Rare Variants in the *CYP27B1* Gene Are Associated with Multiple Sclerosis

Sreeram V. Ramagopalan, DPhil,[1,2] David A. Dyment, MD, DPhil,[3] M. Zameel Cader, MD, DPhil,[2,4] Katie M. Morrison, MSc,[1,2] Giulio Disanto, MD,[1,2] Julia M. Morahan, PhD,[1,2] Antonio J. Berlanga-Taylor, MD,[1,2] Adam Handel, MD,[1,2] Gabriele C. De Luca, MD, DPhil,[1,2] A. Dessa Sadovnick, PhD,[5,6] Pierre Lepage, PhD,[7] Alexandre Montpetit, PhD,[7] and George C. Ebers, MD, FRCP, FMedSci[1,2]

Objective: Multiple sclerosis (MS) is a complex neurological disease. Genetic linkage analysis and genotyping of candidate genes in families with 4 or more affected individuals more heavily loaded for susceptibility genes has not fully explained familial disease clustering.
Methods: We performed whole exome sequencing to further understand the heightened prevalence of MS in these families.
Results: Forty-three individuals with MS (1 from each family) were sequenced to find rare variants in candidate MS susceptibility genes. On average, >58,000 variants were identified in each individual. A rare variant in the *CYP27B1* gene causing complete loss of gene function was identified in 1 individual. Homozygosity for this mutation results in vitamin D-dependent rickets I (VDDR1), whereas heterozygosity results in lower calcitriol levels. This variant showed significant heterozygous association in 3,046 parent-affected child trios ($p = 1 \times 10^{-5}$). Further genotyping in >12,500 individuals showed that other rare loss of function *CYP27B1* variants also conferred significant risk of MS, Peto odds ratio = 4.7 (95% confidence interval, 2.3–9.4; $p = 5 \times 10^{-7}$). Four known VDDR1 mutations were identified, all overtransmitted. Heterozygous parents transmitted these alleles to MS offspring 35 of 35× ($p = 3 \times 10^{-9}$).
Interpretation: A causative role for *CYP27B1* in MS is supported; the mutations identified are known to alter function having been shown in vivo to result in rickets when 2 copies are present. *CYP27B1* encodes the vitamin D-activating 1-alpha hydroxylase enzyme, and thus a role for vitamin D in MS pathogenesis is strongly implicated.
ANN NEUROL 2011;70:881–886

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction.[1] Although the cause of MS is not yet conclusively known, both genetic and environmental factors and their interactions are important.[1]

Despite a strong genetic component, with at least a 15-fold increased risk to first-degree relatives,[1] it is rare to find families with 4 or more individuals with MS. The population-based Canadian Collaborative Study Group has ascertained a large cohort of such families.[2,3] Genome-wide linkage screens of these families showed no evidence of linkage.[3] The major heritable component of MS is found in the human leukocyte antigen (HLA) class II region.[4] The HLA class II as well as non-HLA loci of modest effect identified by genome-wide association (GWA) studies[5] showed greater liability in these familial patients compared with sporadic cases[2]; however,

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.22678

Received Nov 4, 2011, and in revised form Nov 4, 2011. Accepted for publication Nov 15, 2011.

Address correspondence to Dr Ebers, Nuffield Department of Clinical Neurosciences (Clinical Neurology), Level 3, West Wing, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom. E-mail: george.ebers@clneuro.ox.ac.uk

From the [1]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom; [2]Nuffield Department of Clinical Neurosciences (Clinical Neurology), University of Oxford, Oxford, United Kingdom; [3]Department of Medical Genetics, University of Ottawa, Ottawa, Ontario, Canada; [4]Medical Research Council Functional Genomics Unit, Department of Physiology, Anatomy, and Genetics, University of Oxford, Oxford, United Kingdom; [5]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada; [6]Faculty of Medicine, Division of Neurology, University of British Columbia, Vancouver, British Columbia, Canada; and [7]McGill University and Genome Québec Innovation Centre, Montreal, Quebec, Canada.

