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Variation in The Vitamin D Receptor Gene is Associated With Multiple Sclerosis in an Australian Population

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Abstract

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) resulting in accumulating neurological disability. The disorder is more prevalent at higher latitudes. To investigate VDR gene variation using three intragenic restriction fragment length polymorphisms (*Apa I*, *Taq I* and *Fok I*) in an Australian MS case-control population, one hundred and four Australian MS patients were studied with patients classified clinically as Relapsing Remitting MS (RR-MS), Secondary Progressive MS (SP-MS) or Primary Progressive MS (PP-MS). Also, 104 age-, sex-, and ethnicity-matched controls were investigated as a comparative group. Our results show a significant difference of genotype distribution frequency between the case and control groups for the functional exon 9 VDR marker *Taq I* ($p_{Gen} = 0.016$) and interestingly, a stronger difference for the allelic frequency ($p_{All} = 0.0072$). The *Apa I* alleles were also found to be associated with MS ($p_{All} = 0.04$) but genotype frequencies were not significantly different from controls ($p_{Gen} = 0.1$). The *Taq* and *Apa* variants are in very strong and significant linkage disequilibrium ($D' = 0.96$, $P < 0.0001$). The genotypic associations are strongest for the progressive forms of MS (SP–MS and PP–MS). Our results support a role for the VDR gene increasing.

Keywords: MS; multiple sclerosis; VDR gene; RFLP association; linkage disequilibrium

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Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) predominantly affecting young Caucasians. The prevalence of MS is dependent on geographical localization where very low sunlight exposure is directly linked to higher prevalence (Kurland, 1952). MS patients have low serum levels of vitamin D and this condition is reflected by higher rates of bone fractures occurring in MS patients as opposed to healthy individuals (Nieves et al., 1994; Cosman et al., 1998). The vitamin D receptor (VDR) is a nuclear receptor that, once bound to 1,25-Dihydroxyvitamin D3 [1,25-(OH)2D3], leads to transcriptional suppression or activation of genes. Experiments on an MS animal model, experimental allergic encephalomyelitis (EAE), have shown a complete inhibition of disease course after the injection of the biologically active form 1,25-(OH)2D3 (Cantorna et al., 1996). This inhibition of disease course was accompanied by enhanced activation of transforming growth factor beta (TGFβ), a known protective factor in EAE mouse models (Cantorna et al., 1998; Cautain et al., 2001) To induce

EAE, animals are immunized with CNS myelin antigens. If such immunization is preceded by 1,25-(OH)2D3 intake, this hinders EAE induction (Cantorna et al., 1996). Additionally, in Swiss Jim Lambert (SJL) mice, the vitamin D analog, TX527 was shown to decrease EAE disease severity and to postpone disease onset (Van Etten et al., 2003). Furthermore, studies of VDR null mice show that VDR presence is necessary for suppressing EAE activity (Meehan & DeLuca, 2002). Considering these data, VDR and its ligand show relative immunosuppressive and anti-inflammatory potential. VDR is expressed in peripheral leukocytes including activated T lymphocytes, which may explain the immunological role of VDR in suppressing EAE. Vitamin D decreases antigen expression by antigen-presenting cells and inhibits the proliferation of activated T cells and the production of proinflammatory cytokines such as interferon gamma (IFN-γ) (Tsoukas et al., 1984; Muller & Bendtzen, 1992, 1996; Nataf et al., 1996). IFN-γ, a type II interferon, is found in high amounts in sera, plasma and lesions of patients with MS (Schrijver et al., 2004). This interferon activates a transduction cascade involving STAT1 factor and is detrimental in MS. STAT1 interaction with VDR antagonises vitamin D action (Vidal et al., 2002).

