ORIGINAL CONTRIBUTION

Genome-Wide Pharmacogenomic Analysis of the Response to Interferon Beta Therapy in Multiple Sclerosis

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Objective: To identify promising candidate genes linked to interindividual differences in the efficacy of interferon beta therapy. Recombinant interferon beta therapy is widely used to reduce disease activity in multiple sclerosis (MS). However, up to 50% of patients continue to have relapses and worsening disability despite therapy.

Design: We used a genome-wide pharmacogenomic approach to identify single-nucleotide polymorphism (SNP) allelic differences associated with interferon beta therapy response.

Setting: Four collaborating centers in the Mediterranean Basin. Data Coordination Center at the University of California, San Francisco.

Patients: A cohort of 206 patients with relapsing-remitting MS followed up prospectively for 2 years after initiation of treatment.

Intervention: DNA was pooled and hybridized to Affymetrix 100K GeneChips. Pooling schemes were designed to minimize confounding batch effects and increase confidence by technical replication.

Main Outcome Measures: Single-nucleotide polymorphism detection. Comparison of allelic frequencies between good responders and nonresponders to interferon beta therapy.

Results: A multianalytical approach detected significant associations between several SNPs and treatment response, which were validated by individual DNA genotyping on an independent platform. After the validation stage was complete, 81 additional individuals were added to the analysis to increase power. We found that responders and nonresponders had significantly different genotype frequencies for SNPs located in many genes, including *glypican 5*, *collagen type XXV α1*, *hyaluronan proteoglycan link protein*, *calpastatin*, and *neuronal PAS domain protein 3*.

Conclusions: The reported results address the question of genetic heterogeneity in MS and the response to immunotherapy by analysis of the correlation between different genotypes and clinical response to interferon beta therapy. Many of the detected differences between responders and nonresponders were genes associated with ion channels and signal transduction pathways. The study also suggests that genetic variants in heparan sulfate proteoglycan genes may be of clinical interest in MS as predictors of the response to therapy. In addition to new insights into the mechanistic biology of interferon beta, these results help define the molecular basis of interferon beta therapy response heterogeneity.

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MULTIPLE SCLEROSIS (MS) is a leading cause of neurologic disability in young adults.[1] No curative therapy is available, and approximately 80% of individuals with MS are ultimately disabled. Recombinant interferon beta, a small protein with antiviral, antiproliferative, antiadhesion, and proapoptotic activity, is widely used as treatment to reduce clinical activity and possibly slow disease progression.[2-4] Despite interferon beta therapy, up to 50% of patients with MS continue to experience relapses and worsening disability. In addition, adverse effects, such as flulike symptoms and depression, are common, leading many patients to discontinue therapy.

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The mechanism of action of interferon beta is incompletely understood, and there are no reliable clinical or biological markers that accurately forecast

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response to therapy. In this setting of variable responsiveness and clinical heterogeneity, pharmacogenomic research could uncover unexpected mechanistic processes and potentially achieve the elusive goal of personalized medicine in MS. So far, a few candidate-based studies have investigated germline variation in genes hypothesized to influence interferon beta therapy response, but findings await replication.[5-9] Since MS is a complex genetic trait and interferon beta, a pleiotropic agent, it is likely that allelic variation at multiple genes contributes to the overall pharmacogenomic response. In contrast to candidate-based methods, the genome-wide pharmacogenomic approach described herein allows for the unbiased detection of DNA variants associated with interferon beta therapy response.

METHODS

STUDY POPULATION AND DATA SET

The screening group consisted of 206 subjects selected from a well-characterized and homogeneous cohort followed up at 4 collaborating centers in the Mediterranean Basin: Hospital Vall d'Hebron, Barcelona, Spain; University of Navarra, Pamplona, Spain; MS Centre, Toulouse, France; and Hospital Regional Universitario Carlos Haya, Malaga, Spain. We focused on southern Europeans to minimize differences in population structure.[10] Patients were followed up prospectively for 2 or more years after the initiation of interferon beta therapy. Disability data were collected at 3-month intervals by neurologists experienced in Expanded Disability Status Score (EDSS) scoring, with low interrater and intrarater variability. Relapses were defined as a new symptom or worsening of a preexisting symptom attributable to MS activity, confirmed by examination within 3 days of onset. After the validation stage was complete, we identified 81 additional samples from the Pamplona, Barcelona, and Malaga centers that met our strict criteria for response. These additional subjects were added to the original study population to be included in the joint analysis.

Inclusion criteria were (1) clinically definite relapsing-remitting MS[11] treated with interferon beta (Betaseron [Bayer HealthCare Pharmaceuticals, Wayne, New Jersey], Avonex [Biogen Idec, Cambridge, Massachusetts], or Rebif [Pfizer, New York, NY]) for at least 2 years, (2) at least 2 documented relapses over the 2 years previous to treatment onset, and (3) 2 years of follow-up clinical data. We focused on extreme clinical phenotypes to maximize the ability to detect differences. Responders had no relapses and no increase in EDSS over the 2-year follow-up period; nonresponders had at least 2 relapses or an increase in EDSS of at least 1 point. The EDSS was required to have been stable over at least 3 consecutive visits.[12] Changes of 0.5 point were not considered significant. Magnetic resonance imaging was performed at the time of diagnosis but was not used to monitor treatment. Experimental protocols were approved by the committees on human research at each institution, and informed consent was obtained from all participants.

