A sensitive method for validation of PIGG variant pathogenicity assessed in families with bi-allelic PIGG variants.


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Phosphatidylinositol Glycan Anchor Biosynthesis, class G (PIGG) is an ethanolamine phosphate transferase that catalyzes the modification of the second mannose of glycosylphosphatidylinositol (GPI). GPI serves as an anchor on the cell membrane for over 150 surface proteins called GPI anchored proteins (GPI-APs). Pathogenic variants in genes involved in the biosynthesis of GPI, like PIGG, cause inherited GPI deficiency (IGD). Herein, we report nineteen individuals from seventeen new unrelated families with bi-allelic variants in PIGG. Affected individuals showed developmental delay, intellectual disability, hypotonia, seizures, ataxia, nystagmus, strabismus and tremor. Diminished deep tendon reflexes and minor dysmorphisms were found in four individuals each. Microcephaly, autistic features or autism spectrum disorders, as well as short stature were each found in three individuals. We observed cerebellar atrophy in five individuals and normal alkaline phosphatase in all tested individuals. We suggest nystagmus, strabismus, tremor, diminished deep tendon reflexes, microcephaly, autism spectrum and short stature to be novel PIGG deficiency associated features. Mitochondrial dysfunction was noted for two individuals, a feature increasingly recognized in IGDs for unknown reasons. We analyzed GPI-AP surface levels on granulocytes and fibroblasts for two individuals respectively. GPI-APs were unchanged on granulocytes whereas FLAER, CD73 and CD87 levels in fibroblasts were decreased in both analyzed individuals. Furthermore, we demonstrated enzymatic activity defects for PIGG variants in vitro in a PIGG/PIGO double knockout system. The results were consistent with pathogenicity prediction tools. Phenotype severity partially correlates with the degree of enzymatic activity loss. These methods could be used to determine the pathogenicity of new variants of uncertain signification in PIGG and to better understand PIGG physiological functions.
Introduction

Phosphatidylinositol Glycan Anchor Biosynthesis, class G (PIGG) is an ethanolamine phosphate transferase that catalyzes the modification of the second mannose of glycosylphosphatidylinositol (GPI). It is one of the 22 phosphatidylinositol glycan (PIG) genes involved in the biosynthesis of GPI\(^1\). GPI serves as an anchor for over 150 surface proteins on cell membrane. These proteins are called GPI anchored proteins (GPI-APs). GPI-APs play a variety of essential roles throughout the human body but are especially critical for development and neurogenesis\(^2\). GPI and GPI-AP are linked together by a bridge of ethanolamine phosphate (EtNP) on the third mannose catalyzed by PIGO\(^3\). Variants in genes involved in the biosynthesis of GPI, like PIGG and PIGO, are known to cause inherited GPI deficiency (IGD). Typically, IGDs cause a decrease of GPI-AP levels on blood cell surface\(^3\). They are also associated with hyperphosphatasia or hypophosphatasia because the lack of, or abnormal, GPI anchor on alkaline phosphatase triggers its secretion out of the cell or its degradation\(^3\)–\(^5\). There are currently 21 genes associated with IGDs\(^6\). The main clinical characteristics of IGDs are intellectual disability, seizures and facial dysmorphisms\(^7\).

As of today, three studies have reported individuals with bi-allelic PIGG variants suffering from intellectual disability, developmental delay, hypotonia, early-onset seizures, cerebellar hypoplasia, ataxia and minor facial dysmorphology\(^1\),\(^3\),\(^8\). These studies, including one by some of the authors of the present article, however, described normal surface levels and normal structure of GPI-APs on granulocytes despite an almost complete inactivation of PIGG. A significant number of individuals described in these studies also showed normal alkaline phosphatase (5 out of 8). This outlined the need for further characterization of PIGG's physiological role. Moreover, there is a need to find new methods to study the impact of genetic variants on PIGG function for clinical diagnosis and research.

