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RESEARCH ARTICLE

MYORG is associated with recessive primary familial brain calcification

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Abstract

Objective: To investigate the genetic basis of the recessive form of primary familial brain calcification and study pathways linking a novel gene with known dominant genes that cause the disease. **Methods**: Whole exome sequencing and Sanger-based segregation analysis were used to identify possible disease causing mutations. Mutation pathogenicity was validated by structural protein modeling. Functional associations between the candidate gene, *MYORG*, and genes previously implicated in the disease were examined through phylogenetic profiling. **Results**: We studied nine affected individuals from two unrelated families of Middle Eastern origin. The median age of symptom onset was 29.5 years (range 21–57 years) and dysarthria was the most common presenting symptom. We identified in the *MYORG* gene, a homozygous c.1233delC mutation in one family and c.1060_1062delGAC mutation in another. The first mutation results in protein truncation and the second in deletion of a highly conserved aspartic acid that is likely to disrupt binding of the protein with its substrate. Phylogenetic profiling analysis of the MYORG protein sequence suggests co-evolution with a number of calcium channels as well as other proteins related to regulation of anion transmembrane transport (False Discovery Rate, FDR $< 10^{-8}$) and with PDCD6IP, a protein interacting with PDGFRβ which is known to be involved in the disease. **Interpretation**: *MYORG* mutations are linked to a recessive form of primary familial brain calcification. This association was recently described in patients of Chinese ancestry. We suggest the possibility that *MYORG* mutations lead to calcification in a PDGFRβ-related pathway.

Introduction

Primary familial brain calcification (PFBC), also known as Fahr disease, is a progressive neurological disorder with extensive brain calcification, lacking known metabolic causes such as calcium or phosphorus disorders.[1] In PFBC, calcification of different brain structures, mainly the basal ganglia, thalami, and cerebellar nuclei, leads to progressive cognitive and psychiatric impairment, hypo- and hyper-kinetic movement disorders, dysarthria, dysphagia, cerebellar ataxia, and seizures.[2] The familial nature of the disorder,[3] and the use of next-generation sequencing facilitated the discovery of genes associated with PFBC.

Previously, four genes were associated with autosomal dominant inheritance of PFBC: *SCL20A2*,[4] *PDGFRB*,[5] *PDGFB*,[6] and *XPR1*.[7] Homozygous mutations in Myogenesis-regulating glycosidase (*MYORG*) were recently suggested as a cause of PFBC in individuals of Chinese

ancestry.[8] Presently, *MYORG* is the only gene known to be associated with an autosomal recessive PFBC.

In this study we confirm the suggested association between recessively inherited mutations in *MYORG* and PFBC in two unrelated families of Middle Eastern ancestry. Using phylogenetic profiling, a computational method that identifies functionally related proteins based on co-evolution, and known protein-protein interactions, we further suggest that the MYORG protein may interact with calcium channels expressed in astrocytes and with several other proteins that interact with PDGFRβ, the protein encoded by *PDGFRB*.

Materials and Methods

Subjects

Two families were recruited for this study based on the following criteria: (1) PFBC affecting more than a single family member, (2) suspected recessive inheritance, and (3) lack of known metabolic cause for brain calcification (confirmed by measurement of blood and urine calcium, phosphorous, vitamin D, parathyroid hormone, and thyroid hormone). Affected individuals with brain calcification in both families were regularly followed in the outpatient clinic at Hadassah Medical Center (Jerusalem, Israel). Because of the familial nature of brain calcification in these individuals, genetic workup was also performed in additional family members. The study was approved by the local institutional review board and all participants signed informed consent forms before beginning the study.

Whole exome sequencing

DNA samples, obtained from peripheral blood, were prepared and processed for whole exome sequencing as previously described.[9] Briefly, exonic sequences were enriched in the DNA sample using SureSelect Human All Exon 50 Mb Kit v4 or v5 (Agilent Technologies, Santa Clara, CA) and sequenced using HiSeq2500 (Illumina, San Diego, CA) as 100-bp paired-end runs. Reads were aligned to the reference human genome assembly hg19 (GRCh37) using the Burrows-Wheeler Alignment (bwa) Tool v 0.7.10.[10] Variants were called using the GATK-lite pipeline v2.3[11] and were annotated using Ensembl Variant Effect Predictor.[12] Following alignment to the reference genome and variant calling, variants were filtered out if they were off-target (>8 bp from splice junction), synonymous (>3 bp from splice junction), or had a minor allele frequency (MAF) > 0.01 in the Exome Aggregation Consortium database[13] or in our in-house exome database comprising 3000 exomes. Exome analysis of the analyzed individuals yielded 52.3 million mapped reads on average with an average coverage of 92.0× for all samples.

