Bi-allelic variants in the GPI transamidase subunit PIGK cause a neurodevelopmental syndrome with hypotonia, cerebellar atrophy and epilepsy.

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Running title (40 characters): *PIGK* variants cause epilepsy and cerebellar atrophy.

Abstract

Glycosylphosphatidylinositol (GPI)-anchored proteins are critical for embryogenesis, neurogenesis, and cell signaling. Variants in several genes participating in GPI biosynthesis and processing lead to decreased cell surface presence of GPI-anchored proteins (GPI-APs) and cause Inherited GPI Deficiency disorders (IGDs). In this report, we describe twelve individuals from nine unrelated families with ten different bi-allelic *PIGK* variants. *PIGK* encodes a component of the GPI transamidase complex, which attaches the GPI anchor to proteins. Clinical features found in most individuals include global developmental delay and/or intellectual disability, hypotonia, cerebellar ataxia, cerebellar atrophy and facial dysmorphisms. The majority of the individuals have epilepsy. Two individuals had slightly decreased levels of serum alkaline phosphatase, while eight did not. Flow cytometric analysis of blood and fibroblasts from affected individuals showed decreased cell surface presence of GPI-anchored proteins. The overexpression of wildtype-*PIGK* in fibroblasts rescued the levels of cell surface GPI-APs. In a knockout cell line, transfection with wildtype *PIGK* also rescued the GPI-AP levels, but not transfection with the two tested mutants. Our study not only expands the clinical and genetic spectrum of IGDs, but it also expands the genetic differential diagnosis for cerebellar atrophy. Given the fact that cerebellar atrophy is seen in other IGDs, flow cytometry for GPI-APs should be considered in the work-up of individuals presenting this feature.

Keywords (5): glycosylphosphatidylinositol (GPI), PIGK, GPI8, transamidase, Inherited GPI Deficiency disorders (IGDs)

Main text

Introduction

The synthesis and modification of the GPI anchor proteins involves at least 31 enzymes. This process includes multiple steps of synthesis of GPI precursor molecules taking place in the endoplasmic reticulum (ER) membrane, then transfer of the entire precursor GPI to newly synthesized proteins by a multi-protein transamidase complex, which also simultaneously cleaves the C-terminal consensus sequence of the GPI target. Finally, the GPI-bound protein is modified to exit from the ER to the Golgi for further modification to be functional.[1; 2] Gpi8p was first characterized in yeast as a glycosylated transmembrane protein of the ER and was hypothesized to be part of the GPI-transamidase complex.[3] Then, in GPI-AP class K mutant cells, where endogenous human GPI8 (hGPI8) is mutated leading to failure of incorporation of GPI anchors into nascent polypeptides, the overexpression of hGPI8 restored the transamidation ability. This protein, later renamed phosphatidylinositol glycan (PIG) anchor biosynthesis class K (PIGK) is coded by a 25-kb gene, *PIGK* (MIM # 605087), that resides on chromosome 1 in humans.[4] It is a cysteine protease and forms a disulfide bridge with PIGT in the GPI-transamidase complex, which is composed of glycosylphosphatidylinositol anchor attachment 1 protein (GPAA1), PIGU, PIGT, PIGS and PIGK, and this bridge is required for normal transamidase activity.[5]

More than 150 GPI-APs are known, making up about 1% of the human proteome, and play important roles as hydrolytic enzymes, adhesion molecules, receptors, protease inhibitors, and complement regulatory proteins.[1] The disruption of GPI anchor biosynthesis and remodelling has been linked to many inherited GPI Deficiency disorders (IGDs). With the development of next generation sequencing, nineteen genes in the GPI-AP biosynthesis pathway have been linked to human diseases.[6; 7] In this study, we report ten individuals from eight unrelated families who presented a neurodevelopmental syndrome associated with biallelic variants in *PIGK*. We further demonstrate GPI-AP deficiency at the cell surface in affected individual cell lines. This deficit could be rescued by overexpression of wildtype (WT) human *PIGK*. *PIGK* loss of function therefore shares common phenotypic and biochemical features with *PIGT* and *PIGS* deficiencies, as well as other IGDs.

Materials and methods

Identification of affected individuals and collection of samples.

Individual 1A. Clinical exome sequencing was performed at Baylor Genetics, and the individual was identified after a search for individuals with potentially bi-allelic variants in PIGK and neurological features suggestive of an IGD. The family was enrolled in a protocol approved at the CHU Sainte-Justine after informed consent and samples obtained. The rest of cohort was then assembled with the help of GeneMatcher.[8]

Individuals 2A and 2B. The family was enrolled in an ongoing research program dedicated to individuals affected by undiagnosed diseases performed at the Ospedale Pediatrico Bambino Gesù, Rome. Clinical data and DNA samples were collected from the participating family after written informed consent was obtained, stored and used following the Institutional Review Board recommendations. Permission was obtained to publish the photographs.

Individual 3 had a research trio exome sequencing in a Dijon UHC research project after sequencing of a cerebellar atrophy gene panel and a clinical solo exome which were negative. Individuals 4 and 5 had exome sequencing after informed consent as part of a study in the Gleeson lab on brain malformations. Individuals 6 and 7 had clinical exome sequencing at GeneDx after informed consent.

Individual 8 had research trio whole genome sequencing (WGS) through the 100,000 Genomes Project (see link at the end), which is a national UK project described in detail elsewhere[9]. Clinical data and samples were collected from the participating family after written informed consent was obtained. Permission was obtained to publish the photographs and MRI images.

After informed consent was obtained prior to genetic testing from the family of individual 9, genomic DNA was extracted from peripheral blood samples (of the proband, parents, 1 unaffected sister and 1 affected brother) according to standard procedures of phenol chloroform extraction.

For GPI studies, blood samples were collected from five probands in three families 1, 2 and 3. Parental blood samples and other family members were used to assess co-segregation between variants and the trait. Skin fibroblasts were established from individual 1a.