The VDR gene is located on chromosome 12q 12–14; and, interestingly, a Sardinian genome-wide association study has shown a significant associative empirical *p*-value, at the 5% level, for the D12S1653 marker (marker for 12q12 position) in MS (Coraddu et al., 2003). In addition, a second genome screen on a Spanish population has shown a significant association with a *P*-value for the same marker, D12S1653, of $p = 0.000001$ (Goertsches et al., 2003). The D12S1653 marker is <3cM from the VDR gene, which contains multiple allelic variants some of which may lead to alterations in VDR function. Such variants may contribute to pathologies such as rickets or susceptibility to autoimmune diseases such as primary biliary cirrhosis (PBC) and autoimmune hepatitis (AH) (Peter et al., 1997; Arndt et al., 2002). Specifically, the VDR gene contains 11 exons, with 3 exons (1a, 1b and 1c) found in the 5′ noncoding sequence and 8 exons (2–9) encoding the structural portion of the protein (Miyamoto et al., 1997). The gene contains 3 restriction fragment length polymorphisms defined as *Taq I*, *Apa I*, and *Fok I* (Figure 1). The *Fok I* polymorphism (a ACG to ATG transition) introduces an initiation codon leading to the addition of three amino-acids to the VDR protein. The *Apa I* and *Taq I* polymorphisms are located towards the 3′ end of the VDR gene more precisely within an intron flanked by exons 8 and 9 for *Apa I* and within exon 9 for *Taq I*. These VDR polymorphisms have been found to be associated with an increased risk of several autoimmune diseases including systemic lupus erythematosus and diabetes type 1 (Huang et al., 2002; Skrabic et al., 2003). Interestingly, a Japanese study reported an association of the vitamin D receptor gene b (*Bsm I*) and gene a (*Apa I*) alleles with MS (Fukazawa et al., 1999; Niino et al., 2000). Prompted by these genetic findings, and the potential significance of the VDR in MS, we decided to investigate three VDR sequence polymorphisms, one from the 5′ region (*Fok I*) and two from the 3′ region (*Apa I* and *Taq I*), for their potential involvement in MS susceptibility in the Australian population.

MATERIALS AND METHODS

Subject Groups

The participants of the study were unrelated and of Caucasian (Northern European) origin. The study protocol was approved by Griffith University's ethics committee for experimentation on humans. The test groups consisted of 104 MS patients and 104

controls, matched for ethnicity, sex and age (+/-5 years). The MS population was obtained from patients from the Multiple Sclerosis Clinic at the Royal Brisbane and Women's Hospital, mainly from the South East Queensland region. This case group consisted of 75% women and 25% men and were subdivided into three groups according to the clinical course: Relapsing-Remitting MS (RR-MS), Secondary Progressive MS (SP-MS) and Primary Progressive MS (PP-MS) with frequencies of 40%, 36%, 24%, respectively. The control group was also obtained from the South East Queensland region through the Genomics Research Centre, Southport, with each control matched to an MS patient for the variables age (+/-5 years), sex, and ethnicity. All individuals gave informed consent before participating in the research. Genomic DNA was extracted from peripheral blood leucocytes using a standard salting-out protocol.

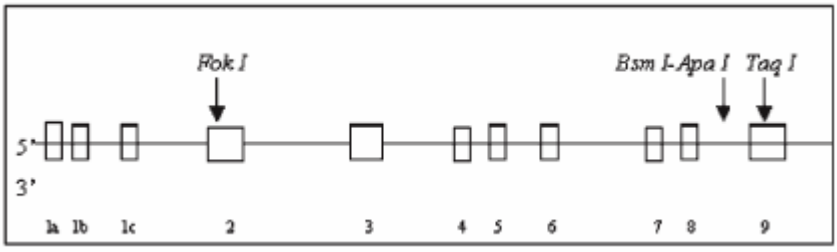

Figure 1. Restriction polymorphism of VDR DNA sequence. Schematic of the VDR gene, showing the array of exons (squares) and relative locations of the polymorphisms tested in this study (arrows).

