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ORIGINAL ARTICLE

Association of IFNL3 and IFNL4 polymorphisms with hepatitis C virus infection in a population from southeastern Brazil

Ana Catharina de Seixas Santos Nastri[1,2] · Fernanda de Mello Malta[2,3] · Márcio Augusto Diniz[4] · Alessandra Yoshino[1] · Kiyoko Abe-Sandes[5] · Sidney Emanuel Batista dos Santos[6] · André de Castro Lyra[7] · Flair José Carrilho[2] · João Renato Rebello Pinho[2,3,8]

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Abstract Hepatitis C virus (HCV) infection is a major cause of chronic liver disease and associated complications such as liver cirrhosis and hepatocellular carcinoma (HCC). Viral and host factors are known to be predictors for antiviral therapy. Host factors that are predictors of sustained viral response (SVR) were discovered by genome-wide association studies (GWAS), including single-nucleotide polymorphisms (SNPs) in or near the interferon lambda gene (rs8099917, rs12979860 and rs368234815). The aim of the present study was to verify the genotype frequencies of SNPs rs8099917, rs12979860 and rs368234815 and to evaluate the association between SNPs and the outcome of HCV infection, taking into account the population ancestry. In this study, there was an association of the three polymorphisms with both clinical outcome and response to treatment with PEG-IFN and RBV. The polymorphisms rs12979860 and rs368234815 were associated with increased sensitivity (97.7 %, 95 % CI 87.2-100, and 93.3 %, 95 % CI 81.3-98.3; respectively) and with a greater predictive value of a positive response to treatment. In multivariable analysis adjusted by gender, age and ancestry, the haplotype G/T/ΔG was related to non-response to treatment (OR = 21.09, 95 % CI 5.33-83.51; $p < 0.001$) and to a higher chance of developing chronic infection (OR = 5.46, 95 % CI 2.06-14.46; $p = 0.001$) when compared to the haplotype T/C/TT. These findings may help to adjust our treatment policies for HCV infection based on greater certainty in studies with populations with such genetic characteristics, as well as allowing us to get to know the genetic profile of our population for these polymorphisms.

✉ Fernanda de Mello Malta
femalta@yahoo.com

1 Department of Infectious and Parasitic Diseases, School of Medicine, University of São Paulo, São Paulo, Brazil

2 Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, Brazil

3 Institute of Tropical Medicine, LIM 07, University of São Paulo, Av. Dr. Enéas Carvalho Aguiar, 500, 2nd floor IMT-II, São Paulo, SP, Brazil

4 Samuel Oschin Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

5 Laboratory of Immunology, Health Sciences Institutes (ICS), Federal University of Bahia (UFBA), Salvador, Bahia, Brazil

6 Department of Medicine, Federal University of Bahia (UFBA), Salvador, Bahia, Brazil

7 Human Genetics and Medical laboratory, Federal University of Pará (UFPA), Belém, Pará, Brazil

8 Hospital Israelita Albert Einstein, São Paulo, Brazil

Introduction

According to a recent study, approximately 115 million people worldwide are infected with hepatitis C virus (HCV). Among these individuals, it is estimated that about 80 million have detectable viremia [1]. There has been an increase in morbidity and mortality caused by chronic hepatitis C (CHC), with an increasing number of cases of cirrhosis and hepatocellular carcinoma and around 500,000 deaths per year due to the disease [2]. It is expected that this number will increase in most countries by 2030. In countries where this tendency has been reversed, such as France, this was achieved by a successful diagnostic policy and early treatment of infected individuals [3]. Until 2011 the standard treatment consisted of pegylated interferon and ribavirin (PEG-IFN + RBV).

Due to the cost and the large number of side effects of PEG-IFN+RBV, viral and host factors related to better response to treatment have been used to aid the optimization of public health policies. One of the factors that could affect the reduction of the future disease burden of HCV infection is the improvement in the rate and of treatment effectiveness. Recently, this has been achieved with the introduction of new treatment regimens using highly effective direct-acting antiviral agents (DAAs) [4–6].