SINGLE-NUCLEOTIDE POLYMORPHISM MICROARRAYS AND POOLING

We used pooled DNA on single-nucleotide polymorphism (SNP) microarrays to assess population allele frequencies.[13,14] DNA was extracted and samples were quantitated in duplicate at the University of California, San Francisco, using the PicoGreen dsDNA quantitation reagent (Molecular Probes, Inc, Eugene, Oregon). Samples were diluted to 20 ng/μL, requantitated, and pooled into groups of 20 subjects. Responders and nonresponders were always pooled separately. Each sample was included in 3 different pools on separate Affymetrix GeneChip 100K arrays (Affymetrix, Santa Clara, California) to provide technical replicates. Thirty-six microarrays were used for SNP detection using manufacturer-recommended procedures to process each chip (Oklahoma research facility). The Oklahoma research facility in-house proprietary software and algorithms were used to generate quantitative estimates of each pool's allele frequency from the raw data, and differential hybridization was corrected using individual allele profiles.

RANKING OF CANDIDATE SNPs

Promising candidate SNPs were selected on the basis of significance-based ranking or a clustering algorithm that considered genomic distance between SNPs. The clustering method assumes that significant SNPs are likely to segregate together in a haplotype. To find clusters of significant SNPs along each chromosome, we used a custom-designed algorithm to group SNPs with a Z^2 P value $<.05$ in each replicate. Single-nucleotide polymorphisms were considered part of a common cluster if they were positioned within 30 kilobases of each other. To be significant, a SNP was required to be a member of a cluster of 4 or more SNPs in all 3 replicates.

VALIDATION OF RESULTS FROM POOLING STAGE USING AN INDEPENDENT PLATFORM

The top 35 candidate SNPs selected using the ranking methods were individually genotyped in each DNA sample from the original 206-subject data set using TaqMan assays (Applied Biosystems, Foster City, California). The DNA of 2 subjects was unavailable for individual genotyping. As an additional confirmatory measure, 5 nonsignificant SNPs were genotyped.

STATISTICAL ANALYSIS

We used t tests to compare age at disease and treatment onset between responders and nonresponders and to compare origin, treatment duration, and number of relapses in the 2 years prior to treatment. Differences in allele frequency were tested using Z^2 binomial proportion tests. The Fisher exact test was used to compare genotype frequencies. Results were not adjusted for multiple comparisons. Logistic regression was used to determine odds ratios for response; analyses were adjusted for baseline EDSS and number of relapses prior to study entry. Statistical tests were completed using Intercooled Stata 7.0 (StataCorp, College Station, Texas). The MSSS test program was used for calculation of the Multiple Sclerosis Severity Score (http://www-gene.cimr.cam.ac.uk/MSgenetics).[15]

GENE ONTOLOGY

We used the Web-based GoStat program to assess which categories of SNPs were overrepresented or underrepresented in our list of pharmacogenomic candidates (http://gostat.wehi.edu.au).[16] The GoStat program annotates genes with gene ontology terms; each gene can be associated with 1 or more gene ontology terms. We compared (1) genes associated with the top significant SNPs (average $P<.05$ over the 3 replicates) with (2) all genes detected by the microarray. Significance for the gene ontology analysis was set at .05; the Benjamini-Hochberg method was used to correct for multiple comparisons.[17]

Table 1. Clinical Characteristics of the Study Population[a]

Characteristic	No. (%) Stage 1 Group (Original Pooled) Responders (n=99 [48%])	Nonresponders (n=107 [52%])	*P* Value	No. (%) Stage 4 Group (Joint Analysis) Responders (n=143 [49.7%])	Nonresponders (n=142 [50.3%])	*P* Value
Center			.42			.77
Toulouse, France	8 (8.1)	12 (11.2)		8 (5.6)	12 (8.5)	
Pamplona, Spain	16 (16.2)	14 (13.1)		30 (21.0)	26 (18.3)	
Barcelona, Spain	64 (64.7)	62 (57.9)		64 (44.8)	62 (43.7)	
Malaga, Spain	11 (11.1)	19 (17.8)		41 (28.7)	42 (29.6)	
Female	68 (68.7)	75 (70.1)	.83	103 (72.0)	98 (69.0)	.58
HLA-DRB1*1501	37 (38.1)	40 (37.4)	.91	53 (37.6)	52 (36.9)	.90
Age at disease onset, y, mean (SD)	25.3 (7.4)	25.7 (7.0)	.66	25.5 (7.5)	25.0 (6.5)	.53
Age at treatment onset, y, mean (SD)	30.5 (7.2)	31.0 (7.5)	.67	31.4 (8.3)	30.5 (7.8)	.40
Duration of MS prior to treatment, y			.34			.36
< 2	27 (27.3)	41 (38.3)		32 (26.9)	46 (36.5)	
2-4	23 (23.2)	19 (17.8)		24 (20.2)	21 (16.7)	
5-8	28 (28.3)	24 (22.4)		33 (27.7)	35 (27.8)	
> 8	21 (21.2)	23 (21.5)		30 (25.2)	24 (19.0)	
Relapses in 2 y prior to treatment			.04			.004
2	57 (57.6)	44 (41.1)		88 (61.5)	63 (44.4)	
3	23 (23.2)	28 (26.2)		34 (23.8)	38 (26.8)	
> 4	19 (19.2)	35 (32.7)		21 (14.7)	41 (28.9)	
EDSS at treatment onset, mean (SD)	2.0 (1.0)	2.4 (1.3)	.01	1.8 (1.1)	2.2 (1.4)	.003
Relapses during 2 y of treatment, mean (SD)	0	2.4 (1.4)		0	2.5 (1.5)	
EDSS change over 2 y, mean (SD)	–0.4 (0.7)	0.8 (1.1)		–0.3 (0.6)	0.8 (1.1)	

Abbreviations: EDSS, Expanded Disability Status Score; MS, multiple sclerosis.

