



PIGF deficiency causes a phenotype overlapping with DOORS syndrome

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Abstract

DOORS syndrome is characterized by deafness, onychodystrophy, osteodystrophy, intellectual disability, and seizures. In this study, we report two unrelated individuals with DOORS syndrome without deafness. Exome sequencing revealed a homozygous missense variant in *PIGF* (NM_173074.3:c.515C>G, p.Pro172Arg) in both. We demonstrate impaired glycosylphosphatidylinositol (GPI) biosynthesis through flow cytometry analysis. We thus describe the causal role of a novel disease gene, *PIGF*, in DOORS syndrome and highlight the overlap between this condition and GPI deficiency disorders. For each gene implicated in DOORS syndrome and/or inherited GPI deficiencies, there is considerable clinical variability so a high index of suspicion is warranted even though not all features are noted.

Introduction

More than 150 proteins are anchored to the cell membrane through GPI. The synthesis of GPI from phosphatidylinositol involves several reactions, and GPI is then attached to nascent proteins. Phosphatidylinositol glycan anchor biosynthesis class F (MIM# 600153) (*PIGF*) encodes a protein

that is involved in the GPI biosynthesis (Inoue et al. 1993). Many variants have been identified in genes that function in the GPI biosynthesis leading to inherited GPI deficiencies (IGDs). The main characteristic features of IGDs are global developmental delay, intellectual disability, seizures, hypotonia, and facial dysmorphisms. Another rare multiple congenital anomaly syndrome with features overlapping with IGDs is DOORS syndrome which is characterized by deafness, onychodystrophy, osteodystrophy, intellectual disability (previously called mental Retardation), and seizures. We have previously reported the causal link between DOORS syndrome and *TBCID24* variants in half of the recruited patients (Campeau et al. 2014), and have reported individuals with phenotype overlapping DOORS syndrome who had IGDs (Alessandri et al. 2018; Murakami et al. 2019).

With a constant aim to identify the genetic basis of DOORS syndrome, here we report *PIGF* variants in two individuals with DOORS syndrome from unrelated families of Indian origin from the same geographic location. We have identified a biallelic variant in *PIGF*, which encodes a protein involved in GPI-anchor biosynthesis, shared by both the individuals. Additionally, we have shown a defective GPI biosynthesis due to variants in *PIGF*, further highlighting its importance in DOORS syndrome (Murakami et al. 2019).

