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Pharmacogenomic analysis of interferon receptor polymorphisms in multiple sclerosis

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Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system characterized by progressive neurological dysfunction. No curative therapy is currently available, and approximately 80–90% of afflicted individuals are ultimately disabled. Interferon beta (IFNβ) has been shown to decrease clinical relapses, reduce brain disease activity, and possibly slow progression of disability. However, the overall effect of treatment is partial and a substantial number of patients are considered poor or nonresponders. For this report, we tested the pharmacogenomic effects of eight polymorphisms in the interferon receptor genes (IFNAR1 and IFNAR2) in a group of 147 patients undergoing open-label IFNβ therapy. Overall, no significant differences in the distribution of responders and nonresponders, classified based on prospectively acquired primary and secondary clinical end points, were observed when stratified by any of the studied IFNAR gene polymorphisms. A trend detected with a single nucleotide polymorphism SNP 16469 (A/T) located at the third intron of the IFNAR1 gene, suggesting modest association with relapse-free status, will require confirmation in an independent data set. In addition, no significant association was observed of any of the IFNAR gene polymorphisms with susceptibility to MS, as studied by a family-based association analysis.

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Introduction

Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system characterized by myelin loss, gliosis, varying degrees of axonal pathology, and progressive neurological dysfunction. MS pathogenesis is complex and multifactorial with a strong genetic component that is not strictly Mendelian. Interactions with infectious, nutritional, climatic, and/or other environmental influences affect susceptibility considerably.[1] Perhaps the most significant advance in MS therapeutics has been the approval of interferons. Interferon beta (IFNβ) has been shown to decrease clinical relapses, reduce brain MRI activity, and slow progression of disability.[2–4] However, the effect of this treatment is partial, a substantial number of patients do not respond to therapy, and others report side effects. In the absence of predictive clinical, neuroradiological and/or immunological markers of response, and given that 15–20% of patients have relatively mild MS, questions such as whom to treat and when to treat remain to be addressed, particularly as side effects, inconvenience, and cost of the drug are significant.

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Pharmacogenomic studies have established the importance of polymorphisms in drug targets or receptors that mediate interindividual differences in the efficacy and toxicity of many medications.[5] Some examples include polymorphisms in the β2-adrenoreceptors and sensitivity to β2-agonists in asthmatics,[6] polymorphisms in neurotransmitter-receptor-related genes and response to clozaphine in schizophrenic patients,[7] and polymorphisms in angiotensin II T1 receptor and vascular reactivity to phenylephrine.[8] The type 1 interferons, which include about 15 cytokines (13 isotypes of IFNα, one IFNβ, one IFNω), all share the same receptor, IFNAR, for binding.[9] The two subunits of the receptor, IFNAR1 and IFNAR2, are coded by two different genes located close to each other on chromosome 21q22.1. To address directly the pharmacogenomic impact of polymorphisms in the receptor, we analyzed its genomic diversity in a robust and well-characterized data set of IFNβ-treated patients. No significant effects of the genotypes were observed on the distribution of prospectively acquired clinical end points after two years of careful clinical follow-up.

Results

We studied the pharmacogenomic effect of *IFNAR*-gene-associated polymorphisms (Figure 1, Table 1) in 147 MS patients on IFNβ therapy. The IFNβ cohort had a mean age of 32.6 ± 9.1, the female : male ratio was 2.1 : 1, the

