CASE REPORT

Novel *GLIS3* mutations demonstrate an extended multisystem phenotype

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Abstract

Introduction: Mutations in the GLI-similar 3 (*GLIS3*) gene encoding the transcription factor GLIS3 are a rare cause of neonatal diabetes and congenital hypothyroidism with six affected cases from three families reported to date. Additional features, described previously, include congenital glaucoma, hepatic fibrosis, polycystic kidneys, developmental delay and facial dysmorphism.

Subjects: We report two new cases from unrelated families with distinct novel homozygous partial *GLIS3* deletions. Both patients presented with neonatal diabetes mellitus, severe resistant hypothyroidism in the presence of elevated thyroglobulin and normal thyroid anatomy, degenerative liver disease, cystic renal dysplasia, recurrent infections and facial dysmorphism. These novel mutations have also resulted in osteopenia, bilateral sensorineural deafness and pancreatic exocrine insufficiency, features that have not previously been associated with *GLIS3* mutations. Gene dosage analysis showed that the parents were carriers of a deletion encompassing exons 1–2 (case 1) or exons 1–4 (case 2) of the 11 exon gene. Genome-wide SNP analysis did not reveal a common ancestral *GLIS3* haplotype in patient 2.

Conclusions: Our results confirm partial gene deletions as the most common type of *GLIS3* mutations, accounting for four of five families identified to date. We propose that mutations in *GLIS3* lead to a wider clinical phenotype than previously recognised. We also report the first case of a recessive *GLIS3* mutation causing neonatal diabetes and congenital hypothyroidism in a child from a non-consanguineous pedigree, highlighting the importance of molecular genetic testing in any patient with this phenotype.

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Introduction

Mutations in *GLIS3* (9p23–24.3, OMIM#610192) have been described in the literature as a rare cause of neonatal diabetes. GLI-similar 3 (*GLIS3*) is a recently identified transcription factor containing five Krüppel-like zinc finger motifs (1). *GLIS3* expression occurs early in embryogenesis and is thought to play a critical role in the cellular regulation of development by functioning as a repressor or activator of transcription (1, 2). The first patients presented with a frameshift mutation in *GLIS3* resulting in a truncated non-functional protein leading to intrauterine growth retardation, neonatal diabetes and hypothyroidism, progressive hepatic fibrosis, renal cystic dysplasia, facial dysmorphism and congenital glaucoma. These patients died from infection in infancy, but recently, patients with deletions within the *GLIS3* gene have presented with a milder phenotype consisting

of diabetes and hypothyroidism in the neonatal period, but with no hepatic or renal involvement. All children to date with *GLIS3* mutations were born to consanguineous parents (3, 4).

The variation in the *GLIS3* phenotype is attributed to the tissue expression of variable length transcripts derived from the 11 exon *GLIS3* gene. Anomalies in pancreatic *GLIS3* expression in animals and humans have resulted in isolated endocrine dysfunction with relative sparing of the exocrine pancreas (3). *In vitro* studies suggest that *GLIS3* may regulate osteoblast differentiation although skeletal abnormalities have not previously manifested in patients (5). We describe two new cases in which deletions in *GLIS3* have resulted in severely affected patients with an extended multisystem phenotype including exocrine pancreatic dysfunction and skeletal abnormalities that have not been previously described.

Case 1

Patient 1 was born to first cousins of Bangladeshi origin, after 35 weeks of gestation. She was proportionately growth retarded, weighing 1.17 kg (-3.6 SDS) with a head circumference of 27.5 cm (-3.2 SDS). Neonatal diabetes was diagnosed with blood glucose of 27 mmol/l on day 3, with normal electrolytes and haemoglobin. The patient was started on i.v. insulin with continuous feeds although her blood glucose remained labile. An s.c. insulin infusion was subsequently commenced with insulin requirements ranging from 0.5 to 0.7 units/kg per day over a 1-year period. Blood glucose has remained difficult to control despite strict adherence to her feed regime. During times of intercurrent illness marked hyperglycaemia and insulin resistance has occurred, needing three to four times normal insulin requirements to normalise blood glucose. Despite difficult blood glucose control, her glycosylated haemoglobin at 1 year of age was 7.8% (62.0 mmol/mol).