[a]The responders and nonresponders in the original (stage 1) group were similar in most respects. However, responders tended to have fewer relapses in the 2 years prior to treatment and a slightly lower mean EDSS at baseline. These differences were also seen in the joint analysis (stage 4). The joint analysis group consisted of the original group plus 81 additional subjects who were identified after stage 3 (validation) was complete.

RESULTS

This project was completed in 4 major stages: stage 1, DNA pooling on SNP microarrays for population allele frequencies (n=206 individuals); stage 2, ranking of top candidate SNPs; stage 3, validation of association through individual genotyping of candidate SNPs (n=204 individuals); and stage 4, joint analysis using new and original subjects (n=285 individuals).

STAGE I: POOLED DNA ON SNP MICROARRAYS

The genome-wide screening included 99 patients who met the criteria for positive response to interferon beta treatment and 107, for nonresponse. Responders and nonresponders were very similar in all respects, except with regard to baseline disability and relapses prior to onset of therapy (**Table 1**). On average, nonresponders had a baseline EDSS 0.4 point greater than responders, a pattern that has been observed previously.[18] Responders also had a lower Multiple Sclerosis Severity Score than nonresponders, which adjusts the EDSS for duration ($P=.01$). Overall, there was a high degree of correlation between the 3 biological replicates (eTable 1, available at http://archneur.ama-assn.org).

Gene ontology analysis from the microarray data using GoStat showed that top-ranked SNPs that differed between responders and nonresponders were more likely to be related to ion channels and signal transduction pathways (**Table 2**). For example, γ-*aminobutyric acid* and *glutamate receptor* genes were overrepresented in the group of significant genes; 42.1% and 53.8% of the microarray's SNPs in this category were in the most significant subset, compared with their expected prevalence of 13.5%.

STAGE 2: RANKING OF TOP CANDIDATE SNPs

We used 3 different methods to rank top candidate SNPs:

1. *P* value significance ranking. We chose $P<.0005$ as our cutoff, yielding 13 candidate SNPs. We also considered 2 SNPs with $P<.00005$ in 2 of 3 replicates (rs952084 and rs1493663).

2. Cluster ranking 1. Hap-cluster ranking is based on the premise that significant SNPs in linkage disequilibrium are likely to segregate together, and the detection of multiple significant SNPs near each other increases the likelihood that the finding is not spurious (see "Methods" section). We found 45 clusters consisting of 172 SNPs. For follow-up in stage 3, we selected 10 candidate SNPs with a $P<.05$ in each of the replicates, with a preference for larger clusters and SNPs showing linkage with others within the cluster.

3. Cluster ranking 2. We selected 10 SNPs with a $P<.01$ for follow-up from the list that remained after cluster 1 selection.

Table 2. Gene Ontology Categories Overrepresented in the $P < .05$ Subset of the SNP Microarray Chip[a]

Category	Gene Examples	Gene Ontology *P* Value
Overrepresented		
Extracellular ligand-gated ion channels (glutamate, glycine, GABA)	*GABRA2*, *GABRB1*, *GLRA2*, *GRIA1*, *GRID2*	.0001
Cell surface receptor–linked signal transduction	*PRKCE*, *PTK2*, *KCNMA1*, *SYK*, *PTGER3*	.0003
Ion channels	*KCNJ6*, *GPR158*, *TGFBR2*, *GPC5*, *SYK*	.0010
Sensory perception of sound	*CDH13*, *GAD1*, *SLC24A2*, *HTR2A*	.0019
Anion transporter activity	*SLC22A8*, *SLC1A2*, *GLRA2*, *GABRA4*	.0081
Lipid binding	*GPC5*, *NEGR1*, *DGKH*, *DGKB*, *DGKE*	.0148
Underrepresented		
Intracellular membrane bound organelle, cytoskeleton (actin, myosin)	*MYPN*, *FRMD3*, *STK39*, *SEP15*	.0210

Abbreviations: GABA, γ-aminobutyric acid; SNP, single-nucleotide polymorphism.
[a]We compared (1) the genes associated with the top significant SNPs (Z^2 *P* average $<.05$ over the 3 replicates; n=666) with (2) all genes detected by the microarray (n=4951).

STAGE 3: VALIDATION OF ASSOCIATION OF CANDIDATE SNPs WITH RESPONSE TO INTERFERON BETA THERAPY

In all SNPs selected for follow-up with individual genotyping, minor allele frequency differences between responders and nonresponders were significant (Z^2 binomial proportion tests, $P < .05$, data not shown). The average minor allele frequencies estimated using the triplicate microarrays were similar to the true frequencies obtained by individual genotyping (mean [SD] difference, 0.034 [0.03]). eTable 2 (available at http://archneur.ama-assn.org) lists the frequencies from DNA pooling on SNP microarrays and individual genotyping. In addition, there was little difference in the performance of each of the biological replicates (vs average triplicate data) in estimating absolute allele frequencies (range of mean [SD] absolute difference was 0.034 [0.029] to 0.039 [0.036]). The absolute estimates generated with DNA pooling on SNP microarrays were robust enough that we did not detect a considerable improvement in the estimation of allele frequency differences over absolute frequencies.

Using individually genotyped data, we were able to determine genotypes for each of the subjects. Genotype differences between responders and nonresponders to interferon beta therapy using individual genotyping were found in 29 of the 35 candidate SNPs (**Table 3**). We also genotyped 5 SNPs that were not significant in the pooled microarray screening data; these controls remained nonsignificant in the validation stage. Altogether, population allele frequency differences detected on SNP microarrays accurately represented differences between responder and nonresponder subjects.

