Mutation analysis in glutaric aciduria type I

Johannes Zschocke, Elfriede Quak, Per Guldberg, Georg F Hoffmann

Abstract
Glutaric aciduria type I (GA1), resulting from the genetic deficiency of glutaryl-CoA dehydrogenase (GDH), is a relatively common cause of acute metabolic brain damage in infants. Encephalopathic crises may be prevented by carnitine supplementation and diet, but diagnosis can be difficult as some patients do not show the typical excretion of large amounts of glutaric and 3-hydroxyglutaric acids in the urine. We present a rapid and efficient denaturing gradient gel electrophoresis (DGGE) method for the identification of mutations in the glutaryl-CoA dehydrogenase (GCDH) gene that may be used for the molecular diagnosis of GA1 in a routine setting. Using this technique, we identified mutations on both alleles in 48 patients with confirmed GDH deficiency, while no mutations were detected in other patients with clinical suspicion of GA1 but normal enzyme studies. There was a total of 38 different mutations; 27 mutations were found in single patients only, and 21 mutations have not been previously reported. Fourteen mutations involved hypermutable CpG sites. The commonest GA1 mutation in Europeans is R402W, which accounts for almost 40% of alleles in patients of German origin. GCDH gene haplotypes were determined through the analysis of polymorphic markers in all families, and three CpG mutations were associated with different haplotypes, possibly reflecting independent recurrence. The high sensitivity of the DGGE method allows the rapid and cost efficient diagnosis of GA1 in instances where enzyme analyses are not available or feasible, despite the marked heterogeneity of the disease.

Keywords: glutaric aciduria type I; glutaryl-CoA dehydrogenase; mutation; denaturing gradient gel electrophoresis

Over the last decade, glutaric aciduria type I (GA1, McKusick 231670) has been recognised as a major inherited cause of acute metabolic brain damage in early childhood. The autosomal recessive disease is caused by a deficiency of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GDH, EC 1.3.99.7) in the catabolism of lysine, hydroxylysine, and tryptophan. It is characterised by the accumulation of glutaric and 3-hydroxyglutaric acids and secondary carnitine depletion. Affected children are generally well in the first months of life or show only mild neurological symptoms, but usually display increasing macrocephaly. Typically during a non-specific illness or vaccination between 6 and 18 months of age, they suffer a severe encephalopathic crisis that causes necrosis of the striatum in the basal ganglia and results in a severe dystonic dyskinetic movement disorder. Intellectual function is relatively unaffected. The metabolic crisis can usually be prevented by carnitine supplementation and diet, and early presymptomatic diagnosis is therefore essential. As some children show a normal pattern of urinary organic acids, enzymatic or molecular analyses may be necessary in the diagnostic process.

The GCDH gene stretches over 7 kb on chromosome 19p13.2 and comprises 11 exons. The gene product is a polypeptide of 438 amino acids, of which 44 N-terminal residues are removed after mitochondrial import; 63 different disease causing mutations in the gene have been described so far. Single common mutations are found in genetically homogeneous communities such as the Amish of Pennsylvania, but GA1 in general is quite heterogeneous; the most frequent mutation in whites, R402W, has been identified on 10-20% of alleles. Routine GA1 mutation analysis in a diagnostic setting therefore requires a sensitive and efficient method for the detection of novel DNA alterations. We have devised a denaturing gradient gel electrophoresis (DGGE) system to analyse the whole GCDH gene in genomic DNA. Uniform PCR conditions and a single polyacrylamide gel are used for all exons, and both mutations in a patient can be identified within three days. Presented here are the results of the molecular analysis in 57 subjects with confirmed or suspected GA1.

Subjects and methods
A total of 57 families was investigated, including most known patients with GA1 in Germany. Most patients in the study had classical GA1 but some displayed atypical clinical or biochemical features, such as consistent isolated urinary excretion of 3-hydroxyglutaric acid, or were referred for exclusion of GA1. GDH deficiency was previously or subsequently proven by enzyme analysis in 46 patients (E Christensen, Copenhagen). Normal GDH activity was found in eight patients, while no enzymatic data were available in three patients. Patients and, where available, both parents were investigated; in one family no patient DNA was available. The families studied were unrelated, but the parents of some patients were related or came from areas with a high degree of consanguinity. The ethnic or national background of each parent over several generations was recorded. The origin of
families with proven GDH deficiency was as follows: 18 German, nine Turkish (seven living in Germany), four Austrian, six Chilean, and 11 from different European countries and the United States.