Sanger-based segregation analysis

Genomic DNA was extracted from whole blood of all participating family members. Amplicons containing potential pathogenic variants were amplified by conventional PCR. PCR products were purified and analyzed by Sanger di-deoxy nucleotide sequencing, according to standard procedures. Primer sequences are available upon request.

Protein structure modeling

HHPred[14] was used to identify PDB structure 2F2H (Structure of the YicI thiosugar Michaelis complex) as a suitable template for homology modeling of the MYORG protein. 2F2H chain A has 22% sequence identity and 41% sequence similarity to the query sequence. Both the wild-type protein and the del354D mutant were modeled using MODELLER.[15] Protein structures were visualized using PyMOL Molecular Graphics System versions 1.70 and 1.762.

Normalized phylogenetic profiling and protein-protein interactions

Phylogenetic profiling is a representation of the occurrence or absence of homologous protein across many species.[16] This approach relies on the assumption that if two, or more, genes (or proteins) share a similar phylogenetic profile they are also likely to be functionally coupled.

We generated the normalized phylogenetic profiles of 19,520 human proteins across 578 eukaryote species as previously described.[17] Briefly, protein sequences from all species were downloaded from Ensembl release 83[18] or Ensembl genomes release 30.[19] Similarity scores between each protein and the most similar protein in each species were calculated using blastp and were then normalized to the blast score of the human protein compared to itself. To avoid biases due to phylogenetic distance, we scaled the score of all proteins in each species to their overall distribution by transforming the values corresponding to a species into z-scores.[17] Pearson's correlation coefficients between the normalized phylogenetic profile of MYORG and the normalized profiles of all other human proteins were used to identify the 200 proteins most tightly co-evolved with MYORG. Enrichment analysis of the list of co-evolved genes was done using the Database for annotation, Visualization and Integrated Discovery, DAVID.[20] Finally, we used the VarElect phenotype prioritization

tool[21] with the search term "brain calcification" to identify proteins within this list that are linked with the four known PFBC genes or the proteins encoded by them.

Results

Brain imaging and clinical description

We studied two unrelated families with PFBC (F1 and F2, respectively, Fig. 1A) of Middle Eastern origin. Ancestral consanguinity was likely in F1 (members were part of a closed community), and was reported in F2. CT scans of nine individuals demonstrated extensive calcification involving the cerebellum, the basal ganglia and the thalami (individuals F1/I-1, F1/II-1, F1/II-2, F1/II-4, F1/II-6, F1/II-9, F1/II-10, and F2/II-4, F2/II-5, Fig. 1B and Fig. S1). Additional calcification of the brainstem, the deep midbrain nuclei and of cortical areas was variably demonstrated in these nine affected individuals (Table S1). CT scans of three other individuals demonstrated punctuate calcification limited to the internal part of the globus pallidus (F2/I-1, F2/II-1 and F2/II-2, Fig. 1C). Four additional healthy individuals had normal scans (F1/I-2, F2/I-2, F2/II-3 and F2/II-7).

All clinically affected individuals had extensive brain calcification. The neurological examination of all individuals with normal brain imaging was unrevealing. Eight of the nine (89%) individuals with extensive calcification were symptomatic. In these individuals, the age of onset of symptoms ranged from 21 to 57 years (median 29.5 years). The most common presenting sign was dysarthria. On examination, hyperkinetic movement disorder was documented in all clinically affected individuals and ranged from mild facial grimacing to chorea of the limbs and severe general dystonia that was not necessarily symmetrical. Other neurological signs, also with a wide range of severity, included pyramidal signs, parkinsonism, and appendicular or truncal cerebral signs. We did not formally test for cognitive deficits but cognitive complaints were common among affected individuals. Table S2 summarizes the clinical spectrum of neurological findings in the nine affected individuals with extensive brain calcification.