Exome and genome sequencing

Family 1. Exome sequencing was performed as described previously.

Family 2. Targeted enrichment (SureSelect ClinicalExome V.2, Agilent) and massively parallel sequencing (NexSeq550, Illumina) were performed on genomic DNA extracted from leukocytes, using a trio-based approach. About 65[11] million reads per sample were obtained. Data analysis was performed using an *in-house* implemented pipeline, which

mainly take advantage of the Genome Analysis Toolkit (GATK V.3.7) framework, as previously reported. [10]Reads mapping was performed by Burrows-Wheeler Aligner BWA V.0.7.12, [11] and GATK tools were used for base quality recalibration and variants calling. SNVs and small INDELs were identified by means of the GATK's HaplotypeCaller tool used in gVCF mode, followed by family-level joint genotyping and phasing; finally, variants were quality-filtered, according to GATK's 2016 best practices. To retain private and clinically associated variants, we selected annotated variants with unknown frequency or having MAF <1% (dbSNP150 and gnomAD V.2.0), and occurring with a frequency <1% in an *in-house* database including frequency data from approximately 1,300 population-matched exomes. SnpEff toolbox (V.4.3) was used to predict the functional impact of variants, which were filtered to retain only those located in exons with any effect on the coding sequence, and splice site regions (variants located from −3 to +8 with respect to an exon-intron junction). Moreover, functional annotation of variants was performed using SnpEff and dbNSFP (V.3.5). The functional impact of variants was eventually analyzed by Combined Annotation Dependent Depletion (CADD) V.1.4, M-CAP V.1.0, and InterVar V.2.0 algorithms, to obtain clinical interpretation according to ACMG/AMP 2015 guidelines.Variant validation and segregation were performed by Sanger sequencing.

Family 3. Genomic DNA extracted from the probands blood leukocytes was used for a targeted exon enrichment with the SureSelect Human All Exon V4 kit (Agilent) on a HiSeq 2000 instrument (Illumina), according to the manufacturer's recommendations for paired-end reads. Raw data were processed as previously described. Files were aligned to the reference human genome (GRCh37/hg19) using BWAv0.6.7, and potential duplicate paired-end reads were removed by Picard v1.109. Indel realignment and base quality score recalibration were conducted with GATK v3.3-0. Variants with a quality score >30 and alignment quality score >20 were annotated with SeattleSeq SNP Annotation. Rare variants present at a frequency above 1% in dbSNP 138 and the NHLBI GO Exome Sequencing Project, Exome Variant Server, ExAC, or present from local exomes of unaffected individuals were excluded. Variant prioritization focused on variants *de novo* heterozygous; compound heterozygous or hemizygous affecting the coding sequence (missense, nonsense, and splice-site variants and coding indels). Candidate variants were then inspected with the Integrative Genomics Viewer andwere validated by Sanger sequencing.

Families 4 and 5. Exome sequencing was performed as described previously.

Families 6 and 7. Diagnostic trio whole exome sequencing (WES) was performed on extracted genomic DNA from the peripheral blood of individual 6 and both her unaffected parents. The pipeline involved exon targeting with the Agilent SureSelect XT2 All Exon V4 kit, sequencing with Illumina HiSeq 2000 sequencing system with 100bp paired-end reads, and data analysis with XomeAnalyzer in comparison to the published human genome build UCSC hg19 reference sequence. All variants were confirmed by Sanger sequencing**.**

Family 8. WGS was performed on extracted genomic DNA from the peripheral blood of individual 8 and both her unaffected parents. Sequencing was performed on a HiSeq2500 (Illumina, San Diego, CA, USA) and alignment was performed by Illumina's Isaac aligner against the reference human genome GRCh37. The length of paired-end reads was 150bp and the mean depth of coverage across individuals was 30×. Clinical genome interpretation was performed using Omicia's Opal platform.

WES on individual 9 was performed as described elsewhere in Macrogen, Korea.[12] Briefly, target enrichment was performed with 2 μg genomic DNA using the SureSelectXT Human All Exon Kit version 6 (Agilent Technologies, Santa Clara, CA, USA) to generate barcoded whole-exome sequencing libraries. Libraries were sequenced on the HiSeqX platform (Illumina, San Diego, CA, USA) with 50x coverage. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (see link at the end). The bioinformatics filtering strategy included screening for only exonic and donor/acceptor splicing variants. In accordance with the pedigree and phenotype, priority was given to rare variants (<0.01% in public databases, including 1,000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and Exome Aggregation Consortium [ExAC v0.2]) that were fitting a recessive (homozygous or compound heterozygous) or a de novo model and/or variants in genes previously linked to developmental delay, intellectual disability and other neurological disorders.

Fluorescence-Activated Cell Sorting (FACS)

Fresh blood samples from the affected children and healthy controls were stained with the GPI-AP markers: PE-conjugated anti human CD16 (BioLegend), FITC-conjugated mouse anti human CD55 and CD59 (BD Pharmingen), or FLAER-Alexa 448 (Cedarlane) for 1 hour on ice. Red blood cells were lysed in FACS Lysing Solution (BD Bioscience). For fibroblasts, cells were harvested at 80-90% confluency, stained with FLAER-Alexa 448, FITC-conjugated mouse anti human CD73 or PE- conjugated mouse anti human CD109 (BioLegend) for 1 hour on ice in the incubation buffer containing 0.5% BSA, then fixed in 3.7% formaldehyde. For all assays, non-specific binding was washed off before analyzing by a BD FACSCanto II system (BD Biosciences) followed by Cytobank software analysis.

Rescue assays of GPI-APs on fibroblasts.

