Research Article

MYRF is associated with encephalopathy with reversible myelin vacuolization (75 characters)

Running head

MYRF is associated with myelin vacuolization (44 characters)

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ABSTRACT

Objective

Reversible myelin vacuolization is associated with variable conditions including mild encephalitis/encephalopathy with a reversible splenial lesion (MERS), which is characterized by mildly impaired consciousness and transient splenial lesion. Familial and/or recurrent cases with a clinical diagnosis of MERS suggest the presence of genetic factors.

Methods

We examined a family in which the proband presented with a history of recurrent encephalopathy with extensive but reversible cerebral myelin vacuolization and neurological symptoms similar to those of MERS spanning over three generations. Whole-exome sequencing was performed in family members.

Results

Eight rare nonsynonymous single-nucleotide variants shared by all patients were identified. By filtering genes expressed in the corpus callosum, we identified a heterozygous c.1208A>G predicting p.Gln403Arg in the highly conserved DNA-binding domain in the myelin regulatory factor (*MYRF*) gene. We subsequently screened the coding regions of *MYRF* by Sanger sequencing in our cohort comprised of

33 sporadic cases with MERS and three cases in another family with extensive myelin vacuolization, and identified the same heterozygous c.1208A>G in all affected members in the second family. Luciferase assay revealed that transcriptional activity of N-terminal region of MYRF was significantly diminished by introducing the c.1208A>G variant.

Interpretation

MYRF is a transcriptional regulator that is necessary for oligodendrocyte differentiation and myelin maintenance. Functional defects of MYRF are likely to be causally associated with encephalopathy with extensive myelin vacuolization. We propose the term "*MYRF*-related mild encephalopathy with reversible myelin vacuolization (MMERV)". Our findings provide a new perspective on the pathogenesis of myelin vacuolization.

INTRODUCTION

Myelin vacuolization is associated with several forms of disorders affecting white matter of the brain. It is triggered by variable pathologic mechanisms, and clinical manifestation is reversible in some patients. Several forms of reversible myelin vacuolization have been reported in association with genetic etiology. X-linked Charcot-Marie-Tooth disease (CMT) X1 is caused by pathogenic variants in *GJB1,*[1] and transient white matter lesion has been reported in children and adults with CMTX1.[2] Megalencephalic leukoencephalopathy with subcortical cysts (MLC) 2B is caused by dominant variants in *GLIALCAM*. [3,4] In MLC2B patients with the remitting clinical phenotype, improvement and normalization of white matter abnormalities have been reported.[5]

Mild encephalitis/encephalopathy with a reversible splenial lesion (MERS) is a subtype of acute encephalopathy characterized by a transient white matter lesion suggesting myelin vacuolization.[6,7] Central nervous system (CNS) symptoms in children with MERS are impaired consciousness and/or delirious behavior lasting for several days.[6,8] Brain lesions observed in MERS patients are characterized by transiently reduced diffusion in the splenium of the corpus callosum, which usually disappears within one week. Brain lesions sometimes extend to the entire corpus

callosum and bilateral subcortical white matter in the centrum semiovale.[9,10,11] Familial cases with MERS have been reported, suggesting the presence of genetic factors.[12]

We examined familial cases of encephalopathy with extensive but reversible cerebral myelin vacuolization including splenium in which the proband had a history of recurrent reversible myelin vacuolization spanning over three generations. All the affected individuals showed CNS symptoms similar to those of MERS. To explore the responsible gene, we performed whole-exome sequencing in the family. By focusing on rare nonsynonymous variants in genes expressed in the CNS, especially in the white matters, we identified a single-nucleotide variant (SNV) in the *MYRF* gene in all affected family members. We also sequenced the *MYRF* gene in another family that was previously described as familial MERS[12] and 33 additional patients with sporadic MERS. Here, we report the clinical and genetic features of these patients.

SUBJECTS AND METHODS

Subjects

The proband of Family A was admitted to a hospital affiliated with Juntendo University Faculty of Medicine. Their clinical information is described in the following section. We previously reported on Family B.[12] Blood samples of the participants from

Family B were obtained by the Study Group of Acute Encephalopathy among Children in Japan, supported by a grant from the Ministry of Health, Labour and Welfare in Japan (principal investigator, Masashi Mizuguchi) for the purpose of exploring genetic factors related to acute encephalopathy. Blood samples from the additional 33 individuals with MERS were also provided by the Study Group of Acute Encephalopathy among Children in Japan. In total, 17 participants were males and 16 were females. Informed consent was obtained from all participants. The study protocol received prior approval by the Ethics Review Committees of Aichi Medical University, Juntendo University, and the University of Tokyo.

In this study, MERS was defined as acute encephalopathy associated with both (1) reversible CNS symptoms such as impaired consciousness, delirious behavior, and seizures and (2) a reversible splenial lesion observed on diffusion magnetic resonance imaging (MRI).

