Title: Mutations in *PIGY*: expanding the phenotype of inherited glycosylphosphatidylinositol (GPI) deficiencies.

Biljana Ilkovski, PhD[1], Gina L. O'Grady, MBChB[1,2], Taroh Kinoshita, PhD[3], Alistair T. Pagnamenta, PhD[4], Malcolm F. Howard[4], MSc, Monkol Lek, PhD[5,6], Brett Thomas, PhD[5,6], Anne Turner, MD[7], John Christodoulou[2,8,9], David Sillence, MD[9], Samantha J.L. Knight, PhD[4], Consuelo Anzilotti, MD/PhD[10], Anne Goriely, PhD[11], Leigh B. Waddell, PhD[1,2], Fabienne Brilot, PhD[1,2], Kathryn N. North, MD[1,2,12], Noriyuki Kanzawa, PhD[3], Daniel G. MacArthur, PhD[5,6], Jenny C. Taylor, PhD[4], Usha Kini, MD[13‡], Yoshiko Murakami, MD, PhD[3], Nigel F. Clarke, PhD, MBChB[1,2‡]

1. Institute for Neuroscience and Muscle Research, The Children's Hospital at Westmead, Sydney, NSW, Australia.
2. Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia.
3. Department of Immunoregulation, Research Institute for Microbial Diseases, and WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
4. National Institute for Health Research Biomedical Research Centre, Wellcome Trust Centre for Human Genetics, University of Oxford, OX3 7BN Oxford, United Kingdom.
5. Analytic and Translational Genetics Unit, Massachusetts General Hospital, Boston, MA, USA
6. Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA
7. Department of Clinical Genetics, Sydney Children's Hospital, Sydney, Australia.
8. Western Sydney Genetics Program, Children's Hospital at Westmead, Australia.
9. Discipline of Genetic Medicine, Children's Hospital at Westmead Clinical School, Sydney, Australia.
10. The Henry Wellcome Building for Molecular Physiology, University of Oxford, OX3 7BN Oxford, United Kingdom
11. Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, United Kingdom.
12. Murdoch Children's Research Institute, The Royal Children's Hospital, Flemington, Road, Parkville, Victoria, Australia
13. Department of Clinical Genetics, Oxford University Hospitals NHS Trust, OX3 9DU Oxford, United Kingdom.

Running Title: *PIGY* mutations associated with variable phenotypes

Corresponding Author:
Associate Professor Nigel Clarke
Institute for Neuroscience and Muscle Research
The Children's Hospital at Westmead
Locked Bag 4001, WESTMEAD, NSW 2145, Australia.
Email: nigel.clarke@health.nsw.gov.au
Tel: 61-2-9845 1453
Fax: 61-2-9845 3078

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Abstract

Objective: Glycosylphosphatidylinositol (GPI) anchored proteins are ubiquitously expressed in the human body and are important for various functions at the cell surface. Mutations in various GPI biosynthesis genes have been described to date in patients with multi-system disease and together these constitute a subtype of congenital disorders of glycosylation.
Methods: We used whole exome sequencing in two families to investigate the genetic basis of disease and studied mRNA levels and cell surface expression of GPI-anchored proteins on patient derived cells to investigate the functional consequences of mutations.
Results: Two families with different phenotypes had homozygous recessive mutations in the GPI biosynthesis gene *PIGY*. Two sisters with a c.137C>T (p.Leu46Pro) *PIGY* mutation had multi-system disease including dysmorphism, seizures, severe developmental delay, cataracts and early death. There were significantly reduced levels of GPI-anchored proteins (CD55 and CD59) on the surface of patient-derived skin fibroblasts (~50-80% compared to controls). In a second, consanguineous family two siblings had moderate development delay and microcephaly. A *PIGY* promoter variant (c.-540G>A) was detected within a 7.7 Mb region of autozygosity. This variant disrupted a SP1 consensus binding site and was shown to be associated with reduced gene expression.
Interpretation: Mutations in *PIGY* can occur in coding and non-coding regions of the gene and cause variable phenotypes. This paper contributes to understanding of the range of disease associated with deficiencies of the GPI-anchor biosynthesis pathway and also serves to highlight the potential importance of analysing variants detected in 5'-UTR regions despite their typically low coverage in exome data.