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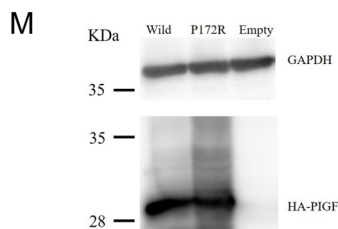
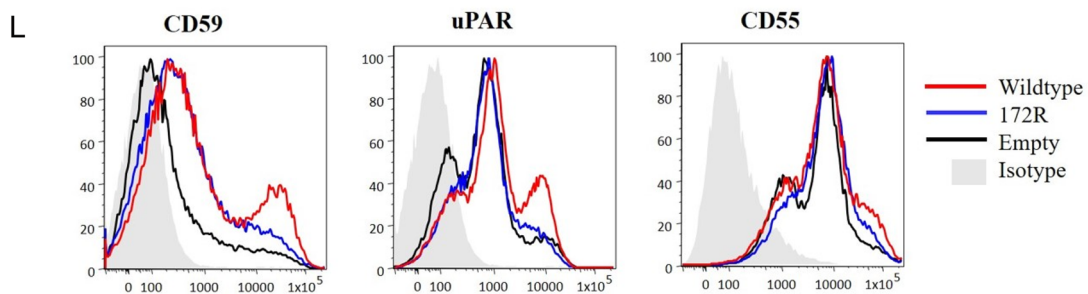
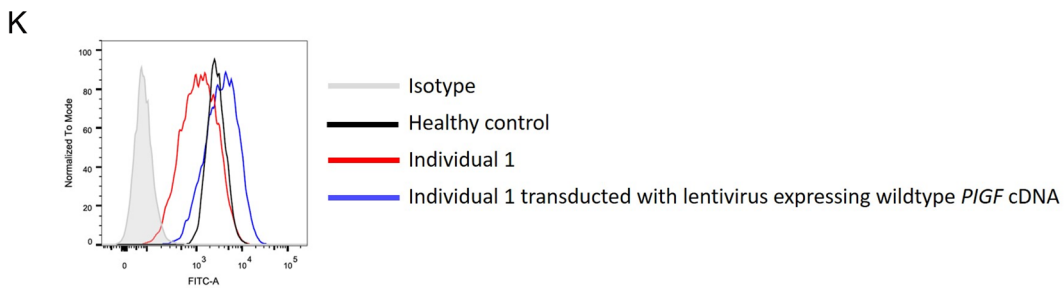
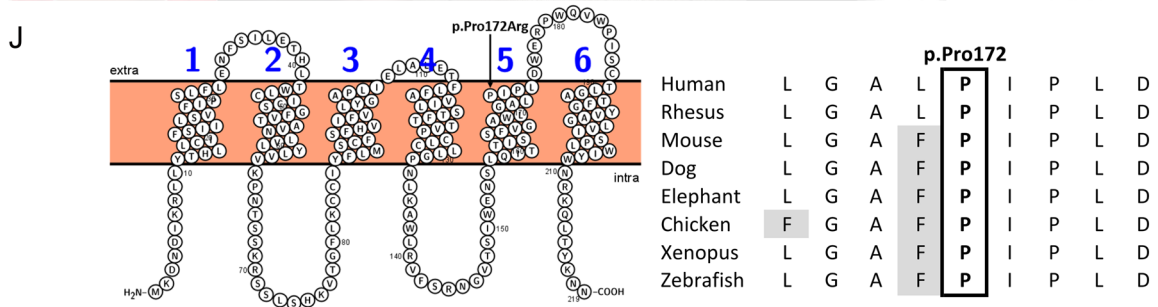
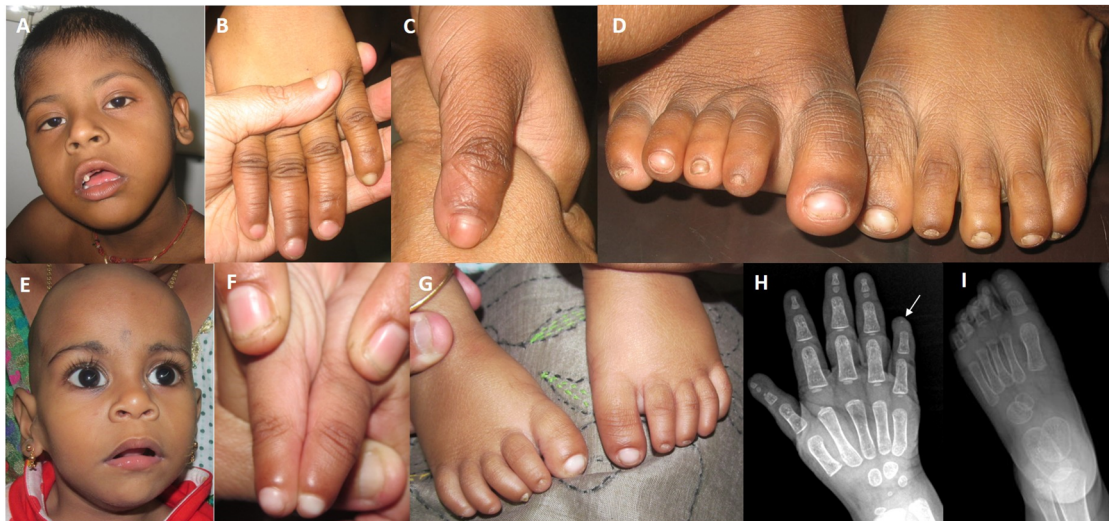


