Diazoxide-responsive hyperinsulinemic hypoglycemia caused by HNF4A gene mutations

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Objective: The phenotype associated with heterozygous HNF4A gene mutations has recently been extended to include diazoxide responsive neonatal hypoglycemia in addition to maturity-onset diabetes of the young (MODY). To date, mutation screening has been limited to patients with a family history consistent with MODY. In this study, we investigated the prevalence of HNF4A mutations in a large cohort of patients with diazoxide responsive hyperinsulinemic hypoglycemia (HH).

Subjects and methods: We sequenced the ABCC8, KCNJ11, GCK, GLUD1, and/or HNF4A genes in 220 patients with HH responsive to diazoxide. The order of genetic testing was dependent upon the clinical phenotype.

Results: A genetic diagnosis was possible for 59/220 (27%) patients. K_ATP channel mutations were most common (15%) followed by GLUD1 mutations causing hyperinsulinism with hyperammonemia (5.9%), and HNF4A mutations (5%). Seven of the 11 probands with a heterozygous HNF4A mutation did not have a parent affected with diabetes, and four de novo mutations were confirmed. These patients were diagnosed with HH within the first week of life (median age 1 day), and they had increased birth weight (median +2.4 SDS). The duration of diazoxide treatment ranged from 3 months to ongoing at 8 years.

Conclusions: In this large series, HNF4A mutations are the third most common cause of diazoxide responsive HH. We recommend that HNF4A sequencing is considered in all patients with diazoxide responsive HH diagnosed in the first week of life irrespective of a family history of diabetes, once K_ATP channel mutations have been excluded.
the intra-mitochondrial enzyme, glutamate dehydrogenase. The majority of patients with GLUD1 mutations have hyperammonemia (HA) (7, 8). Rarer causes of diazoxide responsive HH include mutations in the GCK or HADH genes (9, 10). Activating GCK mutations show a variable phenotype but patients may be diagnosed outside the neonatal period, and some are responsive to diazoxide (11). HADH mutations have been reported in five patients, and these mutations typically cause HH with associated defects in fatty acid oxidation (10, 12, 13).

We have recently shown that loss-of-function mutations in the HNF4A gene can also cause HH (14). The clinical severity ranges from mild transient hypoglycemia that does not require pharmacological treatment to persistent HH treated with diazoxide for up to 3 years (15, 16). Heterozygous HNF4A mutations result in increased birth weight (median increase 790 g), macrosomia in 56%, and a form of maturity onset diabetes of the young (HNF4A MODY) that shows sensitivity to low-dose sulfonlureas (14, 16). All previous studies have described patients with hypoglycemia in families with known HNF4A mutations recruited because of their history of diabetes (14, 15), or selected for testing due to neonatal HH in a proband, where the family history was consistent with HNF4A MODY (16). The prevalence of HNF4A mutations in patients referred for genetic testing due to a diagnosis of HH has not been investigated. We now report the genetic and clinical characteristics in a large cohort (n=220) of patients with diazoxide responsive HH.

Subjects and methods

We studied 220 patients with diazoxide responsive HH who did not require pancreatectomy. Diazoxide responsiveness was defined as the ability to come off i.v. glucose and maintain normoglycemia. Patients with evidence of perinatal asphyxia were excluded from the cohort. The cohort included referrals via the UK Genetic Testing Network (http://www.ukgtn.nhs.uk) and international cases (n=111). Clinical data were provided via a standard request form (www.diabetesgenes.org), clinical letter of referral, or by case note review. The age at diagnosis ranged from birth to 15 years (median 1 week), and 61% of the cohort were male (see Table 1 for clinical characteristics of the cohort). Macrosomia was defined as a birth weight of $\geq 1.3$ SDS (equivalent to the 90th centile). The study was conducted in accordance with the Declaration of Helsinki (2000).