Affected individuals were tested for possible metabolic causes for their calcification (blood and urine calcium, phosphorous, vitamin D, parathyroid hormone, and thyroid hormone), and all values were within the normal range.

Whole exome sequencing

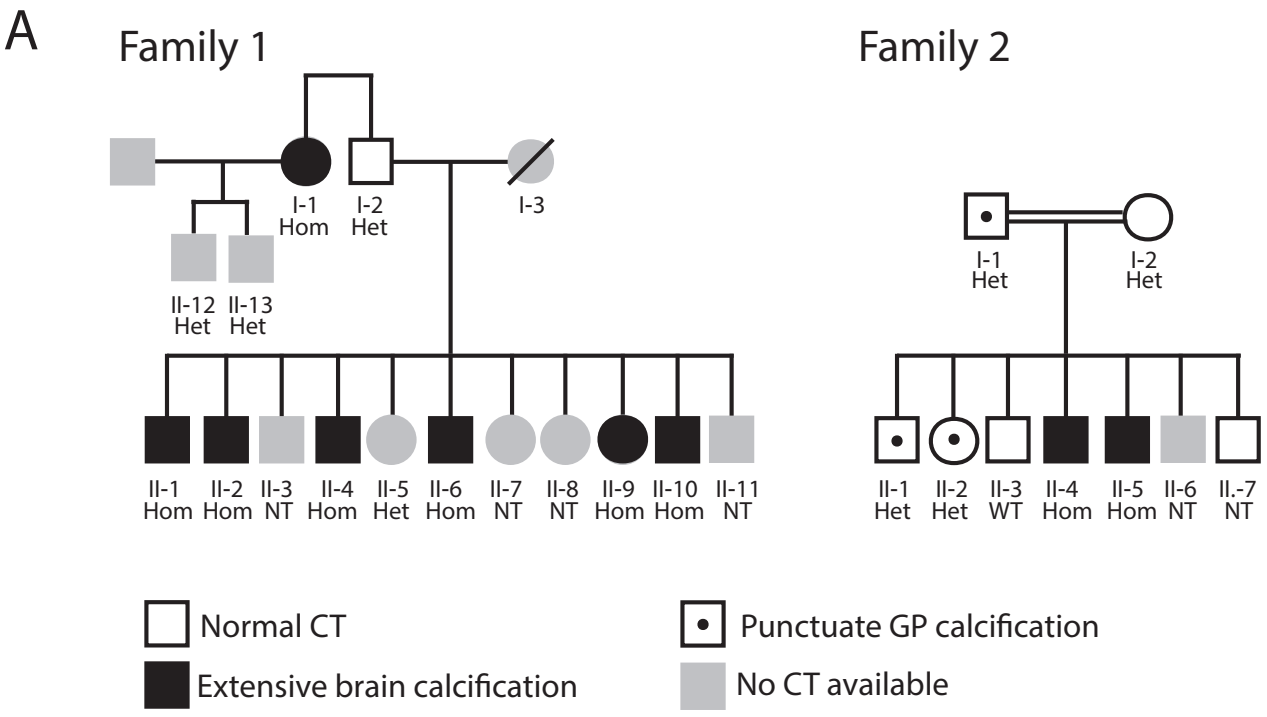
In F1, exome sequencing was performed on DNA extracted from three affected individuals (F1/I-1, F1/II-6, F1/II-10). These exomes detected a shared homozygous frameshift deletion NM_020702.4:c.1233delC (p.F411L fs*23) in the *MYORG* gene (HGNC: 19918). This frameshift mutation is predicted to cause protein truncation or nonsense-mediated mRNA decay (NMD). The mutation was not found in the gnomAD database, encompassing exome sequences from over 120,000 individuals.[13] Exome coverage did not indicate large deletions that include *MYORG*.

