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Role of interferon gamma gene polymorphism in spontaneous viral clearance versus chronicity in hepatitis C infected Egyptian patients

Maha R Habeeb[1,*], Rokia Anwar Saad[1], Dina Elhammady[2], Maysaa ElSayed Zaki[3], Neven Farouk Abbas[1].

[1] Department of Internal Medicine, Faculty of Medicine, Mansoura University, Mansoura, Egypt. [2] Department of Tropical Medicine, Faculty of Medicine, Mansoura University, Mansoura, Egypt. [3]Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt

Abstract

Background: Polymorphisms in the cytokine gene involving IFN gamma (IFN-γ) have been implicated in many infections including HCV. The aim of this study is to evaluate the association between IFN-γ polymorphism in three regions, (+2109A/G, +874A/T, -183G/T) and either chronicity or spontaneous viral clearance (SCV) in HCV infected patients. **Materials and Methods:** The study included 200 HCV-infected patients divided into *Group I* of 100 patients with spontaneous virus clearance (SVC) and *Group II* of 100 patients with persistent chronic hepatitis C infection (PI) who did not receive any therapy. These patients were subjected to history taking, full clinical examination and laboratory investigations included analysis of IFN-γ gene polymorphisms. **Results:** At locus +2109 of the IFN-γ gene, patients with A/A genotype had a significantly higher rate of spontaneous hepatitis C clearance while the G/G genotype was more prone to persistent infection. No statistically significant difference was found between both groups regarding loci +874 and -183 of the IFN-γ gene, but column proportion comparison using Bonferroni method at locus +874 revealed a higher proportion for T/A genotype in SVC group. Both haplotypes AAT and TGT were more susceptible to chronic HCV infection, as were heterozygote T/A at locus +874 and G/G genotype at locus +2109s. A/G and A/A genotypes at locus +2109, TLC at cut off value ≤7.15, and AST at cut off value ≤27.5 were considered independent predictors for development of SVC. **Conclusions***:* Polymorphisms in the IFN-γ gene may play role in sequelae following HCV infection, possibly determining whether the virus will be cleared spontaneously or not.

Introduction

Chronic hepatitis C virus (HCV) infection is a universally prevalent pathogen leading to liver cirrhosis and liver cancer [1,2]. Hepatitis C viral infection is endemic in Egypt with the highest prevalence rate in the world [3, 4].

Because acute HCV infections are typically asymptomatic they are rarely diagnosed and often missed. Hepatitis C virus appears in blood within 2-14 days following initial exposure with antibody production becoming evident in the subsequent 20-150 days [5, 6]. Approximately 25% of infected patients undergo spontaneous clearance within about 6 months [7], while an overwhelming majority of patients (70–80%) progress to chronic HCV infection with persistence of the virus associated with ongoing hepatic inflammation and necrosis, ultimately resulting in development of liver cirrhosis and hepatocellular carcinoma [8]. The complex array of interactions between the host and virus that establish the outcome of HCV infection, whether spontaneous clearance of the virus or its persistence into chronicity, is a poorly understood dynamic [7,9,10], but it has been definitively demonstrated that a strong host immune response is a fundamental determining factor for spontaneous viral clearance [11,12].

Control of HCV infection has been shown to be associated with polymorphisms in host proteins, such as HLA class I and II , natural killer, and interferon-stimulated gene [13]. Interferon (IFN) release is triggered by the host cell upon HCV entry so as to hinder viral replication either directly or by stimulation of immunoregulatory mechanisms that control the infection [15]. Most important of these interferons is interferon gamma (IFN- γ), a secretory protein produced primarily by natural killer and T cells. The role of IFN-γ in developing liver fibrosis, and possibly providing a potential avenue towards new treatment methods, is emphasized by the recognition of a cluster of IFN-γR2 variants which are markedly associated with progression of liver fibrosis in chronic HCV infection. Reduced function of IFN-γ receptors may be associated with increased production of IFN-γ, as seen in cases with complete IFN-γ receptor deficiencies [16].

Polymorphisms in cytokine genes, including interferon gamma (IFN-γ), have been implicated in numerous infections [17,18], autoimmune diseases, and chronic inflammatory conditions [18,19]. Some polymorphisms reported in the regulatory and coding regions of IFN-γ gene include -183G/T, +764C/G, +874A/T, +2109A/G, +3810G/A, and + 5944G/A loci [20].