STAGE IV: INCREASING THE POWER OF ASSOCIATION USING JOINT ANALYSIS

At this stage, we added an additional 81 subjects who were not available for the original screening to the analysis, recruited to the study using identical stringent inclusion criteria at the Barcelona, Pamplona, and Malaga centers (stage 1). This was possible because, overall, the clinical characteristics and genotypes of subjects were very similar between centers. Furthermore, southern Europeans from Spain, Italy, and southern France can be considered 1 genetic population.[10] With the original group of 206, we had 44% power to detect an odds ratio effect size of 2 when the minor allele frequency was 0.1, whereas the power was 60% in the joint population. With larger minor allele frequencies, for example 0.2 and 0.3, the power increased to 85% and 99% in the original population and 95% and 99% in the joint population.

In the joint analysis,[19] more than half of the SNPs remained significantly different between responders and nonresponders, and the significance of the effect increased in 5 SNPs (Table 3). Odds ratios for good interferon beta therapy response were calculated, comparing heterozygotes (Aa) or homozygote minors (aa) with homozygotes for the major allele (AA). The adjusted odds ratios were similar in effect size to unadjusted odds (data not shown). After adjustment for the baseline differences in relapse rate and baseline EDSS, candidate pharmacogenomic SNPs remained significant.

Two-thirds of the joint analysis group had information available on type of interferon beta therapy used. Among these subjects, there was no difference between responders and nonresponders in type of therapy (Avonex, Betaseron, or Rebif; $P = .35$).

COMPARISON WITH PREVIOUS STUDIES

We probed our screening data set to determine whether previously reported interferon beta candidate genes were significant in our population. We considered SNPs near or within 112 candidate genes investigated by others.[5-9] We searched for differences in SNP allele frequencies within the (1) 112 candidate genes (intragenic) and (2) 75 kilobase pairs to each side of their transcriptional start site (neighboring area), which captured SNPs in haplotype and promoter regions of genes. For 100 genes, we detected both intragenic (n=35) and neighboring (n=65) SNPs; other genes had no SNP representation on the microarray. We found 15 significantly different SNPs within or near 11 genes (**Table 4**). Five interferon-receptor SNPs were detected (1 *IFNAR1* and 4 *IFNAR2* SNPs), none of which distinguished between responders and nonresponders. The *IFNAR1* SNP rs1041429 has been studied previously; consistent with other studies, there is no strong pharmacogenomic relationship.[5,8] We did not detect dif-

Table 3. Top-Ranked Pharmacogenomic SNPs[a]

SNP ID	Chromosome	Gene	Stages 1-3: Original Population[b] DNA Pooling (Proportion Test)	Stages 1-3: Original Population[b] Individual Genotyping (Fisher Exact Test)	Stage 4: Joint Analysis[c] Individual Genotyping (Fisher Exact Test)	Adjusted Odds Ratio (95% Confidence Interval)[d] Aa:AA	Adjusted Odds Ratio (95% Confidence Interval)[d] aa:AA
		***P* Value Ranked**					
rs4466137	5	**Hyaluronan proteoglycan link protein (*HAPLN1*)**	0.0002	0.0010	**0.0040**	0.50 (0.29-0.84)[e]	0.22 (0.06-0.72)[e]
rs10492503	13	**Glypican 5 (*GPC5*)**	0.0000	0.0000	**0.0070**	0.51 (0.29-0.89)[e]	0.32 (0.13-0.81)[e]
rs1493663	11	Bone morphogenetic protein 8b (*BMP8B*), transfer RNA isopentenyltransferase 1 (*TRIT1*)	0.0017	0.0050	0.0740	0.61 (0.36-1.04)	3.85 (0.73-20.4)
rs4698555	4	Similar to 40S ribosomal protein S7 (*LOC645097*), hypothetical protein (*LOC729006*)	0.0000	0.0020	**0.0060**	0.62 (0.34-1.15)	0.30 (0.15-0.70)
rs986393	15	Leucine-rich repeat-containing 49 (*LRRC49*, linkage)	0.0000	0.0010	**0.0230**	0.64 (0.36-1.12)	0.39 (0.19-0.78)[e]
rs1172902[f]	9	*DIRAS* family, guanine nucleotide triphosphate–binding Ras-like 2 (*DIRAS2*), spleen tyrosine kinase (*SYK*)	0.0002	0.0460	0.3910	0.68 (0.37-1.21)	0.90 (0.28-2.87)
rs1327464	1	**Prostaglandin E receptor 3 (*PTGER3*)**	0.0001	0.0170	0.3270	0.79 (0.47-1.32)	0.60 (0.28-1.29)
rs952084	1	**WD-repeat domain (*FLJ32978*)**	0.0010	0.0170	**0.0050**	0.93 (0.55-1.57)	0.15 (0.42-0.56)[e]
rs10506738	12	E2F transcription factor 7 (*E2F7*), neuron navigator 3 (*NAV3*)	0.0002	0.0600	0.0740	1.34 (0.80-2.24)	3.28 (1.20-8.97)[e]
rs9301789	13	**GPC5 glypican 5 (*GPC5*)**	0.0001	0.0030	**0.0100**	1.44 (0.83-2.50)	2.83 (1.41-5.66)[g]
rs794143	4	**Collagen type XXV α1 (*COL25A1*)**	0.0002	0.0120	**0.0370**	1.63 (0.94-2.83)	2.27 (1.17-4.40)[e]
rs230275	1	Family with sequence similarity 5, member C (*FAM5C*), regulator of G-protein signaling 18 (*RGS18*)	0.0001	0.0060	0.0610	1.64 (0.92-2.94)	2.70 (0.91-8.01)
rs10512706	5	Mitogen responsive phosphoprotein (*DAB2*)	0.0001	0.0070	**0.0130**	1.73 (1.03-2.91)[e]	3.14 (1.33-7.42)[e]
rs1421784	5	Small nucleolar RNA, H/ACA box 6 (*LOC574040*)	0.0002	0.0010	**0.0030**	1.98 (1.15-3.39)[e]	3.87 (1.02-14.68)[e]
rs10494649	1	Similar to 60S ribosomal protein L6 (*LOC39118*, linkage)	0.0002	0.0080	**0.0020**	2.29 (1.33-3.94)[g]	2.75 (1.19-6.37)[e]
		Cluster Ranked 1					
rs2212774	21	Peptidylprolyl isomerase A pseudogene (*LOC653214*, linkage)	0.0140	0.0200	**0.0070**	0.47 (0.26-0.85)[e]	0.13 (0.02-0.68)[e]
rs1573400	18	Coiled-coil domain containing 102B (*CCDC102B*), docking protein 6 (*DOK6*)	0.0029	0.0030	0.0800	0.56 (0.33-0.94)[e]	0.49 (0.17-1.42)
rs538307	4	RasGEF domain family, member 1B (*RASGEF1B*), cytochrome c oxidase subunit Vb-like 1 (*COX5BL1*)	0.0271	0.5530	0.3300	0.70 (0.41-1.20)	0.79 (0.33-1.88)
rs1999333	21	Protein O-fucosyltransferase 2 (*POFUT2*, linkage)	0.0124	0.0280	**0.0120**	0.75 (0.45-1.25)	0.21 (0.07-0.61)[g]
rs1389357	X	Dachshund homologue 2 (*DACH2*), kelchlike 4 (*KLHL4*)	0.0265	0.3440	0.4310	1.23 (0.69-2.20)	1.73 (0.85-3.52)
rs239586	6	**TTK protein kinase (*TTK*)**	0.0236	0.0990	0.1840	1.49 (0.88-2.54)	1.34 (0.62-2.90)
rs7169847	15	Multiple C2 domains, transmembrane 2 (*MCTP2*),similar to glioma tumor suppressor candidate region gene 2 protein (*LOC220311*)	0.0123	0.0310	**0.0260**	1.65 (0.97-2.79)	2.30 (1.02-5.16)[e]
rs479341	1	**Selenoprotein (*SEP15*), heparan sulfate 2-O-sulfotransferase 1 (*HS2ST1*, linkage)**	0.0053	0.0140	0.0930	1.74 (1.00-3.01)[e]	1.65 (0.57-4.80)
rs10504026	8	Chromosome 8 open reading frame 4 (*C8ORF4*), zinc finger, matrin type 4 (*ZMAT4*)	0.0143	0.0280	**0.0370**	1.92 (1.14-3.24)[e]	1.29 (0.51-3.31)
rs10510779	3	**Calpastatin (*CAST*)**	0.0217	0.0140	**0.0420**	1.95 (1.09-3.48)[e]	0.85 (0.18-4.05)

