



# Newborn screening for Pompe disease in Japan: report and literature review of mutations in the *GAA* gene in Japanese and Asian patients

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## Abstract

A newborn screening program for Pompe disease using dried blood spots (DBSs) was initiated in Japan. Here, we summarized this screening program and described the results of the *GAA* gene analysis. From April 2013 to November 2016, 103,204 newborns were screened; 71 had low acid alpha-glucosidase (A $\alpha$ Glu) activity. *GAA* sequencing showed that 32 (45.1%) and 37 (52.1%) of these newborns were homozygous and heterozygous for pseudodeficiency alleles c.[1726G>A; 2965G>A], respectively. Moreover, 24 of 32 newborns with homozygous c.[1726G>A; 2965G>A] alleles had no mutations, and the other eight had one mutation each. Thirty-five of 37 newborns with heterozygous c.[1726G>A; 2965G>A] alleles had one mutation, and the other two had two mutations each. Only one newborn who had two mutations did not harbor c.[1726G>A; 2965G>A] alleles. Thus, it was difficult to distinguish newborns with c.[1726G>A; 2965G>A] alleles from newborns with pre-symptomatic Pompe disease using A $\alpha$ Glu assays in DBSs or fibroblasts; *GAA* gene sequencing was necessary. Seventy-one newborns had 50 variants, including 21 mutations or predictably pathogenic variants, and 29 polymorphisms or predictably non-pathogenic variants. Four of 21 mutations or predictably pathogenic variants and four of 29 polymorphisms or predictably non-pathogenic variants were novel. No infantile-onset Pompe disease was detected, and three newborns were diagnosed with potential late-onset Pompe disease. In the literature, 156 variants have been reported for 296 patients from 277 families in 41 articles from Japan, Korea, Taiwan, and China. Our results provide insights into *GAA* gene mutation profiles and the relationship between *GAA* and Pompe disease in Asian populations.

## Introduction

Glycogen storage disease type II (OMIM 232300), also known as Pompe disease, is an autosomal recessive disorder caused by a deficiency of A $\alpha$ Glu (EC 3.2.1.20/3), resulting in the accumulation of lysosomal glycogen in the skeletal muscles and heart [1]. The rates of accumulation and tissue damage vary depending on the residual enzyme activity. Patients with infantile-onset Pompe disease (IOPD) with an almost complete absence of A $\alpha$ Glu activity present with hypotonia and hypertrophic cardiomyopathy within a few months after birth. Massive amounts of glycogen accumulate in skeletal and heart muscle, and these patients eventually die due to cardiorespiratory failure. Patients with late-onset Pompe disease (LOPD) who exhibit marked reduction of A $\alpha$ Glu activity are predominantly characterized by skeletal muscle dysfunction, but rarely show an involvement of cardiac muscle. The onset time and phenotypes of LOPD are variable, and patients often develop symptoms in the

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fifth decade or later in life [2]. Enzyme replacement therapy (ERT) is often used for the treatment of IOPD and LOPD [3, 4]. ERT should be started before symptoms are apparent, prior to the development of irreversible damage, to achieve optimal outcomes [5, 6]. Early initiation of ERT in IOPD markedly improves survival, reduces the need for ventilation, results in earlier independent walking, and enhances patient quality of life [7, 8].

Newborn screening (NBS) is often the best approach for early diagnosis and treatment. Asian populations have a high frequency of pseudodeficiency alleles c.[1726G>A; 2065G>A] in the *GAA* gene, and these pseudodeficiency alleles reduce  $\text{A}\alpha\text{Glu}$  activity into the abnormal range [9]. The frequency of pseudodeficiency alleles in the Japanese population is estimated to be 3.9% for homozygous alleles and 30.5% for heterozygous alleles [10]. Therefore, the presence of pseudodeficiency alleles interfered with NBS for Pompe disease in our previous pilot program [10]. Recent reports have suggested that the combination of  $\text{A}\alpha\text{Glu}$  assays using dried blood spots (DBSs) and *GAA* gene mutation analysis could be used to distinguish false-positive cases from patients with Pompe disease in an NBS program for Pompe disease [11].

We have conducted a large-scale NBS program for Pompe disease at Kumamoto and Fukuoka prefectures in Japan. Overall, 103,204 newborns were screened from April 2013 to November 2016 using DBS analysis with fluorometric assays [12]. Accordingly, in this study, we describe the analysis of  $\text{A}\alpha\text{Glu}$  activity and *GAA* gene sequencing in this population. We also discuss the usefulness of  $\text{A}\alpha\text{Glu}$  assays in skin fibroblasts, which are considered to be the gold standard for the diagnosis of patients with suspected Pompe disease [13]. Moreover, we summarize previous reports of *GAA* mutations in Japanese, Taiwanese, Korean, and Chinese populations and discuss the mutation profiles in Asian patients.

## Materials and methods

### Study population and materials

The study population consisted of 103,204 newborns born in Kumamoto and Fukuoka prefectures between April 2013 and November 2016. The DBSs were prepared in each maternity clinic or obstetric department using standard procedures at 4–6 days after birth for Newborn Mass Screening in the public health system. After dropping blood spots onto filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), the DBSs were dried for at least 4 h at room temperature and sent to the Newborn Screening Center at KM Biologics Co., Ltd. (Kumamoto, Japan) within 1 week after preparation. After analysis, the DBSs were transferred to Kumamoto University.

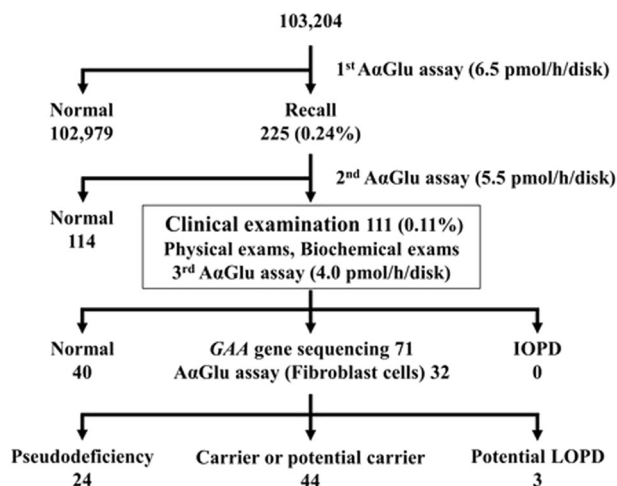


Fig. 1 Flow chart of newborn screening for Pompe disease

### NBS program for Pompe disease

The NBS using  $\text{A}\alpha\text{Glu}$  assays with DBSs for Pompe disease was performed in three steps (Fig. 1). In the first step, newborns with  $\text{A}\alpha\text{Glu}$  activity under the cutoff value of 6.5 pmol/h/disk were recalled, and their DBSs were prepared again. In the second step with the Ba/Zn method, newborns with  $\text{A}\alpha\text{Glu}$  activity under the cutoff value of 5.5 pmol/h/disk were called to the hospital within two months for the clinical examination. The newborns were examined by physical and biochemical assays to confirm symptomatic signs of IOPD, and the third  $\text{A}\alpha\text{Glu}$  assay also was performed. Finally, *GAA* gene sequencing was performed in newborns with  $\text{A}\alpha\text{Glu}$  activity under the cutoff value of 4.0 pmol/h/disk. Additionally, the  $\text{A}\alpha\text{Glu}$  activity of the fibroblast in the newborns was measured. The period after birth until the result of the first  $\text{A}\alpha\text{Glu}$  assay was acquired was 1–2 weeks, and the period until the result of the second  $\text{A}\alpha\text{Glu}$  assay was acquired was within 4 weeks, the period from birth until clinical examination was performed was within 2 months, and the period from birth until *GAA* gene analysis and final diagnosis was achieved was up to 6 months.

