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Novel *CTSA* Variant Identified in a Thai Family With Late-Infantile Galactosialidosis

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ABSTRACT: Galactosialidosis (GS) is a rare lysosomal storage disease (LSD) with variable onset caused by a defect in protective protein/cathepsin A (PPCA) encoded by the *CTSA* gene. The late-infantile onset is characterized by developmental delay, visceromegaly, coarse facies, and cherry-red macula. We report cases of late-infantile GS in a Thai-Lahu family, with affected members initially presenting with recurrent infections due to T-cell defects. The clinical features of LSD and cherry-red macula led us to perform lysosomal enzyme assays, which showed undetectable activity of PPCA. A novel homozygous missense *CTSA* variant (NM_000308.4): c.1307A > G (p.Gln436Arg) was identified in affected individuals. In vitro functional analysis suggested that the variant may impair the dimerization process of PPCA, potentially disrupting proper protein maturation or function and leading to significantly reduced PPCA activity. Exome sequencing did not reveal any variants in other genes associated with primary immunodeficiencies. To date, our cases represent the first reported patients with GS and T-cell defects. Our study broadened the clinical and genotype spectrum of this rare disease.

1 | Introduction

Galactosialidosis (GS, OMIM#256540) is a rare lysosomal storage disease (LSD) caused by a defect in protective protein/cathepsin A (PPCA; EC 3.4.16.5), which forms a multienzyme complex essential for stabilizing lysosomal β -galactosidase (GLB1; EC 3.2.1.23) and activating of neuraminidase (NEU1; EC 3.2.1.18), leading to deficiency of these enzymes (van der Spoel et al. 1998). Variants in the *CTSA* gene encoding PPCA were first identified in Japanese GS patients (Shimmoto et al. 1993). To date, almost 50 variants have been identified in the *CTSA* gene (<https://www.hgmd.cf.ac.uk/>, as of July 29, 2024). GS presents with variable clinical manifestations and can be classified into three subtypes based on the age of onset and severity: early infantile form, late-infantile

form, and juvenile/adult form. Patients with early infantile form typically exhibit hydrops fetalis, hepatosplenomegaly, telangiectasias, and skeletal dysplasia. The late infantile type usually presents within the first few years of life and is characterized by hepatosplenomegaly, coarse facial features, cherry red spot, and dysostosis multiplex (Okamura-Oho et al. 1994; Sláma et al. 2019). Survival of patients with the late infantile type varies from childhood to adulthood (Caciotti et al. 2013). The juvenile/adult type is the most common form and is predominantly found in Japanese patients. Patients with this phenotype exhibited myoclonus, ataxia, neurological deterioration, angiokeratoma, and absence of visceromegaly (Darin et al. 2009). Enzyme replacement therapy, bone marrow transplantation, pharmacological chaperone treatment, and gene therapy are currently under

clinical trials, so there is no specific treatment available at present (Annunziata and d’Azzo 2017).

Here, we report two Thai siblings affected with late-infantile GS and novel clinical features of T-cell defects due to a novel homozygous missense variant in the *CTSA* gene. Furthermore, we confirm the underlying mechanism of this novel variant by in vitro functional study.

2 | Methods

The parents of Patients 1 and 2 provided written informed consent for the publication of photographs and their clinical history. This study was approved by the institutional review board of the Faculty of Medicine Siriraj Hospital, Mahidol University (Si. 348/2024) and was conducted in accordance with the Declaration of Helsinki.

2.1 | Leukocyte Enzyme Assays and Variant Analysis

Blood samples were collected from the patients, their parents, and the maternal aunt after informed consent. Leukocytes were isolated from the whole blood samples as previously published (Ngiwsara et al. 2018). The activities of PPCA, NEU1, and GLB1 were measured as previously described (Hindman and Cotlier 1972; Koli and Garman 2014; Potier et al. 1979). Genomic DNA was extracted from whole blood samples with the QIAamp DNA Mini kit (Qiagen GmbH). All coding exons and their flanking intron sequences of the *GLB1*, *NEU1*, and *CTSA* genes of Patient 1 were PCR amplified by gene-specific primers, and the products were sent for direct Sanger sequencing at Macrogen Inc. (Seoul, Korea). Direct sequencing of exon 13 of the *CTSA* gene for the c.1307A > G variant was performed in Patient 2, the parents, and the affected maternal aunt.