The aim of this study is to evaluate the role of interferon gamma (IFN-γ) gene polymorphism in three

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* Corresponding author. Email: maha_ragab68@yahoo.com

regions, namely +2109A/G, +874A/T, -183G/T, in different HCV infection outcomes, whether clearance or chronicity, in Egyptian patients.

Materials and methods

This prospective case-control study recruited subjects from the outpatient clinics of the Internal Medicine and Tropical Medicine Departments at Mansoura University Hospital during the period from October 2017 till March 2019. The 200 recruited patients were divided into two groups. *Group I* included 100 patients with hepatitis C who achieved spontaneous vial clearance (SVC) as determined by positive HCV IgG and negative PCR for HCV-RNA on two occasions separated by a six-month period. *Group II* comprised 100 patients with persistent chronic hepatitis C infection (PI), characterized by positive HCV IgG and positive PCR for HCV-RNA, who did not receive any therapeutic interventions. Criteria for exclusion were patients with HBV or HIV co-infection, alcohol ingestion and patients with other etiologies of chronic liver disease. The study protocol was approved by the Ethics Review Board of Faculty of Medicine, Mansoura University, and informed written consent was obtained from all participants according to the Declaration of Helsinki. The committee's reference number is MD/17.08.92. All participants were subjected to thorough history taking and full clinical examination.

Laboratory assessment.

Blood samples of 10 ml were withdrawn from each subject and separated into three aliquots, two in plain tubes and one on ethylenediaminetetraacetic acid (EDTA). Sera from the plain tubes was separated for liver functions tests (including AST, ALT, total and direct bilirubin, albumin), serological markers for HCV antibodies, HBs antigen, HIV antibody, and HCV RNA detection by real time PCR. The third aliquot on EDTA was used for DNA extraction from peripheral blood mononuclear cells by DNA extraction kit (Qiagen-Germany).

DNA Extraction and Analysis of IFN-γ Gene Polymorphism

A 5mL blood sample was collected from each participant in EDTA anticoagulant tubes. DNA analysis was performed using Qiagen kit (Qiagen-Germany) according to the manufacturers' guidelines.

PCR for IFN-γ Gene

Polymorphisms of IFN-γ gene was studied at positions +874, +2109 and -183. Gene polymorphism at position +878 was performed by allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) [21], while those at positions +2109 and -183 was assessed using polymerase chain reaction – restriction length fragment polymorphism (PCR-RLFP) technique using the two restrictions enzymes Arthrobactercitreus I and Arthrobacterluteus I, respectively. [22, 23]. The primers used are summarized in Table 1. Internal control primers were used in each PCR as positive control. The quality control primers are for amplification of β-actin-specific primers (F-5' ACA CAA CTG TGT TCA CTA GC-3' and R-5' CAACTT CAT CCA CGT TCA CC-3'). The amplified products were subjected to electrophoresis on 2% agarose gel with 0.5 μg/mL ethidium bromide to determine the base pairs (bp) of the products [24].

Statistical analysis

Software used:

Data were entered and analyzed using Statistical Package for the Social Sciences (SPSS) software (version 21) and SNPStats software (http://bioinfo.iconcologia.net/SNPstats).

Data expression:

Qualitative data were expressed as count and percentage. Quantitative data were initially tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk's test with data being normally distributed if $p>0.05$. Quantitative data were expressed as mean ± standard deviation (SD) if normally distributed or median and interquartile range (IQR) in case of abnormal distribution.

Data comparison:

Qualitative data for two groups (2X2 table) used Chi-Square test (or Fisher's exact test). Qualitative data for more than two groups (e.g. 2X3 table) used Chi-Square test (with Bonferroni method to adjust p values when comparing column proportions). Quantitative data between two groups: Data that was normally distributed in both groups was analyzed using independent sample t-test, while abnormally distributed data required the alternative Mann-Whitney U test. Quantitative data between more than two groups: Assessment of normally distributed data in all groups was performed using one-way ANOVA test and abnormally distributed data using the alternative Kruskal-Wallis H test. Significant results were further analyzed with pairwise comparisons to detect where that significant difference existed

Results

(Table 1) shows primary sequences and methods used for detection of cytokine gene polymorphisms**.**

(Table 2) shows a statistically significant difference for SNP+2109 with predominance of A/A genotype in the spontaneous hepatitis C viral clearance (SVC) group and G/G genotype in patients with persistent infection (PI). For SNP +874, there was no statistically significant difference found between the two groups, but comparison of column proportions using Bonferroni method revealed a higher proportion for T/A genotype in SVC group. In addition, SVC patients demonstrated statistically significant lower values for total leucocyte count (TLC), neutrophils, bilirubin, AST, and ALT as well as a statistically significant higher levels for fasting blood glucose and platelet count when compared to patients who did not achieve SVR.