Introduction

Glycosylphosphatidylinositol (GPI) acts as a membrane anchor for many cell-surface proteins including receptors, enzymes and adhesion molecules. At least 25 genes are involved in the step-wise production of mature GPI-anchored proteins (GPI-APs)[1]. GPI biosynthesis is initiated in the endoplasmic reticulum from phosphatidylinositol (PI) in a reaction mediated by the GPI-N-acetylglucosaminyltransferase (GPI-GnT) complex. The GPI-GnT enzyme complex consists of seven proteins including PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, DPM2 and PIG-Y. Phosphatidylinositol glycan class Y (PIG-Y), the smallest subunit of this complex, is a 71 amino acid protein containing 2 putative transmembrane domains and it directly associates with the catalytic subunit PIG-A. In the absence of PIG-Y activity, the cell surface levels of GPI-anchored proteins are severely decreased[2].

The first disorders to be described in association with deficiencies of the GPI-anchor biosynthesis pathway were paroxysmal nocturnal haemoglobinuria (PNH), caused by somatic mutations in *PIGA*[3], in hemopoietic cells, and inherited GPI deficiency caused by a hypomorphic promoter mutation in *PIGM*[4]. Whole exome sequencing (WES) technology has facilitated the discovery of pathogenic mutations in several other GPI biosynthesis genes. Germline mutations in *PIGL*[5], *PIGM*[4], *PIGN*[6], *PIGO*[7], *PIGT*[8], *PIGV*[9], *PIGW*[10], *PIGQ*[11] and *PIGA*[12] have all been reported in a small number of families in association with multisystem disease. Common clinical features include moderate to severe developmental delay, seizures and dysmorphic facial features. Reduced levels of GPI-anchored proteins CD59, or membrane attack complex-inhibitory protein**,** and FLAER (fluorescently labelled inactive toxin aerolysin which binds to GPI- linked structures) at the cell surface have been described in many of these patients[13].

We have identified two different homozygous recessive mutations in *PIGY* in two separate families. In family A, we identified a c.137C>T mutation in two affected sisters presenting with a multi-system disease encompassing dysmorphism, developmental delay, seizures, cataracts and gastro-intestinal dysmotility. Levels of two GPI-anchored proteins on patient fibroblasts were reduced, providing evidence that these mutations impair GPI synthesis and are pathogenic. In Family B, we identified a

c.-540G>A mutation in the promoter region in a consensus SP1 site. The affected siblings from Family B have primary microcephaly, moderate developmental delay and are mildly affected in comparison to Family A. This report identifies *PIGY* as a new disease-causing gene with variable expressivity and increases our knowledge of the phenotypes that can arise from abnormal GPI biosynthesis.

Methods

Genetic analysis

This research was approved by the Human Research Ethics Committee of the Children's Hospital at Westmead, Australia (10/CHW/45) and the Wales Research Ethics Committee, UK (12/WA/0001). WES was performed on genomic DNA from two sisters and their parents (Family A) in collaboration with the Broad Institute using methods previously reported[14]. In Family B, SNP-array based autozygosity mapping was performed on all family members. WES of the proband (II-2) and data analysis were performed as previously described[15]. Segregation of both variants was confirmed by Sanger sequencing.

Cell culture and flow cytometry studies

All tissue culture media and reagents were purchased from Life Technologies unless otherwise stated. Skin fibroblast cells were grown in a Dulbecco's Modified Eagles Media (DMEM)/F12-HAMS supplemented with 10% foetal bovine serum (FBS) and gentamicin (50 μg/ml) and grown in 5% CO_2 incubator. To investigate levels of GPI-anchorage proteins, cultured skin fibroblasts derived from Patient II-1 in Family A (5 months), Patient II-2 in Family A (8 days) and three controls (ages 3.5 months, 5.5 months, 5.5 years) were treated with versene and incubated with allophycocyanin (APC)-conjugated mouse anti-human CD55 and phycoerythrin (PE)-conjugated mouse anti-human CD59 antibodies (BD Biosciences) in phosphate buffered saline (PBS) without calcium or magnesium and supplemented with 2% FBS for 30 minutes at 4°C. Cells were then centrifuged at 250 g for 5 minutes and washed twice in the above solution. Labelled cells were analysed using a CantoII flow cytometer (BD Biosciences) and analysed using FlowJo™ v 7.6.5 software. Experiments were performed in triplicate and results were averaged. Fibroblasts were not available from members of Family B and so whole blood samples were used instead. These were treated with ACK lysis buffer and stained with anti-human CD16 (Invitrogen); samples were run on a BD FACSCanto and data analysed by FlowJo. Granulocytes were identified according to FSc and SSc profile.

