Chapter 3

Characterization of seven novel mutations in seven patients with GAMT deficiency


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Characterization of Seven Novel Mutations in Seven Patients with GAMT Deficiency

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INTRODUCTION

Guanidinoacetate methyltransferase (GAMT) deficiency is an autosomal recessive error of creatine synthesis characterized by cerebral creatine deficiency, accumulation of guanidinoacetate, mental retardation, epilepsy and extrapyramidal signs. So far, six mutations have been identified in seven patients. We investigated seven new patients by screening the promoter, 3’UTR, and six exons and exon/intron boundaries using direct sequencing and denaturing gradient gel electrophoresis. The clinical and biochemical phenotype was characterized by scoring the degree of main clinical manifestations and by determination of urinary guanidinoacetate concentrations and of GAMT activity in fibroblasts / lymphoblasts, respectively. We identified 7 novel mutations, including c.64dupG (exon 1; 4/14 alleles); c.59G>C (exon 1; 3/14 alleles); c.491delG (exon 5; 2/14 alleles); c.160G>C (exon 1; 2 / 14 alleles); c.160G>C (exon 1; 2 / 14 alleles); and c.152A>C (exon 1; 1 / 14 alleles); c.526dupG (exon 5; 1/14 alleles); c.521G>A (exon 5; 1/14 alleles), and two polymorphisms c.626C>T (exon 6) and c.459+71G>A (intron 4). Frameshift and missense mutations in exon 1 were prevalent in the 4 patients with the severe phenotype, however a clear genotype-phenotype correlation has not been established in the limited number of patients characterized so far. ©2004 Wiley-Liss, Inc.

KEY WORDS: creatine deficiency; GAMT; creatine synthesis; mutation screening
extrapyramidal signs and symptoms (Stöckler et al., 1994; Stromberger et al 2003; von Figura et al., 2000). Biochemical findings include high urinary excretion of guanidinoacetate (GAA; immediate precursor of creatine and substrate to deficient enzyme activity), low urinary excretion of creatinine (conversion product of intracellular creatine), and depletion of creatine in brain and muscle (Stöckler et al., 1997; Schulze et al, 2003). Enzymatic diagnosis is possible by the demonstration of deficient GAMT activity in liver, cultivated skin fibroblasts and virus-transformed lymphoblasts (Stöckler et al., 1996b; Ilas et al., 2001). Patients with GAMT deficiency have shown favourable response to oral supplementation of creatine monohydrate and dietary restriction of arginine, but in none of them complete reversal of symptoms was observed (Stöckler et al., 1996 a; Stöckler et al 1997; Schulze et al 2001). Inheritance of GAMT deficiency is autosomal recessive. So far, only 6 mutations have been reported in 7 GAMT patients recognized so far (Schulze et al., 2003; Item et al., 2002). In this study, we describe 7 new mutations in seven newly diagnosed patients with GAMT deficiency and compare their clinical phenotype by scoring the degree of the main clinical manifestations and handicaps.

MATERIALS AND METHODS

Patients

Seven newly diagnosed patients with GAMT deficiency (P7 – P13) (Table 1) and their parents were investigated. Patient numbers were given according to the order of the patient files from our own laboratory. Numbers 1–6 (P1-P6) were assigned to the six patients reported previously (Item et al., 2002); and numbers 7-13 (P7-P13) were assigned to the 7 newly diagnosed patients reported here.

Mutation analysis was performed in DNA extracted from dry blood filter paper samples, from cultivated skin fibroblasts and virus transformed lymphoblasts, respectively.

Reagents

All chemicals were purchased from Sigma, St.Louis, Missouri, U.S.A and Bio-Rad Laboratories, Munich, Germany respectively. Oligonucleotides and Ampli-Taq Gold DNA polymerase were purchased from MWG-Biotech, Ebersberg, Germany, and Roche, New Jersey, USA respectively. Chelex-100 particles were purchased from Promega, Madison, WI, U.S.A., and Microcon-50 concentrators from Millipore, Bedford, MA, U.S.A.

DNA extraction

DNA extraction from dried blood spot samples, cultivated skin fibroblasts and lymphoblasts was carried out as described using chelex particles and reagent kits (Item et al., 2002).

Nested polymerase chain reaction and DGGE

Two rounds of PCR using primer pairs covering the promoter, 3’UTR, exons 2-6 and the surrounding intron boundaries, and eventually DGGE of the PCR products were carried out as described (Item et al., 2002). Direct sequencing of PCR products showing putative sequence variations as pointed out by DGGE analysis was carried out by double stranded automated cycle sequencing of the PCR products on a ABI 370 sequencer (Applied Biosystems, Foster City, CA).

