Polymorphisms in the IFNAR1 gene in patients with chronic hepatitis C: outcome of combined IFN-α therapy

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ABSTRACT. ***Aims*. Interferon-α (IFN-α) alone or in combination with ribavirin has been used for the last decade in the treatment of chronic hepatitis C, although the achievement of a sustained virological response (SVR) has not been very satisfactory. The treatment outcome depends on viral genotypes and host genetic polymorphisms in genes involved in the IFN-α signaling cascade. In this paper, we investigated the distribution of two variants of the IFNAR1 gene, G17470C and L168V, in two patient groups having received IFN-α alone or in combination with ribavirin.** ***Methods.*** **The analysis was performed using DNA sequencing of the relevant gene fragments.** ***Results and conclusions*. This study suggests that when combination therapy with high dose IFN-α and ribavirin is administered, HCV genotypes and age rather than the IFNAR1 polymorphisms are the predictors of a sustained response.**

Keywords: interferon-alpha receptor-1 (IFNAR1), hepatitis C virus (HCV), interferon-α, ribavirin, combined therapy, polymorphism

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, which can progress to fatal liver cirrhosis and hepatocellular carcinoma. Worldwide, an estimated 170 million people are persistently infected with the virus, indicating the ability for HCV to escape innate immune response mechanisms [1].

The first treatment to be approved was IFN-α, but a sustained virological response (SVR), defined as an undetectable HCV-RNA level at six months after discontinuation of therapy [2], was only achieved in 20-35% of patients with IFN-α monotherapy. Combination therapy with IFN-α and ribavirin is currently the standard regimen for the treatment of chronic hepatitis C virus (HCV) infection, but again only 40-50% of suitable candidates treated with this regimen are sustained virological responders (SVRs) [3]. Since IFN-α therapy is expensive and may cause serious adverse effects, it would be clinically useful to identify the factors predictive of the response to IFN therapy in patients with HCV infection [4, 5].

The response to IFN-α is influenced by both viral virulence factors and efficacy of the host's innate immune response against HCV [4]. Viral factors, such as a high viral load in the serum or viral genotype 1 are strongly associated with an unfavorable response to IFN [6]. Also, several host factors adversely influencing the response to IFN treatment have been reported. These include age, degree of fibrosis and duration of the disease [7].

Genetic factors attributed to each individual may play an important role in HCV infection and subsequent immune responses [8]. A number of studies have indicated the possibility of a positive association between HCV disease outcome and host genetic polymorphisms. For example, many immunorelevant proteins play a role in the response to anti-HCV treatment, in particular cytokines and chemokines. Single nucleotide polymorphisms in the interleukin-10 (IL-10) [9], cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [2], myxovirus resistance protein-1 (MxA) [10] and immunoproteasome subunit low-molecular-mass polypeptide (LMP) 7 genes [7] may influence the response to the IFN-α treatment in patients with chronic HCV, along with variants of the major histocompatibility complex (HLA) system [11].

IFN-α itself has no antiviral effect [5, 12]. Antiviral activity is mediated by its affinity to specific cellular receptors (IFNAR) and subsequent triggering of the signaling pathways that induce the antiviral state in the cell [4]. IFN stimulates gene factor-3 (ISGF-3) *via* the formation of transcription factor complex, and it activates transcription of the so-called type I IFN-regulated genes including those with antiviral effector activities such as myxovirus resistance protein-1 (MxA) and many others [13].

Expression of the type I IFN receptor in the liver is likely to be involved in the pathogenesis of viral hepatitis and the effectiveness of IFN therapy in patients with HCV infection. In fact, recent studies in chronic hepatitis C patients have demonstrated a significant correlation between hepatic expression of the type I IFN receptor and response to IFN therapy [12, 14].

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This observation is interesting considering the action of IFN in viral elimination: IFN mediates antiviral activity by binding to a specific receptor on the cell surface and subsequently induces signal transduction. Thus, the number of IFN receptors may be directly associated with the efficacy of treatment in patients with chronic hepatitis C [14].

