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Interleukin-4 receptor alpha polymorphisms in autoimmune myasthenia gravis in a Caucasian population

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ABSTRACT

Autoimmune myasthenia gravis is a T-cell–dependent, antibody-mediated, rare neuromuscular disorder. Interleukin-4, acting via interleukin-4 receptor alpha, plays a pivotal role in B-cell differentiation and antibody production and has been implicated to influence disease progression in experimental autoimmune myasthenia gravis. Polymorphisms of the *interleukin-4 receptor alpha* gene have been shown to be associated with various autoimmune diseases. We compared the distribution of three polymorphisms of the *interleukin-4 receptor alpha* gene (S503P, rs1805015, Q576R, rs1801275, I75V, rs1805010), all affecting interleukin-4 signaling, in two cohorts of myasthenia gravis patients with ethnically matched controls. Although the distribution of the S503P and Q576R polymorphisms did not differ significantly between the groups, the frequency of the GG rare homozygote genotype of the I75V polymorphism was significantly higher in patients with myasthenia gravis. Our data suggest that the reduced responsiveness to interleukin-4 because the I75V polymorphism may contribute to the pathogenesis of myasthenia gravis.

1. Introduction

Pathologic muscle fatigue, the characteristic symptom of autoimmune myasthenia gravis (MG), is caused by autoantibodies directed against different proteins of the neuromuscular junction, most commonly antinicotinic acetylcholine receptor (AChR) antibodies in 70% to 90% of the cases, less frequently anti–muscle-specific kinase (MuSK) antibodies.

By modulating the synthesis of high affinity antibodies, $CD4^+$ T cells and their secreted cytokines play a vital role in the pathomechanism of MG. T helper 1 (Th1) cytokines may induce the synthesis of IgGs capable of fixing complement, and thus, facilitating the destruction of the neuromuscular junction. Th2 cytokines have anti-inflammatory properties, and have diverse effects on MG (for review, see Conti-Fine et al. [1]). Among these cytokines, interleukin-4 (IL-4) is a potent growth and differentiation factor for B cells and stimulates class-switching and autoantibody production [2] but has also been suggested to take part in the modulation of regulatory T-cell functions [3,4]. The signal of IL-4 is mediated by the interleukin-4 receptor alpha (IL4Rα) chain which is part of both the IL4R and the IL13R. Of the seven nonsynonymous polymorphisms found in the coding sequence of *IL4R*α gene in the Caucasian population, three (I75V, S503P, Q576R) have been shown to modulate the signal transduction pathway of IL-4 via STAT6 protein or by synergizing with STAT6 actions [5–7]. The role of these polymorphisms in different immune disorders has been well established, and associations have been demonstrated in exacerbation of allergic asthma [8], multiple sclerosis [9] and erosive rheumatoid arthritis [10]. Here we report a case-control study, where we investigated the association of these polymorphisms with MG.

2. Subjects and methods

2.1. Patients and controls

For the initial genotype analysis clinical data and blood samples were collected from 164 Hungarian MG patients treated in 2 major Hungarian centers, the Neurology Department of Debrecen University and the Centre for Molecular Neurology at Semmelweis University, Budapest. The clinical data and samples of these patients were used to build the NEPSYBANK (Hungarian Neurological and Psychiatric Biobank) [11]. To confirm data, a second cohort consisting of further 96 Hungarian MG patients was analyzed; these samples and clinical data were obtained from 2 other major MG centers, the Neurology Departments of Pécs University and the Jahn Ferenc Teaching Hospital, Bu-

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dapest. The controls included 299 Hungarian blood donors and individuals participating on routine check-ups. These people also filled out a questionnaire about their previous diseases. Subjects with autoimmune disorders were excluded from the study. Diagnosis of MG was based on standard criteria, including symptoms of fluctuating muscle weakness supported by an electromyographic pattern of neuromuscular transmission dysfunction by repetitive stimulation as well as by anti-AChR antibody positivity. Age at onset was determined using previous medical documentation or the patient's recollection. Data of patients and controls are summarized in Table 1. This study was carried out according to the Declaration of Helsinki and was approved by the local Ethical Committees.

2.2. Determination of anti-AChR and anti-MuSK antibody concentration

AChR-specific and MuSK-specific antibody levels were measured by radioimmunopercipitation assays (both from DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. Briefly, ^{125}I-labeled α-bungarotoxin or ^{125}I-labeled MuSK, respectively, was incubated with test sera. Complexes of labeled receptor and antibodies were immunoprecipitated with anti– human IgG and counted in a γ-counter. The measured radioactivity was directly proportional to the concentration of anti-AChR or anti-MuSK autoantibodies of the sample.