Host factors that are predictors of sustained viral response (SVR) were discovered by genome-wide association studies (GWAS). In 2009, some GWAS showed that single-nucleotide polymorphisms (SNPs), rs12979860 genotype CC, and rs8099917 genotype TT in the interferon lambda 3 gene (*IFNL3*, previously named interleukin 28B gene), located on chromosome 19q13.13, are associated with SVR when patients with CHC are treated with PEG IFN + RBV [7–10].

In 2013, Prokunina-Olsson et al. discovered a novel functional dinucleotide polymorphism, rs368234815 (previously designated as ss469415590), located in exon 1 of the *IFNL4* gene, between *IFNL3* and *IFNL2*. In addition, they showed that the rs12979860 variant is located in intron 1 of the same gene [11]. The presence of the variant IFNL4-ΔG allows *IFNL4* transcription and the production of a new interferon, IFN-λ4, which is not detected in individuals who are IFNL4-TT homozygotes. The mechanism of action of IFNL4-ΔG has not been fully elucidated, but it is associated with the expression of intrahepatic interferon-stimulated genes (ISGs) [12]. The rs368234815-ΔG variant is in strong linkage disequilibrium (LD) with the unfavorable allele rs12979860-T. This LD value is very high in Asian ($r^2 = 1.0$) and European ($r^2 > 0.9$) individuals and consequently does not provide predictive values for response to treatment much better than the isolated analysis of SNP rs12979860. In people of African descent, this correlation is moderate ($r^2 \approx 0.7$), and the SNP rs368234815 is a better predictor of the response to treatment than is the SNP rs12979860 [11]. In a recent study, it was shown that rs368234815 is the primary region associated with impaired viral clearance in African American and European American populations [13].

Since the frequencies of the SNPs near the *IFNL3* and *IFNL4* genes are associated with ethnicity, it is important to know their frequency in the different regions of the world; this information will be relevant in establishing treatment guidelines for hepatitis C virus. The Brazilian population is an admixed population formed by ancestors of three different ethnic groups (European, African and Amerindian), thus the aim of this study was to determine the genotype frequencies of SNPs rs8099917, rs12979860 and rs368234815 and to evaluate the association between SNPs and the outcome of HCV infection, taking into account the population ancestry.

Material and methods

Patients

A total of 145 patients were enrolled in the present study. The patients were divided in three groups based on clinical outcome: spontaneous clearance (SC; n = 51), sustained virological response (SVR; n = 49) and non-responder (NR; n = 45). Patients in the SC group had two negative HCV RNA test results with a six-month interval, had positive ELISA and recombinant immunoblot assay (RIBA 3.0) results for anti-HCV, and had not undergone treatment for HCV. All CHC patients were infected with HCV genotype 1 and had been treated with PEG-IFN and RBV for 48 weeks. Patients in the SVR group had undetectable HCV RNA for 24 weeks after the end of treatment. NR patients were positive for HCV RNA at weeks 12 and 24 of treatment. Liver biopsies were performed prior to initiation of treatment. Fibrosis was staged according to the METAVIR score (F0-4). None of the patients were infected with hepatitis B virus (HBV) or human immunodeficiency virus (HIV), made abusive use of alcohol, or had autoimmune diseases.

In addition to the previously described groups, 157 individuals who were not infected with HCV, HBV or HIV were selected from among the general population. This group, called the control group, was used for frequency analysis of the studied polymorphisms. All patients were Brazilians with no reported Asian ancestry and were recruited from hepatitis units from Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and Centro de Referência e Treinamento–AIDS, São Paulo, SP, Brasil. All of the individuals signed a written informed consent to participate in the study, which was approved by the Ethics Committee of the University of Sao Paulo Medical School CAPPESQ (protocol 0462/11).

DNA extraction

Blood samples were collected from each patient for genetic analysis. Human genomic DNA was extracted from 140 μL of whole blood using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following the protocol provided by manufacturer.

