Immunoglobulin GM and KM Allotypes Influence on the outcome of Hepatitis C Virus Treatment

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Abstract

Hepatitis C virus (HCV) is an endemic problem in Egypt with genotype 4 affecting about 90% of the infected patients. The treatment with interferon (IFN) and ribavirin (RBV) is costly and has various side effects, so there is an urgent need for predictors of IFN response. IL-28B genotype was confirmed as a predictor of response to IFN treatment, but there are needs for other predictors. Patients and methods: DNA samples from 100 patients with chronic hepatitis C were allotyped for alleles at two GM loci: GM3/GM17, GM23+/GM23- and the KM locus: KM1/KM3, using PCR amplification, restriction analysis and sequencing. Based on sustained virological response (SVR), two groups were studied; group I consisted of 50 responder patients to pegylated IFN + RBV therapy while group II comprised of 50 non-responder patients to standard HCV therapy . Aim of the study: To detect immunoglobulin GM and KM allotypes—genetic markers of γ and κ chains, respectively— in Egyptian patients with chronic HCV and to assess their influence on treatment outcome. Results: There was a significant association between the KM genotypes and the treatment outcome. KM3 homozygosity was associated with five fold increase in response to treatment compared to KM1 allele carriers. Conclusion: Our findings suggest that KM genotypes can be used as genetic markers for prediction of chronic HCV treatment outcome.

Keywords — *GM/KM allotypes, Chronic hepatitis C, Sustained virological response (SVR), Genotyping.*

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic hepatitis. It affects an estimated 170 million people worldwide. Only about 20% of those with acute infection will experience spontaneous viraemia resolution. Both viral and host genetic factors play important roles in the clearance of HCV. Among the host genetic factors, genes of the major histocompatibility complex (HLA) have been the primary focus of studies. Associations with several HLA alleles have been reported, but the results are conflicting [1]. Polymorphic determinants of interleukin-10 and interferon-γ have also been implicated in spontaneous recovery from HCV [2,3]. Allelic variations at these loci, however, do not account for the total inter individual variation in the outcome of HCV infection [4].

Results of recent genome-wide association studies (GWAS) demonstrated involvement of a single nucleotide polymorphism (SNP) very close to the IL28B gene on chromosome 19 in both spontaneous and treatment induced HCV clearance. Allelic variations near the IL28B locus account for only about half of the interracial variation [5], suggesting involvement of additional genetic factors in treatment outcomes [6]. GM and KM allotypes are associated with the susceptibility to and outcome of infection by several infectious agents. GM allotypes are strongly associated with IgG subclass concentrations, making them relevant to viral immunity, as the antibody responses to most viral epitopes appear to be IgG subclass (IgG1 and IgG3) restricted [7]. Numerous studies have identified particular GM genes as risk factors for many diseases [8], but most of these findings have not been confirmed by modern GWAS. One contributing factor might be the absence of GM gene probes in the genotyping platforms commonly used in GWAS [9].

KM3 could be a marker for enhanced HCV specific CD8+T-cell activity through significant linkage disequilibrium with a cis-acting enhancer element in the CD8 gene complex. Increased HCV specific CD8+ T cell responses are associated with SVR to peginterferon - ribavirin therapy (peg IFN+RBV) for chronic HCV infection [10]. In the present report; we have examined the contribution of particular GM and KM alleles to the outcome of chronic HCV treatment in Egyptian population.

MATERIALS AND METHODS

Patients

This study included 100 patients with chronic HCV, presenting at outpatient internal medicine clinics of Ain-Shams University Hospitals. A written informed consent was taken from all participants prior to enrolment in this study which was approved by the Ain Shams Medical Research Ethics Committee. Criteria for inclusion in the study were the following: serum anti-HCV and HCV RNA positivity for at least 6 months. None of the studied patient had obesity (BMI > 30), decompensated cirrhosis, autoimmune disorders, impaired renal function, concomitant HBV or HIV infection or cardiac insults. 6 months after treatment, a SVR was assessed (SVR was defined as an undetectable serum HCV RNA level at 24 weeks after stopping antiviral therapy) and accordingly the patients were divided into 2 groups: Group I included 50 patients with SVR. They received treatment in the form of interferon α2a or α2b and ribavirin. Group II included 50 patients though received the same treatment as group I; they had not responded to the treatment.

