Association of FOXP3 polymorphisms rs3761547 and rs3761548 with multiple sclerosis in the Slovak population

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Abstract

OBJECTIVES: Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), which develops mainly due to autoimmune inflammation. Regulatory T (Treg) cells play a key role in the control of autoimmunity, and their development and function depend on proper expression and action of transcription factor forkhead box P3 (FoxP3). The aim of the current study was to analyze the association between FOXP3 rs3761548 and rs3761547 polymorphisms and MS risk, including their possible gender-specific effects.

METHODS: Polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) was used to genotype both FOXP3 single nucleotide polymorphisms in 536 unrelated MS patients and 667 healthy controls. Their association with MS was evaluated by logistic regression analysis.

RESULTS: Comparison of FOXP3 allele frequencies did not show any significant differences between female MS patients and female controls (p=0.68 for rs3761548; p=0.80 for rs3761547). Furthermore, no association with MS could be detected in females when FOXP3 rs3761548 and rs3761547 genotypes were analyzed by logistic regression analysis adjusted for HLA-DRB1*15:01 carrier status and age. Similarly, no association between MS and studied FOXP3 polymorphisms was observed in males when the differences in allele frequencies between the patients and controls were analyzed (p=0.61 for rs3761548; p=0.17 for rs3761547).

CONCLUSION: The results of our study suggest that FOXP3 polymorphisms are not associated with increased risk of multiple sclerosis. However, further studies are required to fully understand the role of various FOXP3 gene variations in the genetic predisposition to MS.
**INTRODUCTION**

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), affecting approximately 2.5 million people worldwide and resulting in chronic progressive disability for the majority of patients with the disorder (Sawcer et al 2014; Dendrou et al 2015). The disease typically starts between 20 and 40 years of age, and affects women 3 times more frequently than men (Sawcer et al 2014). Recent studies have indicated a key role for immune dysregulation and aberrant lymphocyte responses in the pathogenesis of multiple sclerosis (Yadav et al 2015). The activation of both innate and adaptive arms of an immune response leads to the formation of typical inflammatory infiltrates, breakdown of myelin sheaths, activation and proliferation of astrocytes and microglia, and development of axonal degeneration (Dendrou et al 2015; Hollenbach & Oksenberg 2015; Yadav et al 2015).

MS is a complex multifactorial disease, with numerous genetic and enviromental factors playing an essential role in its immunopathogenesis (Weissert 2013). The major histocompatibility complex (MHC) class II genes are by far the most important determinants of MS genetic susceptibility, with HLA-DRB1*15:01 allele conferring the highest risk in Caucasians (Schmidt et al 2007; International Multiple Sclerosis Genetics Consortium et al 2011). Recent advent of genome-wide association studies (GWAS) and follow-up Immuno-chip-based studies has led to discovery of 110 additional novel MS risk polymorphisms in 103 discrete loci outside of the MHC region (International Multiple Sclerosis Genetics Consortium et al 2013; Sawcer et al 2014; Hollenbach & Oksenberg 2015). The majority of identified MS risk variants are located within immunologically related genes, including those which are linked to lymphocyte activation, differentiation, and proliferation, thus emphasizing the prominent role of the immune system in disease development (Dendrou et al 2015; Bos et al 2016). However, together they explain only roughly 30% of the genetic component of the disease. Multiple explanations of this missing heritability in MS have been proposed, including neglecting the analysis of sex chromosomes (Chang et al 2014; Hollenbach & Oksenberg 2015). Indeed, while successful for the autosomes, the vast majority of GWAS have either incorrectly analyzed or ignored the X chromosome, thus leaving its role largely unknown (Chang et al 2014; Gao et al 2015). As multiple sclerosis shows sexual dimorphism in disease prevalence and course (Bearoff et al 2015), the need for analysing X-linked variants as potential contributors to disease susceptibility becomes more obvious.

FOX3 gene localized on chromosome Xp11.23 appears as one of several potential candidates for genetic susceptibility to MS. It encodes transcription factor forkhead box P3 (FoxP3), which plays a pivotal role in the development and function of FoxP3+ regulatory T (Treg) cells. Tregs represent an important mechanism for the control of autoreactive T cells and the maintenance of peripheral tolerance to self and foreign antigens through various immunosuppressive mechanisms (Hori et al 2003; He et al 2013). It is well established that the development of various autoimmune diseases (AD) is associated with changes in the proportion and/or function of FoxP3+ Tregs (Pesenacker et al 2016). Tregs have also emerged as key players in the pathogenetic scenario of CNS autoimmune inflammation, as altered Treg function and homeostasis was shown to be detrimental to the pathogenesis of multiple sclerosis (Viglietta et al 2004; Yadav et al 2015).