Genotyping

Three restriction fragment length polymorphisms (RFLPs) were genotyped; the *Fok I* polymorphism in the initiation codon at the 5′ end and two polymorphisms (*Apa I* and *Taq I*) in the 3′ region of VDR. These polymorphic regions were amplified by standard, unlabeled oligonucleotides followed by restriction enzyme digestion corresponding to each RFLP. Oligonucleotide primers used for *Apa I* and *Taq I* RFLP were: forward primer 5′ CAG AGC ATG GAC AGG GAG CAA G 3′; reverse 5′ GCA ACT CCT CAT GGG CTG AGG TCT CA 3′; and for *Fok I* RFLP were: forward 5′ GAT GCC AGC TGG CCC TGG CAC TG 3′ and reverse 5′ ATG GAA ACA CCT TGC TTC TTC TCC CTC 3′. For detection of the initiation codon polymorphism, 50–100 ng genomic DNA was amplified with 1 X polymerase chain reaction (PCR) buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer and *Taq* polymerase in a 20-μL final volume on a Corbett (Sydney, Australia) PC-960 thermocycler. Cycles consisted of a 4-min denaturation at 94ºC followed by 30 cycles of 94ºC for 1 min and 60ºC for 1 min then a final extension at 60ºC for 7 min. PCR products were digested with *Fok I* (1 U at 37ºC) and electrophoresed on 2% ethidium bromide stained agarose gels. Genotypes were denoted as FF (272 bp), Ff (272, 198, 74 bp) or ff (198, 74 bp). For detection of the *Apa I* and *Taq I* RFLPs, amplification required 50–100 ng genomic DNA with PCR Premix Optimisation Buffer E (Epicentre Technologies, Madison, WI), 0.2 μM of each primer and *Taq* polymerase in a 25-mL reaction volume. Amplification was then performed on a Perkin Elmer (Foster City, CA) thermocycler with a 94ºC initial denaturation for 4 min followed by 5 cycles of 94ºC for 45 sec, 64ºC for 60 sec and 72ºC for 2 min; and a further 25 cycles of 94ºC for 30 sec, 64ºC for 30 sec and 72ºC for 45 sec. Following amplification, PCR products were digested with *Apa I* (2 U at 37ºC) or *Taq I* (2 U at 65ºC) and electrophoresed on 2% agarose gels stained with ethidium bromide.

Genotypes were determined as *AA* (740 bp), *Aa* (740, 515, 225 bp) or *aa* (515, 225 bp) for *Apa I* polymorphism; and *TT* (490, 245 bp), *Tt* (490, 290, 245, 205 bp) or *tt* (290, 245, 205 bp) for *Taq I* polymorphism (dominant alleles denoting absence of restriction site).

Statistics

Genotype and allele frequencies for the VDR variants were calculated from observed genotype counts. As a statistical control for systematic genotyping error and population stratification, the expected genotype proportions according to the Hardy-Weinberg law were calculated and compared to observed genotypes. Genotype and allele frequencies were initially assessed for association with MS using conventional contingency table analyses incorporating the standard chi-squared test for independence. This analysis produces a χ^2 statistic with one or two degrees of freedom and corresponding *p*-values for allele and genotype distributions, respectively. Genetic risk magnitudes (effect size) were estimated by calculating odds ratios (ORs) with 95% confidence intervals. Linkage disequilibrium (haplotype) analysis of the VDR variants was performed using the 2LD program (Zhao, 2004).

RESULTS

The genotype and allele frequencies for all three variants are shown in Tables I through III. All three genotypes for the case-control association studies conform to Hardy-Weinberg equilibrium (HWE) expectations ($p > 0.05$) in both case and control groups. Both genotype and allele frequencies for the *Taq I* variant were significantly different between the MS population and the control population ($p = 0.016$ and $p = 0.0072$, respectively). A significant difference was also observed for the allelic distribution of the *Apa I* variant ($p = 0.04$) although the genotype distribution was not significantly different ($p = 0.1$). Neither the genotype nor the allele frequency distribution was significantly different between the MS and control populations for the *Fok I* variant ($p > 0.2$).