On day 4 of life, hypothyroidism was identified (initial serum TSH concentration was >150 (normal range 0.30–5.88) mIU/l and free thyroxine (T_4) 4.3 (10.3–21.9) pmol/l). She was initially managed on 20 $\mu\text{g/kg}$ per day of oral T_4 with adequate TSH suppression. At 2 months the TSH level exceeded 150 mIU/l. Despite using 75 $\mu\text{g/kg}$ per day of T_4 , TSH remained over 150 mIU/l. Subsequent thyroid function and treatment is summarised in Fig. 1. A trial of i.v. liothyronine was commenced at 2–3 $\mu\text{g/kg}$ per day on day 87 to exclude possible T_4 malabsorption. Despite this, hyperthyrotropinaemia persisted. A reduction in TSH was observed when oral T_4 was divided into three to four doses. No overt clinical signs of hypothyroidism were observed during the administration of liothyronine and oral T_4 .

Despite normalising serum T_4 , serum TSH remained at 50–100 mIU/l. Thyroid gland anatomy and echotexture appeared normal on ultrasound scan. At 99 days of age, the thyroglobulin level was 170 (16.0–24.0) $\mu\text{g/l}$, TSH was >150 mIU/l, free T_4 was 3.7 pmol/l and free tri-iodothyronine (T_3) was 11.2 pmol/l. T_4 uptake was assessed at 6 months of age when the patient had a free T_4 of 25.0 pmol/l and a thyroglobulin of 23.4 $\mu\text{g/l}$. T_4 uptake was 44.10 (26.8–39.4 IU) with a total T_4 index of 214 (62–141) nmol/l and a total T_3 index of 3.1 (1.2–2.8) nmol/l. The elevated T_4 uptake suggested fewer unoccupied thyroglobulin-binding sites consistent with the high free T_4 level.

The patient also developed neonatal hepatitis evidenced by markedly elevated gamma glutamyl transferase (GGT – 630 IU/l (11–149)), aspartate transaminase (AST – 380 IU/l (18–92)) and alanine transaminase (ALT – 75 IU/l (0–38)), which has subsequently progressed to cirrhosis with portal hypertension (reversed portal vein flow and portal varices on hepatic ultrasound with Doppler) and associated oesophageal varices. She also developed a pancreatic exocrine

Figure 1 Thyroid function tests in patient 1 commencing from day 80 to day 329. (A) Oral thyroxine at 75 $\mu\text{g/kg}$ once daily. (B) i.v. liothyronine (T_3) commenced at 2–3 $\mu\text{g/kg}$ per day on day 87 to exclude possible thyroxine malabsorption. (C) Oral thyroxine at 30 $\mu\text{g/kg}$ per day divided into four doses on day 114. (D) Oral thyroxine divided into three doses commenced on day 127 at 15 $\mu\text{g/kg}$ per day and increased to 25 $\mu\text{g/kg}$ per day. (E) 37.5 μg thyroxine three times daily (27 $\mu\text{g/kg}$ per day) on day 345.

deficiency (identified by a low faecal elastase at 6 $\mu\text{g/g}$ (200–500)) requiring enzyme supplementation. Renal ultrasonography confirmed renal cystic dysplasia although renal function has remained normal. She has had a number of intercurrent sinopulmonary and urinary tract infections that have responded appropriately to i.v. antibiotics. However, serum immunoglobulins were within normal range (IgG 3.79 (2.4–8.8) g/l, IgA 0.50 (0.1–0.5) g/l and IgM 1.12 (0.2–1.0) g/l).

