responder to PEG-IFN /RBV therapy in chronic HCV patients so as to avoid useless, expensive and side effects of this regimen, so as to improve patient care.

Patients and Methods

The study included 77 patients attending in Ismailia fever hospital, known to have CHC (HCV RNA genotype 4), chosen according to inclusion criteria (age between 18 and 60 years, CHC patients for at least 6 month proved by detectable HCV RNA in serum, ALT level above the upper limit of normal and intake of pegylated interferon alpha / ribavirin therapy for 12 weeks) and exclusion criteria (HBV co-infection or other hepatotropic virus, decompensated cirrhosis, any other contraindication to therapy with the combination of PEG-IFN /RBV, history of hepatotoxic drugs intake, history of alcohol intake (>40 g/day), pregnancy and breast feeding, autoimmune diseases and irregular therapy.

The patients were subclassified according to early virological response into responder group (decline 2 log HCV RNA from the initial viral load or absence of HCV RNA at the end of 12^{th} week of treatment) [8] (n=70) and non-responder (no or slight drop in HCV RNA load at the end of 12^{th} week of treatment) (n=7). The study was approved by the ethical committee of Suez Canal University.

Methods

After obtaining a written informed consent, all participants were asked to document the following information in a questionnaire: age, sex, alcohol history, past medical, family history and concomitant medications taken during the past three clinic visits. The results of complete blood picture, ALT, AST and initial and 12^{th} week viral load were all obtained from patient's files.

Laboratory investigations

After taking PEG-IFN /RBV for 12 weeks, peripheral blood samples were collected on EDTA tubes and DNA extraction and amplification were done by QIAamp DNA Blood Mini Kit for DNA isolation from blood and body fluids supplied by Qiagen (Qiagen, Hilden, Germany). Amplification reaction of extracted NOS2A gene was done by Corbett Research PCR Rotorgene 6000 (Qiagen, Hilden, Germany). The Primers used for genotyping the three single nucleotide polymorphism (SNPs) (NOS2A -277 (A/G), NOS2A -1026 (G/T), NOS2A -1026 (G/T)) in the promoter region of the NOS2A gene are shown in Table "1" [9]. The amplification reaction tubes was comprised of 0.2 mM of specific primers (as described below) Table "1" 2.5U of Stoffel Gold Polymerase (Qiagen, Hilden, Germany); 1_Stoffel Gold buffer (10mM Tris-HCl, 10mM KCl at pH 8.0); an additional 30mM KCl for a final concentration of 40mM; 3mM MgCl2; 50mM each dATP, dCTP, and dGTP; 25mM TTP; 75mM dUTP: 2U of UNG (PE), 0.2_SybrGreen I; 2 mM ROX; 5% DMSO; and 2.5% glycerol (Roche Molecular Probes, Mannheim, Germany). Biotin gene, One of the housekeeping gene, was used as the internal control in each reaction. (The extracted DNA, primers & master mix were left at room temperature until thawed and 6 tubes of each sample were prepared according to:

Tube "1": 5 µl master mix, 0.5 µl, 277(A/G) forward common, 0.5 µl G-specific reverse, 2.5 µl DNA sample and 1.5 µl distilled water.

Tube "2": 5 µl master mix, 0.5 µl, 277(A/G) forward common, 0.5 µl 277(A/G) A-specific reverse, 2.5 µl DNA sample and 1.5 µl distilled water.

Tube "3": 5 µl master mix, 0.25 µl 1026(G/T) forward common, 0.5 µl 1026 (G/T) T- specific reverse, 2.5 µl DNA sample and 1.75 µl distilled water.

Tube "4": 5 µl master mix, 0.25 µl 1026(G/T) forward common, 0.5 µl 1026 (G/T) G- specific reverse, 2.5 µl DNA sample and 1.75 µl distilled water.

Tube "5": 5 µl master mix, 0.5 µl 1659(C/T) reverse common, 0.5 µl 1659(C/T) C- specific forward, 2.5 µl DNA sample and 1.5 µl distilled water.

