

Glutaryl-CoA Dehydrogenase Deficiency in Spain: Evidence of Two Groups of Patients, Genetically, and Biochemically Distinct

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ABSTRACT

Glutaryl-CoA dehydrogenase (GCDH) deficiency causes glutaric aciduria type I (GA I), an inborn error of metabolism that is characterized clinically by dystonia and dyskinesia and pathologically by neural degeneration of the caudate and putamen. Studies of metabolite excretion allowed us to categorize 43 GA I Spanish patients into two groups: group 1 (26 patients), those presenting with high excretion of both glutarate and 3-hydroxyglutarate, and group 2 (17 patients), those who might not be detected by routine urine organic acid analysis because glutarate might be normal and 3-hydroxyglutarate only slightly higher than controls. Single-strand conformation polymorphism (SSCP) screening and sequence analysis of the 11 exons and the corresponding intron boundaries of the GCDH gene allowed us to identify 13 novel and 10 previously described mutations. The most frequent mutations in group 1 were A293T and R402W with an allele frequency of 30% and 28%, respectively. These two mutations were also found in group 2, but always in heterozygosity, in particular in combination with mutations V400M or R227P. Interestingly, mutations V400M and R227P were only found in group 2, and at least one of these mutations was found in 11 of 15 unrelated alleles, accounting together for 53% of the mutant alleles in group 2. Therefore, it seems clear that two

genetically and biochemically distinct groups of patients exist. The severity of the clinical phenotype seems to be closely linked to the development of encephalopathic crises rather than to residual enzyme activity or genotype. Comparison of GCDH protein with other acyl-CoA dehydrogenases (whose x-ray crystal structure has been determined) reveals that most of the mutations identified in GCDH protein seem to affect folding and tetramerization, as has been described for a number of mutations affecting mitochondrial β -oxidation acyl-CoA dehydrogenases. (*Pediatr Res* 48: 315–322, 2000)

Abbreviations

GA I, glutaric aciduria type I
GCDH, glutaryl-CoA dehydrogenase
FAD, flavin adenine dinucleotide
ETF, electron-transfer flavoprotein
GC-MS, gas chromatography-mass spectrometry
MCAD, medium-chain acyl-CoA dehydrogenase
SCAD, short-chain acyl-CoA dehydrogenase
VLCAD, very-long-chain acyl-CoA dehydrogenase
IVD, isovaleryl-CoA dehydrogenase
SSCP, single-strand conformation polymorphism

GA I (MIM 231670) is an autosomal recessive disorder caused by deficient or nonfunctional GCDH (EC 1.3.99.7). The

metabolic block leads to the accumulation of glutaric and 3-hydroxyglutaric acids as well as glutarylcarnitine in body fluids. The clinical picture is characterized by the sudden onset of a severe dystonic-dyskinetic disorder, hypotonia, irritability, macrocephaly, and degeneration of the caudate and putamen, which generally appear between 5 and 14 mo of age, but mild

Received November 11, 1999; accepted March 1, 2000.
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This work was supported by Fondo de Investigaciones Sanitarias (FIS) grant 97/0049–01.

symptoms such as motor delay and hypotonia can be observed at earlier ages (1–4). However, clinical variability is common and some patients remain asymptomatic (2, 4). The condition is not exceedingly rare, and a frequency of 1:40,000 in Caucasians has been proposed (2).

The GCDH enzyme functions in the mitochondrial matrix as a homotetramer. The monomer is made up of 438 amino acids (48.2 kD), of which the 44 N-terminal residues are cleaved after being imported into the mitochondria. It also contains one molecule of FAD, which is reduced by ETF (5).

The human GCDH gene has been mapped to chromosome 19p13.2 (6), spans about 7 Kb, and comprises 11 exons and 10 introns. The genomic structure and most of the intronic sequences have been published (5, 7, 8) and the amino acid sequences of mouse, human, and pig GCDH are highly conserved (9). Single prevalent mutations, IVS1+5G>T and A421V, have been found in the Ojibway Indians of Canada (10) and in the Amish of Pennsylvania (7), respectively. However, mutations of the GCDH gene in general population are heterogeneous. To date, 66 disease-causing mutations and 6 polymorphisms and neutral mutations have been identified in the GCDH gene (7, 8, 10–16), the most common mutation being R402W with an allele frequency of 12 to 16% (7, 8).

In this study, we have analyzed metabolite excretion, GCDH activity, and the mutations responsible for GCDH deficiency in 43 Spanish patients with GA I. Our studies showed that there were two genetically and biochemically distinct groups of patients. We have attempted to establish a correlation between the GA I genotype and the clinical or biochemical phenotype.

