Analysis of Plasminogen Genetic Variants in Multiple Sclerosis Patients

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ABSTRACT Multiple sclerosis (MS) is a prevalent neurological disease of complex etiology. Here, we describe the characterization of a multi-incident MS family that nominated a rare missense variant (p.G420D) in plasminogen (PLG) as a putative genetic risk factor for MS. Genotyping of PLG p.G420D (rs139071351) in 2160 MS patients, and 886 controls from Canada, identified 10 additional probands, two sporadic patients and one control with the variant. Segregation in families harboring the rs139071351 variant, identified p. G420D in 26 out of 30 family members diagnosed with MS, 14 unaffected parents, and 12 out of 30 family members not diagnosed with disease. Despite considerably reduced penetrance, linkage analysis supports cosegregation of PLG p.G420D and disease. Genotyping of PLG p.G420D in 14446 patients, and 8797 controls from Canada, France, Spain, Germany, Belgium, and Austria failed to identify significant association with disease (P = 0.117), despite an overall higher prevalence in patients (OR = 1.32; 95% CI = 0.93–1.87). To assess whether additional rare variants have an effect on MS risk, we sequenced PLG in 293 probands, and genotyped all rare variants in cases and controls. This analysis identified nine rare missense variants, and although three of them were exclusively observed in MS patients, segregation does not support pathogenicity. PLG is a plausible biological candidate for MS owing to its involvement in immune system response, blood-brain barrier permeability, and myelin degradation. Moreover, components of its activation cascade have been shown to present increased activity or expression in MS patients compared to controls; further studies are needed to clarify whether PLG is involved in MS susceptibility.

Keywords: multiple sclerosis genetics linkage association plasminogen

Materials and Methods

Participants
A total of 2160 MS patients and 886 unrelated healthy controls from Canada, which includes 1857 multi-incident families, collected through the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS), were included in this study (Sadovnick et al. 1998). Five independent European cohorts consisting of 2391 MS patients and 672 healthy controls from France, 4288 patients and 4018 controls from Spain, 3733 patients and 2722 controls from Germany, 1006 patients and 504 controls from Belgium, and 925 patients from Austria, were used for replication. All patients were diagnosed with MS according to published criteria (Poser et al. 1983; McDonald et al. 2001; Polman et al. 2005), and the demographics for each cohort are presented in Table 1. The ethical review board at each institution approved the study, and all participants provided written informed consent.

Exome sequencing
We performed exome sequencing in three patients diagnosed with MS (pedigree A; II-1, II-4, and III-1) from a multi-incident family (Figure 1). Exonic regions were enriched using an Ion AmpliSeq exome kit (57.7 Mb), and sequenced in an Ion Proton sequencer (Life Technologies, Carlsbad, CA) with a minimum average coverage of 50 reads per base, and an average read length of 150 bases. The Ion Torrent Server v4 was used to map reads to NCBI Build 37.1 reference genome using the Torrent Mapping Alignment Program (TMAP), and to identify variants differing from the reference. Sequences with a mapping Phred quality score under 20, fewer than five reads, or over 95% strand bias were excluded from further analysis.