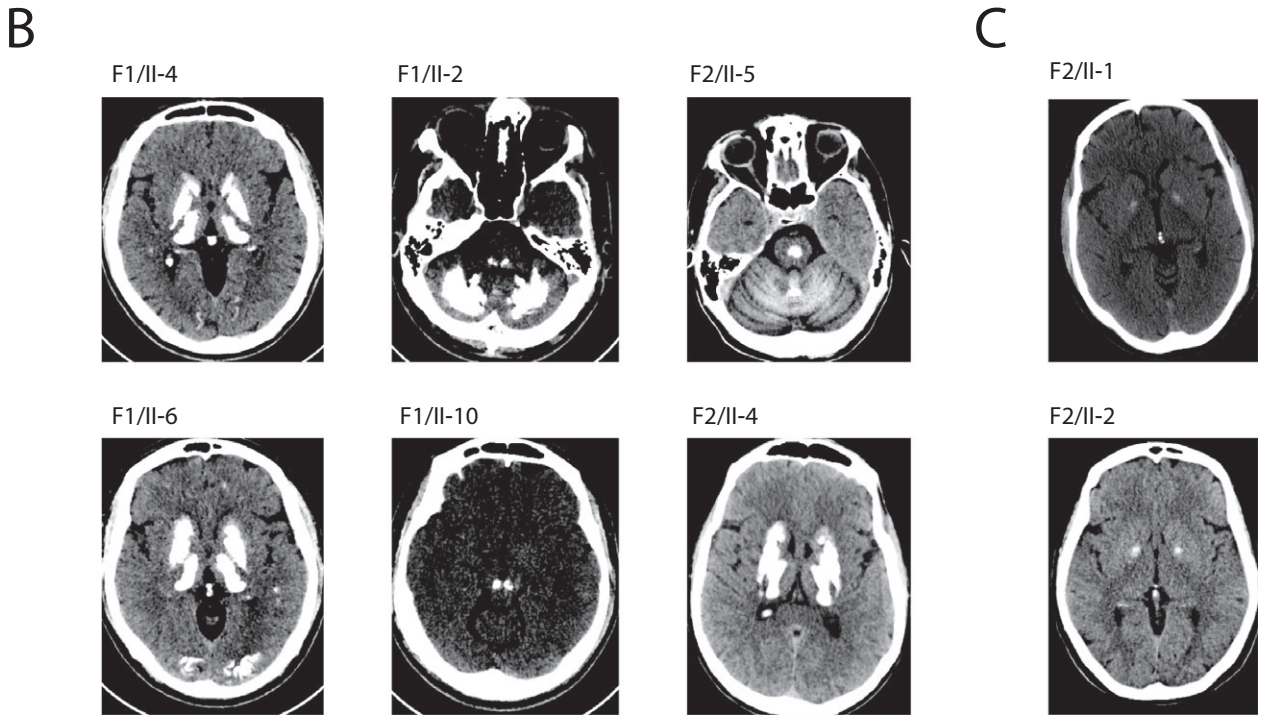
In F2, we performed whole exome sequencing in two affected individuals (F2/II-4, F2/II-5). These two affected individuals shared a homozygous in-frame deletion, NM_020702.4:c.1060_1062delGAC (p.354delD), in the *MYORG* gene. This deletion results in a loss of a highly conserved aspartic acid residue at position 354 (Fig. 2A). This variant is rare in the gnomAD database (heterozygous allele frequency 0.001%). The affected F2 members shared two additional homozygous variants co-segregated with the phenotype in this family: one in *RAI1* (NM_030665.3:c.3479G>A; R1160Q) and another in *SLC4A9* (NM_001258426.1:c.191T>C; L64P). The first variant was reported in 10 individuals in the gnomAD database (heterozygous allele frequency 0.004%) and was predicted to be benign by several pathogenicity prediction programs (FATHMM, LRT, MetaLR, MetaSVM, MutationAssessor, MutationTaster and PROVEAN). The second variant was found in 43 individuals reported in gnomAD (heterozygous allele frequency 0.02%), and was ruled out as a potential PFBC causing variant as it resides in a gene which is negligibly expressed in the brain.[22]

Sanger-based segregation analysis

We performed Sanger sequencing for the *MYORG* NM_020702.4:c.1233delC mutation in 11 F1 members who were also clinically examined by a neurologist (Fig. 1A). This analysis yielded seven homozygous individuals (three of whom were already identified by the exome sequencing); all of whom harbored extensive brain calcification confirmed by CT. Four healthy family members were heterozygous carriers. Brain imaging performed for one of these (F2/I-2) did not show brain calcification.

