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Case Report

GAA variants associated with reduced enzymatic activity but lack of Pompe-related symptoms, incidentally identified by exome sequencing

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ABSTRACT

Pompe disease is a rare metabolic myopathy caused by pathogenic variants affecting the activity of the lysosomal glycogen-degrading enzyme acid alpha-glucosidase (GAA). Impaired GAA function results in the accumulation of undegraded glycogen within lysosomes in multiple tissues but predominantly affects the skeletal, smooth and cardiac muscle. The degree of residual enzymatic activity appears to roughly correlate with the age of onset and the severity of the clinical symptoms.

Here, we report four siblings in which the *GAA* variants NM_000152.5:c.2237G > C p.(Trp746Ser) and NM_000152.5:c.266G > A p.(Arg89His) were identified as an incidental finding of clinical exome sequencing. These variants are listed in the ClinVar and the Pompe disease *GAA* variant databases but are reported here for the first time in compound heterozygosity. All four siblings displayed normal urine tetrasaccharide levels and no clinical manifestations related to Pompe disease. Nevertheless, GAA enzymatic activity was within the range for late onset Pompe patients.

Our report shows an association between a novel genotype and attenuated GAA enzymatic activity. The clinical significance can only be established by the regular monitoring of these individuals. The study highlights the major challenges for clinical care arising from incidental findings of next generation sequencing.

1. Introduction

Pompe disease (PD), also known as glycogen storage disease type II (MIM #232300) is a rare, autosomal recessive metabolic disorder caused by the complete or partial deficiency of acid alpha-glucosidase (GAA), resulting in the lysosomal accumulation of undegraded glycogen [1]. To date >640 disease-associated variants in the *GAA* gene have been identified and are listed in the "Pompe disease *GAA* variant database" (http://www.pompevariantdatabase.nl) [2]. These are distributed throughout the whole gene and mainly include missense variants, small deletions and nonsense variants. PD displays a broad spectrum of clinical phenotypes with significant variations in the age of onset, the severity of symptoms and the rate of progression [3]. Disease presentations are mainly classified in two major forms, infantile-onset PD (IOPD) and late-onset PD (LOPD) which appear to be roughly determined by the amount of residual enzyme activity [3,4]. IOPD is characterized by a nearly complete lack of enzyme activity (<1% of normal mean activity) and manifests during the first year of life with hypertrophic cardiomyopathy, generalized muscle weakness, hepatomegaly, macroglossia and respiratory insufficiency [1,3,4]. Without enzyme replacement therapy (ERT) IOPD patients usually die within the first year of life due to cardiopulmonary failure. On the other hand, LOPD is caused by pathogenic *GAA* variants which do not completely abolish enzyme activity and presents with an onset of symptoms

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anytime from infancy to adulthood. These include slowly progressive muscle weakness and respiratory dysfunction without cardiomyopathy [1,3,4].

Enzyme replacement therapy (ERT) is the standard treatment for patients with PD [4]. Despite the variations in the response of tissues and the heterogeneity of the therapeutic outcome among patients, ERT prolongs survival and improves cardiac structure, muscle performance and respiratory function. The success of ERT in ameliorating PD pathology and improving the quality of life of patients led to the inclusion of PD in newborn screening programs. A consequence of newborn screening for PD is that a number of potential LOPD patients are nowadays identified while being asymptomatic and for which it cannot be predicted if and when they will develop disease-related symptoms. This poses a considerable challenge for the management of these individuals since no data are currently available on whether ERT on asymptomatic LOPD patients delays or prevents the onset of symptoms [4]. These individuals therefore require regular clinical follow-up so that they are placed on ERT as soon as symptoms develop, according to consensus guidelines [5].

Here we report four siblings from a Greek Cypriot family in whom *GAA* variants have been incidentally detected by clinical exome sequencing. All four subjects displayed significantly attenuated GAA enzymatic activity, within the range of LOPD patients but normal levels of urine tetrasaccharide and no clinical manifestations related to PD.

