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Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese

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Abstract

1,25-Dihydroxyvitamin D3 ($1,25(OH)_2D_3$), the biologically active form of vitamin D, exerts an immunosuppressive effect and can completely prevent experimental autoimmune encephalomyelitis (EAE). $1,25(OH)_2D_3$ exerts most of its actions only after it has bound to its specific nuclear receptors. To investigate the possible role of vitamin D receptor gene (VDRG) polymorphism in susceptibility to or disease-modulation of MS, we evaluated 77 Japanese patients with 'conventional' MS and 95 controls. A VDRG allelic polymorphism was assessed by *Bsm*1 endonuclease restriction after specific PCR amplification. Genotypic polymorphism was clearly defined as BB (absence of restriction site on both alleles), bb (presence of restriction site on both alleles), or Bb (heterozygous). We found overexpression of the b allele (92.9 vs. 84.2%; $P=0.0138$) and homozygote bb (85.7 vs. 71.6%; $P=0.0263$) in MS patients compared with controls. The results indicate for the first time an association of MS with VDRG polymorphism, which may be involved in pathogenesis of MS, or in the linkage disequilibrium of VDRG to another pathogenic gene loci. The role of VDR gene polymorphism should be further studied in other populations, and the distribution of other polymorphism, such as Apa I, Taq I, should be also analyzed to confirm another susceptibility gene for MS and to obtain more adequate strategies for treatment of MS.

Keywords: Multiple sclerosis; Vitamin D receptor; Polymorphism; Susceptibility gene; Japanese

1. Introduction

Recent studies have clarified the molecular basis of the immunomodulatory activity of $1,25(OH)_2D_3$, the biological active form of vitamin D [1]. This hormone inhibits T-cell activation both in vitro and in vivo, and inhibits the secretion of interleukin (IL)-1, IL-2, IL-6, IL-12, tumor necrosis factor (TNF) and interferon (IFN)-gamma [2–4]. These cytokines play important roles in the development of T-helper 1 (Th1) cells, which are believed to be involved in the pathogenesis of chronic inflammatory autoimmune diseases [5]. $1,25(OH)_2D_3$ exerts most of its actions only after it has bound to its specific nuclear receptors, and which are present in monocytes and activated T lymphocytes [6,7]. IL-2 and IL-12 were reported to be downregulated via vitamin D receptor (VDR)-dependent inhibition of NFATp/AP1 or NF-kB activation [8,9]. Furthermore, in appropriate murine models, $1,25(OH)_2D_3$ prevents the development of some autoimmune diseases such as lupus [10], type 1 diabetes [11] and experimental autoimmune encephalomyelitis (EAE) [12–14].

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system that is dependent on both genetic and environmental susceptibility factors, and is widely believed to have a T-cell-mediated autoimmune etiology [15]. Despite evidence for a strong

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genetic effect, a weak major histocompatibility complex (MHC) association is the only consistently observed genetic feature of MS [16,17]. Conversely, recent studies of genome scans suggest that no single MS susceptibility locus is necessary and sufficient to cause disease [18–20]. Furthermore, MS is a heterogeneous disorder, so that different genes might influence the course or the presentation of the disease.

To investigate the possible role of VDRG in MS susceptibility, we analyzed whether there is any association between a VDRG polymorphism and MS. We also investigated the possible interactions of VDRG with clinical course and severity, with MRI findings, with another genetic marker (HLA antigens), and with oligoclonal bands (OCB) in the cerebrospinal fluid (CSF).

