

## CLINICAL STUDY

# Hyperinsulinism–hyperammonaemia syndrome: novel mutations in the *GLUD1* gene and genotype–phenotype correlations

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## Abstract

**Background:** Activating mutations in the *GLUD1* gene (which encodes for the intra-mitochondrial enzyme glutamate dehydrogenase, GDH) cause the hyperinsulinism–hyperammonaemia (HI/HA) syndrome. Patients present with HA and leucine-sensitive hypoglycaemia. GDH is regulated by another intra-mitochondrial enzyme sirtuin 4 (*SIRT4*). *Sirt4* knockout mice demonstrate activation of GDH with increased amino acid-stimulated insulin secretion.

**Objectives:** To study the genotype–phenotype correlations in patients with *GLUD1* mutations. To report the phenotype and functional analysis of a novel mutation (P436L) in the *GLUD1* gene associated with the absence of HA.

**Patients and methods:** Twenty patients with HI from 16 families had mutational analysis of the *GLUD1* gene in view of HA ( $n=19$ ) or leucine sensitivity ( $n=1$ ). Patients negative for a *GLUD1* mutation had sequence analysis of the *SIRT4* gene. Functional analysis of the novel P436L *GLUD1* mutation was performed.

**Results:** Heterozygous missense mutations were detected in 15 patients with HI/HA, 2 of which are novel (N410D and D451V). In addition, a patient with a normal serum ammonia concentration (21  $\mu\text{mol/l}$ ) was heterozygous for a novel missense mutation P436L. Functional analysis of this mutation confirms that it is associated with a loss of GTP inhibition. Seizure disorder was common (43%) in our cohort of patients with a *GLUD1* mutation. No mutations in the *SIRT4* gene were identified.

**Conclusion:** Patients with HI due to mutations in the *GLUD1* gene may have normal serum ammonia concentrations. Hence, *GLUD1* mutational analysis may be indicated in patients with leucine sensitivity; even in the absence of HA. A high frequency of epilepsy (43%) was observed in our patients with *GLUD1* mutations.

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## Introduction

The hyperinsulinism/hyperammonaemia (HI/HA) syndrome is a form of congenital HI (CHI) in which affected children have recurrent symptomatic hypoglycaemia together with a persistently elevated serum ammonia concentration. It has been shown that this disorder is caused by activating mutations in the *GLUD1* gene that encodes for the intra-mitochondrial enzyme, glutamate dehydrogenase (GDH) (1). GDH is highly expressed in the liver, pancreatic  $\beta$ -cells, kidney and the brain (2).

It catalyses the oxidative deamination of glutamate to  $\alpha$ -ketoglutarate and ammonia. In the pancreatic  $\beta$ -cells,  $\alpha$ -ketoglutarate enters the tricarboxylic acid cycle and leads to insulin exocytosis.

GDH is allosterically activated by leucine and inhibited by GTP (3). Activating mutations in the *GLUD1* gene reduce the sensitivity of the enzyme to allosteric inhibition by GTP and ATP (4). The loss of inhibition by GTP leads to increased leucine-induced glutamate oxidation to  $\alpha$ -ketoglutarate. Hence, leucine sensitivity is manifested by postprandial hypoglycaemia

(following protein-rich meals), which is a classical feature of this condition (5). The mechanism of the persistent HA, a striking and consistent feature of this condition, is not completely understood.

GDH is also inhibited by sirtuin 4 (SIRT4), an intramitochondrial enzyme (6) that is highly expressed in the pancreatic islets (6, 7). Hence, *SIRT4* would be an ideal candidate gene to analyse in patients with HI/HA syndrome, where no mutations in the *GLUD1* gene are found.

In this study, we report the phenotype and genotype of 20 patients with HI/HA syndrome. We also report a novel *GLUD1* mutation in a patient with severe leucine sensitivity but a completely normal serum ammonia concentration. We highlight the high risk of epilepsy in patients with *GLUD1* mutations and we discuss our findings of the *SIRT4* gene analysis in a cohort of patients with HI/HA syndrome, where no mutations are identified in the *GLUD1* gene.

