SHORT REPORT

Glycosylphosphatidylinositol (GPI) anchor deficiency caused by mutations in *PIGW* is associated with West syndrome and hyperphosphatasia with mental retardation syndrome

Tomohiro Chiyonobu,[1] Norimitsu Inoue,[2] Masafumi Morimoto,[1] Taroh Kinoshita,[3,4] Yoshiko Murakami[3,4]

[1]Department of Pediatrics, Kyoto Prefectural University of Medicine, Kamigyo, Kyoto, Japan
[2]Department of Molecular Genetics, Osaka Medical Center for Cancer, Osaka, Japan
[3]Department of Immunoregulation, Research Institute for Microbial Diseases, Suita, Osaka, Japan
[4]Laboratory of Immunoglycobiology, WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka, Japan

Correspondence to
Dr Yoshiko Murakami, Department of Immunoregulation Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; yoshiko@biken.osaka-u.ac.jp

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ABSTRACT

Background Glycosylphosphatidylinositol (GPI) is a glycolipid that anchors 150 or more kinds of proteins to the human cell surface. There are at least 26 genes involved in the biosynthesis and remodelling of GPI anchored proteins (GPI-APs). Recently, inherited GPI deficiencies (IGDs) were reported which cause intellectual disability often accompanied by epilepsy, coarse facial features and multiple anomalies that vary in severity depending upon the degree of defect and/or step in the pathway of affected gene.

Methods and Results A patient born to non-consanguineous parents developed intractable seizures with typical hypsarrhythmic pattern in electroencephalography, and was diagnosed as having West syndrome. Because the patient showed severe developmental delay with dysmorphic facial features and hyperphosphatasia, characteristics often seen in IGDs, the patient was tested for GPI deficiency. The patient had decreased surface expression of GPI-APs on blood granulocytes and was identified to be compound heterozygous for NM_178517:c.211A>C and c.499A>G mutations in *PIGW* by targeted sequencing.

Conclusion Here we describe the first patient with deficiency of PIGW, which is involved in the addition of the acyl-chain to inositol in an early step of GPI biosynthesis. Therefore, IGD should be considered in West syndrome and flow cytometric analysis of blood cells is effective in screening IGD.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) is a complex glycolipid that acts as a membrane anchor of 150 or more human cell surface proteins. GPI is synthesised in the endoplasmic reticulum (ER) and transferred by GPI transamidase en bloc to a protein that has a C-terminal GPI attachment signal sequence. During this post-translational modification, the C-terminal GPI attachment signal peptide that transiently anchors the proprotein to the ER membrane is cleaved and replaced by GPI. There are at least 26 genes involved in the biosynthesis and remodelling of GPI anchored proteins (GPI-APs).[1] Complete GPI deficiency should cause embryonic lethality because all GPI-APs with important roles in embryogenesis cannot be expressed on the cell surface without GPI anchoring. This is supported by *Piga* gene knockout in mice.[2] Therefore, inborn GPI deficiency must be due to partial deficiency; in fact, the reported cases of inherited GPI deficiency (IGD) are all due to hypomorphic mutations. The first case of IGD reported in 2006 had a homozygous mutation in the promoter region of *PIGM*, which encodes the first mannosyltransferase essential for the GPI biosynthesis,[3] and caused decreased cell surface expression of GPI-APs.[4] Recently, IGDs due to *PIGV*, *PIGN*, *PIGL*, *PIGA*, *PIGO*, *PGAP2*, and *PIGT* deficiency[5–11] were found mainly using whole exome sequencing. The common symptoms are intellectual disability, epilepsy, and coarse facial features; sometimes multiple organ anomalies are present, the severities of which are correlated with the degree of defect in each responsible gene.

Additionally, hyperphosphatasia is commonly seen in IGDs caused by mutations in *PIGV*, *PIGO*, and *PGAP2*, and these deficiencies are termed hyperphosphatasia with mental retardation syndrome 1, 2 and 3 (HPMRS1-3), respectively. Alkaline phosphatase (ALP), a GPI-anchored enzyme, is released under certain conditions of GPI deficiency. Mutations in *PIGV* and *PIGO* affect the late GPI synthesis steps and result in cleavage of the GPI attachment signal peptide by the GPI transamidase in the ER, leading to generation of non-GPI anchored soluble proteins, which are secreted and cause hyperphosphatasia.[12] In contrast, mutations in *PGAP2* affect the re-acylation step in the fatty acid remodelling of the GPI anchor that occurs in the Golgi apparatus, resulting in generation of lyso-forms of GPI-APs due to a lack of re-acylation, that are rapidly released from the cell surface and cause hyperphosphatasia.[13] Here, we report on a patient with HPMRS who had compound heterozygous mutations in *PIGW*. The patient was initially diagnosed as having West syndrome, a type of infantile epilepsy.