Functional analysis of mutant PIGY cDNA

Daudi cells (PIG-Y deficient human Burkitt's lymphoma cell line expressing EBNA1 antigen[2, 16] were permanently transfected with wild type or Leu46Pro mutant PIG-Y constructs containing Epstein–Barr virus oriP. For low level expression, *PIGY* cDNA was driven by minimum TATA box promoter in pTAL oriP puro PIGY. Cells (10^7) were suspended in 0.8ml of Opti-MEM and electroporated with 20 μg each of the plasmids at 350 V and 500 μF using a Gene Pulser (Bio Rad, Hercules, CA). After selection with 0.5mg/ml of puromycin for 2 weeks, restoration of the surface expression of GPI-anchored proteins was determined by staining cells with mouse anti-human CD59 (5H8), human CD55 antibodies and each isotype IgG followed by a PE-conjugated anti-mouse IgG antibody (mouse IgG1 and IgG2a, and secondary antibody were purchased from BD Biosciences) and analyzed by flow cytometer (Cant II) using Flowjo software (Tommy Digital Inc., Tokyo, Japan). For high level expression, PIG-Y deficient Daudi cells were transiently transfected with wild type or mutant (p.Leu46Pro) strong promoter driven pME-oriP HA-PIGY. Two days later, restoration of the surface expression of CD59 was assessed by flow cytometry. Lysates were applied to SDS-PAGE and western blotting was performed using anti-HA antibody (HA-7, Sigma, St. Louis MO). The levels of protein expression were normalized with the intensities of glyceraldehydephosphate dehydrogenase (GAPDH), the loading control, and luciferase activities used for evaluating transfection efficiencies.

GPI mannolipid analysis
Patient and control fibroblasts were transformed using telomerase gene and SV40 large T gene and metabolically radiolabeled with tritiated mannose in the presence of tunicamycin to enhance incorporation into GPI mannolipids by preventing incorporation into more abundant N-glycan precursors. Radiolabeled GPI mannolipids were treated with 0.1 N KOH to cleave ester linkages and then analyzed by thin layer chromatography on silica gel followed by phosphoimaging to detect tritiated GPI[17].

Gene expression analysis
RNA was extracted from 2.5ml of blood using the PAXgene kit (Qiagen) and RT reactions were performed using the QuantiTect reverse transcriptase kit (Qiagen). Relative allelic expression of *PIGY* was measured by analysing cDNA using both Sanger sequencing and a 314 chip run on the Ion Torrent PGM platform. As the c.-540G>A variant is only 4 bp from the transcription start site, expression levels had to be measured indirectly using the T allele at rs3177413 (c.-222C>T) which familial transmission showed was *in cis* with c.-540G>A. RT-PCR primer sequences are shown in Table 1. The 1st round of amplification (25-30 cycles) and 2nd round of amplification (15 cycles, with barcoding primers), were both performed with the FastStart Taq DNA polymerase kit (Roche). Samples were pooled, cleaned using Ampure beads, quantified using the BioAnalyser (Agilent) and diluted to 10pM. Emulsion PCR and sequencing were carried out according to manufacturer instructions.
Quantitative PCR was performed using IQ SYBR Green Supermix (BIO-RAD) and the iQ5 Real-Time PCR Detection System (BIO-RAD) and the following primers: PIGY-V5-F 5'-AGGGATGTTCATCTCCAACCA-3', PIGY-V5-R 5'-TGCGCATATCAGGCTTAGGA-3', RPL30-F 5'-CAGACAAGGCAAAGCGAAAT-3' and RPL30-R 5'-TGGACACCAGTTTTAGCCAAC-3'. PCRs were performed in triplicate and the experiment was carried out three times.