In F2, segregation analysis for the *MYORG* NM_020702.4:c.1060_1062delGAC mutation was conducted in seven family members using Sanger sequencing. This analysis confirmed homozygosity for this mutation in the two affected individuals (F2/II-4, F2/II-5). None of the other individuals was homozygous for the mutation. Four other family members were heterozygous carriers and one sibling was a non-carrier. All heterozygous carriers, and the non-carrier, had normal neurological examinations.





Brain CTs were done for these four heterozygous carriers and revealed symmetrical punctuate calcification limited to the globus pallidus in three of them. Brain imaging of the non-carrier sibling was normal.

Modeled mutant protein structure

To evaluate the effect of NM_020702.4:c.1060_1062del-GAC (in F2) on the function of MYORG protein we

Figure 1. Pedigrees, genotypes, and brain imaging findings of the studied families. (A). Pedigrees of the two studied families. Individuals with extensive brain calcification are marked with black symbols, these with punctuate globus pallidus (GP) calcification are marked with white dotted symbols and these without calcification are marked with white symbols. Gray symbols represent individuals with no CT available; all of them are clinically asymptomatic. Genetic status of *MYORG* mutation is depicted for homozygotes (Hom), heterozygotes (Het), wild type (WT), and untested (NT) individuals. (B). Representative CT scans. All individuals homozygous for *MYORG* mutations had extensive calcification of the basal ganglia and thalamus (e.g., F1/II-4, F1/II-6, F2/II-4), cerebellar lobes, and vermis (e.g., F1/II-2, F2/II-5). In addition to these involved brain areas, some individuals also presented calcification of the cortex (e.g., F1/II-4), sub-cortex (e.g., F1/II-6), and brainstem (e.g., F1/II-2, F1/II-10, F2/II-5). (C). In three (out of five) individuals heterozygotes for *MYORG* mutation, punctuate calcification limited to the globus pallidus were seen (e.g., F2/II-1, F2/II-2).

