Polygenic Disease Associations in Thymomatous Myasthenia Gravis

Christian Amdahl; Espen H. Alseth; Nils E. Gilhus, MD, PhD; Hanne L. Nakkestad; Geir O. Skeie, MD, PhD

Background: Relevant genetic markers for myasthenia gravis (MG) include tumor necrosis factors \( \alpha \) and \( \beta \), Fc\( \gamma \) receptor IIa, and interleukin 10. The corresponding gene products are thought to be involved in MG pathogenesis.

Objectives: To investigate whether MG susceptibility correlates with specific combinations of genetic markers and to compare the contribution of each marker.

Participants: Forty-seven patients with MG and 92 healthy blood donors.

Main Outcome Measures: Presence of tumor necrosis factors \( \alpha \) and \( \beta \), Fc\( \gamma \) receptor IIa, and interleukin 10 genotypes and autoantibodies against nicotinic acetylcholine receptor, titin, and ryanodine receptor.

Results: Susceptibility to MG increases with an increasing number of genetic markers in both thymomatous MG and MG with titin antibodies but not in early-onset MG. In thymomatous MG, Fc\( \gamma \) receptor IIa allelic variants seem to be the most important determinant of disease.

Conclusion: Specific combinations of allelic variants individually associated with MG synergize in predisposing to thymomatous MG and MG with titin antibodies.

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Myasthenia gravis (MG) is an autoimmune disease characterized by fluctuating pathologic weakness involving one or several skeletal muscle groups. It is primarily caused by antibodies (Abs) to the nicotinic acetylcholine receptor (AChR) at the postsynaptic site of the neuromuscular junction. The disease is heterogeneous and is classified by age at onset and pathologic findings in the thymus. In 30% of patients with MG, onset is early (EO-MG; onset before age 50 years), and in 60%, onset is late (LO-MG; onset at age 50 years or older), and 10% of patients have a thymoma. Patients with LO-MG and thymoma have autoantibodies against the muscle proteins titin (its myasthenia gravis titin 30-kDa region) and ryanodine receptor. Their presence correlates with more severe disease and should prompt the search for a thymoma.

Several polymorphic sites in immunoregulatory genes influence the immune response, including encoding tumor necrosis factor \( \alpha \) (TNFA), encoding tumor necrosis factor \( \beta \) (TNFB), encoding Fc\( \gamma \) receptor IIa (FCGR2A), and encoding interleukin 10 (IL-10). Susceptibility to MG is linked to a number of such allelic variants. Early-onset MG is associated with HLA-A1\*B8\*DR3, TNFA\*T2, TNFB\*1, FCGR2A 131R/R, and IL-10 genotype ATA/ATA (G.O.S., unpublished data, 2007). Late-onset MG is associated with HLA-A3\*B7\*DR2 and HLA-DR4. In thymomatous MG, there are no strong HLA associations, although some investigators have reported a higher frequency of HLA DQB1*0604 in thymomatous MG and of HLA Dw15 in young women with thymoma. Thymomatous MG is also associated with TNFA\*T1, TNFB\*2, GM 1, 2, 3 23 5, 21, FCGR2A 131H/H, and IL-10 genotype ACC/ACC (G.O.S., unpublished data, 2007). Nonthymomatous titin Ab–positive MG is associated with HLA-DR7, and titin Ab–negative MG is associated with HLA-DR3. Because most associations are rather weak and MG is probably a polygenic disease, we examined allelic variants in several MG-associated genes to look for synergy in predisposition.

Methods

Patients and Control Subjects

The study included 47 patients with generalized MG (18 with EO-MG, 19 with LO-MG, and 10 with thymomatous MG) and 92 healthy
blood donors. All participants were white Norwegians; none were related. The diagnosis of MG was based on typical clinical features, the presence of AChR Abs in all patients, positive results of edrophonium chloride testing, and typical findings at neurophysiologic examination (decrement >10% at 3 Hz after repetitive motor nerve stimulation, increased jitter on a single-fiber electromyogram, or both). The diagnosis of thymoma was based on computed tomographic findings in the mediastinum and confirmed at thymectomy (10 patients). Both EO-MG and LO-MG were determined by age at first symptom of MG (age <50 or ≥50 years).

LABORATORY STUDIES

Antibodies to AChR were analyzed using a radioimmunoassay with [125I]-labeled AChR as antigen.2 Antibodies to titin were analyzed using an enzyme-linked immunosorbent assay with MGT-30 as antigen.19 Genomic DNA from each person tested was purified using QIAquick (Qiagen GmbH, Hilden, Germany) as described by the manufacturer.

