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Research paper

A pilot study to estimate incidence of guanidinoacetate methyltransferase deficiency in newborns by direct sequencing of the *GAMT* gene

S. Mercimek-Mahmutoglu [a,b,*], A. Pop [c], W. Kanhai [c], M. Fernandez Ojeda [c], U. Holwerda [c], D. Smith [c], J.G. Loeber [d], P.C.J.I. Schielen [d], G.S. Salomons [c,**]

[a] Division of Clinical and Metabolic Genetics, Department of Pediatrics, University of Toronto, Canada
[b] Genetics and Genome Biology, Research Institute, The Hospital for Sick Children, Toronto, Canada
[c] Metabolic Unit, Department of Clinical Chemistry, VU Medical Center, Neurosciences Campus, Amsterdam, The Netherlands
[d] National Institute for Public Health and the Environment, Centre for Infectious Diseases Research, and Screening, Bilthoven, The Netherlands

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ABSTRACT

Background: GAMT deficiency is an autosomal recessive disorder of creatine biosynthesis causing developmental delays or intellectual disability in untreated patients as a result of irreversible brain damage occurring prior to diagnosis. Normal neurodevelopmental outcome has been reported in patients treated from neonatal period highlighting the importance of early treatment.
Methods: Five hundred anonymized newborns from the National Newborn Screening Program of The Netherlands were included into this pilot study. Direct sequencing of the coding region of the *GAMT* gene was applied following DNA extraction. The disease causing nature of novel missense variants in the *GAMT* gene was studied by overexpression studies. GAA and creatine was measured in blood dot spots.
Results: We detected two carriers, one with a known common (c.327G>A) and one with a novel mutation (c.297_309dup (p.Arg105Glyfs*) in the *GAMT* gene. The estimated incidence of GAMT deficiency was 1:250,000. We also detected five novel missense variants. Overexpression of these variants in GAMT deficient fibroblasts did restore GAMT activity and thus all were considered rare, but not disease causing variants including the c.131G>T (p.Arg44Leu) variant. Interestingly, this variant was predicted to be pathogenic by in silico analysis. The variants were included in the Leiden Open Variation Database (LOVD) database (www.LOVD.nl/GAMT). The average GAA level was 1.14 μmol/L ± 0.45 standard deviations. The average creatine level was 408 μmol/L ± 106. The average GAA/creatine ratio was 2.94 ± 0.136.
Conclusion: The estimated incidence of GAMT deficiency is 1:250,000 newborns based on our pilot study. The newborn screening for GAMT deficiency should be implemented to identify patients at the asymptomatic stage to achieve normal neurodevelopmental outcome for this treatable neurometabolic disease. Biochemical investigations including GAA, creatine and GAMT enzyme activity measurements are essential to confirm the diagnosis of GAMT deficiency. According to availability, all missense variants can be assessed functionally, as in silico prediction analysis of missense variants is not sufficient to confirm the pathogenicity of missense variants.

1. Introduction

Guanidinoacetate methyltransferase (GAMT) deficiency (MIM #612736) is an autosomal recessively inherited disorder of creatine biosynthesis (Stöckler et al., 1996b). GAMT enzyme (EC#2.1.1.2) deficiency caused by mutations in the *GAMT* gene (MIM# 601240) results in the depletion of creatine and accumulation of guanidinoacetate (GAA). Creatine has a buffering and transport function of high-energy

Abbreviations: GAMT, guanidinoacetate methyltransferase; GAA, guanidinoacetate; EGFP, enhanced green fluorescent protein; GABA, gamma amino butyric acid; ^{1}H-MRS, proton magnetic resonance spectroscopy; CSF, cerebral spinal fluid; GDD, global developmental delay; CNS, central nervous system; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ORF, open reading frame; PEI, polyethyleneimine; GC–MS, gas chromatography–mass spectrometry; UPLC, ultra high performance liquid chromatography; SD, standard deviation; SDM, site-directed mutagenesis; MS/MS, tandem mass spectrometry; UPLC–MS/MS, ultra performance liquid chromatography–tandem mass spectrometer; SIFT, sorting intolerant from tolerant; LOVD, Leiden Open Variation Database.

