IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF *GAA* GENE MUTATIONS IN THAI PATIENTS WITH INFANTILE-ONSET POMPE DISEASE

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Pompe disease (glycogen storage disease type II, acid maltase deficiency, OMIM #232300) is an autosomal recessive lysosomal glycogen storage disorder caused by a deficiency of the lysosomal enzyme acid α-glucosidase (GAA). The build up of glycogen causes progressive muscle weaknesss throughout the body, particularly in the heart, skeletal muscles, liver and nervous system. Pompe disease occurs in approximately 1 per 40,000 births around the world. This is broadly divided into two forms based on the age symptoms occurs. Infantile-onset form is usually diagnosed at 4-6 months with muscles appear normal but are limp and weak preventing from lifting head and rolling over. Late onset form occurs later one to two years and progresses more slowly. The muscle strength decrease with the legs and then to the respiratory muscle. Since the incidence of this disease in Thailand is very low, molecular study are rarely investigated and no laboratory diagnosis with the biochemical test.

In this study, we examined the molecular characteristics of 12 patients, who was classified as infantile-onset pompe disease. The patients showed predominant hypertrophic cardiomyopathy, generalized hypotonia, muscle weakness, and mild hepatomegaly. Biallelic mutations of the *GAA* gene were identified in all patients. The GAA activity, genotypes and effect on protein phenotype are shown in **Table 1**.

Table 1 GAA enzyme activity and mutations of 12 patients with IOPD

Patient	GAA activity[a] (% of normal)	Nucleotide changes	Exons	Effects on coding protein	GAA domains	Inheritance
1	0	c.876C>G	5	p.Y292X[b]	N-terminal β-sheet	FA
		c.1003G>A	6	p.G335R	N-terminal β-sheet	MO
2	0	c.1935C>A	14	p.D645E	Catalytic GH31	FA
		c.1933G>C	14	p.D645H	Catalytic GH31	MO
3	0	c.1099T>C	7	p.W367R	Catalytic GH31	FA
		c.1942G>A	14	p.G648S	Catalytic GH31	MO
4	0	c.1226insG	8	p.D409GfsX95	Catalytic GH31	FA
		c.2024_2026delACA	14	p.N675del	Catalytic GH31	MO
5	0	c.1538A>G (hom)	10	p.D513G	Catalytic GH31	MO, FA
6	0.43	c.781G>A (hom)	4	p.A261T	N-terminal β-sheet	MO, FA
7	0.05	c.1411_1414delGAGA	9	p.E471fsX5	Catalytic GH31	MO
		c.1933 G>C	14	p.D645H	Catalytic GH31	FA
8	1.46	c.877G>A (hom)	5	p.G293R	N-terminal β-sheet	MO, FA
9	NA	c.1941C>G (hom)	14	p.C647W	Catalytic GH31	MO, FA
10	NA	c.876C>G (hom)	5	p.Y292X	N-terminal β-sheet	MO, FA
11	NA	c.1895T>G (hom)	14	p.L632R[c]	Catalytic GH31	MO, FA
12	0.10	c.1327-2A>G	IVS8	Splicing	-	MO
		c.1437G>C	9	p.K479N	Catalytic GH31	FA

[a] The activity is expressed as a percent relative to normal control (glucose/hr/mg protein); [b] the mutation creates a *BfaI* restriction site; [c] the mutant creates *SfcI* restriction site. PCR-restriction digest with respective enzyme revealed no mutation in 50 healthy control. IOPD, infantile-onset Pompe disease; NA, not available. Hom is homozygous mutation and FA and MO mean father and mother, respectively.

According severity rating system, two types of assay were performed to analyze the impact of sequence variations as expressed in cells transfected with mutant compared to wild type GAA: 1. GAA enzyme activity detected in transiently transfected COS-7 cells (C%) and in culture media (M%); 2. The combination of outcome data in term of quantity and quality of apparent molecular mass of the secreted precursor 110 kDa (M110), the intracellular precursor 110 kDa (C110), the intracellular intermediate 95 kDa (C95) and intracellular mature 76 kDa (C76) forms of GAA, as they present after SDS-PAGE and immunoblotting. The mutants were assigned to class 'B' or potentially less severe mutation except p.A261T was characterized as class D/E (**Table 2**). The position of the novel mutations were showed in **Fig.3**.

