A novel protein truncating mutation in \textit{L2HGDH} causes L-2-hydroxyglutaric aciduria in a consanguineous Pakistani family

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Abstract

Background L-2-hydroxyglutaric aciduria (L2HGA) is a rare neurometabolic disorder that occurs due to accumulation of L-2-hydroxyglutaric acid in the cerebrospinal fluid (CSF), plasma and urine. The clinical manifestation of L2HGA includes intellectual disability, cerebellar ataxia, epilepsy, speech problems and macrocephaly.

Methods In the present study, we ascertained a multigenerational consanguineous Pakistani family with 5 affected individuals. Clinical studies were performed through biochemical tests and brain CT scan. Locus mapping was carried out through genome-wide SNP genotyping, whole exome sequencing and Sanger sequencing. For in silico studies protein structural modeling and docking was done using I-TASSER, Cluspro and AutoDock VINA tools.

Results Affected individuals presented with cognitive impairment, gait disturbance, speech difficulties and psychomotor delay. Radiologic analysis of a male patient revealed leukoaraiosis with hypoattenuation of cerebral white matter, suggestive of hypomyelination. Homozygosity mapping in this family revealed a linkage region on chromosome 14 between markers rs2039791 and rs781354. Subsequent whole exome analysis identified a novel frameshift mutation NM_024884.3:c.180delG, p.(Ala62Profs*24) in the second exon of \textit{L2HGDH}. Sanger sequencing confirmed segregation of this mutation with the disease phenotype. The identification of the most N-terminal loss of function mutation published thus far further expands the mutational spectrum of \textit{L2HGDH}.

Keywords L-2-hydroxyglutaric aciduria · Whole exome sequencing · Intellectual disability · \textit{L2HGDH} · Leukoaraiosis · N-terminal frameshift mutation

Introduction

L-2-hydroxyglutaric aciduria [L2HGA (OMIM #236,792)] is a rare autosomal recessive neurodegenerative metabolic disorder, that occurs due to accumulation of L-2-hydroxyglutaric acid in the cerebrospinal fluid (CSF), plasma and urine (Duran et al. 1980; Chen et al. 1996). Phenotypic features of the affected individuals are variable and may include developmental delay, moderate to severe intellectual disability, epilepsy, behavioral problems, spasticity, macrocephaly, speech disorders and cerebellar ataxia (Barth et al. 1992; Barth et al. 1998; Hanefeld et al. 1994). Age of onset of L2HGA (OMIM #236,792) is variable and may occur at an early age with severe epilepsy and intellectual disability or in adulthood with moderate to mild symptoms. Different studies have documented that an elevated level of L2HGA (OMIM #236,792) in the
L2HGDH is expressed in various tissues with highest expression in brain, followed by muscles and testis (Vilarinho et al. 2005). The corresponding protein consists of 463 amino acids, which contains two domains i.e. a mitochondrial targeting sequence and a FAD dependent oxidoreductase domain (UniProtKB: Q9H9P8) (Goffette et al. 2006). L2HGDH acts as a mitochondrial enzyme which is involved in glutamate and glutamine metabolism pathways. Its prime function is to catalyze the oxidation of L-2-hydroxyglutarate (L2HG) to α2-ketoglutarate (α2KG) (Topçu et al. 2004; Vilarinho et al. 2009). Exact prevalence of L2HGA (OMIM #236,792) is unknown, but approximately 140 cases have been reported to date (Goffette et al. 2006; Topçu et al. 2004; Vilarinho et al. 2009; Jellouli et al. 2014; Larnaout et al. 2008; O’Connor et al. 2009). Although there is no established treatment of L2HGA, Samuraki et al. reported effective treatment of a late onset patient with flavin adenine dinucleotide sodium (FAD) and levocarnitine chloride (Samuraki et al. 2008).