MATERIALS AND METHODS

Patients. We studied 43 patients (35 unrelated families) with GA I from different areas of Spain. Diagnosis was established by clinical symptoms, characteristic findings on neuroimaging, and quantitative analysis of organic acids in urine. The disease was confirmed by analysis of GCDH activity in cultured skin fibroblasts, as previously described (17). Fibroblasts from nine patients were not available, but in six of them GCDH activity could be predicted on the basis of other patients belonging to the same GCDH genotype. Only in four families were the parents known to be consanguineous. Case histories of patients 4, 13, 19, 22, 23, 25, 26, 27, 28, 34, 38, 39, and 42 have been previously described (18–23).

In 24 patients the disease began abruptly, during infancy, with focal seizures or generalized convulsions, vomiting, and obtundation or lethargy, usually after an acute infectious illness. In 18 patients the onset was “insidious,” with slowly developing psychomotor delay, hypotonia as dystonic postures, lethargy, or macrocephaly. The phenotype was classified according to the following criteria: “severe,” when the patient showed dystonia (either static or progressive) and was unable to stand up, walk, or speak (25 patients); “mild,” when dystonia was static or improving and the child was able to walk without help (11 patients); and “slight,” when there were only minimal neurologic signs, and the child was able to lead a normal life (5 patients). Two patients were asymptomatic. The

siblings of patients presented the same phenotype and clinical onset as their corresponding index case, except patient 11, who is an elder asymptomatic brother of patient 10, and patient 17, who is a younger brother of patient 16 with a milder clinical phenotype (Table 1).

The ethics committees of the participating hospitals and organizations approved the collection of samples on which these studies were performed.

Quantitative analysis of glutarate and 3-hydroxyglutarate.

Organic acids were isolated by strong anion exchange (SAX) column extraction as described (24) and were analyzed by GC-MS (Hewlett-Packard 5890, Palo Alto, CA, U.S.A.) as trimethylsilyl (TMS)-derivatives. For quantitation, the mass spectrometry was operated in the single ion monitoring mode, and the following ion currents were measured: glutarate m/z 261, 3-hydroxyglutarate m/z 259, and undecanedioate (internal standard) m/z 345. The sodium salts of glutaric and undecanedioic acids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 3-hydroxyglutaric acid was from Dr. Brunet (Organic Chemistry Department, Universidad Autónoma de Madrid, Spain).

Mutation analysis. Genomic DNA was prepared from fresh blood or from cultured skin fibroblasts by standard methods. Total RNA was prepared from cultured fibroblasts by the UltraspecTM RNA Isolation System (Biotech, Laboratories, Houston, TX, U.S.A.).

Mutation screening of genomic DNA was performed by PCR amplification and SSCP analysis. PCR conditions and primers used were as described elsewhere (7, 14).

SSCP was performed at room temperature or at 4°C. DNA fragments were detected after silver staining. The bands with mobility shifts were excised from the gel, purified (QIAquick Gel Extraction Kit; QIAGEN, GmbH Germany, Hilden, Germany) and reamplified, followed by sequencing using a Sequenase Version 2.0 Kit (USB, Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and ³⁵S-dATP. In some cases, PCR products were purified (QIAquick PCR Purification Kit; QIAGEN) and sequenced with the DNA sequencing kit, dye terminator cycle sequencing ready reaction (Perkin Elmer Applied Biosystems Division, Foster City, CA, U.S.A.).

For cDNA analysis, about 5 µg of total RNA was transcribed into the first strand of cDNA in a 25-µL reaction volume containing avian myeloblastosis virus (AMV) 1× buffer, 400 µM dinucleotide triphosphate (dNTP), 10 U AMV reverse transcriptase (Promega, Madison, WI, U.S.A.), 33 U RNA in ribonuclease inhibitor (Promega), and 400 nM of primer for 45 min at 52°C. PCR amplification of cDNA was carried out using primers described elsewhere (10). PCR products were studied by SSCP analysis and sequenced as described above.

When possible, sequence changes were confirmed by restriction enzyme analysis as summarized in Table 2. To detect mutation A293T, a mismatch PCR was designed. We introduced an *Hga*I restriction site into the mutant PCR product. The two primers used were located on exon 8. The forward mismatch primer was 5'-CCTTCGGCTGCCTGAACGAC-3', where the mismatch nucleotide is underlined, and the reverse primer was as described (7).