2.2 | Exome Sequencing

The genomic DNA of Patient 1 was sequenced using exome sequencing services from Macrogen Inc. (Seoul, Korea). To exclude variants causing primary immune deficiency, we used the Human Phenotype Ontology (HPO, <https://hpo.jax.org/>) terms: “abnormality of the immune system” (HP:0002715: 2085 genes) and “cellular immune defect” (HP:0002843; 145 genes) and searched for disease-causing variants in VCF file of Patient 1’s exome sequencing.

2.3 | Functional Study in COS-7 Cell and Western Blot Analysis

The full-length *CTSA* cDNA of the normal healthy control and Patient 1 was PCR amplified and cloned into the pcDNA3.1/CT GFP TOPO plasmid (Invitrogen; Thermo Fisher Scientific Inc.). The methods for transient transfection of COS-seven cells and Western blotting were performed under reducing conditions as described previously (Ngiwsara et al. 2018). Following 24 h of incubation, the cells were used for Western blot analy-

sis and PPCA activity measurement. The primary antibodies used were anti-PPCA (cat.no. ab184553; 1: 3000; Abcam), anti-Neuraminidase (cat.no. ab197020; 1: 2000; Abcam), anti-Beta galactosidase (cat.no. ab128993; 1: 3000; Abcam), and anti-ACTB (cat.no.3700; 1: 20,000; Cell Signaling Technology). Following primary antibody incubation, the membranes were washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Dako; Agilent Technologies Inc., Santa Clara, CA, USA). These experiments were performed in triplicate.

2.4 | Molecular Visualization of PPCA

The structures of human PPCA were downloaded from the Protein Data Bank (<http://www.rcsb.org/pdb/>) (Rudenko et al. 1995). PyMOL Molecular Graphics System (Schrödinger LLC) was utilized to visualize the structural features of the PPCA protein (PDB: 1IVY), with particular emphasis on the newly identified variant.

3 | Results

3.1 | Clinical Report

3.1.1 | Patient 1

He was born to consanguineous parents of Thai-Lahu descent, an ethnicity from Thailand’s northern border with Myanmar. Despite an unremarkable prenatal and birth history, he presented with recurrent pneumonia at one month of age and was diagnosed with pulmonary tuberculosis at 11 months of age. Global developmental delay, coarse facies, hepatosplenomegaly, inguinal hernia, claw hands, gibbus deformity, excessive Mongolian spots, and cherry-red spots were noted (Figure 1a–c). A skeletal survey showed dysostosis multiplex (Figure 1d–f), and an echocardiogram revealed patent ductus arteriosus (PDA) and left ventricular hypertrophy. A peripheral blood smear showed vacuolated lymphocytes. Immunologic studies indicated persistently low CD4-lymphocyte counts (Table S1). He had multidrug-resistant tuberculosis, herpes labialis, and asthma but no opportunistic infections. Co-trimoxazole prophylaxis was started and stopped at 11 years of age after no further infections. Despite severe developmental delay, he could speak in short sentences and walk independently. He developed progressive weakness at 11 years of age, became bedridden probably from myelopathy, and died from aspiration pneumonia at 12 years of age. An eye examination performed at 12 years of age revealed no evidence of corneal clouding. Exome sequencing in Patient 1 could not identify pathogenic/likely pathogenic variants causing primary immunodeficiency.

The patient’s mother experienced three miscarriages and had one female offspring who died in the neonatal period. The maternal aunt of the patient presented with similar clinical features, including coarse facial features, delayed development, and hepatosplenomegaly, and died at 18 years of age without a definitive cause of death. Immunologic investigations were not performed on the maternal aunt; however, there was no reported



FIGURE 1 | Clinical pictures of affected individuals. (a–c) Patient 1 at 11 years of age showing coarse facies, claw hand, and excessive Mongolian spots. (d–f) Skeletal radiographs demonstrating dysostosis multiplex. (g) Patient 2 at 2 years of age exhibiting early coarse facial features.

history of recurrent or opportunistic infections suggestive of immunodeficiency.

3.1.2 | Patient 2

She was the younger sister of Patient 1, born with bilateral club feet, which were treated with serial casting and tendon-Achilles lengthening. At 19 months of age, she exhibited similar features to those of Patient 1, including global developmental delay, coarse facial features, hepatosplenomegaly, cherry-red spots, and dysostosis multiplex (Figure 1g). An echocardiogram revealed only a functional patent foramen ovale. She also had recurrent infections, low CD4 lymphocyte counts (Table S1), nephrotic-range proteinuria, and hypoalbuminemia, though a renal ultrasound showed normal kidneys.