Results

Clinical descriptions
Family A
Two affected sisters were the only children of a non-consanguineous Caucasian Australian couple. II-1 was born at 32 weeks gestation and had a complicated course with necrotising enterocolitis and chronic lung disease. She was dysmorphic and had brachyphalangy (Figure 1), 2-3 toe syndactyly, flexion contractures and severe bilateral hip dysplasia. At 5 months of age she developed an intractable seizure disorder with multifocal spike and slow wave activity on EEG. This was followed by developmental regression and death at 2 years of age from a respiratory infection.

II- 2 had abnormal antenatal scans from 25 weeks gestation with enlarged echogenic kidneys and bowel and long-bone growth failure. Delivery was induced at 28 weeks gestation due to polyhydramnios. Similar dysmorphic features, brachyphalangy, proximal limb shortening, contractures and left hip dysplasia were present. She had bilateral inguinal hernias and bilateral dilatation of the renal collecting systems, with markedly increased echogenicity of the renal parenchyma. Intractable seizures commenced at 6 weeks of age, her development regressed and at 5 months she was mostly unresponsive with poor vision. She died at 7 months of age secondary to an aspiration.

In addition, both sisters had congenital cataracts, intermittent episodes of abdominal distress and vomiting, and growth failure. Head growth was normal. MRI brain was normal in both neonates, but showed loss of white matter and cerebral volume in II-1 at 9 months. No abnormalities were present on diffusion-weighted images. Skeletal survey showed osteopenia and brachytelephalangy. Creatine kinase (CK) measurements were persistently elevated in both siblings (554-3640). Rectus abdominus

muscle biopsy taken at 9 months (II-1) and 8 days (II-2) showed moderate variation in fibre size with many small, rounded atrophic fibres, increased fibrosis and adipose tissue. No degenerating or regenerating fibres were seen, but in II-2, acid phosphatase staining was increased suggestive of active degeneration.

Alkaline phosphatase levels were elevated in both patients (443-853u/L). Extensive metabolic work up in both patients was otherwise non-contributory. Erythrocyte plasmalogen levels were mildly elevated in II-2 and the phytanic acid level was normal.

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Family B

Two siblings, born to consanguineous parents of Pakistani descent were referred with global developmental delay and microcephaly. II-2 was born following an uncomplicated pregnancy and delivery, but had microcephaly at 6 weeks of age. At 5 years this was 46 cms (-3 to -4SD). His developmental milestones were delayed. He sat with support at 15 months and walked at 3 years of age. His speech was delayed with short 2-3 word sentences at the age of 5 years. He was short-sighted with a strabismus and had behavioural difficulties with aggressive outbursts.

II-3 was born following an uncomplicated pregnancy and delivery. She had microcephaly noted at 2 weeks of age and at 2 years had a head circumference of 42.1cm (-4 to -5SD). Her development was delayed. She sat with support at 18 months and was not walking at the age of 3 years. Her speech was better than her brother's with 10-15 single words at age 3 years. She had poor concentration and was hyperactive.

The parental head circumferences were within the normal range. Neither child had seizures. There was mild facial dysmorphism with long palpebral fissures, a bulbous tip to the nose and a wide mouth. Brachytelephalangy was not present.

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Genetic analysis

WES of DNA from II-1 and II-2 from Family A identified a homozygous missense variant c.137T>C (p.Leu46Pro) in the coding region of the *PIGY* gene (NM_001042616.1). Sanger sequencing confirmed variant segregation was consistent with a recessive inheritance pattern (Figure 1c). *In silico* analysis predicted the variant to be pathogenic (*PolyPhen-2 score of 1.0*)[18] and the variant was not present in the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/ accessed April 2014) or 1000 genomes[19, 20].

For Family B, an autozygosity mapping approach identified candidate regions on 4q22.1-q22.3 and 10p14-pter, both 7.7 Mb in size. WES was performed for the proband (II-2) and the resulting variants were intersected with these two candidate loci. This revealed a c.-540G>A variant in a conserved region of the *PIGY* 5'-UTR. This variant is not in either of the publically available databases mentioned above and was not detected in 274 in-house genomes of mixed ancestry (see www.well.ox.ac.uk/wgs500) or 108 Punjabi individuals from Lahore. Sanger sequencing confirmed segregation consistent with autosomal recessive inheritance (Figure 1c). Analysis of the exome outside of the homozygous region revealed no likely causative coding variants.