Genotyping

Two polymorphic loci in the promoter region of the TNFA gene were studied. Both polymorphisms involve a G-to-A transition, one at position −238 and the other at position −308. The TNFA region incorporating these 2 sites was amplified using a polymerase chain reaction (PCR) kit (GeneAmp; Applied Biosystems, Foster City, California) and the following primers: 5'-AGCCAAATAGTTTTGAGGGCAT-3' and 5'-GAAGAGACGTTCCAGTGTTGTCAT-3', respectively. Amplification was undertaken by 35 cycles of incubation at 95°C for 15 seconds and at 65°C for 30 seconds.9

Encoding Fc Receptor IIa

The FCGRA2A genotypes were determined using an amplification refractory mutation system PCR modified from Botto et al.20 Two PCRs with 2 allele-specific primers were carried out for each sample. To verify the presence of genomic DNA, internal control primers amplifying a 270-base pair sequence from the TCR Vα2 gene were added. The PCR primers used were as follows: EC2-131R, 5'-CCAGAATGAAATCTCCAGAATTCCTCTCG-3'; EC2-131H, 5'-CCGAGATGGGAAATCTCCAGAATTCCTCTCA-3'; reverse primer (TN1), 5'-CCATTGTGGAAGACTGGCCTATGCTGGGCA-3'; control 1, 5'-GATTCCAGTACCCAGATGGGAAGG-3'; and control 2, 5'-ACGCAAGACTGACCCCTGAGGCGCT-3'. The PCR conditions were 94°C for 3 minutes; 45 cycles of 94°C for 45 seconds; 63°C for 30 seconds; 72°C for 1 minute 30 seconds; and 72°C for 10 minutes. For control, PCR was performed on DNA from a patient homozygous for the 131H allele and on the cell line U937, homozygous for the 131R allele, and K562, which is heterozygous.10

Encoding IL-10

Polymerase chain reaction was performed using the following primers: 5'-ATCCAAGAAACAGACTCTA-3' (upstream) and 5'-AATATATCTCCTGAAAGGTTCC-3' (downstream). The PCR product was purified using QIAquick (Qiagen GmbH) and sequenced using BigDye ThermoSequenase (Applied Biosystems).

Statistical Analysis

All statistical analyses were performed using commercially available software (SPSS Inc, Chicago, Illinois). The χ2 and Fisher exact tests were used to compare groups. Differences were considered statistically significant at P < .05.

Results

Overall, in the patients with MG, the IL-10 genotype ACC/ACC occurred with significantly increased frequency.

Table 1. Gene Allelic Variants in Patients With Thymomatous MG vs Control Participants and Patients With Nonthymomatous MGa

<table>
<thead>
<tr>
<th>Allelic Variant</th>
<th>Patients With Thymomatous MG</th>
<th>Control Participants</th>
<th>P Value</th>
<th>Patients With Nonthymomatous MG</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFA* T1</td>
<td>7/7 (100)</td>
<td>59/92 (64.1)</td>
<td>.09</td>
<td>12/23 (52.2)</td>
<td>.03</td>
</tr>
<tr>
<td>TNFB* T1</td>
<td>6/7 (85.7)</td>
<td>32/90 (35.6)</td>
<td>.01</td>
<td>9/23 (39.1)</td>
<td>.08</td>
</tr>
<tr>
<td>FCGRA2A 131H/H</td>
<td>5/9 (55.6)</td>
<td>11/50 (22)</td>
<td>.05</td>
<td>8/36 (22.2)</td>
<td>.09</td>
</tr>
<tr>
<td>IL-10 ACC/ACC</td>
<td>1/10 (10)</td>
<td>0/50</td>
<td>1.7</td>
<td>5/37 (13.5)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>TNFA* T1 + TNFB T1</td>
<td>6/7 (85.7)</td>
<td>32/90 (35.6)</td>
<td>.01</td>
<td>9/23 (39.1)</td>
<td>.08</td>
</tr>
<tr>
<td>FCGRA2A 131H/H</td>
<td>5/9 (55.6)</td>
<td>9/67 (13.4)</td>
<td>.009</td>
<td>2/34 (5.9)</td>
<td>.002</td>
</tr>
<tr>
<td>TNFA* T1 + FCGRA2A 131H/H</td>
<td>5/9 (55.6)</td>
<td>5/77 (6.5)</td>
<td>.001</td>
<td>1/34 (2.9)</td>
<td>.001</td>
</tr>
<tr>
<td>TNFA* T1 + TNFB* T1 + FCGRA2A 131H/H</td>
<td>5/9 (55.6)</td>
<td>5/77 (6.5)</td>
<td>.001</td>
<td>1/34 (2.9)</td>
<td>.001</td>
</tr>
</tbody>
</table>