2. Materials and methods

2.1. Clinical exome and sanger sequencing

Genomic DNA was extracted from peripheral blood samples using the QIAmp DNA Blood Midi Kit (Qiagen, Toronto, ON, Canada), after obtaining written informed consent. DNA libraries for clinical exome sequencing (CES) for parents and subjects 1 and 2, were prepared using the TruSight One sequencing panel (Illumina, San Diego, CA, USA) according to the manufacturer's protocol guidelines. Paired-end sequencing of the pooled libraries was performed on a NextSeq 500 system (Illumina) using the High Output Kit v2.5 (300 Cycles) according to the manufacturer's guidelines (Illumina, San Diego, CA, USA). Demultiplexing and adapter trimming was performed automatically using BaseSpace Sequencing Hub Apps (Illumina, San Diego, CA, USA). Bioinformatics processing, analysis, annotation and interpretation was performed by VarSome Clinical platform (Version: 11.4) using the human reference genome build hg19. Variants were classified according to the ClinGen Lysosomal Storage Disorders Expert Panel Specifications to the ACMG/AMP Variant Interpretation Guidelines Version 2 (https://clinicalgenome.org/site/assets/files/6993/clingen_lsd_acmg_specifications_v2-1.pdf).

The two variants were confirmed by conventional polymerase chain reaction (PCR) followed by automated sequencing of exons 2 and 16 of the *GAA* gene. PCR amplification was carried out using Amplitaq Gold DNA polymerase (Applied Biosystems, Thermo Fisher Scientific) and specifically designed primers (Primer 3 web tool), flanking the two variants. Amplification products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Thermo Fisher Scientific) and bidirectional Sanger sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific). The sequencing reactions were subjected to clean-up using Performa® DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD, USA) and capillary electrophoresis was performed on the Applied Biosystems 3500XL Genetic Analyzer. The resulting data were compared to the normal *GAA* sequence as listed in the GenBank database (NM_000152.5). Primers and PCR conditions used are available upon request.

2.2. Measurement of creatine kinase (CK) levels in serum

CK levels in serum were determined by means of a kinetic spectrophotometric assay using the CK-NAC FS kit (Diagnostic Systems) according to the instructions of the manufacturer.

2.3. Determination of GAA activity in leukocytes

GAA activity was determined in leukocytes, using either rabbit liver glycogen or 4-methylumbelliferyl-α-D-glucoside (4-MU-αGlc) as substrate, essentially as described previously [6]. For the assay with glycogen as substrate, cell homogenates were incubated with 3.3% *w/v* glycogen in the presence of 3 μM acarbose in McIlvain's Pi/Ci buffer, pH 4.0 for 2 h. The reaction was terminated by boiling for 2 min. The amount of liberated glucose was determined using a glucose oxidase/peroxidase system and 2,2′-azino-di-[3-ethylbenzthiazolinsulfonate-6 as substrate and by measuring the absorbance at 420 nm. GAA activity with 4-MU-αGlc as substrate was determined by measuring the fluorescence emission (excitation 365 nm, emission 448 nm), following the incubation of cell homogenates with 1.5 mM 4-MU-αGlc in the presence of 3 μM acarbose.

2.4. Determination of glucose tetrasaccharide levels in urine

Quantitative determination of the tetrasaccharide 6-α-D-glucopyranosyl-maltotriose (Glcα1-6Glcα1-4Glcα1-4Glc) was performed by means of ultraperformance liquid chromatography, tandem mass spectrometry (UPLC-MS/MS), as previously described [7]. Briefly, urine samples were diluted in 0.1% ammonium hydroxide and filtered. Samples were introduced into a BEH amide column and the glucose tetrasaccharide was identified and quantified by tandem mass spectrometry by monitoring the mass transition *m/z* 665/179 in negative ionization mode.

3. Case report

Subject 1, currently at the age of 13 years is the firstborn male child of unrelated parents. He was referred to the genetics clinic at the age of 5 years for developmental delay, decreased social interaction, lack of eye contact, limited speech and toe-walking. He was born with a birth weight of 2470 g after an uneventful pregnancy and delivery. On physical examination he was found to have a triangular face and hypospadias but otherwise nothing remarkable. The family history was also unremarkable.