Also, in peripheral blood mononuclear cells (PBMCs) isolated from patients responding to IFN treatment had higher basal levels of IFNAR1-mRNA than non-responders and controls ($p < 0.05$) [4]. However, whether upregulation of the type I IFN receptor results in enhanced antiviral activity by IFN therapy has not been confirmed. It has been shown that soluble human IFNAR1 and IFNAR2 inhibit IFN-α antiviral activity *in vitro*, and suppress the effectiveness of IFN-α therapy in patients with chronic hepatitis C [15-17]. Mutants that are truncated, and produce probably soluble forms that do not function as IFN receptors, may act as dominant-negative inhibitors, and may lead to interception of the IFN signaling.

In the present report, we investigated whether the two variants of the IFNAR1 gene that play a role in the resistance against cerebral malaria [18] can also influence the susceptibility and the treatment outcome in hepatitis C patients.

METHODS

Patients

From July 2003 to April 2005, 103 patients diagnosed with chronic hepatitis C and 195 healthy controls were enrolled in this study at the Hospital de Clinicas in Curitiba, Brazil. No patients had a history of alcohol or drug abuse or past treatment with interferon of any kind. Patients were included in the study if they were positive for HCV antibodies and serum HCV RNA, but negative for HBV and HIV antibodies. Biopsies were taken from all patients, liver histology was assessed blindly by two pathologists and staging of fibrosis was scored following the METAVIR method, in which classification of fibrosis is described as follows: F0 corresponding to no fibrosis, F1 to periportal fibrosis, F2 to portal-portal fibrosis with occasionally bridging, F3 to portal-central fibrosis with marked bridging and incomplete cirrhosis, and F4 corresponding to cirrhosis [19] *(table 1A)*.

Blood from all the participants was collected in EDTA tubes and plasma was separated, aliquoted and stored at -20°C. Genomic DNA was extracted from blood samples using QIAamp DNA blood kit (Qiagen, Hilden, Germany), as described in the user manual.

Viral RNA was extracted from plasma using the Amplicon® Hepatitis C virus-HCV-Test, version 2.0 (Roche, Branchburg, NY, USA) following the manufacturer's instructions.

Virus genotyping was done by Line Probe Assay (LIPA) using VERSANT™HCV Genotype Assay kit (Bayer, Tarrytown, NY, USA). HCV virus was classified into subtypes 1 and 3.

Study design

Of the 103 patients, only 93 patients were treated with combination therapy. These were divided in two groups receiving ribavirin therapy combined with either conventional IFN-α or pegylated IFN-α, respectively.

The first group consisted of 65 patients who received 3 million units (MU) of conventional IFN-α (BLAUFERON 2a or 2b, Blausiegel, Cotia, Sao Paulo, Brazil) administrated subcutaneously three times/week for 24 weeks in patients carrying HCV genotype 3, or for 48 weeks if the patients carried the HCV genotype 1. The treatment was stopped if, after 24 weeks, RNA-HCV was still detected in the serum of the HCV genotype 1 carriers. Twelve patients who did not achieve a SVR were then included in the second group, which received pegIFN-α: 180 µg/week (PEGASYS, Roche, Nutley, NJ, USA) or 1.5 µg/kg/week (PEG-INTRON, Schering-Plough, Kenilwort, NJ, USA) subcutaneously. The other 28 patients who had not received treatment before were also included in this group. The treatment schedule was the same as for conventional IFN-α.

Table 1

A) Clinical characteristic of all patients recruited for the study. **B**) Clinical and virological parameters associated with response to combination therapy with interferon-α and ribavirin in 86 patients with chronic hepatitis C virus (HCV) infection

A	**All patients n = 103 (100%)**		
Sex (male/female)	65 (63.1)/38 (36.9)		
Age, average ± SD, years	51 ± 9.4		
HCV (genotype 1/genotype 3)	40 (38.8)/51 (49.5)		
Not determined	12 (11.7)		
Fibrosis (F2/F3/F4)	21 (20.3) /37 (35.9)/45 (43.7)		
B	**Non-responders n = 47 (100%)**	**Patients with a SVR n = 39 (100%)**	**p value**
Sex (male/female)	29 (62)/18 (38)	23 (59) /16 (41)	0.83*
Age, average ± SD, years	52.3 ± 9.2	49 ± 8.4	0.05**
HCV (genotype 1/genotype 3)	26 (55)/20 (43)	6 (15)/25 (64)	< 0.01*, ***
Not determined	1 (2)	8 (21)	n.d.
Fibrosis (F2/F3/F4)	8 (17) /18 (38)/20 (43)	9 (23)/16 (41)/14 (36)	n.s.