Genetic factors are one of several mechanism that can lead to the relative lack of functional Treg cells in the human body (Pesenacker et al 2016). Several polymorphisms with potential impact on gene expression or functional protein activity have been identified in coding and regulatory regions of the FOX3 gene (Oda et al 2013), and some of them have been implicated in the development of various autoimmune diseases (He et al 2013; Oda et al 2013). The role of FOX3 gene polymorphisms in the genetic predisposition to MS is, however, still unclear as the results of association studies have been inconsistent so far (Chang et al 2014; Jafarzadeh et al 2015; Eftekharian et al 2016; Gholami et al 2016). Therefore, the aim of the current study was to examine whether two selected polymorphisms in the FOX3 gene, namely rs3761548 C/A and rs37615487 A/G, are associated with an increased risk of developing MS in the Slovak population.

**MATERIAL AND METHODS**

**Study subjects**

For the purpose of the study, 536 unrelated MS patients (375 females and 161 males; mean age 40.9±10.6 years) were recruited at neurology departments of university hospitals in Bratislava and Martin in Slovakia. The diagnosis of MS was based on the revised McDonald criteria (Polman et al 2011), and only patients with bout-onset MS were included in the study: 472 individuals were classified as having relapsing-remitting (RR) MS, and 64 had secondary-progressive (SP) form. The degree of neurological disability at the time of examination was quantified using Kurtzke’s Expanded Disability Status Scale (EDSS) (Kurtzke 1983); EDSS score and disease duration were subsequently used to determine the Progression Index (PI) and Multiple Sclerosis Severity Score (MSSS) as means to evaluate the rate of disability accumulation and clinical severity, respectively (Roxburgh et al 2005). The control group consisted of 667 unrelated adults (426 females and 241 males; mean age 39.6 ± 15.4 years) without any personal and family history of MS and other autoimmune and neurological diseases. Basic demographic and clinical data of MS patients are summarized in Table 1.
DNA extraction and genotyping
Genomic DNA was extracted from whole EDTA-anticoagulated blood with standard phenol-chloroform method (Sambrook & Russell 2001). FOXP3 rs3761547 A/G and rs3761548 C/A genotyping was performed by a restriction fragment length polymorphism analysis of PCR-amplified product (PCR-RFLP) according to slightly modified protocols of Inoue et al (2010). PCR was performed in a final volume of 10 μl containing 50–100 ng of genomic DNA, 10 x PCR buffer with KCl, 1.5 mM of MgCl2, 0.4 μM of each primer, 0.2 mM of dNTPs and 0.5 U of Taq polymerase (Thermo Fisher Scientific Biosciences, Germany). The PCR cycling conditions for rs3761547 were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. The PCR cycling conditions for rs3761548 were as follows: initial denaturation at 94 °C for 4 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 63.2 °C for 30 s and extension at 72 °C for 30 s, and final extension at 72 °C for 10 min.

The PCR products were subsequently digested overnight with \textit{PvuII} (for rs3761547) and \textit{PstI} (for rs3761548) restriction enzymes at 37 °C (Thermo Fisher Scientific Biosciences, Germany). PCR fragments were separated by electrophoresis in 3% agarose gel stained with ethidium bromide and visualized under the UV-transilluminator.

The PCR-RFLP method was also used to genotype rs3135388 SNP, which serves as a specific surrogate marker for the major MS risk allele \textit{HLA-DRB1*15:01} (de Bakker et al 2006; International Multiple Sclerosis Genetics Consortium et al 2007). The primer sequences with PCR cycling and restriction conditions were described in detail elsewhere (Benešová et al 2013).