(Table 3) shows that patients with A/A, A/G, TLC ≤7.15, bilirubin ≤0.75 and AST ≤27.5 had 4.8, 8.6, 5.8, 3.9 and 6.7 higher odds, respectively, of achieving SVC while no significance was detected for values of platelet count or FBG level. Binomial logistic regression was performed to ascertain the effects of SNP +2109 genotypes, TLC ≤7.15,

bilirubin ≤0.75, and AST ≤27.5 on the likelihood that participants may have a logistic regression model that was statistically significant ($\chi^2(5) = 77.257$, $p < 0.0005$). The model correctly classified 75% of cases, with sensitivity of 67% and specificity of 83%. Positive predictive value was 79.8% while negative predictive value was 71.6%.

All entered predictor variables, except bilirubin, were statistically significant as shown in **Table 4,** and were considered as 'independent' predictors of the likelihood of occurrence of SVC. Presence of A/A, A/G, TLC ≤ 7.15, and AST ≤ 27.5 was associated 5.3, 8.9, 5.3, and 5.9 higher odds, respectively, of achieving SVC.

SNP analysis:

(Table 5) depicts multiple inheritance models showing that the 'best' model for +874 was over-dominant and for +2109 was recessive and codominant. Heterozygosity in the over-dominant model of SNP +874 analysis was more susceptible to SVC by 1.79 higher odds more than the others. G/G genotype in the recessive model of +2109 SNP analysis was more susceptible to lack of SVC by 0.19 odds than the others. There was no minor group in SNP -183 and, hence, no model. Study of linkage disequilibrium showed that none existed between +874 and +2109 (D=0.0039, D'=0.0388, r=0.0188, P=0.71). Haplotypes AAT and TGT were significantly associated with absence of SVC (P=0.0300, P= 0.0094, respectively) as shown in **(Table 6)**.

Table 1. Primary sequences and methods used for detection of cytokine gene polymorphisms.

Gene locus	Sequences of the primer	Method
IFN +874	**Common primer:** 5'-TCA ACA AAG CTG ATA CTC CA-3' **T allele primer:** 5'-TTC TTA CAA CAC AAA ATC AAA TCT-3' **A allele primer:** 5'-TTC TTA CAA CAC AAA ATC AAA TCA-3'	ASO –PCR
IFN +2109	F5'-AAT CGC TGA AGT ATG TAA T-3' R 5'-GCA TTG TAG AGT TTT GCA G-3'	RFLP-PCR (Restriction enzyme: Arthrobactercitreus)
IFN -183	F5'- AAT GTG CTT TGT GAA TGA-3' S5'- CTC CTC TGC CTG CTG CTA-3'	RFLP-PCR (Restriction enzyme: Arthrobacterluteus)

Table 2. Comparisons of data between the two study groups.

Parameter	Groups		P
	Group I: Spontaneous viral clearance (SVC) (N=100)	**Group II: Persistent infection (PI) (N=100)**	
*Sex:			0.777
– Male	50	52	
– Female	50	48	
*Smoker:	25	33	0.213
*DM:	17	28	0.063
*Schistosomiasis:	18	15	0.568
**Age	55 (46-61)	55.5 (45-63)	0.333
**Weight	89 (80-97.75)	89 (80-95)	0.663
**Height	167 (160-172)	167 (161-172)	0.886
**BMI	30.7 (28.1-35.5)	31.2 (28.7-35.2)	0.799
**TLC	5.4 (4.8-6.6)	6.45 (5-8.4)	0.001
**Neutrophils	47 (39-55)	50 (42-58)	0.033
**Lymphocytes	43 (34-48)	39 (30.5-48)	0.185
**Hemoglobin	12.8 (11.3-13.7)	12.6 (11.4-14.2)	0.192
**Platelet count	203 (175-263)	178 (131.25-216.25)	0.001
**INR	1.05 (1.0-1.195)	1.035 (1.0-1.12)	0.320
**Albumin	4.20 (3.9-4.5)	4.25 (3.9-4.5)	0.589

**Bilirubin	0.6 (0.5-0.7)	0.7 (0.6-0.9)	<0.0005
**AST	22 (18-30.75)	35 (25-63.75)	<0.0005
**ALT	27 (19.25-35)	35 (25-63.75)	<0.0005
**FBG	107 (96-128.75)	101 (90-125.75)	0.030
**Creatinine	0.8 (0.7-0.975)	0.8 (0.7-1.0)	0.498
*SNP+874:			0.135
– A/A	20 A	25 A	
– T/A	50 **B**	36 **A**	
– T/T	30 A	39 A	
*SNP+2109:			<0.0005
– A/A	78 **B**	65 **A**	
– A/G	15 A	07 A	
– G/G	07 **B**	28 **A**	
*SNP-183:			0.552
– G/T	95	93	
– T/T	05	07	

Data are expressed as *frequency (P value by Chi-square test) or as **median (IQR) (P value by Mann-Whitney test). Comparisons of column proportions are expressed in capital letters (similar letters = insignificant difference, different letters = significant difference).