3.2 | Leukocyte Enzyme Assays and Molecular Analysis

Infantile GM1 gangliosidosis was initially suspected in Patient 1, and GLB1 activity in the leukocytes decreased to 14.2% of normal controls. However, no disease-causing variant was identified in the *GLB1* gene which led to suspicion of GS. Therefore, PPCA enzyme assay in the leukocytes was performed which revealed undetectable activity of PPCA (Table S2). The activity of NEU1

also decreased to 24.3% of normal controls (Table S2). Sanger sequencing identified a novel homozygous missense variant c.1307A > G (p.Gln436Arg) in the *CTSA* gene (NM_000308.4) in Patients 1 and 2, and their maternal aunt (Figure S1). Both parents of the patients were heterozygous for this variant. This variant was classified as a variant of uncertain significance (VUS) (PM2_Support, PP3_Moderate, and PP4_Moderate) according to the ACMG 2015 classification system (Richards et al. 2015).

3.3 | Western Blot Analysis and Functional Study

To analyze PPCA expression in leukocytes, the lysates were probed with the anti-54 antibody, and the results revealed a 54 kDa PPCA precursor band in both control and the patient's lanes (Figure 2a). A lower band was observed in the control lane possibly due to partial degradation of protein. Western blot using anti-GLB1 and anti-NEU1 demonstrated decreased amounts of both GLB1 and NEU1 proteins in the patient's leukocytes compared to the control lanes, suggesting a loss of stability in these proteins (Figure 2a). The impact of variant p.Gln436Arg on PPCA protein was assessed through transient expression in COS-7 cells. Western blot analysis demonstrated that wild-type PPCA exhibited the expected band pattern of 54 kDa precursor; however, PPCA protein expression in p.Gln436Arg-expressing cells was increased up to twofold compared to the wild-type