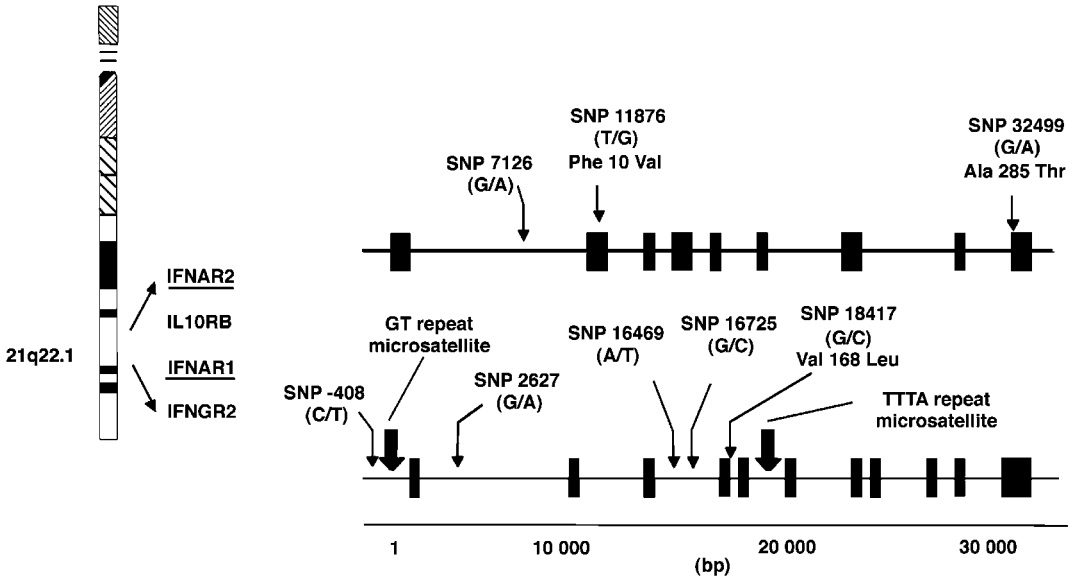

Figure 1 Map of *IFNAR* genes showing the locations of the SNPs and the microsatellites on chromosome 21. The panel on the right shows the expanded *IFNAR2* (top) and *IFNAR1* (bottom) loci. The black shaded boxes in the expanded genome represent the exons.

mean baseline relapse rate was 1.4 ± 0.7, the mean EDSS score at entry was 2.5 ± 1.3, the mean EDSS score at 24 months was 2.5 ± 1.7, and The mean relapse rate during IFNβ therapy was 0.5 ± 0.7. After the 2-year follow-up period, a decrease in the relapse rate from 1.5 to 0.5 (>66% decrease) was observed in the full cohort. From the original cohort ($N=151$), four individuals were dropped from the study owing to accelerated disease course and death ($n=1$), development of psychiatric disease ($n=1$), or premature departure from study before the end of 2-year follow-up period ($n=2$). Additional baseline clinical characteristics of this data set are described elsewhere.[10]

The analysis of *IFNAR1* and *IFNAR2* genotypes and response to IFNβ therapy are shown in Table 2. According to the primary outcome categorization, there were 57 responders, 48 nonresponders, and 42 were designated as an undefined group. According to secondary clinical outcome, 116 out of 140 patients had >30% decrease in relapse rate after 2 years and 67 out of 139 patients were found to be relapse-free for 2 years. To look for associations between *IFNAR* genotypes and clinical outcomes, the individual alleles were compared and tabulated under Genotype 1 (binary variable where 11 and 12 are compared to 22; so allele 1+ *vs* allele 1−) and Genotype 2 (binary variable where 12 and 22 are compared to 11; so allele 2+ *vs* allele 2−). Genotypes were analyzed either for all three categories (responder, nonresponder, and undefined) using the primary end points or only two categories (responder and nonresponder) (Table 2). No significant deviation in the frequencies of any of the genotypes was observed when only two categories of patients were considered. Modest differences in the frequencies of the alleles were observed when the undefined category was also included for SNPs 16469 and 16725 (Genotype 1—SNP 16469, $P=0.03$, SNP 16725, $P=0.05$; Genotype 2—SNP 16469, $P=0.04$). Independent effects due to *HLA-DRB1*1501* status or gender were not observed (data not shown). The genetic effects of *IFNAR1* and *IFNAR2* on response to therapy that can be excluded on the basis of the results of this study are given by the upper 95% confidence limits for the odds ratios (ORs) shown in Table 2. For example, only ORs>4.5 for the association of *IFNAR1*-Prom408 (Genotype 1) with the primary end point can be excluded with 95% confidence. In contrast, for *IFNAR1*-16469 (Genotype 1), ORs>1.2 can be excluded. The variation across polymorphisms in the certainty or precision of the negative, as reflected in the ORs that can be excluded, was because of both genotype frequencies and distribution of patients within clinical end point categories. No significant associations were observed with any of the alleles based on the secondary clinical end points also (data not shown). However, allele A (Genotype 1) of SNP 16469 showed a trend towards association with the relapse-free status of the patients ($P=0.03$).

