Investigation of the $[-/A]_8$ and C1236T genetic variations within the human toll-like receptor 3 gene for association with multiple sclerosis

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Multiple sclerosis (MS) is a serious cause of neurological disability among young adults. The clinical course remains difficult to predict, and the pathogenesis of the disease is still modestly understood. Autoimmunity is thought to be a key aspect of the disease, with autoreactive T cells thought to mediate central nervous system (CNS) inflammation to some extent. Toll-like receptors are known to mediate cellular recognition of pathogens by way of patterns of molecular presentation. Toll-like receptor 3 is coded by the gene TLR3 and is recognized as an important factor in virus recognition and is known to be involved in the expression of neuroprotective mediators. We set out to investigate two variations within the TLR3 gene, an 8 bp insertion–deletion $[-/A]_8$ and a single base-pair variation C1236T, in subjects with MS and matched healthy controls to determine whether significant differences exist in these markers in an Australian population. We used capillary gel electrophoresis and TaqMan genotyping assay techniques to resolve genotypes for each marker, respectively. Our work found no significant difference between frequencies for TLR3 $[-/A]_8$ by genotype ($\chi^2 = 1.03, p = 0.60$) or allele ($\chi^2 = 1.09, p = 0.30$). Similarly, we found no evidence for the association of TLR3 C1236T by genotype ($\chi^2 = 0.35, p = 0.84$) or allele frequency ($\chi^2 = 0.31, p = 0.58$). This work reveals no evidence to suggest that these markers are associated with MS in the tested population. Although the role of TLR3 and the wider toll-like receptor family remain significant in neurological and CNS inflammatory disorders, our current work does not support a role for the two tested variants in this gene with regard to MS susceptibility.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of uncertain etiology. Associations between genetically determined childhood susceptibility to viral infections and the risk of developing MS in early adulthood have been described. Predispositions to autoimmunity appear to play an important role central to the evolution of the disease pathology. Autoreactive T cells directed against myelin basic and proteolipid proteins appear to contribute to pathogenesis, with viruses being possible triggers in individuals with a susceptible predisposition. The role of human endogenous retroviruses in autoimmune diseases has received increasing attention in recent times. Toll-like receptor 3 (TLR3) plays a fundamental role in pathogen recognition, and human neurons express TLR3 to facilitate innate immune responses to viral double-stranded RNA. TLR3 is known to be expressed by human astrocytes to trigger the production of neuroprotective mediators and anti-inflammatory cytokines, including interleukin (IL)-9, IL-10 and IL-11. Polymorphisms in TLR3 have previously been investigated in other diseases in the context of altered innate immune response. A recent study found that activation of human microglia via TLR3 signaling can change the profile of local central nervous system (CNS) immune responses (by translation of Th1-polarizing signals to CD4 T cells). The importance of TLR3 in both innate and adaptive immune responses has been repeatedly demonstrated. Mounting evidence suggests that the ability of individuals to respond to TLR ligands may be impaired by variations such as single nucleotide polymorphisms (SNPs) within TLR genes resulting in altered susceptibility and course of infection particularly in inflammatory diseases. Immunohistochemical
analyses of MS brains and spinal cords have shown enhanced expression of TLR3 (and TLR4) particularly in inflamed tissues. Furthermore, animal models have previously taught us that infections with viruses, such as Theiler's murine encephalomyelitis virus, are sufficient to induce TLR3-mediated MS-like pathologies. We set out to investigate an 8 bp adenine insertion/deletion [−/A]8 (rs3081264) and a SNP C1236T (rs3775291) to determine frequencies of these variants within an Australian white population comparing MS affected and matched healthy control DNA by capillary gel electrophoresis and TaqMan genotyping assay methods. Identifying TLR3 variations associated with MS susceptibility may lead to a better understanding of the etiology of MS.

**EXPERIMENTAL PROCEDURE**

**Subjects**

Griffith University’s Ethics Committee approved the research involving human experimentation. Informed consent was obtained from all participants. The case control populations consisted of 205 MS patients and 196 healthy controls for [−/A]8 (rs3081264) and 199 MS patients and 198 healthy controls for C1236T (rs3775291), which had been matched for age (±5 years), gender and ethnicity (white). The MS population was recruited from the Multiple Sclerosis Clinic at the Royal Brisbane and Women’s Hospital; all samples were procured from patients residing in the South East Queensland region. The population consisted of ~75% women and 25% men and was subcategorized based on clinical course: relapsing-remitting (RR), secondary-progressive (SP) and primary-progressive (PP). Frequencies were the following: RR remitting (RR), secondary-progressive (SP) and primary-progressive (PP). Genotypes were the following: RR, secondary-progressive (SP) and primary-progressive (PP). Frequencies were the following: RR, secondary-progressive (SP) and primary-progressive (PP).