2. Materials and methods

2.1. Patients and controls

Since 1986, we have maintained special research charts for MS patients in our MS clinic, and detailed clinical and demographic records have been kept. Family history, CSF analyses, evoked potential and neuroimaging data (brain CT, brain and spinal cord MRI) have also been recorded when available. Hematological and biochemical studies, collagen screenings and serologic tests for syphilis and HTLV-1 antibodies were performed in all patients. Patients with only recurrent transverse myelopathy, recurrent optic neuritis, or recurrent brainstem symptoms were excluded from this study. Patients with the optic-spinal form of MS (OpS-MS) whose lesions were clinically confined to the optic nerve and spinal cord, and patients with acute transverse myelopathy (ATM), were also excluded because these subgroups of MS constitute a distinct subgroup among patients with a clinical diagnosis of MS [21–26]. Patients who were serologically positive for anti-nuclear antibody (ANA) on Hep-2 cells [27], without any other evidence of collagen disease, thrombosis, or systemic vasculitis, were enrolled in the study. All of the patients were negative for anti-HTLV-1 antibody, rheumatoid factor, anti-double stranded DNA antibody, anti-Ro antibody, anti-La antibody, anticentromere antibody, and antiribonucleoprotein antibody, anti-cardiolipine antibody (aCL), and anti-neutrophil cytoplasmic antibody (ANCA) by previously described methods [28,29]. Ultimately, 77 unrelated patients with the relapsing and remitting type or secondary progressive type of MS, who had been observed for at least 1 year, and who were diagnosed with clinically definite MS according to the criteria of Poser et al. [30], participated in this study. Ninety-five unrelated healthy hospital staffs who undergo yearly medical examination served as controls. All of the patients and controls were Japanese and were residents of Hokkaido, the northernmost island of Japan [31].

2.2. Analysis of VDR gene polymorphism

After obtaining informed consent, high molecular weight DNA was extracted from peripheral blood cells. Referring to the original VDRG sequence (accession no. I33554), polymerase chain reaction (PCR) amplification of a region containing the polymorphism was done using forward primer, Fuka-1 5′-gggagacgtagcaaaagg and reverse primer, Fuka-2 5′-agaggtcaagggtcactg. After PCR amplification of genomic DNA using a PJ9600 thermal cycler (Perkin Elmer), PCR products were digested by the restriction enzyme *Bsm*1 (BioLabs Inc.). The polymorphism was analyzed using an Mupid®-3 electropheresis system (Cosmo Bio). According to the paper by Morrison et al. [32], *Bsm*1 detects a dimorphism with either one band at 359 bp (allele B) or two bands at 182 and 177 bp (allele b).

PCR was carried out in a total volume of 15 μl, containing 50 ng of genomic DNA, 5 pmol of each primer, 250 μM dNTP, 10 mM KCl, 20 mM Tris–HCl pH 8.2, 1.5 mM $MgCl_2$, 2% formamide and 0.12 U Taq polymerase (AmpliTaq®; Perkin Elmer). The PCR reaction mixtures were then denatured at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and final elongation for 10 min. Thereafter, each PCR product was digested by *Bsm*1 at 65°C for 1 h. Eight microliters of each PCR product was mixed with 1 μl loading buffer (25% Ficoll, 0.25% bromophenol, 0.25% xylene cyanol FF, containing 1 mM of EDTA) and electrophoresed through a 2% agarose gel at 100 V in 1× TAE buffer containing 5 μl of ethidium bromide (10 mg/ml) for 40 min. The polymorphism was determined using a Thermal Imaging System FTI-500® (Fuji film).

2.3. Genomic HLA-DRB1, -DQB1, and -DPB1 typing

A nonisotopic oligotyping method using reverse dot blot hybridization was used for HLA class II DRB1, DRB3,4,5, DQB1, DPB1 alleles (INNO-LiPA HLA kit; Innogenetics). This method allows discrimination of the 69 DRB1, 4 DRB3, 2 DRB4, 5 DRB5, 23 DQB1, and 47 DQB1 alleles. In the current study DRB1 alleles were determined in 71 MS patients, DRB3, DRB4 and DRB5 alleles in 71, DQB1 alleles in 71, and DPB1 alleles in 61 patients.