Herein, we report seventeen new unrelated families with bi-allelic PIGG variants. These individuals presented several common characteristics such as developmental delay, intellectual disability, hypotonia, seizures, ataxia, nystagmus, strabismus, tremor, diminished deep tendon reflexes, minor dysmorphisms, microcephaly, autism spectrum and short stature. Cerebellar atrophy was observed in five individuals. As reported before, alkaline phosphatase was normal in all tested individuals. We suggest nystagmus, strabismus, tremor, diminished deep tendon reflexes, microcephaly, autism spectrum and short stature to be novel PIGG deficiency associated features. We analyzed granulocytes as well as fibroblasts from two affected individuals respectively and compared GPI-AP surface levels to those of controls. Furthermore, almost all PIGG variants were tested in vitro in a new PIGG/PIGO double knockout system to determine variants’ effect on enzymatic activity. PIGO knockout cells show partial loss of surface GPI-APs which is completely removed by further knockout of PIGG. Wild-type and variant PIGG are then used to rescue GPI-AP expression (measured via DAF and CD59 expression) on PIGO/PIGG double knockout cells. This system could be used to validate new PIGG variants and to study its physiological role.
Results
Clinical information and genetic analysis of the affected individuals:

Seventeen unrelated families were recruited for this study, including 19 individuals with twenty different PIGG variants. Family number was given according to enzymatic activity reduction of variants (null activity to no decreased). Consanguinity was present in nine families (See Figure 1 for pedigree and genotypes of families). Individuals with bi-allelic PIGG variants all presented developmental delay and/or intellectual disability (See Table 1 for main phenotypic characteristics from this cohort compared to previously published cases, a blue color gradient shows degrees of enzymatic activity reduction for each individual). Developmental delays were mixed, affecting motor, language and social aspects. Eleven individuals presented hypotonia and seizures (61% of this cohort). Hypotonia was particularly severe in individuals 3, 7 and 9 and was generalized. For all affected individuals, seizures were mostly febrile and occurred mainly in the first two years of life. Almost all individuals are now seizure free (except individual 3 and 17). EEG showed mostly diffuse slowing and generalized spikes. Ataxia and nystagmus were also prominent features in this cohort, affecting nine (50%) and seven (39%) individuals respectively. Individual 4B was wheelchair-bound because of frequent falls and wide-based gait. Individual 16 had acute ataxic attacks with regression around 2 years old which responded to corticosteroids. Strabismus and tremor were also observed in five individuals each (28%). Tremors were intentional and/or resting. Individual 12b had esotropia in infancy that resolved. Four individuals had diminished deep tendon reflexes (22%). In individual 3, reflexes were normal in the upper limbs but diminished in the lower limbs, unlike individual 8 who had brisk reflexes. Minor dysmorphism were observed in four individuals (22%). They variably had smooth and long philtrum, thin upper lip, shallow nose tip, tented mouth, coarse face and mild bilateral fifth fingers clinodactyly (Figure 2a). Three individuals had microcephaly under the 2nd percentile (17%). Individual 15, however, had macrocephaly (head circumference in the 99th percentile). Autism spectrum disorders were diagnosed in 2 individuals and suspected in individual 14 who had autistic features (17%). Three individuals had short stature under the 10th percentile (17%). MRI demonstrated cerebellar atrophy in five individuals (28%) and normal alkaline phosphatase was found in all tested individuals (eight/44%). Common MRI findings included cerebellar and vermis hypoplasia/atrophy which was progressive for individuals 10 and 11 (Figure 2b shows the MRI for individual 12B). Individual 17 was put separately in Table 1 and Table S1 because we were unable to demonstrate that the phenotype was due to PIGG bi-allelic variants (See Discussion). Hearing loss and congenital heart defect were not common findings in this cohort, contrary to other IGDs. Only one individual (individual 6) showed mild unilateral hearing loss and small ventricular septal defect. Detailed history, laboratory findings, clinical findings and phenotype descriptions are presented in Table S1 for each individual. Globally, most individuals seemed to clinically improve with age, with respect to developmental delay, seizures, nystagmus and ataxic features. Many affected individuals had extensive biochemical and genetic investigations with normal results, including mainly CGH microarray, karyotype, mitochondrial DNA sequencing, Fragile X DNA
testing, transferrin isoelectric focusing, acylcarnitine profile, creatine kinase, lactate, pyruvate, ammonia, alpha-foetoprotein, TSH, biotinidase, urinary organic acids and purines/pyrimidines, plasmatic amino acids and cerebrospinal fluid autoantibodies, amino acids, 5-methyltetrahydrofolate and neopterin. Individual 12b had 6% absence of heterozygosity in her genome indicating consanguinity of the parents. Individual 13 was found to have a maternally inherited variant in the ELN gene which was predicted to be pathogenic. He had hyperelastic skin with easy bruising, bilateral congenital hip dysplasia and joint hypermobility with Beighton score at 8/9. However, his ECG and echocardiogram were normal and the mother had a normal skin and joint exam. We therefore considered this variant to be a variant of uncertain significance and included this individual in the study. Individual 4a had a muscle biopsy. Light microscopy and histopathology were normal. Electron microscopy showed a marked accumulation of subsarcolemmal mitochondria, some of which were enlarged. Spectrophotometry showed significant decrease in both complex I and II. Mitochondrial DNA sequencing and metabolites were normal. Individual 7 also had a muscle biopsy at the age of three years showing subsarcolemmal aggregates of normal mitochondria on histopathology and electron microscopy and moderately low respiratory chain complex II enzymatic activity. Mitochondrial complex II deficiency was confirmed in skin fibroblasts. Other mitochondrial complex activities were normal in skin fibroblasts. This individual had decreased muscle bulk and weakness in the four limbs.