Whole-exome sequencing analysis in Family A

Using DNA extracted from blood, whole-exome sequencing was performed in five individuals (II-3, III-2, III-3, III-4, and IV-1) from Family A using a SureSelect Human All Exon v4 Kit (Agilent Technologies, Santa Clara, CA, USA) and a HiSeq

2500 sequencing system (Illumina, San Diego, CA, USA). Reads were first mapped against the hg19 reference genome using BWA[13] (http://bio-bwa.sourceforge.net), and unmapped reads were mapped again using BLAT[14] (http://www.molecularevolution.org/software/genomics/blat) (default parameters for both methods). PCR duplicates were removed using Picard tools (http://picard.sourceforge.net). Sequence variants were called using VarScan2[15] (http://dkoboldt.github.io/varscan/) with the threshold of coverage ≥ 8, variant call ≥ 2, and ratio of variant ≥ 0.2.

Sequencing of MYRF *in the MERS cohort*

The candidate disease-associated SNV within the *MYRF* gene identified by whole-exome sequencing in Family A was confirmed by Sanger sequencing in affected members in Families A and B, as well as in 33 additional MERS patients. A total of 28 exons and their flanking intronic regions were amplified by PCR using the primers shown in Supplementary Table 1. PCR products were sequenced on an Applied Biosystems 310 Genetic Analyzer or a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The position of human *MYRF* cDNA was obtained from GenBank (accession number NM_001127392).

Luciferase assay

We performed a luciferase assay to examine the transcriptional activity of MYRF with the candidate disease-associated SNV. To construct the luciferase reporter vector with the previously identified enhancer in the rat *Rffl* gene (pGL3P-Rffl),[16] which is recognized and activated by MYRF, the intron of *Rffl* was amplified by PCR using the following primer pair: forward, 5′-ACGTGGTACCTCTTAGGAGACTGCCGCT-3′ and reverse, 5′-ACTGCTCGAGTTTGCCATAGGCTCTCACTGT-3′, where the restriction endonuclease sites are underlined. Amplified DNA was ligated into the KpnI and XhoI sites of the pGL3-promoter vector (Promega, Madison, WI, USA).

To construct expression vectors for the N-terminal fragment of MYRF containing the DNA-binding domain (DBD) and nuclear localization signals (pRBG4-MYRF-N), the human *MYRF1* cDNA (AB023171; NBRC, NITE, Kisarazu, Japan) was amplified by PCR using the following primer pairs; forward, 5′-ATATGCGGCCGCCATGGAGGTGGTGGACGAGACGGA-3′: reverse, 5′-GACTGGTACCGAGGGGTGCATAAGCGAGCCCAT-3′, where the restriction endonuclease sites are underlined. The amplified DNA was ligated into the NotI and KpnI sites of the CMV-driven expression vector pRBG4.[17] The c.1208A>G mutation

was introduced into pRBG4-MYRF-N using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA).

HEK293 cells were grown in Dulbecco's' modified Eagle's medium with 10% fetal bovine serum. HEK293 cells seeded on a 96-well plate were transfected with 5 ng pGL3P-Rffl and 5 ng pRL/SV40 (Promega) along with 40 ng of the pRBG4 empty vector or pRBG4-MYRF-N using FuGENE 6 (Roche, Indianapolis, IN, USA). At 48 h after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions.

RESULTS

Clinical information

Family A

The proband (IV-1, Fig 1A) was a female and the first child of unrelated parents. She experienced a prolonged seizure with normal MRI findings at 32 months of age. At 38 months of age, she experienced a seizure following a subsided fever within two days. Loss of consciousness, eye deviation, and smacking-like oral movement lasted for 30 minutes. After her seizure had been controlled by intravenous diazepam,

she could not comply with simple verbal directions and was in a mildly excited state. Laboratory data were unremarkable. Electroencephalogram (EEG) showed generalized slowing, and brain MRI revealed high intensities in the entire corpus callosum and bilateral white matter in the centrum semiovale on diffusion-weighted and T2-weighted images (Fig 2A). At that time, she was diagnosed with MERS and was treated with methylprednisolone pulse therapy. She recovered consciousness within three days. Brain MRI one week after admission revealed complete recovery of brain lesions (Fig 2B). She experienced repeated episodes of altered consciousness with similar MRI abnormalities at 75, 86, and 99 months of age. However, no neurological sequelae were observed at the last follow-up at 106 months of age.

The relatives of the proband had also experienced episodes of disturbed consciousness or movement lasting for a few days. Her mother (III-2) had experienced a clinical event characterized by speech difficulty and spontaneous movement for three days after a febrile illness at 11 years of age. Her aunt (III-3) had experienced a seizure followed by impaired consciousness for three days associated with febrile illness at 6 years of age. In addition, her uncle (III-5) had experienced an episode of delirious behavior, which included meaningless speech and laughter associated with febrile illness at 8 years of age. No recurrence of similar clinical events was observed in these

individuals. Moreover, the proband's grandmother (II-2) had experienced four clinical events between 5 and 13 years of age associated with febrile illness, characterized by difficulty in spontaneous movement, dysarthria, and mildly impaired consciousness. Her grandaunt (II-1) had also experienced a single clinical event associated with difficulty in spontaneous movement accompanied by pyrexia during her childhood. EEG and MRI were not performed on any other family member, except for her uncle (III-5), who showed no remarkable abnormality. No individual with a history of clinical events exhibited neurological sequelae.