Patients with p.Leu46Pro substitution in PIG-Y have reduced cell expression of GPI–anchored proteins CD55 and CD59

Given the known function of PIG-Y, we investigated the effects of the p.Leu46Pro substitution on the surface expression of two GPI-anchored proteins, CD55 and CD59. Skin fibroblasts derived from both affected sisters had a 50-80% reduction of cell surface expression of CD55 and CD59 compared to 3 different controls (Figure 2a) [For CD55-APC, Patient 1 p=0.0055 (mean MFI=2809 +/- 521), Patient 2 p=0.0449 (mean MFI=2393+/-582) (pooled controls mean MFI= 3042 +/- 485), for CD59-PE, Patient 1 p=0.0165 (mean MFI= 2422+/-921), Patient 2 p=0.0164 (Mean MFI= 1927 +/- 582) (Pooled control mean MFI= 3988+/- 485) using a standard Students' T-test]. This data suggest that the

p.Leu46Pro substitution disrupts GPI biosynthesis or interferes with GPI anchoring capacity, associated with reduced expression of CD55 and CD59 on patient cells. –Skin fibroblasts were not available from Family B.

Transfection of p.Leu46Pro PIG-Y partially rescues PIG-Y deficient cell line.
We permanently transfected Daudi cells (PIG-Y-deficient human Burkitt's lymphoma cell line) with wild-type (WT) or p.Leu46Pro mutant vector constructs driven by the weak promoter followed by assessment of cell surface expression of CD55 and CD59 by FACS analysis. WT-PIG-Y transfection restored the cell surface expression of both CD55 and CD59, but the p.Leu46Pro mutant PIG-Y did not (Figure 3a). Levels of their expression by the mutant PIGY were similar to the empty vector control. To test if the lack of activity is due to the instability of the mutant PIG-Y protein, stronger expression was needed for detection by western blotting. For this, Daudi cells were transiently transfected with wild type or mutant (p.Leu46Pro) pME-oriP HA-PIGY. With this strong promoter driven vector the mutant *PIGY* restored the surface expression of CD59 similarly to WT *PIGY*, indicating that the mutant PIG-Y has some activity (Figure 3b). As demonstrated by western blotting, the mutant protein expression was significantly decreased compared to WT-PIGY (Figure 3b). This data suggests that the p.Leu46Pro mutation reduces PIG-Y protein stability leading to lower protein levels and a reduced capacity to synthesize GPI anchors for correct protein targeting to the cell surface.

Patients with p.Leu46Pro substitution in PIG-Y have normal lipid remodeling
Radiolabelling of tritiated mannose into tunicamycin-treated transformed patient fibroblast cells was performed to investigate whether GPI protein anchors in Family A were abnormal in their structure as well as their quantity, as occurs in peroxisomal disorders. GPI precursors in fibroblasts derived from Patients 1 and 2 (Family A) had KOH-resistant alkyl bonds similar to that observed in similarly transformed control fibroblasts (Figure 3c, bands in KOH lanes). These data suggest that patient cells harbouring the p.Leu46Pro *PIGY* mutation can remodel GPI lipid to the alkyl-acyl form.

Effect of c.-540G>A variant on gene expression and GPI anchor biosynthesis
As the c.-540G>A variant lies in a consensus SP1 transcription factor binding site (GG**G**GCGGGGC > GG**A**GCGGGGC) in which there are no other variants reported in dbSNP138, we hypothesised that it might influence gene expression. Sanger sequencing of genomic DNA confirmed that the father (I-1) and the unaffected sibling (II-1) were heterozygous for the A:T haplotype (c.-540G>A:rs3177413) and so could be used to directly compare the relative allelic expression. RT-PCR and Sanger sequencing of two independent blood samples obtained from each of these individuals indicated that expression was consistently from the wild-type allele (data not shown). However Sanger sequencing does not have a high sensitivity for variant detection when one allele is present at low levels[21]. Therefore to better quantify the relative expression, sequencing was repeated using the Ion Torrent PGM and this showed that only 7-11% of transcripts originate from the mutant allele (Table 1). These results were consistent with qPCR which showed that for both affected individuals (II-2 and II-3), the *PIGY* expression was at 6-10% compared to an unrelated control (Figure 4), whereas for the three heterozygote carriers in family B, expression levels were intermediate. Despite this decrease in the *PIGY* transcript levels, we could detect no significant reduction in granulocyte CD16 surface expression for the two individuals homozygous for the c.-540G>A mutation (data not shown).

