eP014

Establishment of a clinically validated in vitro functional assay to score pathogenicity of novel *GAA* variants in Pompe patients

Shelly Goomber[1], Erin Huggins[1], Catherine Rehder[2], Jennifer L. Cohen[1], Deeksha Bali[1], Priya Kishnani[1]

[1]*Department of Pediatrics, Division of Medical Genetics, Duke University Medical Center;* [2]*Department of Pathology, Duke University Medical Center*

Introduction: Pompe disease was added to the Recommended Uniform Screening Panel in the United States in 2015. Currently, 28 states screen for Pompe disease at birth, and patients with late-onset Pompe disease (LOPD) represent about 80% of all confirmed cases. While infantile-onset Pompe disease (IOPD) can often be rapidly diagnosed based on absent or severely deficient GAA enzyme activity and notable clinical features such as cardiomegaly and muscle weakness, diagnostic confirmation of LOPD is often more challenging due to the lack of obvious clinical features in the newborn period. Once a diagnosis of Pompe disease is suspected based on low GAA enzyme activity, *GAA* sequencing is performed to confirm the diagnosis and better understand a patient's expected disease course. After a positive newborn screen (NBS), sequencing can reduce the rate of false positives by identifying carriers or those with pseudo-deficiency alleles, and can predict cross reacting immunological material (CRIM) status and timing of disease onset (late vs. infantile). Many cases are identified in which the patient carries one pathogenic or likely pathogenic variant, and one variant of uncertain significance (VUS) which has never been reported.

Of novel variants, missense variants are especially challenging to classify clinically and of the 626 missense *GAA* variants annotated in ClinVar, 448 (70%) of these are classified as VUS. Thus, with the increase of novel and unclassified *GAA* variants due to NBS, there is an urgent need for a reliable and reproducible in vitro functional analysis expression system that can be used to aid in classification of these variants which will enable appropriate clinical management. We established a HEK293 mammalian cell line system for transient in vitro expression of *GAA* VUS using site-directed mutagenesis in order to analyze the impact of a VUS on acid alpha glucosidase (GAA) enzyme activity. Using this assay, we analyzed eight VUS in six individuals with confirmed or suspected Pompe disease.

Methods: pcDNA3.1 cloned *GAA* open reading frame (NM_0000152.5) was used as a reference sequence to generate targeted variants in the laboratory. Our expression system was validated using a set of twelve known *GAA* variants including seven known pathogenic variants (five missense and two nonsense) and five known benign variants (three missense and two synonymous change). Transfected cloned *GAA* (NM_000152.5) open reading frame was used as a positive (+ve) control, while un-transfected HEK293 cell line cultures were used as negative (–ve) controls. Intra- and inter-assay reproducibility was established.

pcDNA3.1-*GAA* targeted variants (Clinical controls and VUS) were generated by QuickchangeII XL-site directed mutagenesis (Agilent Technologies). Cloned *GAA* variants were transiently expressed in HEK293 cell lines and cultured for forty-eight hours for expression studies. Sonicated cell lysates were used to assess the residual GAA activity for each VUS. Artificial substrate 4-Methylumbelliferone (4MU-G) (Sigma-Aldrich) was used to measure residual GAA enzyme activity indirectly using a fluorescence plate reader. Mutant protein was further characterized by western blot analysis using anti-GAA antibodies (Abcam).

Results of these studies were used as evidence toward classification or reclassification of each selected VUS.

Results: Of the seven known pathogenic control variants, resulting enzyme activity level ranged from 0-11% as compared to the wild type (WT) +ve control. Of the five known benign variants, resulting enzyme activity ranged from 54-100% of the WT +ve control. Thus, our assay fulfills the criteria set forth by the Clinical Genomics Sequence Variant Interpretation Working Group for functional studies that can be used at the supporting level of evidence for variant classification (PS3/BS3).

Three *GAA* missense VUS: c.1721T>C (p.Leu574Pro), c.1880C>T (p.Ser627Phe), and c.2450A>G (p.His817Arg) were expressed and assayed separately and were shown to have complete loss of GAA catalytic activity. Using Western blot analyses, these deleterious variants demonstrated only an inactive precursor protein band at 110kDa. Mature protein (76 and 70kDa) bands were absent, indicating a failure of protein processing attributable to these VUS. Four *GAA* VUS: c.266G>A (p.Arg89His), c.316C>T (p.Arg106Cys), c.664G>A (p.Val222Met), and c.1103G>A (p.Gly368Asp) were demonstrated to be damaging with reduced residual activity and reduced protein amounts. The final VUS, c.1642G>T (p.Val548Phe) demonstrated normal GAA expression and enzyme activity.