Nomenclature

DNA nomenclature is according to published GAMT cDNA sequence, version 1, accession number Z49878.1. Nucleotide positions are counted from the A of the ATG of the initiator methionine codon, which is denoted nucleotide +1. Intronic nomenclature is according to published GAMT genomic DNA sequence, version 1, accession number AF188893.1. Nucleotide positions are counted from the first intronic nucleotide at the exon-intron junction.

Characterisation of the clinical phenotype

For characterisation of the clinical phenotype, the main features of GAMT deficiency (mental retardation, epilepsy and motor handicap) were subdivided into 3 possible severity degrees and scored with one (1), two (2) or three (3) score-points. Mental retardation was defined as mild (1), moderate (2) and severe (3) according to standardized definition by ICD 10. Epilepsy was classified as occasional (1), drug responsive (2), and drug resistant (3) seizures. Motor handicap was classified according to the patients’ ability to walk: able to walk freely without specific neurologic signs (1); able to walk with help and specific neurologic signs such as spasticity and dystonia (2); and not able to walk at all (3). Maximum points were 9, indicating the most severe phenotypical manifestation, and minimum score points was 1, indicating the mildest phenotypical manifestation. Patients with 1-
3 points were considered to have mild phenotype, patients with 4-6 points were considered to have intermediate phenotype, and patients with 7-9 points were considered to have severe phenotype.

**Confirmation of diagnosis**

Diagnosis was suspected on the ground of clinical symptoms and on the absence of brain creatine as shown by in vivo proton magnetic resonance spectroscopy (data not shown). Diagnosis was confirmed by demonstration of undetectable *GAMT* activity in fibroblasts/lymphoblasts and/or by demonstration of characteristically elevated GAA concentrations in 24-hour urine samples. Accumulation of GAA was also found in plasma and CSF, but as plasma and CSF were available only from single patients, respective values are not shown here.

**Determination of *GAMT* activity and urinary GAA**

Measurement of *GAMT* activity was carried out in homogenates of cultured skin fibroblasts and/or EBV transfected lymphoblasts as described (Ilas et al., 2001). Urinary GAA concentrations were measured in 24-h urine samples by GC-MS and stable isotope dilution (Hunneman and Hanefeld, 1997; Struys et al., 1998).

**Deduction of effects of mutations on protein level**

The effects of all mutations at the protein level, as described in the result and discussion section, were deduced theoretically.

**RESULTS**

The clinical, biochemical and molecular findings of the 7 newly diagnosed *GAMT* deficiency patients are summarized in Table 1.

**Patients 7 and 8** (P7, P8) are sisters, homozygous for a novel single G duplication at nucleotide position 64 in exon 1 of the cDNA (c. 64dupG). The duplication is deduced to produce a frameshift with Ala22 as the first affected aminoacid and the new reading frame ending in a stop after 19 codons (p.A22fsX19) in exon 1. Sequence analysis of the parents DNA showed both to be heterozygous for c. 64dupG. Phenotype in patients 7 and 8 was classified as severe *GAMT* deficiency.

**Patient 9** (P9) was compound heterozygous for a novel A>C transition at nucleotide position 152 in exon 1 of the cDNA (c.152A>C), which is deduced to result in the replacement of His (CAC) by Pro (CCC) at codon 51 (p.His51Pro), and a novel single G duplication at nucleotide position 526 in exon 5 of the cDNA (c.526dupG), which is deduced to produce a frameshift with Glu 176 as the first affected aminoacid and the new reading frame ending in a stop after 13 codons (p.E176fsX13) in exon 5. Sequence analysis of the parents showed the mother to be heterozygous for c.526dupG and the father for c.152A>C. Phenotype of patient 9 was classified as mild *GAMT* deficiency.

**Patient 10** (P10) was homozygous for a novel single G deletion at nucleotide position 491 in exon 5 of the cDNA (c.491delG), which is deduced to produce a frameshift with Gly 164 as the first affected aminoacid and the new reading frame ending in a stop after 13 codons (p.G164fsX13) in exon 5. Sequence analysis of the parents DNA showed both to be heterozygous for c.491delG. Phenotype of patient 10 was classified as intermediate GAMT deficiency.

**Patient 11** (P11) was compound heterozygous for a novel G>C transition at nucleotide position 97 in exon 1 of the cDNA (c.59 G>C), which is deduced to result in the replacement of Trp (TGG) by Ser (TCG) at codon 20 (p.Trp20Ser), and a novel G>A transition at nucleotide position 521 in exon 5 of the cDNA (c.521G>A), which is deduced to result in the replacement of Trp (TGG) by Stop (TAG) at codon 174 (p.Trp174Ter). Sequence analysis of the parents showed the mother to be heterozygous for c.521G>A and the father for c.59G>C. Phenotype of patient 11 was classified as severe *GAMT* deficiency.
mutations identified in this study occurred at such repetitive GC-rich sequences and not at CpG dinucleotides which are considered as main hotspots in particular for missense mutations (Cooper and Youssoufian, 1988).