Individuals from families 1 and 7 had siblings with concordant phenotypes but were excluded from this study because they were not tested for PIGG variant (Figure 1). We had no clinical details concerning the sibling of individual 1. Sibling of individual 7 had a significantly milder phenotype compared to her older brother. She had mild developmental delay, diminished deep tendon reflexes and proven superior vermian atrophy of cerebellum. She had no seizure, hypotonia, ataxia, nystagmus, tremor, strabismus, dysmorphisms, autistic features and had normal height and head circumference. Consanguinity increases the risk for another recessive disease in this sibling. Individuals from families 5 and 9 had siblings with proven bi-allelic PIGG variants and seizures but clinical information was insufficient to include them in the study.

PIGG variants were dispersed throughout the protein length affecting amino acids positions 46 to 957 (See Figure 3). There was a higher density of variants from amino acid positions 270 to 344 in the GPI ethanolamine phosphate transferase 2 domain near the second active site. Variants were eleven missenses, eight nonsenses and one splice variant. Variants p.Asp786AlafsTer6, p.Gln851Pro and p.Leu875Ter were already reported in ClinVar as likely pathogenic or pathogenic. Variant p.Trp547Ter was the only one previously published\(^1\). Missense variants were mostly located in luminal loops but two (p.Val531Met and p.Gln851Pro) were cytoplasmic. Variants p.Tyr934Ter and p.Tyr957Ter were the only two nonsense variants that were predicted to escape nonsense-mediated decay and to result in a truncated protein because of their position in the last exon. Amino acids 934 to 961 were strongly conserved across vertebrates suggesting they may play an important role in PIGG physiology and that a nonsense
variant in this region may have a deleterious impact (amino acid conservation across vertebrates for the C-terminal tail of PIGG is shown in Figure 4). Individuals 10 and 11, who were respectively homozygous for p.Tyr934Ter and p.Tyr957Ter, showed incapacitating ataxia and progressive cerebellar atrophy. Conservation of residues affected by missense variants is also shown in Figure 4. Seven of them appeared to be strongly conserved, whereas four were poorly conserved (Met344, Ser497, Val531, Glu696). These four non-conserved residue variants were all found as compound heterozygous in individuals 16, 17, 14, 15, respectively. Individual 17 had a severe phenotype with respect to developmental delay, intellectual disability and epilepsy (See Discussion). Individual 16 had severe acute ataxia. Individuals 14 and 15 had global developmental delay, moderate intellectual disability and severe language delay.