Genomic DNA was extracted from peripheral lymphocytes using standard methods. Primer sequences for all exons are given in table 1. For DGGE analysis, a 40–65 bp GC clamp was attached 5′ to the specific primers. PCR conditions were 95°C for five minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for one minute; followed by 72°C for five minutes, 95°C for one minute, 65°C for 30 minutes, 37°C for 30 minutes, 4°C hold (Taq polymerase purchased from 1-86 Technologies, Rockville, MD, USA; thermocycler 9700, Perkin Elmer, Norwalk, CT, USA). Amplification of exon 1 for DGGE required the addition of 5% DMSO. Broad range DGGE was performed on a vertical electrophoresis system (modified Protein II, BioRad, Hercules, CA, USA) using essentially the same procedure and electrophoresis conditions as previously described for the PAH gene. All exons were run in parallel on the same gel containing a denaturing gradient from 0–80% electrophoresis time was six hours at 170 V. The relatively short PCR product of exon 8 was loaded 90 minutes after start of the electrophoresis run. The results were visualised by ethidium bromide staining.

Exons displaying variant gel patterns were amplified from genomic DNA with primers containing a universal 20mer tail (table 1), cycle sequenced in both directions with dye labelled universal primers (ThermoSequenase, Amersham Pharmacia, Uppsala, Sweden), and electrophoresed on an ALF express fluorescent sequencer (Amersham Pharmacia, Uppsala, Sweden). Inheritance in the families was confirmed by DGGE or sequencing. Several mutations including R402W were confirmed by appropriate restriction enzyme analyses of the respective exon.

The inheritance of three polymorphic silent mutations in the GCDH gene was investigated with restriction enzyme assays within families. IVS2+63G>C creates an AcI site in intron 2, while c.1173G>T (G391G) in exon 10 and c.1482A>G, respectively. Primers for sequencing and DGGE of exons 9 and 11 were at different positions.
Results

The result of broad range DGGE analysis of all exons of the \textit{GCDH} gene in four GA1 patients is illustrated in fig 1. Mutations were found on all 96 GA1 alleles in 48 patient families with unequivocal diagnosis of the disease, including two of the three patients in whom enzyme studies were not performed (table 2). There were 38 different mutations including 20 that have not been previously reported. All but one small deletion were single nucleotide changes; there were three splice site mutations. R402W was confirmed to be the commonest mutation in Europeans, accounting for one quarter of mutant alleles (24/96) in our study. Ten other mutations were also found in more than one family. Fourteen mutations, most of them previously described, were C>A or G>T alterations at potentially methylated CpG sites that are known to be hypermutable.\textsuperscript{10} Origin of alleles and haplotype associations are included in table 2. Among both normal and mutant alleles, there were two major (a and h) and one minor (b) haplotype; the other haplotypes were rare or absent. There was no significant difference in the haplotype distribution between normal and mutant alleles (table 3). With regard to individual mutations, more than half were associated with haplotype a. No mutations were found in the nine patients with atypical clinical or biochemical findings in whom GDH enzyme analysis was normal (eight) or not available (one).

R402W was more common in patients of German origin in whom it had a relative frequency of almost 40\% (14/36 alleles), but it was also identified in patients from various other European countries as well as Chile and the USA. The mutation is usually associated with haplotype h but was found once on a haplotype a background; as a CpG mutation it may have arisen independently in different founders. Of the four Austrian patients, three were heterozygous and one was homozygous for R88C, a mutation that was not identified in patients from other countries. This clearly reflects a strong regional founder effect. The common “Amish” mutation A421V was detected in patients from Germany, Austria, Croatia, and Italy, indicating a central European origin of the mutation in line with the known historical background of the Amish people. In 22 patients, mutations were found in homoallelic constellations; blood samples from both parents were available in 15 of these families. The father in one Chilean family did not carry the mutation (Y113H) for which his child was homozygous, reflecting either non-paternity or hemizygosity of Y113H with a large deletion on the paternal chromosome.\textsuperscript{11} Five of the eight homozygous German patients carried R402W, including one who was heterozygous for \textit{GCDH} haplotypes. Seven of the nine Turkish patients were homozygous, and the comparatively high rate of consanguinity probably contributes to the relatively

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\end{figure}
large number of Turkish patients with GA1. The commonest Turkish mutations with relative frequencies above 20% were P248L (5/18 alleles) and E365K (4/18 alleles). P248L was also identified in a compound heterozygous patient from Sicily, while the CpG mutation E365K was found in association with a different haplotype in a North American patient, again possibly reflecting independent recurrence. DiVerering haplotype associations were also found for one other CpG mutation, R132Q, in two patients of German and Turkish origin.