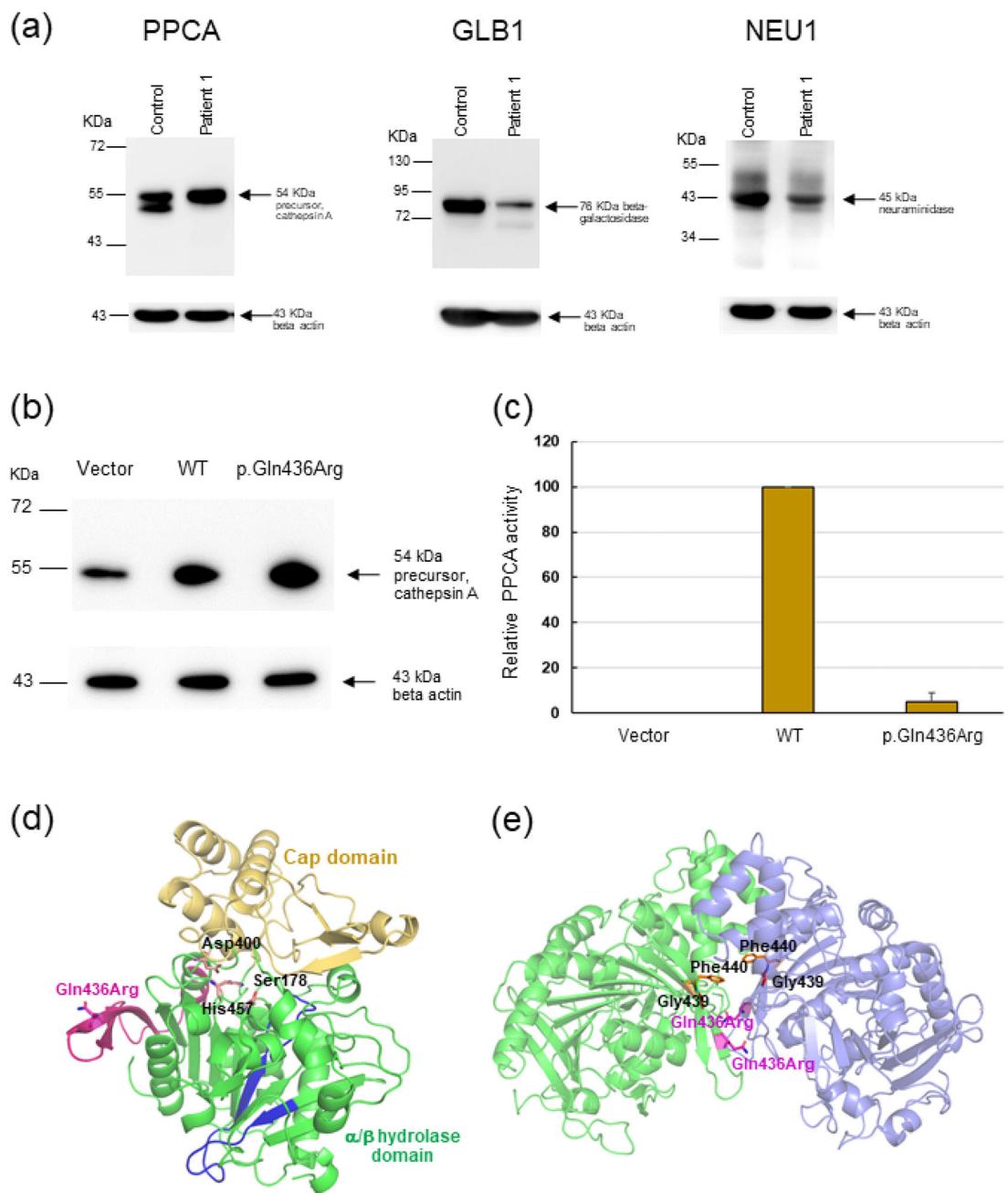


FIGURE 2 | (a) Western blot analysis of PPCA, GLB1, and NEU1 protein levels in leukocytes of Patient 1 and the healthy control. Beta-actin was used as a loading control. (b and c) Functional study of mutant PPCA Gln436Arg in COS-7 cell lines. (b) Western blot analysis of PPCA protein in extracts of cells expressing the wild type and Gln436Arg. (c) PPCA activity in COS-7 cells transfected with the wild type and Gln436Arg. (d and e) Locations of the variants in the three-dimensional structure of PPCA (PDB: 1IIVY). (d) A monomer of PPCA is composed of an alpha/beta hydrolase domain (green) containing the catalytic residue (Ser178, Asp400, and His457) and the cap domain (yellow). The position of the Gln436Arg is indicated by residue sticks with a pink color. The internal repeated motif in 32-kDa subunits (dark blue) and in 20-kDa subunits (pink) with yellow color. (e) Dimer of PPCA, the position of novel variant Gln436Arg, and reported variants, Gly439 and Phe440, on the dimer interface.

protein (Figure 2b). PPCA activity in COS-7 cell extracts indicated that p.Gln436Arg had extremely low activity ($5.11\% \pm 3.71\%$) compared with the wild-type enzyme (Figure 2c).

4 | Discussion

We report late-infantile GS cases with atypical initial presentations, including recurrent infections linked to T-cell defects.

Despite these atypical features, the patients exhibited classic GS symptoms such as coarse facies, hepatosplenomegaly, dysostosis multiplex, cherry-red spots, and proteinuria. Although T-cell lymphopenia has not been previously reported in GS, exome sequencing did not identify any variants in genes associated with primary immunodeficiency. However, other potential causes of T-cell defects cannot be excluded, including inborn errors of immunity that may not be detected by exome sequencing due to its limitations, such as an inability to identify large deletions,

as well as immunodeficiency secondary to viral infections or malnutrition. Therefore, while T-cell defects might be related to PPCA deficiency, further investigation is needed to elucidate the underlying etiology. GS is characterized by a significant reduction in immunoregulatory NEU1 activity and excessive accumulation of sialyl glycans in tissues, including blood cells. In contrast, GLB1 activity is only partially decreased in GS patients (Annunziata and d'Azzo 2017). Therefore, the reduction in T cells and natural killer (NK) cells observed in GS patients may be attributed to the accumulation of sialyl glycans rather than GM1 gangliosides. Various immunological abnormalities have been described in other LSDs. Low numbers of CD4+ lymphocytes and NK cells were found in patients with Type 1 Gaucher disease (Burstein et al. 1987; Sotiropoulos et al. 2015). Mucopolysaccharidosis (MPS) Type VI patients had a decreased percentage of NK cells and monocytes when compared with controls (Lopes et al. 2023). GS results in a combined phenotype of GM1 gangliosidosis (GLB1 deficiency) and sialidosis (NEU1 deficiency). Neither of these disorders has been reported to be associated with T-cell defects. However, cats affected with GM1 gangliosidosis had reduced thymic mass, as well as reduced CD4 and CD8 lymphocyte subpopulations which could be related to GM1 ganglioside-mediated thymic apoptosis (Cox et al. 1998; Zhou et al. 1998), but this phenomenon has not been demonstrated in affected humans. Further studies are required to elucidate the immunodeficiency in GS.