Family B

Family B was partially described previously as familial MERS[12] and is not related to Family A. The proband (II-1) was a previously healthy female and the first child of unrelated parents. At 6 years of age, she presented with impaired consciousness and seizures following 1 day of febrile illness. Laboratory data were unremarkable, and the EEG showed no abnormalities. MRI revealed reduced diffusion in the entire corpus callosum and bilateral white matter in the centrum semiovale (Fig. 2C). At that time, she was diagnosed with MERS and treated with methylprednisolone pulse therapy. She completely recovered in 2 days. MRI on day 6 of the illness showed resolution of

diffusion abnormalities (Fig. 2D). Her younger sister (II-3) had a similar clinical event, characterized by mildly impaired consciousness following a 1 day febrile illness at 2 years of age. She was diagnosed with MERS with similar MRI findings (Fig 2E). She was treated with dexamethasone and recovered completely, with resolution on MRI (Fig. 2F). In addition to the two previously reported sisters, the second eldest sister (II-2) had experienced similar clinical symptoms and MRI findings at 9 years of age. Their mother (I-2) had presented with aphasia and convulsions during measles at 6 years of age, but no definite diagnosis had been made. Their father (I-1) had no history of convulsions or impaired consciousness.

Identification of disease-associated variants in Family A

The average amount of exome coverage ranged from 68× to 81× in five samples, and over 80,000 variants were called in each sample. A total of 31,613 variants were shared by all five patients. Intronic or synonymous variants were eliminated, as well as variants with a minor allelic frequency ≥ 0.01. Eight rare non-synonymous SNVs were shared by all five patients (Table 1). We next examined the expression of candidate genes in the cerebral cortex using the GTEx Portal[18] (http://gtexportal.org/home/) and in the corpus callosum using Fantom5[19]

(http://fantom.gsc.riken.jp/5/) (Supplementary Table 2). Among the eight candidate genes, the expression level of *MYRF* was highest in the corpus callosum in 889 human tissues/cells according to Fantom5, whereas the expression levels of the other seven genes were either zero or not top-ranked in the corpus callosum. We thus assumed that an SNV in *MYRF* is the causative mutation. The missense variant c.1208A>G, predicting p.Gln403Arg, in *MYRF* has not been reported in any SNV database (NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), 1000 Genomes (http://www.internationalgenome.org/), Human Genetic Variation Database including a Japanese cohort (http://www.hgvd.genome.med.kyoto-u.ac.jp/), or ExAC (http://exac.broadinstitute.org/).[20] The Gln at codon 403 is highly conserved across species (Fig 3). FATHMM (http://fathmm.biocompute.org.uk/), MetaSVM, MetaLR, and VEST3 (http://karchinlab.org/apps/appVest.html), which are the most dependable tools used to predict the deleterious effects of amino acid substitution,[21] all indicated that p.Gln403Arg is the most deleterious missense SNV in the eight candidate SNVs. The c.1208A>G variant was verified by Sanger sequencing in all individuals (Fig 1C and D).

Exploration of MYRF variants in Family B

We explored the *MYRF* variant in three affected individuals (I-2, II-1, and II-2) in Family B by Sanger sequencing. The same c.1208A>G variant was identified in all affected members in Family B.

Transcriptional activity of the c.1208A>G variant

The N-terminal region of MYRF contains a DBD, which regulates the transcription of myelin genes.[16,22] The c.1208A>G variant found in our study is located in the DBD. We prepared a luciferase reporter construct harboring the enhancer element of the *Rffl* gene, which is recognized and activated by MYRF,[16] and performed the luciferase assay. Our analysis revealed that overexpression of the N-terminal region of MYRF increased luciferase activity 14-fold, which was significantly diminished by introducing the c.1208A>G variant (Fig 5).

Exploration of MYRF variants in sporadic cases with MERS

To identify additional deleterious variants in *MYRF* among other individuals with MERS, we performed Sanger sequencing in the coding regions and exon-intron boundaries of *MYRF* in 33 individuals. This cohort consisted of four sporadic cases with recurrent episodes of MERS and 29 sporadic cases with a single episode. Variants with

an allelic frequency < 0.01 were not identified in these 33 individuals.