## Research design and methods

We studied 20 patients with HI who were referred for mutational analysis of the *GLUD1* gene either in view of HA ( $n=19$ ) or leucine sensitivity ( $n=1$ ). Sixteen patients were unrelated and four were related demonstrating vertical transmission of disease consistent with a dominant pattern of inheritance. The diagnosis of HI was based on diagnostic criteria described previously, i.e. inappropriately elevated insulin concentrations at the time of hypoglycaemia with corresponding low concentrations of plasma  $\beta$ -hydroxybutyrate and fatty acids. Clinical information (birth weight, age of presentation, treatment details, seizure history and family history) was collected from case notes and the referring clinicians. The clinical characteristics are presented as means and summarised in Table 1.

The study was approved by the regional ethical committee and written consent was obtained from the families.

## Molecular genetics

DNA was extracted from peripheral leukocytes using standard procedures. The exons of the *GLUD1* gene, which encode the catalytic and allosteric domains of GDH (exons 6, 7, 10, 11 and 12), were amplified by PCR (for primer sequences see Supplementary Table 1, which can be viewed online at <http://www.eje-online.org/supplemental/>) and sequenced using BigDye Terminator v3.1 (Applied Biosystems, Warrington, UK). Sequencing reactions were analysed on the ABI 3730 (Applied Biosystems) and compared with the published sequence (M37154) using Mutation Surveyor software (SoftGenetics, Philadelphia, PA, USA). Mutation testing was performed on parental samples and microsatellite analysis of six markers on chromosome 20 was undertaken to confirm family relationships when a novel *de novo* mutation was identified (primers available on request).

When no *GLUD1* mutation was identified, exons 1–3 of the *SIRT4* gene were analysed. All exons were amplified by PCR using a single set of primers with the exception of exon 1, which was amplified in two overlapping fragments (for primer sequences see Supplementary Table 1). PCR products were sequenced and compared with the published sequence (NM\_012240.1) using Mutation Surveyor software (SoftGenetics).

## Functional analysis of GDH activity

Lymphocytes were isolated from peripheral blood of the patient with the novel P436L mutation and transformed with Epstein–Barr virus to establish lymphoblast cultures. The activity of GDH in lymphoblast homogenates and the effect of added GTP were determined

**Table 1** Summary of the clinical characteristics of patients with a mutation in the *GLUD1* gene.

Family	No	Sex	Age of presentation	Current age	Epilepsy	Ammonia ( $\mu\text{mol/l}$ ) (<50 $\mu\text{mol/l}$ )	Mutation	Novel
A	1	F	2 days	7 years	Yes	58–95	R221C (c.833C>T)	No
B	2	M	24 weeks	1.6 years	Yes	258–307	R265K (c.966G>A)	No
C	3	F	32 weeks	2.5 years	Yes	128	R269H (c.978G>A)	No
D	4	M	28 weeks	1.4 years	No	165	R269H (c.978G>A)	No
E	5	F	24 weeks	2.5 years	No	96	R269H (c.978G>A)	No
E	6	F	52 weeks	35 years	No	78	R269H (c.978G>A)	No
E	7	F	Not known	56 years	No	Not known	R269H (c.978G>A)	No
E	8	M	52 weeks	26 years	No	103	R269H (c.978G>A)	No
F	9	M	72 weeks	5.9 years	No	60–100	R296H (c.978G>A)	No
G	10	M	16 weeks	0.9 years	No	250	N410T (c.1401A>C)	No
H	11	F	1 week	10 years	Yes	120	N410D (c.1400A>G)	Yes
I	12	F	52 weeks	13.8 years	No	21	P436L (c.1479C>T)	Yes
J	13	F	6 weeks	18 years	Yes	244	S445L (c.1506C>T)	No
K	14	F	1 week	1.6 years	Yes	125–161	S445L (c.1506C>T)	No
L	15	M	8 weeks	4.8 years	Yes	150–190	S445L (c.1506C>T)	No
M	16	F	28 weeks	2.6 years	No	73–144	D451V (c.1524A>T)	Yes

spectrophotometrically by the oxidation of NADH at 25 °C in Tris–0.01 M acetate buffer, pH 8, containing 10 µM EDTA according to the protocol of de Lonlay *et al.* (8). Protein was measured according to the method of Lowry *et al.* (9).

## Results

### Molecular genetics

Seven different heterozygous mutations in the *GLUD1* gene were identified in 13 out of 17 screened probands (76%; see Table 1). Three mutations were novel: N410D, D451V and P436L. These mutations were not present in the probands' unaffected parents and affected residues were conserved across species. Mutations at residue N410 have previously been reported in patients with HI/HA, including one patient within this cohort, suggesting that N410 is a functionally important residue.

