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Title**: IFNAR1 and IFNAR2 polymorphisms confer susceptibility to multiple sclerosis but not to interferon-beta treatment response**.

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Abstract

We investigated the role of three polymorphisms in the IFNAR1 (SNPs 18417 and -408) and IFNAR2 (SNP 11876) genes in multiple sclerosis (MS) susceptibility and in the IFNβ treatment response in a group of 147 patients and 210 controls undergoing interferon therapy during the last 2 years. Only the 18417 and the 11876 SNPs showed an association with disease susceptibility ($p = 0.001$ and 0.035, respectively) although no differential genotype distribution were observed between interferon responders and non-responder MS patients. No alteration of the expression level of IFNAR-1 was observed with respect to the -408 genotypes or to interferon treatment response. These data suggest a role for the IFNAR pathway in susceptibility to MS.

Keywords: Type I interferon receptor; Polymorphism; Multiple sclerosis; Association; Treatment

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory–demyelinating disease characterized by multifocal damage of the central nervous system (CNS) and caused presumably by an autoimmune process that induces demyelination, destruction of oligodendrocytes, and axonal injury (Trapp et al., 1998).

IFNAR1 and *IFNAR2* encode two subunits of the heterodimeric type I interferon receptor (IFNAR) and are important because interferon-beta (IFNβ) is known to exert its biological activities by the interaction with these subunits. In MS controlled clinical trials, IFNβ therapy has been shown to decrease clinical relapses, reduce brain MRI activity and possibly slow progression of disability (McCormack and Scott, 2004). However, a long-term shift in the natural history of the disease has not been demonstrated (IFNβ Multiple Sclerosis Study Group, 1993; PRISMS study group, 1998; European Study Group on Interferon h-1b in secondary progressive MS, 1998). A significant number of patients are refractory to interferon therapy. MS lymphocytes tend not to be responsive to type I IFN actions and, in some circumstances, circulating IFN may not transduce a signal via IFNAR. Abnormalities ranging from the transmembrane receptor genes *IFNAR1* and *IFNAR2*, interactions between both chains of the receptor, cytoplasmic proteins associated with this heterodimer, or the ultimate complex that interacts with IFN sensitive response elements in the nucleus, may be responsible for IFN resistance (Brod, 1998). The mechanisms by which IFNβ exerts its disease-modifying effect are not completely understood, but among other activities, IFNβ is known to reduce T cell activation, inhibit IFNγ effects and blood–brain barrier leakage, and induces an immune deviation,

either by inhibiting Th-1 or by promoting Th-2 cytokine production (Dhib-Jalbut, 2002). The biological responses of IFNβ are initiated by the interaction of this cytokine with its cell surface heterodimer receptor (IFNAR), shared by all type 1 interferons which include about 15 cytokines (13 isotypes of IFNα, one IFNβ, one IFNω), (Novick et al., 1994). This binding brings together two receptor chains, IFNAR1 and IFNAR2 (Croze et al., 1996), whose interaction induces a cascade of signalling pathways resulting in the secretion or production of a number of proteins called IFN-stimulated gene products with antiviral, antiproliferative and immunomodulatory activities (Dhib-Jalbut, 2002; Yang et al., 2000). These subunits are encoded by two different genes located on chromosome 21q (Lutfalla et al., 1992), where several polymorphisms have been described (McInnis et al., 1991; Muldoon et al., 2001; Sriram et al., 2003). Two of these polymorphisms induce amino acid substitutions in the mature proteins (*IFNAR1* 18417 [G⇨C] V168L and *IFNAR2* 11876 [T⇨G] F10V) and the third is located at the IFNAR1 promoter region. The object of this study was to assess the role of these cytokine receptor subunits *IFNAR1* and *IFNAR2* polymorphisms in the genetic control of MS and address the pharmacogenomic impact on IFNβ treated patients.

