Original Article

Molecular Determination of Glutaric Aciduria Type I in Individuals from Southwest Iran

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Abstract

Background: Glutaric Aciduria type 1 (GA1) is a metabolic inborn error and is characterized by increasing excursion of glutaric acid and its derivates, presented in microcephaly and dystonia. The disease is resulted from mutational inactivation in the *GCDH* gene encoding the glutaryl-CoA dehydrogenase. The defective enzyme causes the accumulation of an excessive level of intermediate breakdown products that leads to the brain damage. In spite of the clinical features, diagnosis of GAI has been often confusing, because of variability in the clinical manifestations of patients. Early diagnosis and treatment can though prevent irreversible disease progression and consequent brain damage; otherwise the affected individuals will die in their first decade of lives.

Methods: The GCDH gene was also analyzed to (detect or identify) disease causing mutations using gene amplification and direct sequencing in 18 patients.

Results: Among 18 patients, 10 patients (55.5%) were homozygous or compounded heterozygous for the recurrent mutation E181Q, three patients (16.7%) were homozygous for the known mutation R402Q and one patient (5.6%) was compound heterozygous for S255L. All three detected missense mutations are pathogenic, which cause structural changes in the binding site and tetramerization or functional deficiency. Four other individuals (22.2%) with a preliminary diagnosis of GAI were negative for any pathogenic mutations.

Conclusion: Most GA1 affected persons in southwest Iran are with Persian ethnicity and the most common mutation in Khuzestan Province is prominent in comparison to previous reports from Iran.

Keywords: *GCDH* gene, glutaric aciduria type 1, metabolic disease, southwest Iran

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Introduction

Glutaric aciduria type 1 is an autosomal recessive inherited disorder that was firstly described by Goodman, et al.[1] The estimated prevalence of this disease is 1 in 30.000 – 100.000 newborns.[2,3] It might be much higher in some isolated population,[4,5] especially in the Middle East countries with high rates of consanguinity and first-cousin marriages. Mutations in the mitochondrial *GCDH* gene lead to a deficiency of Glutaryl-CoA Dehydrogenase causing the abnormal processing of lysine, hydroxylysine and tryptophan metabolism. Generation of toxic by-products might affect acute encephalopathic crisis, if the disease continues untreated in the period of brain development.[6] Very late diagnosis may also lead to irreversible defects.[7]
In spite, clinical features of the GA1 are widely variable from asymptomatic patients to sever cases of encephalopathy.[6] For instead, close to half of patients with GA1, develop microcephaly during the first 8 month.[8]
Generally, radiologic imaging such as CT scan or MRI can be a useful tool to detect neurological symptoms of disease development.[9] Regardless; radiological changes are not highly specific among GA1 patients and vary in each case. However, some neurological signs such as hydrocephalus, brain atrophy, structural changes of basal ganglia, and demyelization could be used as strong signs of disease.[10]
Because of relative wide symptom variability among GA patients,[11] as well as slow and steady progression of disease,[12] a rapid screening method is of great importance.
From this reason, Knowledge about the distribution and the frequency of disease causing mutations within the GCDH gene appears to be the first considerable step for this concept.
However, we aimed to screen the entire *GCDH* gene on a genomic level by direct sequencing of exons and flanking intron regions in a number of patients from unrelated families in southwest Iran that have been diagnosed with GA1.

Materials and Methods

A total of 18 unrelated families with at least one affected patient with GA1 were investigated. Samples were collected in a time period of 16 months from the neurological department of the Golestan hospital of Ahvaz city. The diagnosis was assessed by biochemical and clinical signs typically for classic GA1 individuals. Six patients were born with moderate microcephaly. Brain MRI with and without contrast injection in some patients revealed prominent subarachnoid spaces and enlarged (bat wing) dilatation of Sylvain fissures. Mild increased signal was also observed in the white matter and basal ganglia with

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Table 1. Details of primers have been used for amplifying and sequencing of entire coding areas of the GCDH gene, which were designed by the primer3out software.