**Discussion**

GA1 is very heterogeneous, and the pattern of mutations is typical for a disease that does not confer heterozygous advantage. Most mutations are private to individual patients, and the few more common mutations probably achieved their higher relative frequencies through recurrence, founder effect, and genetic drift. There is one relatively common mutation, R402W, that may be partly responsible for the relatively high incidence of GA1 in European populations. A large number of mutations involve hypermutable CpG sites in the gene, as would be expected for a disease in which a strong negative selection pressure (homozygosity for GA1 alleles is usually lethal if untreated) is counterbalanced by new mutations. The high proportion of homozygosity and consanguinity among GA1 families indicates that the frequency of mutant alleles (carrier status) in the general population is probably smaller than would be calculated using the Hardy Weinberg formula.

Previous reports have suggested that compound heterozygosity for mutation R227P and another mutation in the GCDH gene is associated with lower urinary excretion of free glutarate, and this observation was confirmed in our study. All three patients who carried R227P on one allele had atypical biochemical

### Table 2  Spectrum of GA1 mutations, haplotype associations, and origin of alleles

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Base change</th>
<th>No</th>
<th>Haplotype</th>
<th>CpG</th>
<th>Novel</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>IVS2-2A&gt;T</td>
<td>c.138-2A&gt;T</td>
<td>1</td>
<td>b</td>
<td></td>
<td></td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td>W50C</td>
<td>c.150G&gt;C</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x Chile</td>
</tr>
<tr>
<td>5</td>
<td>Y113H</td>
<td>c.337T&gt;C</td>
<td>3</td>
<td>h</td>
<td></td>
<td></td>
<td>x Chile</td>
</tr>
<tr>
<td></td>
<td>R129Q</td>
<td>c.383G&gt;A</td>
<td>3</td>
<td>a</td>
<td></td>
<td></td>
<td>x x Turkey*, Germany</td>
</tr>
<tr>
<td></td>
<td>R131Q</td>
<td>c.395G&gt;A</td>
<td>2</td>
<td>a, b</td>
<td></td>
<td></td>
<td>x x Turkey, Germany</td>
</tr>
<tr>
<td>6</td>
<td>R148T</td>
<td>c.442G&gt;A</td>
<td>1</td>
<td>h</td>
<td></td>
<td></td>
<td>x England</td>
</tr>
<tr>
<td></td>
<td>R161Q</td>
<td>c.482G&gt;A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x Chile</td>
</tr>
<tr>
<td>7</td>
<td>R138K</td>
<td>c.413G&gt;A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x England</td>
</tr>
<tr>
<td></td>
<td>V133M</td>
<td>c.397G&gt;A</td>
<td>1</td>
<td></td>
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<td></td>
<td>x Chile</td>
</tr>
<tr>
<td>8</td>
<td>R138K</td>
<td>c.413G&gt;A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x England</td>
</tr>
<tr>
<td></td>
<td>V148I</td>
<td>c.442G&gt;A</td>
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<td></td>
<td></td>
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<td>x England</td>
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<td>R161Q</td>
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<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x Chile</td>
</tr>
<tr>
<td>9</td>
<td>V551+1G&gt;A</td>
<td>c.515+1G&gt;A</td>
<td>2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>C176R</td>
<td>c.526T&gt;C</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x Indonesia</td>
</tr>
<tr>
<td></td>
<td>E181Q</td>
<td>c.541G&gt;C</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>x Turkey, Italy</td>
</tr>
<tr>
<td>11</td>
<td>R227P</td>
<td>c.680G&gt;C</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>USA, England</td>
</tr>
<tr>
<td></td>
<td>P248L</td>
<td>c.743C&gt;T</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>x x Turkey, Italy</td>
</tr>
<tr>
<td>12</td>
<td>R355H</td>
<td>c.1064G&gt;A</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>x x Germany, Turkey</td>
</tr>
<tr>
<td>13</td>
<td>R402W</td>
<td>c.1204C&gt;T</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td>x x Germany, Europe</td>
</tr>
<tr>
<td>14</td>
<td>R402Q</td>
<td>c.1205G&gt;A</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>x x Germany</td>
</tr>
</tbody>
</table>

Haplotype = haplotype as denoted in table 3; CpG = hypermutable CpG mutation; Novel = novel mutation. * = homozygous in one patient; † = homozygous in more than one patient; ‡ = apparent homozygosity in one patient that could also be the result of hemizygosity with a large deletion on the paternal chromosome. Haplotypes in parentheses were found on single chromosomes only. The numbering of cDNA nucleotides (c.) follows the recommendation of the Nomenclature Working Group and starts with 1 at the initiator codon; values are therefore 36 nucleotides smaller than those used in previous publications on GA1 mutations.