Table 3. **Predictors of SVC (Crude Odds Ratio [OR] for significant variables)**

Variable	Crude OR (95% CI)	P-Value
SNP+2109:		
– **G/G**	R*	
– **A/G**	4.800 (1.969-11.703)	0.001
– **A/A**	8.571 (2.528-29.062)	0.001
TLC	0.770 (0.664-0.893)	0.001
Neutrophils	0.969 (09.45-0.994)	0.015
Platelet count	1.0 (1.0-1.0)	0.004
Bilirubin	0.070 (0.014-0.347)	0.001
AST	0.955 (0.937-0.974)	<0.0005
ALT	0.966 (0.950-0.982)	<0.0005
FBG	1.001 (0.995-1.007)	0.653
****TLC cutoff**		<0.0005
– **>7.15**	R	
– **≤ 7.15**	5.754 (2.672-12.390)	
****Bilirubin cutoff**		<0.0005
– **>0.75**	R	
– **≤ 0.75**	3.938 (1.998-7.760)	
***AST cutoff**		<0.0005
– **27.5**	R	
– **≤ 27.5**	6.677 (3.592-12.414)	
***Platelet cutoff**		1.000
– **< 183.5**	R	
– **≥ 183.5**	1.000	

***R=Reference category.**
****Cutoff values were created by ROC curve analysis.**

Table 4. Predictors of the likelihood of occurrence of SVC.

Predictors	B	SE	Wald test	P	OR (95% CI)
SNP+2109G/G					R
SNP+2109 A/G	1.663	0.518	10.306	0.001	5.276 (1.911-14.566)
SNP+2109A/A	2.190	0.715	9.380	0.002	8.936 (2.200-36.290)
TLC>7.15					R
TLC≤ 7.15	1.660	0.445	13.890	<0.0005	5.257 (2.196-12.581)
Bilirubin > 0.75					R
Bilirubin≤ 0.75	0.693	0.414	2.810	0.094	2.000 (0.889-4.498)
AST > 27.5					R
AST≤ 27.5	1.774	0.360	24.295	<0.0005	5.897 (2.912-11.941)
B=Logistic regression coefficient					
SE=Standard error					

Discussion

Various studies on human and animal models of HCV infection have reported on the role of host immune responses following viral entry [25,26]. However, assessment of spontaneous viral clearance is difficult on account of the many controlling factors, including sex, age, ethnicity, genetic background and viral genotype [20].

IFN-γ is a multifunctional cytokine produced by effector T and natural killer cells that is important for host defense against many intracellular pathogens, including HCV, in addition to its recognition as an inhibitor of HCV replication in vitro [27]. Several studies have established an association between low levels of IFN-γ and spontaneous clearance of HCV [28]. Moreover, cytokine gene polymorphism, including that of IFN-γ, have been confirmed to be involved in evolution of several diseases [29].

The current study showed that at locus +2109 of IFN-γ gene, patients with A/A genotype had a significantly higher rate of spontaneous hepatitis C viral clearance while G/G genotype was associated with more persistent infection. This is in concordance with another study similarly demonstrating that A/A genotype and A allele of IFN-γ at +2109 locus was detected at higher rates in treatment-responder patients infected by HCV genotype 1 than in non-responders [24]. However, a further study demonstrated that G/G genotype at +2109 locus was higher in patients who cleared hepatitis C virus when compared to chronically infected patients, with presence of G allele at 2109 locus possibly attributing to HCV clearance, and additionally reporting that presence of A/A genotype was higher in chronically infected patients. Furthermore, +2109 A/G locus IFN-γ gene polymorphism was found to be associated with increased response to therapy in patients with genotype 1 HCV infection in this study, although the association of this polymorphism with outcome of HCV infection was not demonstrated [20].