Table I. Distribution of VDR *Taq* Variant (Genotype and Allele) frequencies in MS case and control groups

	Genotypes*				Alleles#	
Group	*T/T*	*T/t*	*t/t*	N (genotypes)	*T*	*t*
Total MS[a]	27 (26%)	57 (55%)	20 (19%)	104	111 (53%)	97 (47%)
Control[b]	42 (45%)	40 (43%)	11 (12%)	93	124 (67%)	62 (33%)

* genotype distribution comparison for total MS($\chi^2 = 8.27$; $p = 0.0016$), #allele distribution comparison for total MS ($\chi^2 = 7.22$; $p = 0.0072$).
[a]Hardy-Weinberg equilibrium $p = 0.302$.
[b]Hardy-Weinber equilibrium $p = 0.756$.

The *Taq* and *Apa* variants were found to be in very strong and significant linkage disequilibrium (LD) ($D' = 0.96$; $p < 0.0001$). Neither of these two variants was in LD with the *Fok* variant. The disease-associated haplotype A-t was shown to be present in 28% of MS cases compared to only 16% of controls. We decided to focus our subsequent genotypic analyses on the *Taq* variant because a) there is near complete LD between the *Taq* and *Apa* variants and b) the *Taq I* variant is exonic and c) this variant yielded the strongest independent association with MS. For the entire case control group, comparison of the *T/t* plus *t/t* genotypes combined against the *T/T*

genotype yielded an OR of 2.35 (95% CI: 1.29–4.27, $p = 0.0048$) for MS. Analysis of this genotype composition in the clinical subtypes (Figure 2) indicated that the association of the *Taq I* variant is stronger with the SP and PP subtypes (abbreviated as in the legend to Figure 2) of MS (ORs > 3; $p < 0.05$).

Table II. Distribution of VDA *Apa* Variant (Genotype and Allele) frequencies in MS case and control groups

	Genotypes*				Alleles#	
Group	*A/A*	*A/a*	*a/a*	N (genotypes)	*A*	*a*
Total MS[a]	35 (34%)	55 (53%)	14 (13%)	104	125 (60%)	83 (40%)
Control[b]	23 (23%)	54 (54%)	23 (23%)	100	100 (50%)	100 (50%)

* genotype distribution comparison for total MS($\chi^2 = 4.604$; $p = 0.01$), #allele distribution comparison for total MS ($\chi^2 = 4.2$; $p = 0.04$).
[a]Hardy-Weinberg equilibrium $p = 0.295$.
[b]Hardy-Weinber equilibrium $p = 0.424$.

Table III. Distribution of VDR *Fok* Variant (Genotype and Allele) frequencies in MS case and control groups

	Genotypes*				Alleles#	
Group	*F/F*	*F/f*	*f/f*	N (genotypes)	*F*	*f*
Total MS[a]	47 (48%)	40 (41%)	11 (11%)	98	134 (68%)	62 (32%)
Control[b]	34 (37%)	48 (51%)	11 (12%)	93	116 (62%)	70 (38%)

* genotype distribution comparison for total MS($\chi^2 = 2.69$; $p = 0.26$), #allele distribution comparison for total MS ($\chi^2 = 1.52$; $p = 0.218$).
[a]Hardy-Weinberg equilibrium $p = 0.577$.
[b]Hardy-Weinber equilibrium $p = 0.337$.

DISCUSSION

The aim of this study was to investigate three sequence polymorphisms in the VDR gene, one from the 5′ region and two from the 3′ region, for their involvement in MS susceptibility within an Australian cohort. The 3 polymorphisms investigated were distinguishable by *Fok I, Apa I* and *Taq I* restriction enzymes for genotyping using 104 MS patients and 104 matched controls. *Taq I* is localized in exon 9 of the VDR gene with a *T/C* nucleotide substitution (ATT to ATC) although this doesn't result in amino acid sequence change from codon 352 (isoleucine). However, the presence or absence of *Taq I* restriction sites has been found to be associated with variable mRNA stability of VDR (Morrison et al., 1994).