Primer Name	Exon	Sequence (5̀ to 3̀)	Product Size	Tm
GCDH-1/2F	1 & 2	GCACTGTAGCCTCGGCAGTGAACC	564 bp	60.4 ℃
GCDH-1/2R	---	TAGTGCGAGCTGCAAGCGAGACTG	---	---
GCDH-3F	3	ATAGCCACCCCACCTCAAG	210 bp	60.6 ℃
GCDH-3R	---	CAGAGGGTTCTGCAGTGTGT	---	---
GCDH-4F	4	CTCCCTCCCTTTCTTCCTTC	254 bp	60.6 ℃
GCDH-4R	---	CCAACACAGTGAAACCCTGTC	---	---
GCDH-5F	5	CACCCTCTGAAAGTGGCTGTGGA	493 bp	59.6 ℃
GCDH-5R	---	TCAGATCTCCAGGTGAAGCCCA	---	---
GCDH-6/7F	6 & 7	AGTAAGGGGATGTATCAGGGACCA	532 bp	59.4 ℃
GCDH-6/7R	---	ATCCGCAGGTGACCCAACAC	---	---
GCDH-X8-F	8	CCCTGCTTCAGAGTTGGTTC	289 bp	60.6 ℃
GCDH-X8-R	---	TCCAGAGCAAGGAACTCAGG	---	---
GCDH-X9-F	9	AAGCTTGGGGGCACTGAG	311 bp	59.4 ℃
GCDH-X9-R	---	TCTCCAGGAAGGACACAAGG	---	---
GCDH-X10-F	10	GGCTAGGTTTGCTTGGAGC	321 bp	60.6 ℃
GCDH-X10-R	---	GTTGGACTCAGACCTGTCCC	---	---
GCDH-X11-F	11	ACTTCTGAAGCAGTGGCCTG	678 bp	60.6 ℃
GCDH-X11-R	---	CCCAGACGTGTTCGTTTCTT	---	---

delayed myelination.

Biochemical tests of patients showed markedly elevated acylcarnitine in the blood sample. The level of ammonia and lactate was over the norm, as well.

Genomic DNA was extracted from peripheral blood collected from patients and their parents. Amplification of exons and the flanking introns of the *GCDH* gene were performed using designed primer pairs and Primer3 software (Table 1). PCR reactions were performed at 93 °C for 5 minutes , followed by 35 cycles at 93 °C for 1 min, at 59 – 61 °C for 30 seconds, and at 72 °C for 45 seconds as well as a final extension cycle at 72 °C for 5 minutes. PCR buffer, primers (each 10 pmol), dNTP, MgCl2, DMSO, and Spermidine (Sinagen, Iran) was used in a final volume of 25 ul, (Figure 1).

Direct sequencing of PCR products was done using an automated sequencer (Applied Biosystems ABI, USA) according to the manufacturer's instruction. Sequences were read using software Chromas version 6.2. They were also analyzed using the software NCBI Blast and Bio edit.

VMD, a molecular visualization program, was used to display and to analyze macromolecule interchange. It uses 3-D graphics and built-in scripting.[13,14]

Results

Mutation screening of the entire coding *GCDH* gene and the splice sites in patients' samples revealed that 55.5% of patients were homozygous for the reported mutation E181Q. One other patient was compounded heterozygous for E181Q change that was transmitted from Persian father. The second disease causing mutation S255L was originated from Arabian mother. This mutation has been exclusively found in this family. The second most mutations in this assay with more than 16.7% was R402Q in exon 10. Four individuals suspecting with GA1 were

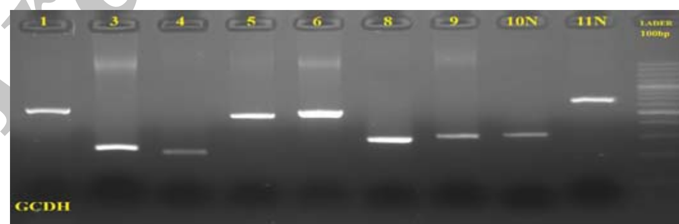

Figure 1. PCR products were loaded on a 1.5% agarose gel. The lines show the appropriate exons of the human GCDH gene. The size of PCR products was verified by the 100 bp DNA size marker (Sinaclon, Iran). Some exons were amplified together with one primer pair showing as 1 (for exons 1 and 2) and 6 (for exons 6 and 7).