In order to capture potential biological interactions between the two subunits of the heterodimer receptor, an extended genotype analysis was also performed including the SNPs in the exonic regions—exon 2 and exon 9 of the *IFNAR2* gene (SNPs 11876, 32499) and exon 4 of the *IFNAR1* gene (SNP 18417). The most common genotypes were, in the order of the SNPs 11876, 32499, 18417, 11, 11, 11 (33%), 11, 11, 12 (16%), 12, 12, 11 (27%), 12, 12, 12 (12%) and the rest comprised a total of 12%. None of the haplotypes showed any association with either the primary outcome categories (responder *vs* nonresponder, $P=0.81$) or the secondary outcome categories (>30% decrease in relapse rate, $P=0.64$; relapse-free after 2 years, $P=0.57$).

Although primarily designed as a pharmacogenomic study, we extended our analysis to assess the influence of *IFNAR1* and *IFNAR2* genes on disease susceptibility in 197 MS simplex families. Based on the currently accepted inflammatory/autoimmune model of disease pathogenesis, IFN receptor genes can be considered as reasonable candidate disease genes. The study group included 98 probands from the IFNβ data set. The global *P*-values from PDT and haplotype analysis using TRANSMIT are shown in Table 3. No significant association of any of the

Table 1 Details of polymorphisms and primers for SNPs and microsatellites in the *IFNAR* genes

Gene/polymorphism (Ref.)	*Base change*	*Primers*[a]	*Sequence*
IFNAR1-SNP PROM−408 (Ref. 27)	C/T	RP1	5′-GGC GCG GGT GGG CTC CTG-3′
		RP2	5′-(AT tail)GG CGC GGG TGG GCT CCT A-3′
		F1	5′-CCG GGC GGA GAA GGG CGA GGA CGA A-3′
IFNAR1-SNP 2627 (rs#1041429)	G/A	RP1	5′-CCC TTA ACA TAC AAG TGG AC-3′
		RP2	5′-(GC tail)C CCT TAA CAT ACA AGT GGA T-3′
		F	5′-ACC AGACTA GAC TGT TTT CCC AA-3′
IFNAR1-SNP 16469 (rs#1012334)	A/T	P1	5′-AAG ACT TAG TTG CCT TTT TA-3′
		P2	5′-(GC tail)A AGA CTT AGT TGC CTT TTT T-3′
		R	5′-TAT CAC CAC ATC ACA CAT CAA-3′
IFNAR1-SNP 16725 (rs# 1012335)	G/C	RP1	5′-CAA CAA GAA CAA AAC TCT GTC-3′
		RP2	5′-(GC tail)C AAC AAG AAC AAA ACT CTG TG-3′
		F	5′-TGG CAA ATG TTT GTT ACC ATA A-3′
IFNAR1-SNP 18417 (rs# 2257167)	G/C	RP1	5′-CTG AAG AGT TTT TCC AGA TAA C-3′
		RP2	5′-(GC tail)C TGA AGA GTT TTT CCA GAT AAG-3′
		F	5′-TCC TCC AGA AGT ACA TTT AGA A-3′
IFNAR2-SNP 7126 (rs# 3153)	G/A	RP1	5′-GAC CTC TGA CAT GTG GC-3′
		RP2	5′-(GC tail)G ACC TCT GAC ATG TGG T-3′
		F	5′-CCT GCA GAT ATA TAA CAA AGA TGA A-3′
IFNAR2-SNP 11876 (rs#1051393)	T/G	P1	5′-CCA GAA TGC CTT CAT CT-3′
		P2	5′-(GC tail)C CAG AAT GCC TTC ATC G-3′
		R	5′-GGC ATC ACA GCT TGC TTC TAT AA-3′
IFNAR2-SNP 32499 (rs#1131668)	G/A	P1	5′-TTT AGA GGC AAG GTC TCG-3′
		P2	5′-(GC tail)T TTA GAG GCA AGG TCT CA-3′
		R	5′-AGG GAT GCA CGC TTG TAA-3′
IFNAR1-GT repeat Microsatellite (Ref. 27)		Forward	5′-ATA GGC CGG AAA GAG TGA GGA A-3′
		Reverse	5′-CCG CAG ATC CCA CCA GTT-3′
IFNAR1-TTTA repeat Microsatellite (Ref. 28)		Forward	5′-TGC TTA CTT AAC CCA GTG TG-3′
		Reverse	5′-CAC ACT ATG TAA TAC TAT GC-3′