In the present study, we report a strong positive association of the *Taq I* VDR marker in MS susceptibility. Both genotype and allele frequency distributions for the *Taq I* variant were significantly different between the MS and control populations. The allelic association was observed to be more significant than the genotypic association. These findings demonstrate that the rarer allele (*t*) is more predominant in MS cases than in controls. Moreover, these results suggest that those who carry the *t* allele are at least 2 times more likely to have MS than those who do not carry this allele. Further analysis of the clinical subtypes of MS indicates that the association with the *Taq* variant is derived primarily from the progressive clinical subtypes (SP–MS and PP–MS) and that the risk of progressive MS is increased 3-fold for *t* allele carriers.

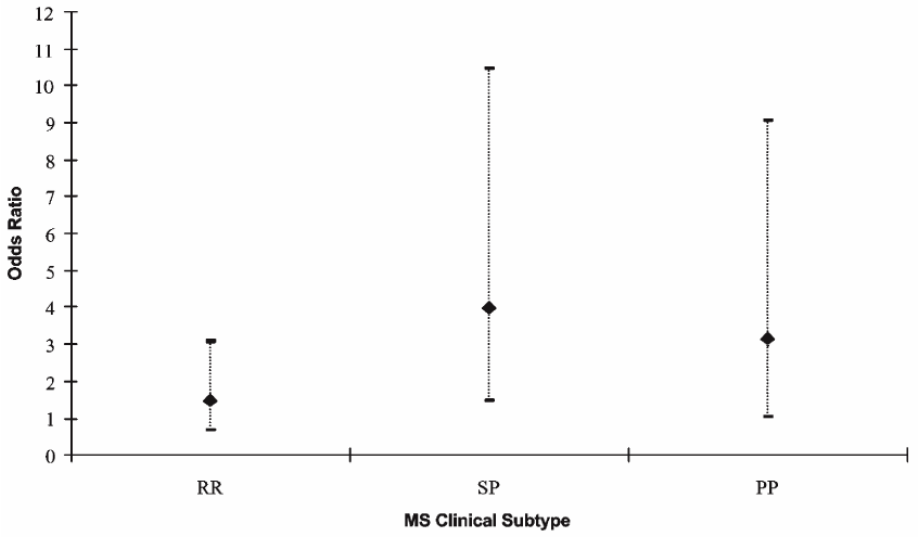

Figure 2. Odds Ratios (ORs) for the three clinical subtype groups. Relapse Remitting (RR), Secondary Progressive (SP), and Primary Progressive (PP), compared with controls involving the *Taq1* variant for the *T/t* and *t/t* genotypes combined vs. *T/T*. The ORs for both progressive subtypes (SP, PP) are significantly greater than 1 ($p < 0.05$).

The second VDR marker investigated, *Apa I*, was also found to be associated with MS. *Apa I* allele frequency distribution comparison was significant at the 0.05 level with a predominant susceptibility for the *A* allele in MS. Interestingly, allele *A* was found associated with the progressive subtypes of MS (SP–MS and PP–MS). Both positive marker *Taq I* and *Apa I* variants were found to be associated with MS with a stronger susceptibility role for the functionally associated variant *Taq I*. The *Taq I* and *Apa I* variants were found to be in strong and very significant linkage disequilibrium ($D' = 0.96$; $p < 0.0001$). This strong LD has been observed in several other studies (Curran et al., 1999). Therefore we suggest that the *Taq I* variant, representing a tag single nucleotide polymorphism for this haplotype, should be the only variant requiring genotyping in future disease- association studies of this region of the VDR gene. Neither of these 3′ variants was in LD with the *Fok I* variant. Thus, the LD breakdown among the VDR variants has dissociated the susceptibility haplotype (A-t) of the 3′ region from the 5′ *Fok I* variant region.