Tube "6": 5 µl master mix, 0.5 µl 1659(C/T) reverse common, 0.5 µl 1659(C/T) T- specific forward, 2.5 µl DNA sample and 1.5 µl distilled water.

The final reaction volume was 10 µl. Good mixing was done for each tube then tubes were put in the thermal cycler according to the following program: Initial denaturation: at 95 ºC for 7min, denaturation: at 95 ºC for 10s, annealing/extension: 60 ºC for 30s, Number of cycles: 40, melting curve: 98 ºC

Analysis

Melting curve analysis was included in the analysis software Rotor-Gene 6000 series software 1.7 (Qiagen, Hilden, Germany) of real-time fluorescence detection instrument Corbett Research PCR Rotor-Gene 6000 (Qiagen, Hilden, Germany). The melting point of the product depends on base composition & length. When the decrease in SYBR Green fluorescence during temperature increase is plotted as a negative first derivative, the temperature of the peak is defined as Tm (melting temperature of the product).

Statistical Analysis

Data were analyzed using software package for statistical analysis SPSS version 16. Data were described in the form of mean ± Standard deviation (SD) and Range. Comparison between two groups regarding quantitative data was performed using independent student t test, chi-square with Yates correction test or Fisher Exact Probability Test according to normality of the data.

Table 1. The three Single nucleotide polymorphisms of NOS2A gene

SNP	Primer specificity	Primer sequence
NOS2A -277 (A/G)	Forward common primer	5'-CTGGCTCCGTGGTGCC-3'
	A-specific reverse primer	5'-CAGGGTGGCTGCTAAGAT-3'
	G-specific reverse primer	5'-CAGGGTGGCTGCTAAGAC-3'
NOS2A -1026 (G/T)	Forward common primer	5'-CTGGCTCCGTGGTGCC-3'
	G-specific reverse primer	5'-CAGGGTGGCTGCTAAGAT-3'
	T-specific reverse primer	5'-CAGGGTGGCTGCTAAGAC-3'
NOS2A -1659 (C/T)	Reverse common primer	5'-GGGATGGTATGGTGCTGATG-3'
	T-specific forward primer	5'-CCTTGAACAAGGCAGAACT-3'
	C-specific forward primer	5'-CCTTGAACAAGGCAGAACC-3'

Results

Regarding age and gender of the patients, there were no statistically significant differences between studied groups (P<0.05). At baseline, there were statistically significant higher mean values of AST and viral load in non-responder than in responder patients (P<0.0001), otherwise no statistically significant difference was detected regarding ALT, white blood cells count (WBCs) or platelets count between both groups, while at 12th week. there were statistically significant higher mean values of ALT, AST, WBCs, hemoglobin (HB) and platelets count in non-responder than in responder patients (P<0.0001). We also found a statistically significant lower mean values of ALT, AST, WBCs, HB and platelets count at 12th week of responder patients at baseline and 12th week (P<0.01) while the opposite were found in non responder patients (P>0.05). The frequency and percentages of distribution of the studied patients according to haplotypes were shown in Table (2).

Table 2. Frequency and percentages distribution of the studied patients according to haplotypes.

Haplotypes	Responders (n=70)	Non-responders (n=7)	*P*-value
	No. (%)	No. (%)	
Unclassified	41 (58.6%)	6 (85.7%)	
ATC/5	10 (14.3%)	0	
GTC/2 & GGC/3	1 (1.4%)	0	
GTT/4	18 (25.7%)	0	<0.0001*
GTC/2 & AGC/1	0 (0.0%)	1 (14.3%)	0.0003*

Used test: χ2=chi-square with Yates correction test, FE=Fisher Exact Probability Test.

* *P*-value <0.05 is significant.

Non-responder patients show statistically significant higher percentages of unclassified haplotypes than responder patients (85.7% versus 58.6%, respectively) (*P*<0.0001), meanwhile, responder patients show statistically significant higher percentages of haplotypes 4 and 5 (GTT and ATC) than non-responder patients (25.7% and 14.3% versus 0% and 0%, respectively) (*P*<0.0001). Correlation between haplotypes and the studied variables were shown in Table (3).

