Genetic Screening of Selected Disease-Causing Mutations in Glutaryl-CoA Dehydrogenase Gene among Indian Patients with Glutaric Aciduria Type I

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Abstract

Keywords

- ▶ genotyping
- ▶ glutaric aciduria type I
- ▶ glutaryl-CoA dehydrogenase

Glutaric aciduria type I (GA-I) is an organic aciduria caused by glutaryl-CoA dehydrogenase (GCDH) deficiency. There are limited studies on GA-I from India. A total of 48 Indian GA-I patients were screened for selected disease-causing mutations such as R402W, A421V, A293T, R227P, and V400M using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Among these patients, 9 (18.8%) had R402W mutation, and none had A421V, A293T, R227P, or V400M mutation. One low excretor mutation (P286S) and several novel mutations (I152M, Q144P, and E414X) were also found in this study. We conclude that among selected mutations, R402W is the most common mutation found among Indian GA-I patients.

Introduction

Glutaric aciduria type I (GA-I; OMIM #231670) is an autosomal recessive disorder of lysine, hydroxylysine, and tryptophan metabolism caused by deficiency of glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7).¹ GCDH catalyzes oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and CO₂. The defective enzyme causes accumulation of excessive levels of intermediate breakdown products such as glutaric acid (GA), 3-hydroxy GA (3-OH-GA), and glutaconic acid (GC) in blood, urine, cerebrospinal fluid (CSF), and brain tissue.^{2–4} With the estimated prevalence of 1 in 30,000 to 100,000 newborns⁵ and much higher in certain ethnic groups, this disorder is recognized as a major cause of acute metabolic brain damage in early childhood.⁶ The characteristic clinical presentation includes macrocephaly, hypotonia, progressive

dystonia, and dyskinesia. The onset of neurologic symptoms occurs following acute illness leading to encephalopathy crisis, resulting in bilateral striatal damage^{7,8} probably due to acute rise in GA or 3-OH-GA levels in body fluids such as serum and/or CSF.^{9,10} Depending on urine GA levels, two distinct biochemical phenotypes are known, high excretors (GA in urine >100 mmol/mol Cr) and low excretors (GA in urine <100 mmol/mol Cr).¹¹

The mammalian *GCDH* gene has been mapped to chromosome 19p13.2 and spans approximately 7 kb comprising 11 exons and 10 introns.¹² The GCDH protein is a 45-kD homotetramer.¹³ More than 150 disease-causing *GCDH* mutations have been described.⁵ Some disease-causing mutations occur in high frequency and belong to the high excretor phenotype, including R402W (p. Arg402Trp), A293T (p. Ala293Thr), and A421V (p. Ala421Val).¹ Other mutations,

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such as R227P (p. Arg227Pro) and V400M (p. Val400Met), are prevalent in closed ethnic groups and are of the low excretor phenotype.^{5,14}

In India, there are few studies and only isolated case reports on GA-I.¹⁵ There is scarcity of data on its genetic etiology. Recent studies on GA-I from this region are contentious and inconclusive due to limited sample size.^{16,17} This study aims to screen selected disease-causing mutations such as R402W, A293T, and A421V (high excretor mutations), and R227P and V400M (low excretor mutations) among GA-I patients from India. These mutations were selected as they are highly prevalent in certain ethnic populations¹ and adversely affect GCDH activity. While screening for selected disease-causing mutations, novel mutations identified were reported, and computational modeling studies were performed to understand the impact of novel mutations on the structure and function of GCDH enzyme.

Materials and Methods

Sample Collection

The Human Ethics Committee of National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, approved this study. Informed consent was obtained from the study subjects. Forty-eight GA-I patients from unrelated families presenting at NIMHANS were recruited based on (1) clinical presentations such as macrocephaly, acute encephalitis-like crisis, spasticity, hypotonia, dystonia, choreoathetosis, ataxia, dyskinesia, and seizures, (2) neuroimaging studies showing generalized frontal cerebral atrophy, subdural hematomas, magnetic resonance imaging suggestive of hyperintensity or atrophy in basal ganglia, or caudate and putamen, and/or computerized tomography changes showing hypoattenuation or atrophy of lentiform nuclei, and (3) elevated levels of GA and 3-OH-GA in urine.

