Genome-Wide Homozygosity Analysis Reveals *HADH* Mutations as a Common Cause of Diazoxide-Responsive Hyperinsulinemic-Hypoglycemia in Consanguineous Pedigrees

Sarah E. Flanagan, Ann-Marie Patch, Jonathan M. Locke, Teoman Akcay, Enver Simsek, Mohammadreza Alaei, Zeinab Yekta, Meena Desai, Ritika R. Kapoor, Khalid Hussain, and Sian Ellard

Institute of Biomedical and Clinical Science (S.E.F., A.-M.P., J.M.L., S.E.), Peninsula Medical School, University of Exeter, Exeter EX2 5DW, United Kingdom; London Centre for Paediatric Endocrinology and Metabolism, Great Ormond Street Hospital for Children, National Health Service Trust, and the Institute of Child Health (R.R.K., K.H.), University College London, London WC1E 6BT, United Kingdom; Sisli Etfal Educational and Research Hospital, Department of Pediatric Endocrinology (T.A.), Sisli, 34377 Istanbul, Turkey; Osmangazi University School of Medicine, Department of Paediatrics, Division of Paediatric Endocrinology (E.S.), 26480 Eskisehir, Turkey; Mofid Children's Hospital (M.A., Z.Y.) Shahid Beheshti University of Medical Sciences, Tehran, 15468-155514 Iran; and Sir Hurkisondas Nurrotumdas Hospital and Research Center (M.D.), Mumbai 400 004, India

Context and Objective: Recessive mutations in the hydroxyacyl-CoA dehydrogenase (*HADH*) gene encoding the enzyme 3-hydroxyacyl-CoA dehydrogenase are a rare cause of diazoxide-responsive hyperinsulinemic hypoglycemia (HH) with just five probands reported to date. HADH deficiency in the first three identified patients was associated with detectable urinary 3-hydroxyglutarate and raised plasma 3-hydroxybutyryl-carnitine levels, but two recent cases did not have abnormal urine organic acids or acylcarnitines.

Research Design and Methods: We studied 115 patients with diazoxide-responsive HH in whom the common genetic causes of HH had been excluded. No patients were reported to have abnormal acylcarnitines or urinary organic acids. Homozygosity mapping was undertaken in probands from 13 consanguineous pedigrees to search for regions harboring mutations that are identical by descent.

Results: *HADH* sequencing was performed after genome-wide single nucleotide polymorphism analysis revealed a large shared region of homozygosity spanning the *HADH* locus in six unrelated probands. Homozygous mutations were identified in three of these patients and in a further two probands from consanguineous families. *HADH* analysis in the remainder of the cohort identified mutations in a further six probands for whom consanguinity was not reported, but who originated from countries with high rates of consanguinity. Six different *HADH* mutations were identified in 11/115 (10%) patients tested.

Conclusion: *HADH* mutations are a relatively common cause of diazoxide-responsive HH with a frequency similar to that of *GLUD1* and *HNF4A* mutations. We recommend that *HADH* sequence analysis is considered in all patients with diazoxide-responsive HH when recessive inheritance is suspected. **(*J Clin Endocrinol Metab* 96: E498–E502, 2011)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-1906 Received August 13, 2010. Accepted December 13, 2010.
First Published Online January 20, 2011

For editorial see page 617

Abbreviations: GDH, Glutamate dehydrogenase; HADH, hydroxyacyl-CoA dehydrogenase; HH, hyperinsulinemic-hypoglycemia; SNP, single nucleotide polymorphism; UTR, untranslated region.

Hyperinsulinemic-hypoglycemia (HH), which is characterized by unregulated secretion of insulin despite a low blood glucose concentration, most commonly presents in the neonatal period with the phenotype ranging from mild to severe medically unresponsive hypoglycemia (1). Diazoxide targets the ATP-sensitive potassium (K_{ATP}) channel in the pancreatic β-cell and is often the first line of treatment. Patients who show a poor response to diazoxide therapy are likely to require a pancreatectomy.