On day 101, osteopenia with thoracolumbar lordosis and multiple left-sided healing rib fractures were identified (Fig. 2a). No radiological evidence of metaphyseal bone abnormality was identified. Parathyroid hormone (PTH) level was 62.6 ng/l (11–35) with a 25-hydroxyvitamin D of 34.6 (50–90) nmol/l. Serum calcium, phosphate and bone alkaline phosphatase concentrations measured 2.56 (2.13–2.72), 2.27 (1.10–2.40) and 297.3 (9–28) mmol/l respectively. Despite treatment with ergocalciferol and calcium, and normalisation of the PTH and vitamin D level, the patient sustained a further rib fracture on the left side. Calcium supplementation continued at 1 mmol/kg daily in four divided doses combined with ergocalciferol at 3000 units daily to maintain vitamin D levels. At 1 year of age, the callus formed around the previously sustained rib fractures has remained and her ribs appear gracile on X-ray with thin long bone cortices (Fig. 2b and c).

Case 2

Patient 2, a male, was born with a weight of 1.43 kg and head circumference of 30.1 cm after 35 weeks of gestation, to non-consanguineous parents of Welsh origin. Oligohydramnios was noted at 29 weeks of gestation. He developed diabetes on day 4, requiring continuous i.v. insulin (0.8 IU/kg per day) and was subsequently treated with a continuous s.c. insulin infusion. His current daily dose of insulin is 0.6 IU/kg per day. Glycosylated haemoglobin was difficult to interpret due to persistently high levels of fetal haemoglobin. Target blood glucoses have been difficult to achieve and as with patient 1, severe insulin resistance has been noted during periods of illness.

Initial serum TSH concentration was 898 mIU/l and serum-free T_4 was 2.7 pmol/l with thyroglobulin concentrations > 500 µg/l. Maternal thyroid function was normal. As with patient 1, suppression of TSH proved difficult despite consistently normal free T_4 measurements on 14 µg/kg per day T_4 . Serum TSH concentration remains at 40–60 mIU/l despite normal free T_4 on 15 µg/kg per day T_4 . Thyroid anatomy was normal on ultrasound scan.

As with patient 1, patient 2 presented with early hepatitis (serum GGT on day 44 was 429 mmol/l, conjugated bilirubin fraction = 42%). Abdominal ultrasound scan performed at 2 months demonstrated a cyst adjacent to the porta hepatis. Subsequent liver biopsy suggested moderate parenchymal cholestasis. Despite low levels of faecal elastase in the neonatal period (< 15 µg/g) he initially thrived. However, at 3 months of age he required pancreatic enzyme supplementation due to exocrine dysfunction. At 1 month of age, he was noted to have a patent ductus arteriosus with volume overloading of the left ventricle, which has since resolved. He also had bilateral sensorineural deafness requiring hearing aids, bilateral renal cystic dysplasia and cystic change in the head of the pancreas (largest cyst is 2.4 mm). Ophthalmic exam and serial cranial ultrasound scans were consistently normal.

Genetic analysis

Consent was obtained from both families to perform familial genetic analysis. *GLIS3* gene mutations were sought by PCR amplification (primer sequences available on request) and sequence analysis of exons 1–11 by comparison with the reference sequence NM_001042413. Exon 1 is non-coding (the 5' UTR) and the start codon is located within exon 2. Failure of PCR amplification for exons 1–2 (patient 1) and exons 1–4 (patient 2) suggested the possibility of a homozygous partial gene deletion. No mutations were identified by sequence analysis of the remainder of the gene. Parental samples were investigated by real-time quantitative PCR on an ABI 7900 (TaqMan assay with

Figure 2 (a) Anteroposterior chest X-ray of patient 1 performed on day 101, demonstrating thoracolumbar scoliosis and left rib fractures located on ribs 7, 8 and 9. (b and c) Anteroposterior chest X-rays performed on days 250 and 412, demonstrating worsening thoracolumbar scoliosis and persistence of callus formation in left ribs 7, 8 and 9 with a new fracture at left rib 6.