Comparison of other filtered variants between the two families

We examined the other seven rare coding SNVs originally identified in Family A in Family B using Sanger sequencing (Supplementary Table 2). Two SNVs in *C11orf94* and *MS4A10* located upstream of *MYRF* were detected in all affected members of Family B. The other five rare coding SNVs in Family A on different chromosomes were absent in Family B. We also observed a rare intronic SNV in *EML3* (rs14942456) downstream of *MYRF* in both families. The SNVs shared between Families A and B spanned 15.6 Mbp upstream and 0.8 Mbp downstream of the missense SNV in *MYRF* on chromosome 11 (Fig 4), suggesting that affected members in Families A and B have a founder haplotype. Among the rare SNVs in three genes (*C11orf94*, *MS4A10*, and *MYRF*) in the shared haplotype region, only *MYRF* was likely to be pathogenic because *C11orf94* and *MS4A10* are scarcely expressed in the cerebral cortex and are not expressed in the corpus callosum.

DISCUSSION

We identified the same c.1208A>G variant in *MYRF* in eight individuals with

mild encephalopathy with extensive white matter myelin vacuolization in two families. Tissue specificity of gene expression and reported functions of the gene product led us to assume that the novel missense SNV in *MYRF* was the genetic cause of encephalopathy with myelin vacuolization in Family A. In addition, no previous report has indicated an association among the other seven genes, where rare SNVs were identified, with human diseases affecting the CNS, metabolism, or inflammation. Further analysis of our cohort revealed that the three affected sisters in Family B carried the same SNV of *MYRF*. We also found that the affected members in both families carried two other rare SNVs in *C11orf94* and *MS4A10* on chromosome 11. However, *C11orf94* and *MS4A10* are not expressed in the corpus callosum and are expressed only in extremely limited testis and intestinal tissues, respectively.[18,19] Thus, these genes are unlikely to be associated with myelin vacuolization. The affected members in Families A and B shared at least 16 Mbp spanning the three rare SNVs and an intronic SNV in *EML3* (rs14942456) on chromosome 11. The presence of the founder haplotype exhibiting the same phenotype in two unrelated families sharing no common ancestors over four generations suggests that the genetic cause is within the shared region that includes *MYRF*. Mutations in 24 genes in this region cause 44 disease phenotypes according to the Online Mendelian Inheritance in Man (OMIM) database

(https://omim.org/) (Supplementary Table 3); however, none is reminiscent of recurrent selective white matter injuries. The prediction of pathogenicity of the amino acid substitution, conservation of the mutated amino acid across species, lack of the identified SNV in multiple databases including a Japanese cohort, lack of other disease-causing genes in the founder haplotype, phenotypic similarities between encephalopathy with myelin vacuolization and the conditional knockout of *Myrf*, as stated below, and suppression of transcriptional activity of the *Rffl* promoter, all support that c.1208A>G in *MYRF* is causally associated with encephalopathy with reversible myelin vacuolization.

MYRF encodes a myelin regulatory factor that is necessary for oligodendrocyte differentiation and the maintenance of mature oligodendrocytes and myelin structure.[23,24] MYRF interacts with Sox10, another transcriptional regulator of myelination.[25,26] No *MYRF* variants have been reported in association with human diseases. In mouse models, conditional knockout of *Myrf* in mature oligodendrocytes and oligodendrocyte precursors causes downregulation of myelin protein expression and blocks formation of new oligodendrocytes, leading to impaired motor skill learning.[24,26,27] Consequently, the mice exhibit remyelination failure and breakdown of myelin sheaths. This demyelination is associated with the infiltration of activated

microglia/macrophages into the white matter. These observations indicate that *MYRF* may play an important role in myelin maintenance in the white matter, whereby its dysfunction results in insufficient maintenance of the myelin sheath, leading to myelin vacuolization.

MYRF contains several domains that are highly conserved among a range of eukaryotic organisms from fungi to mammals.[16] It contains DBD, an intramolecular chaperone auto-processing (ICA) domain, a transmembrane domain (TMD), and a Sox10-binding domain (SBD). MYRF protein is first expressed as a transmembrane protein and subsequently undergoes proteolytic processing, which results in a release of the N-terminal region containing the DBD from the endoplasmic reticulum into the nucleus to regulate the transcription of myelin genes.[16,22] The c.1208A>G variant found in our study is located in the DBD and is highly conserved across species. The luciferase analysis showed a significant reduction in MYRF-induced luciferase activity after introducing the c.1208A>G mutation. The c.1208A>G variant compromises the ability of the DBD domain to regulate the transcription of myelin genes. The magnitude of functional changes of the c.1208A>G variant is presumed to be less serious because all individuals with this variant exhibit normal psychomotor development with no irreversible neurological sequelae, even after recurrent neurological events. We

speculate that the function of MYRF is relatively preserved under usual circumstances, and that its function becomes insufficient when the demand is increased under various pathological conditions such as infection. To clarify the effects of the c.1208A>G variant, the interaction between MYRF and the targeted myelin genes should be investigated under several conditions that mimic the triggers of reversible myelin vacuolization.