[a]P1, P2, allele-specific primer sets on the forward strand; R, common reverse primer; RP1, RP2, allele-specific primer sets on the reverse strand; F, common forward primer.
Sequence of GC tail—GCG GGC AGG GCG GCG GGG GCG GGG CC.
Sequence of AT tail—TAT TTA TAA AAT TTA TTT TAT TTT AAA ATA T.
The SNPs are underlined; reference to (rs#)—NCBI SNP ID.

SNPs or the microsatellite alleles with MS was observed. Stratification of subjects according to their *HLA-DRB1*1501* status (positive, $n=71$; negative, $n=126$) did not show any significant association of any of the *IFNAR* SNPs or microsatellite alleles with either *DRB1*1501* or any specific gender. SNP 11876 (exon 2, *IFNAR2* gene) showed some trend towards association with *HLA-DRB1*1501*-positive families ($P=0.03$).

Discussion

The beneficial effects of IFNβ therapy in MS have been shown in several studies,[2–4] decreasing the relapse rate by approximately 30%. However, a long-term shift in the natural history of the disease has not been demonstrated. Furthermore, a significant number of patients are refractory or show adverse reactions following IFN therapy. Our study was designed to address the role of genetic heterogeneity in the response to immunotherapy, by analysis of the correlation between different *IFNAR* genotypes and clinical outcome, employing end points validated in double-blinded clinical trials. The *IFNAR* genes have been mapped to chromosome 21q22.1 (GenBank Acc. 7768717), located in the vicinity of the IL10 receptor (*IL10R-2*) and the interferon gamma receptor 2 chain (*IFNGR2*) genes (together they form the gene cluster of human class II helical cytokine receptors)[11] (Figure 1). The two subunits of the receptor, IFNAR1 and IFNAR2, are coded by two different genes located close to each other: the *IFNAR1* gene spans about 31 kb (comprising 11 exons)[12] and the *IFNAR2* gene about 32 kb (comprising nine exons).[9] To achieve full coverage of the locus, we have analyzed the frequencies of eight SNPs and two microsatellites. In addition, the availability of unaffected family members provided an opportunity to assess the role of these polymorphisms in conferring susceptibility. Family-based association analysis yielded no significant results, confirming previously published exclusion results using flanking microsatellites.[13]

Currently available disease-modifying agents for MS include recombinant IFNβ (Avonex®, Betaseron/Betaferon®, and Rebif®), glatiramer acetate (Copaxone®),

Table 2 Analysis of *IFNAR1* and *IFNAR2* genotypes and clinical outcome to IFNβ therapy in Spanish MS patients[a]