2. Materials and methods

2.1. Study subjects

The study included 147 patients with clinically defined MS according to Poser's criteria (Poser et al., 1983) (100 relapsing–remitting and 47 secondary progressive), on IFNβ therapy: 42 received IFNβ1b subcutaneously (Betaferon®, Schering) at 250 μg/48 h, 47 IFNβ1a intramuscularly (Avonex®, Biogen) at 30 μg/week, and 58 IFNβ1a subcutaneously three times weekly (Rebif®, Serono), 53 with 22 μg and 5 with 44 μg. Criteria to classify patients as non-responders to IFNβ were: an increment of one or more relapses with respect to the previous year or an increase in EDSS score of 0.5 points or more after the first year of treatment. Demographic characteristics of MS patients and controls are shown in Table 1. A control group of 210 healthy subjects with similar genetic background was included in the study. Studies were performed after obtaining written informed consent from all participants under protocols approved by the Institutional Review Board of the Hospital Carlos Haya and Blood bank of Málaga, Spain. All the patients and controls were Caucasians from Malaga and Granada, Spain.

2.2. PCR amplification and restriction fragment length polymorphisms (RFLP)

The DNA was extracted by standard procedures (Ausubel et al., 1990). PCR reactions were carried out in a final volume of 25 μl, in a mixture of 5 pM of each primer, 50 ng of genomic DNA, 250 μM dNTPs and 1 U *Taq* DNA polymerase in the 10 X KCl buffer provided by the manufacturer (Roche). For the *IFNAR1* SNP 18417 determination, each DNA sample was PCR amplified with forward primer IP18 (AGAAGTACATTTAGAAGCTG) and reverse primer

IP19 (CAATCCTTTCCTATAACACAA), yielding a band of 261 bp. The digestion of PCR amplified products with *Dde*I restriction enzyme (Roche) produced two fragments of 155 and 106 bp from the SNP 18417-G allele product.

The *IFNAR2* SNP 11876 was amplified with forward primer IP20 (5´-TCACCTAATGTTGATTTCAG-3´) and reverse primer IP21 (5´-ATCACAGCTTGCTTCTATAA-3), yielding a band of 158 bp, whose digestion with *Mbo*II (Fermentas) produced two fragments of 110 and 48 bp for the SNP 11876-G allele product and three fragments of 83, 48 and 27 bp from the SNP 11876-T allele product.

Polymorphism of the -408 C/T SNP involved amplification of a 328 bp fragment with primers forward IP22 (5'-TCTCGCCCCTCAGCCAAGTC-3') and reverse IP23 (5'-CCTTGACCTTCACAGGATCG-3'). The digestion of PCR amplificates with MvaI produced two fragments of 204 and 124 bp for the T allele, and three fragments of 124, 106 and 98 bp for C allele. The fragments from each digestion were separated on 12% polyacrilamide gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light.

2.3. Expression of IFNAR1 mRNA

Briefly, total RNA was isolated from peripheral blood mononuclear cells (PBMC) using a classical method (Chomczynsky and Sacchi, 1987) and was reverse-transcribed to cDNA with MMLV reverse transcriptase. *IFNAR1* and a low number copy housekeeping gene porphobilinogen deaminase (*PBGD*) (Chretien et al., 1988) mRNA expression was measured by real time QT-PCR in a LightCycler.

The following primers (Proligo, France), designed using OLIGO6.0 software (Medprobe, Sweden), were used in the reactions: *IFNAR1* forward 5'-AGAAGTACATTTAGAAGCTG-3' and *IFNAR1* reverse 5'-AGTGCTGCTTTAACTTT-3', *PBGD* forward 5'-TCCAAGCGGAGCCATGTCTG-3' and *PBGD* reverse 5'-AGAATCTTGTCCCCTGTGGTGGA-3'. Reaction mixtures contained 1X LightCycler-Fast Start DNA Master SYBR green I (Roche, Spain), 3 mM $MgCl_2$, 0.2 mM forward and reverse primer and 20 ng of cDNA. PCR reactions were set up in a total volume of 20 μl,in duplicate, and each run included its standard curve. Data evaluation was performed using the LightCycler data analysis software (version 3.5).

IFNAR1–mRNA expression in PBMC was assessed as the *IFNAR1*–mRNA/*PBGD*–mRNA ratio. *PBGD* was used to verify comparability of RNA loading between samples and to normalise PCR products.