Table 1. Genotype-phenotype correlation in 35 unrelated Spanish families with GA I

| Patients | Genotype | Metabolite excretion* | | %GCD activity in fibroblasts | Clinical onset | Phenotype |
|----------|------------------------|-----------------------|----------------|---------------------------------|---------------------------|---------------------|
| | | Glutarate | 3-OH-Glutarate | | | |
| Group 1 | | | | | | |
| 1, 2† | A293T /A293T | 3336/2120 | 825/543 | <0.5 | Acute encephalopathy | Severe |
| 3 | A293T /A293T | 1773 | 340 | <0.5 | Acute encephalopathy | Severe |
| 4, 5† | A293T /A293T | 4135/2166 | 752/420 | <0.5 | Insidious | Mild |
| 6, 7† | A293T /A293T | 500/3200 | 461/843 | n.d. | Insidious | Slight |
| 8 | A293T/E90K | 1098 | 148 | 6 | Acute encephalopathy | Severe |
| 9 | A293T /R402W | 2982 | 415 | <0.5 | Insidious | Slight |
| 10, 11 | A293T /R402W | 772/2083 | 264/262 | n.d. | Acute encephalopathy/none | Severe/asymptomatic |
| 12 | A293T /R402W | 8188 | 3573 | n.d. | Insidious | Severe |
| 13 | R402W /R402W | 1288 | 75 | <0.5 | Insidious | Severe |
| 14 | R402W /R402W | 2625 | 262 | n.d. | Insidious | Mild |
| 15‡ | R402W /R402W | n.d. | n.d. | n.d. | Acute encephalopathy | Severe |
| 16, 17 | R402W /R128X | 4885/6644 | 713/603 | <0.5 | Acute encephalopathy | Severe/mild |
| 18 | R402W /1209delG | 4097 | 212 | <0.5 | Insidious | Severe |
| 19 | R372K /1209delG | 8955 | 2262 | <0.5 | Insidious | Mild |
| 20 | R132Q /1209delG | 3571 | 232 | n.d. | Insidious | Mild |
| 21 | S119L /A195T | 204 | 143 | 2 | Acute encephalopathy | Severe |
| 22 | E181K /E181K | 1028 | 291 | <0.5 | Insidious | Mild |
| 23 | 423-424delGC /R383C | 7932 | 1074 | <0.5 | Insidious | Asymptomatic |
| 24 | A304T/ L309W | 1673 | 795 | <0.5 | Insidious | Slight |
| 25, 26† | A316D /A316D | 6164/3556 | 626/420 | <0.5 | Acute encephalopathy | Severe |
| Group 2 | | | | | | |
| 27 | V400M /V400M | 2 | 18 | 10 | Acute encephalopathy | Severe |
| 28 | V400M /V400M | 2 | 185 | 5 | Acute encephalopathy | Severe |
| 29 | V400M /V400M | 5 | 114 | n.d. | Acute encephalopathy | Severe |
| 30 | V400M /R402W | 25 | 571 | 1 | Acute encephalopathy | Severe |
| 31 | V400M /R402W | 12 | 175 | 2 | Acute encephalopathy | Mild |
| 32 | V400M /R402W | 69 | 429 | 3 | Acute encephalopathy | Severe |
| 33 | V400M /unknown | 4 | 16 | 34 | Insidious | Severe |
| 34, 35† | V400M /R227P | 7/6 | 99/36 | 30 | Insidious | Mild |
| 36 | V400M /R227P | 24 | 221 | 22 | Acute encephalopathy | Severe |
| 37 | A293T /R227P | 6 | 23 | 7 | Insidious | Mild |
| 38 | Y155H /R227P | 4 | 43 | 9 | Acute encephalopathy | Severe |
| 39, 40† | A293T /S255L | 16/10 | 77/31 | <0.5 | Acute encephalopathy | Severe |
| 41 | F71S /1209delG | 48 | 101 | 0.8 | Acute encephalopathy | Severe |
| 42 | A433V /A433V | 35 | 268 | n.d. | Acute encephalopathy | Slight |
| 43 | R161Q /884delT | 84 | 312 | n.d. | Acute encephalopathy | Severe |

n.d., not done.

* Values are expressed in mmol/mol creatinine. Median and range for glutarate and 3-hydroxyglutarate in 42 independent controls were 3 (2–10) and 7 (2–14), respectively.

† Siblings with the same enzyme activity, clinical onset and phenotype than the corresponding index case.

‡ Patient 15 died and no samples were available; the presumptive diagnosis of GA I was established due to clinical features and demonstration of heterozygosity in the parents.

Mutations were named according to suggested nomenclature (25). Throughout this article, all mutations except frameshift are referred to by their amino acid-based name.

