REPORT

Genetic Mapping of Glutaric Aciduria, Type 3, to Chromosome 7 and Identification of Mutations in *C7orf10*

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While screening Old Order Amish children for glutaric aciduria type 1 (GA1) between 1989 and 1993, we found three healthy children who excreted abnormal quantities of glutaric acid but low 3-hydroxyglutaric acid, a pattern consistent with glutaric aciduria type 3 (GA3). None of these children had the *GCDH* c.1262C→T mutation that causes GA1 among the Amish. Using single-nucleotide polymorphism (SNP) genotypes, we identified a shared homozygous 4.7 Mb region on chromosome 7. This region contained 25 genes including *C7orf10*, an open reading frame with a putative mitochondrial targeting sequence and coenzyme-A transferase domain. Direct sequencing of *C7orf10* revealed that the three Amish individuals were homozygous for a nonsynonymous sequence variant (c.895C→T, Arg299Trp). We then sequenced three non-Amish children with GA3 and discovered two nonsense mutations (c.322C→T, Arg108Ter, and c.424C→T, Arg142Ter) in addition to the Amish mutation. Two pathogenic alleles were identified in each of the six patients. There was no consistent clinical phenotype associated with GA3. In affected individuals, urine molar ratios of glutarate to its derivatives (3-hydroxyglutarate, glutarylcarnitine, and glutarylglycine) were elevated, suggesting impaired formation of glutaryl-CoA. These observations refine our understanding of the lysine-tryptophan degradation pathway and have important implications for the pathophysiology of GA1.

Glutaric aciduria type 1 (GA1) is one of the most common genetic disorders of the Old Order Amish of Lancaster County, Pennsylvania. Infants with GA1 have elevations of glutarate, 3-hydroxyglutarate, and glutarylcarnitine in blood and urine. Without timely diagnosis and therapy, GA1 results in striatal degeneration and severe dystonia.[1,2] Between 1989 and 1993, we screened 1223 Amish infants for GA1 by urine organic-acid analysis with gas chromatography-mass spectrometry. In the process, we identified three healthy children who excreted large quantities of glutarate but low 3-hydroxyglutarate,[1] consistent with the phenotype of glutaric aciduria type 3 (GA3 [OMIM 231690]), first described in 1991.[3] These children received no therapy and remained healthy over more than 15 years of follow-up.

In their original description of GA3,[3] Bennett et al. postulated that glutaryl-CoA degradation in vivo occurred in two compartments, mitochondria and peroxisomes, corresponding to the GA1 and GA3 phenotype, respectively. They showed that fibroblast homogenates from a GA3 patient did not produce hydrogen peroxide in the presence of labeled glutaryl-coenzyme A (CoA) and took this as evidence of a defective peroxisomal glutaryl-CoA oxidase. This was a compelling idea; it suggested that neurodegenerative consequences of GA1 (in contrast to the benign phenotype of GA3) were rooted in the mitochondrial locus of the disturbance.

However, no gene encoding a glutaryl-CoA oxidase has been identified, and subsequent work showed that, in vitro, a small amount of glutaryl-CoA is oxidized in peroxisomes by an inducible acyl-CoA oxidase (*ACOX1*, a.k.a. palmitoyl-CoA oxidase), whereas this enzyme probably mediates little or no glutaryl-CoA degradation in vivo.[4,5] As a further complication, case reports in 1975 described disabled children with alpha-ketoadipic and alpha-aminoadipic aciduria who were thought to have a defect in the oxidative decarboxylation of alpha-ketoadipate to form glutaryl-CoA.[6–8] However, no enzyme mediating this reaction was ever found.