For genotyping studies, 2-mL blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) vacutainer (Becton Dickinson) from GA-I patients. Urine organic acid analysis (UOA) for GA and 3-OH-GA was done by gas chromatography-mass spectrometry (GC-MS), and for this purpose, midstream urine sample of 10 mL or urine spots were collected in a sterile urine container or on Schleicher & Schuell (S&S) 903 filter paper, respectively, from GA-I patients. Prior to urine analysis, samples were stored at -20°C . Demographic details, clinical history, and laboratory findings were recorded from the study participants. The statistical methods such as mean and standard deviation were used to represent demographic data performed using SPSS 17.0.

Preparation of Dried Blood Spots for Genomic Deoxyribonucleic Acid Extraction

For long-term storage, easy handling and meticulous use of patient samples, dried blood spots (DBS) were prepared using the whole blood collected from GA-I patients by spotting three nonoverlapping drops of blood (50 μL for each spot) on S&S 903 filter paper. The spots were then air-dried for 2 hours and stored at -20°C until further process.

Extraction of Genomic Deoxyribonucleic Acid

Genomic deoxyribonucleic acid (gDNA) was extracted from DBS by using an in-house gDNA extraction protocol called single lysis-salting out process.¹⁸

Genotyping Studies

GA-I patients were screened for high excretor mutations R402W (rs121434369), A293T (rs121434371), and A421V (rs121434367), and low excretor mutations R227P (p.Arg227Pro, rs121434373) and V400M (p.Val400Met, rs121434372) by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Exon-specific oligonucleotide primers were used for amplification of exons to screen selected disease-causing mutations (► **Table 1**). Thermal cycler (Mastercycler nexus, Eppendorf AG Hamburg, Germany) program was optimized to 95°C for 5 minutes followed by 30 cycles of 95°C for 5 minutes, 60°C for 1 minutes 30 second, 72°C for 2 minutes, and final extension at 72°C for 10 minutes. Specific restriction enzymes were used to digest PCR amplicons, and restricted products were electrophoretically separated on 2% agarose gels. Depending upon the band pattern on the agarose gel, the genotypes of GA-I patients were identified. Due to nonavailability of appropriate restriction enzyme, direct sequencing of PCR product was done for screening A293T mutation (Chromous Biotech Pvt. Ltd., Bangalore, India). The GA-I patients carrying heterozygous mutations were further screened for all the 11 exons to find the presence of other mutations in *GCDH* gene. The genotype and allelic frequency of mutant alleles was calculated by the Hardy-Weinberg formula.

In Silico Studies

In silico analysis was performed for novel Q144P and I152M missense mutations. PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>) and Protein Variation Effect Analyzer (PROVEAN; http://provean.jcvi.org/seq_submit.php) tools were used for predicting the impact of mutations on protein function based on comparative analysis and physicochemical properties of amino acid. I-Mutant 2.0 (<http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant2.0/I-Mutant2.0.cgi>) was used to depict the impact of point mutations on the protein stability. Swiss PDB viewer V. 4.0 (SPDBV) and Swiss-Model server (<http://swissmodel.expasy.org/interactive#deepview>) were used for homology modeling to build three-dimensional (3D) models of mutant GCDH proteins by using crystal structure of native GCDH (PDB code:1SIR). The ERRAT (<http://services.mbi.ucla.edu/ERRAT/>) and Modloop (<https://modbase.compbio.ucsf.edu/modloop/>) servers were used for refining the loops of mutant models. Structural validation of mutant models was performed using ERRAT, ProSA (<http://prosa.services.came.sbg.ac.at/prosa.php>), and Procheck (<http://swissmodel.expasy.org/workspace/?functoolsstructureassessment1>). The 3D protein models were compared with native GCDH protein to recognize the changes in counterpart amino acids residues within 5 Å radius of the mutant amino acid. The UCSF CHIMERA (<http://www.cgl.ucsf.edu/chimera/>)¹⁹ was used for predicting unfavorable

Table 1 Details of PCR and RFLP for screening mutations

Mutation	Forward 5'-3' primer ^a	Reverse 5'-3' primer ^a	Exon	PCR product (bp)	Restriction enzyme ^a	Restriction pattern		
						Wild	Homozygous	Heterozygous
R402W	TGTTACCTCATGTGCCAC	TGCCTTCGGAGCTTACCTGT	10	481	<i>Msp</i> I(+)	146bp, 335bp	146bp	146bp, 335bp & 481bp
A293T	TGCATCTTCTGGCATCGTC	GGGACACCAAACTGCATCCT	8	663	Direct sequencing	-	-	-
A421V	TGTCAGCATTCACCATCTCTGT	CACAGACCGGCACTGCTAA	11	821	<i>Mae</i> II(+)	703bp, 92bp	555bp, 148bp and 92bp	703bp, 555bp, 148bp, and 92bp
R227P	GACCTGTAAGGGTTCTGGGT	AGCGAGAACTTGCCTGGAT	7	221	<i>Nla</i> IV(+)	195bp	131bp	131bp and 195bp
V400M	TGTTACCTCATGTGCCAC	TGCCTTCGGAGCTTACCTGT	10	481	<i>Eco</i> 72I(-)	140bp, 341bp	481bp	140bp, 341bp, and 481bp