Table 3. Correlation between haplotypes and the studied variables

Variables	Haplotypes	
	Correlation Coefficients (r)	*P*-value(2tailed)
Age	-0.466	<0.0001*
Gender	-0.160	NS
ALT	0.100	NS
AST	0.148	NS
WBCs	0.127	NS
HB	0.069	NS
PLT	0.152	NS
HCV RNA	-0.206	NS
ALT12	-0.093	NS
AST12	-0.072	NS
WBC12	0.023	NS
HB12	0.008	NS
PLT12	0.438	0.001*
Response	0.143	NS

*Correlation is not significant at the *P*-value >0.05 (NS).

We also found a significant correlation between higher platelets count at baseline and 12th week and EVR of the patients and there was significant correlation between lower viral load at baseline and EVR of the patients, but we could not detect correlation between haplotypes and response (Table 4). Using linear regression analysis model, the significant predictors of good response at 12th week EVR were higher platelets count and lower viral load at baseline. Haplotypes has no role as a predictor of response (Table 5).

Table 4. Correlation between early response and the clinical and laboratory findings

Variables	Early response	
	Correlation Coefficients (r)	*P*-value (2-tailed)
Age	0.094	NS
Gender	0.019	NS
ALT	0.157	NS
AST	0.153	NS
WBCs	-0.094	NS
HB	-0.059	NS
PLT	0.653	<0.0001*
HCV RNA	-0.838	<0.0001*
ALT12	-0.417	0.001*
AST12	-0.203	NS
WBC12	-0.525	<0.0001*
HB12	-0.303	0.028*
PLT12	0.682	<0.0001*
Haplotypes	0.143	NS

*Correlation is not significant at the *P*-value >0.05 (NS).

Table 5. Predictors of early response using linear regression analysis model

Model	Standardized Coefficients (β)	*P*-value
(Constant)		0.039*
Age	0.141	NS
Gender	-0.049	NS
ALT	0.082	NS
AST	-0.021	NS
WBCs	-0.066	NS
HB	0.027	NS
PLT	0.316	0.008*
HCV RNA	-0.501	<0.0001*
Haplotypes (Other than Haplotype 4 &5)	-0.277	NS
Haplotype 4	0.265	NS
Haplotype 5	0.156	NS

* *P*-value >0.05 is not significant (NS).

Discussion

Over 170 million people worldwide are infected with HCV leads to progressive and life-threatening sequelae such as end-stage cirrhosis and liver cancer [4]. About 25% of infection with it will be anticipated to proceed to rigorous liver disease, which may take more than 30 years to develop [10]. The striking genetic heterogeneity of RNA genome of HCV is well recognized, Smith *et al*. mentioned that there are seven HCV genotypes, and are further subclassified into subtypes except genotypes 5 and 7 [11]. HCV genotype differences had considerable clinical significance because they affect the responses to the antiviral treatment [12]. The HCV genotype 4 is the most prevalent genotype in the Middle East and Central Africa, mainly in Egypt [13], Combination therapy regimen with pegylated-IFN-α and ribavirin is the gold standard treatment module [14], however the total percentage of a SVR with these regimens ranges from only 54% to 63% [15] and this limited therapeutic efficacy might be due to the poor virological response in particular patients or to undesirable consequences of the IFN-based management, leading to low treatment tolerance [16]. Predictive aspects concomitant with the virological response to IFN-based therapy include viral and host elements. Numerous studies mentioned a possible association between the efficacy of IFN-based treatment and polymorphisms in genes encoding cytokines, chemokines, or their receptors [17, 18, 19, 20, 21, 22]. One of these genes is NO as it is an important signaling molecule that is involved in combating microbial infections. NO possess potent antimicrobial effects, including the capacity to restrain the growth of numerous infectious organisms *in vitro* [5]. Given the potent antimicrobial properties of NO, we examined the association between NOS2A gene haplotypes and HCV treatment response in chronic HCV patients. Seventy seven patients were recruited from outpatient clinic of treatment center of viral hepatitis in Ismailia Fever Hospital. They