Lentiviruses carrying a wild type (WT) *PIGK*-pEZ-Lv105 or an empty- pEZ-Lv105 construct (GeneCopoeia) with the presence of packaging plasmids pMD2.G and psPAX2 (AddGene) were produced in HEK293T cells. Fibroblasts were transduced with the lentiviruses and selected by Puromycin resistance. These cells, untransduced cells, as well as control cells were subjected to FACS analyses as described above for fibroblasts.

***In vitro* functional assays**

PIGK deficient CHO cells (clone 10.2.2 previously published) were transfected by electroporation with mutant or wild type PIGK cDNA expressing strong (SR alpha) or weak (thymidine kinase) promoter driven plasmids. After two days, the cells were analyzed by FACS and western blotting was performed.

Results

Clinical descriptions

The individuals with bi-allelic pathogenic *PIGK* variants present several clinical features common to other known IGDs (see table 1). Hypotonia was present in all affected individuals, and all affected individuals presented with developmental delay or when it could be assessed, intellectual disability (from mild to severe, see Table S1 for details). Cerebellar atrophy was noted in eight individuals and was progressive, ataxia was noted in five and other movement disorders in three. Epilepsy was seen in 4 out of 8 individuals and was well controlled in 3 of the 4. One individual had 3 episodes of fever-related seizures and no afebrile seizures by the age of 3 years and 5 months. Facial dysmorphisms were noted in 7 individuals and varied between individuals (see Figure 2 and Table S1). Other malformations or anomalies were relatively infrequent, and include brachydactyly, hydronephrosis and teeth anomalies. These issues are also seen in other IGDs.[6]

Molecular analyses

By WES and WGS, we identified eight different *PIGK* variants in ten affected individuals in a homozygous or compound heterozygous state (Table 1). Individuals 1a and 1b had two missense changes, c.823T>C (p.Cys275Arg) and c.158C>T (p.Ser53Phe), which had been inherited from their father and mother, respectively (Figure 1, NM_005482.3). Note that p.Cys275 is not the cysteine forming the aforementioned disulfide bridge with PIGT (Cys92). The two affected individuals in family 2 were homozygous for the c.260C>T nucleotide substitution (p.Ala87Val), while individual 3 was compound heterozygous for the c.97C>T (p.Gln33*) and c.479A>C (p.Tyr160Ser) changes. Notably, the same c.479A>C variant was also found to be heterozygous in individual 4, and homozygous in individual 5, and all three families have African Arab origins, thus the variant might represent a founder mutation in these populations. Individual 4 also carries maternal c.94-1G>C, which is an essential splice acceptor site variant (Figure 3). Individual 6 has a homozygous variant, c.257T>C, p.Leu86Pro. Individual 7 is compound heterozygous for c.551C>T, p.Ala184Val and c.737T>A, p.Met246Lys. Individuals 8 and 9 have a homozygous variant, c.262G>A, p.Asp88Asn, and both families are of Indian origin. As shown in figure 3, all the variants causing missense mutations affect highly conserved residues.

Cell surface abundance of GPI-AP in blood cells

Flow cytometry on blood samples from affected individuals of families 1, 2 and 3 showed very low level of cell surface CD16 on granulocytes (the most sensitive marker for inherited GPI deficiency) (Figure 4). The two individuals in family 1 have 21 to 26% of CD16 cell surface abundance compared with their parents. These levels were found to be about 35% and 25% in individuals 2a and 2b, respectively versus unrelated healthy controls and a similar diminution was also seen in individual 3. In individual 8, CD16 is significantly less found at the cell surface, with only 5% compared to control were seen.

For FLAER (marker for all GPI-APs), a low signal was also found in families 1 and 2 whereas this marker appeared to be normal in family 3. Individual 1a and 1b have 50% lower levels compared to parents while a more moderate decrease of 30 to 35% was observed in the individuals of family 2 Individual 8 has a 60% decreased compared to an unrelated control (Figure 4). There was also a decrease of CD24 in granulocytes and CD14 in monocytes (data not show). No significant decrease was noted for CD55 or CD59 in all tested families (Figure S1)

Overexpression of wildtype PIGK in fibroblasts from affected individuals can rescue its GPI-AP deficiency.

Examination of fibroblasts from individual 1a and individual 7 revealed, for individual 1a, 40% signal for FLAER compared to healthy fibroblasts, and 15% for CD73 and 50% for CD109 while these marker levels in individual 7 cells are 80%, 50% and 40% for FLAER, CD73 and CD109, respectively (Figure 5). We therefore stably transduced these cells with a lentivirus which expresses wildtype PIGK. The results indicate that while the empty vector did not change the GPI-AP cell surface levels, the overexpression can increase the cell surface abundance of all these GPI-APs in individual 1a fibroblasts to similar levels as seen in healthy control fibroblasts. For individual 7 cells, FLAER and CD73 were completely restored but only partial restoration can be seen for CD109.

Effect of PIGK variants in GPI-AP cell surface abundance ***in vitro***

The effect of the variants found in family 1 were also studied by using a *PIGK*-deficient CHO cell model to further demonstrate that they lead to a protein with a loss of function. The cells were transfected with wild-type or mutant pME-*hPIGK GST* (pME has a strong SRα promoter) and with pTK-*hPIGK GST* (pTK has a weaker promoter, thymidine kinase promoter). FACS analysis was performed two days post-transfection to check the cell surface abundance of CD59, CD55 (DAF) and CD87 (uPAR). As shown in Figure 6, even using the strong promoter-driven pME vector, PIGK cDNA bearing the Ser53Phe variant could not rescue the surface abundance of GPI-APs such as CD59, DAF and uPAR whereas the protein with the p.Cys275Arg variant could. However, using the weak promoter-driven pTK vector, the p.Cys275Arg PIGK protein failed in rescuing the surface abundance of GPI-APs completely, providing evidence of the hypomorphic behavior of this mutant. These mutant PIGK proteins were expressed at similar levels as the wildtype protein suggesting that the variant did not affect the protein cell surface abundance (Figure 5B).