Statistical analyses
Differences in categorical variables (gender, MS course, \textit{HLA-DRB1*15:01} positivity, FOXP3 allele frequencies) between the study groups were compared using the chi-square test, whereas differences in continuous variables (age, age of onset, disease duration, EDSS, MSSS and PI) were assessed by Welch’s corrected \textit{t} test. The analyses were performed with InStat statistical software (GraphPad Software, Inc. San Diego, USA). Genotypes of both FOXP3 polymorphisms were tested for departure from Hardy-Weinberg equilibrium (HWE) using the \chi² goodness-of-fit test with 1 degree of freedom. The association between FOXP3 polymorphisms and susceptibility to MS was examined separately in males and females by logistic regression analysis with adjustment for age and \textit{HLA-DRB1*15:01} carriage status as possible confounding variables. The \textit{P}, odds ratio (OR) and 95% confidence interval (CI) values were computed for the effects of genotypes in co-dominant, dominant and recessive inheritance models. The analyses were performed with SNPStats web software available at http://bioinfo.iconcologia.net/snpstats/start.htm (Solé et al 2006). Correction for multiple testing was performed by a matrix spectral decomposition method (http://neurogenetics.qimrberghofer.edu.au/matSpD/), which employs linkage disequilibrium (LD) data to set a new significance threshold adjusted for number of independent test performed (Nyholt 2004).

Results
The presence of \textit{HLA-DRB1*15:01} allele as the single strongest known genetic risk factor for MS was determined by genotyping the specific tag polymorphism rs3135388 C/T. Carriers of rs3135388 T allele that
corresponds with HLA-DRB1*15:01 were significantly more frequent among MS patients than in healthy controls (51.68% vs. 19.79%, p<0.0001), and the carriage of at least one copy of HLA-DRB1*15:01 allele was associated with significantly increased risk of MS (OR=4.34, 95% CI=3.36–5.59). The HLA-DRB1*15:01 carriage status was therefore used as a possible confounding variable in subsequent association analyses of FOXP3 polymorphisms.

The comparison of clinical parameters between male and female MS patients revealed no significant differences in age at disease onset, MS form, EDSS, MSSS and PI scores, although a tendency towards higher rate of disability accumulation (p=0.10 for PI) and higher MS severity (p=0.17 for MSSS) could be seen in male MS subgroup (Table 1).

As males carry only one copy of the X-chromosome, the deviation of genotype distribution from Hardy-Weinberg equilibrium was tested in females only. Both rs3761547 and rs3761548 showed no significant departure from HWE neither in patients (p=0.975 for rs3761547, p=0.552 for rs3761548) nor in the control group (p=0.127 for rs3761547, p=0.450 for rs3761548).

Matrix spectral decomposition method was used to correct for multiple comparisons, utilizing the LD correlation matrix value $r$ for the calculation of effective number of independent variables (Veff). The new significance threshold was then determined by dividing the nominal significance value 0.05 by Veff. As Veff computed for the two FOXP3 polymorphisms was 1.914, only $p$-values ≤0.026 were further considered statistically significant. Comparison of allele frequencies showed no significant differences between female patient and control groups neither in FOXP3 rs3761548 polymorphism (minor allele A frequency 43.87% in patients vs. 42.84% in controls; $p=0.68$; OR=1.04) nor in rs3761547 (minor allele G frequency 10.40% vs. 10.80%; $p=0.80$; OR=0.96). In accordance, logistic regression analysis adjusted for HLA-DRB1*15:01 carrier status and age revealed no significant association between MS and studied FOXP3 polymorphisms in female subjects (Table 2). Similarly to females, the regression analysis of both FOXP3 polymorphisms in males found no significant association with the disease neither in rs3761548 (allele A frequency 45.34% in patients vs. 44.40% in controls; $p=0.61$; OR=1.11) nor in rs3761547 (minor allele G frequency 8.70% vs. 12.03%; $p=0.17$; OR=0.61) (Table 3).

### Table 2. Allele and genotype frequencies of FOXP3 rs3761548 and rs3761547 polymorphisms in female patients with multiple sclerosis and control subjects.