Abbreviations: FCGRA2A, Fcγ receptor IIa; IL-10, interleukin 10; MG, myasthenia gravis; TNFA and TNFB, tumor necrosis factors α and β, respectively.

aValues are given as number of patients and control participants (percentage). Total numbers of patients and control participants differ because of incomplete data sets for some.

bP values not italicized compare patients with thymomatous MG vs control participants. P values in italics compare patients with thymomatous MG with those with nonthymomatous MG. P values statistically significant at .05 are in boldface.
As expected, patients with thymomatous MG had a higher frequency of TNFB*2 (P = .01) and FCGR2A 131H/H (P = .05) compared with controls (Table 1). Of patients with thymomatous MG, 55.6% had all 3 MG-related allelic variants, that is, TNFA*T1, TNFB*2, and FCGR2A 131H/H, a gene combination found in only 6.5% of the controls (P = .001) and in only 2.9% of patients with nonthymomatous MG (P = .001) (Table 1). The risk of having thymomatous MG correlated with the number of thymomatous MG–associated allelic variants. Variants of FCGR2A seem to be the most important determinants of disease (Figure).

The gene association profile in patients with titin Ab–positive MG (n = 19) was similar to that in patients with thymomatous MG. The combination TNFA*T1, TNFB*2, and FCGR2A 131 H/H was found in 31.6% of the patients with titin Ab–positive MG vs 6.5% of the controls (P = .007) and no patients with titin Ab–negative MG (P = .02) (Table 2).

Patients with EO-MG had an increased frequency of TNFB*1 (40%) compared with controls (7.8%) (P = .01) and also the IL-10 ATA/ATA genotype (16.7% vs 2%; P = .05). No combinations of allelic variants showed significant differences in distribution between patients with EO-MG and controls. It was rare to find more than one disease-associated allelic variant in both the EO-MG and control groups.

Patients with thymomatous MG exhibited significantly different combinations of alleles at TNF and FCGR2A loci compared with other patients with nonthymomatous MG and controls. Having more than one disease-associated allele increased disease susceptibility. These allelic variants, therefore, can be used as markers for a thymoma in the MG group, in whom thymomas are more common than in controls (Figure). However, in our study, the 5 patients with thymomatous MG for whom records were available all had findings indicative of a thymoma at preoperative computed tomography of the mediastinum.

Our findings demonstrate how thymomatous MG is a polygenic disorder. Whether the association is with the development of MG in the population with thymoma or with the development of the thymic tumor per se remains to be determined.

This study confirms earlier MG associations with specific allelic variants in TNFA, TNFB, FCGR2A, and IL-10 genes.9,10,21,22 The genetic profile in patients with thymomatous MG leads to a typical phenotype of low TNFA expression (homozygous for TNFA*T1), low TNFB expression (homozygous for TNFB*2), low IL-10 expression (IL-10 genotype ACC/ACC), and optimal interaction between Fcγ receptor and IgG2 (homozygous for

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**Table 2. Gene Allelic Variants in Patients With Titin Ab–Positive MG vs Control Participants and Patients With Titin Ab–Negative MG**