**Toll-like receptor 3 ([−/A]8) genotyping assay**

An 8 bp adenine insertion/deletion [−/A]8 (rs3081264) was chosen for analysis. This is the only known insertion–deletion within the TLR3 gene. DNA concentrations were adjusted to 10 ng/μl for each sample. Specific forward and reverse polymerase chain reaction (PCR) primers were designed: forward, 5′-GAGGCTGAGATTGCACTGCTG-3′ and reverse, 5′-CTTCCAATGGCCTGAAAAACA-3′. The reverse primers were labeled with the fluorescent reporter dye FAM. Primers were ordered from GeneWorks, Australia. Genomic DNA was used as a template to generate PCR products for subsequent genotyping by capillary gel electrophoresis.

Assay conditions were empirically determined. Efficient thermal cycling consisted of 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, with a final extension of 72°C for 3 minutes. A total of 40 ng of genomic DNA was amplified with 10× PCR buffer, 3 mM MgCl2, 0.2 mM deoxynucleotide triphosphate, 10 μM total primers (5 μM forward, 5 μM reverse) and 0.2 μM Taq polymerase in a 25 μl final volume on a Corbett (Sydney, Australia) PC-960 thermocycler. Polymerase chain reaction products were electrophoresed on a 2% w/v agarose gel to confirm the presence of DNA fragments (either 175 or 183 bp in size). As agarose gel electrophoresis lacks the sensitivity to resolve an 8 bp difference, the PCR products were genotyped using ABI PRISM 310 Genetic Analyzer. Appropriate DNA dilutions were determined with regards to relative fluorescence from the GeneScan-350 Size Standard marker. For capillary gel electrophoresis, equimolar concentrations of PCR product were combined with Hi-DiTM formamide (Applied Biosystems) and ABI310 Genetic Analyzer Performance Optimized Polymer-4 (Applied Biosystems) according to the manufacturer’s instructions and then run at 60 V for ~20 minutes per sample.

**Toll-like receptor 3 C1236T TaqMan genotyping assay**

Nearby base sequence variations to the marker of interest (TLR3 C1236T rs3775291) influenced the selection of the current assay. A specific TaqMan Pre-designed Genotyping Assay was ordered from Applied Biosystems, Australia (part number 4351379). The TaqMan SNP assay incorporates probes labeled with FAM or VIC dyes at the 5′ end and a minor groove binder and non-fluorescent quencher at the 3′ end. Reactions consisted of 2 × TaqMan Universal PCR Master Mix, 20 × TaqMan SNP Genotyping Assay Mix, dH2O and 10 ng starting template (DNA) in a total volume of 20 μl. Thermal conditions consisted of 95°C for 10 minutes once, followed by 92°C for 15 seconds and 60°C for 1 minute repeated for 40 cycles. Reactions were performed on a Corbett Life Science Rotor-Gene 6000 real-time quantitativeycler collecting fluorescence on green (470 nm source, 510 nm detect) and yellow (530 nm source, 555 nm detect).

**Statistical analyses**

Data for the 8 bp adenine insertion/deletion [−/A]8 (rs3081264) were collected on the ABI PRISM 310 Genetic Analyzer (Macintosh) platform. Each genotype was determined based on distinct peak size (base-pair). Descriptive statistics were assessed using SPSS Standard Package with follow-up analysis using Microsoft Excel. Genotype distributions were checked for Hardy–Weinberg equilibrium. Observed genotype and allele frequencies were investigated using χ2 independence test at α=0.05.

Results for the TLR3 C1236T marker (TaqMan Assay) were resolved on the Corbett Rotor-Gene 6000 real-time cycler software collecting fluorescence on two separate channels (yellow and green) providing cycling information. A common threshold was applied for analysis and cycle threshold values were tabulated into discrimination plots to determine genotypes.
Genotyping summary
For the TLR3 marker [-/A]₈ (rs3081264), 205 (99%) cases and 196 (94%) controls were genotyped successfully. For the marker TLR3 C1236T, 199 (96%) cases and 198 (95%) matched controls were genotyped successfully. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorized during analysis.