2.4. Magnetic resonance imaging (MRI)

MRI analyses were performed in all 77 patients, and all scans were evaluated by the same neuroradiologist (KM), without knowledge of the clinical profiles of the patients, by previously described methods [22]. In brief, the distribution, sizes, shapes and numbers of lesions were recorded for each subject, then the severity of the cerebral

abnormality present was graded using the following six-point scale: grade I, no cerebral lesions in the white matter; grade II, only subcortical lesions without periventricular lesions; grade III, one to several periventricular lesions of small or moderate size; grade IVa, grade III plus subcortical lesions, with a total number less than 10; grade IVb, grade IVa plus one or two large lesions; grade V, widely distributed subcortical or periventricular white matter lesions, with a total of ten or more lesions with more than two large confluent lesions.

2.5. Oligoclonal IgG band (OCB) analysis

OCB analyses were performed in 52 patients. CSF and serum samples were coded and sent for blind analysis to the laboratory at the Division of Clinical Chemistry of Vancouver Hospital and Health Sciences Center in Canada. An experienced observer, who had detected OCB in numerous patients, tested all of the samples for OCB by a previously described method [33]. The samples were tested by isoelectric focusing and silver staining visualization with a Resolve CSF kit (Isolab Inc., USA).

2.6. Statistical analysis

Comparisons between the various alleles in patients with MS and controls were made using the chi-square test for two-by-two or two-by-three tables and Fisher's exact test. The Student's *t*-test, the chi-square test and Fisher's exact test were used to compare the clinical characteristics and the MRI findings in the MS subgroups.

3. Results

3.1. Clinical profiles

There were 21 men and 56 women. The mean age at blood sampling was 36.2 years (S.D. 11.2; range 16–58). The mean age at onset was 25.6 years (S.D. 8.9; range 15–48), and the mean duration of disease was 10.6 years (S.D. 8.6; range 1.0–48.0 years). The expanded disability status scale of Kurtzke (EDSS) ranged from 0.0 to 9.5 (mean 3.4; S.D. 2.7). MRI ratings of cerebral white matter were: grade I in three, grade II in three, grade III in ten, grade IVa in nine, IVb in seven, and grade V in 45 patients. Forty-three patients (55.8%) had relapsing-remitting MS and 34 (44.2%) had secondary progressive MS. The clinical features of these cases of 'conventional MS' were, as previously described [21,22,24], and were quite similar to those of Western MS patients. The OCB positive rate was 53.8% (28/52), with correspondence to our previous study [33]. In the control group, there were 33 men and 62 women ranging from 20 to 61 years old (mean 34.4; S.D. 10.2). The differences in sex ratio and age between the patient and the control group were not significant ($P=0.3246$, $P=0.2813$).

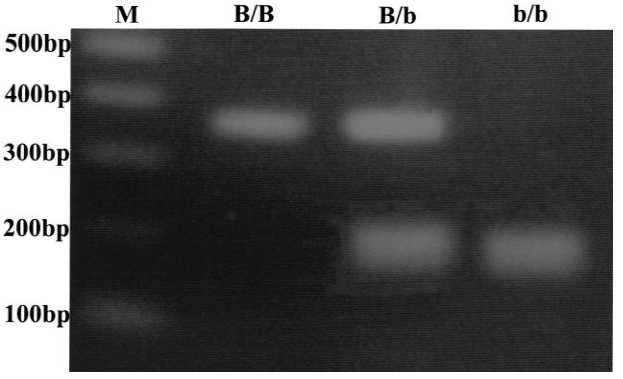

Fig. 1. Agarose gel electrophoresis. *Bsm*1 detected a dimorphism with either one band at 359 bp (allele B) or two bands at 182 and 177 bp (allele b). M=dsDNA length markers.

3.2. VDR gene polymorphism in MS patients and controls

We could clearly determine the type of VDRG polymorphism in all the patients and controls (Fig. 1). The proportions of the three VDR genotypes (BB, Bb, bb) were tabulated in Table 1. We conducted the 2×3 chi-square test for patient and control groups and found the association to be statistically significant ($\chi^2=6.041$, df=2, $P=0.0488$). A total of 85.7% of the patients carried homozygous bb, and this percentage was significantly higher than in controls (71.6%) (odds ratio=2.38, 95% CI=1.11–5.11, $P=0.0263$ by chi-square test). The b allele was excessively represented in the MS patient group (92.9%) compared with control group (84.2%) (odds ratio=2.45, 95% CI=1.20–5.00, $P=0.0138$ by chi-square test).