Additional supporting information can be found in the online version of this article.

a full explanation for the strong familial aggregation observed was not uncovered. Although more common variants may exist that together may explain the increased familial aggregation, attention must also turn to rarer variants (minor allele frequencies of <1%) as possibly explaining disease clustering, although shared familial environments cannot be ignored.

One strategy to identify rare variants might be to resequence associated genomic regions identified by GWA studies. There is some evidence to support the use of this approach having been recently applied successfully to Crohn disease[6]; however, it was thought that rare coding variants in positional candidates did not make a large contribution to inherited predisposition to Crohn disease.[6] The challenges for rare variant association differ from those faced by common variant GWA studies.[7] Using families with many affected individuals may be more powerful and fruitful in finding rare causal variants.[7]

Whole-exome sequencing (ie, sequencing of all protein-coding regions of the genome) has been responsible for the identification of several autosomal recessive and dominant genes in Mendelian disease.[8] Given the relatively high penetrance observed in our multiplex MS families, we applied whole-exome sequencing technology to 43 randomly selected individuals with MS from these families (1 individual from each family) to identify any rare coding variants involved in MS etiology.

Subjects and Methods

Ascertainment of the families has been described elsewhere.[3] In brief, families were ascertained by the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS).[9] More than 30,000 families have been ascertained, of which 82 (0.2%) had 4 or more individuals with MS. For this study, a subset of 43 multicase families were selected based upon having 4 or more individuals affected with MS, with affected individuals present in >1 generation. In 51% of the families, all affected individuals were first-degree relatives. Informed consent was obtained from all subjects, and the experiments performed for this study comply with current guidelines and ethics.

DNA was extracted by standard methods. Exome capture was performed with the SureSelect Human All Exon kit (Agilent Technologies, Santa Clara, CA). Exon-enriched DNA was sequenced by the Illumina Genome Analyzer II platform following the manufacturer's instructions (Illumina, San Diego, CA). Raw image files were processed by the Illumina pipeline (version 1.3.4) for base calling with default parameters. The sequencing reads were aligned to the National Center for Biotechnology Information (NCBI) human reference genome (NCBI36.3) using Bowtie 0.12.7[10] (options "-a –best –strata"). Single nucleotide polymorphisms (SNPs) were subsequently called using SAMTools (v0.1.8, r613)[11] with the following quality criteria: ≥×20 coverage, a Phred-like[12] consensus quality of ≥30, and an SNP quality score of ≥100. Variants were defined as heterozygous when ≥25% of all nucleotides at the position showed nonreference bases and as homozygous according to the initial SAMTools classification (using default parameters). We used PolyPhen2 to assess nonsynonymous variants for a likely functional impact[13]; both the HumDiv and HumVar data sets were screened.

Reads for which Bowtie was unsuccessful in identifying an ungapped alignment were mapped to the human reference genome with Burrows–Wheeler Aligner,[14] using default parameters. Insertion–deletion polymorphisms (indels) of up to 4 bases were subsequently called using SAMTools (v0.1.8, r613)[11] with the following quality criteria: ≥×20 coverage, a Phred-like[12] consensus quality of ≥50, and an SNP quality score of ≥100. Variants were defined as heterozygous when ≥25% of all nucleotides at the position showed nonreference bases and as homozygous according to the initial SAMTools classification (using default parameters).

Additional cohorts of 3,046 parent-affected child trios, 422 parent-affected sibling pairs, and 1,873 healthy individuals (in total 12,579 people) from the CCPGSMS (Table 1) were available for validation studies. Genotyping of additional families was performed using TaqMan (Applied Biosystems, Foster City, CA) assays. Genotyping was performed blinded to affection status. The first pass genotyping success rate was >99%. To obtain a 100% genotyping success rate, many samples were genotyped in duplicate (>3,000); the genotyping concordance rate was >99.9%. Serum calcitriol measurement was performed as described.[15] Sequencing of *CYP27B1* was performed as described.[16]

TABLE 1: Patient Demographics

Group	Sex Ratio (F:M)	Relapsing–Remitting MS at Onset, %	Mean Age at Onset, yr (SD)
43 probands	1.4:1	100	29.8 (5.9)
3,046 trios	2.3:1	73	30.4 (10.7)
844 affected sib pairs	2.5:1	74	31.4 (11.1)

F = female; M = male; MS = multiple sclerosis; SD = standard deviation.