Regarding SNP in +874 T/A locus of the IFN-γ gene, the present study demonstrated that A/A and T/T genotypes were present more in patients with persistent infection while T/A genotype was more evident in SVC group, but no statistically significant difference between the study groups could be detected despite reports of higher T/A genotype in SVC group on comparison of column proportions. A number of previous studies had reported similar findings of frequency of alleles and genotypes of IFN-γ at +874 T/A locus being statistically insignificant in the treatment non-responder group when compared to responders infected by HCV genotypes 1 and 3 [24, 30]. However, other studies found that frequency of T/T genotype was significantly higher than T/A in persistent chronic hepatitis C [31-34]. A meta-analysis on Asian and Caucasian patients demonstrated that the T allele was protective in liver disease while A/A genotype at locus +874 T/A was associated with about 1.4-fold increased risk of hepatitis C virus-related disease [35].

Conversely, an additional study found that T allele of +874 T/A was significantly higher in patients with chronic HCV infection than in patients who had cleared the virus spontaneously and in non-infected individuals, suggesting that T allele was associated with increased risk of chronic HCV while A allele was associated with decreased risk of chronicity [36]. These results align with numerous different studies demonstrating that T allele had a significantly higher rate of association with liver cirrhosis or HCC [36-38].

Due to the constant need for repeated blood transfusions, patients with thalassemia are at a higher risk for contracting HCV infection. A study conducted on thalassemic patients infected with HCV found that these patients had lower T/A genotype at locus +874 when compared to their healthy counterparts (77% vs. 80%, respectively). In addition, patients with A/A genotype had lower viral loads than those with T/A and T/T genotypes [39]. IFN-γ +874 A/A genotype was also associated with increased risk of occurrence of chronic hepatitis and cirrhosis while T/A genotype had lower risk. On the other hand, T/A allele showed no difference between these groups [41].

It is possible that IFN-γ +874 T/A polymorphism may affect the outcome of HCV infection based on several observations, namely that IFN-γ +874 T/T genotype is

associated with higher levels of IFN-γ production which facilitate host antiviral defense mechanisms, while A/A and T/A genotypes were accompanied by lower IFN-γ secretion with increased risk of hepatitis infection [32].

Study of SNP AT -183 G/T locus of IFN-γ gene showed no statistically significant difference between the study groups, with only G/T and T/T genotypes detected in both study groups and no G/G genotype. Previous studies had reported that chronic HCV patients all had G/T genotype at -183 G/T locus of IFN-γ gene [20, 24].

Haplotype analysis based on linkage disequilibrium between the studied SNPs is important for detection of predisposing genes in serious diseases, including chronic liver disease. Haplotype analysis in the present study reported that AAT and TGT were more susceptible to lack of SVC. A study conducted in Iran reported that polymorphism in +874 and +2109 loci of IFN-γ were under linkage disequilibrium with no significant association between different haplotypes and response to therapy [24]. However, another study found that haplotype A/G (A allele at +874 and G allele at +2109 loci) was associated with clearance of virus [20].

In the present study, the best model to study SNP +874 was over-dominant which revealed the association of heterozygote T/A with increased risk of not achieving SVC by 1.79 higher odds ratio than others. SNP +2109 recessive model was best showing that G/G genotype had higher risk of lack of SVC by 0.19. IFN-γ gene -183 G/T locus had no minor group and, hence, could not have a model of study. Previous studies on the association of IFN-γ and HCV infection are limited and reported results are conflicting.

The current study reported that patients with A/A and A/G at locus +2109, TLC ≤7.15 and AST ≤27.5 were considered independent predictors for development of SVC (sensitivity=67%, specificity=83%, PPV=79%, NPV=71.6%), with other parameters, including age and sex, showing no statistically significant difference. Similar findings that age and sex had no association with response to therapy have been reported [24]. An Iranian study recently amended its previous findings that age and sex could affect the therapy outcome in HCV infected patients [42,43], by stating that sex had no effect on outcome of HCV infection [20]. However, contradictory findings that women significantly clear virus more often than men in a cohort of the Chinese population have also been reported [44]. A limitation of the current study is the absence of a control group of healthy population.

Conclusion

Genotype A/A at locus +2109 of IFN-γ gene was more likely to achieve SVC while G/G genotype more prone to not to achieve SVC. Both haplotype AAT and TGT were more susceptible to chronic HCV infection, with heterozygote T/A at locus +874 and G/G genotype at locus +2109 having greater risk of HCV chronicity. A/G and A/A genotypes at locus +2109, TLC at cutoff value ≤7.15, and AST at cutoff value ≤27.5 were considered independent predictors for development of SVC.

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