Methods

HCV RNA quantitation

2mL of venous blood was collected under aseptic conditions from every patient in plain vacutainer tube. HCV RNA was extracted from serum using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), then, the extract was added to Qiagen One Step RT-PCR Master

Mix and real-time RT-PCR was performed by Stratagene Mx3000P device (Corbett Research, Australia).

GM and KM genotyping

5mL of venous blood was collected under aseptic conditions from every patient in sterile EDTA vacutainer tube. Genomic DNA was isolated from human blood cells using QIAamp DNA Mini Kit (Qiagen).

For the determination of IgG1 allelic markers GM3 and GM17 (arginine-to-lysine substituation, a G→A transition in the CH1 region of the γ1 gene), oligonucleotides primers 5'-CCCCTGGCACCCTCCTCCAA-3' and 5'-GCCCTGGACTGGGGCTGCAT-3' (Bioneer, USA) were used as the primer set for amplification of a 364 bp

fragment from the CH1 encoding exon of the γ1 gene. The PCR conditions were an initial denaturation at 96 °C for 6 minutes and 35 cycles consisting of denaturation at 96 °C for 1 minute, annealing at 70 °C for 30 seconds, then extension at 72 °C for 1 minute [11]. Amplification product was analyzed by 2% agarose gel electrophoresis. Amplicons (364 bp) were purified with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Then, the purified PCR product was sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) using a Big Dye 3.1 terminator ready reaction kit (Applied Biosystems, USA) (Figure 1).
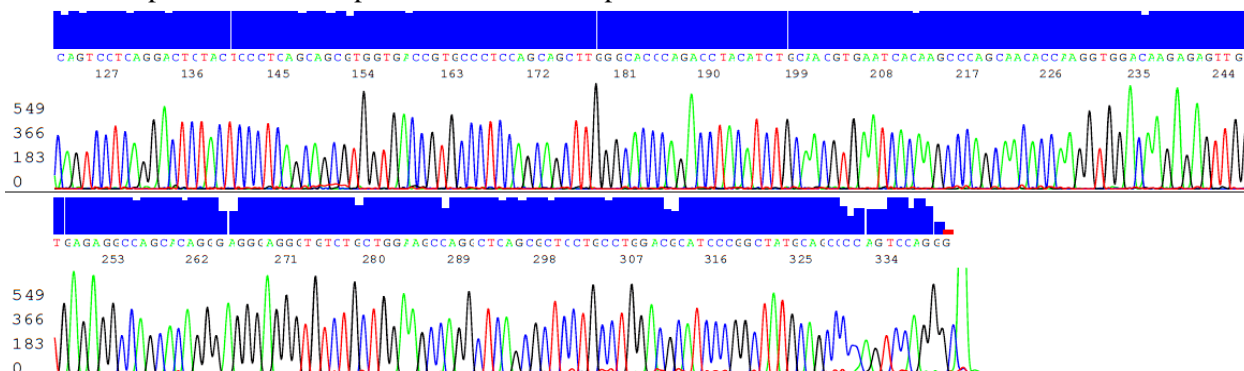

Figure (1): DNA sequencing results showing homozygous GM3 (a single G peak = a single black color peak is present at position 238)