(continued)

ferences in the frequency of SNPs in or near *LMP7*, *CTSS*, or *MxA*. Our microarray did not contain the same promoter SNPs studied by Cunningham and colleagues[7] and Nicolae and colleagues[20] in the interferon-stimulated response elements of *LMP7*, *CTSS*, or *MxA*, so a direct comparison was not possible.

COMMENT

We completed a nonbiased genome-wide pharmacogenomic screen of interferon beta therapy response in relapsing-remitting MS, using a DNA pooling strategy on microarrays. This method was shown to be reliable, with outstanding correlation in frequency between our 3 replicates. In addition, allele frequencies were similar to the true population allele frequencies obtained using individual genotyping. Of 35 SNPs selected in stage 2, 18 SNPs maintained significance in a follow-up analysis with additional subjects: 10 of 15 of those significance ranked, 5 of 10 of those cluster ranked with a $P < .05$ cutoff, and 3 of 10 of those cluster ranked with a $P < .01$ threshold. Single-nucleotide polymorphisms selected using the sig-

Table 3. Top-Ranked Pharmacogenomic SNPs[a] (cont)

SNP ID	Chromosome	Gene	Stages 1-3: Original Population[b] DNA Pooling (Proportion Test)	Stages 1-3: Original Population[b] Individual Genotyping (Fisher Exact Test)	Stage 4: Joint Analysis[c] Individual Genotyping (Fisher Exact Test)	Adjusted Odds Ratio (95% Confidence Interval)[d] Aa:AA	Adjusted Odds Ratio (95% Confidence Interval)[d] aa:AA
		Cluster Ranked 2					
rs9294145	6	**Diazepam binding inhibitorlike 2 (*DBIL2*)**	0.0097	0.0210	0.1100	0.49 (0.26-0.93)[e]	0.50 (0.08-3.27)
rs6944054	7	**Similar to dynein, protein inhibitor of NOS (*LOC442331*)**	0.0007	0.0040	**0.0140**	0.51 (0.29-0.88)[e]	0.43 (0.19-0.97)[e]
rs10521996	X	**Dystrophin (*DMD*)**	0.0046	0.2030	0.3380	0.65 (0.34-1.22)	1.25 (0.47-3.33)
rs9287889	2	**Serine threonine kinase (*STK39*)**	0.0009	0.2620	0.1280	0.66 (0.36-1.19)	0.48 (0.24-0.94)[e]
rs1109542	16	**Cadherin (*CDH13*)**	0.0057	0.0030	0.0750	0.79 (0.44-1.41)	0.49 (0.25-0.97)[e]
rs137219	22	**Similar to circumsporozoite protein precursor (*LOC402059*)**	0.0025	0.0100	0.0990	0.88 (0.51-1.50)	0.40 (0.18-0.90)[e]
rs9325096	5	**F-box protein 38 (*FBX038*)**	0.0070	0.0300	0.1180	1.58 (0.93-2.69)	0.65 (0.20-2.18)
rs581405	1	**Selenoprotein (*SEP15*)**	0.0045	0.0280	0.0910	1.72 (0.99-2.98)	2.20 (0.66-7.32)
rs4855469	3	**Chemokinelike (C-C motif) (*TAFA1*)**	0.0003	0.0000	**0.0100**	1.76 (0.93-3.33)	3.04 (1.47-6.28)[e]
rs4128599	14	**Neuronal PAS domain protein 3 (*NPAS3*)**	0.0047	0.0040	**0.0240**	1.85 (1.07-3.17)[e]	2.30 (0.85-6.27)
		Nonsignificant Controls					
rs966087	5	Lamin B1 (*LMNB1*)	0.0315	0.2900	NA	NA	NA
rs968355	5	Lamin B1 (*LMNB1*)	0.3431	0.5700	NA	NA	NA
rs484888	1	Immunoglobulin superfamily member 3 (*IGSF3*)	0.3108	0.6100	NA	NA	NA
rs2395182	6	Major histocompatibility complex, class II DR alpha (*HLA-DRA*)	0.2362	0.8500	NA	NA	NA
rs546354	1	Eukaryotic translation initiation factor 2B(*IEF2B3*)	0.2773	1.0000	NA	NA	NA

