Novel GLIS3 mutations demonstrate an extended multisystem phenotype


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Abstract

Introduction: Mutations in the GLIS3 gene encoding the transcription factor GLI-similar 3 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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Mutations in GLIS3 (9p23-24.3, OMIM#610192) have been described in the literature as a rare cause of neonatal diabetes. GLIS3 (GLI-similar 3) 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 GLIS 3 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, at 35 week’s 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 a blood glucose of 27 mmol/l on day 3, with normal electrolytes and haemoglobin. The patient was started on intravenous insulin with continuous feeds although her blood glucose remained labile. A subcutaneous insulin infusion was subsequently commenced with insulin requirements ranging from 0.5-0.7 units/kg/day over a one-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 3-4 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 T4 4.3 (10.3-21.9) pmol/l). She was initially managed on 20 mcg/kg/day of oral thyroxine with adequate TSH suppression. At 2 months the TSH level exceeded 150 mIU/l. Despite using 75mcg/kg/day of thyroxine, TSH remained over 150 mIU/l. Subsequent thyroid function and treatment is summarised in figure 1. A trial of intravenous liothyronine was commenced at 2-3 mcg/kg/day on day 87 to exclude possible thyroxine malabsorption. Despite this, hyperthyrotropinaemia persisted. A reduction in TSH was observed when oral thyroxine was divided into 3-4 doses. No overt clinical signs of hypothyroidism were observed during the administration of liothyronine and oral thyroxine.

Despite normalising serum T4, 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) µg/l, TSH was >150 mIU/l, freeT4 was 3.7 pmol/l and free T3 was 11.2 pmol/l. Thyroxine uptake was assessed at 6 months of age when the patient had a free T4 of 25.0 pmol/l and a thyroglobulin of 23.4 µg/l. Thyroxine uptake was 44.10 (26.8-39.4 IU) with a total T4 index of 214 (62-141) nmol/l and a total T3 index of 3.1 (1.2-2.8) nmol/l.

The elevated thyroxine uptake suggested fewer unoccupied thyroglobulin binding sites consistent with the high free T4 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)), that 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 deficiency (identified by a low faecal elastase at 6 µg/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 intravenous 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, 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 (figure 2a). No radiological evidence of metaphyseal bone abnormality was identified. Parathyroid hormone level was 62.6 (11-35) ng/l 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) mmol/l, 2.27 (1.10-2.40) mmol/l 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 1mmol/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 (figure 2b and 2c).
Case 2

Patient 2, a male, was born with a weight of 1.43 kg and head circumference of 30.1 cm at 35 weeks’ gestation, to non-consanguineous parents of Welsh origin. Oligohydramnios was noted at 29 weeks gestation. He developed diabetes on day 4, requiring continuous intravenous insulin (0.8 IU/kg/day) and was subsequently treated with a continuous subcutaneous insulin infusion. His current daily dose of insulin is 0.6 IU/kg/day. Glycosylated haemoglobin was difficult to interpret due to persistently high levels of foetal haemoglobin. Target blood gluoses 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 mU/l and serum free T4 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 T4 measurements on 14 µg/kg/day thyroxine. Serum TSH concentration remains at 40-60 mIU/l despite normal free T4 on 15 µg/kg/day thyroxine. 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 has 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 has bilateral sensorineural deafness requiring hearing aids, bilateral renal cystic dysplasia, cystic change in the head of the pancreas (largest cyst is 2.4 mm). Ophthalmic exam and serial cranial ultrasound scans were consistently normal.
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 SYBR Green detection) and the copy number of exons 1-11 determined by the $2^{-\Delta\Delta CT}$ 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) for 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 412kb in patient 1 (chr9:4182610 – 4594192) and 482 kb in patient 2 (chr9:4092663-4575167). 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 (figure 3). These results suggest that in both families the partial *GLIS3* gene deletions originate 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, 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 which 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 thyroxine uptake or thyroid anatomy that may help to explain this.