GM23 (valine-to methionine substitution, a G→A transition in the CH2 region of the γ2 gene) was determined by the use of a nested polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. This was done as described by Brusco et al. [12]. The primers used were 5'-AAATGTTGTGTCGAGTGCCC-3' and 5'-GGC TTG CCG GCC GTG GCAC-3'. PCR conditions were an initial denaturation step at 94°C for 4 minutes and 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 61°C for 1 minute and extension at 72°C for 1 minute, then final extension at 72 °C for 10 min. A 197 bp segment was further amplified from this 915 bp fragment, by use of primers 5'-GCACCACCTGTGGCAGGACC-3' and 5'-TTGAACTGCTCCTCCCGTGG-3'.PCR conditions

were an initial denaturation step at 94°C for 4 minutes and 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds and extension at 72°C for 15 seconds, then final extension at 72 °C for 10 minutes. Restriction digestion of the amplified product (197 bp) was performed using 1 μl (10 units∕ μl) Hsp92 II restriction enzyme supplied by promega (Madison Wi, USA). Site of restriction: (5′…CATG↓… 3′) (3′↓GTAC…5′). The enzyme was added to 5 μl of PCR amplified product, 10 μl deionized water, 2 μl of buffer (10 x of 60mM tris-Hcl) and 2 μl of acetylated bovine serum albumin (BSA) (10μg/μl), followed by gentle mix. Then the tubes were placed on a heat block for 2 hours at 37°C. The enzyme was inactivated at 65°C for 15 minutes. Amplified product of DNA samples and restriction

fragments were run on 2% agarose. DNA molecular weight marker was also run to identify the site of bands (50 bp DNA ladder supplied by Fermentas). Digestion of the amplified product (197 bp) by the restriction enzyme Hsp92 II resulted in the following products corresponding to the following 3 genotypes according to Pandey et al [4]: GM23+, 90 bp, 63 bp, and 44 bp; GM23-, 134 bp and 63 bp; and GM23+/23-, 134 bp, 90 bp, 63 bp, and 44 bp (Figure 2).
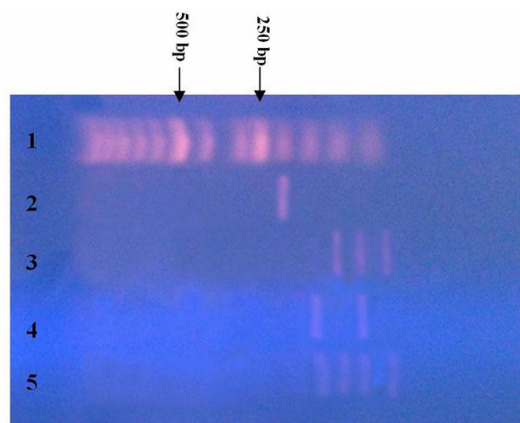
Figure (2): Electrophoresis pattern of PCR fragments after digestion with Hsp92 II for GM23$^+$,23$^-$polymorpism:

Lane (1) DNA ladder (50bp).

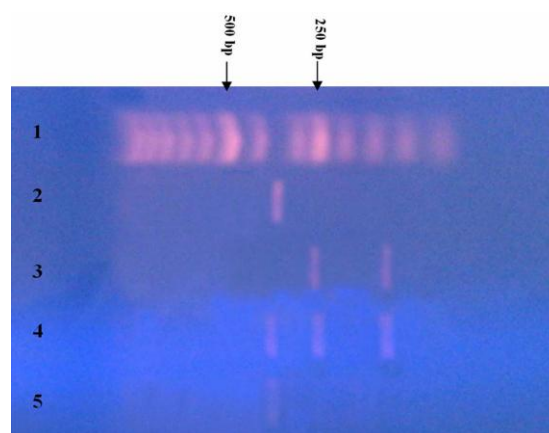Lane (2) PCR product (unrestricted fragment) →1 band (197 bp).

Lane (3) GM 23$^+$→ 3 bands (90bp, 63bp and 44bp).

Lane (4) GM 23$^-$ → 2 bands (134bp and 63bp).

Lane (5) GM 23$^+$, 23$^-$ → 4 bands (134bp, 90bp, 63bp and 44bp).