Table 2 Specific activity of GAA mutants in transiently transfected COS-7 cells and severity rating

Variant	GAA activity*		Severity rating						
	Medium	Cell lysates	M110	C110	C95	C76	M%Wt	C%Wt	Class
Wild type	60.5	631.3 (± 44.5)	4,4	4,4	4,4	4,4	100	100	-
A261T	3.31	478.3	3,4	4,4	4,4	4,4	5.47	75.8	D/E
Y292X	0	0	1,1	2,4	2,4	2,4	0	0	B
D513G	0	4.8	1,1	3,4	2,4	2,4	0	0.76	B
L632R	0.05	0.1	1,1	3,4	2,4	2,4	0.83	0.02	B

* Specific activity of the wild type GAA and its mutants in transiently transfected COS-7 cells (nmol 4-MU/hr/mg protein). M%Wt and C%Wt mean the relative GAA activity normalized by the wild type GAA activity of medium and cell lysates, respectively. M110, C110, C95, C76 are the GAA protein masses detected by western blotting at 100, 95, and 76 kDa from medium (M) and cell lysate (C), respectively. Two digit numbers indicate the quantity and quality of GAA protein; 4 represents as normal while 3, 2 and 1 mean lower quantity and quality of GAA protein, respectively.

Of those mutations found, the novel p.Y292X, p.D513G, p.L632R showed evolutionary conservation among vertebrate (**Fig. 1**) and all mutants including p.A261T were transiently transfected in COS-7 cells for functional verification. Western blot analysis demonstrated that the p.A261T mutant exhibited three major bands: 110 kDa precursor (C110), 95 KDa intermediate form (C95) and 76 kDa mature forms (C76), in its intracellular protein pattern, similar to the wild type GAA. As predicted, the p.Y292X mutant peptide resulting from early termination of protein synthesis, migrated as a major form at an aberrant molecular mass of 36 kDa.

Species	Sequence 216	513	632	Accession no.
Homo sapiens	ITGLAEHLS__QVPFDGMWI__VPEILQFNL			ABI53718.1
Pan troglodytes	ITGLAEHLS__QVPFDGMWL__VPEILQFNL			JAA43384.1
Bos taurus	IYGLGEHIH__QVNYDGLWI__ITGMLEFGL			DAA33199.1
Mus musculus	ITGLGEHLS__QVPFDGMWL__VPDILQFNL			AAH10210.1
Rattus norvegicus	ITGLGEHLS__QVPFDGMWI__VPEILQFNL			AAH61753.1

Fig. 1 Protein sequence alignment of vertebrate GAA

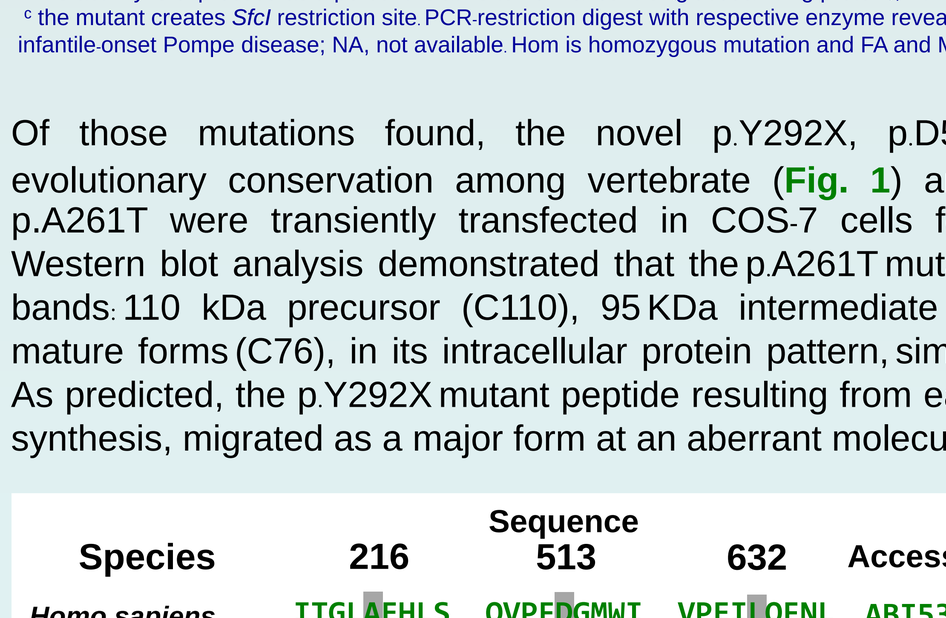

Fig. 2 Western blot analysis of GAA protein in the wild type and mutants

The p.D513G and p.L632R substitutions appeared to affect the maturation of the 110 kDa precursor to intermediate and mature forms. The 110 kDa precursor was found in culture medium (M) only in p.A261T but band intensity was obviously less than the wild type GAA, possibly due to an effect on protein transport, while p.Y292X, p.D513G and p.L632R prohibited secretion of the precursor form completely (**Fig. 2**).