Flow cytometric analysis of blood cells and fibroblasts:
We analyzed GPI-AP surface levels on granulocytes for individuals 7 and 12a. FLAER, CD16, CD55 and CD59 were all normal in both individuals (Figure 5). We analyzed fibroblasts from individuals 14 and 15. FLAER, CD73 and CD87 were lower in both individuals compared to controls whereas CD109 was unchanged (Figure 6).

Functional analysis using PIGO/PIGG DKO cells and genotype-phenotype correlation:
Eighteen different variants were tested in the PIGO/PIGG double knock-out system. Restoration of the GPI-AP expression on PIGO/PIGG DKO cells by transfection with wild type or variant PIGG cDNA were compared (Figure 7). PIGG variants’ expression was analyzed by western blotting (Figure S1). Variants p.Phe580LeufsTer2 and p.Ala46GlnfsTer28 were not tested but both activities were expected to be null, as both were frameshift variants. Ten variants had a null enzymatic activity, one had severe decrease in activity, two had a decreased activity, four had a mild decrease in activity and two had slight or no decreases in activity (Figure 7). The prediction tools could clearly differentiate pathogenic variants with null or decreased activity from benign variants with no or slight decreased activity, but they could not predict the pathogenicity of the variants with mild decreased activity (Table 2). The variants with null, severely decreased and decreased activity variants had a mean pathogenicity CADD score of 30.1 (Table 2). Variants causing a slight or no decrease were predicted to be likely benign variants and had a mean CADD score of 11.7. Both variants of individuals 10 and 11 (p.Tyr934Ter and p.Tyr957Ter) truncated the strongly conserved C-terminal end of PIGG resulting in a null enzymatic activity (Figure 4). Prediction tools predicted these variants to be likely pathogenic and CADD scores were 31 and 36, respectively. Individuals 14, 15 and 16 had one of their variants located in a non-conserved residue (Met344, Val531 and Glu696) (Figure 4), with slight/no or mild decrease and CADD scores of 0.61, 9.0 and 6.0 respectively (Table 2). All individuals with PIGG deficiency showed developmental delay and/or intellectual disability but the severity did not correlate with the degree of decreased activities (Table 1). Individuals 7 and 8 showed severe DD/ID, whereas individuals 2, 10 and 11 showed mild DD/ID, although their PIGG variants were all with null activity. On the contrary, individual 15, the two variants of which had mildly decreased activity showed moderate DD/ID. One of its variants (p.Arg681Trp) had a
pathogenicity CADD score of 32. Affected individuals with null to severely decreased activity showed high probability of cerebellar atrophy (individuals 2, 10, 11, 12a and 12b), seizures, tremor, nystagmus, diminished tendon reflexes, strabismus, dysmorphism, microcephaly and short stature (*Figure S2*, P-value were calculated using one-tailed spearman correlation). The most severely affected individuals regarding DD/ID (individuals 7 and 8) and hypotonia (Individuals 3, 7 and 9) were homozygous for null activity variants. Individuals with milder activity decrease showed higher probability of autistic features (individuals 13, 14, 15).
Discussion
Focusing on the characteristic of PIGG deficiency (Differences from other IGDs):

Our study confirms the phenotype of individuals with PIGG deficiency reported before in smaller studies. Individuals with bi-allelic variants in PIGG show intellectual disability, developmental delay, hypotonia, early-onset seizures, cerebellar atrophy, ataxia and minor facial dysmorphism. Here, we also report new PIGG deficiency associated features. Nystagmus, strabismus, tremor, diminished deep tendon reflexes, microcephaly, autism spectrum and short stature were commonly found in this cohort. Strabismus was also reported in one individual in Zhao et al. Hyporeflexia, autistic features and growth retardation were also described in Makrythanasis et al but were not present in a significant proportion of individuals. Contrarily to several other IGDs, individuals with PIGG deficiency have normal levels of alkaline phosphatase and often normal levels of GPI-APs on granulocytes. Classical IGD phenotypes usually include developmental delay, intellectual disability, seizures, hypotonia and facial dysmorphisms, which is common with PIGG deficiency symptoms. Other characteristics of IGDs include anorectal and finger anomalies, hearing loss and kidney hypoplasia. These last characteristics were not found in individuals with bi-allelic PIGG variant in this cohort.