Fig. 1 Individual 1 (**a**) and 2 (**e**) have full lips and anteverted ear lobes, as well as hypoplastic fingers nails (**b**, **c** and **f**) and toes nail (**d** and **g**). **h–i** Radiographs of individual 2 show a triphalangeal thumb and brachytelephalangy (indicated by the arrow). **j**. Schematic representation of PIGF protein, made with Protter. The affected amino acid lies in a transmembrane domain, after an alpha-helix. **k** Flow cytometry analysis of fibroblasts derived from individual 1 compared to healthy control. The individual cells were further transduced with lentiviruses expressing a wildtype *PIGF* cDNA. CD73 antibody was stained for GPI-AP. **l** Functional analysis of mutant *PIGF* cDNA (P172R). Flow cytometry analysis of GPI-APs in *PIGF*-deficient CHO cells transiently transfected with wildtype and mutant HA-tagged *PIGF* cDNAs subcloned in pTK vector. Note with the wildtype protein (red) the higher peak on the right. **m** Cell lysates from the transfected cells were used for Western blot analysis using the HA-tag antibody, thus confirming proper cDNA expression

Individuals and methods

Individuals and exome sequencing, including ethics statement

This study was approved by the CHU Sainte-Justine institutional review board. Samples were collected from individuals and families after taking appropriate, written, informed consent. Detailed clinical presentations, magnetic resonance imaging (MRI) findings and family history were recorded. Trio exome sequencing and data analysis were carried out as described previously (Murakami et al. 2019).

Rescue assays of GPI-APs on fibroblasts

Lentiviruses generated using a pLenti-GIII-CMV plasmid carrying a wildtype (WT) *PIGF* (ABM, Richmond, BC, Canada) were produced in HEK293T cells with the presence of packaging plasmids pMD2.G and psPAX2 (Addgene), then were transduced into the individual fibroblasts and selected by Puromycin resistance. These cells—untransduced cells and control cells—were cultured and harvested at 80–90% confluency, stained with FITC-conjugated mouse anti-human CD73 (BioLegend) for 1 h on ice in the incubation buffer containing 0.5% BSA, then fixed in 3.7% formaldehyde. The nonspecific binding was washed off before analyzing in a BD FACSCanto II system (BD Biosciences) followed by FlowJo software analysis. CD73 was reduced to 50% versus healthy controls.

Western blot for CD73

For Western blot analysis, protein lysate from patient fibroblast, transduced fibroblast with a lentivirus expressing wildtype *PIGF* and control fibroblast were separated by electrophoresis on Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad, catalog #4569033), transferred to polyvinylidene difluoride membranes. The membrane was blocked with

5% milk and incubated with primary antibody, mouse anti-CD73 monoclonal antibody (1:500; MyBioSource, catalog #MBS668166) and mouse anti-GAPDH monoclonal antibody (1:5000; Santa Cruz Biotechnology and catalog #SC-47724). Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000; Cell Signaling Technology, catalog #7076) was used as secondary antibody. Clarity Western-enhanced chemiluminescence (ECL) substrate (BIO-RAD, catalog #361059) was used to detect the immunocomplexes.

Functional analysis in knockout cells

A construct encoding HA-tagged human *PIGF* cDNA and its mutant were subcloned into the thymidine-kinase-promoter-driven pTK vector. These constructs were transfected into *PIGF*-deficient CHO cells by electroporation. Co-transfection with a luciferase-expressing plasmid was used to monitor transfection efficiency. Restoration of GPI-APs was analyzed by FACS, staining cells with anti-CD59 and anti-CD55, anti-uPAR antibody. Levels of expressed wildtype and mutant HA-tagged *PIGF* in the transfected cells with the strong promoter-driven pME vector were analyzed by Western blotting using an anti-HA antibody (MBL) and glyceraldehyde 3-phosphate dehydrogenase GAPDH for the loading controls. We also measured luciferase activity and confirmed comparable transfection efficiencies (data not shown).