### AαGlu assay

The procedures for  $\text{A}\alpha\text{Glu}$  assays using DBSs and fibroblasts were described in our previous report [13]. Briefly, a single 3.2-mm diameter disk punched from DBSs was incubated in the well of a 96-well clear microwell-plate (Corning, NY, USA) with 100  $\mu\text{L}$  of 0.8 mM citrate/24 mM potassium phosphate buffer (pH 6.0) containing 0.1% Triton X-100 for 1 h at room temperature with gentle mixing. A 20  $\mu\text{L}$  aliquot of the extract was then added to 40  $\mu\text{L}$  of 2.0 mM 4-methylumbelliferyl  $\alpha\text{-D-}$

glucopyranoside (4MU- $\alpha$ Glc) in 0.12 M citrate/0.15 M potassium phosphate buffer (pH 4.0) containing 4.5  $\mu$ M acarbose (3.0  $\mu$ M final concentration) in a 96-well black microwell-plate (PerkinElmer). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was stopped by adding 190  $\mu$ L of 0.2 M glycine/NaOH buffer (pH 10.7) containing 0.1% Triton X-100 and 0.2% SDS to measure fluorescence intensity. For the Ba/Zn method, a 3.2-mm diameter disk was placed in a 1.5 mL reaction tube and gently mixed for 10 min at room temperature in 60  $\mu$ L of 0.12 M citrate/0.15 M potassium phosphate buffer (pH 4.0) containing 2.0 mM 4MU- $\alpha$ Glc and 3.0  $\mu$ M acarbose. The reaction mixture was then incubated at 37 °C for 24 h. Next, 30  $\mu$ L of 0.15 M barium hydroxide was added. The reaction tubes were mixed by vortexing and incubated at room temperature for 5 min. Thirty microliters of 0.15 M zinc sulfate was then added, and the tubes were mixed by vortexing and incubated for 10 min at room temperature. The tubes were then centrifuged for 5 min at 12,000 rpm and 5 °C. Finally, 90  $\mu$ L of the supernatant was transferred to a 96-well black microwell-plate, and 160  $\mu$ L of 0.4 M glycine/NaOH buffer (pH 10.7) containing 0.1% Triton X-100 was added to measure fluorescence intensity. Stock solutions of 0, 6.25, 12.5, 25, 50, and 100  $\mu$ M 4-methylumbelliferone (4MU) in 20 mM sodium phosphate buffer (pH 7.0) were used to calibrate the measurement of the liberated 4MU. The enzyme activity was expressed as picomoles of 4MU released per hour per disk (pmol/h/disk). Each assay was performed in duplicate.

Fibroblasts were prepared from a skin biopsy and cultured under standard conditions in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (50 kU/L penicillin and 50 mg/L streptomycin). After growing to confluence, the fibroblasts were harvested and washed with phosphate buffered saline. The cell pellet was stored at -80 °C until use. Fibroblasts ( $2\text{--}4 \times 10^6$  cells) were homogenized in 150  $\mu$ L water by sonication on ice, and 10  $\mu$ L of the cell homogenate was added to 40  $\mu$ L of the substrate solution containing 2.0 mM 4MU- $\alpha$ Glc in 0.12 M citrate/0.15 M potassium phosphate buffer (pH 4.0) containing 3.75  $\mu$ M acarbose (3.0  $\mu$ M in the final concentration) in a well of a 96-well black microwell-plate. The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by the addition of 200  $\mu$ L of 0.2 M glycine/NaOH buffer (pH 10.7) containing 0.1% Triton X-100 to measure fluorescence intensity, with correction for the substrate blank. The stock solution of 250  $\mu$ M 4MU in 20 mM sodium phosphate buffer (pH 7.0) was used to calibrate the measurement of liberated 4MU. The enzyme activity was expressed as nanomoles of 4MU released per hour per milligram of cellular protein (nmol/h/mg protein). Each assay was performed in duplicate.

## Sequencing of the *GAA* gene by NGS

The procedures for sequencing of the *GAA* gene by NGS were described in our previous report [14]. Briefly, genomic DNA was extracted from total blood using a Genra Puregene Blood Kit (Qiagen, Hilden, Germany) and stored at -80 °C until use. The 22-kbp region including the *GAA* gene was amplified by dividing the genomic DNA into three fragments using long-range polymerase chain reaction (Supplementary Fig. S1). The reaction was performed using a DNA polymerase (KOD FX; Toyobo, Osaka, Japan) and Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified using an Agencourt AMP XP PCR Purification Kit (Beckman Coulter, Brea, CA, USA) and quantified with a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) using a Qubit 2.0 Fluorometer (Life Technologies). Simultaneous fragmentation of PCR products and adaptor ligation were performed using a Nextera XT Kit (Illumina, San Diego, CA, USA). Indexed DNA was purified using an Agencourt AMP XP PCR Purification Kit (Beckman Coulter). Each library was validated using High Sensitivity D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA) with an Agilent 2200 TapeStation and quantified using a Qubit dsDNA HS Assay Kit with a Qubit 2.0 Fluorometer to allow for library normalization. Sequencing was performed with a MiSeq Reagent Kit v3 and a MiSeq sequencer (Illumina) using the "paired-end" sequencing run method. After sequencing runs, the data were aligned to target sequences on the human reference genome sequence using MiSeq Reporter software (Illumina). Sequence data analysis, mapping, and variant calling were streamlined using MiSeq Reporter v2 (Illumina). Briefly, reads were aligned to the reference sequence, from positions 80,101,882 to 80,123,207 of the genome sequence of chromosome 17 (NC\_000017.11), using bwa-0.6.1. Single-nucleotide polymorphism and insertion/deletion identifications were performed using the Genome Analysis Toolkit (GATK v1.6; Broad Institute, Cambridge, MA, USA). Visualization of sequencing reads was performed with IGV\_2.3.40 (Broad Institute).

## Resequencing of the *GAA* gene by the Sanger method

Variants detected in the *GAA* gene by NGS were resequenced by the Sanger method [15]. Briefly, a region including the variant was amplified by PCR using an appropriate set of primers. PCR products were sequenced on an ABI3500xl auto sequencer (Applied Biosystems) and analyzed using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

## Mutation analysis of the variants

The mRNA reference sequence (RefSeq) NM\_000152.4 was used for this study, whereby the “A” nucleotide of the ATG codon at nucleotide position 398 of the RefSeq constituted +1 numbering of the cDNA sequence. The ATG codon also represented +1 for the amino acid numbering as set forth by the A $\alpha$ Glu preprotein sequence NP\_000143.2. Mutation nomenclature followed the guidelines established by the Human Genome Variation Society (<http://varnomen.hgvs.org/>). The public databases, Pompe Center (<http://cluster15.erasmusmc.nl/klgn/pompe/mutations.html>, updated May 2016), and ClinVar [16] (<http://www.ncbi.nlm.nih.gov/clinvar>) were used for the classification of each variant. The software PolyPhen-2 [17] (<http://genetics.bwh.harvard.edu/pph2>) was used for missense mutations to predict the potential impact of an amino acid alteration on the function of A $\alpha$ Glu. Online bioinformatics tools, e.g., Human Splicing Finder [18] (<http://www.umd.be/HSF3/>) and Mutation Taster [19] (<http://www.mutationtaster.org>), were used to estimate the effects of mutations on splicing signals. The database of the 1,000 Genome Project (<http://browser.1000genomes.org>) and the Web application Mutation@A Glance [20] (<http://harrier.nagahama-i-bio.ac.jp/mutation/>) were used to estimate the variants as polymorphisms.