Among 77 patients, the male to female ratio, age at blood sampling, age at onset, clinical course, disease duration, EDSS, MRI findings, and OCB-positive rate were similar between patient groups with and without bb genotype (Table 2). Clinical course and disability, considered together with the disease duration from disease onset

Table 1
VDRG[a] polymorphism in patients with MS and controls

	Controls ($n=95$)	MS ($n=77$)
Genotype frequencies[b]		
BB	3 (3.2%)	0 (0.0%)
Bb	24 (25.3%)	11 (14.3%)
bb	68 (71.6%)	66 (85.7%)
Allele frequencies[c]		
bB	30 (15.8%)	11 (7.1%)
b	160 (84.2%)	143 (92.9%)

[a] VDRG, vitamin D receptor gene.
[b] Chi-square test of heterogeneity between controls and MS: $\chi^2=6.041$, df=2; $P=0.0488$.
[c] $\chi^2=6.058$, df=1; $P=0.0138$; odds ratio=2.44, 95%CI=1.20–5.00.

Table 2
Clinical profiles of MS patients with or without homozygotes bb genotype

	BB or Bb (n=11)	bb (n=66)	Total (n=77)
Male/Female	1:1.8	1:2.9	1:2.7
Age (mean±S.D., years)	34.9±12.9	36.4±10.9	36.2±11.2
Age at onset (mean±S.D., years)	23.7±11.5	25.9±8.4	25.6±8.9
Rel-Rem[a] course	6 (54.5%)	37 (56.1%)	43 (55.8%)
Sec-prog[a] course	5 (45.5%)	29 (43.9%)	34 (44.2%)
Duration (mean±S.D., years)	11.2±9.1	10.5±8.6	10.6±8.6
EDSS[a] (mean±S.D.)	2.9±2.7	3.5±2.7	3.4±2.7
MRI			
I	1 (9.1%)	2 (3.0%)	3 (3.9%)
II	0 (0.0%)	3 (4.6%)	3 (3.9%)
III	1 (9.1%)	9 (13.6%)	10 (13.0%)
IVa	2 (18.2%)	7 (10.6%)	9 (11.7%)
IVb	1 (9.1%)	6 (9.1%)	17 (22.1%)
V	6 (54.5%)	39 (59.1%)	45 (58.4%)
OCB positivity	3/8 (37.5%)	25/44 (56.8%)	28/52 (53.8%)

[a] Rel-Rem, relapsing-remitting course; Sec-prog, secondary progressive course; EDSS, expanded disability status scale of Kurtzke.

to blood samplings by a previously described method [33], were also similar in the two groups (data not shown). HLA allele frequencies were distributed equally between the two groups.

4. Discussion

We found, for the first time, an association of a VDRG polymorphism with MS. Since the incidence of MS is very low in Japan and the clinical features of Japanese MS have been thought to be distinct from those of Caucasians, the disease entity of MS in Japan may mask several distinct diseases with different etiologies. In this study, however, all patients had been followed-up and confirmed as having clinically definite MS for at least 1 year at the same hospital, and diagnoses other than MS had been ruled out. Conversely, patients with OpS-MS, which is relatively common in Japanese MS, remains problematic. Previous reports indicated that OpS-MS constitutes a distinct subgroup among whole MS patients [21,22,24], and there are different immunogenetic backgrounds between OpS-MS and 'conventional' MS in Japan [23,25,26]. Accordingly, we investigated this VDRG polymorphism in 'conventional' MS patients independently. Clinical features of 'conventional' MS are similar to those of Caucasians as described previously [24]. We found a significantly higher frequency of the occurrence of the homozygotic type of b allele (bb) in 'conventional' MS patients. We did not detect any difference in clinical course, clinical severity or patterns of brain MRI findings and occurrence of VDRG polymorphism. This might suggest that the VDRG variants affect the development of the autoimmune phenomena leading to MS, rather than modulating disease.