Abbreviations: bp, base pair; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

^aAccording to reference sequence NG_009292.1, (+) mutation creates the restriction site and (-) mutation destroys the restriction site.

interactions of point mutations with close amino acid residues. Docking studies were performed using AutoDock Vina²⁰ to examine the changes in the binding affinity of flavin adenine dinucleotide (FAD) and substrate, S-4-nitrobutyryl-CoA (NBC), to mutant models of GCDH protein.

Results

The mean age of the GA-I patients in our study was found to be 1.8 ± 1.5 years (ranged between 1 month and 5 years). The male-to-female ratio was found to be 0.9:1. Parental consanguinity was seen in 30% of the patients. In this study, 40% GA-I patients were from Northern and 60% were from Southern parts of India. Six (12%) patients had family history of GA-I. The most common clinical manifestations in Indian GA-I patients were developmental delay (56.3%), seizures (23%), dystonia (13%), and macrocephaly (13%).

The genotyping analysis revealed that 9 (18.8%) of 48 GA-I patients had the R402W mutation. Among them, seven (14.6%) were found to be homozygous with the "TT" genotype and two (4.2%) were found to be heterozygous with the "CT" genotype. The heterozygous R402W patients were screened for all the 11 exons of GCDH gene and were found to be compound heterozygous for novel mutations. One was found to be heterozygous for novel missense mutation, Q144P, and another was heterozygous for a novel nonsense mutation, E414X. The demographic data revealed 7 ± 0.3 months as the mean age of disease onset among patients with R402W mutation. The parental consanguinity was seen in 9% of the patients with this mutation. The common clinical presentations among these patients are found to be global developmental delay (60%), dystonia (30%), and macrocephaly (20%).

The A421V, A293T, R227P, and V400M mutations were not found in our cohort (→ **Table 2**). While interpreting the sequencing data, a single patient was found to be compound heterozygous for recently identified low excretor mutation, P286S (g.10754 C > T),¹⁷ and novel mutation, I152M (g.7445 C > G); both these mutations were present in exon 8 of GCDH gene. This patient presented with global developmental delay, dystonia, and macrocephaly with elevated blood glutaryl-carnitine (C5DC) levels. Unfortunately, urine sample collected on filter paper from this patient did not give good results accompanied by lack of follow-up.

In silico analysis by PolyPhen-2 and PROVEAN tools for missense mutation, Q144P and I152M, predicted that these mutations are "probably damaging" or "deleterious" to the GCDH enzyme, respectively. Changes in negative free energy (DDG or ΔG) were seen for these mutations when analyzed by I-Mutant 2.0 (→ **Table 3**).

The mutant homology models for Q144P and I152M were generated using SPDBV and Swiss-Model. The structural validation for homology mutant models was done by ERRAT, Procheck, and ProSA. All the homology models showed quality factor of >95%. The mutant models were analyzed for changes in protein tertiary structure against the native GCDH model. The native GCDH model with Q144 showed the presence of I97, L106, A140, M141, S142, V143, S145, S146,

Table 2 Mutations identified in GA-I patients from India

Sl. no.	Mutations	Exon	Nucleotide	Patients	Wild type, n (%)	Mutants, n (%)	Homozygous, n (%)	Heterozygous, n (%)	Mutant allele frequency
1.	R402W	10	g.11665 C > T	48	39 (81.25)	9 (18.8)	7 (14.6)	2 (4.2)	0.17
2.	V400M	10	g.11659 G > A	48	48 (100)	-	-	-	-
3.	R227P	7	g.10090G > C	48	48 (100)	-	-	-	-
4.	A421V	11	g.13327C > T	48	48 (100)	-	-	-	-
5.	A293T	8	g.10775G > A	48	48 (100)	-	-	-	-
Compound heterozygotes									
6.	R402W + Q144P	10 and 5	g.11665 C > T & g.7420A > C	48	47(97.9)	1(2.1)	-	1(2.1)	0.18, 0.01
7.	R402W + E414X	10	g.11665 C > T & g.11701G > T	48	47(97.9)	1(2.1)	-	1(2.1)	0.18, 0.01
8.	I152M + P286S	5 and 8	g.7445C > G & g.10754C > T	48	47(97.9)	1(2.1)	-	1(2.1)	0.01, 0.001