## Literature search

An electronic literature search of the PubMed database was performed for current and past findings of the *GAA* mutation in Japanese, Taiwanese, Korean, and Chinese patients for the years 1990 through 2018 using the keywords “acid alpha-glucosidase deficiency”, “acid maltase deficiency”, “Pompe disease”, “GAA”, “mutation”, “variant”, etc. When there were overlaps between multiple studies, the more relevant articles were used.

## Results

### NBS for Pompe disease

The flow chart and results for the NBS program are shown in Fig. 1. In total, 103,204 newborns were screened, 225 newborns were recalled for a second A $\alpha$ Glu assay, and 111 newborns with low A $\alpha$ Glu activities under the cutoff at the second A $\alpha$ Glu assay were examined at the outpatient clinic. These patients were evaluated to detect IOPD using physical and biochemical examinations (creatinine kinase, alanine transaminase, aspartate aminotransferase, and lactate dehydrogenase) and echocardiogram assessments at Kumamoto University Hospital or Fukuoka University Hospital, and the third A $\alpha$ Glu assay was performed. In this study, no

patients were found to have IOPD. For the 71 newborns with low A $\alpha$ Glu activity under the cutoff at the third A $\alpha$ Glu assay, *GAA* gene sequencing was performed using NGS. Moreover, the A $\alpha$ Glu activities in fibroblasts were measured in 32 of the 71 newborns.

### Variants detected in the newborns

The *GAA* gene is highly polymorphic, and many novel mutations are still being discovered. The Pompe Center contains a list of over 550 sequence variations in the version from May 2016. In this study, 50 variants were detected in the 71 newborns (Table 1). Thirty-seven of these 50 variants were registered at the Pompe Center. The other 13 variants, i.e., c.317G>A (p.R106H), c.705G>A (p.T235T), c.1082C>A (p.P361Q), c.1244C>T (p.T415M), c.2003A>G (p.Y668C), c.2055C>G (p.Y685\*), c.-260G>C, c.547-67C>G, c.547-39T>G, c.692 + 38C>T, c.858 + 8G>A, c.1552-52C>A, and c.1638 + 43G>T, were not registered. Five of the 13 variants, i.e., c.317G>A (p.R106H), c.705G>A (p.T235T), c.-260G>C, c.547-39T>G, and c.858 + 8G>A were registered at the ClinVar. Eight of the 13 variants, i.e., c.1082C>A (p.P361Q), c.1244C>T (p.T415M), c.2003A>G (p.Y668C), c.2055C>G (p.Y685\*), c.547-67C>G, c.692 + 38C>T, c.1552-52C>A, and c.1638 + 43G>T, were novel. Additionally, six missense variants, i.e., c.317G>A, c.1082C>A, c.1124G>A, c.1244C>T, c.1562A>T, and c.2003A>G, were predicted by PolyPhen-2 as “probably damaging”. The silent variant c.705G>A was predicted by the Human Splicing Finder [18] as a “potential alteration of splicing”.

Although Qiu et al. reported that the c.2132C>G variant was responsible for LOPD [21], and the polyphen-2 predicted the c.2132C>G variant as probably damaging (Table 1), we considered c.2132C>G as a non-pathogenic variant because this variant is registered as “non-pathogenic” at the Pompe Center, and the mother of subject ID 106, with homozygous variants of c.2132C>G, has never presented signs of LOPD. Therefore, we considered these seven variants (c.317G>A, c.705G>A, c.1082C>A, c.1124G>A, c.1244C>T, c.1562A>T, and c.2003A>G) as predicted pathogenic mutations. As a result, we classified 21 of the 50 variants as mutations or predicted pathogenic variants, and the other 29 variants were classified as polymorphisms or predicted non-pathogenic variants (Table 1). The most common mutation was c.752C>T+c.761C>T, which accounted for 20 alleles (14.1%, 20/142). The pseudodeficiency alleles c.1726G>A (p.G576S) and c.2065G>A (p.E689K) were detected in 71.8% (102/142) and 72.5% (103/142) of all newborns with low A $\alpha$ Glu activity, respectively. Additionally, 32 newborns had the homozygous c.1726G>A (p.G576S) mutation, 33 newborns had the homozygous c.2065G>A (p.E689K) mutation, and 32 newborns (45.1%)

**Table 1** The variants detected in NBS program for Pompe disease

Variant No.	Nucleotide change	Amino acid change	Location	Allele frequency (%)	Classification		ClinVar	PolyPhen2
					Pompe center			
mutation or predictably pathogenic variant	c.317G>A	p.R106H	Exon 2	7/142 (4.9)	NR	uncertain significance		1.000 (probably damaging)
	c.546G>T	p.T182=	Exon 2	4/142 (2.8)	r.spl potentially mild	likely pathogenic		most probably affecting splicing#
	c.705G>A	p.T235=	Exon 4	1/142 (0.7)	NR	uncertain significance		potential alteration of splicing#
	c.752C>T	p.S251L	Exon 4	20/142 (14.1)	[c.752C>T+c.761C>T] potentially less severe	likely pathogenic		0.004(benign)
	c.761C>T	p.S254L	Exon 4	20/142 (14.1)		likely pathogenic		0.997 (probably damaging)
	c.796C>T	p.P266S	Exon 4	1/142 (0.7)	potentially mild	NR		0.001 (benign)
	c.1082C>A	p.P361Q	Exon 7	1/142 (0.7)	NR	NR		1.000 (probably damaging)
	c.1124G>A	p.R375H	Exon 7	1/142 (0.7)	unknown	NR		1.000 (probably damaging)
	c.1190C>T	p.P397L	Exon 7	1/142 (0.7)	less severe	NR		1.000 (probably damaging)
	c.1244C>T	p.T415M	Exon 8	1/142 (0.7)	NR	NR		1.000 (probably damaging)
	c.1316T>A	p.M439K	Exon 8	4/142 (2.8)	potentially mild	likely pathogenic		0.096(benign)
	c.1562A>T	p.E521V	Exon 11	1/142 (0.7)	unknown	NR		1.000 (probably damaging)
	c.1669A>T	p.I557F	Exon 12	1/142 (0.7)	potentially less severe	NR		0.069(benign)
	c.1726G>A	p.G576S	Exon 12	102/142 (71.8)	pseudodeficiency allele	benign/likely benign		1.000 (probably damaging)
	c.1857C>G	p.S619R	Exon 13	1/142 (0.7)	less severe	NR		1.000 (probably damaging)
	c.2003A>G	p.Y668C	Exon 14	1/142 (0.7)	NR	NR		1.000 (probably damaging)
	c.2055C>G	p.Y685*	Exon 15	1/142 (0.7)	NR	NR		stop codon
	c.2065G>A	p.E689K	Exon 15	103/142 (72.5)	pseudodeficiency allele	Conflicting interpretations of pathogenicity		0.092(benign)
	c.2238G>C	p.W746C	Exon 16	1/142 (0.7)	potentially mild	pathogenic		1.000 (probably damaging)
	c.2560C>T	p.R854*	Exon 18	1/142 (0.7)	very severe	pathogenic		stop codon
	c.2647-7G>A	-	Intron 18	3/142 (2.1)	r.spl potentially mild	NR		most probably affecting splicing#
	c.-260G>C	non-coding	Exon 1	3/142 (2.1)	NR	likely benign		-
	c.324T>C	p.C108=	Exon 2	136/142 (95.8)	non-pathogenic	benign		-
	c.547-67C>G	-	Intron 2	101/130 (77.7)	NR	NR		no impact on splicing#
	c.547-39T>G	-	Intron 2	101/130 (77.7)	NR	benign		-
	c.547-4C>G	-	Intron 2	110/142 (77.5)	non-pathogenic	benign		-
	c.596A>G	p.H199R	Exon 3	111/142 (78.2)	non-pathogenic	benign		0.000(benign)
	c.668G>A	p.R223H	Exon 3	111/142 (78.2)	non-pathogenic	benign/likely benign		0.206(benign)
	c.692 + 38C>T	-	Intron 3	24/142 (16.9)	NR	NR		no impact on splicing#
	c.858 + 5 ins7(GCAGCGG)	-	Intron 4	39/132 (29.4)	r.spl? unknown	NR		no impact on splicing#
	c.858 + 8G>A	-	Intron 4	38/142 (26.7)	NR	benign		-
	c.858 + 30T>C	-	Intron 4	104/142 (73.2)	non-pathogenic	benign		-
	c.955 + 12G>A	-	Intron 5	110/142 (77.5)	non-pathogenic	benign		-
	c.1203G>A	p.Q401=	Exon 8	113/142 (79.6)	non-pathogenic	benign		-
	c.1327-18A>G	-	Intron 8	137/142 (96.5)	non-pathogenic	benign/likely benign		-
	c.1438-19G>C	-	Intron 9	111/142 (78.2)	non-pathogenic	benign		-
	c.1552-52C>A	-	Intron 10	103/142 (72.5)	NR	NR		no impact on splicing*
	c.1581G>A	p.R527=	Exon 11	5/142 (3.5)	non-pathogenic	benign/likely benign		-
c.1638 + 43G>T	-	Intron 11	23/142 (16.2)	NR	NR		probably no impact on splicing#	
c.1888 + 21G>A	-	Intron 13	102/142 (71.8)	non-pathogenic	benign		-	
c.2040 + 20A>G	-	Intron 14	139/142 (97.9)	non-pathogenic	benign		-	
c.2132C>G	p.T711R	Exon 15	1/142 (0.7)	non-pathogenic	NR		0.999 (probably damaging)	