SYBR Green detection) and the copy number of exons 1–11 was determined by the $2^{-\Delta\Delta C_t}$ method (6). Dosage analysis was consistent with a heterozygous partial gene deletion encompassing exons 1–2 (c.-?_388 + ?del) in the parents of patient 1 and exons 1–4 (c.-?_1710 + ?del) in the parents of patient 2. The deletion mutations were further investigated by genome-wide SNP analysis (Affymetrix Genome-Wide Human SNP Array 6.0) of the patients' DNA samples. This revealed a minimal deleted region of 412 kb in patient 1 (chr9: 4 182 610–4 594 192) and 482 kb in patient 2 (chr9: 4 092 663–4 575 167). These deletions are located within a homozygous region extending 27.63 Mb in patient 1 whose parents are first cousins and 3.48 Mb in patient 2 whose parents were not known to be related (Fig. 3). These results suggest that in both families the partial *GLIS3* gene deletions originated from a common ancestor. The smaller size of the homozygous region in patient 2 is consistent with this child's parents being more distantly related. The deleted regions in patients 1 and 2 encompass the *SLC1A1* gene and result in loss of translation of the *SLC1A1* protein. In humans, the deletion of the *SLC1A1* gene does not result in any detectable pathological consequences. Patients who are null for *GLIS3* and *SLC1A1* do not have any additional clinical features beyond those who carry a null *GLIS3* and an intact *SLC1A1* gene (3).

Discussion

We have described two new unrelated patients with neonatal diabetes due to deletions in *GLIS3* at exons 1–4 and 1–2 with novel phenotypic features. These include osteopenia with skeletal deformity and fractures,

Figure 3 Schematic representation of the chromosome 9p region defined using NCBI36/HG18 co-ordinates to show the minimal homozygous and deleted regions in the two patients. These homozygous and deleted regions were defined using the Affymetrix Genome-Wide Human SNP Array 6.0. The minimal deleted region in patient 1 is defined by rs12237673–CN_1327065. The minimal deleted region in family 2 is defined by CN_1320225–CN_1327052. Hashed boxes represent minimal regions of homozygosity, grey boxes correspond to the minimal deleted regions and black boxes represent the NCBI RefSeq genes.

bilateral sensorineural deafness and exocrine pancreatic dysfunction, together with the previously described neonatal diabetes and hypothyroidism, renal cystic dysplasia, progressive hepatic fibrosis, intrauterine growth retardation, developmental delay and characteristic facial dysmorphism but with absent ocular involvement. This suggests that mutations in *GLIS3* result in a phenotype that includes more features than previously described. Phenotypic features of these patients and previously described cases of *GLIS3* mutations are provided in Table 1. In both cases we have described hypothyroidism that has not responded to conventional treatment. However, we have been unable to identify abnormalities in T_4 uptake or thyroid anatomy that may help to explain this.

The original two patients described by Taha *et al.* (4) had high daily T_4 requirements with persistently elevated TSH levels despite normalisation of free T_4 following treatment. Similarly, the second patient described by Taha *et al.* (4) had markedly raised thyroglobulin levels (1016 $\mu\text{g/l}$ – normal up to 55 $\mu\text{g/l}$) despite normal thyroid anatomy and location, similar to our first patient. Three more patients with *GLIS3* mutations described by Senée *et al.* (3) had thyroid ultrasound scan results suggestive of athyreosis or glandular hypoplasia with no radioiodide uptake on scintigraphy. Moreover, these patients responded to conventional daily T_4 supplementation, although subsequent TSH and free T_4 levels on treatment were not reported (3). Although it is clear that all patients with a *GLIS3* mutation present with thyroid dysfunction, the absence of consistent pathological features in patients makes it difficult to ascertain a unifying causative mechanism. It is possible that the markedly elevated levels of TSH observed in our patients and those described previously, combined with the variation in thyroid anatomy are a result of partial to complete TSH resistance. However, this does not explain the variable reduction in TSH, on occasions, values within the normal range following initial T_4 supplementation, and the need thereafter to administer T_4 three times daily to normalise free T_4 in patient 1. *PAX8* and thyroid transcription factor1 (TTF1/NK2 homeobox-1: *NKX2-1*) are involved in thyroid cell differentiation and proliferation and subsequent expression of genes encoding for thyroglobulin, thyroid peroxidase, thyrotropin receptor (*TSHR*) genes and the sodium–iodide symporter (7–10). Mutations in *PAX8* and *NKX2-1* result in significant phenotypic variability, with patients presenting with hypothyroidism, normal serum T_4 with hyperthyrotropinaemia (compensated hypothyroidism) and euthyroidism due to variable gene penetrance and expression, with a thyroid size ranging from normal to severe hypoplasia and athyreosis (11–16). However, despite these phenotypic similarities there is no evidence for conserved GLI transcription-binding sites in the *PAX8*, *NKX2-1* or *TSHR* flanking gene sequences. Although high doses of T_4 may be required to overcome resistance to T_4 at a tissue level,