Genotype-Phenotype correlation:

Genotype partially correlates with affected individual’s phenotype. Siblings from a same family (families 4 and 12) resembled each other in terms of phenotype. Individuals 7, 8 and 9 who all had the same genotype (homozygous p.Leu875Ter) were also similar in the severity of the developmental delay. Some features like nystagmus, seizure, tremor, cerebellar atrophy, strabismus, diminished deep tendon reflexes, dysmorphism, microcephaly and short stature seemed to correlate better with severity of enzymatic activity disruption observed in vitro. However, some features like developmental delay, intellectual disability and hypotonia were more ubiquitous throughout individuals with bi-allelic PIGG variants regardless of enzymatic activity. The most severely affected individuals regarding this two features had homozygous null variants but some individuals with homozygous null variants had milder phenotype. It is possible that small changes in enzymatic activity in vitro may have variable clinical impacts in vivo. This outlines the complexity of interaction between genomic changes and phenotype and the need for further clinical and in vitro characterization of impacts on the GPI biosynthesis pathway and the GPI structure itself.

Individual 17 had a very severe phenotype especially regarding developmental delay, intellectual disability, seizures and ophthalmological anomalies. Our enzymatic activity measurement, however, did not show any decrease for the Ser497Leu variant. Pathogenic predictions were in favor of a likely benign variant and the residue’s conservation in vertebrates was incomplete. Moreover, the second variant for this individual, p.Thr124Met, only resulted in a mild activity decrease but the conservation for this residue was high and pathogenic predictions were unclear. Again, it is possible
that even a mild decrease in enzymatic activity, too mild to be detected by our assays, could affect neurons and have clinical repercussions. However, at this point, we are uncertain as to the involvement of these PIGG variants in the phenotype of this individual. We therefore excluded individual 17 from our phenotype analysis and put it separately in Table 1 and Table S1.

Physiological meaning of PIGG function:

It is puzzling that several individuals with IGDs have features of mitochondrial disease. Here, we report two individuals with a complex II deficiency in addition to a PIGG deficiency (Individual 4a and 7). Individual 4a also had complex I deficiency. There are only two previously published studies describing cases with mitochondrial complex deficiency and IGDs. One study suggested that several mitochondrial membrane proteins may be associated with GPI-APs, or GPI-anchored themselves. This could explain why some individuals with IGDs have mitochondrial complex deficiency or dysfunction. Therefore, there is a need to investigate the mechanism and the strength of the association of mitochondrial dysfunction with different GPI deficiency disorders. In the meantime, it could be reasonable to seek mitochondrial dysfunction in individuals with IGD and to exclude IGDs in individuals with unexplained mitochondrial phenotype.

In PIGG deficiency, as opposed to other IGDs, blood cells are not useful in the diagnosis. In individuals with involvement of other enzymes in the pathway, the most reduced GPI-anchored proteins on flow cytometry are CD16 in granulocytes and CD73 as well as CD109 in fibroblasts. Indeed, the GPI-anchored protein studies of PIGG deficient granulocytes are not typical of those usually seen in other IGDs. There is often no reduction of GPI-anchored protein levels or changes in their structure on granulocytes. Our results are consistent with previous studies as we did not found changes of GPI-AP levels on granulocytes. Indeed, fibroblasts seem to be the best tissue for PIGG deficiency diagnosis both to look at GPI-AP levels and at the consequences on mitochondrial function. Zhao et al. 2017 found that GPI-APs are decreased on PIGG deficient fibroblasts and CD73 was the lowest marker compared to controls. They, however, did not measured CD109 in fibroblasts. Thus, our results confirmed these findings. CD73 was also the most reduced marker in both tested individuals. GPI-AP diminution on fibroblasts also corroborate our findings that PIGG variants from individuals 14 and 15 are pathogenic. Contrarily to other IGDs, CD109 was normal in the two affected individuals.