In an effort to clarify the metabolic pathology of the lysine-tryptophan degradation pathway, we took advantage of the natural occurrence of both GA1 and GA3 among the Pennsylvania Old Order Amish.[9] This study was approved by the Institutional Review Board of Lancaster General Hospital and parents consented in writing to molecular genetic testing. In addition to the three Old Order Amish patients, three other GA3 patients were studied, including an American child of mixed European ancestry and two previously described patients, one German[10] and one Pakistani.[3]

Single-nucleotide polymorphism (SNP) genotyping was performed with the GeneChip Mapping 10K Assay Kit (Affymetrix, Santa Clara, CA, USA) as previously described.[11] Data were analyzed in Microsoft Excel spreadsheets (Microsoft Corporation, Redmond, WA, USA) that were custom formatted at the Clinic for Special Children. SNP positions came from Affymetrix genome annotation files, and genotype data came from the Affymetrix GeneChip Human

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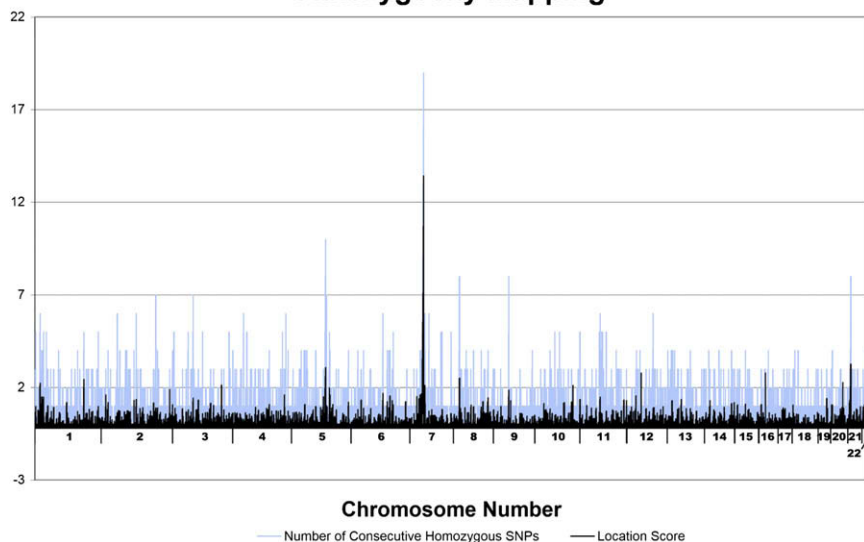*Correspondence: epuffenberger@clinicforspecialchildren.org

Figure 1. Two Mapping Parameters, the Number of Consecutive Homozygous SNPs and the Location Score, Were Maximized on Chromosome 7p14.1-p14.2 in Three Old Order Amish GA3 Patients
The region comprised 19 SNPs and contained 25 known genes including *C7orf10*.

Mapping 10K Xba 142 Arrays. Data analyses were designed for identification of genomic regions that were identically homozygous between all three affected Old Order Amish individuals. Such analyses assume mutation and locus homogeneity. Two-point LOD scores were calculated for each genotyped SNP with an approach similar to Broman and Weber.[12] Cumulative two-point LOD scores for blocks of homozygous SNPs were considered the location score for that region, providing a relative measure that the region harbors the disease gene. Genotype data from 80 healthy Old Order Amish samples as well as from previous studies[11,13–15] were used for estimation of population-specific SNP allele frequencies.

Genome-wide autozygosity mapping using three distantly related Old Order Amish children with GA3 identified a homozygous 4.7 Mb region on chromosome 7p14 bounded by SNPs rs4128395 and rs200463 (Figure 1). The region was queried for candidate genes with both the University of California Santa Cruz (UCSC) and National Center for Biotechnology Information (NCBI) genome browsers. The region contained 25 known or hypothetical genes. For each gene, we assessed function and expression to generate a priority list for sequencing. We chose *C7orf10* (OMIM 609187) as a candidate based on its putative CoA transferase function (NCBI Gene) and mitochondrial targeting sequence (MitoProt).

C7orf10 was subjected to polymerase chain reaction (PCR) amplification and sequencing. PCR primers were designed (Primer3) for amplification of the coding regions and adjacent intron-exon boundaries. DNA sequence from GA3 patients was compared to the human reference sequence and dbSNP so that potential pathogenic sequence variants could be identified. All three Amish GA3 subjects were homozygous for a nonsynonymous c.895C→T (Arg299Trp) change in exon 11. This sequence variant is not a known polymorphism (dbSNP) and is highly conserved in all species tested. Among 150 Amish control individuals, we identified 26 who were heterozygous and one who was

homozygous for the *C7orf10* c.895C→T variant. Retrospectively, we collected a urine sample from this c.895C→T homozygote, a healthy 35-year-old man, and confirmed high glutaric acid excretion (40 mmol/mol creatinine; control 0.9 ± 0.5 mmol/mol Cr).