Table 1 (continued)

Variant No.	Nucleotide change	Amino acid change	Location	Allele frequency (%)	Classification	ClinVar	PolyPhen2
40	c.2133A>G	p.T71I=	Exon 15	107/142 (75.3)	non-pathogenic	benign/likely benign	-
42	c.2331+20G>A	-	Intron 16	138/142 (97.2)	non-pathogenic	benign	-
43	c.2331+24T>C	-	Intron 16	128/142 (90.1)	non-pathogenic	likely benign	-
44	c.2338G>A	p.V780I	Exon 17	139/142 (97.9)	non-pathogenic	benign	0.000(benign)
45	c.2446G>A	p.V816I	Exon 17	26/142 (18.3)	non-pathogenic	benign/likely benign	0.008(benign)
46	c.2553G>A	p.G851I=	Exon 18	10/142 (7.0)	non-pathogenic	benign	-
49	c.2859G>A	non-coding	Exon 20	28/142 (19.7)	non-pathogenic	NR	-
50	c.*232C>T	non-coding	Exon 20	1/142 (0.7)	non-pathogenic	likely benign	-

c.752C>T (p.S251L) and c.761C>T (p.S254L) occurred in *cis* formation [11]. These variants were treated one mutation as c.752C>T+c.761C>T.

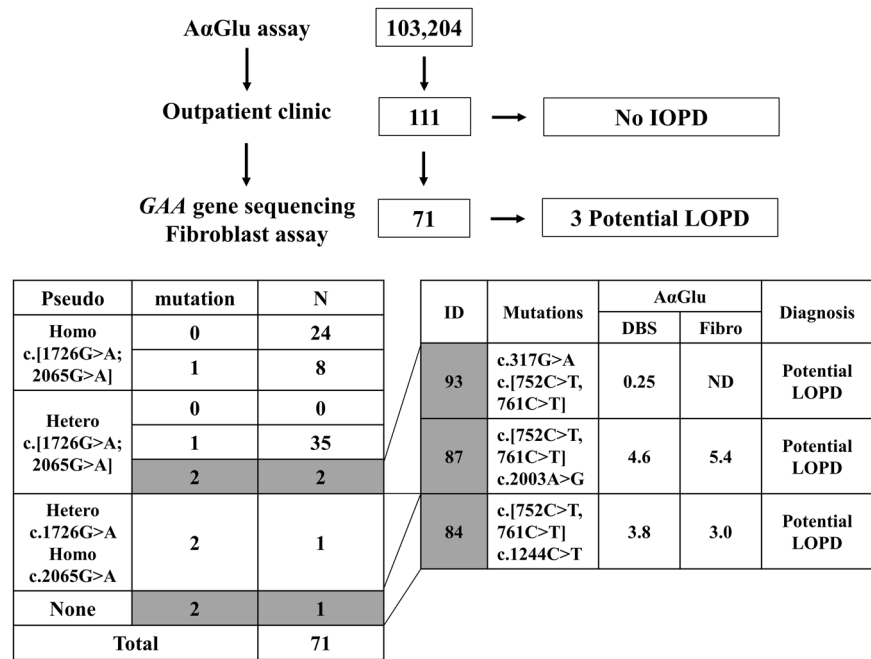
NR not registered, #Splicing Finder

had a combination of homozygous c.1726G>A (p.G576S) and c.2065G>A (p.E689K) mutations. Eight of 32 newborns had one mutation, and the other 24 newborns had no mutations. Additionally, 38 newborns had the heterozygous c.1726G>A (p.G576S) mutation, 37 newborns had the heterozygous c.2065G>A (p.E689K) mutation, and 37 newborns (52.1%) had a combination of heterozygous c.1726G>A (p.G576S) and c.2065G>A (p.E689K) mutations. Thirty-five of 37 newborns had one mutation, and the other two newborns had two mutations. One newborn had two mutations as a combination of heterozygous c.1726G>A (p.G576S) and homozygous c.2065G>A (p.E689K) mutations. Only one newborn with two mutations had no pseudodeficiency alleles.

Although we did not detect IOPD in any of the patients in this study, we did identify three newborns with potential LOPD (subject IDs 93, 87, and 84). Although these three patients developed no symptoms related to Pompe disease and received no treatment, we considered them as patients with potential LOPD based on the impact of variants predicted by PolyPhen-2. The  $\alpha$ Glu activities and gene mutations in the three patients with potential LOPD are shown in Fig. 2 and Table 2. The c.317G>A (p.R106H), c.1244C>T (p.T415M), and c.2003A>G (p.Y668C) mutations were considered novel mutations. The subject ID 8 was not considered a patient with potential LOPD because the  $\alpha$ Glu activity in fibroblasts was relatively normal, although the variants, i.e., c.705G>A (p.T235T) and c.2560C>T (p.R854\*), were considered as mutations by the Splicing Finder or the Pompe Center.