Results

One affected individual from each of the 43 families was selected at random. In total, 25 females and 18 males were sequenced. All individuals had clinically definite MS as defined by the Poser criteria, and all had the relapsing–remitting subtype at disease onset. The average

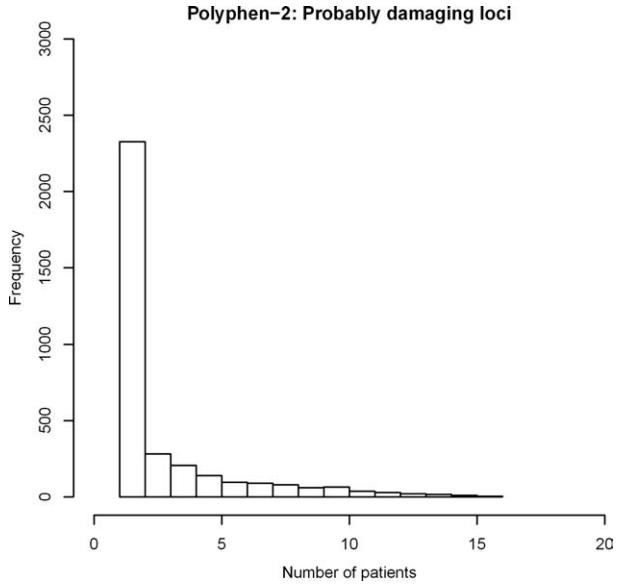

FIGURE : Frequency of probably damaging variants in sequenced individuals.

age of MS onset of these individuals was 29.8 (standard deviation, 5.9) years (see Table 1).

Paired, 76bp reads from postenrichment shotgun libraries were aligned to the reference genome (Supplementary Table 1). On average, 3.7 gigabases of mapped sequence were generated per individual. The average coverage of each exome was 70-fold. On average, 58,400 SNPs were called per individual (range, 45,689–73,486), of which 58% were already annotated in a public database (dbSNP v131). On average, 5,060 indels were identified per sample, of which 14% had been previously reported. A predicted loss of function gene (a missense mutation predicted by PolyPhen to be damaging or frameshift indel; either the same variant or different variants in the same gene) was not present in any of the 43 individuals. The distribution of all probably damaging variants is shown in the Figure. No 2 individuals had any probably damaging variants in common that were novel or had a dbSNP reported allele frequency of <5%.

A recent genome-wide association study provided evidence for modest association of 57 genetic regions with MS, in total implicating >340 candidate genes.[17] By looking for variants that were either (1) novel or present in the 1000 Genomes project but not in the HapMap study and predicted by Polyphen to be damaging or (2) had HapMap allele minor allele frequencies of 5% or less and that were predicted by PolyPhen to be damaging in these candidate genes, we identified 3 variants in *CBLB*, *IL7R*, and *CYP27B1* (Table 2), each present in a different family. No exonic indels were identified in any of these genes. To assess the effect of the variants identified in patients with MS in general, we genotyped the 3 variants in 3,046 parents–affected child trios. This cohort had >99% power to find a fully penetrant dominant acting variant with a frequency of 1%, conferring an odds ratio of 2. We used the transmission disequilibrium test (TDT) to assess significance; the TDT measures the overtransmission of an allele from heterozygous parents to affected offspring.[18]

Only rs118204009 showed evidence of association in the larger MS cohort (see Table 2), and genotyping the variant in the original multiplex family showed that it was present in 100% of affected family members (4 of 4; an affected sib pair and their affected aunt and affected cousin) and in 33% of genotyped unaffected family members (1 of 3; variant present in unaffected mother of the sib pair), highlighting that rs118204009 is an incompletely penetrant variant.