In the present study, we report on a consanguineous Pakistani family displaying mild intellectual disability. Genome-wide homozygosity mapping coupled with whole exome sequencing revealed a novel frameshift mutation NM_024884.3:c.180delG, p.(Ala62Profs*24) in the 2nd exon of L2HGDH. The identified mutation presumably creates a premature stop codon ether leading to nonsense mediated mRNA decay or truncation of the protein, which would distort the local folding of the polypeptide chain and lead to loss of its interacting sites.

Clinical assessment

The clinical assessment of patients was carried out through biochemical tests e.g. liver functioning test (LFTs), renal function tests (RFTs), urine organic acid analysis and plasma amino acid analysis. Radiologic analysis was performed through CT scan of affected individual V:8.

Genome-wide SNP genotyping

Whole genome SNP genotyping was performed through microarray analysis using the Infinium Global Screening Array (Illumina, USA) kit. Raw data analysis was performed at the Life and Brain GmbH, Bonn, Germany. Homozygosity mapping to identify the disease associated locus, was carried out using GenomeStudio 2.0 Software (Illumina, USA).

Whole exome sequencing (WES)

For genetic analysis whole exome sequencing (WES) was performed for a single affected individual (V:4) via Agilent SureSelect V6 human All Exon library preparation, sequencing was conducted using a NovaSeq 6000 with 2 × 150 bp and 100 × coverage (50 × on-target coverage). Sequence alignment of raw fastq files to the human reference sequence (GRCh37/hg19 assembly) and variant calling was performed with the DRAGEN Germline Pipeline 3.2.8 on Illumina BaseSpace (https://basespace.illumina.com/). Variant annotation, analysis and homozygosity mapping was performed using VarSeq™ v2.2 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com).

Segregation analysis

For Sanger DNA sequencing, primers were design through Primer3web tool (version 4.1.0) (Untergasser et al. 2012). Sanger sequencing was performed for available study participants (IV:2, V4-V8). Sequence analysis was performed by online BLAT tool package available in UCSC Genome Browser (Sanger et al.1977; Kent 2002) and offline BioEdit tool (version 7.0.5).
**In silico studies**

Computational analysis of mutant L2HGDH involved protein structural modeling and protein interaction capability.

**Protein structure prediction**

Structural modeling was done on I-TASSER tool (Yang and Zhang 2015) and models with highest confidence score (C-score) were selected for onward interaction studies. To further confirm the efficiency of predicted structure, I-TASSER (Yang and Zhang 2015) results were also crossed checked by SWISS MODEL (Bienert et al. 2016).

**Molecular Docking and visualization**

For interaction studies, ClusPro (Kozakov et al. 2017) tool was used for protein–protein docking between L2HGDH (wildtype and mutated protein) with its close interacting protein, which was predicted through STRING database (Szklarczyk et al. 2018). Protein-substrate docking was performed through AutoDock VINA tool (vina.scripps.edu) (Trott and Olson 2009), and molecular visualization through different offline tools e.g. LigPlot + (Version 2.1) (Laskowski et al.2011), PyMOL 2.3 (Schiffrin et al. 2020), Chimera 1.13.1(Pettersen et al. 2004) and Discovery Studio 2020(https://www.3dsbiovia.com/products/datasheets/discovery-studio-visualizer).

**Results**

The current study describes a consanguineous Pakistani family displaying intellectual disability with gait and speech problems recruited from Dera Ismail Khan City in Khyber Pakhtunkhwa province of Pakistan. The family pedigree and clinical history was assessed for five generations, with 4 affected individuals in the 5th generation and a single affected individual in the 4th generation (Fig. 1a).

Molecular genetic analysis revealed a common linkage region on the q arm of chromosome 14 between SNP markers rs2039791 to rs781354 (45,171,670 bp – 52,879,326 bp). The size of the identified linkage interval spans over 7.7 Mb, which harbors 65 protein-coding genes (Fig. 2a).

Whole genome homozygosity scan revealed a novel frameshift mutation in L2HGDH associated with L-2-hydroxyglutaric aciduria.