Sequencing, genotyping, and statistical analysis
Sanger sequencing was used to genotype amplicons containing exome variants of interest, and all 19 coding exons, and exon–intron boundaries, of plasminogen (PLG, NM_000301.3) by polymerase chain reaction (PCR) as previously described (Sadovnick et al. 2013). Nine tagging SNPs (tSNPs) spanning a 61 kb region encompassing the PLG locus were selected based on HapMap data (version 3, release 27) using Haploview software (Barrett et al. 2005). Selected tSNPs captured over 92% of the polymorphic variation in the region [minor allele frequency (MAF) > 5%, and r² > 0.8] in Caucasian population standards. Genotyping of variants was performed using a combination of TaqMan probes and Sequenom MassArray iPLEX as previously described (Traboulsee et al. 2014; Nishioka et al. 2010). Genotyping success rate was over 99.4% for all variants, and without deviation from Hardy-Weinberg equilibrium expectation (p-value > 0.005). Statistical association was determined using logistic regression analysis adjusted for age and gender, in addition, the combined cohort analysis was adjusted for site. Genotypes were dichotomized as presence vs. absence of the minor allele (dominant model). The combined dataset
was obtained by pooling samples from all populations. Segregation was quantified using nonparametric and parametric linkage analysis. Nonparametric linkage analysis was performed using SimWalk2 software (version 2.91), and NPL-All statistic (Sobel et al. 2001). Two-point parametric logarithm of odds (LOD) scores were obtained with MLINK, assuming a dominant model, with a fully penetrant disease, and without phenocopies (Ott 1989). All MS patients were treated as affected, noncarrier individuals as healthy, and unaffected mutation carriers were treated as having an unknown disease status. The deleterious allele was defined with a 0.0001 frequency, and the marker-allele frequency was determined empirically from genotyped individuals.

### Haplotype analysis

Microsatellite markers spanning the PLG locus between D6S1633 and D6S297 were chosen to define the disease-carrying haplotype (Supplemental Material, Table S1). All family members from those families identified with the PLG p.G420D mutation were genotyped. One primer for each pair was labeled with a fluorescent tag, and PCR reactions were performed under standard conditions. PCR products were run on an ABI 3730xl (Life Technologies, Carlsbad, CA), and analyzed using GeneMapper 4.0. Marker sizes were normalized to those reported in the CEPH database and manually phased within each family.

### Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are fully represented within the article.

### RESULTS

To identify genes and variants of major effect on MS susceptibility, we applied exome sequencing analysis to a multi-incident family consisting of 12 individuals over three generations, with DNA available for nine family members, including six diagnosed with MS (Figure 1A). Exome analysis of II-1, II-4, and III-1, identified 47479, 46545, and 46580 variants, respectively. Of those, 25 missense variants with a MAF below 1% from public and proprietary databases of variants were identified in all three individuals (Table S2). Segregation in additional family members identified 10 variants shared among at least five of the six family members diagnosed with MS for whom DNA was available, and no more than one of the two unaffected blood relatives. Three of these variants were subsequently excluded as they were identified at a frequency over 1% in 366 ethnically matched controls (Table S2). The seven remaining variants were genotyped in a multi-ethnic cohort consisting of 2160 MS patients and 886 unrelated healthy controls from Canada. Three variants [TGFBI, p.V608L (ss1467426521); SPINK13, p.C72R (ss1467426567); OR1E1, p.D96Y (ss1467426912)] appear to be private as they were not observed in any of the other samples genotyped in this study, and have not been described in public databases of variants (Abecasis et al. 2012; Exome Aggregation Consortium et al. 2015). ARHGAP10, p.T518K (rs34242925), with a reported MAF of 5 × 10⁻⁵ in the ExAC database, was also not observed in any additional samples. Segregation of these four variants within the exome sequenced family is provided in Figure S1. Of the remainder, SPATA18 p.P286L (rs150116592) was identified in two MS patients, UNC45B p.R776Q (rs34242925) was identified in one patient and one control, and PLG p.G420D (rs139071351) in 12 MS patients and one control.

Segregation for variants identified in SPATA18 and UNC45B did not support cosegregation with disease in additional families, and were excluded from further analysis (Figure S1). Segregation of PLG p.G420D identified the variant in 26 out of 30 family members diagnosed with MS (87%), 14 parents of MS patients (including eight obligate carriers) not known to suffer from MS, and 12 out of 30 family members not diagnosed with disease (Figure 1, B–M). To quantifiably assess segregation, we performed nonparametric and parametric linkage analysis for PLG p.G420D. The more conservative nonparametric score resulted in a LOD score of 1.29, whereas parametric linkage analysis resulted in a maximum LOD score of 5.48 (0 = 0.05), despite a penetrance estimate of 50%. Additional support for a role in disease susceptibility is provided by the level of conservation for the glycine residue in mammals, indicating the importance of this amino acid for protein function (Figure 2). Haplotype analysis of PLG p.G420D carriers between D6S1633 and D6S297 did not identify a shared haplotype among families (Table S1), thus suggesting that PLG p.G420D is a mutational hotspot that has independently arisen in each family rather than being inherited from a common ancestor.