The original two patients described by Taha et al had high daily thyroxine requirements with persistently elevated TSH levels despite normalisation of free T4 following treatment. Similarly, the second patient described by Taha et al had markedly raised thyroglobulin levels (1016 micrograms/l – normal up to 55 micrograms/l) despite normal thyroid anatomy and location, similar to our first patient (4). Three further patients with *GLIS3* mutations described by Senée et al had thyroid ultrasound scan results suggestive of athyreosis or glandular hypoplasia with no radioiodide uptake on scintigraphy. Moreover, these patients responded to conventional daily thyroxine supplementation, although subsequent TSH and free T4 levels on treatment were not reported (3). Whilst it is clear that all patients with a GLIS3 mutation present with thyroid dysfunction, the absence of consistent pathological features between 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 with on occasions, values within the normal range following initial thyroxine supplementation, and the need thereafter to administer thyroxine three times daily to normalise free T4 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 (NIS) (7-10). Mutations in PAX8 and NKX2-1 result in significant phenotypic variability, with patients presenting with hypothyroidism, normal serum T4 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. Whilst high doses of thyroxine may be required to overcome resistance to thyroxine at a tissue level, and may explain the difficulties in the reduction of TSH at the level of the pituitary, the normalisation of TSH after 3-4 times daily thyroxine in case 1 is difficult to explain given thyroxine has a long half-life.

Abnormalities in osteoblast differentiation in humans could potentially result in undermineralised 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 mineralization 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 possible 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 \textit{Glis3}\textsuperscript{−/−} mice despite a significant reduction in islet cell secretion of insulin, glucagon and somatostatin (18). GLIS3 transcripts are highly expressed in pancreatic beta 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 beta 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 \textit{Glis3} murine models (3, 18). We speculate that the significant insulin requirements observed during periods of illness suggests a possible role of \textit{GLIS3} on end organ response to insulin, possibly at the level of the insulin receptor.

Three mutations of \textit{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 from a Saudi-Arabian kindred with consanguineous parents was caused by a single base insertion at nucleotide 2067 on \textit{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-RE’s) 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 \textit{GLIS3} were born to consanguineous parents. Patient 2 is the first child with a \textit{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 heart, kidney, liver and skeletal muscle (3). In all cases of \textit{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 \textit{GLIS3} transcript which is preferentially expressed in pancreas and thyroid (3). A reduction in the larger (7.5 kb) and smaller (0.8-2.0 kb) \textit{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 \textit{GLIS3}. The new clinical manifestations in our patients are possibly due to a reduction in further \textit{GLIS3} tissue transcripts secondary to these new deletions. Nuclear localisation and subsequent DNA binding to GLI-RE’s is partly dependent on integrity of all five zinc-fingers of \textit{GLIS3}. The zinc fingers are encoded for 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 mal-configuration of the tetrahedral zinc fingers such that \textit{GLIS3} transcript nuclear localisation and action is reduced, as observed in the original cases of \textit{GLIS3} mutation.

In summary, we have described two patients with neonatal diabetes caused by novel deletions in \textit{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 potential roles for 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.

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The authors of this manuscript declare that they have no conflict of interest

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**Figure 1:** Thyroid function tests in patient 1 commencing from day 80 to day 329

**A:** Oral thyroxine at 75mcg/kg once daily

**B:** Intravenous liothyronine (T3) commenced at 2-3 mcg/kg/day on day 87 to exclude possible thyroxine malabsorption.

**C:** Oral thyroxine at 30mcg/kg/day divided into four doses on day 114.

**D:** Oral thyroxine divided into 3 doses commenced on day 127 at 15 mcg/kg/day and increasing to 25 mc/kg/day.

**E:** 37.5 micrograms of thyroxine three times daily (27micrograms/kg/day) on day 345.

**Figure 2a:** 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.

**Figure 2b and 2c:** 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.

**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.
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<th>Thyroid phenotype</th>
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*THR = Thyroid hormone resistance