	P-*value (three categories)*	P-*value (two categories)*	*Adjusted ORs*[b]	*95% CI*	*Logistic regression* P-*value*
Genotype 1					
IFNAR1-SNP PROM−408	0.49	0.62	0.4	0.0–4.5	0.47
IFNAR1-SNP 2627	—	—	—		—
IFNAR1-SNP 16469	0.03	0.70	0.9	0.2–1.2	0.88
IFNAR1-SNP16725	0.05	0.51	0.8	0.3–2.1	0.71
IFNAR1-SNP 18417	0.88	1.00	0.9	0.1–5.7	0.90
IFNAR2-SNP 7126	0.09	0.47	1.7	0.4–8.3	0.50
IFNAR2-SNP 11876	0.22	0.51	1.3	0.3–5.5	0.76
IFNAR2-SNP 32499	0.36	0.73	0.9	0.2–4.4	0.92
Genotype 2					
IFNAR1-SNP PROM−408	0.06	0.55	0.9	0.4–2.1	0.83
IFNAR1-SNP 2627	0.16	0.11	0.3	0.1–1.2	0.09
IFNAR1-SNP 16469	0.04	1.00	1.1	0.3–3.6	0.87
IFNAR1-SNP16725	0.34	0.27	1.7	0.7–4.3	0.23
IFNAR1-SNP 18417	0.74	0.54	1.1	0.5–2.5	0.88
IFNAR2-SNP 7126	0.41	0.85	1.1	0.5–2.6	0.80
IFNAR2-SNP 11876	0.48	0.85	1.1	0.5–2.4	0.90
IFNAR2-SNP 32499	0.52	0.70	1.1	0.5–2.5	0.75

[a]A total of 147 patients were used for analysis; *P*-values for the first two columns were determined using χ^2-test or Fisher's exact test when appropriate, as implemented in SPSS v. 9 or SAS v. 6.12.
[b]DR2 status and gender both included in models. Unadjusted ORs were very similar (not shown). Genotypes were used to predict (good) response to therapy.

Table 3 PDT analysis of *IFNAR1* and *IFNAR2* genes in Spanish MS families

Polymorphism	*Allele frequencies*[a]	*ALL families* N=*197*	*DRB1*1501 positive only* N=*71*	*DRB1*1501 negative only* N=*126*
IFNAR1				
SNP PROM−408	0.73/0.27	0.53	0.76	0.32
PROM $(GT)_n$	0.53	0.58	0.12	0.85
SNP 2627	0.96/0.04	0.92	0.74	0.81
SNP 16469	0.52/0.48	0.06	0.19	0.14
SNP 16725	0.60/0.40	0.16	0.06	0.66
SNP 18417	0.85/0.15	0.77	0.71	0.61
INTRON 5 $(TTTA)_n$	0.24	0.23	0.60	0.11
5 SNP haplotype[b]		0.21	—	—
IFNAR2				
SNP 7126	0.74/0.26	0.47	0.13	0.75
SNP 11876	0.68/0.32	0.29	0.03	0.75
SNP 32499	0.69/0.31	0.49	0.13	0.64
3 SNP haplotype[b]		0.65	—	—

[a]Allele frequencies determined from random unrelated founders in each pedigree; for microsatellite markers, the frequency of the most common allele is shown in the table.
[b]Analysis done for 5 SNP (*IFNAR1* gene, PROM−408, 2627, 16469, 16725, 18417) and 3 SNP (*IFNAR2* gene, 7126, 11876, 32499) haplotypes using TRANSMIT; *P*-values for single marker analyses of SNPs were not significant (data not shown).