SNP genotyping

All DNA samples were typed for three SNPs located near the IFNL3/IFNL4 genes (rs8099917, rs12979860, and

rs368234815). The genotypes of the SNPs were determined using TaqMan SNP genotyping assays (Applied Biosystems, Thermo Fisher Brand, Foster City, USA). The rs12979860 and rs368234815 assays were customized using sequences from the NCBI Entrez SNP Database, and the rs8099917 assay (C_11710096_10) is a predesigned TaqMan SNP genotyping assay from Applied Biosystems. The reactions were performed in a final volume of 13.3 μL containing 2 μL of DNA, 6.3 μL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 4.3 μL of water and 0.7 μl of 20x SNP Genotyping Assay mix (Applied Biosystems) containing primers and MGB TaqMan probes. Real-time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 seconds and at 60 °C for 1 minute.

Ancestry analysis

In populations with recent miscegenation, a populational stratification occurs in which the proportion of the genetic material of the ancestors of each subpopulation varies among individuals in the population. To assess the composition of these populations, the structured association method was used, utilizing a set of genetic markers to estimate the genetic ancestry of the individual and then testing this association with correction for individual admixture [14].

A panel of 48 ancestry-informative markers (AIMs) was used to distinguish continental populations, specifically Europeans, Africans (sub-Saharan) and Native Americans. The protocol used was the same as described previously by Santos et al., briefly described below [15]. The markers were genotyped using an automatic DNA sequencer (ABI PRISM1 3130 Genetic Analyzer, Applied Biosystems) and analyzed using the program GeneMapper1 ID v3.2 (Applied Biosystems). The identification of alleles was performed with reference to the ladder ABIGS LIZ-500 (Applied Biosystems) and a standard of known size. As a quality control, for each allele, a control sample of known size was analyzed simultatneously.

Statistical analysis

Quantitative variables were expressed using median and interquartile range (IQR), and qualitative variables were presented using frequency (percent). Frequencies of demographic characteristics were compared between groups using the χ^2 test and Fisher's exact test with the Holm correction [16]. Genotype frequencies for all investigated polymorphisms were tested for consistency with the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD, r^2 value). The analyses were also performed with the Holm correction [16]. SNPs were tested comparing the presence of two copies of the minor allele versus none or one copy (recessive model). A multivariable logistic model adjusted by age, gender, and ethnicity was applied for genotypes and haplotypes. For the haplotypes, the expectation-maximization (EM) algorithm was used, considering a minimum frequency of 1 % and the HWE assumption satisfied [17]. Statistical analysis was performed using the program R Foundation for Statistical Computing (version 3.1.2, R Development Core Team 2014, Vienna, Austria) and a P-value <0.05 was considered significant.

Results

Study population

The studied population was similar regarding age as a variable ($P = 0.298$), with a median of 45 (35-52.5), 48 (40-57) and 45 (35-52) in the SC, SVR and NR group, respectively. Regarding gender, there was also homogeneity between groups, with a majority of females in the SC, SVR and NR groups, 56.9 %, 59.2 % and 57.9 %, respectively ($P = 0.679$). Ancestry analysis showed that the contribution of European genetic markers was dominant in all three groups. However, the SC group showed lower frequency of these markers when compared to the SVR group ($P = 0.003$). Most of the population of the SVR and NR groups showed a Metavir profile with fibrosis grade 0 to 2, without statistical differences. However, a significant difference was observed in the Metavir profile with fibrosis grade 3 to 4 ($P = 0.037$) (Table 1).