RESULTS
Genotype and allele frequencies were determined as shown in Tables 1 and 2. The Hardy–Weinberg equilibrium held (all \( p > 0.05 \)) for all analyses. Independent \( \chi^2 \) analysis returned no statistically significant difference (all \( p > 0.05 \)) between genotype frequencies of MS and control samples for TLR3 [-/A]₈ (\( \chi^2 = 1.03, p = 0.60 \)) or C1236T (\( \chi^2 = 0.35, p = 0.84 \)). Similarly, no statistically significant difference was observed for allele frequencies for [-/A]₈ (\( \chi^2 = 1.09, p = 0.30 \)) or C1236T (\( \chi^2 = 0.31, p = 0.58 \)). For controls, the deletion was more common (51%) than the insertion (49%), whereas a converse trend was observed in MS cases, with the insertion being more common (53%) than the deletion (47%). For the C1236T marker, the C allele was more common than the T allele for both cases (66%) and controls (68%). Genotypes and allele frequencies were also analysed separately for men and women. The [-/A]₈ marker returned significantly similar frequencies for both men and women (data not shown). The C1236T marker also returned significantly similar frequencies for men and women (data not shown). Previously determined allele frequencies were not available for TLR3 [-/A]₈. Previous frequencies were available for TLR3 C1236T online at both the NCBI SNP database and the Ensembl database; upon comparison, both the case and control frequencies for the C and T alleles were similar to the previously published findings.

DISCUSSION
Human neurons are known to express increased levels of TLR3 during the immune response to viral infections\(^ {15} \). Toll-like receptors play a key role in pathogen recognition, and studies have shown that susceptibility to infections and inflammatory diseases may be regulated in part by genetic variations within the gene (TLR3) that codes for the receptor\(^ {10} \). TLRs are known to be activated by several triggers, including viruses and bacteria upon presentation of pathogen associated molecular patterns with distinct receptors being activated by specific antigens. In general, toll-like receptors participate in modulating a Th2 (anti-inflammatory) to Th1 (inflammatory) shift, which involves the increased expression of proinflammatory cytokines. This process is thought to drive CNS inflammation, while interaction with T and B cells and macrophages is thought to contribute to CNS demyelination (Figure 1). Infections and autoimmune disease have been the topic of intense scrutiny in recent years\(^ {14} \). It is well established that MS is a complex inflammatory T-cell-mediated autoimmune disease\(^ {15} \). The complex mechanisms underlying the processes by which this autoimmunity occurs and the nature of the contributing genetic variations remain interesting points of discussion\(^ {16} \). In this work, we set out to determine whether genotype and allele frequencies differ between MS and matched healthy control DNA for the TLR3 [-/A]₈ and C1236T markers. The findings of this work suggest that the tested variations do not play a significant role in MS susceptibility because no significant differences could be detected between MS patients and controls in either genotype or allele frequencies for the investigated markers within this population (all \( p > 0.05 \)). The relative importance of toll-like receptors in the CNS is well established\(^ {11} \). Furthermore, important roles of TLR3 have been proposed in mechanisms relating to expression of neuroprotective mediators and in mounting innate immune response to infections\(^ {5,14} \). Other variations within TLR3 may still play a role in susceptibility to MS, given the role and function of this receptor. Human microglia, also thought to be key components in the MS pathogenesis cascade, also express toll-like receptors in response to T cell stimulus\(^ {8} \). Recent studies have also proposed that the interaction between inflammatory mediators and neural precursor cells may have important consequences for CNS homeostasis and repair\(^ {17} \). Recent genetic studies have implicated the pathways involving toll-like receptors and their associated transcription factors (such as IRF5) as MS susceptibility candidates\(^ {18} \), while other efforts focus on

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<th>Genotype frequencies</th>
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<td>Genotype frequencies</td>
<td>n</td>
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<tr>
<td>Control</td>
<td>57 (29)</td>
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<td>MS</td>
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Values are expressed as \( n \) (%).
Emediated processes of CNS demyelination (dendritically) as well as macrophages (encircled letter M) facilitate cell-interactions with T and B cells (encircled letters T and B, respectively). This mechanism drives CNS inflammation, while subsequent inflammatory cytokines such as IL-9, IL-10 and IL-11 are decreased.

Inflammatory neurobiology remains complex, further genetic analyses investigating the variations within the toll-like receptor family that are likely to contribute to susceptibility may prove insightful.

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REFERENCES