Although CNS symptoms of the patients with *MYRF* variant are indistinguishable from MERS, the patients with *MYRF* variant present with distinct radiological features. The main clinical features of MERS include mildly impaired consciousness and/or delirious behavior, which resolve within a few days to 1 week. Seizures can be observed in some patients with MERS. The affected individuals in our study presented with symptoms that were similar to those of MERS triggered by febrile illness. On the other hand, MRI lesions of our patients with a *MYRF* variant involved widespread white matter and the entire corpus callosum, which were more extensive than typical MERS. In addition, no variants in *MYRF* are identified in 33 sporadic cases with MERS. It suggests that the cases with *MYRF* variant and the other sporadic cases may represent different conditions. We propose the term "*MYRF*-related mild encephalopathy with reversible myelin vacuolization (MMERV)" to characterize the

condition. Although extensive clinico-radiological studies are required for additional cases with MMERV, our study indicates that a *MYRF* variant should be tested for when MRI shows extensive myelin vacuolization. In contrast, a *MYRF* variant is less likely in patients with restricted lesions in the splenium of the corpus callosum. In patients without extensive white matter lesions or *MYRF* variants, such as sporadic cases in our study, some other factors are presumed to contribute to the development of MERS.

Reversible myelin vacuolization affecting cerebral white matter including the corpus callosum is associated with different gene defects such as *GJB1* and *GLIALCAM*, toxic substances, and infections. Transient and recurrent CNS symptoms with white matter abnormalities involving the corpus callosum and centrum semiovale have been reported in patients with CMTX1.[28,29] CMTX1 is inherited in an X-linked manner, and carrier females may or may not present with mild to moderate symptoms, including CNS symptoms.[30] The causative gene *GJB1* encodes connexin-32 (Cx-32).[1] Cx-32 is a myelin-related protein involved in gap junction formation and is expressed in both the peripheral nervous system and the CNS.[31] Interestingly, the *GJB1* p2 promoter is activated by a combination of Sox10 and Myrf *in vitro*,[25] indicating that *GJB1* and *Myrf* are essential components of the myelin-specific regulatory network in the CNS. In our study, the patients in the two families presented with no peripheral nervous system

symptoms such as depressed tendon reflexes and no rare variants were identified in the *GJB1* coding region. The interaction between *Myrf* and *GJB1*, and the similarity in distribution of the myelin vacuolization in the CNS between MMERV and CMTX, indicates that a shared pathomechanism for those disorders is likely.

In summary, we identified a heterozygous missense variant in *MYRF* in patients with familial encephalopathy with widespread lesions in the cerebral white matter in two families. MYRF is a transcriptional regulator of the myelin gene, which is necessary for oligodendrocyte differentiation and myelin maintenance. The variant affects a highly conserved residue in the DBD of MYRF, and a luciferase assay revealed that the transcriptional activity of the MYRF N-terminal region was significantly diminished after introducing the variant. These results suggest that MYRF dysfunction is causally associated with the development of reversible myelin vacuolization.

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Author Contributions

Study concept and design: H.K., Y.A., K.O., and A.O. Data acquisition and analysis: H.K., Y.A., A.M., T.O., E.N., T.I., M.S., M.M., K.O., and A.O. Drafting the manuscript and Figures: H.K., Y.A., T.O., T.S., K.O., and A.O. All authors edited and approved the final version of the manuscript

Potential Conflicts of Interest

Nothing to report.

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Figure Titles and Legends

Fig 1. Segregation of c.1208A>G in *MYRF* in Families A and B with encephalopathy with reversible myelin vacuolization. In the family trees, +/+ denotes normal individuals with two wild-type alleles, whereas +/m denotes heterozygous carriers of c.1208A>G in *MYRF*. (A) Whole-exome sequencing revealed that affected relatives of the proband (indicated by an arrow) possess the same heterozygous variant in *MYRF*. The relatives shown in gray had a history suggesting encephalopathy, but neuroimaging was not performed (II-1, 3, III-2, and 5) or the results were reported to be unremarkable (III-3). (B) Sequencing analysis of the coding regions of *MYRF* revealed that three cases in Family B (II-1, 2, and 3) possessed the same heterozygous variant as in Family A. The mother (I-2) presented with aphasia and convulsions during measles at 6 years of age, but no definitive diagnosis was made. (C) The heterozygous variant c.1208A>G was confirmed by Sanger sequencing. The mutant arginine residue is highlighted. (D) The control showed a single peak of A at c.1208 on the chromatogram.

Fig 2. Diffusion-weighted imaging of the three patients in two families. Three patients (Family A, patient IV-1, Family B, patient II-1, and II-2) underwent MRI

scanning. Diffusion-weighted imaging in acute phase showed abnormal intensity in the splenium of the corpus callosum with lateral extension into the parietal or frontoparietal white matter (A, C, and E). These abnormal findings disappeared within one week (B, D, and F). C, D, E, and F are reprinted from Imamura T (2010) with permission by Elsevier Inc.

