CLINICAL STUDY

Diazoxide-responsive hyperinsulinemic hypoglycemia caused by *HNF4A* gene mutations

S E Flanagan[1], R R Kapoor[2,3], G Mali[1], D Cody[4], N Murphy[5], B Schwahn[6], T Siahanidou[7], I Banerjee[8], T Akcay[9], O Rubio-Cabezas[1,10], J P H Shield[11], K Hussain[2,3] and S Ellard[1]

[1]Peninsula Medical School, Institute of Biomedical and Clinical Science, University of Exeter, Barrack Road, Exeter EX2 5DW, UK, [2]London Centre for Paediatric Endocrinology and Metabolism, Great Ormond Street Hospital for Children NHS Trust, London WC1N 3JH, UK, [3]Institute of Child Health, University College London WC1N 1EH, UK, [4]Department of Endocrinology, Our Lady's Childrens Hospital, Dublin, 12 Ireland, [5]Children's University Hospital, Dublin, 1 Ireland, [6]Department of Metabolic Medicine, Royal Hospital for Sick Children, NHS Greater Glasgow and Clyde, Glasgow, UK, [7]Department of Pediatrics, Aghia Sophia Children's Hospital, University of Athens, Athens, 115 Greece, [8]Department of Endocrinology, Royal Manchester Children's Hospital, Central Manchester and Manchester Children's University Hospitals NHS Trust, Manchester, M13 9WL, UK, [9]Department of Endocrinology, Bakirkoy Maternity and Child Hospital, Istanbul, 34142 Turkey, [10]Department of Endocrinology, Hospital Infantil Universitario Niño Jesus, Madrid 28009, Spain and [11]Department of Child Health, Bristol Royal Hospital for Children, Bristol BS2 8BJ, UK

(Correspondence should be addressed to S Ellard; Email: sian.ellard@rdeft.nhs.uk)

Abstract

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 HI 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.

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Introduction

Hyperinsulinemic hypoglycemia (HH) is characterized by unregulated secretion of insulin, despite hypoglycemia. HH most commonly presents in the neonatal period with the clinical presentation ranging from mild, moderate to severe medically unresponsive hypoglycemia (1). Diazoxide is the first line of treatment for patients with HH. In the pancreatic β-cell, glucose metabolism is coupled to insulin secretion by the adenosine triphosphate-sensitive potassium channels (ATP-sensitive K_{ATP} channels). Glucose metabolism results in an increase in the concentration of intracellular ATP, which binds to the Kir6.2 subunits to effect channel closure. This results in membrane depolarization and ultimately insulin secretion. Diazoxide binds to the SUR1 regulatory subunits and acts by keeping the K_{ATP} channels open, thereby preventing insulin secretion.

Loss-of-function mutations in the *ABCC8* and *KCNJ11* genes encoding the SUR1 and Kir6.2 subunits of the K_{ATP} channel lead to either a reduction in the number of channels within the β-cell membrane or a decrease in channel activity (2–5), and hence diazoxide treatment is often ineffective. Some of these patients may be managed with octreotide, but many will require a partial or near total pancreatectomy. A minority of cases have mutations with residual function that correlate with response to diazoxide (6).

Diazoxide responsive HH can also result from activating mutations in the *GLUD1* gene which encodes

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 sulfonylureas (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 ≥ 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$_{ATP}$ channel positive	*GLUD1* mutation positive	*GCK* mutation positive	Unknown etiology
Number of patients	220	11	33	13	2	161
Age at diagnosis	1 week (1 day–24 weeks)	1 day (1–2 days)	4 days (1 day–4 weeks)	24 weeks (5 days–30 weeks)	9 years (3–15 years)	1 week (1 day–26 weeks)
Birth weight (SDS)	+0.17 (−1.1±1.28)	+2.4 (+1.4±3.8)	+1.27 (−0.03±2.94)	−0.29 (−1.08±1.13)	+0.74 (−0.88±2.4)	−0.19 (−1.2±0.97)

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_{ATP} 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 (Sian 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.

HNF4A variants of uncertain significance

Four novel heterozygous *HNF4A* variants were identified in a further four probands: c.621 + 4A > G (IVS5nt + 4A > G), V94M (c.280G > A), S371R (c.1113C > A), and H378del (c.1133_1135delACC) (Supplementary Table 1). In one patient, the c.621 + 4A > G variant was inherited from their unaffected father (current age 33 years), *in silico* splicing prediction software suggested no effect on splicing (http://www.fruitfly.org), and it was present in 2/76 ethnically matched (Bangladeshi) control chromosomes. Family member testing for the three remaining patients demonstrated inheritance of the variant from an unaffected grandparent (aged 71, 50, and 52 years respectively) and parent. Normal blood glucose levels and HbAlc were confirmed in two of these grandparents (S371R and H378del; V94M not tested). The penetrance of *HNF4A* mutations causing diabetes is estimated at 74% by the age of 50 years (Sarah Flanagan, Sian Ellard,
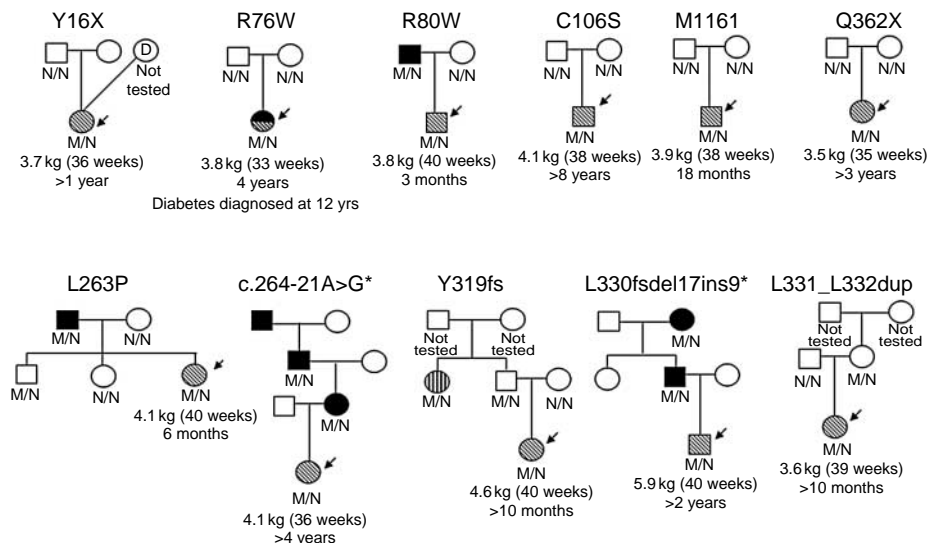

Figure 1 Partial pedigrees showing inheritance of *HNF4A* mutations in the 11 families. Circles represent females, and squares indicate males. A circle with the letter D denotes an ovum donor. Probands are indicated by an arrow. Diagonal hatching denotes patients with hyperinsulinism, vertical hatching 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).

& 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 ≥ 1.3 s.D.s from the mean. The median birth weight for the 11 cases was $+2.4$ SDS (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 $+2.4$ SDS 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 $\sim 50\%$ 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$_{ATP}$ channel mutation who present with diazoxide–responsive HH in the first week of life irrespective of a family history of diabetes.

Supplementary data

Declaration of interest

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