We subsequently analyzed DNA from three non-Amish GA3 subjects and identified *C7orf10* mutations in all of them (Figure 2). One patient was heterozygous for the Amish c.895C→T allele and a second variant, c.424C→T (Arg142Ter). The latter, a nonsense change, is predicted to produce a truncated, nonfunctional protein. A second child, from Germany, was homozygous for the same c.895C→T mutation found in the Amish patients. Surprisingly, the intragenic SNPs indicated that the mutation in this patient resided on a different haplotype (Figure 2). This circumstance could be explained by an old intragenic recombination or a recurrent mutation; however, the observation that the SNP genotypes were identical in patients 1 and 5 at the 3′ end of *C7orf10* (and beyond) lends support to the hypothesis of intragenic recombination. Finally, sequence analysis of the Pakistani child originally described by Bennett et al.[3] revealed homozygosity for c.322C→T (Arg108Ter). This exon 4 nonsense mutation is predicted to result in a nonfunctional protein.

Urine organic acids were measured by gas chromatography-mass spectrometry.[16] Glutarylglycine and glutarylcarnitine were measured with electrospray-ionization tandem mass spectrometry.[16,17] To quantitate 3-hydroxyglutarate and glutarylglycine levels, we added labeled 3-hydroxyglutaric acid and $^{13}C_2$-glutarylglycine, respectively, as internal standards. For biochemical comparisons, we used urine samples from six Amish GA3 patients and 19 Amish GA1 patients (*GCDH* c.1262C→T homozygotes), ages 1 week to 16 years, as well as 13 healthy Amish siblings and parents of GA3 subjects (designated controls). Urine metabolite values for three groups (control, GA3, and GA1) were studied with one-way analysis of variance (ANOVA). For ANOVA p values < 0.05, we used Tukey's posttest to make pairwise comparisons among groups. We log transformed urine-metabolite-concentration ratios to produce normal distributions for ANOVA testing.

Amish GA3 patients had high glutarate excretion (78.5 ± 97 mmol/mol Cr; control 0.9 ± 0.5 mmol/mol Cr) relative to controls (Table 1). Urine glutarate levels were not distinguishable between GA1 and GA3 subjects;

Figure 2. Single-Nucleotide Polymorphism and *C7orf10* Mutation Table for Six GA3 Patients

Patient	rs4128395	rs2392461	rs1986567	rs3986568	rs718015	rs1376256	rs1376264	rs1112743	rs723969	rs953874	rs1568645	c.322C>T, Arg108Ter (Exon 4)	rs3897941 (Intron 6)	--- (Intron 6)	c.424C>T, Arg142Ter (Exon 7)	rs10129803 (Intron 7)	rs10245165 (Intron 8)	c.895C>T, Arg299Trp (Exon 11)	rs1921746 (Intron 14)	rs1921747 (Intron 14)	rs1203569 (Intron 14)	rs2010706 (Exon 15)	rs1053953 (Exon 15)	rs200463	rs1384383	rs21081167	rs720816	C7orf10 Genotype	Ethnicity
1	CC	TT	GG	GG	GG	CC	TT	AA	AA	CC	AA	CC	CC	TT	CC	TT	TT	TT	TT	AA	TT	GG	TT	TT	GG	CC	TT	[c.895C>T + c.895C>T]	Old Order Amish
2	CT	TT	GG	GG	GG	CC	TT	AA	AA	CC	AA	CC	CC	TT	CC	TT	TT	TT	TT	AA	TT	GG	TT	GT	GG	AC	CT	[c.895C>T + c.895C>T]	Old Order Amish
3	CT	TT	GG	GG	GG	CC	TT	AA	AA	CC	AA	CC	CC	TT	CC	TT	TT	TT	TT	AA	TT	GG	TT	GT	GG	CC	TT	[c.895C>T + c.895C>T]	Old Order Amish
4	CT	TT	CC	CC	GT	CC	TT	AG	GG	CC	AA	CC	TT	CT	CT	TT	AT	CT	TT	AA	CT	CG	AT	TT	GG	AC	CT	[c.424C>T + c.895C>T]	Mixed European, USA
5	TT	TT	CG	CG	GT	CT	CC	AA	AG	CT	AA	CC	TT	CC	CC	TT	AA	TT	TT	AA	TT	GG	TT	TT	GG	CC	CC	[c.895C>T + c.895C>T]	German
6	CC	GG	GG	GG	TT	--	CC	AA	--	--	GG	TT	--	TT	CC	TT	TT	CC	--	--	--	GG	TT	TT	GG	CC	CC	[c.322C>T + c.322C>T]	Pakistani