Discussion
We describe two families with homozygous recessive mutations in *PIGY*, establishing *PIGY* as a new disease-causing gene in humans. Two affected sisters from Family A, who were homozygous for a missense mutation in *PIGY (*p.Leu46Pro*),* died from severe multi-system disease while two affected siblings from Family B, homozygous for a mutation in the promoter region of *PIGY (*c.-540G>A), presented with moderate developmental delay and microcephaly. The p.Leu46Pro substitution involves a conserved residue in the second putative transmembrane domain[2]. Amino acids in this domain and the carboxy-terminal cytoplasmic region are relatively more conserved among species and may be important for interaction with PIG-A, a catalytic component of GPI-GnT. The substitution of Leu to less hydrophobic Pro might affect association with PIG-A, thereby causing instability of

PIG-Y protein (Fig. 3b). Fibroblasts derived from both affected sisters from Family A show a 50-80% reduction in protein expression of CD55 and CD59 at the cell surface by flow cytometric assessment, consistent with a defect in GPI-anchor biosynthesis in these patients. Using a cell culture model we show that expression of mutant p.Leu46Pro PIG-Y in the PIG-Y deficient Daudi cell line only partially restores the cell surface expression of the GPI protein markers CD55 and CD59. Taken together, these results provide strong evidence that the p.Leu46Pro substitution leads to defects in GPI anchored proteins in patient cells.

Family A had multisystem disease consistent with previously described disorders of GPI anchor biosynthesis, whilst Family B had less severe disease limited to microcephaly and global developmental delay. This is likely due to differences in the mutations between the two families and their effects on PIG-Y protein function. We have shown that the promoter mutation in Family B markedly reduces *PIGY* transcript levels in blood, which likely leads to reduced expression of functionally normal PIG-Y protein. We hypothesise that the level of PIG-Y expression in most tissues in Family B is sufficient for health, explaining why Family B's phenotype is more limited. The effects of the SP1-binding site mutation on *PIGY* transcript levels may also vary between tissues. Indeed, initial experiments showed no difference in levels of CD16, a GPI-anchored protein, in granulocytes from affected individuals from Family B, suggesting that PIG-Y levels are sufficient for normal GPI biosynthesis in that tissue. In contrast, the mutation in Family A will lead to expression of the Leu46Pro mutant protein in all tissues, which likely accounts for the severe, multi-system involvement.

There is growing recognition that autosomal recessive disorders of GPI biosynthesis are an important subclass of the congenital disorders of glycosylation. Over 150 proteins require GPI anchorage for cell-surface expression and all cell types in the body employ this anchoring mechanism[1]. Previously published defects of GPI anchor biosynthesis present in infancy with multi-system disease, likely due to the wide range of biological processes affected by the reduced expression of GPI-anchored proteins. The findings in our patients help to understand the range of phenotypes that can arise (Table 2). Common dysmorphic features include bi-temporal narrowing, anteverted nares, depressed nasal bridge, long philtrum, high palate and a tented upper lip. The ears are commonly large with fleshy lobes and overfolded helices. Fifth finger clinodactyly and nail hypoplasia are commonly described. Seizures, moderate to severe developmental delay, growth failure and cerebral visual impairment are commonly described. Other common features include genitourinary defects, gastrointestinal dysfunction and cardiac defects (Table 1). The sisters from Family A share many features with other inherited GPI anchor defects but are the first to be described with cataracts. Recently, it has been reported that pyridoxine may be effective in patients with mutations in PIGO[22] and butyrate may be effective in patients with PIGM[23], raising important therapeutic considerations.