Discussion

To date, four genes in the transamidase complex including *PIGT* (MIM# 610272), *GPAA1* (MIM# 603048), *PIGS* (MIM # 610271) and *PIGU* (MIM # 608528) have been reported to cause IGDs. Phenotype clustering was illustrated by a heatmap in the recent paper on PIGU deficiency.[13] The common phenotypes seen in individuals with variants in these genes are brain anomalies (notably cerebellar atrophy), DD/ID and dysmorphic facial features. These characteristics are also found in individuals with biallelic *PIGK* variants. Elevated serum alkaline phosphatase (ALP), a feature found in half of other known IGDs, was not seen in individuals with *PIGK* variants, similarly to what was seen in individuals with variants in *PIGS* and *GPAA1*. In fact, two individuals in our cohort actually had low alkaline phosphatase. As for *PIGT*, seven out of 13 individual with *PIGT* mutations also had low levels of serum ALP. This is caused by the failure of alkaline phosphatase protein precursor processing by the GPI transamidase complex resulting in ER-associated degradation of the protein. Since alkaline phosphatase is critical for the synthesis of hydroxyapatite, this could explain the low bone density found in some individuals with mutations in the transamidase complex (seen with *PIGT, PIGU* and *GPAA1* mutations). A low bone density was not noted with *PIGS* deficiency or in the *PIGK* deficiency we describe here, but bone density studies were not systematically studied in those cohorts. Hypotonia was common in the cohort described here, and ophthalmological anomalies were found in a half of individuals with *PIGK* variants, comparable to individuals with mutations in other components of the transamidase complex. Seizures were a common manifestation in individuals with *PIGT, GPAA1,* and *PIGS* variants, and were observed in half of the individuals with *PIGK* variants.

Flow cytometry analyses showed decreased cell surface presence of CD16 in granulocytes (Figure 4) similar to most previously reported IGD cases. Total cell surface GPI-AP, which is measured by the FLAER signal, is also very low in granulocytes. However, CD55 and CD59, two GPI-AP markers decreased in both granulocytes and lymphocytes of some individuals with *PIGS* variants notably, were not changed in the individuals with *PIGK* variants. This is also seen in other IGD syndromes and may be due to cell type specific effects. Interestingly, in fibroblasts of individual 1a, we detected very low levels for all measured GPI markers, especially of CD73 (5'-nucleotidase).

The variants in our cohort affected all regions of PIGK which lies inside the endoplasmic reticulum, but there was a clustering of three mutations at amino acids 86, 87, and 88. This lies just upstream of the cysteine residue at amino acid 92 previously shown to be important for PIGK to form a disulfide bridge with PIGT. It is possible that these variants affect the interaction of the PIGK cysteine protease with PIGT. Protein structure homology modeling using SWISS-MODEL (based on PDB structure 4FGU[14], see link at the end) showed homology with the cysteine protease legumain. PIGK amino acids 86, 87 and 88 in this model lie within the legumain insertion-loop, a well conserved region thought to perhaps regulate protease activity.[14; 15] These possibilities could be investigated in future biochemical studies.

A wide variety of genes have been associated with cerebellar ataxia and cerebellar atrophy.When occurrence is in adulthood, a dominant form of spinocerebellar ataxia, often caused by trinucleotide repeat expansion disorders has to be considered (note that repeat expansions can be missed by next-generation sequencing). With childhood onset, a recessive form is more common, and Friedrich's ataxia and ataxia telangiectasia are among the most frequent disorders.The initial workup of affected individuals also includes detailed testing for reversible causes of ataxia, including metabolic, toxic, autoimmune, and nutritional disorders, such as measuring vitamin E. Once more common and treatable causes have been screened for, a next generation sequencing panel or exome sequencing is often the next diagnostic test. Cerebellar atrophy is noted with several IGDs (*PIGA, PIGG, PIGL, PIGN, PIGT PIGS,*[7] *GPAA1,* and *PGAP1*) and there does not seem to be a correlation between the location of the protein in the biosynthesis pathway and the presence of cerebellar atrophy. Given that inherited GPI-deficiency can be tested for by flow-cytometry, such a test could be considered in the initial evaluation of cerebellar hypoplasia, or following negative next generation sequencing panel if IGD genes were not included, as is often the case, before moving to an exome which is not always clinically available. This test is available in research laboratories, and could also potentially be available after specifically requesting CD16 in granulocytes from clinical labs performing high-sensitivity flow cytometry testing for paroxysmal nocturnal hemoglobinuria.

In summary, we present an IGD characterized by hypotonia, DD/ID, cerebellar atrophy and epilepsy. We show GPI-AP deficiency in affected individual cells which can be rescued by overexpression of wildtype PIGK and show the effect of individual mutations in *PIGK* KO cells. Functional analyses using PIGK-deficient neuronal cells and perhaps a neuron-specific PIGK knock-out mouse model could be useful next steps to better characterize which GPI-APs are impaired by PIGK deficiency in various neuronal subpopulations, to better elucidate the downstream effects of GPI-AP abnormalities, and ultimately understand the neuropathogenesis of IGDs.

Acknowledgements

We thank Kana Miyanagi and Saori Umeshaita for technical assistance. This work is supported by the grant from Ministry of Health, Labor and Welfare (Y Murakami). Fondazione Bambino Gesù (Vite Coraggiose) to M.T., the Italian Ministry of Health (Ricerca Corrente 2018, 2019 to A.C. and 2019 M.N. and CIHR and FRQS grant to P.M.C.

The identification of the homozygous *PIGK* variant in individual 8 was made possible through access to the data and findings generated by the 100,000 Genomes Project. The 100,000 Genomes Project is managed by Genomics England Limited (a wholly owned

company of the Department of Health and Social Care UK). The 100,000 Genomes Project is funded by the National Institute for Health Research and NHS England. The Wellcome Trust, Cancer Research UK and the Medical Research Council have also funded research infrastructure. The 100,000 Genomes Project uses data provided by individual and collected by the National Health Service as part of their care and support.