The genotype frequency of the three SNPs (rs8099917, rs12979860 and rs368234815) was analyzed according to the clinical course and response to treatment (Fig. 1). The LD between rs12979860 and rs368234815 was strong in all groups (SC, $r^2 = 0.94$; SVR, $r^2 = 0.76$; NR, $r^2 = 0.77$; HC, $r^2 = 0.88$). The genotypes GT and TT (rs8099917), CT and TT (rs12979860), and TT/ΔG and ΔG/ΔG (rs368234815) were associated with a higher chance of chronic infection and non-response to treatment (Table 2). The genotypes CT and TT (rs12979860) were more frequently associated with chronic disease (OR = 47.7, 95 % CI 5.49-414.42, P <0.001) and with failure of therapeutic response (OR = 5.15, 95 % CI 2.25-11.8, P <0.001) (Table 2).

Seven different haplotypes were found (Fig. 1), five of which were observed at a frequency greater than 1 % (Table 3). The haplotype G/T/ΔG was the most frequent in the NR group (40 %), in spite of the fact that HWE was not observed for this group ($P = 0.03$). However, in the SC and SVR groups, the T/C/TT was the most frequent haplotype (78 % and 69 %, respectively). In multivariable analysis

Table 1 Demographic and clinical characteristics of the four study groups

	SC n = 51	SVR n = 49	NR n = 45	HC n = 157	*P*-value
Age, median (IQR)	45 (35-52.5)	48 (40-57)	45 (35-52)	39 (30-51)	0.006[#]
Gender, n (%)					
Female	29 (56.9 %)	29 (59.2 %)	26 (57.8 %)	80 (51 %)	0.679
Male	22 (43.1 %)	22 (48.8 %)	19 (42.2 %)	77 (49 %)	
Ancestry classification, median (IQR)					
European	0.64 (0.47-0.76)	0.75 (0.71-0.87)	0.76 (0.56-0.87)	0.74 (0.78-0.61)	0.003*
Amerindian	0.12 (0.04-0.17)	0.08 (0.05-0.14)	0.07 (0.04-0.12)	0.07 (0.04-0.16)	0.379
African	0.18 (0.08-0.37)	0.12 (0.04-0.18)	0.11 (0.05-0.3)	0.10 (0.04-0.22)	0.002*
HCV genotype	NA	1	1	NA	
Liver fibrosis (Metavir), n (%)					
F0-F2	NA	33 (67.3 %)	34 (75.6 %)	NA	0.037
F3-F4	NA	16 (32.7 %)	11 (24.4 %)	NA	

IQR, interquartile range; *SC vs SVR;[#] SVR vs HC; $P < 0.05$

SC, spontaneous clearance; SVR, sustained virological response; NR, non-response; HC, healthy control

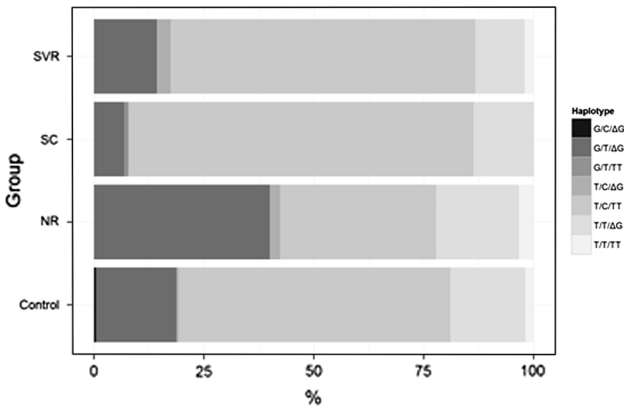

Fig. 1 Haplotype distribution of rs8099917 (T/G), rs12979860 (C/T) and rs368234815 (ΔG/TT). SVR, sustained virological response; SC, spontaneous clearance; NR, non-response; control, healthy patients

adjusted by gender, age and ancestry, the haplotype G/T/ΔG was related to non-response to treatment (OR = 21.09, 95 % CI 5.33-83.51; p <0.001) and to a higher chance of developing chronic infection (OR = 5.46, 95 % CI 2.06-14.46; p=0.001) when compared to haplotype T/C/TT (Table 4).