**Genetic analysis**

Genomic DNA was extracted from peripheral leukocytes using standard procedures, and the coding exons and intron/exon boundaries of the ABCC8, KCNJ11, GCK, GLUD1, and HNF4A genes were amplified by PCR (primers available on request). HNF4A analysis included the coding exons 1d–10 and the P2 pancreatic promoter. PCR products were sequenced using standard methods on an ABI 3730 (Applied Biosystems, Warrington, UK), and were compared to the published sequence NM_000457.3 (exons 2–10) and AY680697 (exon 1d only) (17) using Mutation Surveyor v3.2 (SoftGenetics, State College, PA, USA). The order of genetic testing depended on the clinical phenotype with sequencing of the GLUD1 gene performed in all the patients with HA. ABCC8, KCNJ11, GCK, and HNF4A mutations were excluded in all the patients whose genetic diagnosis was not known. No patients in our cohort were reported to have defects in fatty acid oxidation (increased levels of 3-hydroxyglutaric acid or 3-hydroxybutyryl-carnitine), and therefore genetic analysis of the HADH gene was not indicated.

When an HNF4A mutation was identified, parents were tested (if available) to establish the mode of inheritance, and microsatellite analysis (PowerPlex 16 System, Promega) was undertaken to confirm de novo mutations. Novel non-synonymous variants were tested in ethnically matched control chromosomes.

**Clinical studies**

Clinical characteristics were obtained from patients’ hospital records with assistance from their physician. HH was defined as a blood glucose level $< 3$ mmol/l

| Table 1 Clinical characteristics for the 220 patients with diazoxide responsive hyperinsulinemic hypoglycemia. |
|-----------------------------------------------|
| **Total cohort** | **HNF4A mutation positive** | **K$_{\text{STR}}$ channel positive** | **GLUD1 mutation positive** | **GCK mutation positive** | **Unknown etiology** |
|-----------------------------------------------|
| Number of patients                          | 220 | 11   | 33   | 13   | 2   | 161   |
| Age at diagnosis (weeks)                     | 1 week | 1 day | 4 days | 24 weeks | 9 years | 1 week |
| Birth weight (SDS)                           | $+0.17 \pm 1.28$ | $+2.4 \pm 1.43$ | $+1.27 \pm 0.03$ | $-0.29 \pm 1.08$ | $+0.74 \pm 0.88$ | $-0.19 \pm 1.27$ |

Data are provided for the total cohort and for probands grouped by their genetic etiology. Unless otherwise indicated, the data are represented by the median (interquartile range). SDS for birth weights were calculated by comparing with the data from Child Growth Foundation LMS (19).
with detectable serum insulin and/or c-peptide. Phenotypic data are presented as median (interquartile range), and comparative statistics used the Mann–Whitney U test.

**Results**

**Genetic results**

The genetic etiology was determined in 59/220 (27%) probands (Table 1). Thirty-three patients had a mutation in one of the K<sub>ATP</sub> channel genes (5 KCNJ11 and 28 ABCC8; 4 with biallelic mutations). Thirteen probands were heterozygous for a GLUD1 mutation (patients previously reported (8)), and activating GCK mutations were identified in two cases.

**HNF4A mutations**

A total of 11 different heterozygous HNF4A mutations were identified in 11 probands (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). Two of these patients, with IVS2-21A>G (c.264-21A>G) and L330fsdel (c.987_1003del) mutations have been reported previously (16). One mutation, Y16X (c.48C>G), has previously been identified in another patient with hyperinsulinism (HI) (16), while the remaining eight mutations are novel: R76W (c.226C>T), R80W (c.238C>T), C106S (c.317G>C), M116I (c.317G>A), L263P (c.789T>C), Y319fs (c.953dupA), L331_L332dup (c.992_997dupTGCTGC), and Q362X (c.1084C>T). L331_L332dup is likely to be pathogenic since a single leucine duplication mutation (p.Leu332dup) has been identified in three unrelated MODY probands (Slan Ellard, unpublished data and (18)). Analysis of seven orthologous sequences demonstrated that the five novel missense mutations occurred at residues that are conserved through evolution, and these mutations were not present in 300 ethnically matched (Caucasian) control chromosomes.