The two severely affected patients (Family A) in this study share many clinical features in common with rhizomelic chondrodysplasia punctata type 1 (RCDP1), a peroxisomal disorder which also presents with proximal shortening of the long bones, growth retardation, cataracts, severe intellectual disability, seizures and early death. However the absence of punctate calcification in our patients is inconsistent with this disorder. Plasmalogens were mildly elevated, phytanic acid was normal and DHAP-AT and DHAP-S activity on cultured skin fibroblasts was normal. Lipid remodelling of GPI anchors occurs in a later part of the GPI biosynthetic pathway and is dependent on the alkyl-phospholipid biosynthetic pathway in the peroxisome. Kanzawa et al showed that patients with mutations in genes involved in peroxisomal biogenesis had defective GPI lipid remodelling and were unable to synthesise 1-alkyl-2-acyl GPIs[24]. Unlike patients with RCDP1 and Zellweger syndrome, patients with p.Leu46Pro substitution in PIGY do not have defective GPI lipid remodelling and are able synthesise alkyl-acyl GPI forms. Inherited GPI deficiencies are an important differential for peroxisomal disorders and GPI lipid remodelling might be used as a tool to differentiate between these two clinically similar disorders.

In Family B, even though we could restrict the search space to <1% of the genome based on autozygosity, initial filtering of the 217,641 variants detected by WES did not yield any likely causative variants. It was only after extending our definition of "deleterious" to include 5'-UTR variants that lie in regions annotated by the ENCODE consortium (ref PMID: 22955616) as transcription factor binding sites, that we uncovered the c.-540G>A variant. UTR regions were not targeted by first-generation exome capture kits and even when capture probes are present in these regions, sequence coverage is often low due to high GC content. Variants that are detected in these regions are difficult to interpret. Our detection of a functional homozygous 5'-UTR variant, despite only having 4 good quality reads, thus represents an unanticipated finding that highlights the importance of developing better tools to analyse the non-coding portion of the genome.

In conclusion, we describe the first report of two different homozygous autosomal recessive mutations in the GPI biosynthesis gene *PIGY* that are associated with different phenotypes. The p.Leu46Pro mutation in the coding region of the *PIGY* causes a severe congenital, multi-system disorder resulting in early death, whilst the promoter mutation identified in Family B results in a moderate CNS phenotype commensurate with life. This study contributes to understanding of the phenotype of disorders associated with deficiencies of the GPI-anchor glycosylation pathway, an important differential diagnosis in infants with multisystem disease, epileptic encephalopathy, typical dysmorphic facial features, brachytelephalangy and hyperphosphatasia.

Figure Legends

Figure 1
(a) Clinical features of patients with mutations in PIGY.
Family A, Patient II-1 had proximal limb shortening, left hip dysplasia, and contractures were present at the elbows, hips and knees (i). Dysmorphic features included bitemporal narrowing, upturned nares, depressed nasal bridge, deep-set eyes, a short neck (ii) and ears with thickened helices and fleshy earlobes (iii). Brachyphalange, 5th finger clinodactyly and adducted thumbs were present (v). Brachytelephalange was present on X-ray (iv). Family B Patient II-2 (v) and Patient II-3 (vi) had a milder phenotype, with soft dysmorphic features including long palpebral fissures, a bulbous tip to the nose and a wide mouth. Patient II-2 had a strabismus.
(b) Pedigrees of Families A and B. Shading indicates affected individuals. The probands are indicated with an arrow. Proven heterozygote carriers are shown by a dot. Sanger sequencing validation from Family A confirms the homozygous missense variant c.137T>C (p.Leu46Pro) in the *PIGY* gene (NM_001042616.1) in the two affected sisters. Region shown corresponds to chr4: 89,442,795-813 (hg19) For Family B, Sanger validation and segregation testing of the *PIGY* promoter variant c.-540G>A is shown. The consensus SP1 binding motif is indicated with a black rectangle. Region shown corresponds to chr4: 89,444,938-958.

Figure 2
(a) Analysis of patient tissue by flow cytometric analysis. Cultured skin fibroblasts from Patients II-1 and II-2 from Family A were labelled with anti-Human CD55-APC and anti-Human CD59-PE and analysed by flow cytometry. **(a)** Histograms showing the mean fluorescent intensity (MFI) are depicted for Patient II-1 and show a reduction in MFI for CD55-APC and CD59-PE (red) compared with control (blue). **(b)** Graphical representation of this data shows a statistically significant reduction in MFI for these GPI markers. Skin fibroblast biopsies were taken from Patient II-1 and Patient II-2 at 5 months and 8 days respectively. Primary human fibroblast controls were used from three different individuals at ages 5.5 years, 6 months and 3.5 months.