Clinical impact:
In this study and previously published work, cerebellar atrophy is a prevalent feature across individuals with PIGG deficiency (9/26) as it is in other IGDs such as PIGH, PIGO, GPAA1. IGDs are, therefore, becoming increasingly recognized as a differential diagnosis for cerebellar atrophy. Furthermore, alkaline phosphatase, which has a GPI-anchored form, is important to get vitamin B6 in neurons to help GABA synthesis. Some published individuals with other GPI-biosynthesis defects have B6-responsive seizures. Since
there are hundreds of GPI-anchored proteins expressed in neurons, supplements are unlikely to change the neurological outcome apart from the seizures. For GPI-biosynthesis defects in general, we recommend measuring serum alkaline phosphatase and to try supplementing Pyridoxine and/or Pyridoxal phosphate. In individuals with PIGG deficiency, alkaline phosphatase is not useful in the diagnosis because it is mostly normal. A GPI-AP study on fibroblasts, if possible, is ideal. The same supplements can be try to reduce seizures for these individuals.

In conclusion, this study strengthens our knowledge of PIGG deficient phenotype which comprise developmental delay, intellectual disability, hypotonia, seizures, cerebellar atrophy, ataxia and facial dysmorphism. It also highlights new features such as nystagmus, strabismus, tremor, diminished deep tendon reflexes, microcephaly, autism spectrum, short stature and mitochondrial dysfunction. Because affected individuals show normal alkaline phosphatase and normal GPI-AP on granulocytes, clinical diagnosis is a challenge. GPI-AP deficiency can be seen on fibroblasts. We also propose a new in vitro system for testing of new variant and research.
Material and methods

Identification of affected individuals and clinical information collection
 Subjects were recruited via the Deciphering Developmental Disorders (DDD) study\textsuperscript{17}, GeneMatcher or by an international network of collaborating clinicians. An informed written consent was obtained for every participant from themselves or parents. Ethical approval was granted by Centre Hospitalier Universitaire (CHU) Sainte-Justine Research Center and Sick Kids Hospital. The percentiles for weight, height and head circumference were based on Centers for Disease Control and Prevention (CDC) growth charts for United States for last follow-up measurements and UK-WHO for birth measurements.

Analysis of PIGG variants
PIGG variants were compared in Varsome\textsuperscript{18}. Mean and highest minor allele frequency from gnomAD was extracted. Pathogenicity scores from ACMG and dbNSFP were compiled. CADD scores were obtained from University of Washington, Hudson-Alpha Institute for Biotechnology and Berlin Institute of Health web site\textsuperscript{19}. PIGG variants and its transmembrane and lumenal domains from UniProt\textsuperscript{20} were shown in ProteinPaint from St. Jude Children’s Research Hospital PeCan Data Portal\textsuperscript{21} and in Protter from Wollscheid Lab\textsuperscript{22}. Multiple alignments for residue conservation in vertebrates was done using UCSC genome browser\textsuperscript{23}.

Blood Fluorescence-Activated Cell Sorting (FACS)
Blood samples from affected individuals, unaffected family members and controls were stained on ice with GPI-AP markers for one hour. Markers used were phycoerythrin (PE)-conjugated anti-human CD16 (BioLegend), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD55 and CD59 (BD PharMingen), or FLAER-Alexa 448 (Cedar-lane). FACS Lysing Solution (BD Bioscience) was used to lyse red blood cells. Fibroblasts from affected individuals were cultivated. They were harvested at 80\%–90\% confluency and stained with GPI-AP markers in an incubation buffer containing 0.5\% bovine serum albumin (BSA) for 1 h on ice. Markers used were FLAER-Alexa 448, FITC-conjugated mouse anti-human CD73, PE-conjugated CD87\textsuperscript{27} or PE-conjugated mouse anti-human CD109 (BioLegend). Before analyzing, all samples were washed to remove non-specific binding using a BD FACSCanto II system (BD Biosciences). Analysis was carried out with Cytobank software.