Numbers of patients are given, with percentages in brackets.
SVR: sustained virological response; n.d.: not done; n.s.: not significant. p was calculated by chi2 (*) or Mann Whitney (**) test.
*** in a multivariate analysis, the only statistically significant predictor of therapy outcome was the HCV genotype 3, with a $p < 0.01$.

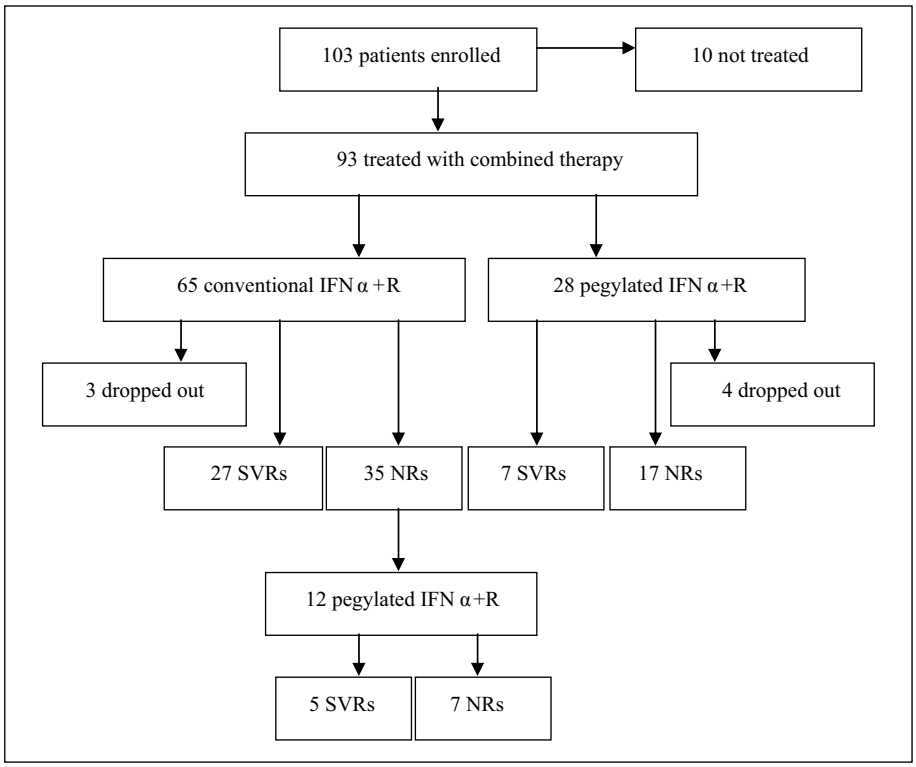

Figure 1

Flowchart of patient recruitment and outcomes of the study.
R: ribavirin; SVRs: sustained virological responders; NRs: non-responders.

In both groups, daily oral ribavirin 1000-1500 µg (VIRIZADOLE, Uci-farma, Sao Bernardo do Campo, Sao Paulo, Brazil) was administrated. RNA-HCV was measured again 24 weeks after treatment had ended.
Individuals were classified into two categories; the first were sustained responders who had no RNA-HCV detectable in serum, at least six months after cessation of the IFN therapy. The second category, who remained viremic after finishing medication or at six months after cessation of the IFN therapy, were termed non-responders (NRs) *(figure 1)*.

PCR and sequencing

Genomic DNA was used as templates for PCR. Two fragments of the IFNAR1 were amplified using the following primer sets:
L168V-Fw 5'AGCTTTCTATCCTATCTGTATG3'
L168V-Rv 5'TTCGCCTAATTTTTCTCT3'
17470-Fw 5'CTTTCCCTGTAGTAGTGGTTCT3'
17470-Rv 5'GTTGGGAGTTTGAGGTCAGC3'
All amplifications were initiated with a denaturing step of 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 30 sec annealing time (54.1°C and 59.3°C for L168V and for 17470 respectively) and 30 sec at 72°C for elongation. The reaction was stopped after a final elongation step at 72°C for 10 min. A PTC-200 Peltier Termal Cycler (Bio-Rad, Munich, Germany) was used to amplify all the samples. After purification, the respective PCR products were sequenced with the same primers described above using BigDye terminator chemistry on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, USA). The sequences were analyzed for polymorphisms in the G17470C and L168V markers in the IFNAR1 gene after DNA alignment of the sequenced products using the Bioedit alignment program (North Carolina State University, USA). The quality of the individual SNPs was assessed by visual inspection of the electrophoretograms.