The variant was further genotyped in 1,873 ethnicity-matched healthy individuals and 422 parent-affected sib pairs. rs118204009 was not found in healthy controls, HapMap samples, or the 1000 Genomes project, and was transmitted from 4 heterozygous parents to 4 affected sib pairs (sib TDT, $p = 0.046$).[19] rs118204009 has been identified in patients with the rare autosomal recessive disorder vitamin D-dependent rickets type 1 (VDDR1).[20] The mutation causes an arginine to histidine change at position 389 of the protein (R389H). *CYP27B1* encodes the 1-alpha-hydroxylase enzyme,

TABLE 2: Candidate Variants from Exome Sequencing Further Genotyped in Additional Samples

SNP ID or hg19 Coordinate	Gene	Reported in 1,000 Genomes	HapMap Minor Allele Frequency	MS TDT (transmitted: not transmitted)	*p*
rs2229232	*IL7R*	Yes	0.9%	27:30	0.23
rs118204009	*CYP27B1*	No	N/A	19:0	1×10^{-5}
chr3:105464791	*CBLB*	No	N/A	2:4	0.41

MS = multiple sclerosis; N/A = not applicable; SNP = single nucleotide polymorphism; TDT = transmission disequilibrium test.

TABLE 3: *CYP27B1* Mutations Identified

Mutation (alleles[a])	Frequency in MS Patients, n = 3,564	Frequency in Controls, n = 1,873	Amino Acid Change	Loss of Function
rs118204009 (C/**T**)	0.67%	0	R389H	In vitro confirmed, associated with VDDR1[20]
rs118204012 (T/**C**)	0.08%	0	E189G	In vitro confirmed, associated with VDDR1[27]
rs118204011 (G/**A**)	0.05%	0	L343F	In vitro confirmed, associated with VDDR1[27]
chr12:58157573 (C/**T**)	0.05%	0	Y413C	Associated with VDDR1[28]
chr12:58158828 (G/**A**)	0.05%	0	R252C	PolyPhen predicted

[a]Risk allele in bold.
MS = multiple sclerosis; VDDR1 = vitamin D-dependent rickets type 1.

which converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, the biologically active form of vitamin D. In vitro studies have shown that the R389H mutation leads to complete loss of enzyme activity of *CYP27B1* and lower levels of activated vitamin D (calcitriol).[16,20] None of the individuals with MS who had the R389H mutation had a clinical history of VDDR1; this is likely due to VDDR1 resulting from compound mutations in *CYP27B1*.

VDDR1 has a higher prevalence in French Canadians,[20] and thus it may be that the results we observed a merely a reflection of population stratification. However, the families in which we observed the R389H mutation did not have French Canadian backgrounds (based on 4 grandparental ethnicities), and our analyses were based on transmissions within families, suggesting a causative role of *CYP27B1* mutations in MS. To see if any other loss of function mutations in *CYP27B1* influence MS risk, we genotyped 9 other nonsynonymous polymorphisms (Supplementary Table 2) causing loss of function of *CYP27B1* in the larger MS cohort of trios and sib pairs as well as controls. Two variants were found, rs118204012 (transmitted 3×, not transmitted 0× in trios) and rs118204011 (transmitted 2×, not transmitted 0× in trios). Again, these trios were also all not French Canadian. rs118204012 causes a change from glutamate to glycine at position 189 and rs118204011 a change from leucine to phenylalanine at position 343, and both lead to partial loss of function of *CYP27B1*.[20] Serum was available from 1 individual carrying the rs118204009 mutation, and this person had a calcitriol level of 38pmol/l (normal range, 50–160pmol/l). As part of ongoing investigations examining vitamin D levels in patients with MS and their relatives,[15] we selected 96 individuals with MS (not included in the above genotyping studies) with persistently low levels of calcitriol when measured on 2 occasions (both values <40pmol/l) for *CYP27B1* sequencing. Sequencing of all 9 exons in these 96 individuals identified 1 individual carrying the R389H mutation, a novel mutation in 1 individual causing an arginine to cysteine amino acid change at position 252, and another VDDR1-associated mutation causing a tyrosine to cysteine amino acid change at position 413. The R252C change is predicted to be loss of function by PolyPhen. These findings connect heterozygote loss of function *CYP27B1* mutations with low serum calcitriol. R252C was transmitted once and not transmitted 0× in the trios, and Y413C was transmitted twice and not transmitted 0× in the trios. These variants were not present in the 1,873 control samples (Table 3).