Subject 2, currently at the age of 11 years, is the second male child of the family and was referred at the same time, at the age of 3 years, to the genetics clinic. Compared to his older brother, he displayed a more severe developmental delay, absence of speech, autistic behavior and symptoms of psychiatric disorder. Possible febrile seizures were also reported. He was also noticed to have a triangular face and hypospadias.

Routine blood analysis and biochemistry were normal for both subjects. Metabolic (plasma amino acids, acylcarnitines, urine organic acids) and genetic (karyotype, array CGH, Fragile-X) investigations did not reveal any abnormality. The children were then referred for clinical exome sequencing which did not identify any pathogenic variants related to the clinical presentation. However, both siblings were found to carry biallelic missense variants in *GAA*, c.266G > A p.(Arg89His) and c.2237G > C p.(Trp746Ser). Subsequently, parent testing showed that the mother carries the c.266G > A p.(Arg89His) and the father carries c.2237G > C p.(Trp746Ser). The above findings raised the suspicion of PD and both siblings underwent a cardiological and neurological examination which did not reveal any pathological findings. No signs of myopathy were noted. Both siblings were able to reach the highest score when evaluated with the quick motor function test (QMFT). In a 6-minute walk test (6MWT) Subject 1 walked a distance of 490 m whereas Subject 2 did not cooperate and was therefore not evaluated. Blood

creatine kinase (CK) levels were within the normal range for both patients (Table 1).

The c.2237G > C missense variant replaces Trp746 with serine. Trp746 lies within the proximal C-terminal domain of the GAA enzyme and is highly conserved across different species (Fig. 1). This variant is present at a very low frequency (<0.0001) in gnomAD databases, only in heterozygosity. It has been previously detected in one PD patient with Caucasian/North European origin [8]. Moreover, a different variant affecting the same amino acid c.2238G > C p.(Trp746Cys) was identified as a common mutation specifically associated with non-infantile PD in China [9]. The c.2237G > C variant was further shown to result in a significant reduction of GAA activity to 8% of the normal, in an *in vitro* functional assay [10]. Additional variants identified in PD patients affecting the same amino acid position include c.2236 T > G p.(Trp746Gly) [11], c.2238G > A p.(Trp746Ter) [12] and c.2236 T > C p.(Trp746Arg) [13], indicating that alterations at the specific amino acid position are clinically significant. The c.2237G > C p.(Trp746Ser) variant is classified as pathogenic or likely pathogenic in ClinVar (with 9 submissions to date) and predicted as "Not tolerated" by SIFT (https://sift.bii.a-star.edu.sg/), "Probably damaging" by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and "Disease Causing" by MutationTaster (https://www.mutationtaster.org/). Additional meta-score tools such as BayesDel [14], MetaRNN [15] and REVEL [16] strongly support the pathogenicity of the variant.

The c.266G > A variant replaces a highly conserved arginine (Arg89) residue with histidine within the trefoil type-P domain of GAA (Fig. 1). According to gnomAD the c.266G > A variant is also very rare with an allele frequency of <0.0001. It has been previously identified in individuals with clinical features of PD [17–19] and also by newborn screening programs [20,21] however, its pathogenicity is equivocal. Furthermore, it was shown to have only a modest impact on GAA activity in *in vitro* studies resulting in a residual activity of 43.5% of the normal [22]. Based on the above, the variant is classified as variant of uncertain significance (VUS) in ClinVar. Nevertheless, similar to the Trp746Arg variant, it is predicted as "Not tolerated", "Probably damaging" and "Disease causing" by SIFT, PolyPhen-2 and MutationTaster, respectively and as moderately pathogenic by meta-score *in silico* tools.

To gain insights into the functional consequences of the identified variants we measured GAA enzymatic activity in the above siblings, their parents as well as the two younger, apparently healthy daughters of the family (subjects 3 and 4, currently at the age of 10 and 1 years, respectively). Subjects 3 and 4 were clinically evaluated and found to display no abnormalities. In addition, their blood CK values were also within the normal range (Table 1). With 4-MU-αGlc as substrate, GAA activity was found to be clearly within the patient range for all four Subjects (Table 1). Activity values using glycogen as substrate were slightly above the upper limit for LOPD patients for three of the subjects but within the range for LOPD patients for Subject 4 (Table 1).