L147, V148, M149, H150, L168, G171, L173, L174, and G175 amino acid residues within 5Å region of Q144 residue. Nevertheless, when replaced by P144, it resulted in the absence of many amino acid residues, that is, I97, H150, L168, G171, L173, L174, and G175 within this region, probably due to alterations in the amino acid polarity from a hydrophilic glutamine to hydrophobic proline at position 144 (►Fig. 1A, B). Similarly, in native GCDH model, I152 showed the presence of V148, M149, H150, P151, Y153, A154, Y155, G156, S157, Q160, R161, Y164, L165, L168, L221, L237, and L277 within 5Å region of I152 residue. When replaced by M152 residue, it causes derangement in surrounding amino acid residues leading to absence of Q160 and L237 residues within 5Å region of M152 (►Fig. 1C, D).

The Q144P and I152M mutants were analyzed by UCSF CHIMERA for clashes or unfavorable interactions. The amino acid residues 144 and 152 lie in region A of GCDH protein. Mutations in this region are linked with residual enzyme activity and are not directly involved in either substrate binding or oligomerization.¹¹ In Q144P mutation, when glutamine at 144 replaced by proline, it forms clashes with alanine at 140, present in region A of GCDH protein (►Fig. 1E). In I152M mutation, when isoleucine at position 152 is replaced by methionine, it forms clashes with leucine at position 237 that lie in close proximity to FAD binding site of region B¹¹ (►Fig. 1F). The docking analysis with FAD and substrate (NBC) by Autodock Vina, for mutant Q144P and I152M GCDH protein models, revealed decreased binding affinity for both the ligands, that is, FAD and substrate (►Table 3).

Discussion

In this study, we aimed to screen the presence of R402W, A421V, A293T, R227P, and V400M mutations in GA-I patients from India. These mutations were chosen as they are highly prevalent in certain ethnic populations¹ and cause dysfunction of GCDH activity either by affecting FAD binding ability to the enzyme or disrupting intersubunit associations or affecting the interactions with electron transfer flavoprotein.¹¹

The mutant “T” allele frequency for R402W mutation in this study was found to be 17% (16/96), which is similar to the frequency range reported among Europeans (10–20%).²¹ A recent study by Gupta et al,¹⁶ with 17 GA-I patients from India, showed mutant allelic frequency of 23.5% (8/34) for R402W mutation and concluded that this is the most commonly occurring mutation in their cohort. Our previous study with 30 GA-I patients screened for R402W mutation showed allelic frequency of 15% (9/60).¹⁸ The difference in allelic frequency between this study, our previous study,¹⁸ and that by Gupta et al,¹⁶ might be due to differences in sample size. A study from southern province of India on GA-I has suggested the absence of R402W mutation in South Indian GA-I patients.¹⁷ To the contrary, this study showed 7 (78%) of 9 South Indian GA-I patients had this mutation, which is indicative of its prevalence in Southern regions as well.

Table 3 Evaluation of the impact of mutations using computational tools

Mutation	PolyPhen-2		PROVEAN		I-mutant 2.0 $\Delta G = <0$ kcal/mol	Binding Affinity (kcal/mol)	
	Prediction	Score (0.1–1.00)	Prediction	Score (< -2.5)		FAD	NBC (substrate)
Native GCDH	–	–	–	–	–	–8.8	–6.9
Q144P	Probably Damaging	1.00	Deleterious	–5.9	–0.16	–11.1	–7.8
I152M	Probably Damaging	1.00	Deleterious	–2.93	–2.00	–10.8	–8.3

The A421V, A293T, V400M, and R227P mutations were not found in this study, suggesting the absence of these mutations in our population. In our study, we found P286S, a low excretor mutation reported by Radha Rama Devi et al,¹⁷ as compound heterozygous with I152M mutation. Though blood C5DC levels (1.41μmoles/L; Ref range: 0.00–0.41μmol/L) were found to be elevated, the biochemical phenotype for this patient was inconclusive due to the absence of UOA results.