![Figure 1 Partial pedigrees showing inheritance of HNF4A mutations in the 11 families. Circles represent females, and squares indicate males. A circle with the letter D denotes an ovum donor. Probands are indicated by an arrow. Diagonal hatching denotes patients with hyperinsulinism, vertical hatching represents gestational diabetes, and filled symbols show diabetic individuals. The genotype is given below each symbol: M/N denotes a heterozygous HNF4A mutation, and N/N denotes a normal genotype. For each proband, birth weight (gestation in weeks) and duration of diazoxide treatment are provided, > indicates the minimum duration when treatment is ongoing. The HNF4A mutation identified in each family is shown above each pedigree. Previously reported pedigrees are denoted by an asterisk* (16).](http://www.eje-online.org)
& Andrew Hattersley, unpublished data), and these variants were not identified in ethnically matched control chromosomes (300 Caucasian control chromosomes tested for V94M; 130 Turkish control chromosomes tested for S371R and H378del). We conclude that these four novel variants are unlikely to be pathogenic.

**Inheritance of HNF4A mutations**

In 4/11 families, the mutation was inherited from a diabetic parent (for pedigrees see Fig. 1). One patient had inherited a Y319fs mutation from her unaffected father (current age 39 years), but her paternal aunt who had gestational diabetes at 30 years was also found to carry the mutation. The proband with the L331_L332dup mutation had inherited it from her unaffected mother, but the maternal grandparents were not available for testing. Four mutations, R76W, C106S, M116I, and Q362X, were proven by microsatellite analysis to have arisen de novo. In the remaining family, with a Y16X mutation, the mode of inheritance could not be established as the child was conceived by ovum donation.

**Clinical characteristics of HNF4A mutation carriers**

Age at diagnosis was provided for 10/11 probands with a HNF4A mutation, and all ten patients were diagnosed within the first week of life (median age 1 day, range 1–7 days; Table 1). The majority of patients (9/11) were macrosomic as defined by a corrected birth weight of $\geq 1.3$ S.D.s from the mean. The median birth weight for the 11 cases was +2.4 S.D.S (see Table 1 and Fig. 1). The duration of diazoxide treatment for all 11 patients ranged from 3 months to ongoing at 8 years, with seven patients having persistent HI as defined by a requirement for diazoxide at the age of 1 year. Two of the remaining probands are currently under 12 months of age, and are still requiring diazoxide. One proband subsequently developed diabetes at the age of 12 years (Fig. 1). Two unaffected parents were found to be heterozygous mutation carriers, but in the absence of a formal OGTT, impaired glucose tolerance cannot be excluded. None of the ten heterozygous relatives reported a history of neonatal hypoglycemia.

**Clinical characteristics by genetic etiology**

The clinical characteristics of the probands were compared according to genetic etiology (Table 1). Patients with a HNF4A mutation presented earlier, and were born heavier than patients with a GLUD1 mutation (1 day versus 24 weeks, $P = 0.0006$ and $+2.4$ SDS versus $-0.29$ SDS, $P = 0.0003$ respectively). No differences in the age at diagnosis or birth weight were observed between patients with an HNF4A or $K_{ATP}$ channel mutations (1 day versus 4 days, $P = 0.084$ and $+2.4$ SDS versus $+1.27$ SDS, $P = 0.052$).

**Discussion**

We identified a genetic etiology in 27% of patients with diazoxide responsive HH. $K_{ATP}$ channel mutations were most common, accounting for 15% of cases. HNF4A mutations have only been reported previously in five probands with diazoxide responsive neonatal hypoglycemia (14, 16), but we found HNF4A mutations in a further nine cases, making this the third most common genetic etiology within the cohort and the second most common cause of isolated diazoxide responsive HH. A further four novel heterozygous HNF4A variants (one intronic and three non-synonymous amino acid substitutions) were identified, but are thought unlikely to be pathogenic mutations.

HNF4A mutations were associated with an early age of diagnosis (median 1 day) and increased birth weight (median birth weight $+2.4$ S.D.S with macrosomia in 9/11) which is likely to result from increased insulin secretion in utero. Seven of the eleven probands (64%) did not have a diabetic parent, and in four cases, a de novo mutation was confirmed. Therefore, the absence of a family history of diabetes should not preclude sequencing of the HNF4A gene in patients presenting with diazoxide responsive HH.