RESULTS

Biochemical and clinical findings. The use of quantitative, as opposed to qualitative, organic acid analysis allowed us to categorize the patients into two groups (Table 1): Group 1 (26 patients) comprised those who could be diagnosed simply by routine GC-MS organic acid analysis in urine. Patients presented with high excretion of both glutarate and 3-hydroxyglutarate, although the excretion of glutarate was always higher than that of 3-hydroxyglutarate. Group 2 (17 patients) comprised those who might go undetected by routine screening because glutarate excretion can be normal

and that of 3-hydroxyglutarate might be only slightly increased. In contrast to group 1 patients, those in group 2 presented with a higher 3-hydroxyglutarate excretion than that of glutarate. The median and range (mmol/mol creatinine) of glutarate and 3-hydroxyglutarate excretion in 42 control urine samples were 3 (2–10) and 7 (2–14), respectively; values for group 1 patients were 2817 (204–8955) and 420 (75–3573), respectively. In contrast, group 2 showed a median value for glutarate, 10 (2–84), which is within the range of controls, whereas the median value for 3-hydroxyglutarate, 101 (16–571), fell within the range of group 1. When possible, only those values of glutarate and 3-hydroxyglutarate obtained at the time of diagnosis were considered to obviate the possible influence of any treatment.

Table 2. Summary of all mutations and their frequencies in 35 unrelated Spanish families with GA I

| Location | Nucleotide change | Effect on protein | Restriction enzyme† | No. alleles | |
|-----------|-------------------|----------------------|---------------------|-------------|---------|
| | | | | Group 1 | Group 2 |
| Missense | | | | | |
| Exon 3 | 248 T>C | F71S* | <i>MnlI</i> (+) | 0 | 1 |
| Exon 3 | 304 G>A† | E90K* | | 1 | 0 |
| Exon 5 | 392 C>T† | S119L* | | 1 | 0 |
| Exon 5 | 431 G>A† | R132Q* | <i>AccI</i> (-) | 1 | 0 |
| Exon 5 | 499 T>C | Y155H | <i>NcoI</i> (+) | 0 | 1 |
| Exon 5 | 518 G>A† | R161Q | <i>AccI</i> (-) | 0 | 1 |
| Exon 6 | 577 G>A | E181K* | | 2 | 0 |
| Exon 6 | 619 G>A | A195T | <i>BanI</i> (-) | 1 | 0 |
| Exon 7 | 716 G>C | R227P | <i>NlaIV</i> (+) | 0 | 4(13%) |
| Exon 7 | 800 C>T† | S255L* | | 0 | 1 |
| Exon 8 | 913 G>A† | A293T | <i>HgaI</i> (-)‡ | 12(30%) | 2(7%) |
| Exon 8 | 946 G>A | A304T* | <i>AluI</i> (-) | 1 | 0 |
| Exon 8 | 962T >G | L309W | <i>EcoPI5</i> (+) | 1 | 0 |
| Exon 8 | 983 C>A | A316D* | <i>HgiEII</i> (+) | 2 | 0 |
| Exon 10 | 1151 G>A | R372K* | <i>MnlI</i> (-) | 1 | 0 |
| Exon 10 | 1183 C>T† | R383C | <i>AccI</i> (-) | 1 | 0 |
| Exon 10 | 1234 G>A† | V400M | <i>NlaIII</i> (+) | 0 | 12(40%) |
| Exon 10 | 1240 C>T† | R402W | <i>MspI</i> (-) | 11(28%) | 3(10%) |
| Exon 11 | 1334 C>T† | A433V* | <i>MwoI</i> (+) | 0 | 2 |
| Deletions | | | | | |
| Exon 5 | 423-424 del GC | Truncated on 185 aa* | <i>AluI</i> (-) | 1 | 0 |
| Exon 7 | 884 del T | Truncated on 289 aa* | <i>MspI</i> (+) | 0 | 1 |
| Exon 10 | 1209 del G | Truncated on 399 aa | | 3 | 1 |
| Nonsense | | | | | |
| Exon 5 | 418 C>T† | R128X* | <i>Sau96I</i> (-) | 1 | 0 |

The shaded region highlights mutations that appear in both groups.

* New mutations found in this study.

† Substitutions found at hypermutable CpG sites; (+) mutation creates the restriction site, (-) mutation destroys the site.

‡ Restriction site created by a mismatch primer (see Materials and Methods).

Glutarylcarbitine in blood spots was tested in six patients from group 2: patient 38 (Dr. Millington, Duke University, Durham, NC, U.S.A.) and patients 30, 34, 36, 37, and 41 (Dr. Hoffmann, Philipps-Universitat, Marburg, Germany). All of them gave negative results (even under carnitine supplementation). This indicates that patients with this metabolite excretion pattern would not be detected in neonatal screening programs employing tandem mass spectrometry.