**Clinical Findings**

**Phenotype**

All affected family members showed mild intellectual disability and developing speech with weak communication skills. The patients had gait disturbance, however, no muscular dystrophy or skeletal anomalies were observed. No digital anomalies were determined, except syndactyly of feet in patient V:8. The affected individuals did not have a feeling of satiety even after excessive eating. Patient V:5 and V:8 had a history of epilepsy during the childhood. The head circumference in all patients was within normal range. Nonetheless, some degree of facial dysmorphism was observed due to drooping mouth (Fig. 1b). The general physique of the patients was normal. Examination of hearing, vision, visceral organs and skin was found normal (a summary of the phenotypic data is given in Table 1).

**Radiological Findings**

A CT scan was performed for a male patient (V:8). Plain CT demonstrates leukoaraiosis with hypopattenuation of cerebral white matter, particularly evident in frontal lobes. Also, a left-hemispheric preponderance becomes apparent (also involving capsula externa). The gyration appears normal, there is slight widening of the lateral ventricles, but no evident atrophy pattern in this 18-year-old male (Fig. 1c).

**Biochemical Findings**

Serum biochemistry reports showed high serum creatinine levels, however, blood urea, bilirubin, alanine transaminase (ALT), and alkaline phosphatase levels were normal. Urine organic acid analysis revealed marked excretion of 2-hydroxylutaric acid with a small peak for glutaric acid. A peak for 2-hydroxyglutaric lactones was not identified. Additionally, plasma amino acid analysis exhibited nonspecific variations in the level of different amino acids. For example, level of glutamate, glycine, alanine, leucine, ornithine and lysine were abnormally high, while, value of the cysteine was below the reference range. Patient’s biochemistry profile is illustrated in Table 2.
Structural Findings

Molecular modeling of Normal and Mutated L2HGDH proteins

After doing molecular modeling, the 3D-structures of both wild type and mutated L2HGDH were superimposed, which failed to overlap due to misfolding. It confirms that identified frameshift mutation results in structural distortion of L2HGDH (Fig. 3).

Protein–Protein Docking

Interaction studies of L2HGDH with D2HGDH have revealed remarkable alteration in docking sites (see Fig. 3). In addition to this, docking of L2HGDH with other close interactors i.e. ALDH4A1 and GLS2 proteins have exhibited significant alteration in the interacting sites (supplementary figure).
Enzyme–Substrate Docking

Interaction studies were also performed for L2HGDH proteins and its substrate i.e. L-2-hydroxyglutarate, which predicted five amino acids i.e. Gln-89, Tyr-195, Val-404, Ala-402 and Gly-403 of wild-type L2HGDH to be involved in interaction with its substrate via conventional hydrogen bonding. All these binding sites are within the FAD dependent enzyme domain. In mutated L2HGDH interacting sites within the FAD domain are lost due to frameshift and protein truncation. However, the mutant enzyme predictably showed interaction with its substrate on different positions i.e. Arg-42, Cys-38, Gly-40 and Cys-27 through conventional hydrogen bond, and Gly-28 through carbon hydrogen bond (see Fig. 3).

Discussion

L-2-hydroxyglutaric aciduria is a rare form of autosomal recessive neuro-metabolic disorder that is caused by mutations in L2HGDH. The corresponding protein acts as a mitochondrial enzyme which bio-oxidizes the L-2-hydroxyglutaric acid to α-ketoglutarate (Olgac et al. 2019), and is involved in butanoate metabolism, glutamate and glutamine metabolism pathways (Olgac et al. 2019; Ma et al. 2017). There are two defined features, the mitochondrial targeting sequence and a FAD dependent oxidoreductase domain (UniProtKB: Q9H9P8). Insufficient enzyme activity leads to toxic levels of L-2-hydroxyglutaric acid in the cerebrospinal fluid (CSF), plasma and urine. The main phenotypical features associated with L2HGA include leukodystrophy, intellectual disability, psycho-motor abnormalities, macrocephaly, intention tremors, abnormal gait, epilepsy and cerebellar atrophy (Haliloglu et al. 2008).