* Correspondence to: S. Mercimek-Mahmutoglu, Division of Clinical and Metabolic Genetics, Department of Pediatrics, University of Toronto, 555 University Avenue, Rm 935, 9th Floor, Toronto, ON M5G 1X8, Canada.
** Correspondence to: G. S. Salomons, Metabolic Unit, Department of Clinical Chemistry, Neuroscience Campus Amsterdam, VU University Medical Center, Amsterdam, The Netherlands.
E-mail addresses: saadet.mahmutoglu@sickkids.ca (S. Mercimek-Mahmutoglu), g.salomons@vumc.nl (G.S. Salomons).

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phosphates in brain and muscle and is essential for growth cone migration, dendritic and axonal elongation, neurotransmitter release, and co-transmission on gamma amino butyric acid (GABA) postsynaptic receptors in the central nervous system (Wallimann et al., 1992; Wyss and Kaddurah-Daouk, 2000; Almeida et al., 2006a). GAA has an interaction with $GABA_A$ receptors in in vitro studies, contributing to the seizures and movement disorder seen in GAMT deficiency (Neu et al., 2002).

Creatine deficiency in brain proton magnetic resonance spectroscopy (^{1}H-MRS), and elevated GAA levels in urine, blood and cerebral spinal fluid (CSF) are the biochemical hallmarks of the GAMT deficiency (Stöckler et al., 1996b; Wyss and Kaddurah-Daouk, 2000). Clinical features include global developmental delay (GDD), hypotonia and seizures in infants and intellectual disability, movement disorder, epilepsy and behavioral problems in children (Mercimek-Mahmutoglu et al., 2009; Viau et al., 2013). Treatment consists of high dose creatine to replenish cerebral creatine deficiency and ornithine supplementation and arginine restricted diet to decrease neurotoxic GAA accumulation in the central nervous system (CNS) (Stöckler et al., 1996a; Schulze et al., 2001; Mercimek-Mahmutoglu et al., 2006, 2009). The current treatment is successful to treat epilepsy and the movement disorder, but not GDD or intellectual disability occurring prior to diagnosis (Stöckler et al., 1996a; Schulze et al., 2001; Mercimek-Mahmutoglu et al., 2006, 2009, 2012a, 2014a; Viau et al., 2013; Stockler-Ipsiroglu et al., 2014). Normal neurodevelopmental outcome has been reported in 3 patients who were diagnosed and treated in the neonatal period due to a positive family history of GAMT deficiency in an older sibling (Schulze et al., 2006; El-Gharbawy et al., 2013; Viau et al., 2013). The effectiveness of early intervention and severe neurodevelopmental outcome in untreated patients, implicate that GAMT deficiency is an excellent candidate for newborn screening and should be included into newborn screening programs.

Since its first description in 1996, 110 GAMT deficiency patients have been reported (Mercimek-Mahmutoglu et al., 2006, 2009, 2012a, 2014a, 2014b; Schulze et al., 2006; Cheillan et al., 2012; Nasrallah et al., 2012; Comeaux et al., 2013; El-Gharbawy et al., 2013; Viau et al., 2013; Akiyama et al., 2014; Stockler-Ipsiroglu et al., 2014). Between 2001 and 2011, five patients were diagnosed with GAMT deficiency among 570,359 live births in Utah giving an approximate incidence of 1:114,072 in Utah (Viau et al., 2013). The carrier frequency of GAMT deficiency was reported 1/1475 in a small cohort of newborns using targeted mutation screening in British Columbia, Canada (Mercimek-Mahmutoglu et al., 2012b). So far, there are no other studies reporting the prevalence or incidence of GAMT deficiency. There are few newborn screening centers performing newborn screening for GAMT deficiency in Europe and in North America. To the best of our knowledge, so far no newborns were identified using blood spot GAA measurements in any of those newborn screening programs. All of the patients with GAMT deficiency were identified either by a selective screening due to symptoms or in the neonatal period due to positive family history.