### Literature search and mutation profiles of the GAA gene in Asian patients

In this study, we collected information on GAA gene variants from 296 patients and 277 families with Pompe disease from 41 studies conducted in Asia. The numbers of families and articles from Japan, Korea, Taiwan, and China were 64 and 12, 13 and 4, 108 and 11, and 92 and 14, respectively. Additionally, 39, 15, 49, and 89 variants, including those in pseudodeficiency alleles, were reported from Japan, Korea, Taiwan, and China, respectively. Some variants were overlapped; thus, 156 variants were reported in Asia (Table 3). Thirty-nine variants were reported in 70 Japanese patients from 64 families (Supplementary Table 1). Five of 39 variants were not registered at the Pompe Center or ClinVar. Three of five were missense mutations and predicted as “probably damaging” by PolyPhen-2. Of the remaining two, c.547-1G>C was predicted as a “potential alteration of splicing” by the Human Splicing Finder, and c.756insT was predicted to be a frameshift mutation resulting in a stop codon. The first, second, and third most common variants were c.546G>T (allele frequency: 19.53%, 25/128), c.1798C>T (p.R600C)

**Fig. 2** Summary of newborn screening for Pompe disease

(14.84%, 19/128), and c.1857C>G (p.S619R) (11.72%, 15/128; Table 4). Among the 64 families, 32 were compound heterozygotes, and 22 were homozygotes, including five consanguineous families. The other ten families had one pathogenic variant and one unknown variant. Eleven and 53 of the 64 families had histories of IOPD and LOPD, respectively.

Fifteen variants were reported in 15 Korean patients from 13 families (Supplementary Table 2). Three of 15 variants were not registered at the Pompe Center or ClinVar. One of these three was a missense mutation and predicted as “benign” by PolyPhen-2, and the remaining two were frameshift mutations resulting in stop codons. The first and second common variants were c.1316T>A (p.M439K) (23.08%, 6/26) and c.1857C>G (p.S619R) (11.54%, 3/26; Table 4). Among the 13 families, 12 were compound heterozygotes, and one family had one pathogenic variant and one unknown variant. Ten and three of the 15 families had histories of IOPD and LOPD, respectively.

Forty-nine variants were reported in 110 Taiwanese patients from 108 families (Supplementary Table 3). All variants were registered at the Pompe Center or ClinVar. The first, second, and third most common mutations were c.1935C>A (p.D645E) (47.69%, 103/216), c.2238G>C (p.W746C) (7.87%, 17/216), and c.1411\_1414del (p.E471Pfs\*5) (5.56%, 12/216), respectively, excluding the pseudodeficiency allele c.1726G>A (19.91%, 43/216; Table 4). Among the 108 families, 72 were compound heterozygotes, and 29 were homozygotes, including one consanguineous family. The remaining seven families had one pathogenic variant and one unknown variant. Out of

108 families, 68 and 33 had histories of IOPD and LOPD, respectively.

Eighty-nine variants were reported in 101 Chinese patients from 92 families (Supplementary Table 4). Twenty-two of 89 variants were not registered at the Pompe Center or ClinVar. Thirteen of 22 were missense mutations and predicted as “probably damaging” for 10 of 13 and “benign” for three of 13 by PolyPhen-2. Eight of 22 were nonsense or frameshift mutations resulting in stop codons. The deletion mutant c.1320\_1322del (p.M440del) was predicted to be “disease-causing” by the Mutation Taster. The first, second, and third most common mutations were c.1935C>A (p.D645E) (10.87%, 20/184), c.2238G>C (p.W746C) (9.24%, 17/184), and c.2662G>T (p.E888\*) (8.15%, 15/184; Table 4). Among the 92 families, 80 were compound heterozygotes, and four were homozygotes. Eight families had one pathogenic variant and one unknown variant. Fifty-two and 40 of 92 families had histories of IOPD and LOPD, respectively.

### Relationship of variants detected in the newborns and patients

Eleven of 50 variants, i.e., c.546G>T, c.752C>T+c.761C>T, c.796C>T, c.1124G>A, c.1562A>T, c.1669A>T, c.1857C>G, c.2238G>C, c.2560C>T, c.2132C>G, and c.2446G>A, were reported in patients with Pompe disease. The c.546G>T mutation, being the most common mutation in Japanese patients (19.53%, 25/128), was present in 2.82% (4/142) of the newborns in our study. The c.1798C>T mutation, the second most common mutation in Japanese patients (14.84%, 19/128),





**Table 3** The mutations reported in Asian patients with Pompe disease

Variant No.	Amino acid change		Location	Classification		ClinVar	PolyPhen2
	Nucleotide change			Pompe center			
1	c.-32-13T>G	p.=, p.?	Intron 1	potentially mild	pathogenic	-	-
2	c.2T>C	p.MIT	Exon 2	potentially less severe	NR	0.276 (benign)	stop codon
3	c.118C>T	p.R40*	Exon 2	very severe	pathogenic	stop codon	stop codon
4	c.169C>T	p.Q57*	Exon 2	NR	likely pathogenic	0.000 (benign)	0.000 (benign)
5	c.199G>A	p.D67N	Exon 2	non-pathogenic	NR	stop codon	stop codon
6	c.241C>T	p.Q81*	Exon 2	very severe	NR	stop codon	stop codon
7	c.309C>A	p.C103*	Exon 2	very severe	NR	1.000 (probably damaging)	1.000 (probably damaging)
8	c.323G>A	p.C108S	Exon 2	unknown	NR	stop codon	stop codon
9	c.378G>A	p.W126*	Exon 2	very severe	NR	stop codon	stop codon
10	c.421C>A	p.L141M	Exon 2	potentially less severe	NR	0.650 (possibly damaging)	0.650 (possibly damaging)
11	c.424_440del	p.S142Lfs*29	Exon 2	very severe	NR	stop codon	stop codon
12	c.483dupC	p.K162Qfs*15	Exon 2	very severe	NR	stop codon	stop codon
13	c.503G>A	p.R168Q	Exon 2	unknown	uncertain significance	0.007 (benign)	0.007 (benign)
14	c.503G>C	p.R168P	Exon 2	unknown	NR	0.795 (possibly damaging)	0.795 (possibly damaging)
15	c.533G>A	p.R178H	Exon 2	unknown	NR	1.000 (probably damaging)	1.000 (probably damaging)
16	c.546G>T	r.sp1	Exon 2	potentially mild	likely pathogenic	-	-
17	c.546 + 5G>T	r.sp1?	Intron 2	unknown	NR	-	-
18	c.547-1G>C	-	Intron 2	NR	NR	Potential alteration of splicing <sup>a</sup>	Potential alteration of splicing <sup>a</sup>
19	c.568C>T	p.R190C	Exon 3	NR	NR	1.000 (probably damaging)	1.000 (probably damaging)
20	c.569G>A	p.R190H	Exon 3	less severe	likely pathogenic	1.000 (probably damaging)	1.000 (probably damaging)
21	c.655G>A	p.G219R	Exon 3	potentially less severe	pathogenic/likely pathogenic	1.000 (probably damaging)	1.000 (probably damaging)
22	c.670C>T	p.R224W	Exon 3	less severe	conflicting interpretations of pathogenicity	1.000 (probably damaging)	1.000 (probably damaging)
23	c.752C>T	p.S251L	Exon 4	less severe	likely pathogenic	0.004 (benign)	0.004 (benign)
24	c.761C>T	p.S254L	Exon 4	NR	likely pathogenic	0.997 (probably damaging)	0.997 (probably damaging)
25	c.756insT	p.P253Sfs*76	Exon 4	NR	NR	stop codon	stop codon
26	c.784G>A	p.E262K	Exon 4	potentially less severe	likely pathogenic	1.000 (probably damaging)	1.000 (probably damaging)
27	c.796C>T	p.P266S	Exon 4	potentially mild	NR	0.001 (benign)	0.001 (benign)
28	c.796C>A	p.P266T	Exon 4	NR	NR	0.018 (benign)	0.018 (benign)
29	c.811A>G	p.T271A	Exon 4	non-pathogenic	NR	0.955 (probably damaging)	0.955 (probably damaging)
30	c.827_845del19	p.I276Tfs*32	Exon 4	very severe	NR	stop codon	stop codon
31	c.859-2A>T	r.sp1	Intron 4	very severe	NR	-	-
32	c.871C>T	p.L291F	Exon 5	potentially less severe	NR	1.000 (probably damaging)	1.000 (probably damaging)
33	c.872T>C	p.L291P	Exon 5	potentially less severe	NR	1.000 (probably damaging)	1.000 (probably damaging)
34	c.875A>G	p.Y292C	Exon 5	potentially mild	likely pathogenic	1.000 (probably damaging)	1.000 (probably damaging)
35	c.893A>C	p.Y298S	Exon 5	unknown	NR	1.000 (probably damaging)	1.000 (probably damaging)
36	c.942C>A	p.N314K	Exon 5	NR	NR	1.000 (probably damaging)	1.000 (probably damaging)
37	c.971dupC	p.S325Efs*5	Exon 6	NR	NR	stop codon	stop codon
38	c.988T>G	p.W330G	Exon 6	potentially less severe	NR	0.955 (probably damaging)	0.955 (probably damaging)
39	c.998C>A	p.T333K	Exon 6	unknown	NR	0.573 (possibly damaging)	0.573 (possibly damaging)
40	c.1057C>T	p.Q353*	Exon 6	NR	NR	stop codon	stop codon
41	c.1062C>G	p.Y354*	Exon 6	very severe	NR	stop codon	stop codon
42	c.1080C>G	p.Y360*	Exon 7	very severe	NR	stop codon	stop codon