A similar MS case-control study, examining the *Apa I* marker, was previously undertaken by a Japanese team (Niino et al., 2000). The authors used 77 Japanese MS patients and 95 controls and found a significant association for *Apa I* allele distribution ($p = 0.032$) (Niino et al., 2001). Additionally they studied *Bsm I* VDR gene variants in MS. This other 3′ untranslated region (UTR) marker of the VDR gene was also found to be associated with MS, both for the allele frequency distribution ($p = 0.014$) and for the genotype frequency distribution ($p = 0.049$). Further data analysis showed that both the *Bsm I* and *Taq I* markers are also in strong LD ($p < 0.0001$) (Wilkinson et al., 2000). Consequently these three markers are all in LD and clustered possibly as a common susceptibility haplotype (BAt) for MS. A Canadian team has also investigated VDR markers in MS, specifically *Apa I* and *Taq I* markers for the VDR gene. However, they reported no preferential transmission of any alleles to affected offspring using a family-based association cohort (*Taq I* and *Apa I*; $p < 0.05$) (Steckley et al., 2000). The genetic heterozygosity (Hu) of the markers based on the genotypes of the parents was very low ($Hu_{TaqI} = 0.488$ and $Hu_{ApaI} = 0.447$).

Reliant upon high heterozygosity values, the use of bi-allelic markers for family-based association analyses (e.g., TDT) will diminish the power to identify susceptibility allele transmission.

MS pathology is associated with the invasion of peripheral mononuclear blood cells into the CNS. These cells induce local inflammation in the CNS with increased synthesis of proinflammatory cytokines, major histocompatibility complex molecules, and oxidative molecules leading to CNS demyelination. Vitamin D inhibits T cell proliferation and IFN-γ cytokine expression (Ball et al., 1986). Vitamin D activation of VDR leads to the transcription of responsive elements of several genes. Certain VDR haplotypes could lead to the reduced expression of VDR protein and alter VDR-induced transcription in the CNS. Vitamin D is an efficient antioxidant and plays a critical role in control of free radical induced tissue damage, another hallmark of MS pathology. VDR also has important roles affecting insulin secretion, hormone production including the parathyroid hormone and also on estrogen biosynthesis with a direct action on the aromatase gene (Haschek et al., 1978; Manolagas, 1987; Wilson, 1992; Kinuta et al., 2000). Of special interest, we have recently undertaken cDNA array-based differential display analysis of MS brain tissues versus healthy control brain tissue (Tajouri et al., 2003). Although our arrays did not contain a VDR cDNA for analysis, results showed a common pattern of activated expression of immunological genes in MS, and interestingly a reduced expression of the insulin receptor gene and insulin-related genes. Of interest, Vitamin D has been shown to prevent reversibly mouse models of type I diabetes (Mathieu et al., 1992). Additional studies show that impaired VDR functionality in mice leads to impaired insulin secretion (Zeitz et al., 2003). An up regulation of parathyroid hormone receptor gene was also found in MS from these microarray data. VDR-responsive elements are also found in the osteopontin gene, an early T cell activator gene found strongly expressed in MS brains (Chabas et al., 2001). Taken together, lower amounts of Vitamin D concentrations by inadequate Vitamin-D intake or ultraviolet radiation (UVR) exposure could contribute to the pathogenesis of MS, and additionally, presence of specific haplotypes for the VDR gene could increase susceptibility to MS, particularly the progressive forms of MS.

Different geographical latitudes of the Japanese, Canadian, and Australian populations could be a factor influencing the differences in *Apa I* and *Taq I* data obtained from each study. Low UVR exposure and reduced Vitamin D synthesis correlate with MS geographical prevalence. The MS Canadian population was pooled around the whole country and Canada is situated at latitudes of around 70º to 50ºN. The MS Japanese population studied was from the northernmost island of Japan, Hokkaido, and is located between 42º and 43ºN whereas South-East Queensland for the MS Australian population is localized at around 25º to 29ºS. It would be interesting to further investigate case-control studies of the *Taq I* VDR marker in extended Japanese populations, in the Canadian population and also in a Tasmanian Australian population (latitude of 43ºS for Hobart, Tasmania).

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