and mitroxantone (Novantrone®). In controlled clinical trials, these immunomodulatory drugs have been shown to decrease clinical relapses, reduce brain MRI activity, and possibly slow progression of disability.[4,14] We were able to detect a clinical effect of IFNβ similar to that found in clinical trials,[4,15] validating the quality of our measures and power of the studied cohort despite the lack of MRI data or placebo group. These analyses showed no significant deviation in the frequency of any of the *IFNAR* SNP genotypes or haplotypes when analyzed according to primary or secondary clinical end points, indicating that genomic variations in the receptor genes do not affect the outcome of IFNβ therapy in a substantial way. A modest effect of allele A in SNP 16469, in the intronic region of the *IFNAR1* gene, on the primary clinical outcome (Genotype 1, 3 categories, $P=0.03$) (Table 2) and secondary clinical outcome (Genotype 1, relapse-free status, $P=0.03$) will require confirmation in an independent data set.

The factors underlying the salutary therapeutic mechanisms of IFNβ are not fully defined. Furthermore, relapses in MS are associated with increases in IFNγ secreting cells, and type I interferons are known to induce, under certain conditions, Th1 differentiation and IFNγ production. A wide number of mechanisms of action of IFNβ in MS have been described.[16] Potential sites of action of IFNβ in MS include antigen

presentation, effects on cytokine profiles, and entry of lymphocytes into the brain parenchyma. The IL10/IL12 regulatory circuit has been suggested as a primary target of IFNβ and treatment.[17] In small cohorts, a reduction in the expression of RANTES, IFNγ and IL4, IL6 and TNF was observed following therapy, as well as increased levels of TGFβ and IL10.[18–22] Exploring the degree of variability of these candidate and upstream genes that regulate their expression and function for direct association with treatment response[23,24] represent a promising approach to clarify the physiological mechanisms of IFNβ administration in autoimmune demyelination.

Subjects and methods

IFNβ cohort

In all, 147 MS patients starting IFNβ treatment during the period of January 1995 to December 1998 were ascertained according to rigorous recruitment criteria at the Neuroimmunology Unit, Hospital Vall d'Hebron, Barcelona, Spain.[10,25,26] Inclusion in the study required a diagnosis of definite MS according to Poser criteria,[25] being of RRMS type, age between 18 and 50 years old, and having suffered two or more relapses in the last 2 years (as regulated by the Spanish Ministry of Health to give access to the drug). Exclusion criteria included pregnancy, inability to give informed consent, dementia, cancer, or recreational drug abuse. The different treatment groups (Betaferon®, $n=111$; Avonex®, $n=25$, and Rebif®, $n=11$) reflect the gradual availability of the three different IFNβ therapies in Spain. All study participants were interviewed, and parental and ancestral information was recorded by province of origin. All known ancestors were Caucasian and Spanish in origin, primarily from the Mediterranean coast of Spain; that is, Catalonia, Valencia, and the Balearic Islands. Age of onset was defined as the first episode of neurological dysfunction suggestive of demyelinating disease.[10] Disability was assessed at entry with the Expanded Disability Status Scale (EDSS).

Patients were followed prospectively for 2 years since initiation of therapy, and clinical data, including EDSS scores, were recorded every 3 months. Neurologists had participated in several clinical trials and are well trained with low inter- and intrarater variability scores, but were blinded to the goals of this experiment. The primary clinical end point was the suppression of relapses during the follow-up (exacerbation-free patients) and no increase in the EDSS score confirmed by two consecutive visits. The secondary clinical end points included (1) a >30% decrease in the relapse rate after 2 years, and (2) relapse-free status for 2 years. MRI was done at the time of diagnosis but not to monitor the treatment. Using the primary end point, patients were categorized as follows: responders were defined as having 0 relapses and no increase in the EDSS in the 2 years follow-up; nonresponders were defined as having suffered two or more relapses and/or having an increase of 1 point in the EDSS score, confirmed by two consecutive visits in the 2 years follow-up. Patients with just one relapse were grouped under the Undefined category. For 98 individuals in this cohort, biological specimens from parents were available and were included in the analysis of genetic susceptibility. All studies were approved by the respective Committees of Human Research at Hospital Vall d'Hebron and UC San Francisco. Informed consent was obtained for all study participants.