SNP genotypes were derived from Affymetrix 10K GeneChips and direct sequencing of *C7orf10*; SNP physical locations were from human genome build 36. The red box delimits the *C7orf10* locus, and the gray highlighting indicates homozygous genotype blocks in each patient.

glutarate excretion was highly variable within these groups (i.e., across three orders of magnitude) and overlapped broadly between them. In contrast, individuals with GA3 had normal urinary 3-hydroxyglutarate, glutarylcarnitine, and glutarylglycine values, whereas GA1 patients had elevations of all three derivatives, particularly 3-hydroxyglutarate. Urine acetylcarnitine levels were also high in GA1 patients (455.8 ± 602.4 mmol/mol Cr; control 0.3 ± 0.3 mmol/mol Cr), possibly reflecting L-carnitine supplementation.[18] Compared to both control and GA1 subjects, molar ratios of glutarate to 3-hydroxyglutarate and glutarylcarnitine were markedly elevated in GA3 (Figure 3), suggesting that the loss of *C7orf10* function interferes with the formation of glutarate derivatives through a glutaryl-CoA intermediate (Figure 4).

In striking contrast to GA1, individuals with GA3 have no consistent disease phenotype. This fact, together with urine metabolite data, provides some insight into the pathophysiology of GA1. Relative to individuals with GA1, those with GA3 produce comparatively little 3-hydroxyglutarate, glutarylcarnitine, or glutarylglycine; in GA3 the ratios of glutarate to these metabolites are 10- to 20-fold higher than they are in GA1 (Table 1 and Figure 3). This suggests that individuals with GA3 produce little or no glutaryl-CoA because this compound is the predicted precursor of glutarylcarnitine, glutarylglycine, and 3-hydroxyglutarate in tissues (Figure 4). Such a finding shows that glutaryl-CoA or one of its downstream derivatives is likely to be the primary neurotoxin in GA1, an idea consistent with recent in vitro work.[19]

We identified four different mutations in *C7orf10* that cause GA3. One of these, c.895C→T, underlies the Amish form of the condition. There is no existing functional data on the C7orf10 protein, but analysis of its amino acid sequence suggests that it functions in mitochondria rather than peroxisomes (MitoProt) and that one of its actions might be to transfer CoA to glutarate (NCBI Gene). On the basis of these findings, we postulate that alpha-ketoadipic aciduria, GA3, and GA1 arise from sequential defects along a common mitochondrial lysine-tryptophan degradation pathway. Within this pathway, C7orf10 may act independently or as part of a multiunit complex (Figure 4). Future studies to ascertain the exact enzymatic function

Table 1. Urine Metabolites as Mean and Standard Deviation for GA3, GA1, and Healthy Control Subjects

Metabolites (mmol/mol Cr)	Controls (n = 13)	GA3 (n = 6)	GA1 (n = 19)	ANOVA p value
Glutarate	0.93 (0.53)	78.50 (97.00)	288.60 (428.60)[a]	0.039
3-hydroxyglutarate	1.37 (1.06)	3.97 (0.83)	161.20 (118.30)[a,b]	<0.0001
Glutarylcarnitine	0.03 (0.02)	0.05 (0.06)	18.65 (16.06)[a,b]	<0.0001
Acetylcarnitine	0.25 (0.27)	0.58 (0.62)	455.80 (602.40)[a]	0.001
Glutarylglycine	0.37 (0.11)	0.39 (0.13)	1.90 (1.37)[a,b]	0.0002
Metabolite Ratios (mol:mol)[c]				
Glutarylcarnitine/ total acylcarnitines	0.03 (0.01)	0.03 (0.01)	0.25 (0.39)[a,b]	0.0002
Glutarate/ 3-hydroxyglutarate	0.57 (0.37)	18.75 (20.61)[a]	1.72 (2.07)[b]	<0.0001
Glutarate/ glutarylcarnitine	38.5 (29.0)	1784.0 (920.0)[a]	95.3 (265.3)[b]	<0.0001