Individual 9 was collected as part of the SYNaPS Study Group collaboration funded by The Wellcome Trust and strategic award (Synaptopathies) funding (WT093205 MA and WT104033AIA). This research was conducted as part of the Queen Square Genomics group at University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

Disclosure

The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing conducted at Baylor Genetics. FMZ and RP are employees of GeneDx, Inc.

Supplementary material

One table with additional clinical details.

Web Resources

100,000 Genomes Project: https://doi.org/10.6084/m9.figshare.4530893.v5

ExAC Browser, http://exac.broadinstitute.org/

GenBank, http://www.ncbi.nlm.nih.gov/genbank/

OMIM, http://www.omim.org/

FastQC: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc

PIGK structure model: https://swissmodel.expasy.org/repository/uniprot/Q92643

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Tables

Table 1. Phenotypic features of individuals with biallelic inactivating variants in *PIGK*.

Individual	1a	1b	2a	2b	3	4	5	6	7	8	9a	9b	Total
Hypotonia	+	+	+	+	+	+	+	+	+	+	+	+	12/12
DD/ID	+	+	+	+	+	+	+	+	+	+	+	+	12/12
Cerebellar atrophy	+	-	+	+	+	+	+	+	-	+	+	+	10/12
Ataxia	-	-	+	+	+	+	+	na[1]	+	na	na	na	6/8
Other movement disorder	+	+	-	-	-	-	-	+	-	-	-	-	3/12
Epilepsy/seizures	+	-	+	+	-	-	-	+	+	+[2]	+	+	8/12
Dysmorphisms	+	+	+	+	+	+	-	+	-	-	+	+	9/12
Ophthalmological anomalies	+	-	-	+	+	+	-	-	+	+	+	+	8/12
Genito-urinary malformation	+	+	-	-	-	-	-	-	-	-	-	-	2/12
Gastrointestinal anomalies including GERD	-	-	-	-	+	-	-	-	+	-	-	-	2/12
Teeth anomalies	+	-	+	+	-	-	-	+	-	-	+	-	5/12
Hand/feet anomalies	+	-	+	+	-	-	-	-	-	-	-	-	3/12
Skeletal findings	-	-	+	+	-	-	-	-	-	-	-	-	2/12
Low serum alkaline phosphatase	-	-	+	+	-	-	-	-	-	-	na	na	2/9

na=not available. Note [1]: Difficult to assess due to non-ambulatory status and no purposeful hand use. However cerebellar dysfunction signs include saccadic ocular pursuit and end-gaze nystagmus. Note [1]: Febrile seizures only.

Table 2. List of the *PIGK* variants identified in the subjects included in the study.

Family	Genomic variant (hg19)	DNA variant (NM_005482.3)	Protein variant	Inheritance	gnomAD minor allele frequency (no homozygotes found for any variant)
1	chr1:g.77620297A>G	c.823T>C	p.Cys275Arg	Compound heterozygous	0
	chr1:g.77672406G>A	c.158C>T	p.Ser53Phe		0
2	chr1:g.77635060G>A	c.260C>T	p.Ala87Val	Homozygous	0.000004129, highest 0.000008978 (European non-Finnish population) rs772948495
3	chr1:g.77632412T>G	c.479A>C	p.Tyr160Ser	Compound heterozygous	0.00001504, highest 0.00004152 (African population)
	chr1:g.77676171G>A	c.97C>T	p.Gln33*		0
4	chr1:g.77632412T>G	c.479A>C	p.Tyr160Ser	Compound heterozygous	0.00001504, highest 0.00004152 (African population)
	chr1:g.77676175C>G	c.94-1G>C	splice site		0
5	chr1:g.77632412T>G	c.479A>C	p.Tyr160Ser	Homozygous	0.00001504, highest 0.00004152 (African population)
6	chr1:g.77635063A>G	c.257T>C	p.Leu86Pro	Homozygous	0
7	chr1:g.77629564G>A	c.551C>T	p.Ala184Val	Compound heterozygous	0.000004011, highest 0.000008837 (European, non-Finnish)
	chr1:g.77627056A>T	c.737T>A	p.Met246Lys		0
8	chr1:g.77635058C>T	c.262G>A	p.Asp88Asn	Homozygous	0.0000124, highest 0.0001071 (South Asian population)
9	chr1: g.77635058C>T	c.262G>A	p.Asp88Asn	Homozygous	0.0000124, highest 0.0001071 (South Asian population)

Figure legends

Figure 1: Pedigrees of families with biallelic *PIGK* variants.

Figure 2: Facial and MRI features of subjects with *PIGK* variants.Clinical features in 2 sibs of family 2: facial dysmorphisms: long face, sparse hair, high anterior hairline, prominent forehead, broad and laterally sparse eyebrows, thin upper lip, antihelix shelf, prominent antitragus, dental crowding, abnormally shaped teeth, brachydactyly in hands and feet. MRI findings: cerebellar atrophy in individuals 2a, 2b, 3, 5, 6 and 8.

Figure 3: PIGK variants and conservation of affected residues. (A) Location of the variants on the *PIGK* gene and corresponding protein. Introns not drawn to scale. (B) Multiple alignment of PIGK orthologs showing conservation of the affected residues in vertebrates.

Figure 4: Cell surface GPI-AP levels in blood cells of affected individuals. Blood samples of the individuals in families 1, 2 and 3 and control cells were stained with FLAER and CD16. Figure shows representative analysis of cell surface GPI-AP levels of granulocytes from triplicate experiments.

Figure 5: Rescue assays in fibroblasts. Skin fibroblasts derived from individuals 1a (upper panels) and 7 (lower panels) were transduced with PIGK-expressing-Lv105 lentivirus or empty Lv105-lentivirus, and non-transduced cells were stained with FLAER, CD73 and CD109. The figure shows representative results from experiments done in triplicate.