Pooled transmission of the 5 variants identified in the trios and sib pairs was highly significant (transmitted, 35; not transmitted, 0; $p = 3 \times 10^{-9}$). Combining all variants identified in unrelated MS patients (34 of 3,564; frequency, 0.95%) and comparing to controls (0 of 1,873) provided a Peto odds ratio of 4.7 (95% confidence interval, 2.3–9.4; $p = 5 \times 10^{-7}$). There appeared to be a strong trend for CYP27B1 variants to be transmitted from the mother as compared to the father (maternal: transmitted, 24; not transmitted, 0; paternal: transmitted, 11; not transmitted, 0; comparison $p = 0.09$).

Discussion

For this study, we reasoned that families with 4 or more affected members in different generations would be more likely than sporadic or sib pair cases to carry variants conferring a greater risk for MS. Initially, we sequenced 1 individual from each family to see if any rare variants were in common between these families. This was not the case, and we therefore investigated candidate MS genes from associated regions in GWA studies. After replication studies, we obtained convincing evidence to

support a role for rare variants in the *CYP27B1* gene in MS. We show here the potential power of using multiplex families to identify the role of rare variants underlying disease, but this required the entire resource of the population-based CCPGSMS.[9] Other variants that influence MS risk likely exist in these families but would not be detected using the current approach, as they do not lie in MS-associated regions.

CYP27B1 has been previously tentatively associated in MS through a GWA study.[17] However, the main associated SNP (rs12368653) actually resides in a gene next to *CYP27B1*, both lying in an associated region containing 25 genes, and thus the primary association remained unclear. We identified no rare variants in the 24 genes next to *CYP27B1*, and thus our data would support *CYP27B1* as being the causative gene for MS in this region. The variants we identified have a striking transmission rate (35 of 35×); however, the variant must be incompletely penetrant, as it is being transmitted by unaffected parents.

Compound or homozygous loss of function mutations in *CYP27B1* cause VDDR1. VDDR1 has a prevalence of approximately 1 in 500,000, whereas that for MS is 1 in 1,000 in Northern Europe or Northern European-derived populations. Accordingly, the prior null expectation would be that 500,000 cases of MS or 1,000 cases of VDDR1 would need to be seen to find 1 case of co-occurrence. In actuality, all 3 patients with VDDR1 identified in Norway had comorbid MS,[21] making a spurious association between *CYP27B1* mutations and MS highly improbable.

Heterozygous loss of function *CYP27B1* mutations lower circulating levels of calcitriol.[16,22] Accordingly, our approach circumvents the often difficult question of relating gene association to function. To this end, we sequenced MS patients who showed consistently low levels of calcitriol and identified a novel putative loss of function mutation in *CYP27B1*. We were unable to access serum from all of our patient samples to show low calcitriol levels for all variants identified. Most biological effects of vitamin D are mediated by calcitriol acting via the vitamin D receptor.[23] We have previously shown that vitamin D regulates >80% of MS-associated genes,[23] and thus it is likely that lower levels of calcitriol as a result of *CYP27B1* mutations lead to a disruption to critical gene–environment interactions important for the developing immune or nervous system, which then predisposes to MS.

The most striking illustration of the importance of the environment in MS has been the observation that incidence and prevalence of MS increase with latitude of childhood and adolescent habitation in both hemispheres.[1] This latitude gradient correlates inversely with ultraviolet radiation exposure, the major determinant of vitamin D levels, and generated the hypothesis that vitamin D influences MS susceptibility.[1] Further attention to climate came from a long series of genetic epidemiological studies showing that the environment acts at a broad population level.[1] The association of loss of function *CYP27B1* mutations with MS links low vitamin D levels directly to disease susceptibility, and validates prior studies showing that MS patients are more likely to be vitamin D deficient prior to disease onset[24] and that MS risk is increased for late spring births and decreased for those in late autumn; in Scotland, which has the world's highest MS rate, risk differences for peak and nadir births can reach an astonishing 50%.[25] In summary, therefore, recent work has made a role for vitamin D in MS broadly unequivocal.[1] Given that an estimated 1 billion people worldwide are deficient to some degree,[26] studies of vitamin D supplementation for disease prevention are strongly warranted.