In group 1, all but two patients had undetectable GCDH activity in fibroblasts (<0.5% of controls), whereas in group 2, the residual activities were usually higher (Table 1).

Acute encephalopathy marked the clinical onset in 24 patients, of whom 21 presented the severe phenotype. Insidious onset was found in 18 patients, of whom only 4 presented the severe phenotype (Table 1). Interestingly, 71% of group 2 patients presented the severe phenotype, whereas it was only found in 50% of group 1 patients (Table 1). Therefore, the severity of the clinical phenotype does not seem to be directly related to the severity of the biochemical phenotype.

Mutation analysis. SSCP screening of the 11 exons and intron boundaries of the GCDH gene allowed us to identify 13 novel (10 missense, 2 deletions, and 1 nonsense) and 10 already known mutations, which account for 99% (69/70) of the total number of mutant alleles (Table 2). Sequence analysis of GCDH cDNA and genomic DNA of patient 33 revealed that mutation V400M was the only consistent change in the entire protein-coding region, suggesting that the other mutations lie

in the promoter region or elsewhere within the intronic sequences. Of the 23 mutations identified only three were common to both groups (A293T, R402W, and 1209delG). However, these mutations were found in group 2 always in heterozygosity, in particular in combination with mutations V400M or R227P. The latter mutations accounted for 53% of the group 2 alleles and were exclusive to this group, whereas R402W and A293T mutations, accounted for 58% of the group 1 alleles (Table 2). None of the missense mutations was found in 100 unrelated control chromosomes. Mendelian inheritance of the mutations was confirmed by use of a restriction-digestion test, where available, or by SSCP pattern, in the DNA of the parents of 25 families.

Flow chart for the diagnosis of GA I. The flowchart depicted in Figure 1, based on the biochemical and mutational results reported here, is now being used in our laboratory. It is especially useful for the diagnosis of group 2 patients, and in most cases means that the measurement of GCDH activity is not necessary. Patients 29, 33, and 36 were, in fact, diagnosed using this flowchart.

DISCUSSION

The aim of this study was to identify the mutations responsible for GCDH deficiency in the Spanish patients to investigate a possible correlation between GA I genotypes and clinical and biochemical phenotypes.

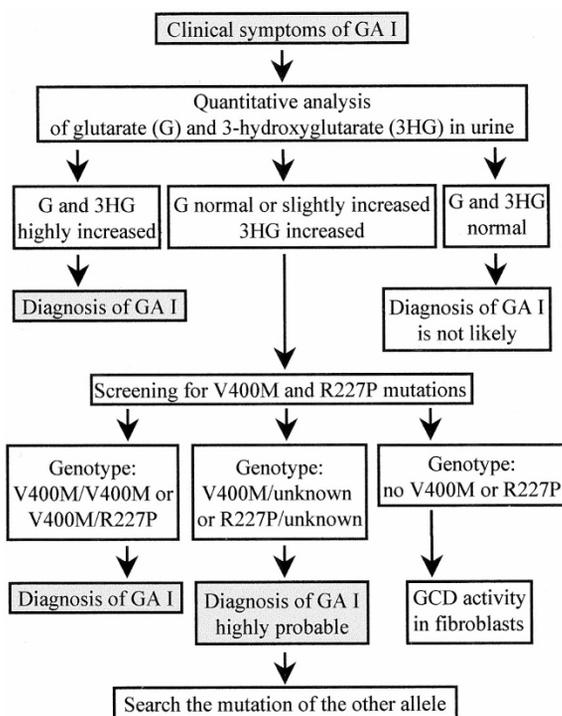


Figure 1. Flow-chart for the diagnosis of GA I.

Mutational spectrum. We report 13 novel mutations in the GCDH gene. This means that, at present, a total of 79 potentially pathogenic mutations are known. Missense mutations are more frequently detected than nonsense mutations or deletions, and here 55% of nucleotide substitutions were found at hypermutable CpG sites in the GCDH gene (Table 2). Deletions and nonsense mutations all result in truncation of the mutant protein before the α -proton-abstracting base (E414) in enzyme catalysis (5); consequently, the encoded protein would not be expected to have any residual activity. Several lines of evidence suggest that missense mutations may be pathogenic: (1) after screening all of the coding region of the GCDH gene, no other mutations were found in the patients; (2) the nucleotide changes were not present in 100 normal chromosomes analyzed; and (3) all the amino acid residues, except R161 and A316, are conserved in pig and mouse GCDH homologs (5, 9).