Figure 6: *In vitro* functional studies for PIGK variants identified in individual 1a and 1b. A. FACS analysis of PIGK deficient CHO cells transfected with mutant or wild type PIGK cDNA driven by a strong promoter, pME vector (upper panels) or a weak promoter, pTK vector (lower panels). B. Expression of wild type and mutant PIGK-GST protein in PIGK deficient CHO cells transfected with mutant or wild type PIGK cDNA driven by a strong promoter, pME vector. GAPDH is a loading control.

Figure S1: Cell surface levels of other GPI-APs in blood cells of affected individuals. Blood samples of the individuals in families 1, 2 and 3 and control cells were stained with CD55 and CD59. Figure shows representative analysis of cell surface GPI-AP levels of granulocytes from triplicate experiments.

Table S1: Detailed phenotypes of the affected individuals.

	1a	1b	2a	2b	3	4	5	6	7	8	9a	9b
Ancestry	Asian Indian	Asian Indian	Italian	Italian	French and Maghreb	Egyptian	Egyptian	Nigerian, Igbo tribe	NA	Pakistani	Pakistani	Pakistani
Gender	Female	Male	Male	Female	Male	Female	Male	Female	Male	Female	Male	Male
Consanguinity?	No	No	Yes	Yes	No	No (parents from same village)	Yes- first cousins	No, but from same village	No	Yes (parents 2nd cousins)	Yes	Yes
Prenatal issues or findings	LSCS	Increased NT of 4mm, hydronephrosis	NA	NA	Single umbilical artery	No	No	No	No	Yes, hydronephrosis (later resolved) and polyhydramnios	No	No
Gestational period	39 weeks and 4 days	39 weeks	40 weeks	39 weeks	40 weeks	40 weeks	38 weeks	40 weeks	40 weeks and 2 days	37 weeks	FT	FT
APGAR scores	8, 9, 9	9, 9	NA	8, 10	10, 10	9	10	NA	8, 9	NA	Immediate cry	Immediate cry
Neonatal complications	Jaundice	No	No	Epileptic seizures, Eating difficulties	No	No	No	No	No	No	Early-onset sepsis at 2nd day of life with seizures for 15 days, discarged on phenobarbitone	No

Birth weight	3120 g (28th %ile)	3935 g (78th %ile)	3600 g (53rd %ile)	3910 g (85th %ile)	3050 g (21st %ile)	3000 g (21st %ile)	4200 g (90th %ile)	3740 g (73rd %ile)	4196 g (86th %ile)	3055 g (19th %ile)	Average (exact not known)	Average (exact not known)
Birth length	48 cm (26th %ile)	53 cm (86th %ile)	51 cm (62nd %ile)	50 cm (58th %ile)	48 cm (21st %ile)	47 cm (14th %ile)	50 cm (48th %ile)	NA	NA	NA	NA	NA
Birth head circumference	34 cm (29th %ile)	37 cm (75th %ile)	NA	35.2 cm (54th %ile)	34 cm (20th %ile)	33 cm (11th %ile)	34cm (20th %ile)	NA	NA	NA	NA	NA
Age at last follow-up	8y 8m	3y 10m	27y	19y	6y 3m	9y 7m	5y 5m	4y 9m	12 months	3y 5m	5y	3y
Weight	19.5 kg (-2.23 SD) Failure to thrive	13.6 kg (7th %ile)	49.5 kg (-2.37 SD)	40 kg (-2.60 SD)	24.3 kg (77th %ile)	20 kg (-2.53 SD)	15 kg (2nd %ile)	20.2 kg (79th %ile)	8.6 kg (5th %ile)	14.2 kg (37th %ile)	18kg (9th %ile)	13kg (9th centile)
Height	127.8 cm (28th %ile)	106.7 cm (91st %ile)	157 cm (-2.72 SD)	148 cm (-2.35 SD)	114 cm (28th %ile)	115 cm (-3.32 SD)	104 cm (6th %ile)	108 cm (at 4y 1mo) (93rd %ile)	79 cm (87th %ile)	96 cm (26th %ile)	102cm (<3rd ile)	89cm (<3rd %ile)
Head circumference	49.5 cm (3rd %ile)	47.8 cm (5th %ile)	57.7 cm (97th %ile)	52.8 cm (8th %ile)	49.2 cm at 3y 2m (35th %ile)	49 cm (-2.46 SD)	49 cm (5th %ile)	53 cm (98th %ile)	47 cm (69th %ile)	50.5 cm (87th %ile)	48cm (<3rd %ile)	41cm (<3rd %ile)
DD/ID	Yes, severe with regression. Global developm ent regression , does not respond to her name at 8y, does not	Global developmental delays, does not respond to name or recognize his parents, can roll from side to side at 3y 10 m and is trying to sit with support, was able to babble at 22 m but no	Yes, severe. At six months head control At 2 years sitting without support At 8 years walking	Yes, severe. At 18 months sitting without support. At 7 years walking with support. She can perform	Mild	Speech delay, but improvi ng now can take steps and speak words, maintain sphincte ric control	Delayed, started to walk few steps with unsteady gait, Speech delay but improving with time (IQ 60)	Severe global developmen tal delay. No expressive or receptive verbal language. Smiles and cries to communicat e needs. Rolls and	Severe global developm ental delay. Controls head but does not sit. No babbling. Smiles. Poor feeding, and	Global develop mental delay. At age 3y 5mo comman do crawling , pulling up to stand and cruising;	Yes, severe GDD, neck holding achieved at 2 years of age, speech delay, can only coo, drooling, sitting with	Yes severe GDD, speech delay, delay in neck holding

	recognize her parents, keeps her mouth open mostly and does abnormal movements with her tongue, does same action repeatedly (head and hand movement). No speech, no ambulation	longer has expressive verbal language	with support. He can perform easy orders. Very poor language Good interaction with environment	easy orders. Very poor language Good interaction with environment		and obey orders. (IQ 58)		sits but does not stand. Lost purposeful hand use after infancy. Feeds orally, but concern for dysphagia.	concern for dysphagia.	non-verbal, points to indicate needs; feeding independently using fingers; picks up bottle and drinks	support, no difficulty in feeding or swallowing	
Seizures?	Yes	No	Yes	Yes	No	No	No	Yes	Yes	Fever-related only; at age 3y 5mo has had 3 in total, brief in duration, 30-40 seconds	Yes	Yes
Seizure age of onset	8 months	-	8 months	At neonatal age	-	-	-	2y 9mo	9 mo	3y 5mo	At neonatal age	8m