Results

Phenotypic details (Fig. 1a–d and e–i) of both individuals are summarized in Table 1. Individual 1 has been previously described as individual 23 in our initial DOORS syndrome study (Campeau et al. 2014). In summary, individual 1 is a 14-year-old male child born to consanguineous parents. His sibling with a similar phenotype died at 1 year 9 months. On examination, his height was 149 cm (4th %ile), his weight was 45 kg (25th %ile) and head circumference was 55 cm (50th %ile). Developmental delay, severe intellectual disability, hypoplastic fingernails, toenails and terminal phalanges, delayed permanent dentition, small teeth, anteverted nares, low-set pinna, open mouth, and atrial septum defect were noted. The individual had generalized tonic–clonic seizures. He has refractory seizures which occur on a daily basis, and is currently treated with three antiepileptics (phenobarbitone, levetiracetam, and clobazam). He has no effective speech, cannot express any of his needs and has no toilet control. He displays head banging and repetitive nonpurposeful rocking movements of head. He also has precocious puberty (sexual maturity rating stage 4, testicular volume of 20 ml bilaterally). Alkaline phosphatase, sometimes decreased or increased in GPI biosynthesis defects, was normal.

Table 1 Summary of clinical, radiological, and biochemical findings in the affected individuals

	Patient 1	Patient 2
Age	14 years	13 months
Gender	Male	Female
Height (SD)	149 cm (4th centile)	74 cm (40th centile)
Weight (SD)	45 kg (25th centile)	7.25 kg (– 2.83 SD)
Head circumference (SD)	55 cm (50th centile)	41.5 cm (– 3.2 SD)
Consanguinity	Yes	Yes
Abnormal fingernails and toenails	Yes	Yes
Triphalangeal thumb	No	Yes
Developmental delay or intellectual disability	Yes	Yes
Seizure	GTCS	None noted, but tonic posturing present
Deafness	No	No
MRI brain	Normal	Normal
Serum alkaline phosphatase level	295 IU/L (normal)	210 IU/L (normal)

MRI magnetic resonance imaging, *GTCS* generalized tonic–clonic seizure, *NA* not available

Individual 2 is a 13-month-old female child, the second child of third-degree consanguineous parents. There was no significant family history. She had a birth weight of 2.9 kg (– 1 SD). Tonic posturing was noticed since birth, but no clear seizures. She has partial head control, does not reach for object nor does she look at faces. On examination, her weight was 7.25 kg (– 2.83 SD), length was 74 cm (– 0.3 SD), head circumference was 41.5 cm (– 3.2 SD). Her anterior fontanelle was closed. Microstomia, micrognathia, coarse face, and short nose were noted. She had hypoplastic convex nails on the thumb and the 5th digit, bilateral triphalangeal thumbs, hypoplastic toenails from 1st to 3rd digit, a complete absence of toenails on 4th and 5th digit. Brainstem evoked response audiometry (BERA), MRI of brain and urine organic acids by gas chromatography–mass spectrometry (GC–MS) were normal. Alkaline phosphatase was normal.

Exome sequencing revealed a homozygous c.515C>G (p.Pro172Arg) missense variant in a predicted transmembrane domain of *PIGF* (NM_173074.3) in both (see Fig. 1j). The variant was validated by Sanger sequencing in individuals and parents were found to be heterozygous carriers thus confirming a recessive mode of inheritance. *PIGF* has never been associated with a genetic disease. This variant was not found in the gnomAD database nor in the GenomeAsia 100k database, lies at a highly conserved position and is predicted to be deleterious by multiple in silico prediction tools (CADD score of 27.6). Changing a proline for an arginine may affect the structure of *PIGF* given the conformation rigidity provided to protein backbones by proline. Membrane protein topology analysis using the SCAMPI tool on the TOPCONS server predicted that the mutation could affect which amino acids

are incorporated in the membrane (Bernsel et al. 2008; Tsirigos et al. 2015).

To analyze the effect of the variant on the GPI-anchor protein (GPI-AP) biosynthesis, we performed flow cytometry for GPI-AP CD73 on fibroblast and rescue assay was also performed to demonstrate *PIGF* specificity. Transduction with a lentivirus expressing wildtype *PIGF* totally restored CD73 expression in the individual cells (Fig. 1k). Western blot analysis of these cells suggests CD73 which does not get GPI-anchored becomes degraded (supplementary Fig. 1). Furthermore, functional analysis using *PIGF*-deficient CHO cells demonstrated that the mutant had low-residual activity compared to wildtype (Fig. 1l and m) while maintaining similar protein levels, thus suggesting an effect on the activity of the complex rather than an effect on *PIGF* protein stability.