Mutations in the *ABCC8* and *KCNJ11* genes, which encode the SUR1 and Kir6.2 subunits of the K_{ATP} channel, most often cause diazoxide-unresponsive HH but rare mutations in these and five other genes (*GCK*, *GLUD1*, *HADH*, *HNF4A*, and *SLC16A1*) have been reported in patients with diazoxide-responsive HH (1). Mutations in these genes are often associated with discrete phenotypes, for example *GLUD1* mutations also result in hyperammonemia while *SLC16A1* mutations cause exercise-induced hyperinsulinism (2, 3). While the clinical characteristics may guide the order of genetic testing, it should be noted that these genotype/phenotype relationships are not absolute. For example, recessive mutations in the hydroxyacyl-CoA dehydrogenase (*HADH*) gene were first identified in patients with specific fatty acid oxidation defects where urinary 3-hydroxyglutarate was present and plasma 3-hydroxybutyryl-carnitine levels were raised (4–6). However, two patients with homozygous *HADH* mutations but with normal acylcarnitines and urine organic acids have recently been reported (7, 8).

Recently, we demonstrated that a genetic diagnosis was possible for 27% of cases in our cohort with diazoxide-responsive HH (59/220 patients) (9). Mutations in *KCNJ11*, *ABCC8*, *GCK*, and *HNF4A* were excluded, but *HADH* was not sequenced because there was no report of any abnormality in the acylcarnitines and urine organic acids (9). Autozygosity analysis is a useful method for identifying novel genetic etiologies within consanguineous pedigrees through the identification of a genetic region harboring a mutation that is identical by descent (10). In the present study we have undertaken genome-wide single nucleotide polymorphism (SNP) analysis on a subset of unrelated consanguineous probands with diazoxide-responsive HH and no genetic diagnosis.

Materials and Methods

We studied 115 patients with diazoxide-responsive HH without mutations in *ABCC8*, *KCNJ11*, *GCK*, and *HNF4A*. Mutations in *GLUD1* had been excluded in patients with hyperammonemia (n = 7).

Clinical data were provided via a standard request form (*www.diabetesgenes.org*) with diazoxide-responsiveness defined as the ability to come off intravenous glucose and maintain normoglycemia. No patients had required a pancreatectomy, and patients with evidence of perinatal asphyxia were excluded from the cohort. No cases were reported to have abnormalities in acylcarnitines and urine organic acids or exercise-induced hyperinsulinism. Consanguinity was reported in 18 probands. The study was conducted in accordance with the Declaration of Helsinki with informed parental consent given on behalf of children.

Homozygosity analysis

Genotyping was undertaken on the Affymetrix 6.0 SNP chip by Medical Solutions (Nottingham, UK), ALMAC Diagnostics (Craigavon, Northern Ireland), or Aros Applied Biotechnology (Aarhus, Denmark) for 13/18 consanguineous patients where there was sufficient DNA. Processing of genomic DNA was performed as per the Affymetrix protocol, and the mean SNP call rate was 98.8%. In-house Perl scripts were developed to automatically identify homozygous segments, defined by at least 20 consecutive homozygous SNPs marking a region that exceeded 3cM (11).

HADH analysis

In all 115 patients the 8 exons of *HADH* (NM_005327.2) were amplified and sequenced as previously described (7). When repeated failure of PCR indicated a homozygous deletion, break points were mapped by sequential PCR and sequencing. Patients with common mutations were further investigated by microsatellite markers (*HADH* flanking markers D4S2859 and D4S2945).

For patients where conventional sequencing failed to identify a mutation but SNP analysis revealed homozygosity over *HADH*, the possibility of a partial gene deletion or a mutation in the promoter, 3′ untranslated region (UTR) or alternative exons [as listed on AceView, accessed June 2010 (12)] was investigated. When a novel variant was identified in an alternative exon the presence/absence of the variant transcript in control islets, whole pancreas, liver, and blood was determined by real-time PCR (RT-PCR) and sequencing (primers available on request).