Neonatal hypoglycemia has been reported in only a minority of patients (11%) with HNF4A mutations who were ascertained by their family history of MODY (14, 15), and none of the ten heterozygous relatives in our study were known to have had neonatal hypoglycemia. The reason(s) for the incomplete penetrance of symptomatic hypoglycemia are not known, although it appears to be a general feature rather than mutation specific. It is also possible that some patients had unrecognized hypoglycemia in the neonatal period. The hyperinsulinemic HNF4A phenotype ranges from increased birth weight (macrosomia in ~50% mutation carriers) to neonatal hypoglycemia managed by i.v. glucose only for 1–9 days (14, 15), or neonatal hypoglycemia requiring diazoxide therapy for between 3 months and 8 years (14, 16). It is therefore likely that other environmental and genetic factors are influencing the severity of the hyperinsulinemic phenotype associated with HNF4A mutations. The mechanism underlying the biphasic phenotype of neonatal hypoglycemia with later diabetes is not known. It has been speculated to result from differences in HNF-4α-dependent temporal gene expression, or early hypersecretion of insulin resulting in later β-cell exhaustion (14).

Patients with an HNF4A mutation were diagnosed with HH within the first week of life (data available for 10/11 patients). The overlap in age at diagnosis and birth weight between patients with HNF4A and $K_{ATP}$ channel mutations means that it is not possible to distinguish between these two etiologies on an individual patient basis. Although diagnosis in the first week of life and a family history of young-onset diabetes suggest a HNF4A mutation, given the higher prevalence of $K_{ATP}$
channel mutations and the high rate of diabetes phenocopies in the population, we recommend sequencing KCNJ11 and ABCC8 first, followed by HNF4A.

The frequency of HNF4A mutations approached that of GLUD1 mutations (5 vs 5.9%). Patients with a GLUD1 mutation were diagnosed later (median 24 weeks), were of normal birth weight, and most (12/13) had HA. However, the recent description of a patient with a GLUD1 mutation, extreme protein sensitivity but normal serum ammonium suggests that this prevalence could be an underestimate (8). A genetic diagnosis was only possible for 27% of patients in this study, suggesting that there are more gene(s) harboring causative mutations which remain to be identified in patients with HH.