Table 3 (continued)

Variant No.	Nucleotide change	Amino acid change	Location	Classification		Clin Var	PolyPhen2
				Pompe center	Pathogenicity		
43	c.1082C>T	p.P361L	Exon 7	potentially less severe	pathogenic	1.000 (probably damaging)	
44	c.1099T>G	p.W367G	Exon 7	NR	NR	1.000 (probably damaging)	
45	c.1115A>T	p.H372L	Exon 7	potentially less severe	likely pathogenic	1.000 (probably damaging)	
46	c.1124G>A	p.R375H	Exon 7	unknown	NR	1.000 (probably damaging)	
47	c.1156C>T	p.Q386*	Exon 7	unknown	likely pathogenic	stop codon	
48	c.1194 + 2T>C	r.0?	Intron 7	very severe	likely pathogenic	–	
49	c.1197_1208del	p.V400_N403del	Exon 8	very severe	NR	–	
50	c.1201C>A	p.Q401K	Exon 8	NR	NR	0.999 (probably damaging)	
51	c.1241delT	p.F414Sfs*26	Exon 8	NR	NR	stop codon	
52	c.1261_1263Tms	p.R422P	Exon 8	NR	NR	0.212 (benign)	
53	c.1280T>C	p.M427T	Exon 8	unknown	NR	0.003 (benign)	
54	c.1286A>G	p.Q429R	Exon 8	unknown	benign/likely benign	0.000 (benign)	
55	c.1298A>C	p.Q433P	Exon 8	NR	NR	0.355 (benign)	
56	c.1309C>T	p.R437C	Exon 8	less severe	likely pathogenic	0.995 (probably damaging)	
57	c.1315_1317delATG	p.M440del	Exon 8	unknown	NR	–	
58	c.1316T>A	p.M439K	Exon 8	potentially mild	likely pathogenic	0.096 (benign)	
59	c.1320_1322del	p.M440del	Exon 8	NR	NR	disease causing <sup>b</sup>	
60	c.1322_1326 + 9del14	–	Exon 8	NR	NR	–	
61	c.1324G>A	p.V442M	Exon 8	unknown	NR	0.942 (possibly damaging)	
62	c.1355delC	p.S454Afs*23	Exon 9	very severe	NR	stop codon	
63	c.1356delC	p.S454Afs*23	Exon 9	NR	NR	stop codon	
64	c.1375G>A	p.D459N	Exon 9	unknown	Conflicting interpretations of pathogenicity	0.995 (probably damaging)	
65	c.1385T>C	p.L462P	Exon 9	unknown	NR	0.998 (probably damaging)	
66	c.1396delG	p.V466Ffs*11	Exon 9	very severe	NR	stop codon	
67	c.1396G>T	p.V466F	Exon 9	potentially less severe	NR	0.996 (probably damaging)	
68	c.1411_1414del	p.E471Pfs*5	Exon 9	very severe	likely pathogenic	stop codon	
69	c.1432G>A	p.G478R	Exon 9	potentially less severe	NR	1.000 (probably damaging)	
70	c.1437G>C	p.K479N	Exon 9	potentially less severe	NR	0.961 (probably damaging)	
71	c.1465G>T	p.D489W	Exon 10	unknown	NR	1.000 (probably damaging)	
72	c.1509_1511del	p.A504del	Exon 10	potentially less severe	NR	–	
73	c.1544T>A	p.M515K	Exon 10	unknown	NR	0.626 (possibly damaging)	
74	c.1551 + 2T>G	r.sp1	Intron 10	very severe	NR	–	
75	c.1551 + 3_1551 + 6delAAGT	p.Val480_Ile517del, p. = ?	Intron 10	potentially less severe	NR	–	
76	c.1561G>A	p.E521K	Exon 11	potentially less severe	pathogenic/likely pathogenic	1.000 (probably damaging)	
77	c.1562A>T	p.E521V	Exon 11	unknown	NR	1.000 (probably damaging)	
78	c.1574T>A	p.F525Y	Exon 11	potentially mild	NR	stop codon	
79	c.1582_3del2	p.G528Lfs*2	Exon 11	NR	NR	0.997 (probably damaging)	
80	c.1585-1586TC>GT	p.S529V	Exon 11	potentially mild	pathogenic	1.000 (probably damaging)	
81	c.1597T>G	p.C533G	Exon 11	NR	NR	stop codon	
82	c.1602_1605delinsAGG	p.N535Gfs*43	Exon 11	NR	NR	stop codon	
83	c.1634C>T	p.P545L	Exon 11	less severe	pathogenic	1.000 (probably damaging)	

Table 3 (continued)