Two mutations, S445L and R269H, were each identified in three unrelated families. All other mutations were identified in a single proband. Testing of unaffected parents demonstrated that the mutation had arisen *de novo* in 11 out of 13 probands. For one patient, with a S445L mutation, a spontaneous mutation could not be confirmed as DNA was unavailable from the unaffected father. In a second patient, the R269H mutation was inherited by the proband from their affected mother. Testing of further family members demonstrated that the R269H mutation was also present in the proband's maternal uncle and grandmother, all of whom have HI/HA.

Sequencing of *SIRT4* in the four patients referred with HI/HA and negative for a *GLUD1* mutation did not reveal any mutations. The mean serum ammonia concentration of this group of patients was 66.7 µmol/l (50–87 µmol/l). All four patients had diazoxide-responsive HI with no clinical symptoms suggestive of protein/leucine sensitivity.

### Clinical characteristics of patients with *GLUD1* mutation

The clinical characteristics of the 16 patients with a *GLUD1* mutation are summarised in Table 1. The mean birth weight of our cohort with a mutation was 3133 g at a mean gestational age of 38.6 weeks. Macrosomia was not a common feature with birth weight >90th centile in only three patients. The age of presentation was delayed (mean of 23.4 weeks) in comparison with the patients with CHI due to  $K_{ATP}$  channel mutations, with only three presenting in the neonatal period. Two patients were successfully managed with manipulation of diet alone; all others required treatment with diazoxide. None of our patients required a pancreatectomy.

Patient 12 presented at 1 year of age with a tonic-clonic seizure associated with HI (insulin 9.6 mU/l and blood glucose 1.7 mmol/l). She had a normal serum ammonia concentration (21 µmol/l) on presentation and on repeated measurements (30 and 41 µmol/l) during childhood. She responded to diazoxide and demonstrated a normal fasting tolerance on a moderate dose of diazoxide (12–15 mg/kg per day). However, she continued to experience intermittent hypoglycaemic episodes in the postprandial period even on a high dose of diazoxide (15–17 mg/kg per day). She was hence evaluated for leucine sensitivity at 13 years of age by an oral leucine tolerance test. Following a fast of 4 h, an oral dose of 0.15 g/kg L-leucine was administered (10). Blood glucose and plasma insulin levels were then measured at –30, 0, 30, 60, 90 and 120 min. Diazoxide was not discontinued for the purpose of the test. The test was stopped at 60 min following the oral leucine load as she developed symptomatic (sweating, disordered consciousness and blurred vision) hypoglycaemia with a blood glucose concentration of 2.2 mmol/l and simultaneous plasma insulin concentration of 69.5 mU/l. Sequencing of the *GLUD1* gene was requested in view of the severe leucine sensitivity. At the age of 13.7 years, she has mild learning difficulties with memory skills and emotional functioning being the areas of most concern.

There was a high incidence of seizures in our cohort of patients with a *GLUD1* mutation. Out of 16 patients, 15 presented with seizures and 7 (43%) of them have developed epilepsy. All of the patients who developed epilepsy had generalised seizures. Out of seven patients with epilepsy, six had mutations in exons 6 and 7. All three patients with the mutation S445L developed epilepsy.

### GDH activity in lymphoblasts

The activity and allosteric response of GDH in lymphoblasts from patient 12 were measured. She had normal basal GDH activities, but the half-maximal inhibitory concentration of GTP expressed in nmol/l (IC<sub>50</sub>) was 470% higher than that of controls ( $n=20$ ) consistent with an excessive activity of GDH.

## Discussion

The phenotype of CHI due to mutations in the *GLUD1* gene is well characterised. It is reported to be milder, not usually associated with macrosomia at birth and tends to escape recognition till later in infancy (4, 5, 11). This was confirmed in our study where the mean age of presentation was 23.4 weeks with only 3 out of 17 patients presenting in the neonatal period. Interestingly, only three patients had a birth weight of >90th centile and all three had the S445L mutation. This may indicate that this mutation causes more severe foetal HI than the other mutations identified in our cohort.