Deficiency refers to non-coding regions/introns/flanking region of the 14 alleles from the 7 patients described here, are in bold face; Darkened fields refer to the coding regions/exons; white fields et al., 2000; Schulze et al., 2003) in 14 patients with the locations of 7 novel mutations and of 6 known mutations identified by ourselves (Item et al., 2002) and others (Carducci Cac.626C>T, and c.459+71G>A, respectively, whilst the father was heterozygous for c.160G>C, c.626C>T, and were found. Sequence analysis of the parents’ DNA showed the mother to be heterozygous for c.160G>C, c.626C>T, and c.459+71G>A, respectively. According to this pattern of inheritance, it is most likely that both c.626C>T and c.459+71G>A are inherited in cis with c.160G>C in patient 12. Phenotype of patient 12 was classified as severe GAMT deficiency.

Patient 12 (P12) was homozygous for a novel G>C transition at nucleotide position 160 in exon 1 of the cDNA (c.160G>C), which is deduced to result in the replacement of Ala (GCC) by Pro (CCC) at codon 54 (p.Ala54Pro). In the patient’s GAMT gene two additional sequence changes c.626C>T (exon 6) and c.459+71G>A (intron 4) were found. Sequence analysis of the parents’ DNA showed the mother to be heterozygous for c.160G>C, c.626C>T, and c.459+71G>A, respectively, whilst the father was heterozygous for c.160G>C, c.626C>T, and c.459+71G>A, respectively. According to this pattern of inheritance, it is most likely that both c.626C>T and c.459+71G>A are inherited in cis with c.160G>C in patient 12. Phenotype of patient 12 was classified as severe GAMT deficiency.

Patient 13 (P13) was homozygous for a novel G>C transition at nucleotide position 59 in exon 1 of the cDNA(c.59G>C), which is deduced to result in the replacement of Trp (TGG) by Ser (TCG) at codon 20 (p.Trp20Ser). Sequence analysis of parents’ DNA showed both to be heterozygous for c.59G>C. Phenotype of patient 13 was classified as intermediate GAMT deficiency.

Table 1: Clinical, Biochemical and Molecular Findings in 7 Newly Diagnosed Patients with GAMT Deficiency

<table>
<thead>
<tr>
<th>Patient Number / Age at diagnosis / Ethnic Origin</th>
<th>Genotype</th>
<th>Urinary GAA levels (n.v: 63-429 micromol/l)</th>
<th>GAMT Activity (F or L) nmol/h/mg</th>
<th>Clinical features, Score of severity (1-3),</th>
<th>Total score &amp; Severity of Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7 (27 y) Kosovo</td>
<td>[c.64dupG] + [c.64dupG]</td>
<td>2100</td>
<td>F:0</td>
<td>severe mental retardation (3), drug responsive epileptic seizures (2), able to walk with help, spastic dystonic gait (2)</td>
<td>(7) severe</td>
</tr>
<tr>
<td>P8 (22 y) Kosovo</td>
<td>[c.64dupG] + [c.64dupG]</td>
<td>n.d.</td>
<td>F:0</td>
<td>severe mental retardation (3) drug resistant epileptic seizures (3) able to walk with help and spastic dystonic gait (2)</td>
<td>(8) severe</td>
</tr>
<tr>
<td>P9 (4 y) Turkish</td>
<td>[c.152A&gt;C] + [c.526dupG]</td>
<td>1963</td>
<td>n.d.</td>
<td>mild mental retardation (1) occasional (febrile) seizures (1) able to walk and normal gait (1)</td>
<td>(3) mild</td>
</tr>
<tr>
<td>P10 (10 y) Turkish</td>
<td>[c.491del G] + [c.491delG]</td>
<td>1007</td>
<td>F:0</td>
<td>severe mental retardation (3) drug responsive epileptic seizures (2) able to walk and normal gait (1)</td>
<td>(6) intermediate</td>
</tr>
<tr>
<td>P11 (14 y) Spanish</td>
<td>[c.59G&gt;C] + [c.521G&gt;A]</td>
<td>2939</td>
<td>F:0</td>
<td>Severe mental retardation (3) drug resistant epileptic seizures (3) able to walk and normal gait (1)</td>
<td>(7) severe</td>
</tr>
<tr>
<td>P12 (4 y) Egypt</td>
<td>[c.160G&gt;C ; c.626C&gt;T; c.459+71G&gt;A] + [c.160G&gt;C; c.626C&gt;T; c.459+71G&gt;A]</td>
<td>1631</td>
<td>L&lt;0.1</td>
<td>Severe mental retardation (3) drug responsive epileptic seizures (2) not able to walk and not able to sit (3)</td>
<td>(8) severe</td>
</tr>
<tr>
<td>P13 (14 y) Turkish</td>
<td>[c. 59G&gt;C] + [c. 59G&gt;C]</td>
<td>1426</td>
<td>n.d.</td>
<td>Severe mental retardation (3) occasional epileptic seizures (1) able to walk and normal gait (1)</td>
<td>(5) intermediate</td>
</tr>
</tbody>
</table>

y= years of age; m= months of age; GAA=guanidinoacetate; L=lymphoblast; F=fibroblast; n.v.= normal values; n.d.= not determined.
The location, the type, and the frequency of the 7 novel mutations are shown in the gene map (Figure 1).