κ -Chain determinants KM1 and KM3 were characterized by PCR-RFLP technique using the method described by Moxley and Gibbs [13]. The primers used were 5'-ACT GTG GCT GCA CCA TCTGTCT-3' and 5'-TCA GGC TGG AAC TGA GGA GCAG-3'. PCR conditions were an initial denaturation step at 94°C for 3 minutes and 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, then final extension at 72 °C for 10 minutes. Restriction digestion of the amplified product (360 bp) was performed using 0.5 µl (10 units/µl) Acc I restriction

enzyme supplied by promega. Site of restriction:(5 ´GT↓AGAC 3´) (3´ CAGA↓TG 5´). The enzyme was added to 5 µl of PCR amplified product, 10.5 µl deionized water, 2 µl of buffer (10 x of 60mM tris-Hcl) and 2 µl of acetylated bovine serum albumin (BSA) (10µg/µl), followed by gentle mix. Then the tubes were placed on a heat block for 2 hours at 37°C. This resulted in the following products corresponding to the following 3 genotypes according to Pandey et al [4]: KM1/1, 360 bp; KM3/3, 247 bp and 113 bp; and KM1/3, 360 bp, 247 bp, and 113 bp (Figure 3)

Figure (3): Electrophoresis pattern of PCR fragments after digestion with AccI for KM1, 3 polymorpism:

Lane (1) DNA ladder (50bp).

Lane (2) PCR product (unrestricted fragment) → 1 band (360bp)

Lane (3) KM 3/3→ 2 bands (247bp and 113bp).

Lane (4) KM 1/3 → 3 bands (360bp, 247bp and 113bp).

Lane (5): KM 1/1 → 1 band (360 bp)

In all the previous amplification reactions, a 50 µl PCR reaction containing 25 µl Taq PCR Master Mix (Qiagen), 2.5 µl of each primer (25 pmol), 5 µl of extracted DNAand 15 µl RNase-free water, was amplified on the Stratagene Mx3000P Real-Time Thermocycler.

Statistical analysis:
Analysis of data was performed using the SPSS program, version 20. Quantitative variables were expressed as mean and SD (standard deviation) and the qualitative variables

were expressed by numbers and percentage. Data were compared using unpaired t-student test, Chi-square test, as appropriate. Univariate and multivariate logistic regression analyses were performed to assess the predictors of SVR, including potentially confounding variables such as: age and gender. A p value of less than 0.05 was considered significant.

RESULTS

Group I (responders) included 50 patients (42 males, 8 females) aged 43±8.2 (range 20–60years).Group II (non-responders) included 50 patients (40 males, 10 females) aged 43±9.3 (range 23–66years). Both groups are comparable as regards age and sex. The baseline demographic and laboratory data of the studied groups are reported in table 1.

Table (1): Baseline demographic and laboratory data for the studied groups.

Variable	Responders (group 1, n=50) (mean ± SD	Non-responders (group 2, n=50) (mean ± SD)	P-value
Age (years)	43±8.2	43±9.3	0.8
ALT(IU/L)	37.51±4.82	59.76±22.98	0.001*
Total bilirubin (mg/dl)	1.0± 0.7	0.8± 0.3	0.06
PT(sec)	13.5±0.8	13.3±1.5	0.2
Albumin(g/dl)	4.06± 0.48	4.25 ± 0.64	0.09
WBCs (10^9/L)	5.917±1.729	5.981±1.706	0.8
HB (g/dl)	13.46 ± 1.38	14.00± 1.86	0.1
Platelet count (10^9/L)	206.48 ±60.04	213.8 ± 65.04	0.6
AFP(IU/µl)	8.55 ± 8.2	10.82 ± 2.3	0.06
Quantitative PCR (HCV RNA, ×10^3IU/mL)	299±5.88	591±8.041	0.001*

Abbreviations: ALT - alanine aminotransferase. PT-prothrombin time. WBC - white blood cells. HB – heamoglobin. AFP - alphafetoprotein. PCR - polymerase chain reaction. HCV - hepatitis C virus.
*Significant.