were divided into responder (n=70) and non-responder (n=7) according to the levels of viral load at 12th week. In our study, the mean age of the total patients (n=77) was 46±8.3 years. The frequency of male patients was higher than female patients (60% versus 40%, respectively) in total population with HCV (male to female ratio of 1.5: 1). This comes in agreement with the results of a study performed in Egypt by Lehman and Wilson, who reported that patients with HCV infection had a mean age ranged from 40 to 48 years [23]. Similar results about higher prevalence in males than in females were found in an African study [24]. From a total of 120 patients with CHC, 76 (63.3%) were males and 44 (36.7%) females (male to female ratio of 1.7: 1). Among Egyptians males the percentage of infection by HCV is higher than that in Egyptians females [23].

In our study, we found a statistically significant higher mean values of AST and viral load in non-responder than in responder patients (P<0.0001). There were statistically significant higher mean values of ALT, AST, WBCs, HB and PLT in non-responder than in responder patients (P<0.0001). There were statistically significant lower mean values of ALT, AST, WBCs, hemoglobin and platelets count at 12th week than at baseline in responder patients (P<0.01). This indicates higher frequency of leucopenia, anemia and thrombocytopenia in responder group. This is in agreement with El-Ahwany *et al.* who reported that liver enzymes (ALT and AST) levels were significantly decreased after 12-weeks interferon-based therapy in responders group (P<0.01) [25]. In line with our results, Irshad et al reported that viral clearance rates are highly variable and between 10–60% of HCV patients clear the virus and show normalization in liver enzymes (ALT and AST) and plasma HCV-RNA clearance [26]. Our study showed that AST levels become normalized in responder group, which is emphasized by the results of DiBisceglie and Hoofnagle study who reported that approximately 50% of patients with cirrhosis had normalized liver enzymes levels during treatment with PEG-IFN [27]. IFN plus RBV combination therapeutic regimens are associated with numerous adverse effects, among which constitutional and neuropsychiatric symptoms and hematological adverse effects in the form of anemia, neutropenia and thrombocytopenia are the primary laboratory abnormalities experienced during IFN plus RBV combination treatment and might demand dose adjustment or even discontinuation of treatment and thus potentially affect outcome. This anemia is attributed to both RBV dose-dependent hemolysis and direct suppressive effect of IFN on erythropoiesis [28]. Moreover, Iqbal *et al*. reported that out of the total population, 30.4% (n=35) individuals developed anemia and 32.2% (n=37) experienced thrombocytopenia [29].

In our study, responder patients show statistically significant higher percentages of haplotypes 4 and 5 (GTT and ATC) than non-responder patients (25.7% and 14.3% versus 0% and 0%, respectively) (P<0.0001).There was significant correlation between lower age of the patients and haplotypes 4 and 5. There was significant correlation between higher platelets count at 12th week and haplotypes 4 and 5. In similar study, Yee et al observed five major NOS2A haplotypes with the subsequent occurrences: haplotype 1, 60.9%; haplotype 2, 16.2%; haplotype 3, 8.7%; haplotype 4, 12.8%; and finally haplotype 5, 1.4%, and is comparable with rates described in other Caucasians [17]. In contrary to our results, Lim et al found that AC NOS2A haplotype comprising two single nucleotide

polymorphisms (rs2248814 and rs2072324) linked with SVR, and its existence might diminish the probability for a successful result of treatment of patients infected with HCV-1 ($P = 0.0053$) [30]. This discrepancy between Lim et al results and our results me be attributed to the difference between the treatment response of HCV genotype 1 and 2 in their study while our patients genotype was HCV genotype 4. Further studies may be needed to find out a predictor of EVR and SVR for HCV.

In conclusions, the NOS2A gene haplotypes are not associated with response to peg IFN/RBV at 12th week EVR. NOS2A gene haplotypes are not considered predictors of response to peg IFN/RBV treatment.

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