Variant No.	Nucleotide change	Amino acid change	Location	Classification		Clin Var	PolyPhen2
				Pompe center			
84	c.1669A>T	p.L557F	Exon 12	very severe	NR	NR	0.069 (benign)
85	c.1696T>C	p.S566P	Exon 12	potentially less severe	NR	NR	0.999 (probably damaging)
86	c.1726G>A	p.G576S	Exon 12	pseudodeficiency allele	benign/likely benign		1.000 (probably damaging)
87	c.1735G>A	p.E579K	Exon 12	potentially less severe	NR	NR	0.995 (probably damaging)
88	c.1760T>C	p.L587P	Exon 13	unknown	NR	NR	1.000 (probably damaging)
89	c.1780C>T	p.R594C	Exon 13	NR	NR	NR	1.000 (probably damaging)
90	c.1781G>A	p.R594H	Exon 13	potentially less severe	NR	NR	1.000 (probably damaging)
91	c.1798C>T	p.R600C	Exon 13	less severe	NR	NR	1.000 (probably damaging)
92	c.1799G>A	p.R600H	Exon 13	potentially less severe	likely pathogenic		1.000 (probably damaging)
93	c.1799G>C	p.R600P	Exon 13	NR	NR	NR	1.000 (probably damaging)
94	c.1802C>T	p.S601L	Exon 13	potentially less severe	pathogenic		1.000 (probably damaging)
95	c.1814G>A	p.G605D	Exon 13	unknown	NR	NR	0.998 (probably damaging)
96	c.1822C>T	p.R608*	Exon 13	very severe	NR	NR	stop codon
97	c.1826dup	p.Y609*	Exon 13	very severe	likely pathogenic		stop codon
98	c.1827C>G	p.Y609*	Exon 13	very severe	NR	NR	stop codon
99	c.1832G>A	p.G611D	Exon 13	potentially less severe	likely pathogenic		1.000 (probably damaging)
100	c.1843G>A	p.G615R	Exon 13	potentially less severe	likely pathogenic		1.000 (probably damaging)
101	c.1844G>T	p.G615V	Exon 13	NR	NR	NR	1.000 (probably damaging)
102	c.1846G>A	p.D616N	Exon 13	unknown	NR	NR	1.000 (probably damaging)
103	c.1857C>G	p.S619R	Exon 13	less severe	NR	NR	1.000 (probably damaging)
104	c.1924G>A	p.V642I	Exon 14	NR	NR	NR	0.245 (benign)
105	c.1927G>A	p.G643R	Exon 14	potentially less severe	pathogenic		1.000 (probably damaging)
106	c.1933G>C	p.D645H	Exon 14	potentially less severe	likely pathogenic		1.000 (probably damaging)
107	c.1935C>A	p.D645E	Exon 14	potentially less severe	pathogenic		1.000 (probably damaging)
108	c.1942G>A	p.G648S	Exon 14	potentially less severe	likely pathogenic		1.000 (probably damaging)
109	c.1958C>A	p.T653N	Exon 14	unknown	NR	NR	1.000 (probably damaging)
110	c.1960T>C	p.S654P	Exon 14	potentially less severe	NR	NR	0.854 (possibly damaging)
111	c.1978C>T	p.R660C	Exon 14	potentially less severe	NR	NR	1.000 (probably damaging)
112	c.1979G>A	p.R660H	Exon 14	potentially less severe	pathogenic/likely pathogenic		1.000 (probably damaging)
113	c.1993G>A	p.G665R	Exon 14	unknown	NR	NR	1.000 (probably damaging)
114	c.2012T>A	p.M671K	Exon 14	unknown	NR	NR	0.785 (possibly damaging)
115	c.2014C>T	p.R672W	Exon 14	less severe	pathogenic/likely pathogenic		1.000 (probably damaging)
116	c.2015G>A	p.R672Q	Exon 14	less severe	likely pathogenic		1.000 (probably damaging)
117	c.2024A>G	p.N675S	Exon 14	NR	NR	NR	1.000 (probably damaging)
118	c.2024_2026del	p.N675del	Exon 14	potentially less severe	likely pathogenic		-
119	c.2040 + 1G>T	r.spl?	Intron 14	very severe	likely pathogenic		-
120	c.2051C>A	p.P684Q	Exon 15	NR	NR	NR	1.000 (probably damaging)
121	c.2065G>A	p.E689K	Exon 15	pseudodeficiency allele	conflicting interpretations of pathogenicity		0.092 (benign)
122	c.2104C>T	p.R702C	Exon 15	potentially less severe	Likely pathogenic		1.000 (probably damaging)
123	c.2105G>A	p.R702H	Exon 15	potentially mild	pathogenic		1.000 (probably damaging)
124	c.2132C>G	p.T711R	Exon 15	non-pathogenic	likely benign		0.999 (probably damaging)

Table 3 (continued)

Variant No.	Nucleotide change	Amino acid change	Location	Classification		Clin Var	PolyPhen2
				Pompe center			
125	c.2161G>T	p.E721*	Exon 15	very severe	NR	stop codon	
126	c.2167G>A	p.V723M	Exon 15	unknown	NR	1.000 (probably damaging)	
127	c.2171C>A	p.A724D	Exon 15	unknown	NR	1.000 (probably damaging)	
128	c.2174G>C	p.R725P	Exon 15	potentially less severe	NR	1.000 (probably damaging)	
129	c.2177C>G	p.P726R	Exon 15	unknown	NR	1.000 (probably damaging)	
130	c.2185delC	p.L729Wfs*8	Exon 15	very severe	likely pathogenic	stop codon	
131	c.2214G>A	p.W738*	Exon 16	very severe	likely pathogenic	stop codon	
132	c.2235dupG	p.W746Vfs*50	Exon 16	NR	NR	stop codon	
133	c.2237G>A	p.W746*	Exon 16	very severe	pathogenic	stop codon	
134	c.2238G>C	p.W746C	Exon 16	potentially mild	pathogenic	1.000 (probably damaging)	
135	c.2274dup	p.G759Rfs*37	Exon 16	very severe	NR	stop codon	
136	c.2297A>C	p.Y766S	Exon 16	NR	pathogenic	0.999 (probably damaging)	
137	c.2297A>G	p.Y766C	Exon 16	potentially less severe	uncertain significance	1.000 (probably damaging)	
138	c.2303C>T	p.P768L	Exon 16	potentially less severe	NR	1.000 (probably damaging)	
139	c.2326C>T	p.Q776*	Exon 16	very severe	NR	stop codon	
140	c.2376_2377insCA	p.P793Hfs*14	Exon 17	NR	NR	stop codon	
141	c.2380delC	p.R794Vfs*12	Exon 17	very severe	NR	stop codon	
142	c.2407_2413del17	p.Q803*	Exon 17	unknown	NR	stop codon	
143	c.2411G>A	p.W804*	Exon 17	NR	NR	stop codon	
144	c.2431delC	p.L811Wfs*37	Exon 17	very severe	NR	stop codon	
145	c.2432delT	p.L811Rfs*36	Exon 17	very severe	NR	stop codon	
146	c.2446G>A	p.V816I	Exon 17	non-pathogenic	benign/likely benign	stop codon	
147	c.2481G>A	p.Q827H	Exon 17	NR	NR	0.008 (benign)	
148	c.2481 + 1G>A	r.0?	Intron 17	very severe	NR	1.000 (probably damaging)	
149	c.2560C>T	p.R854*	Exon 18	very severe	pathogenic	stop codon	
150	c.2563G>C	p.G855R	Exon 18	NR	NR	1.000 (probably damaging)	
151	p.2585delG	p.G862Nfs*24	Exon 18	NR	NR	stop codon	
152	c.2662G>T	p.E888*	Exon 19	very severe	NR	stop codon	
153	c.2738C>T	p.P913L	Exon 19	NR	NR	0.997 (probably damaging)	
154	c.2812_2813delTG	p.C938 = fs	Exon 20	very severe	NR	stop codon	
155	c.2815_2816del	p.V939Lfs*78	Exon 20	very severe	pathogenic	stop codon	
156	c.2842insT	p.L948Sfs*70	Exon 20	very severe	NR	stop codon	

NR not registered

<sup>a</sup>Splicing Finder<sup>b</sup>Mutation taster

**Table 4** Allele frequency of *GAA* gene in IOPD and LOPD in each country

Country	Mutation	Allele frequency			IOPD/LOPD
		Total	IOPD	LOPD	
Japan	c.546G>T	19.53% (25/128)	0% (80/22)	23.58% (25/106)	11/53
	c.1798C>T	14.84% (19/128)	31.82% (7/22)	11.32% (12/106)	
	c.1857C>G	11.72% (15/128)	18.18% (4/22)	10.38% (11/106)	
Taiwan	c.1935C>A	47.69% (103/216)	57.35% (78/136)	27.27% (18/66)	68/33
	c.2238G>C	7.87% (17/216)	0.74% (1/136)	27.73% (15/66)	
	c.1411_1414del	5.56% (12/216)	8.09% (11/136)	0% (0/66)	
China	c.1935C>A	10.87% (20/184)	15.38% (16/104)	5.71% (4/70)	52/35
	c.2238G>C	9.24% (17/184)	0% (0/104)	22.86% (16/70)	
	c.2662G>T	8.15% (15/184)	10.58% (11/104)	5.71% (4/70)	
Korea	c.1316T>A	23.08% (6/26)	20% (4/20)	33.33% (2/6)	10/3
	c.1857C>G	11.54% (3/26)	10% (2/20)	16.67% (1/6)	