According to the pattern of metabolite excretion, patients were categorized into two groups. In group 1, we have found 15 different mutations, of which 9 are novel. In contrast to other reports (7, 8, 15), we found a particular prevalence not only for mutation R402W (28%) but also for mutation A293T (30%). In addition, we have recently found linkage disequilibrium between mutation R402W and a specific haplotype, suggesting a single origin for this mutation (26). In group 2, we have found 11 mutations, of which 4 are novel. The most striking result from this group was the strong association with mutations V400M and R227P, which together account for more than 50% of mutant alleles. We have previously shown that the R227P allele was associated with minimal glutarate excretion when heterozygous (13) and it has recently been confirmed in a patient with genotype E365K/R227P, whereas patients homozygous for mutation E365K present with large amounts of glutarate and 3-hydroxyglutarate in urine (27). The

same applies when mutation A293T was found in homozygosity, whereas in heterozygosity with R227P the biochemical phenotype changed to that of group 2 (Table 1). Consequently, R227P seems to be one of the causative mutations of this phenomena, but it is not clear how this mutation acts (7, 8). It might be the case that in these patients the decarboxylase activity was more severely affected than that of the dehydrogenase, but this was not sustained when mutation R227P was expressed in *Escherichia coli*, and partial reactions (dehydrogenation and decarboxylation) of GCDH activity were measured (28). A similar phenomena has been observed with mutation V400M. Genotypes V400M/R227P (patients 34, 35, and 36) and V400M/unknown (patient 33) presented the highest residual enzyme activities in fibroblasts, similar to those in the heterozygous range. If we take into account that patients homozygous for mutation V400M have lower enzyme activities than those compound heterozygous for V400M/R227P, a complementary effect between these two alleles might be speculated. Other private mutations (F71S, R161Q, and S255L) were also found to be associated with this group of patients.

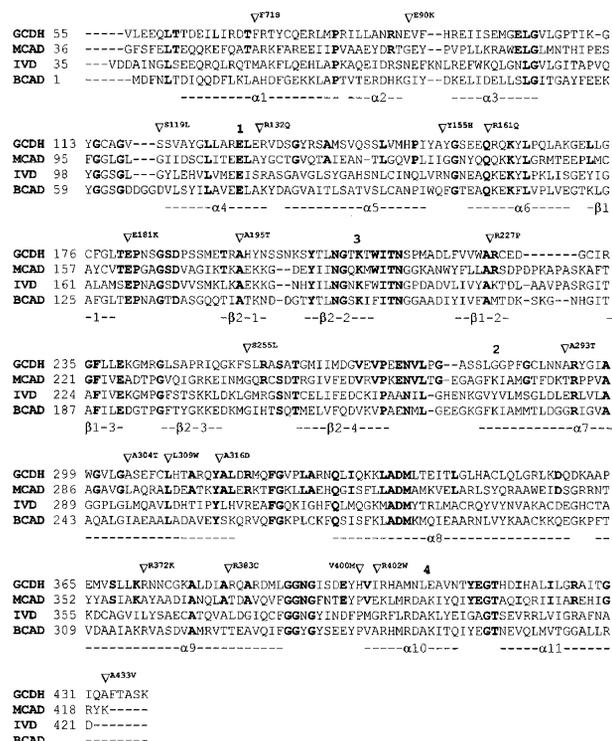


Figure 2. Alignment of the human GCDH sequence with the sequences of three acyl-CoA dehydrogenases (ACD) with known three-dimensional structure: pig MCAD (29), human IVD (30), and bacterial SCAD or BCAD (31). The GCDH sequence was aligned manually with the reference sequences taking into account conserved residues, residues in contact with substrates, and also the suitability of hydrophobicity and secondary structure profiles. All numbering is according to the precursor enzymes. The names of α -helices and β -sheets are given below their sequence. The position of the amino acid change due to each missense mutation identified is shown above its sequence with downward-pointing arrows. Residues shared by all ACD or residues that are identical in GCDH and MCAD are highlighted. Positions marked by a number are described in detail in the text: 1, E-129 in GCDH; 2, G-284 in GCDH; 3, K-164 in mature MCAD (K189 in precursor MCAD); 4, E-408 in GCDH.