The parents displayed comparable GAA activity that was clearly above the patient range but below the values of normal controls, using both substrates (Table 1). The measured GAA activity values suggest that compound heterozygosity for the identified variants significantly reduces enzymatic activity. Consistent with the attenuated GAA activity levels, the two healthy siblings were found to carry the same variants as their brothers. The presence of additional variants associated with reduced GAA activity such as the common K_M-mutant polymorphism c.271G > A also known as the *GAA2* allele [23], and the Asian pseudodeficiency variant c.[1726G > A p.(Gly576Ser); 2065G > A p.(Glu689Lys)] [24] was excluded. To our knowledge the c.2237G > C p.(Trp746Ser) and c.266G > A p.(Arg89His) variants are reported here for the first time in compound heterozygosity.

Although not specific for PD, the levels of the tetrasaccharide 6-α-D-glucopyranosyl-maltotriose (Glc4) in urine appear to correlate with glycogen storage in skeletal muscle and are therefore used as a diagnostic biomarker and also to monitor disease severity and response to treatment in PD patients [25]. Consistent with the lack of clinical manifestations related to PD, urinary Glc4 levels were within the normal range for all four subjects (Table 1).

4. Discussion

The availability of an effective treatment by means of ERT combined with improved laboratory methods for the diagnosis of PD compatible with the use of dried blood spots (DBS) enabled the introduction of newborn screening for PD. Where applied, individuals with low GAA activity are currently identified at a presymptomatic stage and can benefit from regular clinical monitoring and early intervention with the first signs of disease-related symptoms. Determination of GAA activity in DBS is a cheap, fast and non-invasive method which is used as a first line diagnostic test for PD. A drawback of DBS testing is the high rate of false positive results [26]. Therefore, it is recommended that GAA deficiency is confirmed in other tissues such as leukocytes, fibroblasts or muscle. Although these assays provide more reliable results they are associated with minimally invasive or invasive procedures and in the case of fibroblast testing also with a long waiting period [26].

A consequence of screening is the identification of individuals with GAA activity within the range of LOPD patients for which it is uncertain if and when they will develop clinical symptoms. Even within the same family there seems to be significant phenotypic variability for LOPD patients with regards to the onset and the severity of disease-related manifestations [27]. The decision when to start therapy is further complicated by the lack of available data on the potential benefits of ERT when initiated early on asymptomatic LOPD patients. The above uncertainty, in addition to stress and anxiety generated in the family, make the management of these subjects a considerable challenge. Muscle Magnetic Resonance Imaging (MRI) can serve as a useful tool both for the diagnosis and the follow-up of patients with LOPD. Muscle MRI can detect early signs of muscle involvement in LOPD individuals in the absence of clinical symptoms and may facilitate the decision when to start therapy [27].

In this report, four siblings with compound heterozygous *GAA*

Table 1
Biochemical investigations.

Subject	Creatine kinase [U/L]	GAA [nmol/h/mg]		β-Galactosidase [nmol/h/mg][3]	Glc4 [mmol/mol creatinine]
	Value (Ref. range)	4-Mu-αGlc[1]	Glycogen[2]	Value[1] (Ref. range)	Value (Ref. range[4])
#1	48 (25–195)	0.50	12.1	214 (50–326)	1.82 (0–2.2)
#2	112 (25–195)	2.45	13.0	131 (50–326)	2.43 (0.3–3.1)
#3	137 (25–170)	2.06	11.1	200 (50–326)	2.41 (0.3–3.1)
#4	169 (25–170)	1.09	8.3	241 (50–326)	3.76 (0.5–7)
Father	nd	9.48	68.0	276 (50–326)	0.71 (0–2.2)
Mother	nd	9.05	81.7	277 (50–326)	1.35 (0–2.2)
Control #1	nd	20.43	188.5	262 (50–326)	nd
Control #2	nd	18.03	151.5	227 (50–326)	nd

[1]: Mean of 3 measurements; [2]: Mean of 2 measurements; [3]: Control enzyme; [4]: Age related; nd: not determined.
GAA activity, reference range [nmol/h/mg]: 4-MU-αGlc: Controls, (6.7–27); Patients, (1.1–4.9). Glycogen: Controls, (40–250); IOPD, (0–3); LOPD, (3−10).