2.4. Statistical analysis

Comparisons of genotype frequencies between healthy controls and MS patients were performed by Pearson χ^2 test for the independent variables, using the SPSS 11.5 statistical package. Comparisons between genotypes for SNP 18417, SNP 11876 and SNP-408 polymorphisms were performed by Pearson χ^2 test for the categorical variables of gender, clinical form and response to IFNβ treatment, and by Student-t, Mann–Whitney or Kruskal–Wallis tests for the quantitative variables of age at onset of MS, disease duration, EDSS score at entry and *IFNAR1* mRNA expression. We performed haplotype frequency estimation, taking into account *IFNAR1* 18417 and *IFNAR2* 11876 SNPs, and tested for differences between case and controls and responders

and non-responders to IFNβ treatment using the FAMHAP software (Becker and Knapp, 2004).

3. Results

3.1. Influence of the polymorphisms in IFNAR1 and IFNAR2 genes on the response to IFNb in MS patients

We analysed three recently described IFNAR polymorphisms by RFLP as is shown in Fig. 1. One located within the promoter region of *IFNAR1* gene, at -408 bp relative to the transcription start site and another two substitutive polymorphisms, one located in the fourth exon, the *IFNAR1*–V168L and the other in the second exon, the *IFNAR*2–F10V. The patients were classified as responders and non-responders to IFNβ according to the criteria indicated in Materials and methods section which is different from those previously published (Waubant et al., 2003; Villoslada et al., 2004). Thus, 104 patients (70.7%) responded to INFβ and 43 (29.3%) did not. Interestingly, non-responders patients had a longer disease evolution and a higher EDSS score at baseline than responders. Genotype and allelic distribution of the IFNAR1 and IFNAR2 polymorphisms between responders and non-responder to IFNβ are shown in Table 2. No significant associations were observed with any of the genotypes or alleles studied for the IFNAR1 SNP 18417, SNP-408 of and SNP 11876 in exon 2 of IFNAR2, based on the response to IFNβ.

3.2. IFNAR1 and IFNAR2 SNPs association with MS susceptibility

No significant association of IFNAR1-408 genotype distribution, allele and carrier frequencies between MS cases and controls were observed. The most common genotype for this polymorphism was the homozygous (C/C) (55.5% of total subjects) followed by the heterozygous (C /T) (32.8% of total

subjects) as reflected in Table 3, in agreement with previous findings (Muldoon et al., 2001).

Significant differences on *IFNAR1* 18417 genotype and allele frequencies were found by χ^2 analysis in MS cases and controls (p =0.001 and p <0.001, respectively). A significant increase in the C allele carrier frequency in MS patients (OR=1.87, p =0.008) was also observed. Viewed as a positive risk factor, the *IFNAR1* 18417 C/C genotype was associated with a fivefold increased risk of MS in subjects from the south of Spain (χ^2_{df1}=9.81, p =0.002; OR=5.008, 95% CI 1.79–13.99) compared with carriage of the *IFNAR1* 18417G allele (Table 3).

The most common genotype for *IFNAR2* 11876 was the homozygous (T/T) (47.05% of total subjects), followed by the heterozygous (G/T) (43.41% of total subjects). Genotype frequencies at *IFNAR2* 11876 differed between MS patients and controls (p =0.035). Carriage of the SNP 11876 T allele was inversely associated with MS (OR=0.396, p =0.017). Viewed as a positive risk factor, the *IFNAR2* 11876 G/G genotype was associated with a twofold increased risk of MS in Spanish subjects (χ^2_{df1}=5.67, p =0.017; OR=2.53, 95% CI 1.22–5.22,) compared with carriage of the *IFNAR2* 11876T allele (Table 3). No significant associations in the distribution of neither of these genotypes in relation with the sex ratio, clinical form, age at onset of disease, disease duration, and disease progression were observed (data not shown).

The results of the Hardy–Weinberg Equilibrium (HWE) calculations for MS patients and controls population revealed that all polymorphisms were in

HWE with the exception of the polymorphisms -408. This polymorphism showed a heterozygote deficit only in the MS population, with a χ^2 value of 10.26.

3.3. Haplotype analysis and interaction between IFNAR1 18417 and IFNAR2 11876 alleles

Potential genetic interactions between the two subunits of the heterodimer receptor were assessed by performing an analysis that included the *IFNAR1* 18417 and *IFNAR2* 11876 SNPs. The frequency of these genotype combinations is shown in Table 4. Significant differences in the distribution of the nine genotype combinations were found between patients and controls by the χ^2 test.