See Table for complete results of each expression study and final evidence toward classification or reclassification.

Conclusion: We have established a HEK293 mammalian cell line system, which is robust and reproducible, for the in vitro expression of novel *GAA* variants using a site-directed mutagenesis system. This system has allowed us to study the impact of VUS on GAA enzyme activity and protein processing. Our work has allowed reclassification of 6 of the 8 variants studied as either 'likely pathogenic' or 'likely benign' following ACMG-AMP classification guidelines and other published standards, and thus has helped improve the management of these patients.

This work may contribute additional diagnostic information for individuals who present with a concern for Pompe disease with a VUS, especially in the NBS setting.

https://doi.org/10.1016/j.gim.2022.01.052

		Pathogenicity calculation as per LSDVCEP guidelines following ACMG-AMP standards						
Patient/ Case Report	*GAA* missense variant of unknown significance (VUS)	Functional study results [PS3_ supporting/ BS3_supporting]	Population databases Absent /Allele frequency <0.001 in all continental gnomAD populations [PM2_supporting/BS1]	In trans with P/LP variant [PM3/BP2]	Computational prediction SIFT/Polyphen [PP3/BP4]	Clinical phenotype for Pompe disease: DBS glucosidase activity below cut off and absence of pseudodeficiency allele [PP4_moderate/PP4]	Erasmus Mc(# patients/ Phenotype with null allele	Evidence based Pathogenicity calculation
1	**c.316C>T (p.Arg106Cys)** [VCV000971945]	10.7% of normal **PS3_supporting**	allele frequency <0.001 in all continental gnomAD populations **PM2_supporting**	c.-32-13T>G, [VCV000004027] **PM3**	**PP3**	DBS glucosidase activity-1.59µmol/lt, CK upper end of limit **PP4_Moderate**	-	**Likely pathogenic** (No conflict)
2	**c.1103G>A (p.Gly368Asp)** [VCV001037598]	2.8% of normal **PS3_supporting**	Not present in gnomAD **PM2_supporting**	c.-32-13T>G, [VCV000004027] **PM3**	**PP3**	DBS glucosidase activity-0.42µmol/lt CK,AST,ALT elevated **PP4_Moderate**	-	**Likely pathogenic** (No conflict)
3	**c.1721T>C (p.Leu574Pro) —**	No detectable activity **PS3_supporting**	Not present in gnomAD **PM2_supporting**	c.-32-13T>G, [VCV000004027] **PM3**	**PP3**	DBS glucosidase activity-0.61µmol/lt CK,AST,ALT elevated **PP4_Moderate**	1, unknown	**Likely pathogenic** (No conflict)
4	**c.266G>A (p.Arg89His)** [VCV000283219]	21.3% of normal **PS3_supporting**	allele frequency <0.001 in all continental population gnomAD **PM2_supporting**	c.1478C>T (p.Pro493Leu) VCV000379593 **PM3**	**PP3**	DBS glucosidase activity-4.10pmol/punch/hr. Low activity in fibroblast. Biomarkers WNL **PP4_Moderate**	2/unknown	**VUS** Lowered but **s**ignificant activity with normal processing
5	**c.664G>A (p.Val222Met)** [VCV000290223]	11.7% of normal **PS3_supportin**g	South Asian allele freq >0.005 (gnomAD) **BS1**	c.2450A>G (p.His817Arg) **VUS —**	**PP3**	DBS glucosidase activity-8.2% of normal. CK slightly elevated	16, unknown	**VUS** (Evidences in conflict)
	c.2450A>G (p.His817Arg) —	No detectable activity **PS3_supporting**	Not present in gnomAD **PM2_supporting**	**c.664G>A (p.Val222Met) VCV000290220 VUS —**	**PP3**	**PP4 Moderate**	-	**Likely Pathogenic** (No conflict)
6	**c.1642G>T (p.Val548Phe)** [VCV000597381]	100% of normal, **BS3_supporting**	Not present in gnomAD **PM2_supporting**	c.2560C>T; (p.Arg854*) VCV000004034	**BP4**	DBS glucosidase activity-1.52pmol/punch/hr CK, AST,ALT, Hex4 elevated	3, unknown	**Likely Benign** (No conflict)
	c.1880C>T (p.Ser627Phe) [VCV000597382]	No detectable activity **PS3_supporting**	Not present in gnomAD **PM2_supporting**	**PM3**	**PP3**	**PP4 Moderate**	5, classic infantile	**Likely Pathogenic** (No conflict)