Fig 3. A novel variant in *MYRF*, c.1208A>G, identified in Family A. The c.1208A>G variant predicts p.Gln403Arg in the DNA-binding domain, which recognizes the CTGGYAC motif in myelin-related genes and activates their expression.[27] The Gln at codon 403 is highly conserved across species.

Fig 4. Rare variants shared between the two families on chromosome 11. Seven rare nonsynonymous variants in Family A (*FAM110C*, *IAH1*, *MLIP*, *C11orf94*, *MS4A10*, *COLEC12*, and *ATP13A1*;Table 1) and heterozygous variants in *C11orf94*, *MS4A10*, and *MYRF* on chromosome 11 were shared by the three cases in Family B. In addition, a single heterozygous variant in *EML3* downstream of *MYRF* was shared between the two families. Allele frequencies are reported based on the ExAC database. Chromosomal coordinates are shown according to GRCh37/hg19.

Fig 5. Activation of the enhancer element of the *Rffl* gene by overexpression of the N-terminal transactivation domain of wild-type MYRF or the c.1208A>G variant.

The *Rffl* enhancer region was cloned into pGL3 upstream of the SV40 promoter and the firefly luciferase gene, and co-transfected into the HEK293 cells with the overexpression vector of the wild-type MYRF (WT) N-terminal transactivation domain or the c.1208A>G variant. The empty vector was transfected as a control (Empty). Firefly luciferase activity was normalized to the Renilla luciferase activity of co-transfected pRL/SV40, and also to the ratio obtained with Empty. Mean and SD are indicated (n = 9). $P < 0.001$ by one-way ANOVA. *P* values by post-hoc Tukey test are indicated above each pair of bars.

Table 1. The number of variants remaining after each filtering step

	Filter at each step	Number of variants after each step
Step0	Called variants in each patient	81,887/ 83,529/ 83,861/ 84,420/ 84,889
Step1	Shared by all the five patients	31,613
Step2	Nonsynonymous or splice donor/acceptor	5,833
Step3	Allele frequency < 0.01	8

The called variants on whole-exome sequencing in Family A were filtered by the following requirements: 1) shared by all the five cases, 2) predicted to be nonsynonymous or to affect splice donor/acceptor sites, 3) allele frequency < 0.01 in all four databases (NHLBI Exome Sequencing Project, 1000 Genomes, Human Genetic Variation Database including a Japanese cohort, and ExAC).