In addition, Patients 1 and 2 exhibited associated conditions uncommon in GS, including PDA, nephrotic-range proteinuria, and bilateral club feet, respectively. Although proteinuria has been occasionally reported in GS, some cases in the literature have progressed to end-stage renal disease requiring renal transplantation (Kiss et al. 2008). Renal biopsy findings from reported cases revealed foamy macrophages in the glomeruli and renal tubules (Steinke et al. 2020). The pathogenesis of renal involvement in GS is hypothesized to result from progressive substrate accumulation, similar to that observed in Fabry disease (Kiss et al. 2008). PDA has been previously reported in one case of GS and a case of sialidosis (Khan and Sergi 2018; Sharma et al. 2023). However, as PDA is a common congenital heart defect, its association with GS may be coincidental. Clubfoot or equinovarus deformity has been reported in GS patients (Caciotti et al. 2013) and in other neonatal-onset LSDs such as multiple sulfatase deficiency (Santos and Hoo 2006). This malformation could represent a neonatal manifestation of LSDs. The differences in clinical features between Patients 1 and 2 suggest some intrafamilial variability of GS which requires further assessment.

The novel *CTSA* variant, p.Gln436Arg, identified in our cases was initially classified as a VUS. Therefore, we investigated the impact of the novel variant in both clinical samples and transient expression analysis. The p.Gln436 is situated in the internal repeating motif (Figure 2d) which is presented in each subunit of PPCA. These repeated motifs are conserved among PPCA proteins of different species, suggesting an ancient intragenic duplication (Galjart et al. 1991). The residues in this region are postulated to expose surface features essential for the recognition and binding of monomeric molecules with each other. The previous studies reported variants p.Gly439Ser and p.Phe440Val found in early infantile and late-infantile GS, respectively (Zhou et al. 1996), were located in the same β -strand as p.Gln436

(Figure 2e). Expression study of both reported proteins resulted in the synthesis of a mutant protein that lacks PPCA-like activity, and those two mutant precursors do not form a homodimer, causing its partial retention in the endoplasmic reticulum. Our expression analysis revealed a pronounced accumulation of the mutant p.Gln436Arg 54 kDa precursor compared to the wild-type PPCA. This observation suggests that the variant may impair the dimerization process, potentially disrupting proper protein maturation or function. The replacement of glutamine with arginine possibly causes dramatic structural consequences, in agreement with a genetic lesion found in this core domain on the beta-sheet strand at the dimer interface. PPCA plays a role as a molecular chaperone protein associating with GLB1 and NEU1 to form LMC. Loss of PPCA function disrupts the LMC, resulting in a combined deficiency of GLB1 and NEU1, which leads to a broad range of clinical manifestations. Our study proves that the novel missense variant identified in our cases results in PPCA deficiency. Therefore, the variant could be reclassified as a likely pathogenic variant (PM2_Support, PP3_Moderate, PP4_Moderate, and PS3_support) according to the ACMG classification system.

In conclusion, we report a family affected with late-infantile type GS and novel clinical features, T-cell defects. In addition, the novel homozygous missense *CTSA* variant was identified. The functional study confirms the pathogenicity of the variant and explains its molecular mechanism. This knowledge can offer insights into the disease's pathophysiology, potentially paving the way for the development of targeted treatments in the future.

Author Contributions

Lukana Ngiwsara: conceptualization, analysis of the experiments, writing—original draft, review, and editing. **Dhachdanai Dhachpramuk:** data collection (clinical), exome analysis, writing—original draft. **Phannee Sawangareetrakul and Sherry Vongphit:** performed the experiments. **Punchama Pacharn:** data collection and analysis (clinical). **Jisnuson Svasti:** conceptualization, writing—review, and editing. **Nithiwat Vatanavicharn:** conceptualization, data collection (clinical), writing—review, and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.