Epilepsy has been frequently reported in association with HI/HA syndrome (12, 13). The increased frequency of epilepsy is thought to be either the result of a) hypoglycaemic brain injury due to recurrent hypoglycaemia or b) chronic HA or c) decreased concentrations of glutamine and the neurotransmitter  $\gamma$ -aminobutyric acid in the brain due to raised GDH activity (13). Our study confirms that a high risk of epilepsy is associated with HI due to mutations in the *GLUD1* gene, as 7 out of 16 (43%) of our patients developed epilepsy. Six out of the seven patients had mutations in exons 6 and 7; this was consistent with the study by Bahi-Buisson *et al.* (14), which reported that epilepsy is associated more frequently with mutations in exons 6 and 7. The mechanism of this genotype–phenotype association according to the location of the mutations is as yet unclear.

The most consistent feature of the HI due to mutations in *GLUD1* is the persistent HA, and hence the term ‘HI/HA syndrome’ is used synonymously with CHI due to *GLUD1* mutations (4, 8, 15). It is proposed that HA is the result of excessive GDH activity in the hepatocytes leading to an increase in the production of ammonia from glutamate and/or depletion of glutamate with reduction in the production of *N*-acetylglutamate, an allosteric activator essential for the first step of ammonia detoxification (16). The second mechanism is supported by the reduction in the concentration of blood ammonia on administration of *N*-carbamylglutamate (an analogue of *N*-acetylglutamate) (17, 18). There has been one other report of two patients (father and son) with HI due to the R269H mutation in the *GLUD1* gene and normal serum ammonia concentrations (35 and 28  $\mu\text{mol/l}$ ) (19). Our patient with the novel P436L mutation had a completely normal serum ammonia concentration (highest being 41  $\mu\text{mol/l}$ ) that led to a delay in establishing the molecular diagnosis. If the origin of the HA is in the liver, it is possible that our patient is mosaic for the mutation with the mutation being absent or at <50% in the hepatic tissues. And hence this absence of HA may not be genotype specific. However, we cannot prove or disprove this possibility without a liver/pancreatic biopsy. Similarly, mosaicism may be the underlying mechanism in our four patients with HI/HA syndrome and no *GLUD1* mutation with the mutation affecting the liver and pancreas, but not blood.

Enzymatic analysis of the lymphoblastic GDH from our patient with the P436L mutation showed a loss of GTP inhibition with the half-maximal inhibitory concentration of GTP being 470% higher than that of controls. Loss of GTP inhibition is known to increase glutamate oxidation in the presence of leucine causing leucine-sensitive HI, a classical feature of HI/HA syndrome. This was clinically evident in our patient with this novel P436L mutation and was confirmed by the oral leucine tolerance test. The confirmation of leucine sensitivity prompted us to sequence the *GLUD1*

gene and led to the molecular diagnosis. This suggests that analysis of the *GLUD1* gene must be considered in all patients with leucine sensitivity even in the absence of HA.

This study also reports two other novel mutations (N410D and D451V) in the *GLUD1* gene. N410 is located in the antenna-like structure connecting to the pivot helix of the GDH structure. A mutation (N410T) previously described at the same residue is known to cause HI/HA (20). D451 is located in the allosteric domain of the *GLUD1* gene, a common site for mutations causing HI/HA syndrome and hence likely to be pathogenic by interfering with the allosteric binding sites. Conservation data suggest that these residues are functionally important as they are both well conserved among species.

*SIRT4* has been shown to regulate insulin secretion by repressing the activity of GDH (6). *Sirt4* knockout mice have increased glucose-stimulated and amino acid-stimulated insulin secretion in comparison with wild-type mice along with fasting hypoglycaemia in the male knockout mice. We hypothesised that a loss of function mutation of *SIRT4* will cause a phenotype similar to gain of function *GLUD1* mutations, i.e. HI/HA syndrome. However, our cohort of HI/HA patients with no known genetic aetiology is small, and hence the negative findings of our study are not enough to exclude a putative role of this gene in HI/HA syndrome.

## Conclusion

Mutations in the *GLUD1* gene are not always associated with HA; hence mutational analysis of the *GLUD1* gene may be indicated in patients with HI with evidence of leucine sensitivity even in the absence of HA. *GLUD1* gene mutations cause a mild form of CHI associated with normal birth weight, delayed age at presentation and a high risk of epilepsy.

## Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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