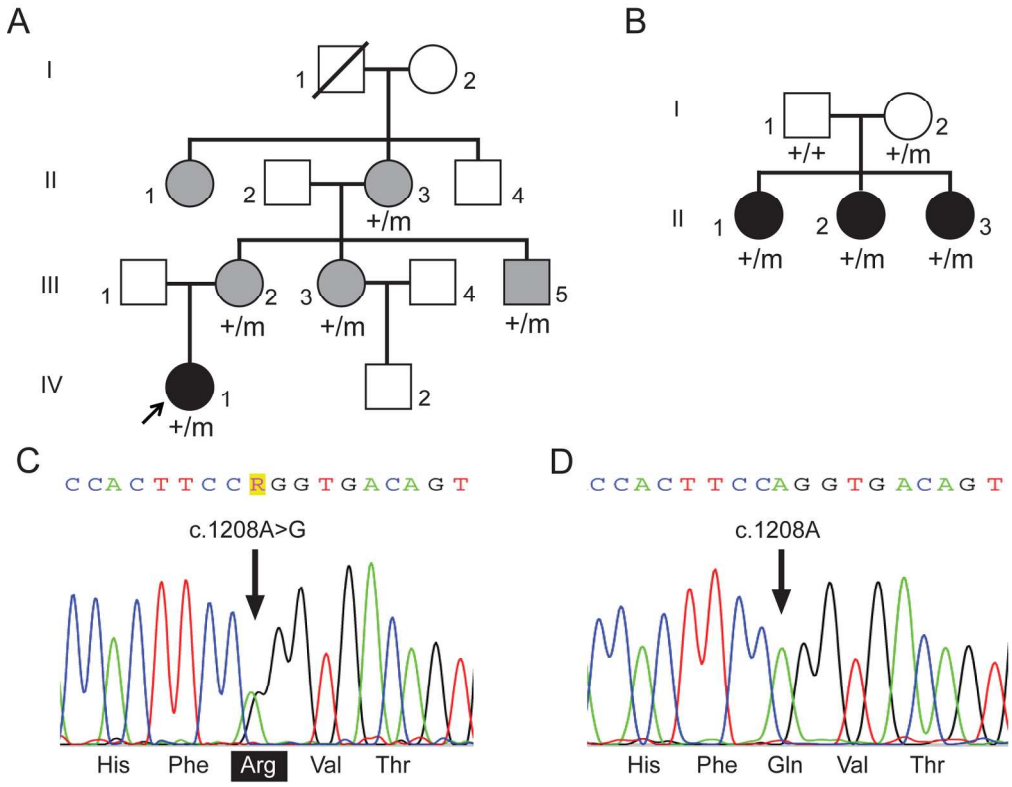

Figure 1

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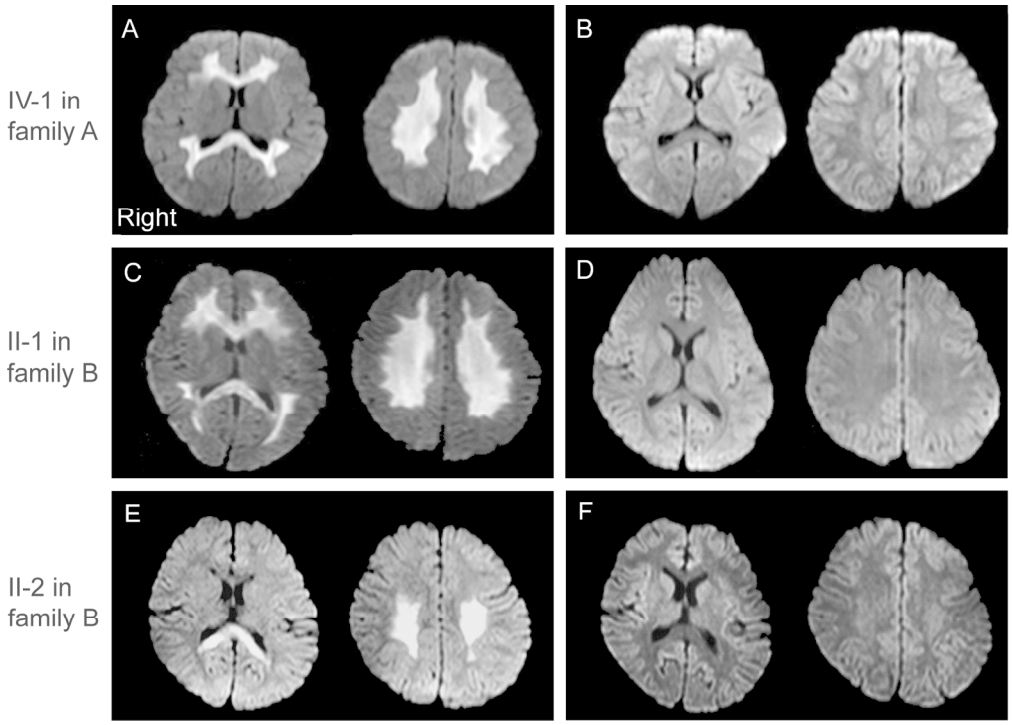

Figure 2

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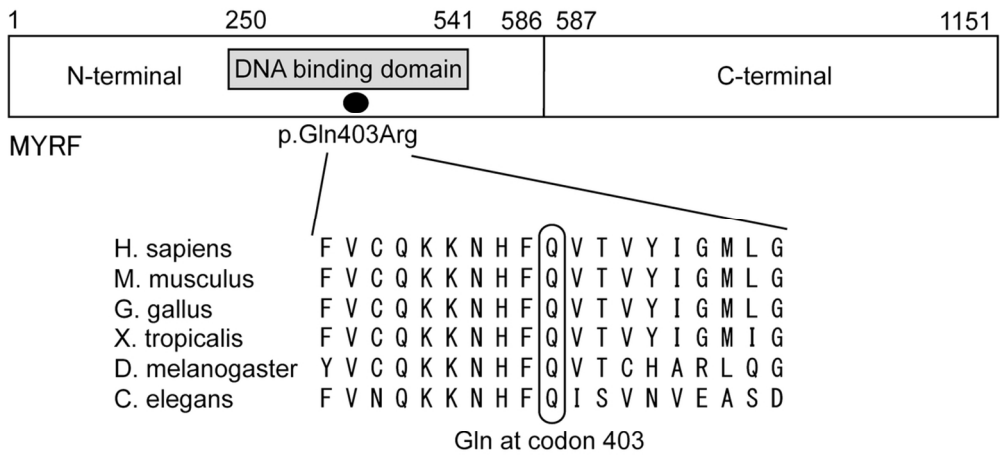