In silico analysis was performed for the novel mutations Q144P and I152M found in our study. The GCDH enzyme belongs to acyl-CoA dehydrogenase (ACDs) family of flavoenzymes. It is unique among ACDs as it catalyzes decarboxylation reaction followed by dehydrogenation to produce crotonyl CoA and CO₂ from glutaryl CoA. The polypeptide folding of GCDH is

similar to the family members belonging to ACDs. The mutations in *GCDH* gene affect protein folding, stability, and/or ligand binding (FAD and/or substrate) ability to the enzyme. The PolyPhen-2 and PROVEAN analysis for Q144P and I152M mutations suggest that these are likely to have an adverse impact on both structure and function of GCDH. Furthermore, the resultant low ΔG (DDG) values for mutant proteins obtained using I-Mutant 2.0 program suggests that these mutations cause structural instability to GCDH.

The structural variations of Q144P and I152M mutations when analyzed using SPDBV and UCSF Chimera showed changes within neighboring 5Å residues and unfavorable interactions between amino acid residues, respectively. Substantially, it can be assumed that Q144P and I152M

Fig. 1 (A) Native GCDH protein with Q144 amino acid at position 144 (green) along with other amino acids I97, H150, L168, G171, L173, L174, and G175 residues within 5 Å region. (B) The absence of amino acids I97, H150, L168, G171, L173, L174, and G175 residues due to the presence of mutant P144. (C) Native GCDH protein with I152 (green) showing G160 and L277 residues (pink), Tyr155 (blue), and S-4-nitrobutyryl-CoA substrate moiety (purple). (D) The absence of G160 and L277 residues due to the presence of I152M mutation. (E,F) UCSF CHIMERA results showing P144 clashes with A140 and M152 clashes with L237 due to the presence of Q144P and I152M mutations, respectively.

mutations cause regional spatial derangements of amino acid residues and structural constraints with neighboring residues due to unfavorable interactions, which might have resulted in nonaccommodative steric hindrance and local structural variations in GCDH protein.

Docking of ligands with GCDH Q144P and I152M mutant proteins has shown decreased affinity for both FAD and substrate, more pronounced in Q144P when compared with I152M GCDH mutant protein. The possible explanation could be that the native GCDH protein with Q144 and A140 residues stabilizes S139 amino acid present at the catalytic site, which is essential to form temporary hydrogen bond with the substrate. Replacement of proline with glutamine at position 144 destabilizes S139 residue, resulting in decreased substrate binding affinity toward P144 mutant GCDH protein. In addition, change in the polarity of amino acid from hydrophilic glutamine to hydrophobic proline might cause disturbances in the amino acid residues (i.e., L173, L147, and G175) in close proximity to FAD binding site in region B. These disturbances could possibly affect the FAD binding ability to Q144P mutant GCDH protein. Similarly, in I152M mutant, when isoleucine at position 152 present in region A is replaced with methionine, it forms an unfavorable interaction with L237 residue in region B. This might result in local folding changes around FAD binding site, reducing the binding ability of FAD to GCDH mutant protein. Also, I152 residue lies in close proximity to Y155, which is present near adenine ring of CoA of the substrate.¹¹ The mutant M152 causes derangement of amino acid residues within 5 Å (including Y155) that might have compromised the substrate binding ability of I152M mutant GCDH protein.

Recent studies on P286S mutation have suggested that this mutation results in local instability and affects the ligand binding to GCDH protein.¹⁷ Previous computational analysis report on R402W mutation has shown that this mutation affects the spatial arrangement and the dynamics, leading to decreased stability of GCDH enzyme.²²

Conclusion

For the first time, we have screened a large number of GA-I patients from India. We conclude that regardless of the genetic and ethnic diversity within this region, R402W is the most common mutation among A421V, A293T, R227P, and V400M in Indian GA-I patients. In addition, we have reported the existence of novel mutations I152M, E414X, and Q144P in our GA-I patients. From computational studies, it is evident that the presence of Q144P and I152M mutations tends to cause local structural variations in GCDH enzyme that might have impact on enzyme stability and impairs its functional ability. This study is limited to genetic screening of certain selected disease-causing mutations in Indian GA-I patients. Further studies undertaking whole gene screening are essential for better documentation of various genotypes prevalent in Indian population, which, in turn, might help in DNA-based prenatal screening of this disorder in high-risk pregnancies.

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Conflict of Interest

None.

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