Effects of GCDH mutations on protein structure. Human GCDH displays a high degree of homology with other acyl-CoA dehydrogenases (Fig. 2), therefore, comparison with the x-ray structure of MCAD (29), IVD (30) and SCAD or butyryl-CoA dehydrogenase (BCAD) (31) may help to explain the molecular consequences of the identified mutations. As described for MCAD (29), the monomer structure of GCDH can be divided in three regions with a distinct secondary structure. Region A, from R-45 to E-172 (Fig. 2), consists of a bundle of six α -helices that does not appear to be directly involved in either substrate binding or oligomerization (except for the first five residues, which might participate in oligomerization). In this region we found six mutations (F71S, E90K, S119L, Y155H, R132Q, and R161Q), most of which are associated with residual activity. Two of these, F71S and S119L, may affect protein stability because of a change in polarity, which is, moreover, maintained among reference structures. In particular, the change in polarity brought about by F71S could prevent the salt bridge formation between residues R72 and E129, impairing GCDH folding, as has been described for mutations R28C and R22W in MCAD and SCAD proteins, respectively (32, 33). Residue Y-155 forms a hydrogen bond with G-284, which lies near the adenine ring of CoA. Therefore, mutation Y155H might compromise substrate binding. The consequences for mutations E90K, R132Q, and R161Q are less evident.

Region B, from L-173 to P-278 (Fig. 2), is a β -sandwich in close proximity to FAD. In this region we found four mutations, two of them (E181K and A195T) were associated with group 1 patients, whereas the other two (R227P and S255L) were found in group 2 patients. The substitution E181K may alter the local fold around the riboflavine moiety of FAD. Moreover, residue E-181 is homologous to residue E-137 in MCAD, the carboxyl of which is crucial for β -domain folding, and which has been described to affect tetramerization (34). A homologue disease-causing mutation, E178K, in VLCAD has also been identified (35). A similar effect could be predicted for mutation S255L. The mutation A195T leads to an increase in the volume and polarity of a conserved residue buried inside the β -sandwich, and this might alter the stability of the whole region. Mutation R227P lies in a region that might be involved in the ETF interaction site, as suggested for MCAD protein (36).

Region C consists of a bundle of four antiparallel α -helices, the latter split in two halves. Loops connecting the helices are directly involved in the interaction with FAD molecule, the loop between α 7 and α 8 is in contact with the adenine ring, and the loop between α 9 and α 10 (including a patch of conserved glycine residues) is in contact with the pyrophosphate group of the dinucleotide (Fig. 3). Moreover, FAD molecules interact at the same time with residues from the two subunits in such a way that FAD binding and subunit aggregation are closely linked. This region is structurally the most important, as regions C of the four subunits form the core of the tetramer. Mutations in this region are expected to compromise both FAD binding and intersubunit contacts. Figure 3 shows the position of the mutations found in this region (A293T, A304T, L309W, A316D, R372K, R383C, V400M, and R402W), together with

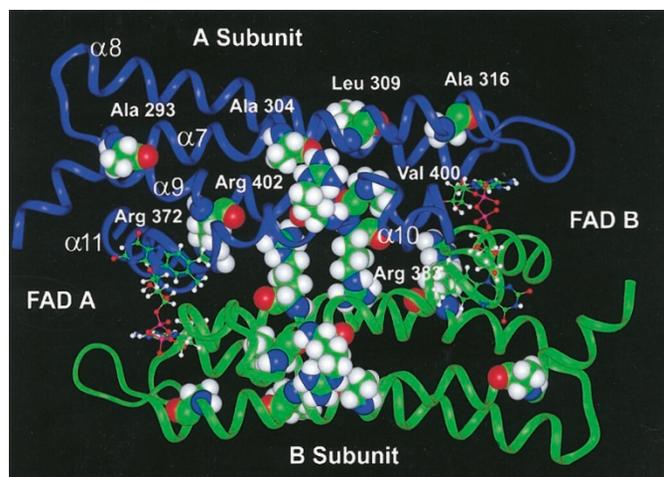


Figure 3. Location of GCDH mutations found in region C. Ribbon representation of region C, comprising α 7 to α 10–11 (from 282 to 438 residues), of two subunits of the GCDH model. The view is oriented along the binary symmetry axis that relates the two subunits. Side chains of the mutated residues are shown as space-fill models and the two FAD molecules are indicated as ball and stick representations. Loops between α 7 and α 8 (adenine ring) and α 9 and α 10 (pyrophosphate) interact directly with the coenzyme. All calculations were performed in a Silicon Graphics R8000 workstation using the molecular modeling programs Insight II, Homology, and Discover (Molecular Simulations, Inc., Princeton, NJ, U.S.A.).

a ribbon representation of regions C of two subunits. All of these mutations, except V400M, were associated with a lack of residual enzyme activity. The residual activity found in patients harboring mutation V400M can probably be accounted for by the fact that this does not constitute a drastic change. Three of these mutations (A293T, R402W, and V400M) were expressed in *E. coli* (15) and the results for R402W and V400M were inconsistent with the biochemical phenotype of our patients. The R402W mutant has been shown to have some residual activity (3%), whereas the residual activity for mutant V400M is almost undetectable (0.8%); the contrary has been observed when GCDH was assayed in fibroblasts. These results reflect the difference between *in vivo* and *in vitro* analyses, as also observed in other diseases (37).