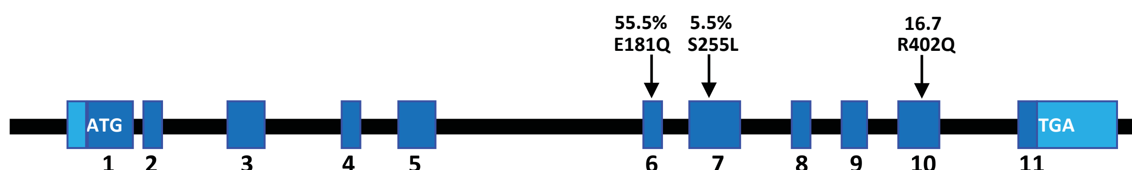

Figure 2. The *GCDH* gene consists of 11 exons. Exon 6, 7 and 10 harbor more than 76% of mutations in GA patients from southwest Iran. Mutation S255L was found in an individual with Arabian Background.

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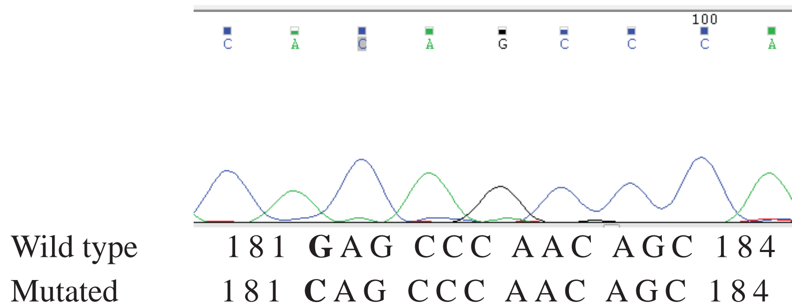

Figure 3. Alignment of mutated and wild type sequences of the human GCDH gene for E181Q mutation. Partial chromogram is shown with marked position of the mutation.

negative for any disease causing mutation. Figure 2, shows schematic positions and frequency of mutations in relation to the gene structure. The parents of the last mentioned patient belong to the Arabian-Persian ethnic group in southwest Iran. The mutation at codon 255 originated from Arabian mother and the mutation at codon 181 from Persian father. The effect of the detected mutations on the gene product was investigated by dynamic analysis and structure modeling showing negative effects of all three mutations on the function of the glutaryl-CoA dehydrogenase, (Figure 3).

Discussion

To date, more than 148 mutations have been detected in the *GCDH* gene from individuals with glutaric acidemia type 1 (www.hgmd.org). However, most of the identified mutations are missense or nonsense, which cause structural or functional deficiency of the gene product glutaryl-CoA dehydrogenase, a mitochondrial enzyme that buildup Glutaric acid. Enzyme deficiency leads to metabolic and neurological crisis in GA1 patients, but some individuals remain undiagnosed, because

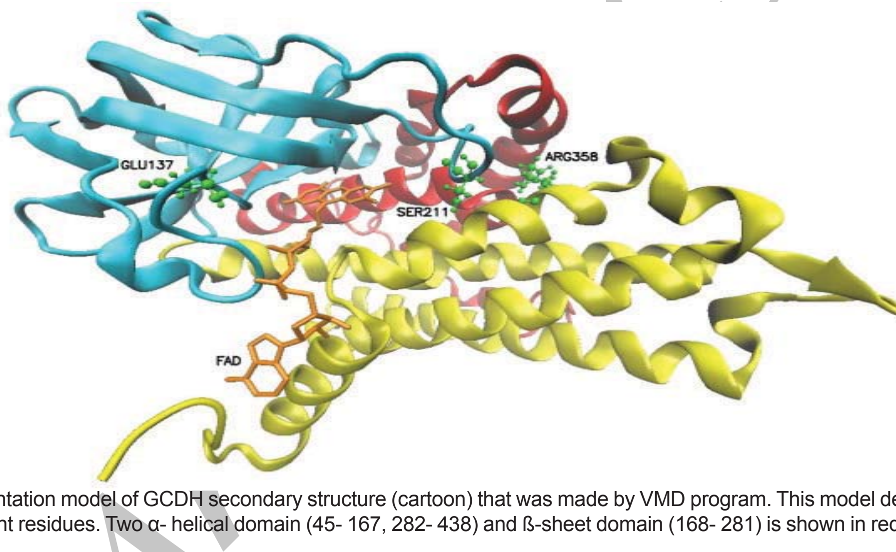

Figure 4. Representation model of GCDH secondary structure (cartoon) that was made by VMD program. This model depicted 3 domain of GCDH and position of all mutant residues. Two α- helical domain (45- 167, 282- 438) and ß-sheet domain (168- 281) is shown in red, yellow and cyan respectively.