Table 1 Comparison of phenotypic features in all patients with *GLIS3* mutation described to date.

Family	Country of origin	<i>GLIS3</i> genotype	IUGR	ND	Thyroid phenotype	CG	LF	RCD	SA	EPI	Deafness	STI	Age at death
1	Saudi Arabia	Frameshift mutation 2067insC	+	+	Thyroid anatomy not documented but THR	+	+	+	—	—	—	+	16 months
1	Saudi Arabia	Frameshift mutation 2067insC	+	+	Thyroid anatomy normal but THR	+	+	+	—	—	—	+	6 months
1	Saudi Arabia	Frameshift mutation 2067insC	+	+	Not described	+	+	—	—	—	—	+	10 days
2	Saudi Arabia	426 kb deletion Chr9: 4 176 077–4 601 776	+	+	Athyreosis	+	—	—	—	—	—	—	Alive
3	France	149 kb deletion Chr9: 4 249 915–4 398 572	+	+	Athyreosis	—	—	—	—	—	—	—	Alive
3	France	149 kb deletion Chr9: 4 249 915–4 398 572	+	+	Thyroid hypoplasia/non-functional tissue	—	—	—	—	—	—	—	Alive
4	Bangladesh	412 kb deletion Chr9: 4 182 360–4 594 467	+	+	Thyroid anatomy normal but THR	—	+	+	+	+	—	+	Alive
5	UK (Welsh)	482 kb deletion Chr9: 4 092 413–4 575 442	+	+	Thyroid anatomy normal but THR	—	+	+	—	+	+	—	Alive

THR, thyroid hormone resistance; ND, neonatal diabetes; CG, congenital glaucoma; LF, liver fibrosis; RCD, renal cystic dysplasia; SA, skeletal abnormalities; EPI, exocrine pancreatic insufficiency; STI, susceptibility to infection.

and may explain the difficulties in the reduction of TSH at the level of the pituitary, the normalisation of TSH after three to four times daily T₄ in case 1 is difficult to explain given T₄ has a long half-life.

Abnormalities in osteoblast differentiation in humans could potentially result in under mineralised bone that is prone to deformity and fracture as observed in patient 1. Recent evidence suggests a role of *GLIS3* in osteoblast differentiation by the upregulation of FGF18 (5). A reduction or absence in FGF18 results in delayed bone mineralisation secondary to diminished osteoblast terminal differentiation and proliferation (17). In addition, the persistence in callus formation suggests a defect in bone remodelling either due to dysfunctional osteoblast signalling to osteoclasts or due to possibly reduced osteoclastic bone resorption.

Previously, patients with aberrations in *GLIS3* have presented with pancreatic endocrine dysfunction with no change in the exocrine pancreas (3). Others have reported normal pancreatic exocrine cell formation in *Glis3*^{−/−} mice despite a significant reduction in islet cell secretion of insulin, glucagon and somatostatin (18). *GLIS3* transcripts are highly expressed in pancreatic β cells, but also to a lesser degree in pancreatic acini (3). As both patients had pancreatic exocrine dysfunction, we propose that in individuals with a severe *GLIS3* phenotype due to alternative gene deletions, progressive pancreatic exocrine dysfunction secondary to altered acini embryogenesis may occur in addition to β cell dysfunction. The neonatal diabetes observed in our patients may be explained by impaired embryonic islet cell development or abnormal gene transcription observed in mutant *Glis3* murine models (3, 18). We speculate that the significant insulin requirements observed during periods of illness suggests a possible role of *GLIS3* on end-organ response to insulin, possibly at the level of the insulin receptor.