METHODS

Genomic analysis by the next generation sequencer

Informed consent was obtained from the patient's parents and ethics approval for this study was obtained from the Osaka University Review Board. Peripheral blood samples were obtained from the patient. Targeted sequencing was performed to detect mutations in 26 genes involved in

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biosynthesis or remodelling of GPI-APs. Custom primer pools for 26 genes (*PIGA*, *PIGB*, *PIGC*, *PIGF*, *PIGG*, *PIGH*, *PIGK*, *PIGL*, *PIGM*, *PIGN*, *PIGO*, *PIGP*, *PIGQ*, *PIGS*, *PIGT*, *PIGU*, *PIGV*, *PIGW*, *PIGX*, *PIGY*, *PIGZ*, *PGAP1*, *2*, *3* and *5*, and *GPAA1*) were designed with Ion AmpliSeq Designer software, and the 460 primer sets divided into two pools were synthesised. Amplicon libraries were prepared by PCR using the Ion AmpliSeq Kit and custom primer pools. The amplicons were sequenced in a PGM Sequencer and the results were analysed using the manufacturer's protocol (Life Technologies, Carlsbad, California, USA). Mutations were validated by Sanger sequencing.

Fluorescence activated cell sorting (FACS) analysis

Surface expressions of GPI-APs were determined by staining cells with Alexa488-conjugated inactivated aerolysin (FLAER), and mouse anti-human CD59 (5H8), anti-human DAF (IA10), anti-human CD24 (ML5), anti-human ALP (B4-78), anti-hamster-urokinase plasminogen activator receptor (uPAR) (5D6) and anti-human CD16 (3G8) monoclonal antibodies, followed by a phycoerythrin (PE) conjugated anti-mouse immunoglobulin G (IgG) antibody, and analysed by flow cytometry (Cant II; BD Biosciences, Franklin Lakes, New Jersey, USA).

Transfection of plasmids into *PIGW* deficient Chinese hamster ovary (CHO) cells

As human *PIGW* cDNA containing plasmid was barely amplified in *Escherichia coli*, we used rat *Pigw* for further analysis. PIGW is well conserved among the species and mutated amino acids are completely conserved in the rat Pigw. Flag-tagged rat *Pigw* cDNA and its mutants generated by site-directed mutagenesis were subcloned into SRα(strong promoter) driven pME vector and thymidine kinase promoter (weak promoter) driven TK vector. Plasmids were transfected by electroporation into *PIGW*-deficient CHO cells (10.14),[14] with the luciferase expressing construct to monitor the transfection efficiency. Surface expression of CD59, DAF, and uPAR was analysed by FACS. Levels of PIGW protein in cells were determined by western blotting using anti-Flag antibody (M2, Sigma) and normalised with luciferase activities for the transfection efficiency and with band intensities of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for the loading controls.

RESULTS

The patient was born from healthy non-consanguineous parents. Development was profoundly delayed from early infancy. Mild dysmorphic facial features (broad nasal bridge and tented upper lip) and inguinal hernia were noted, but abnormalities of the fingers or nails were absent. The patient developed clusters of tonic spasms and was diagnosed as having West syndrome based on a high-amplitude hypsarrhythmic pattern on interictal EEG. Routine laboratory investigations were normal but the serum ALP was constantly elevated at around 2000 U/L. Brainstem auditory evoked response and ophthalmologic examination was normal. Flow cytometry using peripheral blood granulocytes revealed reduced levels of the GPI-APs, supporting the diagnosis of GPI anchor deficiency (figure 1A). CD59 and FLAER on lymphocytes and CD14 on monocytes were also reduced to half that of the control. However, CD59 and DAF on erythrocytes did not show a significant reduction (data not shown).