Discussion

Infection with hepatitis C virus is of great importance worldwide due to the morbidity and mortality it causes, as well as the expense in places on public health systems for monitoring and treatment. Rapid development in the therapeutic arsenal against this infection has been achieved in recent years, culminating in the introduction of direct-act-

Table 2 Statistical analysis of the frequency of each studied polymorphism in different outcome groups

Factor	CHC vs. SC		NR vs. SVR	
	OR (95 % CI)	*P*-value	OR (95 % CI)	*P*-value
rs8099917				
TT	1		1	
GT/GG	4.99 (1.99-12.54)	0.001	10.78 (3.63-32.06)	<0.001
rs12979860				
CC	1		1	
CT/TT	5.15 (2.25-11.8)	<0.001	47.7 (5.49-414.42)	<0.001
rs368234815				
TT/TT	1		1	
TT/ΔG or ΔG/ΔG	4.72 (2.07-10.77)	<0.001	14.84 (3.57-61.68)	<0.001

NR, non-response; SVR, sustained virological response; OR, odds ratio; CI, confidence interval (P<0.05); CHC, chronic hepatitis C; SC, spontaneous clearance

Table 3 Haplotype frequencies of the different polymorphisms in the different groups

Factor	rs8099917	rs12979860	rs368234815	Haplotype frequency
NR	G	T	ΔG	0.40
	T	C	ΔG	0.02
	T	C	T	0.35
	T	T	ΔG	0.19
	T	T	T	0.03
SVR	G	T	ΔG	0.14
	T	C	ΔG	0.03
	T	C	T	0.69
	T	T	ΔG	0.11
	T	T	T	0.02
SC	G	T	ΔG	0.07
	G	T	T	0.01
	T	C	T	0.78
	T	T	ΔG	0.14
HC	G	T	ΔG	0.18
	T	C	T	0.62
	T	T	ΔG	0.17
	T	T	T	0.02

NR, non-response; SVR, sustained virological response; SC, spontaneous clearance; HC, healthy control

ing agents (DAAs) [4, 6, 18]. These drugs have far better efficacy and safety when compared to the previously available treatment based on PEG-IFN and RBV [19, 20], but the cost of these new drugs is still very high for health systems in general. Thus, treatment using PEG-IFN and RBV will still be used for a while until the cost of the new drugs has decreased and their availability to the general population has increased. Knowledge of the predictors of response to treatment enables us to improve strategies to fight the infection. The present study evaluated a resident population in the city of São Paulo, Brazil, where the haplotypes rs8099917 (T/G), rs12979860 (C/T) and rs368234815 (ΔG/TT) are associated with clearance and response to treatment with PEG-IFN and RBV.

Brazil, due to its continental dimensions and its colonization history, has a very high level of ethnic diversity, and thus, the genetic profile of its population is extremely variable. In admixed populations, such as the Brazilian one, there are a few studies that point to SNPs as being the best predictors of clinical outcome of infection and response to treatment [21–23]. Studies describing allele and genotype frequencies in the population are scarce, particularly for the polymorphism rs368234815 (ΔG/TT). Furthermore, there are no studies correlating the haplotypes of the three mentioned SNPs with viral clearance and response to treatment.

In a meta-analysis by Olmedo et al in 2015, studies that included admixed populations were analyzed in order to establish whether individuals with the genotypic profile rs12979860-CC and rs8099917-TT infected with genotype 1 HCV have a higher chance of response to treatment with PEG-IFN and RBV [23]. This was confirmed, and this meta-analysis also included two studies examining the the Brazilian population. Ramos et al., in 2012, demonstrated the correlation between the two genotypes mentioned above with spontaneous viral clearance [22]. Cavalcante et al., in 2011, also analyzed an admixed population infected with genotypes 1, 2 and 3 [21]. This study analyzed ancestry markers showing a highly mixed population where the rs12979860-CC and rs8099917-TT genotypes were also correlated with SVR. Genomic markers that were found mainly in Africans were associated with poorer response to treatment [7, 8].