Abbreviations: AA, homozygote major; Aa, heterozygote major; aa, homozygote minor; NA, not applicable; SNP, single-nucleotide polymorphism.

[a] Thirty-five SNPs were selected as pharmacogenomic candidates using 3 ranking strategies based on triplicate SNP microarray data: (1) *P* value ranking: SNPs with $P<.0005$ in 3 of 3 replicates or $P<.00005$ in 2 of 3 replicates, (2) cluster ranking 1: SNPs with $P<.05$ in clusters of 4 or greater, and (3) cluster ranking 2: SNPs with $P<.01$ in clusters (stages 1-3, DNA pooling). We confirmed the genotype frequency differences between responders and nonresponders by individually genotyping the original pooled population, using Fisher exact tests to evaluate significance (stages 1-3, individual genotyping). We then evaluated whether the same SNPs distinguished responders and nonresponders in a joint analysis that included 81 additional subjects (stage 4, individual genotyping). We confirmed the nonsignificance of 5 control SNPs that did not differ between responders and nonresponders in the SNP microarray data. Bolding indicates that SNPs are associated with the gene, and regular text is used for the nearest upstream and downstream genes or genes in linkage with the SNP.

[b] Total of 204 patients, 107 nonresponders and 97 responders.

[c] Total of 285 patients, 142 nonresponders and 143 responders.

[d] Odds ratios for good response were calculated for (1) heterozygote vs homozygote major and (2) homozygote minor vs homozygote major and were adjusted for baseline disability and relapses prior to therapy.

[e] $P<.05$.

[f] The genotyping results for rs1172902 were difficult to interpret; results are included for completeness.

[g] $P<.005$.

nificance-based ranking system were the most likely to replicate, probably because they were associated with the most extreme allele frequency differences. The magnitude and direction of the estimated effects are reflected in the odds ratios in Table 3. Their persistent significance after adjustment for baseline disability and relapse rate increases confidence that the findings reflect true differences between responders and nonresponders. Given how few microarrays are required for DNA pooling studies, future pooled SNP microarray pharmacogenomic and association studies completed in triplicate could result in cost savings compared with individual microarray genotyping.

Candidate SNPs that significantly differed between responders and nonresponders in the final joint analysis included 7 located within genes: *glypican 5*, *collagen type XXV α1*, *hyaluronan proteoglycan link protein*, *calpastatin*, *TAFA1* (chemokinelike), *neuronal PAS domain protein 3*, and *LOC442331* (similar to dynein). The remaining SNPs are located in intergenic regions.[21] These may represent long linkage disequilibrium with annotated genes or polymorphisms in distant cis-regulatory regions. The results reflect the pleiotropic action of interferon beta and complex nature of MS. Results of the gene ontology classification—suggesting an enrichment of *glutamate* and *γ-aminobutyric acid* receptors in pharmacogenomic candidate genes—are provocative, implying an interaction between neuronal excitation and interferon beta therapy effect. This potential connection needs further exploration.

Glypican 5 polymorphisms arose several times in our candidate SNP lists. Glypicans are 1 of 2 main classes of heparan sulfate proteoglycans. They are implicated in synapse formation and axon regeneration and guidance and are found in dense networks in active MS plaques, where they may be involved in sequestering proinflammatory chemokines.[22,23] In the peripheral nervous system, glypican 1 is required for Schwann cell myelination; glypican 5 is highly expressed in neurons.[24] Interferon beta may affect the expression of glypicans, as occurs with in-

Table 4. Candidate SNPs From Other Studies That Are Significantly Different Between Responders and Nonresponders[a]

Chromosome	Gene	Intragenic		Neighboring Area		Significant SNPs (Associated Gene)
		No. of Significant SNPs/Total SNPs Detected	Z^2 *P* Value Average	No. of Significant SNPs/Total SNPs Detected	Z^2 *P* Value Average	
1	Adenosine deaminase (*ADAR*)	0/1	. . .	1/1	.0015	rs4131514
1	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2) (*PTGS2*)	. . .	. . .	2/14	.0192; .0363	rs10494593; rs1924743
2	Interferon-inducible double-stranded RNA (*PRKR*)	0/1	. . .	1/4	.0212	rs728005 (*OSBPL6*)
3	Synapsin II (*SYN2*)	1/7	.0219	1/2	.0484	rs60834, rs795000
4	Complement factor I (IF [CFI])	. . .	. . .	2/6	.0055; .0093	rs1990250; rs6814789
9	Osteoglycin (*OGN*)	. . .	. . .	1/2	.0119	rs1121979
11	Tumor necrosis factor receptor–associated factor 6 (*TRAF6*)	1/7	.0352	0/2	. . .	rs10501154
11	Caspase 1 (*CASP1*)	1/1	.0369	0/11	. . .	rs580253
12	Interleukin 22 (IL-22)	0/2	. . .	1/14	.0378	rs10506559
12	Interferon gamma (IFN-γ)	. . .	. . .	2/13	.0458; .0343	rs10492199; rs10492198
20	DNA-directed RNA polymerase III 39-kDa polypeptide F (*POLR3F*)	. . .	. . .	1/3	.0305	rs3736775 (*SEC23B*)

Abbreviation: SNP, single-nucleotide polymorphism.