Haplotypes were analysed using the *IFNAR1* 18417 and *IFNAR2* 11876 data to identify those associations with susceptibility to MS. As shown in Table 5, the haplotype *IFNAR1* 18417G/*IFNAR2* 11876T was found to be less frequent in MS patients (0.48) than in controls (0.63). The simulation-based test performed by FAMHAP software for the haplotypes resulted in *p* value of 0.0002 for MS versus control population. However, haplotype frequencies were not significantly different between responders and nonresponders to the IFNβ therapy (data not shown).

3.4. Expression levels of IFNAR1 in PBMCs from MS patients and relation with the IFNAR1 expression pattern to genotypes

Though *IFNAR1*-408 SNP did not map to any recognised transcription factor binding sites in the promoter (Muldoon et al., 2001), we tested the possible impact of this polymorphic site on the regulation of *IFNAR1* transcription levels in 113 MS patients (34 C/T, 66 C/C and 13 T/ T). As even

modest doses of systemic IFNβ are known to induce a significant decrease of IFNAR1 cell surface expression (Dupont et al., 2002), blood was drawn immediately before the administration of IFNβ to ensure a time lapse of 48 h following the last dose and minimize variations in *IFNAR1* mRNA expression due to IFNβ treatment. There was a great individual heterogeneity in *IFNAR1* mRNA expression. In the patients investigated here, we detected no significant differences in the expression at the RNA level among the different genotypes studied for *IFNAR1*-408 when analyzed by the Kruskal–Wallis test (χ^2_{df2}=0.530 and p =0.767) (Fig.2). Also no significant difference was observed at *IFNAR1* transcriptional level between MS patients who responded to IFNβ treatment and those who did not (Fig. 3), when assessed by the Mann–Whitney test (p=0.362).

4. Discussion

This study was aimed to determine the role of *IFNAR1* and *IFNAR2* genetic heterogeneity in the susceptibility to MS, their correlation with the positive or negative response to IFNβ immunotherapy and the effect on the *IFNAR1* expression. We have found that genomic variations in *IFNAR1* and *IFNAR2* genes were not relevant to the IFNβ therapy responsiveness in accordance with a previous report (Sriram et al., 2003). To improve fidelity of predicting therapeutic response to IFNβ it would be useful to assess additional genes of proteins involved in the IFNβ signalling cascade. On the other hand, this is the first study to report an association between allelic variation at *IFNAR1* 18417 and *IFNAR2* 11876 and MS. The *IFNAR1* 18417 C/C genotype was associated with a fivefold higher risk of MS than carriage of the G allele, and the

IFNAR2 11876 G/G genotype was associated with more than a twofold risk of this disease than carriage of the T allele. Interestingly, in cerebral malaria, a complication of Plasmodium falciparum infection characterized by a reversible encephalopathy, a similar association of the *IFNAR1* 18417 G/G genotype to the reduction risk of suffering this complication, compared with individuals sharing at least one C allele, has been described (Aucan et al., 2003; Maneerat et al., 1999).

Several cytokines associated with cerebral malaria have also been associated with the MS. Irrespective of the initiating stimuli, both pathologies seem to share some characteristics of inflammation at the brain that could explain the confluence of susceptibility genes in both diseases, that not only affect *IFNAR* genes but *IL10*, *IFNG* and *TNFA* genes (He et al., 1998; de Jong et al., 2002; Martinez et al., 2004; Takahashi et al., 2003).

The *IFNAR* receptors are localised on a cluster of immune response genes on chromosome 21q22.11 containing *IFNAR1*, *IFNAR2*, *IL10RB* and *IFRGR2*. Therefore, the association of the IFNAR polymorphisms to MS could be due to functional effects on the molecule or linkage to polymorphisms of other members of the cluster. The functional significance of these associations is unclear.

However, in the *IFNAR1* chain, the variation of Val to Leu at position 168 of the peptide sequence, increasing hydrophobicity of the amino acid residue, could alter the binding affinity of the molecule for the ligand. This polymorphic amino acid position is located at the extra-cellular region at the subdomain 2 (SD2) which together with SD3 seems to constitute the core of the ligand-

binding determinants of *IFNAR1* (Kumaran et al., 2000; Cutrone and Langer, 2001).