Three mutations of *GLIS3* have been previously described resulting in neonatal diabetes and hypothyroidism syndrome in six patients (3). Phenotypic differences have resulted from different gene mutations with variations in tissue-specific *GLIS3* transcript expression between probands. The most severe clinical phenotype in a Saudi Arabian family kindred with consanguineous parents was caused by a single base insertion at nucleotide 2067 on *GLIS3* (c.2067insC) that was predicted to result in a truncated protein with an altered C-terminal proline-rich domain, abolishing the DNA-binding capacity of *GLIS3* to GLI response elements (GLI-REs) on the promoter region of target genes. Apart from the consistent presentation of neonatal diabetes and hypothyroidism, additional features included renal cystic dysplasia, likely to result from renal ciliary dysplasia early in renal embryogenesis (19, 20) and progressive hepatic fibrosis culminating in cirrhosis. All three children from this Saudi Arabian family died between 10 days and 16 months from infection. However, skeletal abnormalities, sensorineural

deafness and exocrine dysfunction were not described in this kindred.

As with patient 1, all previously described patients with mutations in *GLIS3* were born to consanguineous parents. Patient 2 is the first child with a *GLIS3* mutation born to apparently unrelated parents. We recognise, however, that the limited area of homozygosity (3.48 Mb) surrounding the 0.48 Mb deletion suggests that parents of patient 2 may be distantly related supporting a likely founder effect in this family.

Twenty-nine putative transcription start sites and five transcription termination sites have been identified (3). Transcripts contain five zinc finger domains with variations in N- and C-terminal regions, which are required for nuclear localisation and binding to GLI-binding sites on the promoter region of target genes (2). Larger 7.5 kb transcripts are predominantly expressed in the pancreas, kidney and thyroid, with smaller transcripts (0.8–2.0 kb) expressed in the heart, kidney, liver and skeletal muscle (3). In all cases of *GLIS3* mutations including ours, the presence of congenital hypothyroidism and neonatal diabetes is likely to result from the absence of the larger 7.5 kb *GLIS3* transcript, which is preferentially expressed in the pancreas and thyroid (3). A reduction in the larger (7.5 kb) and smaller (0.8–2.0 kb) *GLIS3* renal transcripts is likely to cause cystic renal dysplasia observed in our patients and those from the first family described with a mutation in *GLIS3*. The new clinical manifestations in our patients are possibly due to a reduction in further *GLIS3* tissue transcripts secondary to these new deletions. Nuclear localisation and subsequent DNA binding to GLI-REs are partly dependent on the integrity of all five zinc fingers of *GLIS3*. The zinc fingers are encoded on exons 2–4 (1). Given that our patients presented with deletions, in part or this entire region, we speculate that the severe and extended clinical phenotype we observed is the result of malconfiguration of the tetrahedral zinc fingers such that *GLIS3* transcript nuclear localisation and action are reduced, as observed in the original cases of *GLIS3* mutation.

In summary, we have described two patients with neonatal diabetes caused by novel deletions in *GLIS3*, whose clinical phenotype includes more features than previously described and whose hypothyroidism and diabetes have proved relatively resistant to conventional interventions. One of these babies is the first with a *GLIS3* mutation from a non-consanguineous pedigree. Our results combined with those from the other six previously described patients confirm partial gene deletions as the most common type of *GLIS3* mutations. The extension of the phenotype, we have described, suggests that infants with neonatal diabetes and a wider range of clinical manifestations than previously reported should be screened for mutations of the *GLIS3* gene. Furthermore, our findings provide novel insights into the potential roles of the *GLIS3* gene in normal human physiology. Both of our patients to

date are still alive suggesting that children with the severe clinical phenotype associated with mutations of *GLIS3* may have a longer life expectancy than originally described.

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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