<table>
<thead>
<tr>
<th>Allele / genotype</th>
<th>Model</th>
<th>MS patients (n=375)</th>
<th>Controls (n=426)</th>
<th>$p$-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761548</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>allelic</td>
<td>421 (56.13%)</td>
<td>487 (57.16%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>329 (43.87%)</td>
<td>365 (42.84%)</td>
<td>0.68</td>
<td>1.04 (0.86–1.27)</td>
</tr>
<tr>
<td>CC</td>
<td>co-dominant</td>
<td>121 (32.27%)</td>
<td>143 (33.57%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td>179 (47.73%)</td>
<td>201 (47.18%)</td>
<td>0.86</td>
<td>1.08 (0.77–1.51)</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td>75 (20.00%)</td>
<td>82 (19.25%)</td>
<td>1.12</td>
<td>0.73–1.71</td>
</tr>
<tr>
<td>CC</td>
<td>dominant</td>
<td>121 (32.27%)</td>
<td>143 (33.57%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>AC + AA</td>
<td></td>
<td>254 (67.73%)</td>
<td>283 (66.43%)</td>
<td>0.60</td>
<td>1.09 (0.79–1.49)</td>
</tr>
<tr>
<td>CC + AC</td>
<td>recessive</td>
<td>300 (80.00%)</td>
<td>344 (80.75%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td>75 (20.00%)</td>
<td>82 (19.25%)</td>
<td>0.71</td>
<td>0.74–1.56</td>
</tr>
<tr>
<td>rs3761547</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>allelic</td>
<td>672 (89.60%)</td>
<td>760 (89.20%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>78 (10.40%)</td>
<td>92 (10.80%)</td>
<td>0.80</td>
<td>0.96 (0.70–1.32)</td>
</tr>
<tr>
<td>AA</td>
<td>co-dominant</td>
<td>301 (80.27%)</td>
<td>342 (80.28%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td></td>
<td>70 (18.67%)</td>
<td>76 (17.84%)</td>
<td>0.47</td>
<td>1.04 (0.71–1.54)</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td>4 (1.07%)</td>
<td>8 (1.88%)</td>
<td>0.47</td>
<td>0.13–1.70</td>
</tr>
<tr>
<td>AA</td>
<td>dominant</td>
<td>301 (80.27%)</td>
<td>342 (80.28%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>AG + GG</td>
<td></td>
<td>74 (19.73%)</td>
<td>84 (19.72%)</td>
<td>0.93</td>
<td>0.98 (0.68–1.43)</td>
</tr>
<tr>
<td>AA + AG</td>
<td>recessive</td>
<td>371 (98.93%)</td>
<td>418 (98.12%)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td>4 (1.07%)</td>
<td>8 (1.88%)</td>
<td>0.23</td>
<td>0.46 (0.13–1.68)</td>
</tr>
</tbody>
</table>

Allele and genotype frequencies are presented as absolute numbers with percentage in parentheses. OR – odds ratio; CI – confidence interval; $p$, OR and 95% CI values adjusted for age and HLA-DRB1*15:01 positivity.
Allele and frequencies of FOXP3 rs3761548 and rs3761547 polymorphisms in male patients with multiple sclerosis and control subjects

<table>
<thead>
<tr>
<th>Allele</th>
<th>MS patients (n=161)</th>
<th>Controls (n=241)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3761548</td>
<td>14 (8.70%)</td>
<td>29 (12.03%)</td>
<td>0.17</td>
<td>0.61 (0.30–1.24)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3761547</td>
<td>147 (91.30%)</td>
<td>212 (87.97%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Allele and frequencies are presented as absolute numbers with percentage in parentheses. OR – odds ratio; CI – confidence interval
P, OR and 95% CI values adjusted for age and HLA-DRB1*15:01 positivity

**DISCUSSION**

Forkhead box P3 (Foxp3) is a member of the forkhead winged-helix transcription-factor family that acts as a transcriptional regulator required for the development and inhibitory function of thymus-derived natural (nTreg), peripheral (pTreg) and induced regulatory T cells (iTreg). FoxP3 occupies the promoter for various genes involved in T cell function and T cell receptor (TCR) signalling, and can act either as a transcriptional repressor or a transcriptional activator (Wu et al 2006; Bacchetta et al 2016). FoxP3+ Treg lineages play a critical role in immune regulation and maintaining immunological unresponsiveness to self-antigens, and quantitative and functional abnormalities of Treg cells may lead to defects of immune tolerance and abnormal immune responses to self-antigens, thus resulting in various autoimmune diseases (Oda et al 2013; Pesenacker et al 2016; Tao et al 2016). The essential role of FoxP3 and Tregs in the control of autoimmunity can be illustrated by an example of immune dysregulation, of FoxP3 and Tregs in the control of autoimmunity, and the functional impact of the two polymorphisms is unclear, but it can be hypothesized that they may potentially alter gene expression by affecting RNA splicing, epigenetic modification, cis-regulatory elements or binding of transcription factors to their binding sites (Cooper 2010). Recently, in silico transcriptional-factor binding site analysis suggested that rs3761548 SNP occurs within the putative binding site for the transcription factor specificity protein 1 (Sp1). It is possible that C to A substitution interferes with the interaction of Sp1 with its binding site, thereby affecting FoxP3 expression (Song et al 2013). In line with this hypothesis, several studies suggested that rs3761548 is associated with an increased risk of vitiligo (Jahan et al 2013; Song et al 2013; Chang et al 2014), and autoimmune thyroid diseases (Ban et al 2009), Behçet’s disease (Hosseini et al 2015), and autoimmune thyroid diseases (Ban et al 2007; Inoue et al 2010; Bosowsk et al 2014).