Results

Genetic results

Consanguineous cohort

Genome-wide SNP data were obtained for 13/18 consanguineous probands. Six of the 13 patients shared a region of homozygosity (3.3 Mb) on chromosome 4q25, which contained 21 genes including *HADH* (http://genome.ucsc.edu/). No further regions of homozygosity shared by four or more individuals were identified.

HADH sequencing identified mutations in 3/6 patients with homozygous regions encompassing *HADH*. Homozygous mutations were also present in 2/5 patients where insufficient DNA was available for SNP analysis (Table 1). No mutations were identified in the seven patients without homozygous regions spanning *HADH*. Three different *HADH* mutations were identified; two novel mutations, Q163X and K136E (each in a single pa-

TABLE 1. Clinical characteristics of patients with *HADH* mutations

Patient	Gender	Age at diagnosis	Birth weight (gestation)	Current age (y)	Current diazoxide dose (mg/kg/d)	Reported Consanguinity	Country of origin	Mutation detection method	Mutation
1	Male	16 wks	2.9 kg (38 wks)	3	5	Yes	Turkey	Homozygosity analysis, then sequencing	K136E/K136E (c.406A>G)
2	Male	16 wks	2.8 kg (40 wks)	8	2	Yes	Turkey	Homozygosity analysis, then sequencing	Q163X/Q163X (c.487C>T)
3	Male	2 wks	4.1 kg (40 wks)	2	10	Yes	Turkey	Homozygosity analysis, then sequencing	R236X/R236X (c.706C>T)
4	Male	5 days	4.35 kg (40 wks)	2	10	Yes	Turkey	Sequence analysis	R236X/R236X (c.706C>T)
5	Female	1 wk	4.0 kg (40 wks)	7	2.5	No	Turkey	Sequence analysis	R236X/R236X (c.706C>T)
6	Male	2 days	3.2 kg (39 wks)	1	11	Yes	Pakistan	Sequence analysis	R236X/R236X (c.706C>T)
7	Female	12 wks	3.5 kg (40 wks)	2	15	No	Iran	Sequence analysis	R236X/R236X (c.706C>T)
8	Female	1 day	3.7 kg (40 wks)	4	8	No	Iran	Sequence analysis	R236X/R236X (c.706C>T)
9	Male	26 wks	2.95 kg (40 wks)	2	10	No	India	Sequence analysis	K95SfsX3/IVS6 + 39C>G (c.283_293delinsT/c.709 + 39C>G)
10	Male	2 days	4.0 kg (40 wks)	2	10	No	India	Long range PCR and sequencing of breakpoints	Ex1del/Ex1del (c.1-3440_132 + 1943del/ c.1-3440_132 + 1943del)
11	Male	24 wks	3.8 kg (40 wks)	3	10	No	India	Long range PCR and sequencing of breakpoints	Ex1del/Ex1del (c.1-3440_132 + 1943del/ c.1-3440_132 + 1943del)

Mutation nomenclature corresponds to *HADH* sequence accession NM_005327.2.

tient), and the previously reported Q236X mutation (8) in three probands. When DNA was available, mutation testing confirmed the carrier status of the unaffected parents. None of the probands had a sibling affected with HH. The K136E mutation is likely to be pathogenic as *in silico* analysis suggests that it is detrimental to protein function (http://neurocore.charite.de/MutationTaster/), the mutated residue is highly conserved across species, and the variant has not been identified in 362 control chromosomes (http://www.1000genomes.org June 2010). For the three probands with homozygosity over *HADH* but no coding mutation, dosage analysis, and sequencing of the *HADH* promoter, 3′UTR and alternative exons was undertaken but no mutations were identified.

Nonconsanguineous cohort

After the identification of mutations in 5/18 (28%) consanguineous patients, *HADH* sequencing was undertaken in the remainder of the cohort and mutations were identified in 6/97 probands. Three probands were homozygous for the R236X mutation, and a failure to amplify exon 1 by PCR in two probands suggested the presence of a homozygous deletion (Table 1). Mapping of the break points confirmed an identical deletion, which included the minimal promoter and exon 1 (c.1-3440_132 + 1943del). When DNA was available carrier status was confirmed in the unaffected parents. None of the probands had a sibling affected with HH.