We then performed molecular investigations to determine the gene responsible for GPI deficiency. The amplicon libraries of all known GPI-anchor synthesis genes were prepared by multiplex PCR from genomic DNA and sequenced. We identified compound heterozygous variants in the *PIGW* gene, NM_178517: c.211A>C:p.Thr71Pro and c.499A>G:p.Met167Val, located in the second and fifth transmembrane domains of PIGW protein, respectively (figure 1B upper and middle panels). Both amino acids were well conserved among the various species. The variant c.211A>C was a novel variant and c.499A>G was registered as single nucleotide variant (SNV) (rs200024253). A frequency of the latter variant is 0.001 in 1208 Japanese individuals according to the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). We found in the same database that frequencies of two other damaging variations in *PIGW*, c.907C>T and c988C>T, are 0.004 and 0.003, respectively, in Japanese subjects. If we assume that a frequency of the variant c.211A>C is 0.004, a probability of finding c.211A>C and c.499A>G in one individual by chance is 1 in 250 000.

We analysed the function of *PIGW* cDNAs bearing the patient's mutations by transfection into *PIGW* deficient CHO cell line and measuring the restoration of the surface expression of GPI-APs. When the strong promoter (SRα) driven constructs were used, Thr71Pro cDNA completely restored and Met167Val cDNA partially restored the surface expression of CD59, DAF, and uPAR (figure 2A). When the weak promoter (TK) driven constructs were used, Thr71Pro cDNA only partially restored and Met167Val did not restore at all the surface expression of GPI-APs, whereas wild-type cDNA completely restored it (figure 2B). Protein expression of the Thr71Pro mutant was decreased to one-third of the wild-type level, whereas that of the Met167Val mutant was similar to the wild-type (figure 2C). From these results, we conclude that both mutations affect the PIGW activity. These variants most likely caused the phenotype of this affected person, but confirmation of the identification of additional families is awaited.

DISCUSSION

Of 26 genes involved in the GPI pathway, 22 (collectively termed PIG genes) are required for synthesis and protein attachment of GPI, and four (PGAP genes) are required for remodelling of GPI after attachment to proteins.[1] IGDs due to mutations in nine of these genes have been reported (including this report): eight were due to mutations in PIG genes and one was due to mutations in the *PGAP2* gene.[4–11] Whereas intellectual disability was a common symptom, hyperphosphatasia was seen only when mutations occurred in *PIGV*, *PIGO*, *PGAP2*, and *PIGW* genes. Serum ALP values were basically normal in IGDs due to mutations in *PIGA*, *PIGL*, *PIGM*, and *PIGN* genes, with exceptional findings of a possibly accidental mild increase reported in *PIGA*-defective IGD cases. In contrast, deficiency of *PIGT*—a component of GPI transamidase—showed hypophosphatasia and skeletal abnormalities. We previously proposed the mechanism of hyperphosphatasia.[12] GPI transamidase, which cleaves the C-terminal GPI attachment signal peptide of the proprotein, also recognises GPI. Cells deficient in *PIGV* and *PIGO* accumulate GPI intermediates bearing at least one mannose with an ethanolaminephosphate side-branch linked to the first mannose. These GPI intermediates appeared to activate GPI transamidase, enabling it to cleave the GPI attachment signal peptide and generate soluble ALP. In contrast, cells deficient in *PIGL* and *PIGX* accumulate GPI intermediates lacking mannose, which do not activate GPI transamidase leading to intracellular degradation of proprotein—hence no hyperphosphatasia. PIGT is an essential component of GPI transamidase,[15] so in *PIGT* deficient cells, GPI-APs including ALP are not processed and are completely degraded in the cell. There is