In conclusion, we have shown that HNF4A mutations are a relatively common cause of diazoxide responsive HH diagnosed in the first week of life. A genetic diagnosis is important for these patients as it predicts the likelihood of later sulfonylurea-sensitive diabetes and a high risk of having macrosomic babies. We therefore propose that HNF4A should be sequenced in all the patients without a K<sub>ATP</sub> channel mutation who present with diazoxide–responsive HH in the first week of life irrespective of a family history of diabetes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-09-0861.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References
1 Kapoor RR, Flanagan SE, James C, Shield J, Ellard S & Hussain K. Hypoglycaemia hypoglycaemia. *Archives of Disease in Childhood* 2009 94 450–457.
2 Thomas PM, Cote GJ, Wohlhk N, Haddad B, Mathew PM, Rabl W, Aguilar-Bryan L, Gagel R & Bryan J. Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycaemia of infancy. *Science* 1995 268 426–429.
3 Thomas P, Ye Y & Lightner E. Mutation of the pancreatic islet inward rectifier Kir6.2 also leads to familial persistent hyperinsulinemic hypoglycaemia of infancy. *Human Molecular Genetics* 1996 5 1809–1812.
4 Taschenberger G, Mougey A, Shen S, Lester LB, LaFranchi S & Shyng SL. Identification of a familial hyperinsulinism-causing mutation in the sulfonylurea receptor 1 that prevents normal trafficking and function of K<sub>ATP</sub> channels. *Journal of Biological Chemistry* 2002 277 17139–17146.
5 Ashcroft FM. ATP-sensitive potassium channelopathies: focus on insulin secretion. *Journal of Clinical Investigation* 2005 115 2047–2058.
6 Henwood MJ, Kelly A, Macmullen C, Bhatia P, Ganguly A, Thornton PS & Stanley CA. Genotype–phenotype correlations in children with congenital hyperinsulinism due to recessive mutations of the adenosine triphosphate-sensitive potassium channel genes. *Journal of Clinical Endocrinology and Metabolism* 2005 90 789–794.
7 Stanley CA, Lieu YK, Hsu BY, Burulina AB, Greenberg CR, Hopwood NJ, Perlman K, Rich BH, Zammarchi E & Foncza M. Hyperinsulinism and hyperammonemia in infants with regulatory mutations of the glutamate dehydrogenase gene. *New England Journal of Medicine* 1998 338 1352–1357.
8 Kapoor RR, Flanagan SE, Fullon P, Chakrapani A, Chadeaux B, Ben-Cohen T, Shield J, Ellard S & Hussain K. Hyperinsulinism–hyperammonaemia (HH/HA) syndrome: novel mutations in the GLUD1 gene and genotype–phenotype correlations. *European Journal of Endocrinology* 2009 161 731–735.
9 Glaser B, Kesavan P, Heyman M, Davis E, Cuesta A, Buchs A, Stanley CA, Thornton PS, Permutt MA, Matschinsky FM & Herald KC. Familial hyperinsulinism caused by an activating glucokinase mutation. *New England Journal of Medicine* 1997 338 226–230.
10 Clayton PT, Eaton S, Aynsley-Green A, Edginton M, Hussain K, Kryxawycz S, Datta V, Malingre HE, Berger R & van den Berg IE. Hyperinsulinism in short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of beta-oxidation in insulin secretion. *Journal of Clinical Investigation* 2001 108 457–465.
11 Sayed S, Langdon DR, Odili S, Chen P, Buettger C, Schiffman AB, Suchi M, Taub R, Grimsby J, Matschinsky FM & Stanley CA. Extremes of clinical and enzymatic phenotypes in children with hyperinsulinism due to glucokinase activating mutations. *Diabetes* 2009 58 1419–1427.
12 Molven A, Matre GE, Duran M, Wanders RJ, Rishaug U, Njolstad PR, Jellum E & Sovik O. Familial hyperinsulinemic hypoglycaemia caused by a defect in the SCHAD enzyme of mitochondrial fatty acid oxidation. *Diabetes* 2004 53 221–227.
13 Kapoor RR, James C, Flanagan SE, Ellard S, Hussain K & Enton S. 3-Hydroxyacyl-coenzyme A dehydrogenase deficiency and hyperinsulinemic hypoglycaemia: characterization of a novel mutation and severe dietary protein sensitivity. *Journal of Clinical Endocrinology and Metabolism* 2009 94 2221–2225.
14 Pearson ER, Boj SF, Steele AM, Barrett T, Stals K, Shield JP, Ellard S, Ferrero J & Hattersley AT. Macrosomia and hyperinsulinemic hypoglycaemia in patients with heterozygous mutations in the HNF4A gene. *PLoS Medicine* 2007 4 e118.
15 Fajans SS & Bell GI. Macrosomia and neonatal hypoglycaemia in RW pedigree subjects with a mutation (Q268X) in the gene encoding hepatocyte nuclear factor 4alpha (HNF4A). *Diabetologia* 2007 50 2600–2601.
16 Kapoor RR, Locke J, Colclough K, Wales J, Conn JJ, Hattersley AT, Ellard S & Hussain K. Persistent hyperinsulinemic hypoglycaemia and maturity-onset diabetes of the young due to heterozygous HNF4A mutations. *Diabetes* 2008 57 1659–1663.
17 Ellard S & Colclough K. Mutations in the genes encoding the transcription factors hepatocyte nuclear factor 1 alpha (HNF1A) and 4 alpha (HNF4A) in maturity-onset diabetes of the young. Human Mutation 2006 27 854–869.

18 Pearson ER, Pruhova S, Tack CJ, Johansen A, Castleden HA, Lumb PJ, Wierzbicki AS, Clark PM, Lebl J, Pedersen O, Ellard S, Hansen T & Hattersley AT. Molecular genetics and phenotypic characteristics of MODY caused by hepatocyte nuclear factor 4alpha mutations in a large European collection. Diabetologia 2005 48 878–885.

19 Freeman JV, Cole TJ, Chinn S, Jones PR, White EM & Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. Archives of Disease in Childhood 1995 73 17–24.