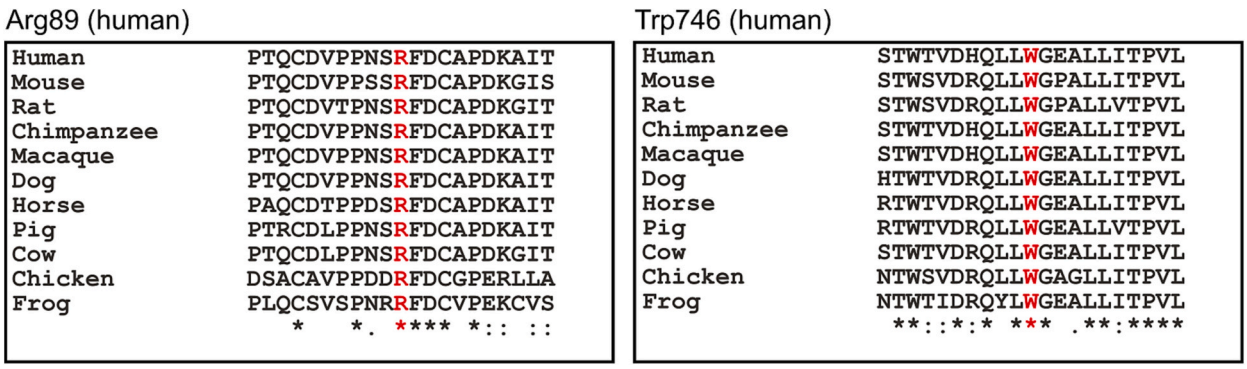

Fig. 1. Multiple alignment of GAA protein sequences (generated by Clustal Omega [29]) from different species featuring the high degree of evolutionary conservation of the amino acid positions Arg89 and Trp746 affected by the identified variants.

variants were identified as an incidental finding of clinical exome sequencing. With the increased application of next generation sequencing (NGS) technologies in clinical diagnostics, the management of incidental findings unrelated to the primary purpose of genetic testing has emerged as a major concern. The present report demonstrates that potential LOPD patients at an asymptomatic stage can also be identified as secondary or incidental findings of NGS. In relation to this, *GAA* is included in the recommendations of the American College of Medical Genetics (ACMG) for the management of secondary findings in clinical exome and genome sequencing which has been recently updated (ACMG SF v3.2) [28].

The variants, c.2237G > C p.(Trp746Ser) and c.266G > A p.(Arg89His) although listed in ClinVar and Pompe disease *GAA* variant database, are reported for the first time in compound heterozygosity in the herein described siblings. Combined with the low GAA activity (within or close to the LOPD range), this raises the possibility that compound heterozygosity for the above variants could be associated with LOPD with an onset and course of symptoms that cannot be predicted.

In summary, the present study reports a new *GAA* genotype with a potential association with LOPD. At the same time, it provides an example of how incidental findings of NGS testing impact on patients and families. For the particular family, NGS testing has generated additional concerns instead of accelerating the diagnostic process.

Accession numbers

The variants reported in this study, c.2237G > C p.(Trp746Ser) and c.266G > A p.(Arg89His), have been classified as pathogenic and likely pathogenic, respectively and submitted to ClinVar with accession numbers SCV003927051 and SCV003927052, respectively.

CRediT authorship contribution statement

Anna Malekkou: Investigation, Writing – review & editing. **Athina Theodosiou:** Formal analysis, Writing – review & editing. **Angelos Alexandrou:** Investigation. **Ioannis Papaevripidou:** Investigation. **Carolina Sismani:** Resources, Writing – review & editing. **Edwin H. Jacobs:** Resources, Writing – review & editing. **George J.G. Ruijter:** Resources, Writing – review & editing. **Violetta Anastasiadou:** Resources. **Sofia Ourani:** Resources. **Emilia Athanasiou:** Resources. **Anthi Drousiotou:** Resources, Writing – review & editing. **Olga Grafakou:** Resources, Conceptualization, Writing – review & editing. **Petros P. Petrou:** Project administration, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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