A C/T polymorphism at position -408 in the promoter region of the *IFNAR1* gene was also used as a marker to test for an association with MS finding no significant allelic association with disease. Therefore, our work does not provide evidence in favour of *IFNAR1*-408 as a candidate for conferring genetic susceptibility to, or protection against, MS in the South of Spain. Furthermore, this polymorphism did not show any correlation with the transcriptional levels of *IFNAR1* on MS patients nor with response to IFNβ treatment. However, the patients included in our study have been treated with IFNβ for at least 2 years. IFNβ therapy could lead to down-regulation of IFNAR1 expression in responders but fail to do it in non-responders, probably due to a primary defect in production of *IFNAR1* transcripts (Massirer et al., 2004). Therefore, because of this problem we cannot rule out the possibility that this promoter polymorphism affects the transcription level of *IFNAR1* and *IFNAR2* gene. In *summary*, this study has demonstrated an association between two *IFNAR1* and IFNAR2 polymorphisms and multiple sclerosis suggesting a role for these receptors/cytokines and their signalling pathway in MS pathogenesis.

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Table 1.

Baseline demographic characteristics of MS patients and controls

	RR MS $n=100$	SP MS $n=47$	Controls $n=210$
Age, mean (range)	36.22 (17–57)	40.8 (24–65)	34.84 (18–62)
Females (%)	71	63.8	45.71
Duration of MS mean (median)	9.18 (7.0)	12.73 (11.0)	
EDSS score mean (median)	1.98 (2.0)	4.49 (4.0)	

RR, relapsing–remitting MS; SP, secondary progressive MS.

Table 2.

Genotype and allele distribution of the *IFNAR1* and *IFNAR2* polymorphisms in responders and non-responders to IFNβ

Polymorphism		Responders $n=104$ (70.7)	Non-responders $n=43$ (29.3)
IFNAR1 18417	C/C	12 (11.5)	4 (9.3)
	C/G	33 (31.7)	13 (30.2)
	G/G	59 (56.7)	26 (60.5)
	C	57 (27.4)	21 (24.4)
	G	151 (72.6)	65 (75.6)
IFNAR1-408	C/C	63 (60.6)	28 (65.1)
	C/T	28 (26.9)	12 (27.9)
	T/T	13 (12.5)	3 (7)
	C	154 (74.1)	68 (79.1)
	T	54 (25.9)	18 (20.9)
IFNAR2 11876	G/G	16 (15.4)	5 (11.6)
	G/T	43 (41.3)	19 (44.2)
	T/T	45 (43.3)	19 (44.2)
	G	75 (36.1)	29 (33.7)
	T	133 (63.9)	57 (66.3)

p-values >0.05 and therefore are non-significant; *n*, number of subjects genotyped; percentages shown in parenthesis.

Table 3.

Genotype, allele and carrier allele distribution of *IFNAR1* and *IFNAR2* polymorphisms in MS patients and healthy controls

		MS $n=147$	Controls $n=210$	
IFNAR1 18417	C/C	16 (10.9)	5 (2.4)	$\chi^2_{df2}=14.18$ $p=0.001$
	C/G	46 (31.3)	54 (25.7)	
	G/G	85 (57.8)	151 (71.9)	
	C	78 (26.5)	64 (15.2)	$\chi^2_{df1}=13.14$ $p<0.001$
	G	216 (73.5)	356 (84.8)	
	G+	131 (89.1)	205 (98)	$\chi^2_{df1}=9.81$ $p=0.002$
	G−	16 (10.8)	5 (2)	OR=5.008 95% CI=1.79–13.99
IFNAR1-408	C/C	91 (61.9)	107 (51)	$\chi^2_{df2}=4.40$ $p=0.111$
	C/T	40 (27.2)	77 (36.7)	
	T/T	16 (10.9)	26 (12.4)	
	C	222 (75.5)	291 (69.3)	$\chi^2_{df1}=3.01$ $p=0.083$
	T	72 (24.5)	129 (30.7)	
	C+	131 (89.1)	184 (87.6)	$\chi^2_{df1}=0.07$ $p=0.791$
	C−	16 (10.9)	26 (12.4)	
IFNAR2 11876	G/G	21 (14.3)	13 (6.2)	$\chi^2_{df2}=6.70$ $p=0.035$
	G/T	62 (42.2)	93 (44.3)	
	T/T	64 (43.5)	104 (49.5)	
	G	104 (35.4)	119 (28.3)	$\chi^2_{df1}=3.67$ $p=0.055$
	T	190 (64.6)	301 (71.7)	
	G+	83 (56.4)	106 (50.5)	$\chi^2_{df1}=5.67$ $p=0.017$
	G−	64 (43.6)	104 (49.5)	OR=2.53 95% CI=1.22–5.22

n, number of subjects genotyped; percentages shown in parenthesis.