Discussion

Here, we describe two independent families where affected children had onychodystrophy, osteodystrophy, developmental delay and seizures associated with a homozygous c.515C>G variant in *PIGF*. Functional analysis by flow cytometry and rescue experiments highlight the importance of *PIGF* in GPI-anchor biosynthesis. Although there is a phenotypic overlap between DOORS syndrome (usually caused by *TBC1D24* variants) (Campeau et al. 2014) and inherited GPI deficiency, as can be illustrated by GPI biosynthesis variants in our initial DOORS syndrome cohort (see Supplementary Table 1) and the literature (Alessandri et al. 2018; Fleming et al. 2016; Murakami et al. 2019), a shared link between the underlying

molecular pathways (TBC1D24 vesicular transport regulation and GPI biosynthesis) has yet to be determined. While 35% of individuals with bi-allelic *TBC1D24* have deafness (Balestrini et al. 2016), only 14% of individuals with GPI biosynthesis defects do (Bellai-Dussault et al. 2019), which may help understand why deafness was not noted in the patients described here.

A *PIGF* partial deletion was previously observed in a child with “short stature with microcephaly and distinctive facies, OMIM #615789”. However, the deletion was heterozygous in the child as it was in the unaffected father, while there were bi-allelic variants in *CRIP1* in the child. Therefore, the article rightly concluded that mutations in *CRIP1* was responsible for the phenotype. Moreover, heterozygous loss-of-function carriers occur at an expected rate in the gnomAD population database (pLI score of 0), thus supporting the fact that haploinsufficiency for this allele is not deleterious (Karczewski et al. 2020).

Regarding the precise role of *PIGF*, a previous report has shown that disruption of Pig-o (phosphatidylinositol glycan anchor biosynthesis class O) in F9 embryonal carcinoma cells resulted in the accumulation of a metabolite which was a GPI intermediate (Hong et al. 2000). The intermediate lacked phosphoethanolamine (EtNP) on the third mannose. Additionally, on the endoplasmic reticulum membrane (ER), *PIGO* and *PIGF* formed a complex and both proteins complemented the stability of each other. It was thus concluded that *PIGF* and *PIGO* function together in transferring EtNP to the third mannose in the GPI backbone. *PIGO* which forms a complex with *PIGF* degrades rapidly in the absence of *PIGF* (Shishioh et al. 2005). *PIGF* is also crucial for the stable expression *PIGG* (Hong et al. 2000). Furthermore, another study demonstrates that *PIGF* has functions beyond a structural role (Stokes et al. 2014). In the present study, analysis of the effect of variant c.515C>G (p.Pro172Arg), on GPI-AP biosynthesis showed reduced levels of GPI-AP on the cell surface compared to control further suggesting its effect in GPI biosynthesis efficiency. Interesting future experiment would include studying the effect of the mutation on the stability and localization of *PIGG* and *PIGO*, and their interaction with *PIGF*.

With regard to the pathophysiology of the neurodevelopmental disease with epilepsy, this can be partly explained by the fact that several GPI-anchored proteins play key roles notably in embryogenesis, neurogenesis, and synaptic specification (Lander et al. 1996; Martinelli and Fan 2007; Um and Ko 2017).

In conclusion, the phenotypic overlap between the two individuals and with inherited GPI deficiency disorders, in silico functional analysis and flow cytometer analysis, strongly suggest the pathogenicity of the *PIGF* variants identified in this DOORS syndrome cohort.

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Availability of data and material Yes.

Code availability Not applicable.

Compliance with ethical standards

Conflict of interest We declare that the authors do not have any conflict of interest and all have read and approved the final manuscript.

Ethics approval This study was approved by the CHU Sainte-Justine institutional review board.

Consent to participate Written informed consent was taken.

Consent for publication Consent for publication has been obtained.

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