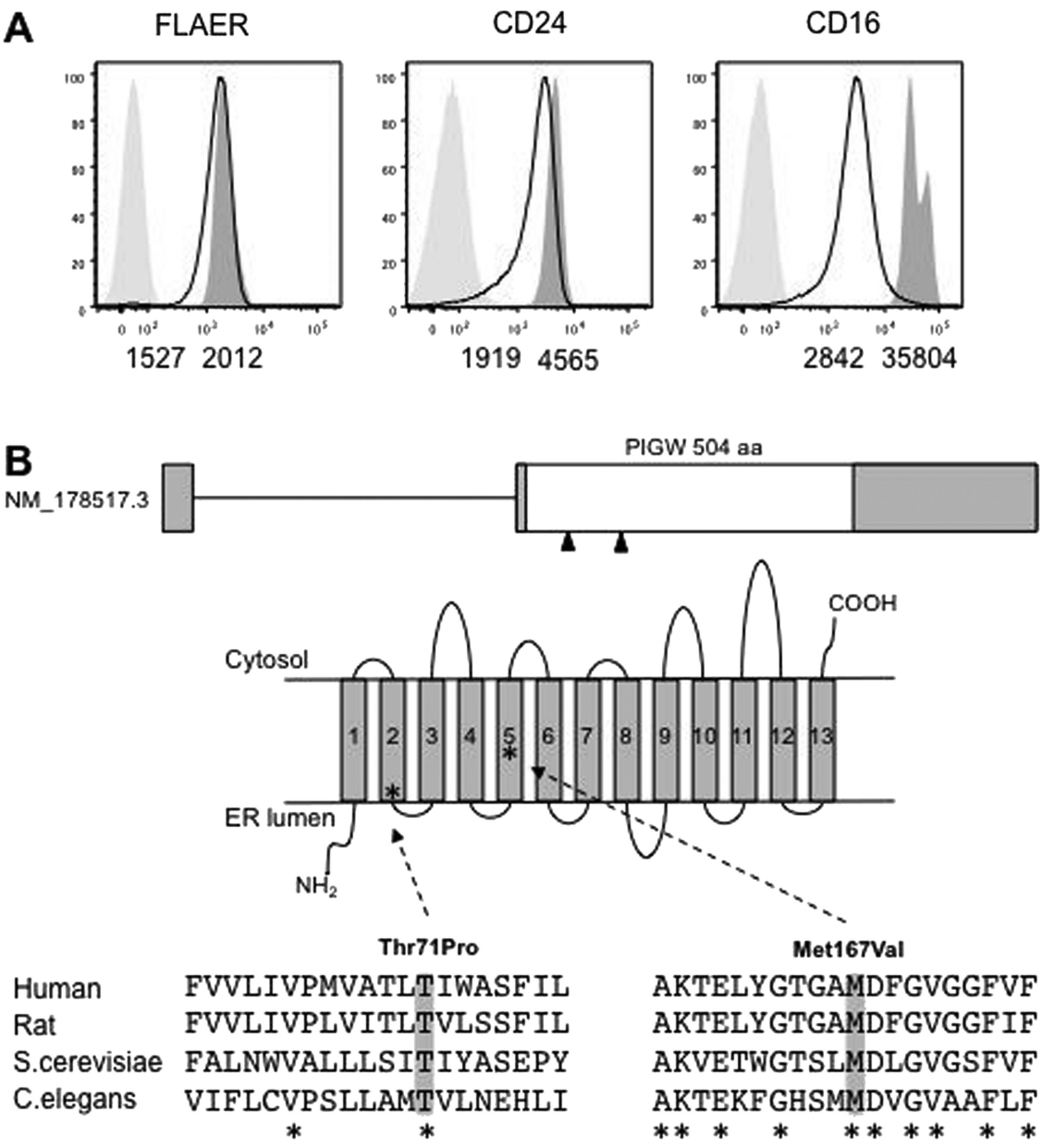

Figure 1 Flow cytometry of the granulocytes and the position of the PIGW mutation. (A) Flow cytometry of the granulocytes. The vertical axis indicates normalised cell number and the horizontal axis indicates the intensity of the fluorescence, which shows the amount of protein expressed on the cell surface. Surface expression of various glycosylphosphatidylinositol anchored proteins (GPI-APs) on the granulocytes from the patient (thick lines) was severely decreased compared with the normal (dark shadows). Light shadows, isotype controls. Mean fluorescent intensities of each sample are shown below each panel (left, the patient; right, normal). (B) Schematic diagram of the PIGW gene (NM_178517.3) and protein (NP_848612.2), showing the position of the mutations (upper panel, arrowheads). Topology of the PIGW protein, showing positions of mutations (middle panel). Multiple sequence alignment of each mutated region of PIGW protein (bottom panel).

also a report clearly showing that for binding of GPI intermediates to GPI transamidase, at least one mannose with an ethanolaminephosphate side-branch is necessary.[16]

PIGW is inositol acyltransferase acting in the third step of GPI biosynthesis. Inositol acylation is not essential for the ensuing mannosylation and addition of an ethanolaminephosphate side-branch, but it is critical for the attachment of bridging ethanolaminephosphate to the third mannose.[14] As a result, *PIGW* deficient cells accumulate the GPI intermediates lacking an inositol-linked acyl chain but bearing mannoses with an ethanolaminephosphate side-branch. It seems that these GPI intermediates activate GPI transamidase, causing hyperphosphatasia. Thus, the patient's symptoms not only confirm our hypothesis based on the in vitro experiments but also further suggest that inositol-acylation is not necessary for activation of GPI transamidase.