[a] Different from control (Tukey, p < 0.05).
[b] Different from GA3 (Tukey, p < 0.05).
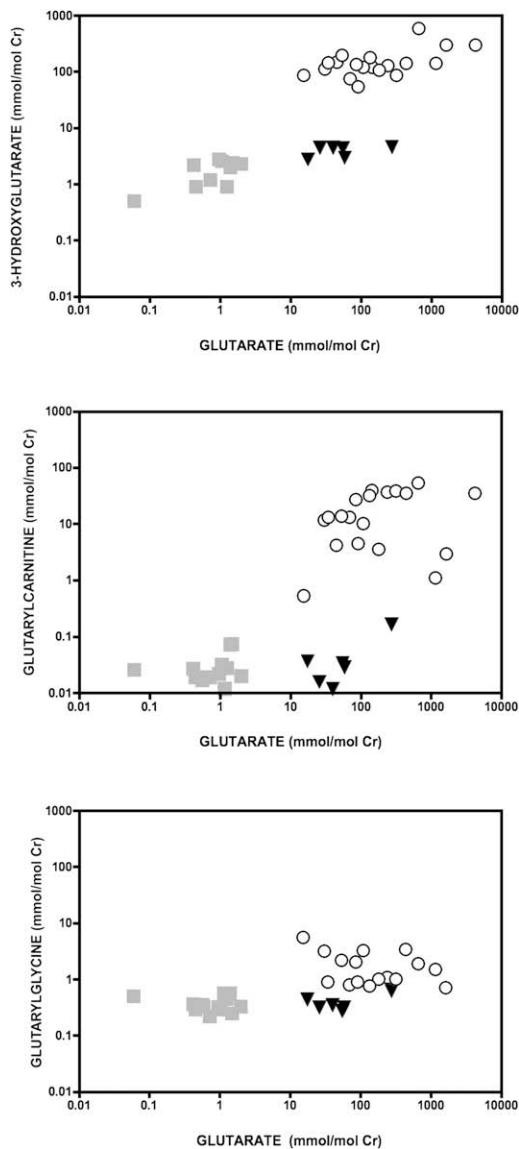[c] Log transformed for ANOVA test.

Figure 3. Urine Metabolite Quantitation in Patients with GA1 and GA3 and Normal Controls

Individuals with GA3 (black triangles) and GA1 (white circles) have elevated urine glutarate relative to controls (gray squares). However, compared to subjects with GA1, those with GA3 have much lower levels of glutarate derivatives: 3-hydroxyglutarate (upper panel), glutarylcarnitine (middle panel), and glutarylglycine (lower panel). Note that all axes are in log 10 scale.

and cellular localization of the C7orf10 gene product are critical to advance our knowledge of this metabolic pathway.

The incidence of GA3 in the general population is unknown. Because these individuals do not produce abnormal quantities of glutarylcarnitine, they are not detected by newborn screening methods based on tandem mass spectrometry analysis of blood spots on dried filter paper. However, clinical laboratories commonly encounter isolated elevations of urine glutaric acid during routine organic-acid analysis and, because GA3 does not appear to

cause disease, the incidence is certainly underestimated. Our findings provide a rationale for sequencing *C7orf10* in individuals with isolated, persistent, and unexplained glutaric aciduria. Perhaps more importantly, however, the present study brings the pathophysiology of GA1 into sharper focus. Future in vitro and in vivo studies of GA1 should concentrate on elucidating precisely how the intra-mitochondrial formation of glutaryl-CoA interferes with the metabolism and survival of striatal neurons.