[a]We interrogated our triplicate microarray data to pursue 112 genes hypothesized in previous articles to have an interferon beta pharmacogenomic effect in multiple sclerosis. One hundred genes had SNP representation on the microarray in or near (75 kilobases upstream or downstream) the gene of interest. Fifteen candidate SNPs in pharmacogenomic areas proposed by Cunningham and colleagues[7] distinguished responders and nonresponders (Z^2 binomial *P* average over 3 replicates $< .05$). Three were located in genes of interest; 12 were in neighboring areas.

terferon gamma and glypican,[25] and the interaction of glypicans with growth factors, chemokines, and extracellular matrix proteins, including those detected in our pharmacogenomic analysis, may affect neuronal growth and repair.

Polymorphisms in extracellular matrix proteins, such as hyaluronan proteoglycans and collagen, were also significant in this study. These polymorphisms may affect the binding of matrix metalloproteinases, which are released by leukocytes and aid in their migration through basement membranes. In vitro, interferon beta inhibits production and secretion of these metalloproteinases,[26,27] and polymorphisms in extracellular matrix proteins could further alter the efficacy of interferon beta therapy.

We also interrogated the microarrays for SNPs in genes previously considered pharmacogenomic candidates. Consistent with other studies, we did not find a pharmacogenomic relationship between the SNPs we detected in or near interferon receptors *IFNAR1* and *IFNAR2*. Single-nucleotide polymorphisms in or near the interferon-stimulated genes *LMP7*, *CTSS*, or *MxA* were not significant in this analysis. We were, however, limited to the SNPs detected by the Affymetrix 100K microarray, which represents a fraction of common polymorphisms in the genome and oversamples intergenic areas.[20]

We did not have the power to detect effects in rare alleles. Odds ratios for effects compare AA individuals with Aa and aa individuals, which may not reflect the true pattern of effect. While results are based on triplicate microarray data to reduce spurious findings, some of our findings are likely false positives, given the lack of stringent correction for multiple comparisons in the individual genotyping stage. In an attempt to address potential confounding by baseline differences between responders and nonresponders, we adjusted odds ratios for baseline EDSS and pretreatment relapse rate. Without a placebo group, however, we cannot definitively distinguish between the natural history of MS and response to treatment.

Neutralizing antibodies (Nabs) may confound the relationship between SNPs and pharmacogenomic response, since Nabs are associated with interferon beta and persistently high titers of Nabs may be associated with decreased effectiveness of interferon beta therapy.[28] Some of the significant SNPs identified herein may be related to Nab status, something we could not examine because Nabs were not measured in all subjects. Finding SNPs related to Nabs would be of great interest, nevertheless, to better understand the mechanism associated with Nab production.

The beneficial outcomes of interferon beta therapy for patients in the relapsing-remitting phase of MS have been clearly shown. On the other hand, the effect of this treatment is partial, and a substantial amount of patients are not responders. Hence, in the absence of prognostic clinical, neuroradiological, and/or immunological markers of response, the question remains who and when to treat when adverse effects, inconvenience, and cost of the drug are significant. The identification of pharmacogenetic polymorphisms provides important new insights into the mechanism of interferon beta action, bringing the paradigms of rational drug design and personalized medicine one step further. These results, however, require replication in a larger, prospective data set and confirmation in functional assays to directly assess the relationship between genotypes, control of cell division, and response to immunotherapy. Because of the heterogeneous and multifactorial nature of MS and the complex role of interferon beta in the

immune response, multianalytical approaches that incorporate and integrate genomic, laboratory, and clinical data will be necessary to predict therapeutic outcomes based on molecular evidence.

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eTable 1. Correlation of Population Allele Frequencies in Microarray Replicates in 206 Patients[a]

	Correlation, r^2		
	Rep 1	Rep 2	Rep 3
Nonresponder Frequencies in the 3 Replicates			
Rep 1	1 [Reference]		
Rep 2	0.99	1 [Reference]	
Rep 3	0.99	0.99	1 [Reference]
Responder Frequencies in the 3 Replicates			
Rep 1	1 [Reference]		
Rep 2	0.99	1 [Reference]	
Rep 3	0.99	0.99	1 [Reference]
Nonresponders, Most Significant SNPs (Top 5%)			
Rep 1	1 [Reference]		
Rep 2	0.99	1 [Reference]	
Rep 3	0.99	0.99	1 [Reference]
Responders, Most Significant SNPs (Top 5%)			
Rep 1	1 [Reference]		
Rep 2	0.99	1 [Reference]	
Rep 3	0.99	0.99	1 [Reference]

Abbreviations: Rep, replicate; SNP, single-nucleotide polymorphism.