2.3. DNA isolation and polymorphism analysis

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA blood and tissue mini kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. Genotypes were determined using TaqMan Allelic Discrimination Assays (Assay IDs: C_8903092_20, C_2351160_20 and C_2769554 _10 for IL4R rs1805015, rs1801275 and rs1805010, respectively; Applied Biosystems, Foster City, CA). Polymerase chain reactions (PCR) were carried out in 96-well format with an ABIPRISM 7000 Sequence Detector (Applied Biosystems) with mixes consisting of 10 ng of genomic DNA, 5 μl of TaqMan Universal PCR Master Mix, 0.25 μl of 40x Assay Mix, and double-distilled water up to 10 μl final volume. Thermal cycle conditions were as follows: denaturation at 95°C 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, and annealing and extension at 60°C for 1 minute. After PCR the endpoint fluorescence intensity was read in each well. The allelic specific intensity data of each plate were analyzed using the ABI PRISM 7000 software (Applied Biosystems) to automatically determine the genotype of each sample.

Table 1
Characteristics of the study population

	Patients, cohorts 1 + 2	Controls
Characteristic	164 + 96	299
Gender, n (% of all)		
Male	33 + 38 (27)	97 (32)
Female	131 + 58 (73)	202 (68)
Age (mean ± SD, years)	49 ± 13.2 and 56.9 ± 15.8 (Ranges: 18–78 and 18–84)	37.9 ±13.31 (Range: 18–86)
Age at onset (mean ± SD, years)	36.02 ± 13.04 and 46.3 ± 13.6 (Ranges: 7–80 and 17–80)	—
AChR antibody positivity n (%)	118 + 96 (82% of all cases)	—
Distribution of weakness n (%) [1]		
Ocular	31 + 0 (12% of all cases)	—
Generalized	133 + 96 (88% of all cases)	

Cohort 1 represents the patients enrolled from the Neurology Department of Debrecen University (Eastern Hungary) and of the Centre for Molecular Neurology of Semmelweis University, Budapest (center part of Hungary). Patients of cohort 2 were enrolled form the Neurology Department of Pécs (southern part of Hungary) and Jahn Ferenc Teaching Hospital, Budapest, Hungary.

2.4. Statistical analysis

Genotype association and Cochran-Armitage tests were performed using an online Hardy–Weinberg equilibrium (HWE) calculator (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) based on Wigginton et al. [12]. As age and gender differed significantly in the control and patients groups; correction was made with logistic regression using SPSS 15.1 (SPSS, Inc., Chicago, IL). The strength of association was represented by exponentiation of the B value (ExB) in logistic regression models with 95% confidence interval (CI). p Values <0.05 were considered significant. Since three loci were analyzed, multiple comparisons have also been done. The Bonferroni significance level was $p < 0.017$.

3. Results

All polymorphisms in this study were in Hardy–Weinberg equilibrium. Distributions of the 3 examined polymorphisms were evaluated in groups of patients with MG and healthy controls. Frequencies of the *IL4Rα* genotypes were also compared among controls and patients grouped according to anti-AChR positivity, gender (women only), age at onset (<40 years), and distribution of muscle weakness (generalized form as first symptom). In logistic regression models containing age and gender, none of the examined SNPs were significantly associated with MG. Different combinations of heterozygous and homozygous groups within one genotype were also analyzed. In case of the S503P and Q576R polymorphisms the number of individuals harboring the rare homozygote form was very low in both the patient and control groups. Thus, for more accurate analysis the combination of the rare homozygote and heterozygote groups was needed, but it did not reveal any association in case of these polymorphisms in the combined analysis either (data not shown).

However, concerning the I75V polymorphism, the rare GG genotype, which results in 2 copies of the V75 allele, was significantly associated with MG (p: 0.023, ExB: 1.78, CI: 1.08–3.08). This association could also be seen in the "women only" group (p: 0.019, ExB: 1.88, CI: 0.136–3.4) and in the subgroup of patients with anti-AChR antibody positivity (p: 0.027, ExB: 1.84, CI: 1.07–3.16). No association could be found in the other investigated groups. The significant associations were also analyzed in a second cohort of MG patients. Here, a significant association could be confirmed with MG (p = 0.042, ExB = 1.91, CI = 1.02–3.55) and the anti-AChR antibody positive subgroup (as in this cohort all MG patients were also anti-AChR antibody positive, p = 0.042, ExB = 1.91, CI = 1.02–3.55). However, no association could be found in the "women only" subgroup (p = 0.297), probably because of the small sample size. Comparison of the distribution of the GG genotype between the combined (merged) MG cohorts and control group also gave a significant difference in the case of the whole MG group (p = 0.017, ExB = 1.74, CI = 1.1–2.72). Similarly, significant differences have been detected in the cases of the anti-AChR–positive (p = 0.018, ExB = 1.77, CI = 1.1–2.84) and the "women only" subgroups (p 0.024, ExB = 1.81, CI = 1.09–3) (association of these subgroups are shown in Table 2). After Bonferroni correction, however, a significant difference in the I75V GG genotype distribution could only be demonstrated when comparing the control group with the merged MG cohorts, but could not be found when either the individual cohorts or subgroups were analyzed separately.