Functional analysis of PIGG variants
PIGG/PIGO double knockout (DKO) cells were generated from HEK293 cells using the CRISPR/Cas System. DKO cells were transiently transfected with a wild-type or mutant PIGG cDNA cloned into a strong promoter-driven (SR\textalpha) expression vector, pME PIGG-GST or a weak thymidine kinase promoter-driven expression vector, pTK PIGG-GST. Restoration of the surface expression of DAF or CD59 was analyzed two days later by flow cytometry. The protein expression of each PIGG-GST mutant was analyzed by western blotting using the cell lysate of each transfectant.
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Author contributions
Conflict of interest
The authors declare no conflict of interest.

Figure legends

Figure 1:
Pedigrees and Genotypes of Families with Bi-allelic PIGG Variants

Figure 2:
A. Photographs from individual 1 showing midly coarse face, strabismus, long philtrum and shallow nose tip
B. MRI from one Affected Individual Showing Cerebellar Atrophy (Individual 12B MRI)
MRI at 12 years old showing cerebellar vermian atrophy predominantly affecting the superior vermis
Cerebellar atrophy was found in individuals 2, 10, 11, 12A, 12B and 17

Figure 3:
PIGG Protein Structure and Location of Variants
Panel A: Figure generated using ProteinPaint. Variants from affected individuals are in blue and matched according to each family’s genotype (F01 to F17). Transmembrane and luminal domains are from UniProt. Already published pathogenic variants are in green\(^1,3,8\). ClinVar likely pathogenic and pathogenic variants are in yellow and red respectively.
Panel B: Figure generated using Protter. Transmembrane domains are from UniProt and numbered 1 to 13. Missense variants are in pink and the two nonsense variants resulting in a truncated protein are in blue (p.Tyr934Ter and p.Tyr957Ter).

Figure 4:
Conservation of Affected Residues and C-Term Region across Vertebrates
Multiple alignment from UCSC genome browser

Figure 5:
FACS from Blood Cells of Affected Individuals
GPI-AP surface levels (FLAER, CD16, CD55 and CD59) on granulocytes for individuals 7 (red) and 12a (green) compared to their unaffected heterozygous mother (purple), father (blue) and unrelated controls (black)

Figure 6:
FACS from Fibroblasts of Affected Individuals
GPI-AP surface levels (FLAER, CD73, CD87 and CD109) on fibroblasts from individuals 14 and 15 (green) compared to control (black)

Figure 7
Functional analysis of PIGG variants
A. HEK293 PIGO/PIGG DKO cells were transfected with strong promoter driven (pME) wild type or mutant PIGG cDNA. Two days later, expression of DAF was analyzed by FACS. Most of the variants could not rescue the expression at all except F12, showing partial rescue. B. HEK293 PIGO/PIGG DKO cells were transfected with strong promoter driven (pME) or a weak promoter driven (pTK) wild type or mutant PIGG cDNA. Two days later, expression of DAF was analyzed by FACS except for F15 (pTK), showing expression of CD59. All variants could rescue similar level to wild type PIGG when using strong promoter. Driven by weak promoter, variants showed various level of restoration of GPI-APs.

Figure S1
Expression of the proteins of PIGG variants
HEK293 PIGO/PIGG DKO cells were transfected with strong promoter driven (pME) wild type or mutant PIGG cDNA with C terminal GST tag. Two days later, expression of PIGG proteins were analyzed by Western blotting; quantity was calculated by the band intensities of PIGG-GST, normalized with those of GAPDH as a loading control and luciferase activities as the transfection efficiency.

Figure S2
Features of affected individuals according to enzymatic activity
Correlation between genotype and phenotype is presented for each feature. Enzymatic activity reduction is noted on a scale of zero to four, four being the most reduced activity (null) and zero being the less reduced (slight or no decrease on one allele). Features are either present/absent (1/0) or noted on a scale of one to three, three being the most severe. P-value were calculated using one-tailed spearman correlation.
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