Genotype-phenotype correlation. Considerable clinical variability in patients with GA I has been reported (2–4). Patient 10 is an extreme example of this heterogeneity. This child presented a severe phenotype and died at 2 y of age, whereas her elder GA I brother is asymptomatic at 4 y of age (data not shown). Another apparent discrepancy of this disease is that a severe phenotype was recorded with greater frequency among group 2 patients (71%), the biochemical phenotype of whom is less severe than that of group 1. Therefore, it seems clear that high residual activity and low metabolite excretion do not prevent the development of a severe clinical phenotype (see patients 33 and 36). The severity of the phenotype seems to be closely linked to the development of encephalopathic crises, often in association with high fever. In our series, 91% of patients with encephalopathic onset presented a severe phenotype, and prevention of the encephalopathic crises is the only treatment available to avoid further deterioration. Thus, environmental factors, such as exposure to metabolic stress, are probably one of the main determinants of the clinical

phenotype, irrespective of the GA I genotype. This is by no means a new finding in the history of acyl-CoA dehydrogenase deficiencies; in MCAD deficiency, patients with the most severe mutations may remain asymptomatic for years and patients with the mildest MCAD mutations may present with the most severe symptoms (38). Similar observations have been made in SCAD deficiency (39).

It is interesting to note that, in contrast to glutarate, the concentration of 3-hydroxyglutarate in urine is similar in both groups of patients. This compound, at concentrations much lower than glutarate, has been reported to induce massive nerve cell lesions *in vitro* (40, 41). Most probably, 3-hydroxyglutarate arises from β -oxidation of the intramitochondrial increased glutaryl-CoA through the β -oxidation enzyme, MCAD (1), but this enzyme is not expressed in brain (42) and, consequently, is unable to produce 3-hydroxyglutarate in this tissue. It is thus difficult to link brain damage to this metabolite, but it might be the case that 3-hydroxyglutarate crosses the blood-brain barrier due to increased permeability during viral or bacterial infections (43–45) and perhaps then provokes brain lesions and encephalopathic crisis. The fact that 3-hydroxyglutarate did not increase in cerebrospinal fluid during clinical stability (A.R., personal observation) and that other authors found it increased during an acute illness (46) favor this hypothesis.

From this study we can conclude the following: (1) For certain diseases it is necessary to perform mutation analysis in every population. In earlier reports (7, 8, 15) many private mutations were reported because, it would seem, the studies were conducted in heterogeneous populations. (2) Recently, it has been speculated that the number of group 2 patients was probably small (3), but this is not the case in our population, where 40% of patients were found to belong to this group. Whether this is a particular genetic characteristic of our population or reflects the results of ongoing attempts to improve diagnosis (18, 22, 23, 47) remains to be established. (3) The knowledge of GA I genotypes might be useful for prenatal diagnosis, particularly for families with high residual enzyme activity. (4) Comparison of GCDH protein with other crystal-solved acyl-CoA dehydrogenases reveals that most of the mutations identified might affect folding and tetramerization, as has been described for a number of mutations affecting mitochondrial β -oxidation acyl-CoA dehydrogenases. However, further experimental studies are needed to demonstrate this definitively. (5) With a few exceptions, a marked correlation between genotype and biochemical phenotype was found. However, no correlation was found between clinical symptoms, enzyme activities, and metabolite excretion in these Spanish GA I patients, which suggests that other factors contribute to the onset and severity of the disease.

Acknowledgments. The authors thank Dr. S.I. Goodman (University of Colorado School of Medicine, Denver, CO, U.S.A.), who provided us with information on GCDH gene structure so that we might undertake our molecular studies. We also thank C. Ogg and C. Llordés for their excellent technical assistance, and Laura Gort for her help and advice concerning mutation studies. We also thank the GA I families and the

following pediatricians and biochemists who contributed to this study by providing fibroblasts or blood samples: J. Artigas, G. Pintos, P. Villanueva, M.A. Ruiz, M. Tallada, M.A. Vilaseca, M.A. Fernández, E. Riudor, M. Aparicio, G. Lorenzo, I. Corral, C. Garzo, G. Bueno, C. Pedrón, and J.C. Cabrera.

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