Figure 3
(a) Daudi cells (a PIG-Y deficient human Burkitt's lymphoma cell line) were transiently transfected with normal (WT) or mutant (Leu46Pro) driven by minimum TATA box promoter in pTAL oriP puro

PIGY (weak promoter). Restoration of the surface expression of CD59 and CD55 was assessed 2 days later by flow cytometry and showed that expression of WT-PIGY restored the GPI surface expression of CD55 and CD59 but p.Leu46Pro mutant PIG-Y did not. **(b)** After transient transfection of Daudi cells with normal (WT) or mutant (Leu46Pro) *PIGY* driven by the strong promoter pME-oriP HA-PIGY, restoration of GPI surface expression was achieved in by WT and p.Leu46Pro constructs, indicating that the mutant has some residual activity (left panel). Cell lysates prepared from Daudi cells transfected with either WT-PIGY, mutant p.Leu46Pro *PIGY*, and empty vector were separated by SDS-PAGE and probed for anti-HA and anti-GAPDH antibodies. Normalisation of the anti-HA signal compared to GAPDH showed a marked reduction in protein expression in the mutant (p.Leu46Pro) compared to WT. **(c)** In vivo labelling of ^{3}H-mannose into transformed fibroblast cells derived from Patients II-1 and II-2 (Family A) was performed to enhance incorporation into GPI mannolipids. GPI precursors in fibroblasts derived from Patients 1 and 2 (Family A) had KOH-resistant alkyl bonds similar to that observed in fibroblasts similarly transformed (Figure 3c, bands in KOH lanes).

Figure 4
Expression analysis in family B using qPCR. Expression analysis was performed using RPL30 as a control gene and results are normalised with respect to the first control individual. qPCRs were performed in triplicate and then the experiment was repeated three times. Error bars represent the standard deviations obtained across the three runs.

Appendix

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Potential Conflicts of Interest
There is no potential conflict of interest.

Authorship Contributions

Sydney, Australia Group
The Sydney Group lead by **Nigel Clarke** (senior author) identified the gene and lead the collaborative studies to ascribe pathogenicity. **Biljana Ilkovski's** (first author) contribution has been to perform the flow cytometric experiments on the patient cells (Figure 2), assemble the figures and take a lead role in the preparation of the manuscript. **Gina O'Grady** (author position #2) identified the gene via WES, confirmed the mutation and family inheritance, reviewed and assembled detailed clinical and pathological information, compiled a detailed clinical table of all known PIG phenotypes, helped write and edit the manuscript. **Leigh Waddell** was involved in mutation identification and confirmed the mutation by Sanger Sequencing and edited the manuscript. **Fabienne Brilot**, helped direct flow cytometeric experiments and edit the manuscript. **David Sillence** and **Anne Turner** were the clinicians who described the clinical phenotypes of the patients and edited the manuscript. **Kathryn North and John Christodoulou.**

UK Group

The Oxford Group lead by **Usha Kini** and **Jenny Taylor** identified the gene in the UK family. **Alistair Pagnamenta** performed SNP array experiments, homozygosity mapping & CNV analysis, submitted exome samples (carried out at the Wellcome Trust in Oxford) and analysed exome data, did RNA extractions, designed and performed RNA sequencing (both Sanger and then high coverage PGM to confirm the results). Alistair also travelled to collect the samples from the family, was involved in the flow cytometry experiments (performed twice) and helped write and edit the manuscript. **Malcolm Howard** performed the qPCR and Sanger validation of the UK Family mutation and analysed exome data.
Samantha Knight
Consuelo Anzilotti
Anne Goriely

Boston, USA Group
The Boston Group, lead by **Daniel MacArthur** identified the gene in the Australian family via WES. **Monkol Lek** and **Brett Thomas** performed the Bioinformatics of WES to ascribe pathogenicity. All authors edited the manuscript.

Japanese Group
The Japanese Group lead by **Taroh Kinoshita**, lead the collaborative studies to examine the functional consequences of the PIGY mutation in the Australian family using cell culture models lipid remodeling studies, helped write and edit the manuscript. **Yoshiko Murakami** and **Noriyuki Kanzawa** were responsible for designing the flow cytometric experiments examining expression of PIG-Y constructs into PIG-Y deficient cells as well as the lipid remodelling studies and, helped write and edit the manuscript.

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