There was a statistically significant difference between the two groups as regards KM genotypes and KM alleles (p value < 0.05*). However, there was no statistically significant difference between the two groups as regards GM genotypes and alleles (p > 0.05) (Table 2). Also the table shows there was no statistically significant difference between the two groups as regards the homozygosity and heterozygosity of different genotypes (p > 0.05), except for the KM genotype homozygosity which showed statistical significance with p value =0.03*.

Table (2): Comparison between the two groups as regards genotypes, homozygosity and alleles.

Genotypes	Responders (group 1, n=50)	Non-responders (group 2, n=50)	Global significance (genotypes)	Significance of homozygosity	Significance of allelic frequencies
KM1/1	4 (8%)	12 (24%)			
KM1/3	15(30%)	28 (56%)	0.01*	0.03*	0.02*
KM3/3	31(62%)	10 (20%)			
GM23 +/+	10(20%)	16 (32%)			
GM23 +/-	28(56%)	24 (48%)	0.1	0.8	0.1
GM23 -/-	12(24%)	10 (20%)			
GM 3/3	26(50%)	21(42%)			
GM 3/17	12(25%)	8 (16%)	0.4	0.5	0.2
GM 17/17	12(25%)	21(42%)			

*Significant

At univariate analysis, KM3 homozygosity [OR 6.5 (3.4-9.8), p < 0.01*], baseline HCV-RNA < 500.000 IU/ml [OR 2.25 (1.82-5.21), p = 0.01*], ALT <40 IU/ml [OR 2.29 (1.29-4.075), p = 0.03*] are related with a higher rate of SVR (Table 3). The multivariate analysis confirmed KM3 homozygosity [OR 5.2 (2.4-8.6), p < 0.01*], baseline HCV-RNA < 500.000 IU/ml [OR 1.9

(1.5-4.21), p = 0.02*], as factors significantly related to a good outcome to anti-HCV treatment (Table 4).

Table 3: Univariate analysis (with SVR as the dependent variable) and other items as independent variables) for predictors of SVR.

Variables	OR	95% CI	P value
Age<40years	0.7	0.45-1	0.11
Gender (male vs female)	0.6	0.4-0.9	0.34
KM3/3 vs.KM1/1 and KM1/3	6.5	3.4 -9.8	<0.01*
Pretreatment HCV RNA <500.000 IU/ml	2.25	1.82-5.21	0.01*
ALT< 40 IU/ml	2.29	1.29-4.075	0.03*

Abbreviations: ALT – alanine aminotransferase; HCV - hepatitis C virus; CI - confidence interval; OR – odds ratio
Significant.

Table 4. Multivariate Analysis vs. SVR.

Variables	OR	95% CI	P value
Age<40years	0.71	0.4-1.26	0.25
Gender (male vs female)	0.64	0.36-1.14	0.13
KM3/3 vs.KM1/1 and KM1/3	5.2	2.4-8.6	<0.01*
Pretreatment HCV RNA <500.000 IU/ml	1.9	1.5-4.21	0.02*
ALT< 40 IU/ml	0.469	0.19-1.14	0.096

Abbreviations: ALT – alanine aminotransferase; HCV - hepatitis C virus; CI - confidence interval; OR – odds ratio
Significant.

DISCUSSION

KM 1,3 polymorphism appeared to predict the rate of response to pegylated IFN + RBV combined therapy in patients with chronic HCV. The present study showed that the KM genotypes (global distribution) were associated with the treatment outcome. This was statistically significant with p value < 0.05*, as it was observed a sharp increase in KM3/3 genotype frequency among chronic HCV patients responding to pegylated IFN + RBV combined therapy vs. non responders (62% vs. 20%). This highlights the protective therapeutic role of KM3/3 in responding of chronic infection to pegylated IFN + RBV

combined therapy. Additionally, KM3 allele was significantly associated with the treatment outcome.