Seizure types	-	-	At the beginning seizures were characterized by: generalized hypotonia and loss of consciousness, with duration of 30 seconds, with a multiple daily occurrences. Treated with Valproic acid Then at 3 years seizures became tonic-clonic and Phenobarbital was added	At 20 months clonic movements on upper limbs and retropulsion of the head, with a multiple daily occurrences with duration of few seconds, more intense in awakening and falling a sleep	-	-	-	Atonic, myoclonic, generalized tonic-clonic	Behavioral arrest, eye rolling, eyelid fluttering	Febrile	Generalized tonic-clonic seizures	Generalized tonic-clonic seizures
Seizure control	2 seizures/6	-	Complete	Complete	-	-	-	Poor. Continues to	Complete	Resolved on	Uncontrolled, 1-2	Well controlled,

	0 days since 16 months old							have clusters of myoclonic seizures daily. Actively titrating medication		their own and did not recur	episodes/ month, occasionally miss dose	last seizure 1 year back, first seizure at 8m of age
Current antiepileptic drugs	Tegretol, Onfi	-	Valproic acid (1000mg x 2) Phenobarbital (2 mg/kg 6 drops x3)	Valproic acid 400 mg	-	-	-	Clonazepam	Levetiracetam	-	Na Valproate (30mg/kg/d) Levitrecitam (55mg/kg/d)	Na Valproate (15mg/kg/d) Levitrecitam (30mg/kg day)
Other antiepileptic drugs tried	Phenobarbital since dec 2010 (7ml BID), keppra, topamax (50mg BID) and clobazam (5mg BID) since age 2	-	Valproic acid, Phenobarbital Clonazepam	-	-	-	-	Keppra (ineffective), Clobazam (effective)	Levetiracetam	-	-	-
Other medications	None	None	None	None	Topiramate	Vitamins and tonics including omega, L-carnitine, vitamin B,	Tonics including omega, L-carnitine, omega and brain stimulants such as Piracetum.	None	None	-	None	None

						vitamin E, coenzyme Q10						
EEG	Left anterior spike and slow wave complex, mild diffuse background slowing	Normal	Diffuse discharges of spikes and spike-wave, prevalent on the anterior regions	During wakefulness symmetrical and reactive, alpha rhythm. Transient isolated rushes of spikes and spike-wave prevalent on the anterior regions	Normal	Normal	Normal	Multifocal bilateral independent spikes and poorly maintained posterior basic rhythm	2-3 Hz generalized spike-wave	At age 10m: wake recording showing an excess of slow activity bilaterally. A degree of diffuse cerebral dysfunction cannot be ruled out. No epileptiform discharges or lateralising features	Not done	No evidence of seizure activity
Hypotonia	Yes, severe generalized hypotonia, axial and appendicular	Yes, severe generalized hypotonia	Yes, hypotonia on lower limbs	Yes, mild generalized hypotonia	Yes, Neonatal	Yes, axial and appendicular since early life, but now can	Yes, Hypotonia since early life both axial and appendicular, can take a few steps	Yes, central	Yes, axial and appendicular	Yes, axial (variable tone in limbs)	Yes, Marked, started tripod sitting at 5.7 yrs. Dependant on mother	Yes, Present but better motor function, self feeds with help, can sit withput support and

						take 10 steps (reflexes were brisk)	(reflexes are brisk)				for all activities	commando crawl. Coos and makes sounds only
Other neurological findings	Hypertonic in extremities, hand flapping, sluggish DTR in upper and lower limbs, generalized muscle wasting	Hypertonic in extremities, spasticity, holding wrists flexed, sudden myoclonic jerks, eyes with jerky saccades with slight nystagmus	Cerebellar ataxia, dysmetria, poor fine motor coordination, wide and unsteady gait, bilateral foot clonus, deep tendon reflexes hyperactive	Cerebellar ataxia, dysmetria, poor fine motor coordination, wide and unsteady gait, bilateral foot clonus, deep tendon reflexes hyperactive	Cerebellar ataxia Headaches	Cerebellar ataxia	Cerebellar ataxia	Gaze-evoked nystagmus, saccadic pursuits, arm tremor in infancy (resolved), loss of purposeful hand use (was able to grasp voluntarily as infant but no longer does), dystonia and choreoathetosis noted transiently at a single visit.	Truncal titubation, nystagmus	Cerebellar ataxia, bilateral alternating convergent squint	Hypopigmented patch on head	Hypopigmented patch around Left eye
MRI	Yes, MRI at 4months was normal, abnormal brain MRI at 1y (possibly reduced white matter volume	Yes, MRI at 6 months showed vague increased diffusion-weighted signal involving the dorsal pons and midbrain, bilateral globus pallidus, and bilateral	Yes. MRI at 2 years: cerebellar atrophy At 14 years was constant	Yes. MRI at 2 years: cerebellar atrophy. At 8 years was constant	Global cerebellar atrophy FLAIR hyper signal of vermis and	Diffuse cerebellar atrophy including both hemispheres with more vermian affection, with	Diffuse cerebellar atrophy including both hemispheres with more vermian affection, with widened cerebellar fissures and	MRI at 4mo normal aside from chronic right subdural fluid and left frontoparietal developmental venous anomaly.	MRI at 5 months normal aside from chronic-appearing bilateral subdural fluid collections	MRI at 3 years (comparison with previous at 11 months): marked interval progression of cerebell	Mild cerebral and cerebellar atrophy	Cerebellar hypoplasia