Clinical details were available for 17 PLG p.G420D carriers, five males and 12 females (Table S3). The disease course observed in these carriers was predominantly consistent with relapsing-remitting MS, or secondary progressive MS, with only two patients presenting primary progressive MS. On average, the age at onset of disease was 35.1 years (SD ± 9.1), with a disease duration of 19.9 years (SD ± 10.4). Disease severity was overall relatively moderate, with an average expanded disability status scale (EDSS) score of 3.92 (SD ± 2.9) and a median of 2.75.

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**Table 1** Logistic regression analysis for PLG p.G420D (rs139071351) and risk of MS

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender M(%)</th>
<th>Age (mean ± SD)</th>
<th>Age at onset (mean ± SD)</th>
<th>Genotypes (GA/GG)</th>
<th>P-Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>Controls</td>
<td>51.0</td>
<td>67.1 ± 9.8</td>
<td>31.0 ± 9.7</td>
<td>1/880</td>
<td>0.046</td>
</tr>
<tr>
<td>MS patients</td>
<td>26.9</td>
<td>46.7 ± 11.7</td>
<td>4.0 ± 3.7</td>
<td>12/2091</td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Controls</td>
<td>39.1</td>
<td>39.3 ± 13.1</td>
<td>30.5 ± 9.7</td>
<td>4/668</td>
<td>0.049</td>
</tr>
<tr>
<td>MS patients</td>
<td>30.0</td>
<td>49.1 ± 11.4</td>
<td>30.0 ± 9.7</td>
<td>32/2359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>Controls</td>
<td>40.5</td>
<td>42.8 ± 12.8</td>
<td>34.0 ± 4.0</td>
<td>34/3984</td>
<td>0.475</td>
</tr>
<tr>
<td>MS patients</td>
<td>34.8</td>
<td>44.5 ± 11.5</td>
<td>30.9 ± 9.8</td>
<td>42/4246</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>Controls</td>
<td>40.3</td>
<td>41.3 ± 16.8</td>
<td>11/2711</td>
<td>0.476</td>
<td>1.31 (0.63–2.84)</td>
</tr>
<tr>
<td>MS patients</td>
<td>29.2</td>
<td>40.5 ± 11.3</td>
<td>30.8 ± 10.3</td>
<td>21/3712</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>Controls</td>
<td>47.2</td>
<td>56.2 ± 14.7</td>
<td>5/499</td>
<td>0.747</td>
<td>0.81 (0.23–3.04)</td>
</tr>
<tr>
<td>MS patients</td>
<td>34.0</td>
<td>48.3 ± 13.1</td>
<td>33.3 ± 10.9</td>
<td>6/1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>MS patients</td>
<td>29.8</td>
<td>49.2 ± 12.1</td>
<td>28.7 ± 9.1</td>
<td>7/918</td>
<td>NA</td>
</tr>
<tr>
<td>Combined</td>
<td>Controls</td>
<td>41.8</td>
<td>44.3 ± 15.9</td>
<td>55/8742</td>
<td>0.117</td>
<td>1.32 (0.93–1.87)</td>
</tr>
<tr>
<td>MS patients</td>
<td>31.0</td>
<td>45.1 ± 12.1</td>
<td>30.9 ± 9.9</td>
<td>120/14326</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M, male; OR, odds ratio; CI, confidence interval; NA, not applicable.
Association analysis of PLG p.G420D was performed in Caucasian samples from Canada already genotyped for the identification of additional PLG p.G420D families. This subset consists of 2103 MS patients and 881 controls, and resulted in a marginally significant association with disease risk ($P = 0.046$), and an odds ratio (OR) of 10.19 (Table 1). In order to validate this association we genotyped PLG p.G420D in five independent cohorts from Europe consisting of 12343 MS patients and 7916 healthy controls. Logistic regression analysis corrected for age and gender identified a similarly marginal association with disease in the French cohort ($P = 0.049$; OR = 2.69), whereas no association was observed for any additional cohort (Table 1). Although the combined dataset did not result in a significant association with disease risk ($P = 0.117$), with the exception of Belgium which is the smallest set, all cohorts resulted in OR greater than 1, indicating a higher prevalence of PLG p.G420D in MS patients than controls.