[a]There was a high degree of correlation in allele frequencies in the replicate microarray pools. This increased when considering the SNPs that are most significantly different in allele frequency between responders and nonresponders. Among the 40 SNPs investigated in this study, the standard deviation of minor allele frequency estimates by the 3 SNP microarray pool replicates was 0.017 for nonresponders and 0.018 for responders.

eTable 2. Comparison of Minor Allele Frequencies Between Pooled Microarray Data and Individual Genotyping[a]

	100K Microarray Pooled DNA							
	Nonresponders			Responders			Individual Genotyping	
SNP ID	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Nonresponders	Responders
***P* Value Ranked**								
rs4466137	0.310	0.269	0.250	0.117	0.112	0.105	0.304	0.149
rs10492503	0.422	0.402	0.348	0.163	0.198	0.159	0.343	0.152
rs1493663	0.254	0.298	0.282	0.142	0.107	0.088	0.210	0.125
rs4698555	0.182	0.205	0.172	0.049	0.022	0.021	0.168	0.057
rs986393	0.448	0.432	0.416	0.338	0.368	0.368	0.457	0.353
rs1172902	0.417	0.389	0.453	0.248	0.220	0.238	0.378	0.263
rs1327464	0.492	0.475	0.457	0.303	0.295	0.275	0.415	0.279
rs952084	0.311	0.248	0.296	0.103	0.130	0.105	0.298	0.180
rs10506738	0.241	0.169	0.223	0.411	0.421	0.411	0.236	0.345
rs9301789	0.393	0.384	0.391	0.390	0.437	0.402	0.369	0.458
rs794143	0.255	0.301	0.304	0.478	0.480	0.483	0.332	0.490
rs230275	0.109	0.133	0.126	0.295	0.293	0.286	0.127	0.258
rs10512706	0.270	0.246	0.274	0.464	0.462	0.460	0.276	0.428
rs1421784	0.121	0.131	0.122	0.276	0.293	0.295	0.119	0.271
rs10494649	0.205	0.227	0.198	0.384	0.395	0.395	0.218	0.366
Cluster Ranked 1								
rs2212774	0.283	0.297	0.310	0.201	0.112	0.169	0.207	0.100
rs1573400	0.367	0.365	0.354	0.211	0.223	0.229	0.308	0.167
rs538307	0.265	0.283	0.305	0.186	0.197	0.187	0.286	0.240
rs1999333	0.357	0.386	0.339	0.241	0.274	0.230	0.330	0.222
rs1389359	0.299	0.280	0.281	0.396	0.379	0.385	0.264	0.339
rs239586	0.367	0.358	0.333	0.480	0.447	0.462	0.325	0.421
rs7169847	0.327	0.296	0.322	0.431	0.460	0.444	0.288	0.411
rs479341	0.176	0.146	0.161	0.286	0.262	0.296	0.120	0.237
rs10504026	0.218	0.215	0.196	0.360	0.299	0.334	0.226	0.328
rs10510779	0.132	0.101	0.111	0.229	0.171	0.199	0.095	0.184
Cluster Ranked 2								
rs9294145	0.108	0.150	0.134	0.044	0.047	0.047	0.264	0.063
rs6944054	0.456	0.429	0.453	0.266	0.278	0.276	0.443	0.282
rs10521996	0.285	0.322	0.280	0.138	0.195	0.171	0.199	0.168
rs9287889	0.397	0.424	0.411	0.448	0.375	0.408	0.533	0.447
rs1109542	0.484	0.494	0.485	0.318	0.378	0.358	0.538	0.365
rs137219	0.454	0.447	0.428	0.319	0.270	0.280	0.432	0.394
rs9325096	0.201	0.174	0.189	0.329	0.302	0.294	0.201	0.288
rs581405	0.140	0.099	0.110	0.245	0.211	0.236	0.127	0.232
rs4855469	0.460	0.478	0.469	0.315	0.355	0.340	0.538	0.326
rs4128599	0.201	0.176	0.189	0.325	0.297	0.317	0.184	0.333
Nonsignificant Controls								
rs968355	0.488	0.456	0.458	0.488	0.456	0.458	0.422	0.402
rs966087	0.453	0.460	0.466	0.453	0.460	0.466	0.396	0.318
rs484888	0.428	0.444	0.383	0.428	0.444	0.383	0.421	0.453
rs2395182	0.347	0.333	0.329	0.347	0.333	0.329	0.340	0.316
rs546354	0.190	0.167	0.177	0.190	0.167	0.177	0.171	0.170

Abbreviations: MAF, minor allele frequency; Rep, replicate; SNP, single-nucleotide polymorphism.

[a]Estimation of absolute MAFs by DNA pooling on SNP microarrays: In general, absolute MAFs were estimated well by SNP microarray. When averaging the triplicate data, the MAF from SNP microarray differed from individual genotyping by 0.035 for nonresponders (SD, 0.033; minimum, 0.001; maximum, 0.134) and 0.033 for responders (SD, 0.028; minimum, 0.000; maximum, 0.142). However, most studies are unlikely to be completed in triplicate. Comparison of each of the replicate SNP microarray frequency estimates with individual genotyping demonstrated similarly accurate MAF estimates. The average difference between each SNP microarray replicate and individual genotyping was 0.034 to 0.039 (SD, 0.029-0.036; minimum, 0.000; maximum, 0.157). Estimation of MAF differences by SNP microarray: Generally, SNP microarray underestimated MAF differences compared with the real (individual genotyping) frequency differences. On average, the difference in MAF between responders and nonresponders was 0.19 across all 40 SNPs examined. For each of the replicates, the average MAF difference between responders and nonresponders across the same SNPs was 0.012 in replicates 1 and 2 and 0.010 in replicate 3. Comparison of MAF differences, instead of absolute values, did not tend to improve the ability to detect reliable differences, because the differences were similarly skewed. There did not appear to be an advantage to comparing MAF differences instead of absolute values because (1) absolute estimates were quite accurate and (2) the error in estimation often only affected 1 of the 2 comparison groups. If differential fluorescence had resulted in absolute MAF estimation errors in both responders and nonresponders, then the effect would have likely canceled out when differences were considered.