In the sixth patient a maternally inherited frame shift mutation, K95Sfs, was identified. Although a second mutation was not found by analysis of the reference sequence, promoter, 3′UTR, or by dosage studies, a novel paternally inherited variant, c.709 + 39C>G (NM_005327.2), which results in a L254V mutation in exon 6 of an alternative splice variant (cDNA accession number BI826991) (Fig. 1, Table 1), was identified. RT-PCR and sequencing confirmed the presence of this variant transcript in normal pancreas, islets, liver, and blood (data not shown). The c.709 + 39C>G variant was not identified in 362 control chromosomes (http://www.1000genomes.org June 2010).

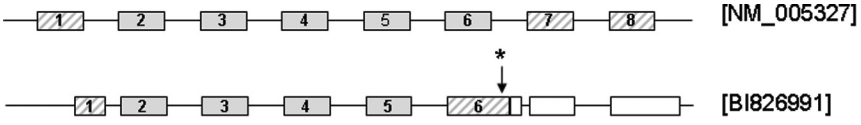

FIG. 1. Schematic representation of the full-length *HADH* gene (Reference sequence NM_005327) and a variant transcript (cDNA accession number B1826991). Exons are represented by *boxes*, and introns are denoted by *lines*. *Hatched boxes* represent coding regions which differ between the two transcripts, and *white boxes* represent UTRs. An *arrow with a star* points to the approximate position of the novel variant, c.709 + 39C>G (nomenclature relating to RefSeq NM_005327) identified in patient 9.

Haplotype analysis

Microsatellite analysis suggested the existence of two R236X founder mutations within the Iranian and Turkish/Pakistani populations. The exon 1 deletion was present on a common haplotype, consistent with a common ancestor within the Indian population (Table 1).

Clinical characteristics

The median age at diagnosis of HH for patients with *HADH* mutations was 7 weeks (range 1 d to 26 weeks) and the median birth weight was 3.6 kg at 40 weeks gestation (range 2.8–4.35 kg) (Table 1). No patients were reported to have defects in fatty acid oxidation, although acylcarnitine profiles and urine organic acid screens were not complete for all patients.

Discussion

HADH sequencing was undertaken after SNP analysis revealed a large shared region of homozygosity spanning the *HADH* locus in six unrelated consanguineous probands. We identified mutations in three of these patients and in a further two probands from known consanguineous families (28% positive). These results prompted us to sequence *HADH* in the remainder of our cohort, and homozygous mutations were identified in a further five probands who were not reported as being consanguineous. However, these patients were all referred from countries with high rates of consanguineous marriages. Only one patient with compound heterozygous mutations was identified. In total, *HADH* mutations were identified in 11/115 (10%) patients with diazoxide-responsive HH of unknown etiology. Our study increases the number of patients reported with *HADH* HH from five probands (4–8) to 16.

In one patient with a maternally inherited frame shift mutation, extensive studies to search for a second mutation of paternal origin were undertaken and a novel variant was found in intron 6 (corresponding to RefSeq NM_005327.2). *In silico* splicing prediction programs suggest that this variant will not alter splicing. However, at least eight transcript variants are predicted to exist for *HADH*, and this substitution results in a missense mutation in exon 6 of a variant transcript (cDNA accession number BI826991) (12) (Fig. 1), which is predicted to encode a protein with an NAD-binding and a C-terminal domain. Although further studies are required to assess the significance of this transcript, its detection in tissues including pancreas and islets suggests that it may be important. While the pathogenicity of the variant is currently unproven, the identification of a frame shift mutation on the opposite allele is consistent with a diagnosis of recessively inherited HH resulting from a *HADH* mutation.