### Table 3  Haplotypes in the GCDH gene

<table>
<thead>
<tr>
<th>IVS2+63 G&gt;C</th>
<th>G391G</th>
<th>c.1482 A&gt;G</th>
<th>Haplotypes</th>
<th>Mutant alleles</th>
<th>Mutations</th>
<th>Normal alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>− − −</td>
<td>− − −</td>
<td>a</td>
<td>40 43%</td>
<td>19 48%</td>
<td>30 42%</td>
<td></td>
</tr>
<tr>
<td>− − +</td>
<td>− − c</td>
<td></td>
<td>8 9%</td>
<td>8 20%</td>
<td>7 10%</td>
<td></td>
</tr>
<tr>
<td>+ − −</td>
<td>d</td>
<td>− − −</td>
<td>1 1%</td>
<td>1 3%</td>
<td>− − −</td>
<td></td>
</tr>
<tr>
<td>+ + −</td>
<td>e</td>
<td>− − −</td>
<td>2 3%</td>
<td>− − −</td>
<td>2 3%</td>
<td></td>
</tr>
<tr>
<td>− + +</td>
<td>f</td>
<td>− − −</td>
<td>2 3%</td>
<td>− − −</td>
<td>− − −</td>
<td></td>
</tr>
<tr>
<td>+ + +</td>
<td>g</td>
<td>− − −</td>
<td>1 1%</td>
<td>− − −</td>
<td>− − −</td>
<td></td>
</tr>
<tr>
<td>+ + +</td>
<td>h</td>
<td>− − −</td>
<td>45 48%</td>
<td>12 30%</td>
<td>30 42%</td>
<td></td>
</tr>
<tr>
<td>Total 46</td>
<td>40*</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Haplotypes were determined from the analysis of three polymorphic markers, IVS2+63G>C, G391G, and c.1482A>G, as indicated. Shown are total numbers and relative frequencies of haplotypes among mutant alleles, individual mutations, and normal alleles. * Three mutations were associated with two different haplotypes, while no haplotype could be assigned to one mutation, explaining the total of 46.
findings in urinary organic acid analysis (data not shown), and one of them had unusual iso-
lated 3-hydroxyglutaric aciduria.13 This is most
probably the result of significant residual
enzyme activity, as has been shown recently by
expression analysis of R227P in E. coli.14 How-
ever, no strict correlation between biochemical and
clinical phenotypes has yet been reported.
It is interesting to note that all three patients
with R227P were of Anglo-Saxon origin, and it
is possible that this mutation may be more
prevalent in this population.
Our results indicate that mutation analysis is
a useful alternative for confirmation of diagno-
sis in patients with suspected GA1 when
enzyme analysis is not available or feasible. We
identified mutations on both alleles in all
patients with enzymatically proven GDH defi-
ciency including several patients with unusual
biochemical features such as isolated excretion
of 3-hydroxyglutaric acid or normal urinary
organic acid profile. No mutation was found in
patients in whom the diagnosis of GA1 was
excluded through normal enzyme studies.
Among these patients there were two with
clinical features compatible with GA1 and
consistent excretion of 3-hydroxyglutaric acid
in the urine, a metabolite that has previously
been regarded as pathognomic for GDH
deficiency. The underlying genetic defect in
these children remains to be identified.

Broad range DGGE using a single, standard
gel for all exons rapidly and reliably detected all
heterozygous mutations in the present study and
should be regarded as the method of
choice for GA1 mutation analysis in a routine
setting. Homozygous mutations that do not
change the melting characteristics are not
always recognised using this method, but this
is easily circumvented by analysing at least one
parent or mixing patient DNA with a normal
control DNA sample. However, we did not
encounter any mutations in exon 1, which is
very GC rich, nor exon 4, and we cannot
therefore exclude the possibility that mutations
in these exons or other mutations in other
exons may be missed by our method. Further-
more, GA1 in some patients may be caused by
large deletions or intron mutations that are not
susceptible to simple PCR analysis.15