	mainly posterior, the corpus callosum is relatively attenuated), MRI at 3y (cerebellar vermian and hemispheric atrophy of moderate degree; focal cortical dysplasia FCD type IB, 4th ventricule appears significantly dilated). progressive atrophy of hippocampus and a decrease in white matter	periatrial white matter			cerebellar hemispheres secondary to gliosis	widened cerebellar fissures and fourth ventricle.	fourth ventricle.	MRI at age 3y 3mo showed interval marked cerebellar atrophy with the vermis more affected than the hemispheres.		ar atrophy and mild callosal dysmorphism, No disproportionate volume loss of brainstem or cord		
Facial dysmorphism	High arched palate	Upturned earlobes, maxillary	Long face, few	Long face, promine	Synophris,	Broad nasal	None	Widely-spaced teeth, down-	None	Broad forehead, short	Triangular/myopathic face	Triangular/myopathic face

	with normal uvula Widely spaced nipples. Flat broad chest with pectus excavatum. low set ears with normal size and shape but with folded ear pinna	alveolus mildly thickened.	sparse hairs, high hairline anterior, prominent forehead, laterally sparse eyebrow, thin upper lip, antihelix shelf, prominent antitragus and lobe, dental crowding, abnormally shaped teeth.	nt forehead, laterally sparse eyebrow, mild hypotelorism, convergent strabismus, antihelix shelf, prominent antitragus and lobe, dental crowding, abnormally shaped teeth, high and narrow palate	Thin upper lip, anteverted nares	root, epicanthic folds, broad nose with prominent nares, long philtrum, thin upper lip, everted lower lip, low set ears, hypotonic, expressionless face		slanting palpebral fissures, low set ears (all mild)		fingers and bilateral 5th finger clinodactyly		
Cranial shape anomalies	No	No	Bifrontal constriction	Bifrontal constriction	No	No	No	No	Positional plagiocephaly	Mild plagiocephaly	Left sided brachcephaly	none
Craniosynostosis	No	No	No	No	No	No	No	No	No	No	No	No

Deafness	No	No	No	No	No	No	No	No	No	No	Normal audiometry	Normal hearing, no format testing done
Ophtalmological anomalies	Delayed visual maturation (left eye had problem to follow the light on the exam of 22/9/2010). Myopia	No	No	Esotropia	Hypermetropia	Hyperm etropia, squint (corrected with surgery)	Normal	No	Strabismus, hyperopia	Convergent squint	Convergent squint	Convergent squint
Cardiac anomalies	Right ventricular hypertrophy on ECG	No	No	No	No	No	No	No	No	No	No	No
Genito-urinary malformation	Bilateral hydronephrosis at birth	Prenatal hydronephrosis, unilateral pelviectasis at 4 months of age	No	No	No	No	No	No	No	Prenatal hydronephrosis, resolved postnatally	No	No
Gastrointestinal issues	No	No	No	No	Paroxystic cyclic vomiting with vertigo and headaches since first mont	No	No	No	Gastrointestinal reflux	No	No	No

					h of life							
Teeth anomalies	Delayed, no teeth at 13m	No	Dental crowding	Dental crowding	No	No	No	Small, widely spaced	Gap between central incisors	No	Widely spaced	No
Nail anomalies	No	No	No	No	No	No	No	No	No	No	No	No
Short fingers or hands	Bilateral single palmar creases, partial syndactyly of D2 and D3	No	Hands and feet brachydactyly, joint hypermobility	Hands and feet brachydactyly, joint hypermobility	No	No	No	No	No	No	No	No
Skeletal anomalies	No	No	Thoracic right-convex and lumbar left-convex scoliosis, bilateral genu valgum	Cubito valgus, pes planus	No	No	No	No	No	No	No	No
Joint contractures	No	No	No	No	No	No	No	No	No	No	No	No
Serum alkaline phosphatase	211 U/L (ref 40-350)	201 U/L (ref 125-320)	44 UI/L Low	29 UI/L Low	340 U/L (ref 156-369)	118 UI/L (ref 0-300)	150 UI/L (ref 0-300)	191 U/L (ref 100-320)	192 U/L (ref 110-400)	173 U/L (ref 145-320)		
Other clinical or laboratory findings	Laughs loudly without any reason (many episodes daily).	Karyotype showed maternally inherited inversion of 46,XY,inv(13)(q14.1q22), normal	No	No	No	Normal extended metabolic screening, acylcarn	Normal karyotyping, extended metabolic screening, acylcarnitine profile, organic	Normal: Lactate, ammonia, plasma amino acids, acylcarnitine profile, TSH, CK.	No	Normal metabolic investigations and SNP array	Normal urinary organic acids,	Ammonia 49 Uric acid 4.1 LDH 342 Bili 0.3, ALT 29

	Spontaneous hypersudation on head since 3 months old, not sleeping at night.,.Normal karyotyping and microarray. Normal GAG, CSF neurotransmitters normal, folate metabolism normal. Elevated Iron serum 174 ug/dL (ref 30-160), elevated iron saturation (55%, ref 14-50), normal total and unsaturated iron-binding capacities	microarray, normal plasma amino acids, normal urine organic acids, normal lactate, normal acylcarnitine profile, negative autism/intellectual disability panel				itine profile, organic acids in urine, echocardiography, abdominal sonar, fundus examination, EEG.	acids in urine, echocardiography, abdominal sonar, fundus examination, EEG.	SNP array showed extended regions of homozygosity, the most significant being 1p32.3p21.2 (54,687,064 - 101,204,644) on GRCh37/hg19 assembly				