In the anti-AChR negative patients, only three patients were anti-MuSK positive with radioimmunoprecipitation, thus this subgroup was not analyzed further. Where appropriate, analysis was also done with excluding this small population, however the results did not change significantly.

4. Discussion

IL-4, acting through IL4Rα, has multiple roles in the pathomechanism of MG [3,4,13,14]. While stimulating isotype switching

Table 2
Association between IL4Rα nonsynonymous coding SNPs

	MG			Women only			Anti-AChR–positive MG		
Genotype	Patients	Controls	*p*	Patients	Controls	*p*	Patients	Controls	*p*
IL4Rα I75V*									
AA (I75/I75)	48 + 28	83		44 + 19	57		32 + 28	83	
AG (I75/V75)	69 + 41	157	0.08	49 + 24	102	0.199	51 + 41	157	0.07
GG (V75/V75)	43 + 26	47		37 + 15	30		32 + 26	47	
IL4Rα I75V**									
GG	43 + 26	47		37 + 15	30		32 + 26	47	
AA + AG	117 + 69	240	0.017	93 + 43	159	0.024	83 + 69	240	0.018
IL4Rα S503P*									
TT (S503/S503)	115 + 76	198		90 + 46	122		83 + 76	198	
TC (S503/P503)	43 + 17	68	0.417	38 + 11	52	0.481	32 + 17	68	0.394
CC (P503/P503)	4 + 1	9		3 + 1	5		2 + 1	9	
IL4Rα Q576R*									
AA (Q576/Q576)	97 + 65	184		79 + 37	116		72 + 65	184	
AG (Q576/R576)	56 + 28	84	0.414	45 + 20	63	0.329	37 + 28	84	0.621
GG (R576/R576)	8 + 1	13		7 + 1	6		6 + 1	13	

Associations were determined with (i) myasthenia gravis (MG), (ii) women MG patients compared with women controls, as well as (iii) anti-AChR positive MG patients compared with controls. Numbers of people (patients of cohorts 1 and 2 and controls) successfully genotyped for the given SNP are shown. The GG variant of the I75V polymorphism is associated with MG, the "women only" subgroup and in patients with anti-AChR positivity. Because of the small sample size, men were not analyzed separately. The numbers represent the successfully genotyped cases of patients of cohorts 1 and 2 as well as people of the control group. The shown *p* values indicate the cases where the 2 MG cohorts were combined and were compared with the control group. Values were calculated using logistic regression.

and antibody production, IL-4 probably also has a protective effect in experimental MG, possibly by the induction of peripheral tolerance via regulatory T-cells [3,4,13,14].

The first *IL-4* gene polymorphism association study in MG investigated a Swedish cohort. Analysis of a dinucleotide polymorphism of the second, and a variable number of tandem repeat of the third intronic regions of the *IL-4* gene, with a putative role in influencing IL-4 levels, showed no association with MG [15].

Here we investigated the association of 3 functional polymorphisms of the *IL4Rα* gene with MG. Although the S503P and Q576R polymorphisms showed no association, and although no allelic association could be found with the I75V SNP either, we found that the homozygous G-allele carriage of the I75V polymorphism, resulting in the V75 variation, was significantly higher in the MG population, the women-only subgroup and in patients with anti-AChR antibodies.

In a study performed in rheumatoid arthritis patients, individuals with such homozygous GG genotype had more severe disease with bone erosions compared with patients carrying zero or one G allele [10]. The I75V amino acid substitution in the extracellular domain of the protein, enhances IL4R signaling [7], with the I75 variant being a "gain of function" mutation and thus having a greater signal transduction capacity via STAT6 compared with the V75 variant [10,16].

In this study performed in myasthenia gravis patients, we report that the GG genotype is associated with the disease. The reduced IL-4 signal transduction on $CD4^+$ T cells from V75/V75 could either cause a shift in the Th balance towards Th1 responses, or reduce the function of regulatory T-cells, which could influence the pathomechanism of the disease. However, the exact role of this polymorphism in the pathogenesis of human autoimmune MG will need to be further investigated. Also, due to the small population size of our anti-MuSK positive MG patients, we could not perform correct statistical analysis. It would be interesting to see however, where there is also association in this subgroup, as this antibody is mostly of the IgG4 subclass, which is an IL-4 driven Th2 isotype [17].

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