<table>
<thead>
<tr>
<th>Allelic Variant</th>
<th>Patients With Titin Ab–Positive MG</th>
<th>Control Participants</th>
<th>P Valueb</th>
<th>Patients With Titin Ab–Negative MG</th>
<th>P Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFA*T1</td>
<td>12/14 (85.7)</td>
<td>59/92 (64.1)</td>
<td>.14</td>
<td>6/14 (42.9)</td>
<td>.046</td>
</tr>
<tr>
<td>TNFB*2</td>
<td>10/14 (71.4)</td>
<td>32/90 (35.6)</td>
<td>.02</td>
<td>4/14 (28.6)</td>
<td>.06</td>
</tr>
<tr>
<td>FCGR2A 131H/H</td>
<td>7/19 (36.8)</td>
<td>11/50 (22)</td>
<td>.23</td>
<td>5/19 (26.3)</td>
<td>.73</td>
</tr>
<tr>
<td>IL-10 ACC/ACC</td>
<td>3/19 (15.8)</td>
<td>0/50</td>
<td>.02</td>
<td>1/20 (5)</td>
<td>.34</td>
</tr>
<tr>
<td>TNFA<em>T1 + TNFB</em>2</td>
<td>10/14 (71.4)</td>
<td>32/90 (35.6)</td>
<td>.02</td>
<td>4/14 (28.6)</td>
<td>.06</td>
</tr>
<tr>
<td>TNFA*T1 + FCGR2A 131H/H</td>
<td>6/19 (31.6)</td>
<td>5/77 (6.5)</td>
<td>.007</td>
<td>0/17</td>
<td>.02</td>
</tr>
<tr>
<td>TNFA*T1 + IL-10 ACC/ACC</td>
<td>2/18 (11)</td>
<td>0/67</td>
<td>.04</td>
<td>0/19</td>
<td>.23</td>
</tr>
<tr>
<td>TNFB*2 + IL-10 ACC/ACC</td>
<td>2/18 (11)</td>
<td>0/77</td>
<td>.03</td>
<td>0/19</td>
<td>.23</td>
</tr>
<tr>
<td>TNFA<em>T1 + TNFB</em>2 + FCGR2A 131H/H</td>
<td>6/19 (31.6)</td>
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<td>0/17</td>
<td>.02</td>
</tr>
<tr>
<td>TNFA<em>T1 + TNFB</em>2 + IL-10 ACC/ACC</td>
<td>2/18 (11)</td>
<td>0/77</td>
<td>.03</td>
<td>0/19</td>
<td>.23</td>
</tr>
</tbody>
</table>

Abbreviations: Ab, antibodies; FCGR2A, Fcγ receptor Iia; IL-10, interleukin 10; MG, myasthenia gravis; TNFA and TNFB, tumor necrosis factors α and β, respectively.

*Values are given as number of patients and control participants (percentage). Total numbers of patients and control participants differ because of incomplete data sets for some.

bP values not italicized compare patients with titin Ab–positive MG vs control participants. P values in italics compare patients with titin Ab–positive MG with those with titin Ab–negative MG. P values significant at .05 are in boldface.
Studies on experimental MG in rodents have shown that IgG2 is an effective inducing agent of MG. Although IgG1 and IgG3 predominate, IgG2 has been identified in serum samples of patients with MG. Even though IgG subclasses do not directly correspond in rodents and human beings, this could imply that IgG2 is involved in the induction of MG in human beings. Inasmuch as low TNFA and TNFB drive the immune system toward a humoral immune response and IL-10 has a general anti-inflammatory effect, our study results indicate that patients with thymomatous MG are predisposed to an enhanced humoral immune response.

In thymomas, expression of several skeletal muscle epitopes has been identified. There is strong evidence for intrathymic immunization against AchR, titin, and other muscle antigens in thymomatous MG. Given that this early immunization involves IgG2, IgG2-antigen complexes will bind to high-affinity FcyRIIa on antigen-presenting cells and epitopes from the antigen will be presented to T cells. Because of the low TNFA and TNFB expression, the resulting immune response will be primarily humoral, and exaggerated because of low IL-10 expression. In contrast, an immunologic profile leading to higher TNFA, TNFB, and IL-10 expression, as well as FcGR2A alleles different from 131HI/H, will reduce both binding to IgG2-antigen complexes and the subsequent humoral immune responses.

In patients positive for titin Abs, the genetic profile seems to resemble that in patients with thymomatous MG. It could be, therefore, that these patients have a similar pathogenesis including an enhanced humoral immune response. Almost all patients with thymomatous MG have titin Abs. One might suggest the possibility that patients with nonthymoma titin Ab–positive MG have already rejected an occult thymoma.

Allelic variants associated with EO-MG and titin Ab–negative MG exhibited few significant differences in allelic distribution. Our results correlate with previous findings on EO-MG, in which the main association was the ancestral haplotype 8.1 (which includes HLA-B8 DR3, TNFA*T2, and TNFB#1). This suggests that EO-MG is more strongly correlated with one specific gene in this region rather than with a specific combination of the multiple genes tested in this study. Individuals with the TNFA*T2 and TNFB#1 genotypes will, in general, have high TNF production, which could contribute to germinal center formation and, thus, thymus hyperplasia in EO-MG.

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REFERENCES


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