**Figure 1.** GAMT gene map: Schematic representation of the coding/non coding regions of the human *GAMT* gene, indicating the locations of 7 novel mutations and of 6 known mutations identified by ourselves (Item et al., 2002) and others (Carducci Ca et al., 2000; Schulze et al., 2003) in 14 patients with GAMT deficiency. Novel mutations and their frequencies identified in the 14 alleles from the 7 patients described here, are in bold face; Darkened fields refer to the coding regions / exons; white fields refer to non-coding regions/introns /flanking region of the *GAMT* gene.

**DISCUSSION**

In the 14 alleles from the seven newly diagnosed patients, 7 novel mutations were identified in their *GAMT* genes. All mutations found in this study are concentrated in exon 1 (4 out of 7 mutations) and exon 5 (3 out of 7 mutations), while the 6 mutations identified in 7 previously reported patients (Item et al., 2002, Schulze, 2003) were mainly concentrated in exon 2 (3 out of 6 mutations) and exon 5 / intron 5 (2 out of 6 mutations) (Figure 1). Analysis of the local DNA environment surrounding all the 13 mutations identified in this and in our previous study (Item et al., 2002), indicates that 12 out of 13 mutations (2 deletions, 4 insertions, and 6 missense) occurred at or within short direct repeats (< 20bp) of nucleotides that are rich in GC. Repetitive GC-rich sequences are hypermutable (Bois, 2003) and are associated with a high deletion and insertion frequency in human genes (Cooper and Krawczak, 1991; Krawczak and Cooper, 1991; Darvai et al., 1995). Interestingly, also the 6 missense mutations identified in this study occurred at such repetitive GC-rich sequences and not at CpG dinucleotides which are considered as main hotspots in particular for missense mutations (Cooper and Youssoufian, 1988).

c.626C>T (exon 6) and c.459+71G>A (intron 4) are most likely polymorphisms, as in a still unpublished study we could show a high frequency of both sequence changes (17.4% and 24 %) in 92 healthy subjects (manuscript in preparation). The pathogenic relevance of the remaining sequence changes identified in this study was supported biochemically by negligible enzyme activity in fibroblasts / lymphoblasts, and / or by the pathognomonic increase of urinary GAA excretion.
Four of the seven patients (P7, P8, P11, P12) had a severe phenotype, 2 patients had the intermediate (P10, P13) and only 1 patient had the mild phenotype (P9). In the 4 patients with the severe phenotype, frameshift and missense mutations in exon 1 were prevalent (7 out of 8 alleles), whereas the intermediate and mild phenotype was correlated both with missense mutations in exon 1 (3 out of 6 alleles) and with frameshift mutations in exon 5 (3 out of 6 alleles). These data suggest that mutations in exon 1 predispose mainly to the severe phenotype, whereas the less severe phenotypes may be associated both with mutations in exon 1 and in exon 5. Due to the heterogeneous spectrum of mutations and due to the limited number of patients identified so far, speculations about possible correlation of genotype to phenotype have to be considered with caution. In particular, in our previous study (Item et al., 2002) a correlation between the phenotype and the genotype could not be established, as 3 patients with the same mutational spectrum (homozygosity for a frameshift mutation c.327 G>A in exon 2) had a phenotype ranging from severe to mild.

Four out of the 7 families investigated in this study, and 2 out of 6 families investigated in our previous study (Item et al., 2002) are immigrants from countries (Turkey and Kosovo) with predominant Turkish and Kurdish population. A main factor for the high prevalence of GAMT deficiency in this particular population group might be the high frequency of consanguinity which is supported by the fact that in most families from the Turkish/Kurdish population group, the single mutations occurred in homozygous form.

Concluding, the mutations causing GAMT deficiency are heterogenous and the corresponding clinical phenotype ranges from severe to mild. Apart from the previously reported c.327G>A mutation, which occurred in 7 of the 28 alleles investigated so far (Figure 1) there appears to be no hotspot in the spectrum of GAMT mutations. More patients need to be characterized in order to establish possible correlations of genotype and phenotype.

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REFERENCES