Table 4.

Genotype combination distribution of *IFNAR1* 18417 and *IFNAR2* 11876 polymorphisms in MS patients and controls

Polymorphisms 18417–11876	MS $n=147$	Controls $n=210$
C/C–G/G	1 (0.7)	0
C/C–G/T	8 (5.4)	2 (1)
C/C–T/T	7 (4.8)	3 (1.4)
C/G–G/G	6 (4.1)	4 (1.9)
C/G–G/T	23 (15.6)	28 (13.3)
C/G–T/T	17 (11.6)	22 (10.5)
G/G–G/G	14 (9.5)	9 (4.3)
G/G–G/T	32 (21.8)	63 (30)
G/G–T/T	39 (26.5)	79 (37.6)
$\chi^2_{df8}=22.063$ and $p=0.005$		

n, number of subjects genotyped; percentages shown in parenthesis.

Table 5

Haplotype frequency estimation for MS and control population obtained with FANHAP software

Haplotype 18417–11876	MS	Controls
C–G	0.092558	0.052990
C–T	0.169347	0.095336
G–G	0.254381	0.214953
G–T	0.483714	0.634329
Global χ^2_{df3} 19.7490 $p = 0.0002$		

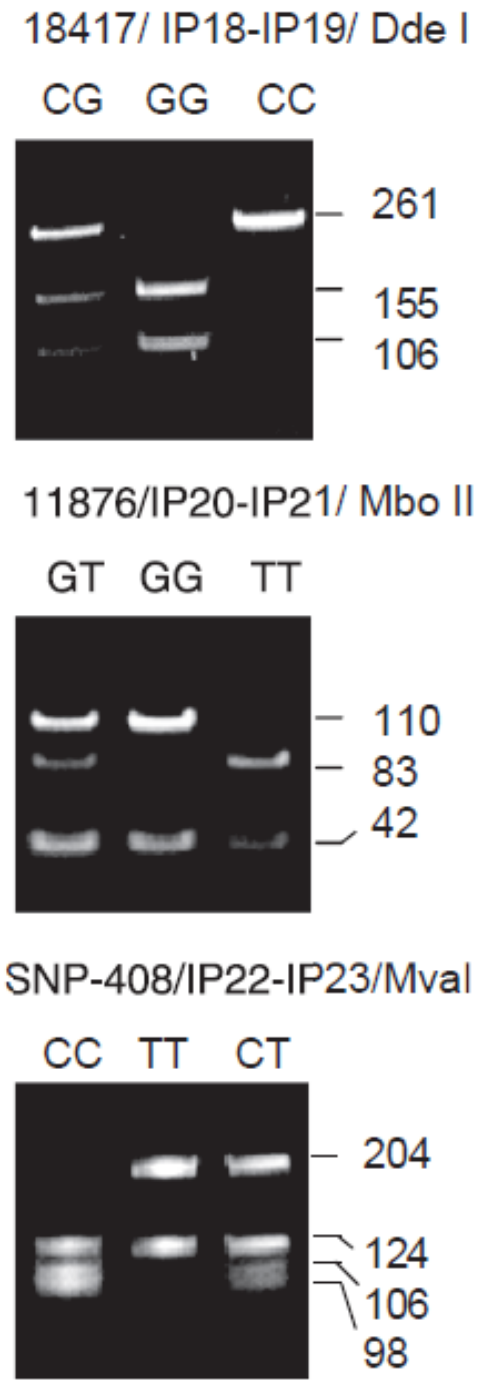

Fig. 1. Detection of IFNAR1 and IFNAR2 polymorphisms in samples of MS patients for case–control association analysis. The three panels are ethidium bromide stained polyacrylamide gels of the PCR-restriction enzyme digestion products of DNA from different individuals. On the top of each gel, the polymorphism identification, primer numbers and restriction enzyme used for the RFLP analysis are indicated. The sizes of the resulting bands for each genotype are indicated at the right side.