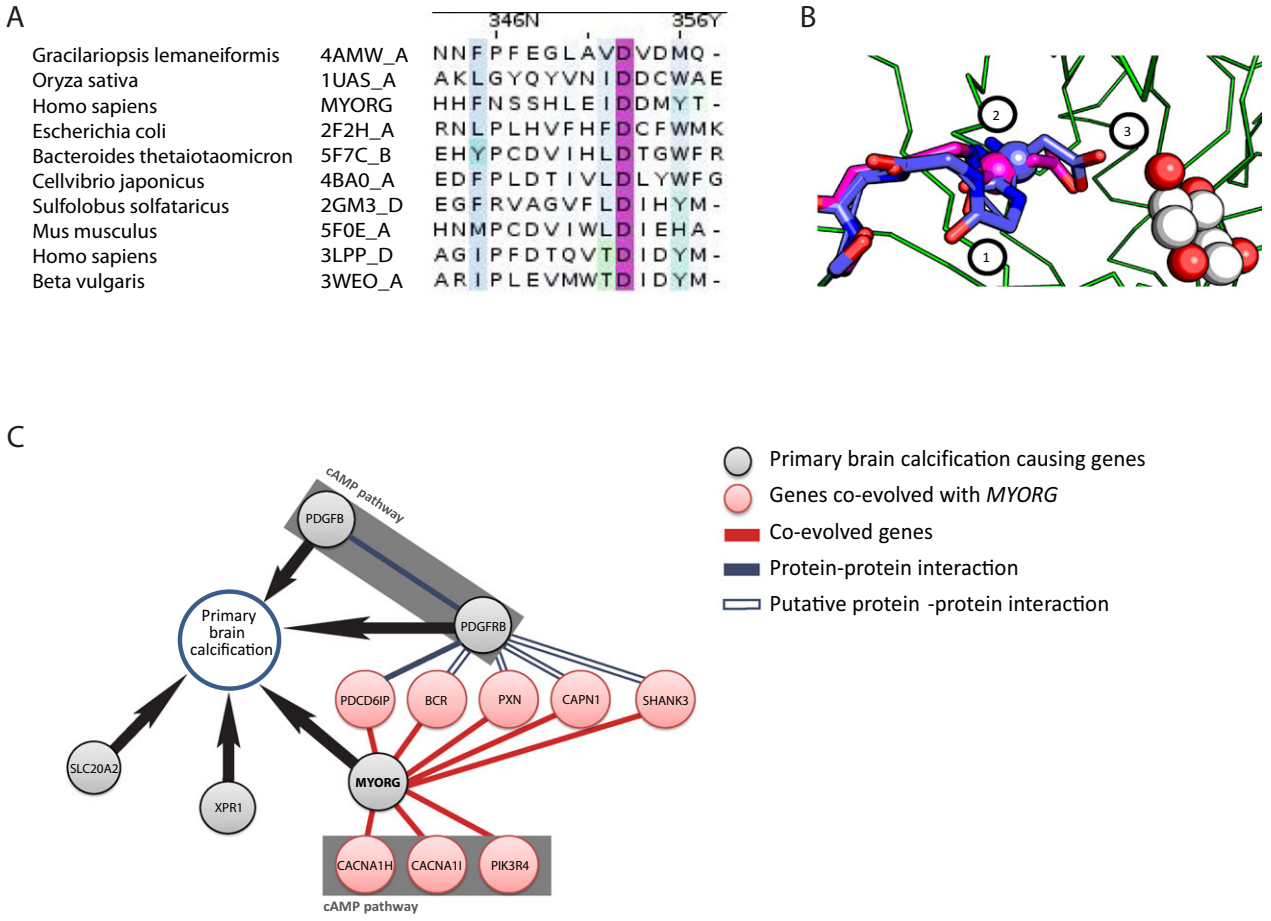


Figure 2. Multiple sequence alignment (MSA), modeled protein conformation and possible link of MYORG to other PFBC related proteins. (A) MSA of the MYORG sequence around the D354 deletion, along with other members of the Glycosyl hydrolase GH31 family. Background indicates the level of conservation of each position in the MSA ranging from white (unconserved) to purple (fully conserved). (B). Modeling of MYORG predicts a conformational change around residue D353 that disrupts substrate binding. Deletion of D354 causes: (1) loop shortening in the mutant protein (magenta sticks) relative to the wild type (blue sticks), (2), deviation of approximately 1 Å of the mutant C-alpha atoms position (magenta versus blue sphere) and, (3) change in the orientation of the D353 terminal oxygens that prevents binding of substrate (red and white spheres). (C). Proteins that co-evolved with *MYORG* and interactions with the proteins they encode suggest a common pathway with PDGFRβ.

generated computational models of the structure of the wild type and the mutant protein. These models are based on homology between the YicI protein of *Escherichia coli* and MYORG. The model predicts that MYORG D353 residue has a similar conformation to that of YicI D306 residue, which participates in hydrogen bonds with the substrate.[23–25] In particular, oxygen atom Oδ2 of the D353 side chain assumes an orientation which is predicted to allow substrate binding (Fig. S2). Strikingly, the orientation of D353 is predicted to be disturbed in the

mutant protein, not allowing hydrogen bonding with the substrate (Fig. 2B).

Functional pathways linking MYORG and other proteins implicated in brain calcification

To examine possible functional links between MYORG and other proteins causing PFBC we identified the 200 proteins (top 1%) most strongly co-evolved with MYORG across 578 eukaryotes by using normalized phylogenetic profiling.[17,26,27] Enrichment analysis of this list revealed that it is significantly enriched in proteins involved in regulation of anion transmembrane transport (False Discovery Rate, FDR $< 10^{-8}$) and regulation of intracellular pH (FDR $< 10^{-6}$). The list also includes several known calcium channels (CUL5, CACNA1I, CACNA1G, CACNA1H, and CACNA1B) that, like MYORG, are also expressed in astrocytes. Two of these calcium channels, as well as PIK3R4, which also co-evolved with MYORG, are known to be involved in cAMP signaling, a cellular process mediated by proteins encoded by other genes associated with brain calcification, namely *PDGFRB* and *PDGFB*.