Penderis et al. (2007) described a spontaneous canine model of L-2-hydroxyglutaric aciduria in outbred bull terriers dogs. All affected dogs exhibited increased urinary excretion of L-2-hydroxyglutarate (L-2-HG), while 12 dogs in which MRI imaging was performed showed symmetric regions of hyper intensity comparable to that seen in humans (Penderis et al. 2007).
Similarly, Ma et al. (2017) developed an L2hgdh null mice and found range of phenotypes i.e. increased level of L-2-hydroxyglutarate (L-2-HG) levels in multiple tissues, especially in the brain and testis. L2hgdh null mice demonstrated white matter deterioration, extensive gliosis, microglia-mediated neuro-inflammation, and an expansion of oligodendrocyte progenitor cells. Additionally, L2HGDH deficiency in the later stages results in hippocampal neurogenesis and late-onset neurodegeneration (Ma et al. 2017). Oldham and coworkers identified L-2-hydroxyglutarate (L2HG) as an important factor for the hypoxia response. Earlier, L2HG was reported to be produced by the malate dehydrogenase via mitochondrial 2-oxoglutarate reduction. Elevated level of 2-oxoglutarate is considered responsible for accumulation of L2HG, which happens due to dysfunction of tricarboxylic acid cycle and increased mitochondrial reducing potential. These changes were associated with homeostasis of cellular redox, because elevated level of L2HG in cell prevents glycolysis as well as electron transport, in order to counterbalance the unfavorable consequences of mitochondrial reductive stress provoked by hypoxia. Therefore, L2HG combines cytoplasmic and mitochondrial based

| S No | Test                          | Normal Range | Patient’s Result                  |
|------|-------------------------------|--------------|-----------------------------------|
| 1    | Renal Functioning test (RFTs) | Blood Urea   | 10–50 mg/dl                       | 47                               |
|      |                                | Serum creatinine | 0.6–1.4 (adult) mg/dl          | 1.7                              |
|      |                                | Bilirubin    | 0.1–1.2 mg/dl                    | 1.1                              |
| 2    | Liver Functioning test (LFTs)  | Alt/SGPT     | 09–45(Male) U/l                  | 37                               |
|      |                                | ALK. Phosphatase | Up to -303 U/l                  | 290                              |
| 3    | Metabolic Screening           | Urine Organic Acid | Marked excretion of 2-hydroxyglutaric acid |
|      |                               | Below normal range amino acids | Cysteine | 32–64 | 15 umol/L |
|      |                               | Border line high range amino acids | Valine | 142–278 | 282 umol/L |
|      |                               |                          | Isoleucine | 38–94 | 104 umol/L |
|      |                               |                          | Histidine | 58–106 | 125 umol/L |
|      |                               |                          | Aspartate | 4–28  | 41 umol/L  |
|      |                               |                          | Serine | 75–175 | 183 umol/L |
|      |                               |                          | Asparagine | 32–64 | 76 umol/L |
|      |                               |                          | Phenylalanine | 38–78 | 106 umol/L |
|      |                               |                          | Taurine | 10–162 | 187 umol/L |
|      |                               | Abnormally high amino acids | Glutamate | 11–59 | 225 umol/L |
|      |                               |                          | Glycine | 148–324 | 406 umol/L |
|      |                               |                          | Alanine | 192–508 | 779 umol/L |
|      |                               |                          | Leucine | 76–168 | 250 umol/L |
|      |                               |                          | Ornithine | 20–84 | 202 umol/L |
|      |                               |                          | Lysine | 105–221 | 414 umol/L |
|      |                               | Normal range amino acids | Threonine | 72–192 | 175 umol/L |
|      |                               |                          | Glutamine | 396–740 | 429 umol/L |
|      |                               |                          | Citrulline | 17–49  | 30 umol/L  |
|      |                               |                          | Methionine | 16–36  | 26 umol/L  |
|      |                               |                          | Tyrosine | 40–92  | 87 umol/L  |
|      |                               |                          | Arginine | 45–125 | 45 umol/L  |
|      |                               |                          | Proline | 75–307 | 291 umol/L |