As mentioned earlier, SVR was influenced by KM3 homozygosity, ALT level and HCV viral load at baseline. After adjusting for viral load at baseline and ALT level, the association between KM3 homozygosity and SVR remained significant. It was found that KM3 homozygotes have 5.2 times the odds of being SVR as those who are KM1 carriers i.e the KM3 homozygosity is associated with five fold increase in treatment response, concluding that the KM3 zygosity is independent predictor for treatment response. This was in line with a study done by Pandey and Kristner-Griffen [6] where, non carriers of KM1 allele, i.e., KM3 homozygosity, was associated with higher SVR in African Americans (odds ratio =2.50, 95% confidence interval=1.12 – 5.60). On the other hand, our results were in disagreement with Pandey et al. [4] who found that none of the KM genotypes was associated with persistence or clearance of HCV in a group of American patients.

At least three mechanisms, which are not mutually exclusive, could explain our observations. The KM3 allele could be directly involved in humoral immunity to HCV. KM allotypes could influence the outcome of HCV infection by regulating the titer and affinity of neutralizing antibodies directed against viral epitopes. Perhaps the B-cell receptors with the KM3 specificities are more compatible with HCV epitopes and thus provoke a vigorous humoral immunity and clear the infection, whereas the non-carriers form a less compatible receptor for the critical epitopes of this agent and remain persistently infected [7]. In addition, it could indirectly contribute to the cellular immunity to HCV epitopes mediated by CD8+ T cells. Genes encoding CD8 glycoprotein's and KM allotypes are very closely linked, both located on the same band (p12) of human chromosome 2. In addition, both CD8 γ and β chains share significant homology with the κ light

chain. Thus, KM3 could be a marker for enhanced HCV-specific CD8+T-cell activity through significant linkage disequilibrium with a cis-acting enhancer element in the CD8 gene complex. Increased HCV-specific CD8+T cell responses are associated with SVR to pegylated IFN + RBV therapy for chronic HCV infection[9]. Molecular mimicry could provide another possible mechanism for the involvement of KM alleles in the treatment-induced clearance of HCV infection. The N-terminal region of HCV envelope protein E2 is antigenically and structurally similar to the conserved variable (V) regions of immunoglobulin κ chains [14].

A good match between E2 and the host V κ region sequences would render the host nonresponsive because of tolerance mechanisms, whereas a poor match will result in the clearance of the virus because of a vigorous immune response. KM alleles are known to be in linkage disequilibrium with particular Vκ region sequences [12], and if E2 is a molecular mimic of these sequences, it follows that anti-HCV responses are likely to be associated with these KM markers [6].

Regarding baseline viral load, other studies [15,16] defined high viral load as HCV-RNA > 800,000 IU/mL. Backus *et al*. identified 5944 hepatitis C patients who were treated at Veterans Affairs Health Care with PEG-INF/ribavirin and found that patients with low viremia (500,000 IU/mL) were more likely to respond than patients with a high viral load [17]. Similarly, our study confirmed low viraemia (500,000 IU/mL) as strong independent predictor of response to therapy.

In the present study, none of the GM allotypes were associated with persistence or clearance of HCV. This was in agreement with Pandey and Kristner-Griffen [6] who studied a group of African Americans. On the other hand, Pandey et al. [4] found that some GM allotypes were involved in the immunobiology of hepatitis C, in several different population groups. Ethnic variation is one potential explanation for the differences observed between the findings obtained here and those reported by some researchers. Pandey et al. [4] explained their findings that the Ig G molecule carrying some GM allotypes (as GM3) bind the Fcγ receptor (FcγR)-like HCV core protein much more strongly than others do. Presumably, the FcγR-like properties of the HCV core protein aid the virus in evading host immunosurveillance[18]. Under this hypothesis, anti-HCV IgG antibodies in GM3-carrying subjects are more likely to have their Fc domains scavenged, thereby reducing their immunological competence to eliminate the virus or circulating nucleocaspids via antibody-dependent cellular cytotoxicity and other Fc mediated effector mechanisms[3].

We recommend that KM allotypes can be used as a predictor to response before treatment, but further studies on large are needed to assess the efficacy of this prediction. This may save time and money and decrease the burden on the patients and the health care system.

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