Importantly, PDCD6IP/ALIX, another protein that co-evolved with MYORG, has been shown to form a constitutive complex with Platelet Derived Growth Factor Receptor Beta (PDGFRβ),[28]which is encoded by *PDGFRB*. Other proteins that co-evolved with MYORG are predicted to interact with PDGFRβ (Fig. 2C) but these interactions are yet to be validated. Notably, we could not find an association between MYORG or proteins co-evolved with it and the two other proteins implicated in primary brain calcification, SLC20A2 and XPR1.

Discussion

Previously it was suggested that PFBC can be inherited recessively[29] but no mutations leading to a recessive inheritance of the disease have been reported until very recently. *MYORG* has recently been associated with a recessive form of PFBC in patients of Chinese ancestry.[8] In this work, we consolidate the evidence that *MYORG* is associated with recessive inheritance of this disease in patients of Middle Eastern ancestry. The two novel mutations described here include one mutation leading to a truncated protein (NM_020702.4:c.1233delC) and one deletion (NM_020702.4:c.1060_1062delGAC) predicted by structural modeling of the protein to impair substrate binding.

The role of *MYORG* is largely unknown. cDNA libraries from the human brain demonstrated that *MYORG* is expressed in all tested brain regions including the cerebellum, caudate, thalamus, and substantia nigra.[30] It was also recently suggested that the gene is expressed in astrocytes and is mainly localized to the ER.[8] The encoded protein, also named KIAA1161 or NET37, was identified as a putative nuclear envelope trans-membrane protein in a large-scale proteomics study.[31] Based on sequence analysis, it is predicted to be a glycosyl hydrolase.[32]

In light of the limited knowledge of the gene function, we used normalized phylogenetic profiling in order to explore pathways in which this protein may be involved. This method, that was already validated in multiple studies,[17,26,27,33] revealed that the protein encoded by *MYORG* co-evolved with multiple calcium channels that are expressed in astrocytes. In addition, the MYORG protein was shown to be co-evolved with PDCD6IP, which encodes a protein that strongly interacts with PDGFRβ.[28] Over expression of PDCD6IP has been shown to inhibit PDGFRβ internalization.[28] Like MYORG, PDCD6IP is also expressed in astrocytes.[34]

In our study, three out of five individuals who were heterozygous for a *MYORG* mutation revealed punctuated calcification limited to the basal ganglia. Two of these three were younger than 40 years at the time the scans were performed. Similar calcification is not a rare finding in brain scans of the elderly, with an estimated prevalence of up to 20%,[35] and their presence is not clinically meaningful. Based on the imaging findings in heterozygous carriers, we speculate that heterozygous mutations in the *MYORG* gene are a contributing factor to this radiological finding, but our cohort of carriers is too small to test this hypothesis.

Patients with PFBC due to recessive mutations in *MYORG* cannot be distinguished clinically from those with PFBC due to mutations in other genes. This gene, therefore, should be included in genetic panels for PFBC, especially if a recessive form is suspected. In addition, the phenotypic variability between affected individuals (age of onset, radiologic findings, mainly in F1) could not be fully explained by the extent of brain calcification and suggests that other environmental or genetic factors (dominant or recessive) affect the clinical phenotype. The validity of this hypothesis, as well as the physiological role of this gene should be examined in future studies.

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Author Contributions

DA, AL, DR, VM: Conception and design of the study, acquisition and analysis of data and drafting a significant portion of the manuscript and figures. MAS, IL:

Acquisition and analysis of data. OSF, SN, BM, YS, YT: Conception and design of the study and analysis of data.

Conflicts of Interest

Nothing to report.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Additional brain CT (A) and MRI images (B) demonstrating hypointense signal in susceptibility weighted images (SWI) and FLAIR hyperintensity.
Figure S2. Protein modeling. D353 of the wild-type MYORG (blue sticks) along with the equivalent position, D306, in the *Escherichia coli* homolog YicI (PDB model 2F2H) in yellow sticks.
Table S1. Region and severity of brain calcification observed in individuals with a homozygous *MYORG* mutation. Extent of calcifications is qualitatively scored from mild (+) to severe (+++).
Table S2. Disease characteristics of individuals with a homozygous *MYORG* mutation. Severity of neurological signs is qualitatively scored from mild (+) to severe (++++).