In this study, we have focused on two selected polymorphisms, namely rs3761547 and rs3761548, and their role in genetic susceptibility to MS. They are both located in the 5’ upstream intron between non-coding exons -2b and -1, in the proximity of conserved non-coding sequences CNS1 and CNS2 that contain histone acetylation domains and CpG methylation sites for epigenetic regulation of gene expression (Marques et al 2015; Tao et al 2016). The functional impact of the two polymorphisms is unclear, but it can be hypothesized that they may potentially alter gene expression by affecting RNA splicing, epigenetic modification, cis-regulatory elements or binding of transcription factors to their binding sites (Cooper 2010). Recently, in silico transcriptional-factor binding site analysis suggested that rs3761548 SNP occurs within the putative binding site for the transcription factor specificity protein 1 (Sp1). It is possible that C to A substitution interferes with the interaction of Sp1 with its binding site, thereby affecting FoxP3 expression (Song et al 2013). In line with this hypothesis, several studies suggested that rs3761548 is associated with an increased risk of vitiligo (Jahan et al 2013; Song et al 2013; Chang et al 2014), and autoimmune thyroid diseases (Ban et al 2009), Behçet’s disease (Hosseini et al 2015), and autoimmune thyroid diseases (Ban et al 2007; Inoue et al 2010; Bosowsk et al 2014).

In support of these findings, a recent meta-analysis of various autoimmune diseases (Graves’ disease, Hashimoto’s disease, Crohn’s disease, primary biliary cirrhosis, myasthenia gravis, alopecia areata, psoriasis, and vitiligo) demonstrated that FoxP3 rs3761548 polymorphism might contribute to AD sus-
ceptibility (He et al 2013). The role of rs3761547 in the genetic susceptibility to autoimmunity is less well known, although some studies reported its association with vitiligo (Birlea et al 2011) and psoriasis (Song et al 2012).

In this case–control study, we have demonstrated that none of the two FOXP3 gene polymorphisms rs3761547 and rs3761548 contributes to the genetic predisposition to MS in the Slovak population. To the best of our knowledge, this is the first study to investigate the relationship between FOXP3 polymorphisms and the risk of developing MS in Slovaks. Our findings are in agreement with the results of recent study which reanalyzed data from 16 GWAS of different autoimmune and related diseases, including two GWAS of MS (Baranzini et al 2009; International Multiple Sclerosis Genetics Consortium et al 2011), and found no evidence of association of FOXP3 gene polymorphisms with MS in Caucasian populations of European origin (Chang et al 2014). In contrast to these studies, Jafarzadeh et al (2015) found an association between FOXP3 rs3761548 and MS in the Iranian population, with allele A being the risk allele. This result was further supported by another Iranian study, which also observed the association between MS and rs3761548 A allele (Eftekharian et al 2016). Interestingly, no such association was found in the third Iranian study (Gholami et al 2016). Moreover, two of these studies also reported that other FOXP3 polymorphism, namely rs2232365, may also contribute to MS susceptibility (Eftekharian et al 2016; Gholami et al 2016). Several factors may account for these observed discrepancies, most notably inter-ethnic genetic differences; other possible explanations include smaller sample sizes in some of the studies resulting in insufficient statistical power and increased risk of false positive results, differences in inclusion criteria, study design and statistical approach, etc.

In summary, the results of the current study suggest that common FOXP3 polymorphisms rs3761548 and rs3761547 do not confer susceptibility to multiple sclerosis in the Slovak population. Although our results support the outcome of recent large GWAS of MS, the role of FOXP3 variations in the genetic architecture of MS cannot be fully ruled out yet. It is possible that their actual impact on disease susceptibility and course will be significantly affected by ethnic and geographical differences, various environmental modifiers and mutual complex interactions between numerous gene variations. Therefore further independent studies with larger cohorts and including other polymorphisms within and outside the FOXP3 gene are warranted to fully disclose its role in the genetic predisposition and pathogenesis of MS.

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