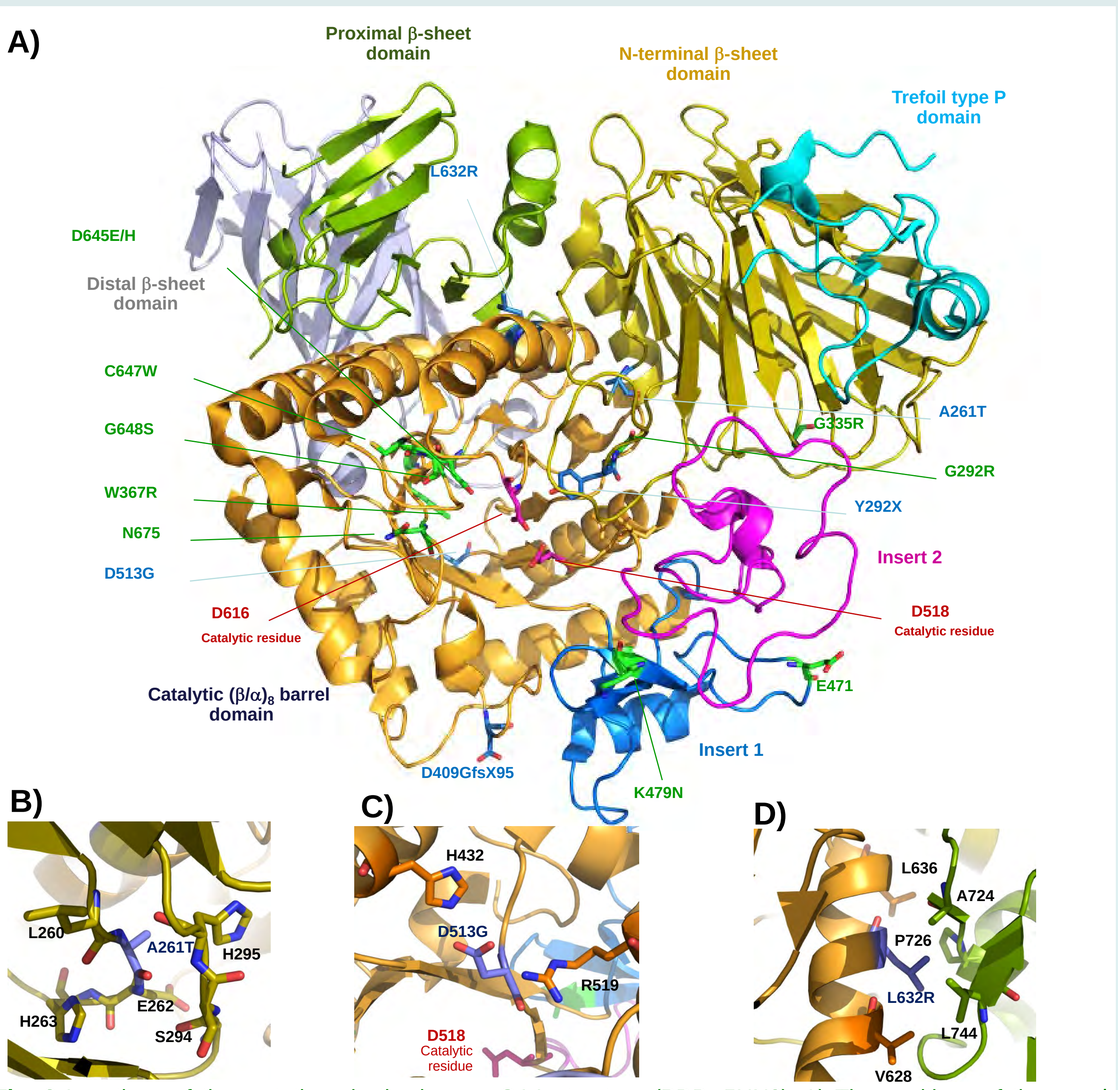

Fig. 3 Locations of the mutations in the human GAA structure (PDB: 5NN8). A) The positions of the novel mutations are indicated by residue sticks with blue color and the previously reported mutations are indicated by residue sticks with green color, respectively. B), C), D) Close-up view of the position p.Ala261Thr, p.Asp513Gly and p.Leu632Arg, respectively.

SUMMARY The broad spectrum of *GAA* mutations described in the present study provide usefulness in confirming the molecular diagnosis of IOPD in the Southeast Asian population of which genetic alterations have been underrepresented in the literature. Almost all the mutations have severity class 'A' or 'B'. The *GAA*-exons 14 and 5 are the hotspots for rapid molecular diagnosis of IOPD especially when enzymatic diagnosis is not a suitable method such as in case of urgent prenatal diagnosis without known familial mutation. These data can benefit rapid molecular diagnosis of IOPD and severity rating of the mutations can serve as a partial substitute for cross reactive immunological material (CRIM) study.

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