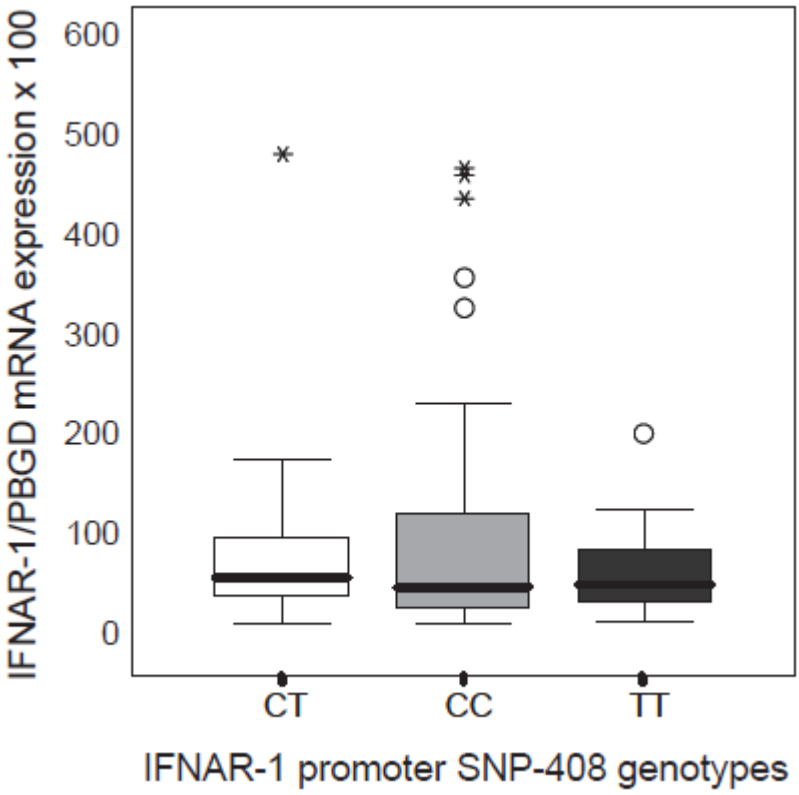

Fig. 2. Box plot showing the difference in IFNAR1 gene expression between MS patients with different genotypes at the -408 promoter polymorphism.

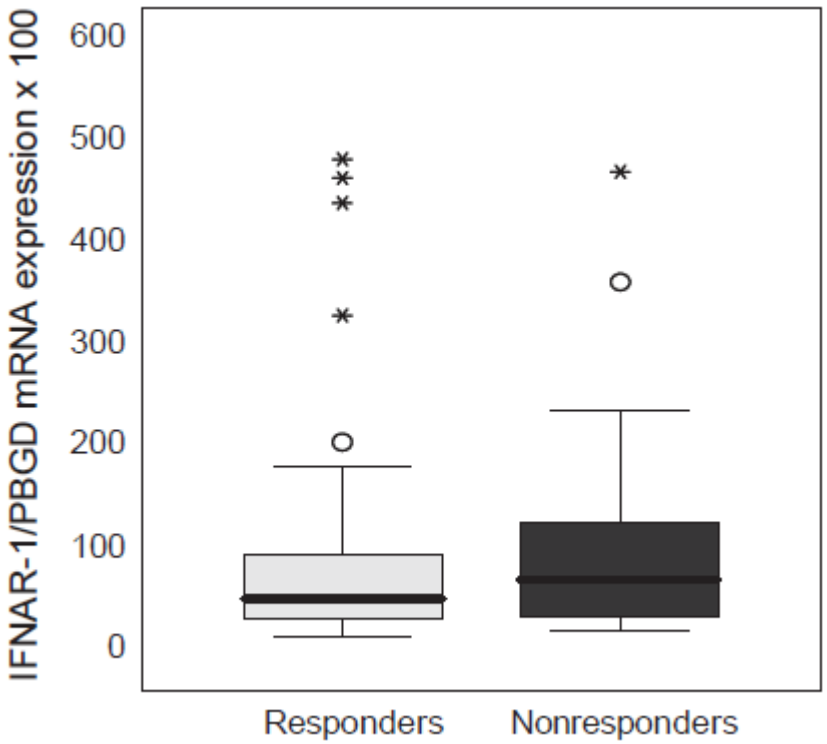

Fig. 3. Box plot showing the difference in IFNAR1 mRNA expression between 104 IFNβ responders and 43 non-responder patients.