Figure 3

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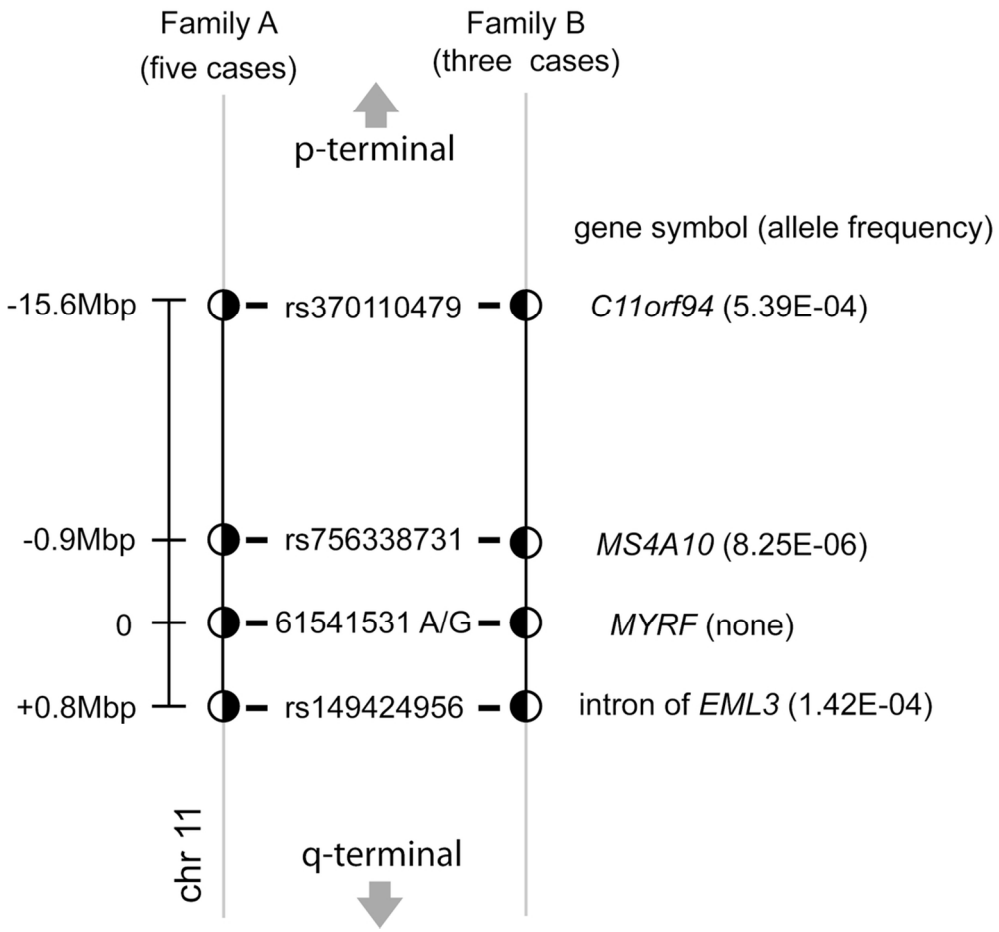

Figure 4

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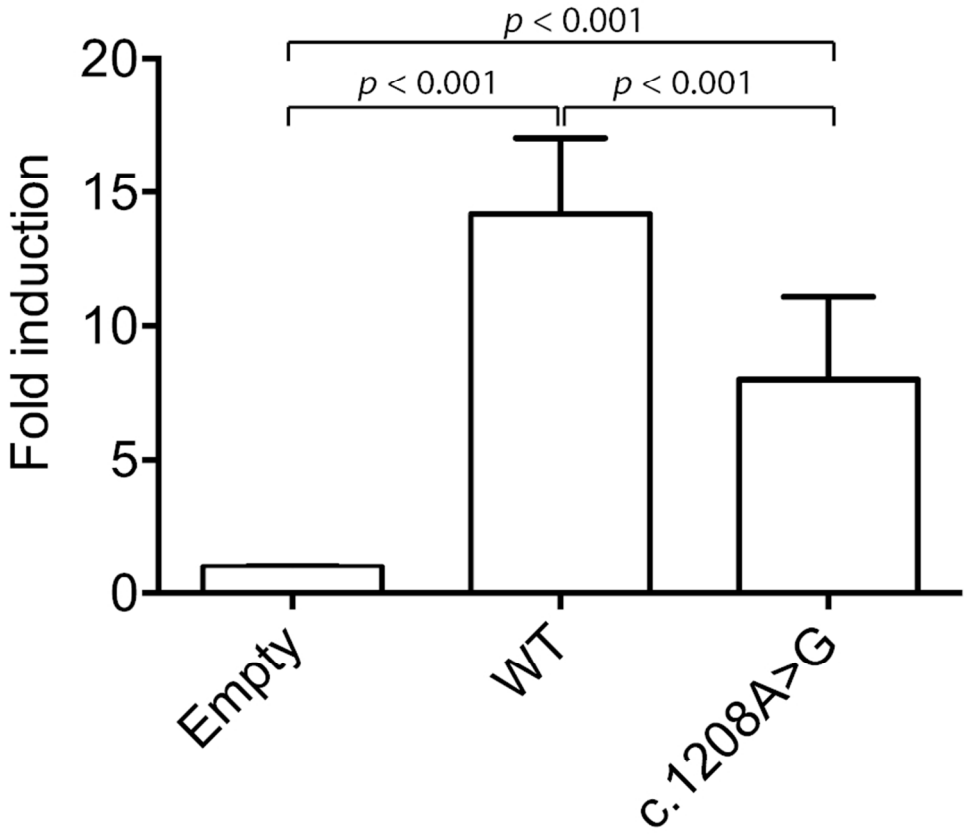

Figure 5

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