DNA extraction

White blood cells were isolated by Ficoll gradient, and high molecular weight DNA was isolated using the standard desalting procedure.

IFN α/β receptor polymorphisms

Eight SNPs and two microsatellites were studied in the *IFNAR1* and *IFNAR2* genes (http://www.ncbi.nlm.nih.gov/SNP/).[27,28] Details of the polymorphisms are shown in Figure 1 and Table 1.

Single-tube T_m-shift SNP genotyping

SNPs were detected using single-tube T_m-shift genotyping-based assays[29] in a GeneAmp® 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA), except for the SNP at −408 (C/T) in the promoter region of the *IFNAR1* gene, which was genotyped separately in two PCRs for the two alleles. The technique combines allele-specific PCR with the discrimination of the amplified products by their melting temperatures (T_m). Two distinct allele-specific primers (designed either in the forward or reverse strands), each of which contains a 3′-terminal base that corresponds to one of the two SNP allelic variants, were combined with a common forward or reverse primer in a single-tube reaction (Table 1). A GC (26 bp) or AT (31 bp) tail was attached to one of the allele-specific primers to increase or decrease, respectively, the T_m of the amplification product from the corresponding allele. Genotypes were read according to the melting or dissociation curve.

All PCR reactions were performed in 96-well plates as follows: 50 μl PCR reaction contained 1 × Stoffel buffer, dNTPs (50 μM dATP, 50 μM dCTP, 50 μM dGTP, 25 μM dTTP, 75 μM dUTP), 2 mM $MgCl_2$, 20 mM KCl, 5U Stoffel polymerase (Applied Biosystems, Foster City, CA, USA), 0.2 μM of allele-specific primer 1, 0.2 μM (SNPs 32499, 16725, 7126, −408, 2627, and 18417) or 0.1 μM (SNPs 11876 and 16469) of allele-specific primer 2 (Table 1), 0.2 μM of common forward or reverse primer, 5% DMSO, 2.5% glycerol, 1 U uracil-*N*-glycosylase (UNG), 20 ng DNA, and 0.2 × Sybergreen. The PCR thermal profile was as follows: initial incubation step for 2 min at 50°C, denaturation for 1 min at 95°C, either a two-step amplification cycle of 20 s at 95°C and annealing/extension for 20 s at 55°C (SNPs 11876, 32499, 16469, and 16725), or an annealing at 55°C (SNPs 2627 and 18417)/57°C (SNPs 7126 and PROM 408) and extension at 72°C for 30 s for 35 cycles. A temperature ramp from 56 to 91°C was programmed at the end of the PCR reaction to melt the PCR products and obtain the dissociation curves.

Microsatellite genotyping

Microsatellite genotyping was performed for the two *IFNAR* repeats[27,28] in an ABI PRISM 377 Automated DNA sequencer. DNA samples were organized in 96-well plates for 30 cycles of 'hot start' PCR amplification using fluorescently labeled oligonucleotide primers (Table 1). The PCR products were resolved by electrophoresis in 6% denaturing acrylamide gels on the ABI sequencer loaded with the ABI PRISM GENESCAN 3.1

software for fluorescent scanning. Genotyper 2.0 was used for peak calling and allele binning.

Statistical analysis

Clinical and genetic data were stored and analyzed in SPSS v. 9.0 (SPSS, Chicago, IL, USA) or SAS v. 6.12. Statistical analyses included χ^2-test or Fisher's exact test for comparisons of categorical variables; logistic regression modeling was used to model binary outcomes. *HLA-DRB1*1501* status and gender were included in the model. Family-based association studies of both *IFNAR* loci were performed in all families using the pedigree disequilibrium test (PDT) v. 3.11 (wwwchg.mc.duke.edu/software/pdt.html).[30] Haplotype analysis was performed using TRANSMIT.[31] The *P*-values have not been corrected for multiple testing.

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