In conclusion, we used 10,000 marker SNP microarrays to genetically map GA3 to chromosome 7p14 and demonstrated that the disorder is caused by mutations in *C7orf10*. On the basis of our findings, we postulate that alpha-ketoadipic aciduria, GA3, and GA1 arise from sequential molecular lesions along a common mitochondrial lysine-tryptophan degradation pathway. Consistent with recent in vitro studies,[19] our results suggest that the formation of glutaryl-CoA in mitochondria is an integral part of the histotoxic process in GA1.

Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/

MitoProt, http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html

NCBI Gene, http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene

NCBI Genome Browser, http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim

Primer3, http://frodo.wi.mit.edu/

UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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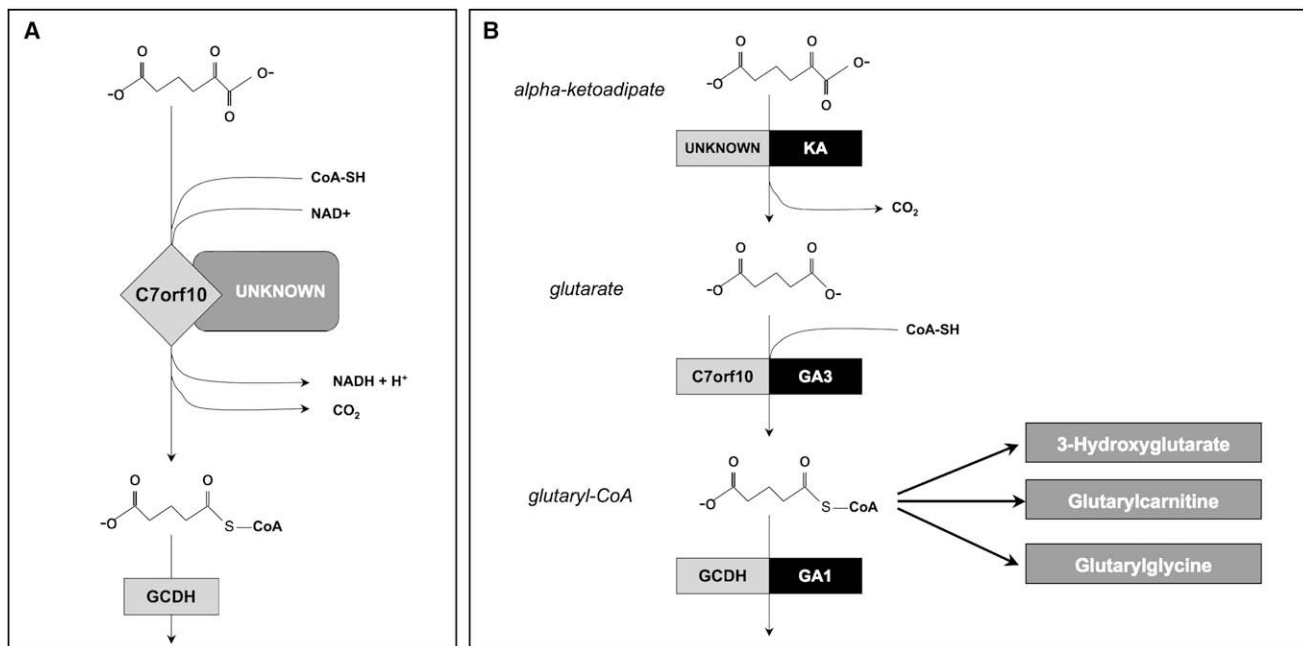
Figure 4.   Alpha-Ketoadipic Aciduria, GA3, and GA1 Possibly Arise from Sequential Enzymatic Defects Along a Common Mitochondrial Lysine-Tryptophan Degradation Pathway

The C7orf10 protein has a putative mitochondrial targeting sequence and a CoA transferase domain. It may function as part of a multiunit enzyme complex (A), similar to the alpha-ketoglutarate dehydrogenase system, or as the second of two independent enzymes, the first of which decarboxylates alpha-ketoadipate to glutarate (B). In either case, C7orf10 defects would block the production of glutaryl-CoA, which we believe to be the source of 3-hydroxyglutarate, glutarylcarnitine, and glutarylglycine in patients with GA1. KA, ketoadipic aciduria; GA3, glutaric aciduria type 3; GA1, glutaric aciduria type 1; and GCDH, glutaryl-CoA dehydrogenase.

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