To assess whether common variants in PLG lead to an increased susceptibility to develop MS, we identified nine tSNPs spanning the entire PLG loci, and genotyped them in 2103 MS patients and 881 controls from Canada (Table S4). Association analysis failed to identify a significant association between any of the tSNPs and susceptibility to MS ($P > 0.05$). Since common variants in PLG do not appear to have an effect on MS disease risk, we assessed for the presence of additional rare PLG substitutions in MS patients. To this end, we sequenced all PLG-coding exons in 293 familial probands from Canada, which identified 11 silent and 11 missense variants (Supplementary Table S5). Of those, nine missense variants with a MAF below 1% in at least two of three publicly available databases (1000G, ExAC, or ESP) were genotyped in cases and controls from Canada (Abecasis et al. 2012; Exome Aggregation Consortium et al. 2015; Exome Sequencing Project 2014). This analysis identified six variants (p.K38E, p.R89K, p.R261H, p.R490Q, p.A494V, and

Figure 1 Simplified pedigrees for families presenting the PLG p.G420D variant. Males are represented by squares and females by circles, the proband is indicated with an arrow head. Patients diagnosed with MS have black filled symbols, and carriers of unknown clinical phenotype have gray filled symbols. Heterozygote carriers (M) and wild-type (wt) genotypes are indicated. An asterisk indicates an inferred carrier. Pedigree A was used for exome analysis, and, with the exception of pedigree E, which is of Asian descent, all families are of Caucasian ancestry.
Table 2 Case-control frequency for rare missense PLG variants identified in MS patients

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Chromosome and Position</th>
<th>Nucleotide Change</th>
<th>Protein Change</th>
<th>ExAC Controls</th>
<th>MS (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs73015965</td>
<td>6:161127501</td>
<td>A/G</td>
<td>p.K38E</td>
<td>0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>rs143079629</td>
<td>6:161128812</td>
<td>G/A</td>
<td>p.R89K</td>
<td>0.007</td>
<td>0.010 (16)</td>
</tr>
<tr>
<td>rs149145958</td>
<td>6:161135876</td>
<td>A/G</td>
<td>p.T200A</td>
<td>0.001</td>
<td>0.002 (8)</td>
</tr>
<tr>
<td>rs4252187</td>
<td>6:161137790</td>
<td>G/A</td>
<td>p.R261H</td>
<td>0.003</td>
<td>0.007 (12)</td>
</tr>
<tr>
<td>rs140537724</td>
<td>6:161152807</td>
<td>G/A</td>
<td>p.R490Q</td>
<td>0.001</td>
<td>0.002 (3)</td>
</tr>
<tr>
<td>rs4252128</td>
<td>6:161152819</td>
<td>C/A</td>
<td>p.A494V</td>
<td>0.008</td>
<td>0.005 (8)</td>
</tr>
<tr>
<td>rs140970354</td>
<td>6:161152837</td>
<td>C/T</td>
<td>p.T500M</td>
<td>0.0002</td>
<td>0.0005 (2)</td>
</tr>
<tr>
<td>rs372603134</td>
<td>6:161152858</td>
<td>C/T</td>
<td>p.A507V</td>
<td>0.0001</td>
<td>0.0002 (1)</td>
</tr>
<tr>
<td>rs4252129</td>
<td>6:161152905</td>
<td>C/T</td>
<td>p.R523W</td>
<td>0.007</td>
<td>0.012 (19)</td>
</tr>
</tbody>
</table>