Acknowledgment

This work was funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland and the Wellcome Trust 075491/Z/04. G.C.E. is the Action Research Professor of Neurology at the University of Oxford.

We thank the patients and their family members who generously participated in this study and Drs H. Hanwell and V. Rakyan for helpful advice.

Potential Conflicts of Interest

Nothing to report.

Authorship

S.V.R. and D.A.D. contributed equally to this article.

References

1. Ebers GC. Environmental factors and multiple sclerosis. Lancet Neurol 2008;7:268–277.
2. D'Netto MJ, Ward H, Morrison KM, et al. Risk alleles for multiple sclerosis in multiplex families. Neurology 2009;72:1984–1988.
3. Willer CJ, Dyment DA, Cherny S, et al. A genome-wide scan in forty large pedigrees with multiple sclerosis. J Hum Genet 2007; 52:955–962.
4. Lincoln MR, Montpetit A, Cader MZ, et al. A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. Nat Genet 2005;37:1108–1112.
5. International Multiple Sclerosis Genetics Consortium. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007;357:851–862.
6. Momozawa Y, Mni M, Nakamura K, et al. Resequencing of positional candidates identifies low frequency IL23R coding variants

protecting against inflammatory bowel disease. Nat Genet 2011; 43:43–47.

7. Cirulli ET, Goldstein DB. Uncovering the roles of rare variants in common disease through whole-genome sequencing. Nat Rev Genet 2010;11:415–425.
8. Ng SB, Buckingham KJ, Lee C, et al. Exome sequencing identifies the cause of a Mendelian disorder. Nat Genet 2010;42:30–35.
9. Sadovnick AD, Risch NJ, Ebers GC. Canadian collaborative project on genetic susceptibility to MS, phase 2: rationale and method. Canadian Collaborative Study Group. Can J Neurol Sci 1998;25:216–221.
10. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.
11. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078–2079.
12. Ewing B, Green P. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res 1998;8:186–194.
13. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7: 248–249.
14. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754–1760.
15. Orton SM, Morris AP, Herrera BM, et al. Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis. Am J Clin Nutr 2008;88:441–447.
16. Kim CJ, Kaplan LE, Perwad F, et al. Vitamin D 1alpha-hydroxylase gene mutations in patients with 1alpha-hydroxylase deficiency. J Clin Endocrinol Metab 2007;92:3177–3182.
17. International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium 2. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011;476:214–219.
18. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet 1993;52: 506–516.
19. Spielman RS, Ewens WJ. A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test. Am J Hum Genet 1998;62:450–458.
20. Wang JT, Lin CJ, Burridge SM, et al. Genetics of vitamin D 1alpha-hydroxylase deficiency in 17 families. Am J Hum Genet 1998;63:1694–1702.
21. Torkildsen O, Knappskog PM, Nyland HI, Myhr KM. Vitamin D-dependent rickets as a possible risk factor for multiple sclerosis. Arch Neurol 2008;65:809–811.
22. Alzahrani AS, Zou M, Baitei EY, et al. A novel G102E mutation of CYP27B1 in a large family with vitamin D-dependent rickets type 1. J Clin Endocrinol Metab 2010;95:4176–4183.
23. Ramagopalan SV, Heger A, Berlanga AJ, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res 2010;20:1352–1360.
24. Munger KL, Levin LI, Hollis BW, et al. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. JAMA 2006;296:2832–2838.
25. Willer CJ, Dyment DA, Sadovnick AD, et al. Timing of birth and risk of multiple sclerosis: population based study. BMJ 2005;330: 120.
26. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357: 266–281.
27. Wang X, Zhang MYH, Miller WL, Portale AA. Novel gene mutations in patients with 1α-hydroxylase deficiency that confer partial enzyme activity in vitro. J Clin Endocrinol Metab 2002;87: 2424–2430.
28. Edouard T, Alos N, Chabot G, et al. Short- and long-term outcome of patients with pseudo-vitamin D deficiency rickets treated with calcitriol. J Clin Endocrinol Metab 2011;96:82–89.