Direct measurement of GDH activity in
fibroblasts or lymphocytes remains the diagnost-
ic method of choice as it reflects the pathophysi-
ology in the patient and provides information
on residual enzyme activity. However, GDH assays
are performed in only a few centres worldwide
and require the analysis of living cells which may
not always be available. In contrast, small
amounts of stored DNA or blood are sufficient
for molecular analysis, and we have successfully
used dried blood spots on old Guthrie cards for
mutation detection by DGGE and sequencing.
Our method also allows genetic testing in patient
families and offers the possibility of reliable car-
rrier analysis in unrelated spouses of GA1
patients. The clinical use of GA1 mutation
analysis is restricted by a large number of private
mutations of which the impact on GDH activity
cannot be predicted. A degree of diagnostic
uncertainty remains when novel mutations are
found, particularly in patients who do not show
the excretion pattern of urine metabolites typical
of GA1. In these patients, mutation analysis
alone is insufficient for confirmation of diagno-
sis, and enzyme analysis should also be
performed. On the other hand, GDH deficiency
in some patients may be caused by defects in the
ETP binding site that would be missed by the
enzymatic assay but detected by molecular
analysis. No such defects have yet been de-
scribed, but mutation analysis should be per-
formed in all patients with clinical and bio-
chemical diagnosis of GA1 but normal enzyme
studies.

Conclusion
The DGGE method presented here may be
used for the rapid and cost efficient molecular
diagnosis of GA1, particularly when GDH
enzyme analysis is not available or feasible.
Comprehensive results in the family can be
obtained within as little as one week, and the
mutation information is particularly useful
when prenatal diagnosis is considered for future
pregnancies. It remains to be seen whether the wide range of genotypes observed
in GA1 can be correlated to differences in
residual enzyme activity, clinical features, and
outcome.

We wish to thank patients, parents, and the referring clinicians for providing blood samples.
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1 Hoffmann GF, Athanassopoulos A, Burlina A, et al. Clinical
course, early diagnosis, treatment and prevention of disease in
glutaryl-CoA dehydrogenase deficiency. Neuropediatrics
2 Baric I, Zschocke J, Christensen E, et al. Diagnosis and
management of glutaric aciduria type I. J Inherit Metab Dis
3 Goodman SI, Kratz LE, DiGiulio KA, et al. Cloning of
glutaryl-CoA dehydrogenase cDNA, and expression of
wild type and mutant enzymes in Escherichia coli. Hum
4 Schwartz M, Christensen E, Superi-Purga A, Brandt NJ.
The human glutaryl-CoA dehydrogenase gene: report of
intrinsic sequences and of 13 novel mutations causing glu-
5 Goodman SI, Stein DE, Schlesinger S, et al. Glutaryl-CoA
dehydrogenase mutations in glutaric acidemia (type I): review and
6 Bierry BJ, Stein DE, Morton DH, Goodman SI. Gene
structure and mutations of glutaryl-coenzyme A dehydro-
genase: impaired association of enzyme subunits that is due to an
A421V substitution causes glutaric acidemia type I in the
7 Guldberg P, Guttler F. ‘Broad-range’ DGGE for single-step
mutation scanning of entire genes: application to human
phenylalaninamidase hydroxylase gene. Nucleic Acids Res
8 Elton HG, Odland E, Borman H, Skjelvikå L, Engebretsen
LJ, Apold J. Application of natural and amplification
created restriction sites for the diagnosis of PKU
9 Antonarakis SE, the Nomenclature Working Group. Rec-
ommendations for a nomenclature system for human gene
10 Bird AP. CpG-rich islands and the function of DNA meth-
11 Zschocke J, Quaak E, Knauer A, Fritz B, Aslan M, Hoff-
man GF. Large heterogeneous deletion masquerading as homo-
zygous missense mutation: a pitfall in diagnostic mutation
12 Christensen E, Ribes A, Busquets C, et al. Compound het-
erozygosity in the glutaryl-CoA dehydrogenase gene with
R227P mutation in one allele is associated with no or very
low free glutarate excretion. J Inherit Metab Dis 1997;20:
383-6.
13 Nyhan WL, Zschocke J, Hoffmann GF, Stein DE, Mao L,
Goodman S. Glutaryl-CoA dehydrogenase deficiency pre-
senting as 3-hydroxyglutaric aciduria. Mol Genet Metab
14 Liesert M, Zschocke J, Hoffmann GF, Mühlhäuser N,
Buchel W. Biochemistry of glutaric aciduria type I:
activities of in vitro expressed wild-type and mutant CDNA
encoding human glutaryl-CoA dehydrogenase. J Inherit