low  $\text{A}\alpha\text{Glu}$  activity was 11.6-times higher than that of normal newborns (3.9%) [10]. Additionally, 24 newborns (33.8%, 24/71) had no mutated alleles but harbored homozygous c.[1726G>A; 2065G>A], defined as a pseudodeficiency. The other eight had one mutation and the homozygous c.[1726G>A; 2065G>A] allele. Moreover, 35 (49.3%) had one mutation and the heterozygous c.[1726G>A; 2065G>A] allele and two (subject IDs 87 and 93) had two mutated alleles with heterozygous c.[1726G>A; 2065G>A]. One subject (subject ID 84) had two mutated alleles without c.[1726G>A; 2065G>A]. These results showed that the pseudodeficiency alleles c.[1726G>A; 2065G>A] significantly affected NBS owing to high recall and false-positive rates, suggesting that a combination of *GAA* gene sequencing and enzyme assays is necessary for NBS in Pompe disease. Interestingly, in newborns with pseudodeficiency alleles, 70 of 71 newborns had compound homozygous or heterozygous pseudodeficiency alleles c.[1726G>A; 2065G>A] in this study. Labrousse et al. [11] analyzed the *GAA* genes of 107 newborns, screened from an NBS of 132,538 newborns, and detected 54 types of variants. Thirty-six of 107 newborns (33.6%) had pseudodeficiency alleles c.[1726G>A; 2065G>A]. Additionally, in Taiwan, the pseudodeficiency alleles c.[1726G>A; 2065G>A] had a significant effect on NBS.

In our study, four newborns (subject IDs 8, 84, 87, and 93) were found to have two mutations (Table 2). Subject ID 8 was diagnosed as a carrier because normal  $\text{A}\alpha\text{Glu}$  activity in fibroblasts was observed. Subject IDs 93 and 84 were confirmed as compound heterozygotes by sequencing their parents' genes. The c.317G>A and c.752C>T+c.761C>T of subject ID 93 were derived from father and mother, respectively. The c.752C>T+c.761C>T and c.1244C>T of subject ID 84 were derived from mother and father, respectively. Unfortunately, informed consent was not approved in subject ID 87. In compliance with diagnosis guidelines [22, 23], we diagnosed these three subjects as

potential LOPD patients. As of November 2018, these patients' ages are 2 years 7 months (subject ID 93), 3 years (subject ID 87), and 3 years 1 month (subject ID 84), and no symptoms related to Pompe disease have been observed. The use of ERT in patients with pre-symptomatic LOPD is controversial. Although ERT is necessary and effective in patients with LOPD who exhibit symptoms related to Pompe disease, further studies are needed to determine whether ERT should be applied in pre-symptomatic patients with Pompe disease.

Bodamer et al. [24] summarized an NBS for Pompe disease in Taiwan ( $n = 473,738$ ) and the USA ( $n = 980,507$ ). The frequencies of IOPD and potential LOPD were 1/52,637 and 1/24,933 (Taiwan) and 1/122,563 and 1/15,563 (USA), respectively. In our study, the frequencies of IOPD and potential LOPD were estimated to be less than <1/103,204 and <1/34,401, respectively. Although further studies are needed to confirm these findings, the onset frequency of IOPD and LOPD is expected to be significantly lower in Japan than those of other countries.

A literature search revealed some of the characteristics of *GAA* mutation profiles in Asian patients with Pompe disease. Some typical mutations were found in patients from Europe and the USA. The c.525delT mutation (p. E176Rfs\*45) in Dutch patients [25], was not detected in Asian patients. The c.2560C>T (p.R854\*) mutation, which is detected in many affected African or African-American patients with IOPD [26], was rarely detected in Asian patients (one allele in a Chinese patient and one allele in this study). The c.-32-13T>G mutation, which has been detected in adult Caucasians [27], also was rarely detected in Asian patients (one allele in a Chinese patient).

The mutation profile of the *GAA* gene was different in each Asian country. In Japan, the first most common mutation, c.546G>T, is a leaky splice mutation that produces a normally spliced transcript and is responsible for the low expression level (approximately 10%) of the active

enzyme [28]. This mutation may also correspond with the phenotype of LOPD. The c.1798C>T mutation, which was the second most common mutation in Japan, was previously reported as a common variant in Japanese patients [15]. The c.1935C>A mutation, the most common mutation in Taiwan, is known as a typical example of the founder effect, which was previously reported in Taiwanese patients [29]. The second most common mutation in Taiwan was c.2238G>C, and a patient with homozygous c.2238G>C alleles was previously reported in Taiwan [30]. The first and second most common variants were the same in Taiwan and China, potentially because Taiwan and China have a common ethnic background and because some Chinese groups have moved to Taiwan. The ratios of IOPD to LOPD (n/n) were 0.21 (11/53), 3.33 (10/3), 2.06 (68/33), and 1.49 (52/35) in Japan, Korea, Taiwan, and China, respectively. The onset frequency of IOPD in Japanese patients was significantly lower than those from Taiwan and China (one-tenth and one-seventh, respectively). This may be a characteristic of the Japanese population or a result of under-diagnosis of LOPD in the other countries. Moreover, the most common variant detected in patients with IOPD, and LOPD differed. In Japan, the most common variant in patients with LOPD (c.546G>T) was not detected in patients with IOPD. Similarly, the second most common variant in LOPD (c.2238G>C) was very rare in patients with IOPD in Taiwan and China. These findings suggested that c.546G>T and c.2238G>C may be characteristic variants in LOPD.

There was one limitation to our literature search, namely, that one mutation was detected, but other mutations were unknown in 26 of 277 families. The reason for this discrepancy may be related to the sequencing method used in these studies; indeed, in all 41 articles, exons and intron/exon boundaries of the *GAA* gene were amplified by PCR and sequenced. Theoretically, some mutations in the 5', 3', and intronic regions are not detectable. Thus, the use of NGS may increase the detection rate and generate more reliable results. Further studies are needed to evaluate the roles of patient ethnicity or characteristics.

The method for A $\alpha$ Glu activity assays with 4MU- $\alpha$ Glc used in this study could not effectively distinguish LOPD from pseudodeficiency or carriers, even if A $\alpha$ Glu activity was analyzed in fibroblasts. Some researchers have reported that liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an effective method for A $\alpha$ Glu activity assays and can permit LOPD to be distinguished from pseudodeficiency or carriers [31, 32]. Therefore, in future studies, it will be necessary to develop a practical screening method using the combination of 4MU- $\alpha$ Glc and LC-MS/MS for analysis of DBSs (e.g., where LC-MS/MS is performed in the second and third A $\alpha$ Glu assays).

In conclusion, in this study, we performed NBS for Pompe disease in 103,204 newborns and detected three patients with potential LOPD, and no patients with IOPD. The *GAA* gene mutation locus, which we detected in the middle Kyusyu area and was reported from patients with Pompe disease in Japan and neighboring countries, was different. Therefore, there are fewer patients with IOPD in Japan than in neighboring countries, and the onset frequencies of IOPD and LOPD in Japan are different from those in the middle Kyusyu area in our study. These results may reflect the differences in genetic backgrounds between Japanese patients and those from other countries as shown by our literature study.

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## Compliance with ethical standards

**Ethical approval** This study was approved by the Ethics Committee of Kumamoto University (approval no. 1537). Written informed consent was obtained from the parents or legal guardians of newborns.

**Conflict of interest** The authors declare that they have no conflict of interest.

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