Statistics

Allele frequencies and genotype distribution, of each SNP were compared using the χ^2 test or Fischer's exact test. Comparisons of the different factors affecting the outcome of therapy among patients were performed using the χ^2 test, Fisher's exact test, or the Mann-Whitney test. Age and response to therapy was compared using the Wilcoxon two sample tests. The Arlequin program (http://lgb.unige.ch/arlequin) was used to estimate frequencies of haplotypes. The software packages used for all of the statistical analyses were Statview (http://statview.com), JMP (http://jmp.com) and STATA (http://stata.com).

RESULTS

Clinical and virological factors

Of the total of 103 patients, 10 individuals received no treatment and seven individuals were not able to complete treatment. Comparison of the clinical and virological characteristics between the sustained responders and non-responders are summarized in *table 1B*.
Thirty nine (45.4%) of the 86 individuals achieved sustained virological response after receiving combination therapy with IFN-α and ribavirin, while 47 (54.6%) did not respond to the treatment.
Possible clinical and associated virological factors for the sustained response to IFN therapy include four variables:

Table 2
Genotype distribution and allele frequency for the two IFNAR1 variants in hepatitis C patients and the healthy control group

G17470C	Genotype			Allele frequency	
	n (%)			n (frequency)	
	GG	GC	CC	G	C
Patients (n = 103)	34 (33)	60 (58)	9 (9)	128 (0.62)	78 (0.38)
Controls (n = 195)	59 (30)	116 (60)	20 (10)	234 (0.60)	156 (0.40)
L168V	**Genotype**			**Allele frequency**	
	n (%)			n (frequency)	
	GG	GC	CC	G	C
Patients (n = 103)	56 (54)	47 (46)	-	159 (0.77)	47 (0.23)
Controls (n = 195)	112 (57)	79 (41)	4 (2)	303 (0.78)	87 (0.22)

Numbers (n), percentages (%) or frequencies are given as indicated above the columns.

age, gender, HCV genotype and staging of fibrosis. In a univariate analysis, responders and non-responders did not differ significantly with respect to gender and stage of fibrosis. However, HCV genotype 3 ($p < 0.01$) and lower age ($p = 0.05$) were associated with a greater chance of achieving a sustained response to the combined therapy. The relative risk of mounting a positive response to the combined therapy was 2.3 [95% confidence interval (CI) 1.36-3.96] when carrying the HCV genotype 3.
When a multivariate analysis was carried out, the only factor associated with the achievement of a sustained response to the treatment was the presence of the HCV genotype 3 ($p < 0.01$), increasing the relative risk of achieving a positive response to 5.8 [95% CI: 1.92-17.29]. The degree of fibrosis was also found to be significantly associated with the age of the patient ($p = 0.05$), in accordance with other studies [20, 21].

Allelic frequencies, genotype and haplotype distributions

For the first analysis, patients and healthy controls were compared in terms of genotype distribution and allele frequency, indicating a possible difference in the distribution of genotypes in HCV patients, regardless of success of treatment. In the patient group, 34 individuals (33%) were homozygous G/G at the position 17470 of the IFNAR1 gene, 60 (58%) were heterozygous and 9 (9%) were found to be homozygous C/C.

For the SNP L168V, there were 56 (54%) individuals carrying the G/G genotype (wild type), having a V at amino acid position 168; 47 (46%) out of 103 patients were found to carry one mutated codon 168, leading to L. We did not find any individual carrying the mutated codon on both chromosomes. The genotype distribution in the control group was similar to the patients group: no significant differences in the distribution of the variants G17470C or L168V were found between the two groups *(table 2)*.
For the second analysis, the group of patients was split in two subgroups: responders and non-responders *(table 3)*. Both SNPs appeared in the non-responding and responding patients at similar frequencies, which did not result in statistically significant differences.

All four possible haplotypes were found in the two populations and the distribution between the hepatitis C patients and the control group was not statistically different. Additionally, no differences were found between responder and non-responder individuals in terms of haplotype distribution.