West syndrome is an age-dependent epileptic encephalopathy that most frequently presents in the first year of life with variable aetiology. The incidence of West syndrome is estimated at between 2.5 and 4.5 per 10 000 live births.[17] One of the mechanisms of the seizure in IGD is a defect in intraneuronal pyridoxal phosphate, a cofactor of γ-amino butylic acid (GABA) synthase, caused by a loss of membrane-anchored ALP and leading to a shortage of GABA. Membrane-bound ALP de-phosphorylates pyridoxal phosphate to generate membrane permeable pyridoxal, which in turn is re-phosphorylated in the cytoplasm to be used by vitamin B6-dependent enzymes including GABA synthase (glutamate decarboxylase). Patients with

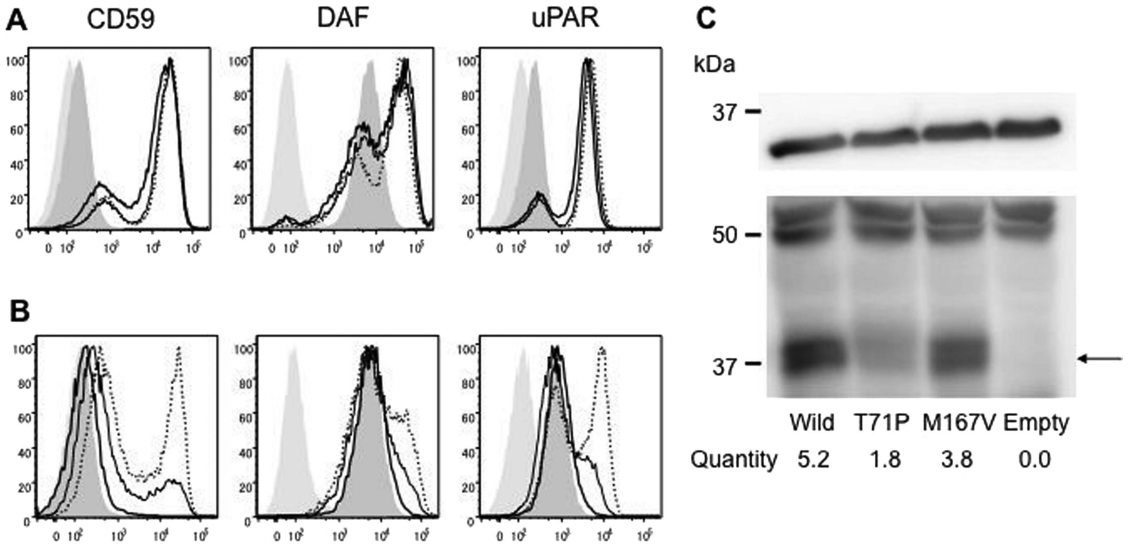

Figure 2 Functional analysis using the PIGW deficient Chinese hamster ovary (CHO) cells. (A) (B) PIGW deficient CHO cells (10.14) were transiently transfected with wild-type (dotted lines) or Thr71Pro mutant (thick lines) or Met167Val mutant (thin lines) pME FLAG-PIGW (A) or pTKFLAG-PIGW (B), stained with anti-CD59, -DAF and -urokinase plasminogen activator receptor (uPAR), and analysed by flow cytometry. Grey shadows, empty vector transfectants; light grey shadows, isotype control. (C) Lysates of transfectants shown in (B) were developed in SDS-PAGE and blotted with anti-Flag antibody for PIGW protein (arrow) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for loading control. Protein quantities are shown below each lane.

hypophosphatasia, caused by a mutation in the tissue non-specific alkaline phosphatase (*TNAP*) gene, also suffer from seizures, the mechanism of which was analysed using TNAP knockout mice.[18] Membrane permeable pyridoxine treatment was very effective for treating intractable seizures in some cases with IGD.[19 20] Additionally, an abnormally high intensity region in the brain stem on T2 MRI was a common characteristic among recent IGD cases.[20] If we regard the response to pyridoxine treatment and the characteristic findings in brain MRI as clues, we can screen patients using FACS analysis of their peripheral blood. More IGD will be found within patients with infantile intractable epilepsy, including West syndrome, which will lead to improved selection of appropriate treatment.

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Contributors TC took care of the patient and wrote the manuscript. NI attended the planning of the screening system of patients with GPI anchor deficiency, analysed the data and wrote the manuscript. MM took care of the patient and wrote the manuscript. TK attended the planning of the screening system of patients with GPI anchor deficiency, discussed the results and wrote the manuscript. YM attended the planning of the screening system of patients with GPI anchor deficiency, performed the experiments, analysed the data and wrote the manuscript.

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Competing interests None.

Parental consent Obtained.

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