Note: Clinical laboratory tests were performed only on patient V.8 due to unavailability and non-cooperativeness of other patients.
energy metabolism in a new cellular redox regulation model (Oldham et al. 2015). Qiu et al. (2020) reported that both mitochondrial enzyme i.e. L2HGDH and D2HGDH catalyzes the oxidation of L2HG and D2HG into α-ketoglutarate. The studies have shown that MYC is the essential factor that regulates the expression of both L2HGDH and D2HGDH. It basically regulates the TET DNA hydroxylases and RNA demethylases, and thereby controls the cellular epigenome and epitranscriptome (Qiu et al. 2020). In addition Ye et al. (2018) have demonstrated the role of 2-HG other than epigenetic control and linked the expression of 2-HG (D- and L-2-Hydroxyglutarates) to T cell regulation and suggest its presumable role in tumor immunity (Ye et al.2018).

To date, 83 mutations in L2HGDH have been published (according to HGMD, Feb. 2021), however, only two mutations i.e. c.1003C > T p.(Arg335*) (Sass et al. 2008) and c.178G > A p.(Gly60Arg) (Ullah et al.2018) have been described in Pakistani families. In this study, we are reporting on a multigenerational Pakistani family presenting with mild intellectual disability, psychomotor retardation, gait disturbance and epilepsy. Whole exome sequencing identified a frameshift mutation NM_024884.3: c.180delG, p.(Ala62Profs*24) in L2HGDH, the most N-terminal loss of function mutation in this gene published thus far. The synopsis of the molecular findings and the clinical presentation of patients, based on the biochemical profile and brain CT findings is in concordance with the diagnosis of L2HGA. Subsequent structural and interaction analysis were conducted to predict the functional impact of the mutation in case of protein truncation. Analysis revealed remarkable changes in the local folding of L2HGDH and interaction with its substrate (L-2-hydroxyglutarate) and close interactors (D2HGDH, ALDH4A1, GLS2). However, as the mutation is located in close proximity to the N-terminus, nonsense mediated mRNA decay cannot be ruled out as the underlying patho-mechanism in this family. Some biochemical studies have shown that 2-hydroxyglutaric aciduria may be associated with elevated levels of lysine (Samuraki et al. 2008). Interestingly, biochemical profiling of one of our patients showed additional abnormally high levels of glutamate, glycine, alanine, leucine and ornithine amino acids, but it remains unclear whether these findings can be attributed to the mutation in L2HGDH. The comparative clinical analysis of the present family with previously reported Pakistani L2HGA family determined partial overlap (Ullah et al. 2018). However, tonic–clonic seizure and macrocephaly was not present in the patients presented here. Further, Peng et al. (2018) have reported a few missense and frameshift mutations in Chinese patients, who exhibited mild phenotypes comparable to the patients included in the current study (Peng et al. 2018).

**Fig. 2** Panel (a) shows SNP genotyping based HBD region and list of candidate genes between markers rs2039791 and rs781354. The causative gene L2HGDH is enclosed in red box (b) The structure of L2HGDH gene in which mutation harboring exon is encircled in red.
In 2018, none of the patients do exhibit cerebral neoplasms thus far.

Based on the findings of the current study, it is suggested that pediatricians in developing countries (especially in Pakistan) should offer screening of metabolic disorders in children, because early diagnosis and therapeutic interventions may effectively reduce the progression of the disease.

**Conclusion**

Herein, we report on the most N-terminal loss-of-function mutation in *L2HGDH* [NM_024884.3: c.180delG p.(Ala62Profs*24)] in a consanguineous family causing L-2-hydroxyglutaric aciduria. This finding further expands the mutational spectrum of L2HGDH.
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