Due to the history of colonization of Brazil, greater miscegenation was expected in the present study, but we found a significantly higher influence of European markers in all subgroups when compared to African and Amerindian markers, with significant difference between the SVR and SC groups ($P = 0.011$, 95 % CI). The State of São Paulo received a large number of European immigrants in the last two centuries, concomitantly with a significant

Table 4 Multivariate analysis of the different haplotypes according to HCV infection outcome

Factor	rs8099917	rs13979860	rs368234815	OR (95 % CI)	*P*-value
CHC vs. SC	T	C	T	1	
	G	T	ΔG	5.46 (2.06-14.46)	0.001
	T	T	ΔG	1.88 (0.84-4.21)	0.129
NR vs. SVR	T	C	T	1	
	G	T	ΔG	21.09 (5.33-83.51)	<0.001
	T	C	ΔG	0.38 (0.05-3.19)	0.378
	T	T	ΔG	4.96 (1.1-22.45)	0.041
	T	T	T	4.15 (0.34-50.35)	0.267

CHC, chronic hepatitis C; SC, spontaneous clearance; NR, non-response; SVR, sustained virological response; OR, odds ratio; CI, confidence interval ($P < 0.05$), adjusted for sex, age and ethnicity

decrease in the number of indigenous peoples on its territory. In addition, since the mid-19th century, even before the abolition of slavery, there has been a significant decrease in the rate of entry of Africans in Brazil [24]. Most of this population came into Brazil through the State of Bahia, and this may be one explanation for the different results of this study and a study by Cavalcante et al., who found a stronger influence of African markers [21].

The studied subgroups were in HWE with each other, with the exception of the NR group. This can be interpreted as another fact supporting the association of this genetic marker with non-response to treatment in a population of recent miscegenation in historical terms [25]. The SC and SVR groups were in HWE with the control group, and this fact also supports it.

In this study, there was association of the three isolated polymorphisms (rs8099917, rs12979860 and rs368234815) with both clinical outcome and response to treatment with PEG-IFN and RBV, similar to the results obtained in previous studies. The polymorphisms rs12979860 and rs368234815 were associated with increased sensitivity (97.7 %, 95 % CI 87.2-100, and 93.3 %, 95 % CI 81.3-98.3, respectively) and greater predictive value of a positive response to treatment, in accordance with the results of studies with European populations. Since the LD between these SNPs in the different groups is high (r^2, 0.76 to 0.94), and the number of individuals is small in some groups, these facts could explain the slightly higher values obtained for the polymorphism rs12979860. In Asian populations, the polymorphism rs8099917 was associated with better predictive values [10]. For us, this polymorphism proved to be a better predictor of non-response when compared to the other polymorphisms (specificity = 73.3 %, 95 % CI 58.8-84.1), although the NR group was too small for a more definitive analysis.

The ΔG allele of rs368234815 encodes a functional IFN-λ4 that impairs HCV clearance by induction of high ISG expression, which inhibits viral replication and leads to an inefficient adaptive immune response [26, 27]. Additionally, a recent study has shown an association between the *IFNL4* TT/TT genotype and increased degranulation activity of lymphocytes [28], and an NS5A resistance-associated variant (Y93H) has been associated with a reduced response to treatment with NS5A inhibitors (daclatasvir/asunaprevir) [29]. Another study by O'Brien and Pfeiffer suggests that the genotypic profile could be useful for decreasing treatment time with DAAs in specific situations [30]. Therefore, even in the era of DAAs, IFNL4 genotyping still proves to be helpful.

Surprisingly, the results of our study strongly resemble those of studies with Caucasian populations. Consequently, in our population, it might perhaps be safe to follow treatment guidelines based on these genetic markers and those obtained from studies with populations of predominantly Caucasian origin. These findings might help to adjust our treatment policies for HCV infection based on greater certainty in studies with populations with such genetic characteristics, as well as allowing us to get to know the genetic profile of our population for these polymorphisms. They also confirm that, in our vast territory, there are populations with different genotypic characteristics that might, depending on the situation, require different approaches to treatment and investigation.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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