In keeping with previous reports a range in birth weights and ages at diagnosis of HH were observed in the patients with *HADH* mutations (5). Interestingly, none of the patients were reported to have abnormalities in plasma acylcarnitines or urine organic acids, a phenotype reported in 3/5 published probands. While the absence of abnormal acylcarnitines and urine organic acids in these patients may be attributable to limitations in laboratory analysis, it is possible that the phenotype is mutation-dependent because none of the mutations identified in this cohort have been found in individuals with abnormal acylcarnitines and urine organic acids. It is unlikely, however, that the disease spectrum reflects the severity of the mutation as the majority of patients with isolated HH have null mutations.

A number of studies have demonstrated that HADH has a pivotal role in regulating insulin secretion (13–16). Most recently a study by Li *et al.* (15) examined the mechanism of insulin dysregulation in mice with a knock-out of the *hadh* gene. Pull-down experiments demonstrated protein–protein interactions between HADH and glutamate dehydrogenase (GDH), and studies on isolated islets showed in increased in the affinity of GDH for its substrate α-ketoglutarate. It is therefore likely that *HADH* mutations cause HH by activation of GDH via loss of inhibitory regulation of GDH by HADH. This finding is of particular interest as activating mutations in *GLUD1*, which encodes GDH, are a common cause of hyperinsulinism and hyperammonemia (2).

In conclusion we have shown that mutations in *HADH* account for 10% of cases with diazoxide-responsive HH without a mutation in the known genes. This study takes the number of *HADH* mutations identified in our cohort to 12 (7), with a prevalence similar to that for HH attributable to *HNF4A* or *GLUD1* (17) mutations. We recommend that analysis of the *HADH* gene is considered in all patients with diazoxide-responsive HH who originate from known consanguineous pedigrees, isolated populations, or countries where inbreeding is frequent, regardless of whether there is evidence of abnormal fatty acid oxidation.

Acknowledgments

We thank Annet Damhuis and Andrew Parrish for their technical assistance. S.E.F. is the Sir Graham Wilkins Peninsula Medical School research fellow. S.E. is a member of the core staff within the National Institute for Health Research funded Peninsula Clinical Research Facility.

Address all correspondence and requests for reprints to: Professor Sian Ellard, Peninsula Medical School, Barrack Road, Exeter, EX2 5DW, United Kingdom. E-mail: Sian.Ellard@rdeft.nhs.uk.

This work was supported by the Wellcome Trust (081188/A/06/Z).

Disclosure Summary: The authors have nothing to declare.