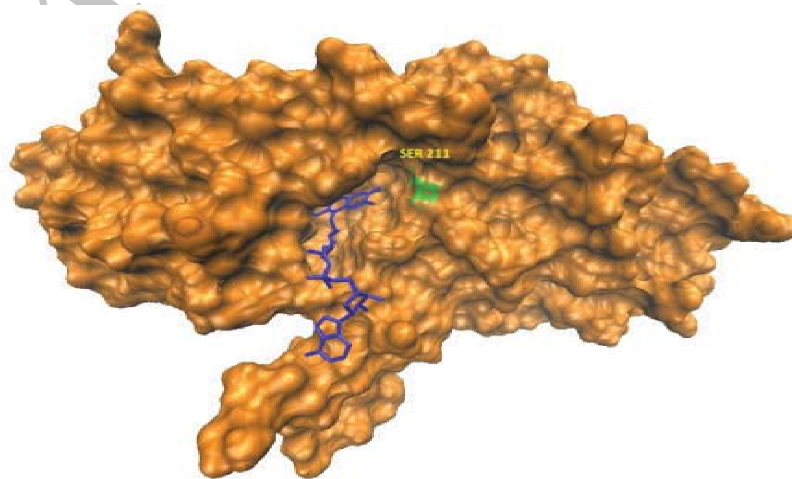

Figure 5. Surface representation of GCDH with the active site for interaction to FAD and surface of Ser 255 (211). FAD is shown in blue. This molecular presentation was made by VMD program.

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of slow progression of the disease.[15] However, molecular test can provide here the chance for rapid diagnosis by mutation screening of the entire *GCDH* gene. On the other hand, molecular genetics analysis is often time and cost intensive. Selective screening of common mutations could be a suitable strategy in distinct population. In this context, we collected 18 patients with a preliminary diagnosis of GA1 from southwest Iran (Province Khuzestan). We hoped to develop a rapid and cost effective diagnosis, especially in this province. Excepting one family with mixed Persian-Arabian parents, all other affected individuals originated from parents with Persian ethnicity (Lor, Bakhtiari). The most common mutation in this study (E181Q) was firstly reported from Turkey.[15] The second frequent mutation R402Q is originated in Germany.[16]

All 3 detected mutations were investigated for their impact of the functionality of the gene product. Crystal structure of GCDH (1SIQ.pdb) contains two N-terminus and C-terminus α-helical and a Beta sheet domain in the middle of two α- helices that bind from right and left of FAD respectively. 3-D model of GCDH was utilized for showing, three mutant residues namely E181Q (137), S255L (211), R402Q (358). Ser 255 (211) is located at the active site in the turn of beta sheet domain and close to the FAD and α-helical of c-terminus, (Figure 4). Arg358 is localized in the α-helical C-terminus and close to the Ser 255 in the turn. Glu137 is localized in the turn of beta sheet and close to the middle of the FAD, (Figure 5). Arg 402 makes H-bond and Salt Bridge with Val 89 and Asp 90. There is a significant difference in the mutant form of R402Q due to substitutions of Arg with positive charge to Gln with a neutral polar side chain that can disturb salt bridges and interaction. This description of each mutant position shows importance of them so mutation of these residues might have influence in the binding and function of GCDH.

We come to the suggestion that screening of just 3 exons (6, 7 and 10) would make a final diagnosis for a great number of GA1 patients from Province Khuzestan. Further, our results can be considered for the development more rapid diagnostic methods such as RFLP and ARMS for postnatal and neonatal screening, but even much more important, for carrier detection preventing high risk pregnancies.

In 2012, Houshmand, et al. reported a molecular investigation of 25 Iranian patients with GA1.[17] Our results showed some interesting differences with their results. They named P304L by 20% as most detected mutations. None of the patients from southwest Iran carried this change. The mutation S255L is absent in mentioned report. However, we just found it in a family with Arabian background. Furthermore, in contrast to previous investigation the mutation E181Q is the most frequent change among our patients.[17] Nevertheless, some individuals suspecting to have GA1 were negative for any mutations in *GCDH* gene. Regarding HGMD, no exonic or whole gene deletion has been reported yet. These have to be ruled out in Iranian patients by CGH array to make a decision.

This is the first molecular validation of Glutaric aciduria type 1 in southwest Iran and the detected recurrent mutations can be used for a countrywide study. In conclusion, larger sample size is needed regarding mutation frequency within the *GCDH* gene in Iran, particularly in Khuzestan Province.

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