\(^a\) dbSNP Build 138
\(^b\) The Exome Aggregation Consortium (ExAC) database.
three additional variants (p.T200A, p.T500M, and p.A507V) not known to cause hypoplasmogenenemia, were observed exclusively in MS patients. Although the allelic frequencies and segregation for rare missense PLG variants do not initially support a role in disease susceptibility, genotyping in additional MS patients is warranted to fully define these preliminary findings. PLG p.T200A seems of particular interest, as it was identified in eight MS patients and no controls (Table 2), it is evolutionarily conserved (Figure 2), and a threonine to proline substitution at the same position has been identified in a patient with severe type I PLG deficiency (Tefs et al. 2006).

PLG is a plausible biological candidate for MS susceptibility as it is involved in the inflammatory response, blood-brain barrier (BBB) permeability, neuronal viability, and myelin degradation (Syrovets et al. 2012; Yao and Tsirka 2011; Chen and Strickland 1997; Cuzner and Opdenakker 1999). PLG has been shown to play a role in the immune response, with plasmin deficiency, the active form of PLG, resulting in a compromised inflammatory response in mouse brain (Hultman et al. 2014). Microglia and astrocytes are the primary mediators of inflammation in the central nervous system, and fibrin has been shown to activate their immune response by stimulating the production of inflammatory mediators, including proinflammatory cytokines and reactive oxygen species, as well as act as a chemotactant for immune cells (Syrovets et al. 2012; Hultman et al. 2014).

Genetic variants in PLG may also have an effect on brain inflammation by altering the BBB permeability. Plasmin alters BBB permeability by inducing morphological changes in brain astrocytes and endothelial cells through the reorganization of the actin cytoskeleton and the redistribution of tight junction proteins (Nigo and Medcalf 2014; Yao and Tsirka 2011). In addition to its effects on the inflammatory response and BBB permeability, plasmin has also been shown to affect neuronal viability, including sprouting, plasticity, and extracellular matrix-related neuronal death (Chen and Strickland 1997; Nakagami et al. 2000; Wu et al. 2000).

Plasmin activates highly active matrix metalloproteinases (MMPs) which are recognized as key proteases in the demyelination process. Synthetic inhibitors of MMPs have been found to ameliorate clinical symptoms and pathological signs in experimental autoimmune encephalomyelitis (EAE) animal models (Cuzner and Opdenakker 1999); minocycline, which has several immunomodulating activities including the inhibition of MMP-9, has been used successfully in clinical trials as an add-on therapy for MS patients (Metz et al. 2009). Microglia and astrocytes are the primary mediators of inflammation in the central nervous system, and fibrin has been shown to activate their immune response by stimulating the production of inflammatory mediators, including proinflammatory cytokines and reactive oxygen species, as well as act as a chemotactant for immune cells (Syrovets et al. 2012; Hultman et al. 2014).

Despite the existence of extended families with a high incidence of MS (Fagnani et al. 2015; Sadovnick 1993), only one rare pathogenic mutations has been reported (Wang et al. 2016). In this study, the implementation of exome sequencing analysis in a multi-incident MS family nominated PLG p.G420D as a potential susceptibility risk for MS. Additional support was provided by 10 additional multi-incident MS families in which the variant segregates with disease, albeit with reduced penetrance. Disappointingly, genotyping of PLG p.G420D in a large European case-control cohort failed to identify a significant association with MS, thus not supporting a role for PLG p.G420D in disease. Despite this lack of association, dysregulation of the PLG/plasmin activation cascade is a plausible pathomechanism of MS, which, in conjunction with the positive segregation of PLG p.G420D in families (Figure 1), the overall higher incidence of PLG p.G420D carriers in European MS patients (Table 1), and the identification of additional rare PLG substitutions in MS patients not observed in controls (Table 2), warrants further genetic and functional characterization of PLG in order to elucidate its potential role on MS susceptibility and pathogenesis.

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