The human VDRG has eight coding exons and three alternative 5′ noncoding exons spanning over 75 kb of DNA on chromosome 12 [32]. The VDR belongs to the nuclear hormone receptor superfamily, and modulates the transcription of target genes in response to $1,25(OH)_2D_3$ [34]. Recent studies focused on the role of $1,25(OH)_2D_3$ beyond its calcemic actions, and the brain is most probably a target tissue for this hormone [35]. $1,25(OH)_2D_3$ is also a potent immunomodulatory hormone. In cultures of peripheral blood mononuclear cells, $1,25(OH)_2D_3$ inhibits mitogen- and antigen-induced lymphocyte proliferation and suppress the production of IL-1, IL-2, IL-6, TNF, and IFN gamma [2–4]. In recent studies, moreover, $1,25(OH)_2D_3$ inhibited IL-12 production by macrophage and dendritic cells by suppressing trancriptional activation of the p35 and p45 genes, which code for subunits of IL-12, and the transcriptional repression of the p40 gene is dependent on expression of VDR [9]. In animal models, 1,25(OH)2D3 can completely prevent EAE [14], a widely accepted mouse model of MS. In clinical settings, conversely, the protective effect of pregnancy for MS may be explained, in part, by increasing levels of $1,25(OH)_2D_3$ [35]. Besides immunosuppressive effects, $1,25(OH)_2D_3$ apparently controls the supply of neurotrophic factors, which may be relevant to myelin biosynthesis [35–37], and controls inducible NO synthetase expression, which may be relevant to the demyelinating process [35,38]. Conversely, an association was reported between common allelic variants in the VDRG, and bone mineral density (BMD) in adults [32]. Individuals with the BB genotype were reported to have lower BMD than individuals with the bb genotype. Interestingly, this polymorphism is also associated with prostate cancer risk, and mortality rates from prostate cancer show a similar north-south gradient, which is well known for MS, and are significantly correlated with MS mortality and MS prevalence [39]. These

findings of BMD changes and prostate cancer risk might be due to an alteration in the function of the VDR leading to differential responsiveness of target cells to the action of $1,25(OH)_2D_3$. Although the functional significance of VDRG in MS remains unknown and we cannot exclude the possibility that the association of this VDRG polymorphism with MS reflects a linkage disequibrium of the alleles with an unidentified gene locus other than VDRG, our current study indicates a possible role for VDRG in development of autoimmune phenomena leading to MS. Furthermore, our findings may account for, in part, a complex interaction of genetic and environmental forces in the development of MS, since average annual sunshine, which catalyzes the product of vitamin D_3 in skin, is significantly and inversely correlated with MS [40]. Previously reported candidate genes (e.g. MHC genes) for susceptibility to MS cannot explain this characteristic geographical distribution of MS. High prevalence of vitamin D deficiency [41,42] may be also due to, in part, the difference in VDRG polymorphism between MS patients and healthy individuals. However, since our evidence for a significant association of MS with VDR gene polymorphism is based on *Bsm*I RFLP alone, the distribution of other polymorphisms in the VDR gene, such as Apa I and Taq I, should be analyzed to confirm our result.

Besides proposing a possible role for VDRG in the development of MS, our results may shed light on the potential importance of $1,25(OH)_2D_3$ or its analogues in the treatment of MS. A combination of interferon-beta or glatiramer acetate with the vitamin D analogues is especially promising, since these combinations may synergistically affect immune function and/or may result in a decrease in side effects, as with retinoic acid or phosphodiesterase inhibitor [43,44]. Conversely, the VDR genotype of individuals may affect the responses to vitamin D analogues in patients with MS, since VDR genotype is associated with the rate of change of lumbar-spine bone density after 1-alfa-hydroxy vitamin D_3 treatment [45].

The role of VDR gene polymorphism should be further studied in other populations to confirm another susceptibility gene for MS and to obtain more adequate strategies for treatment of MS.

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