References

1. **Kapoor RR, Flanagan SE, James C, Shield J, Ellard S, Hussain K** 2009 Hyperinsulinaemic hypoglycaemia. Arch Dis Child 94:450–457
2. **Stanley CA, Lieu YK, Hsu BY, Burlina AB, Greenberg CR, Hopwood NJ, Perlman K, Rich BH, Zammarchi E, Poncz M** 1998 Hyperinsulinism and hyperammonemia in infants with regulatory mutations of the glutamate dehydrogenase gene. N Engl J Med 338: 1352–1357
3. **Otonkoski T, Jiao H, Kaminen-Ahola N, Tapia-Paez I, Ullah MS, Parton LE, Schuit F, Quintens R, Sipila I, Mayatepek E, Meissner T, Halestrap AP, Rutter GA, Kere J** 2007 Physical exercise-induced hypoglycemia caused by failed silencing of monocarboxylate transporter 1 in pancreatic beta cells. Am J Hum Genet 81:467–474
4. **Clayton PT, Eaton S, Aynsley-Green A, Edginton M, Hussain K, Krywawych S, Datta V, Malingre HE, Berger R, van den Berg IE** 2001 Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of beta-oxidation in insulin secretion. J Clin Invest 108:457–465
5. **Molven A, Matre GE, Duran M, Wanders RJ, Rishaug U, Njolstad PR, Jellum E, Sovik O** 2004 Familial hyperinsulinemic hypoglycemia caused by a defect in the SCHAD enzyme of mitochondrial fatty acid oxidation. Diabetes 53:221–227
6. **Hussain K, Clayton PT, Krywawych S, Chatziandreou I, Mills P, Ginbey DW, Geboers AJ, Berger R, van den Berg IE, Eaton S** 2005 Hyperinsulinism of infancy associated with a novel splice site mutation in the SCHAD gene. J Pediatr 146:706–708
7. **Kapoor RR, James C, Flanagan SE, Ellard S, Eaton S, Hussain K** 2009 3-Hydroxyacyl-coenzyme A dehydrogenase deficiency and hyperinsulinemic hypoglycemia: characterization of a novel mutation and severe dietary protein sensitivity. J Clin Endocrinol Metab 94: 2221–2225
8. **Di Candia S, Gessi A, Pepe G, Sogno Valin P, Mangano E, Chiumello G, Gianolli L, Proverbio MC, Mora S** 2009 Identification of a diffuse form of hyperinsulinemic hypoglycemia by 18-fluoro-L-3,4 dihydroxyphenylalanine positron emission tomography/CT in a patient carrying a novel mutation of the HADH gene. Eur J Endocrinol 160:1019–1023
9. **Flanagan SE, Kapoor RR, Mali G, Cody D, Murphy N, Schwahn B, Siahanidou T, Banerjee I, Akcay T, Rubio-Cabezas O, Shield JP, Hussain K, Ellard S** 2010 Diazoxide-responsive hyperinsulinemic hypoglycemia caused by HNF4A gene mutations. Eur J Endocrinol 162:987–992
10. **Lander ES, Botstein D** 1987 Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. Science 236:1567–1570
11. **Woods CG, Cox J, Springell K, Hampshire DJ, Mohamed MD, McKibbin M, Stern R, Raymond FL, Sandford R, Malik Sharif S, Karbani G, Ahmed M, Bond J, Clayton D, Inglehearn CF** 2006 Quantification of homozygosity in consanguineous individuals with autosomal recessive disease. Am J Hum Genet 78:889–896
12. **Thierry-Mieg D, Thierry-Mieg J** 2006 AceView: a comprehensive cDNA-supported gene and transcripts annotation. Genome Biol 7(Suppl 1):S12.1–S12.14.
13. **Martens GA, Vervoort A, Van de Casteele M, Stange G, Hellemans K, Van Thi HV, Schuit F, Pipeleers D** 2007 Specificity in beta cell expression of L-3-hydroxyacyl-CoA dehydrogenase, short chain, and potential role in down-regulating insulin release. J Biol Chem 282:21134–21144
14. **Hardy OT, Hohmeier HE, Becker TC, Manduchi E, Doliba NM, Gupta RK, White P, Stoeckert Jr CJ, Matschinsky FM, Newgard CB, Kaestner KH** 2007 Functional genomics of the beta-cell: short-chain 3-hydroxyacyl-coenzyme A dehydrogenase regulates insulin secretion independent of K+ currents. Mol Endocrinol 21:765–773
15. **Li C, Chen P, Palladino A, Narayan S, Russell LK, Sayed S, Xiong G, Chen J, Stokes D, Butt YM, Jones PM, Collins HW, Cohen NA, Cohen AS, Nissim I, Smith TJ, Strauss AW, Matschinsky FM, Bennett MJ, Stanley CA** 2010 Mechanism of hyperinsulinism in short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency involves activation of glutamate dehydrogenase. J Biol Chem 285:31806–31818
16. **Filling C, Keller B, Hirschberg D, Marschall HU, Jornvall H, Bennett MJ, Oppermann U** 2008 Role of short-chain hydroxyacyl CoA dehydrogenases in SCHAD deficiency. Biochem Biophys Res Commun 368:6–11
17. **Kapoor RR, Flanagan SE, Fulton P, Chakrapani A, Chadefaux B, Ben-Omran T, Banerjee I, Shield JP, Ellard S, Hussain K** 2009 Hyperinsulinism-hyperammonaemia syndrome: novel mutations in the GLUD1 gene and genotype-phenotype correlations. Eur J Endocrinol 161:731–735