We performed a pilot study to estimate incidence of GAMT deficiency in a small Dutch newborn population by direct sequencing of the *GAMT* gene to establish an evidence base for newborn screening for GAMT deficiency. We report estimated incidence of GAMT deficiency, which is 1:250,000 newborns.

2. Methods

The study was approved by The Newborn Screening Research Committee, The Netherlands. Five-hundred anonymized newborn bloodspot cards from The Dutch National Newborn Screening Program were selected randomly between March–April, 2013. Three 3 mm blood spots were punched by an automated puncher (Perkin Elmer, Turku, Finland) into 96-well plates: one for direct sequencing of the *GAMT* gene, one for measurement of GAA and creatine by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and one as reserve.

2.1. Direct sequencing of the GAMT gene

DNA was extracted from dried blood spots by Generation DNA Purification kit. Sequencing of the *GAMT* gene (NM_000156.5) was performed as previously described for mutation analysis (Caldeira Araújo et al., 2005). Nucleotide numbering of variants reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen).

2.2. Functional characterization of missense variants

The open reading frame (ORF) of the *GAMT* gene (NP_000147.1) was cloned as a fusion protein with enhanced green fluorescent protein (EGFP) in a pEGFPN1 vector as described previously (Almeida et al., 2006a). Each missense variant was introduced in pGAMT-EGFPN1 plasmids by site directed mutagenesis using standard molecular biology techniques [see references Almeida et al., 2006b and Mercimek-Mahmutoglu et al., 2014b for details]. A primary GAMT-deficient human fibroblast cell line (homozygous for a frameshift mutation), cultured according to standard procedures to 70% confluence, was used for transient transfections. For the transfections, 45 μg of each plasmid was incubated at room temperature for 10 min with polyethyleneimine (PEI, Polysciences, Omnilabo) in serum-free medium, and subsequently the complex was applied to the cells. Cells transfected with either the wild type pGAMT-EGFPN1 construct or with the pEGFPN1 empty vector as well as untransfected cells were taken along as controls. All transfections were performed in triplicate, and green fluorescence of the EGFP tag was used to estimate the transfection efficiency. Cells were harvested 48 h after transfection, flash-frozen and stored at −80 °C for western blot analysis and GAMT enzyme activity measurement. Immunodetection of the GAMT-EGFP fusion protein was carried out as described previously (Almeida et al., 2006b; Mercimek-Mahmutoglu et al., 2014b) using an EGFP antibody (Abcam). The GAMT enzyme assay was performed with the supernatants from lysed cells as described by (Verhoeven et al., 2004).

2.3. GAA and creatine analysis by LC–MS/MS

Three millimeter punches of dried blood spots were mixed with 200 μL methanol and 20 μL internal standard (containing 20 μM [D3]-creatine and 2 μM [13C2]-GAA) and shaken for 15 min in an ultrasonic bath. The supernatant was transferred in a vial and the methanol was evaporated. Subsequently, the sample was butylated with 100 μL 5% acetylchloride in n-butanol for 15 min at 60 °C. The butanol was evaporated under nitrogen at 30 °C and the residue was dissolved in 500 μL 75% acetonitrile.

The concentrations of creatine and guanodinoacetate were determined by positive electrospray LC–MS/MS, using an API 5000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) in combination with a Nexera LC-30AD solvent system (Shimadzu, Kyoto, Japan). Using a Symmetry Shield RP8 analytical column (3.0 × 100 mm; 3.5 μm; Waters, Milford, MA, USA) 7 μL of the sample was separated using 75% acetonitrile within 5 min with a flow rate of 0.35 mL/min. The following LC–MS/MS transitions were measured: m/z 188.0 → 90.0 and m/z 191.0 → 93.0 for creatine, and m/z 174.0 → 101.0 and m/z 176.0 → 103.0 for GAA. The LC–MS/MS data were acquired and processed using Analyst 1.5.2 software (Applied Biosystems). All samples above the 99.9th percentiles were re-assayed using the second-tier gas chromatography–mass spectrometry (GC–MS) using previously described method (Struys et al., 1998).