Table 3
IFNAR1 variants associated with response to combination therapy with interferon-α and ribavirin in 86 patients with chronic hepatitis C virus (HCV) infection

	Non-responders n = 47 (100%)	Patients with a SVR n = 39 (100%)
IFNAR1 17470 genotype		
GG	17 (36)	13 (33)
GC	28 (60)	23 (59)
CC	2 (4)	3 (8)
IFNAR1 L168V		
GG	27 (58)	22 (56)
GC	20 (42)	17 (44)
IFNAR1 17470.C allele		
Carriers	30 (64)	26 (67)
Non-carriers	17 (36)	13 (33)

Numbers (n) of patients are given, with percentages in brackets. SVR: sustained virological response. The distribution of genotypes between the groups is not significant.

Association of IFNAR1 variants and clinical parameters

To determine the association of defined genotypes or alleles with several clinical and virological parameters, we compared the frequency of IFNAR1 variants in the patient group, according to HCV genotypes and degree of fibrosis. Factors such as HCV genotype or degree of fibrosis were found not to be affected by the SNPs in the IFNAR1 gene. Only a tendency to develop a greater degree of fibrosis was found to be of borderline significance ($p = 0.06$) in individuals heterozygous for the mutation G17470C in the group of hepatitis C patients.

DISCUSSION

Type I IFN receptors are present in most cells and consist of at least two subunits encoded by the IFNAR1 and IFNAR2 genes, both of which are directly involved in signal transduction [12].

Molecules directly involved in ligand receptor binding have not been previously investigated in detail, although IFNAR1 expression in the liver plays an important role in the response to treatment of hepatitis C [5, 14]. The mRNA content of this receptor can be used as predictor of the outcome of IFN therapy [22, 23]. Whether upregulation of the type I IFN receptor results in an enhanced antiviral activity by IFN therapy has not yet been confirmed [12].

This is the second study investigating associations between IFNAR1 polymorphisms and outcome of combined IFN-α therapy against hepatitis C. The first study showed an association of a microsatellite in the promoter of the IFNAR1gene with responsiveness to hepatitis C treatment [24].

Since IFN effectiveness may be determined by the host's genetic background for genes that are involved in IFN-α signaling or effector functions [13], we carried out this study to assess the role of the two variants of the IFNAR1 in the outcome of IFN therapy in chronic hepatitis C patients. We hypothesized that mutations in the IFNAR1 can lead to an impairment of the cellular immune response contributing to the persistence of hepatitis C virus due to a change of affinity with the IFN-α molecule.

The first polymorphisms (G17470C) is situated in intron 3, the second (L168V) is located in exon 4, which codes for the first D200 domain of the extracellular part of the receptor [25] and causes an exchange from Leu to Val. Whether these alterations have functional effects is not yet known. Although the physical properties of these two hydrophobic, non-polar amino acids are very similar, these substitutions could change the solubility of the protein or produce other effects affecting the activity of the proteins as in oestradiol hydrolase [26] or in the enzyme ribulose-biphosphate carboxylase [27].

Polymorphisms in the IFNAR1 gene at the same positions have been found to have a role in reducing the risk of cerebral malaria [18]. In the present study, the group of controls and patients show homogeneity in terms of genotype distributions and allele frequencies. The comparison of the genotypic distribution, allele and haplotype frequencies between responders and non-responders to IFN-α treatment do not show any association between IFNAR1 gene polymorphisms and the outcome of combined therapy. Therefore, the genetic changes caused by G17470C and L168V probably do not interfere with binding or uptake of recombinant IFN-α in these patients. Our results are consistent with the fact that genomic variations in the L168V marker were also not relevant to IFN-β therapy responsiveness in multiple sclerosis (MS), although recessive homozygosity was associated with a high risk of MS [28]. This may suggest that the SNPs investigated here do not produce conformational or functional changes in the IFNAR1 chains, which destroy its affinity with IFN-α completely. We suggest that in the response to recombinant or naturally-produced IFN-α, other molecules involved in IFN-α signal transduction and effector functions, might play a decisive role, especially those whose expression is determined genetically. Thus, other genes, acting alone or in concert with other genetic determinants and environmental factors, need to be identified to elucidate the differences in the host response to the virus [29]. In conclusion, the results of this study suggest that after combination therapy with high doses of IFN-α and ribavirin, HCV genotypes and age but not inheritance of the IFNAR1 polymorphisms G17470C and L168V, are predictors of a sustained response.

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