Novel *FOXA2* Mutation Causes Hyperinsulinism, Hypopitutarism with Craniofacial and Endoderm-Derived Organ Abnormalities

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Abstract

Congenital hypopituitarism(CH) is characterised by the deficiency of one or more pituitary hormones and can present alone or in association with complex disorders. Congenital hyperinsulinism(CHI) is a disorder of unregulated insulin secretion despite hypoglycemia that can occur in isolation or as part of a wider syndrome. Molecular diagnosis is unknown in many cases of CH and CHI. The underlying genetic etiology causing the complex phenotype of CH and CHI is unknown. In this study, we identified a *de novo* heterozygous mutation in the developmental transcription factor, forkhead box A2, *FOXA2* (c.505T>C,p.S169P) in a child with CHI and CH with craniofacial dysmorphic features, choroidal coloboma and endoderm-derived organ malformations in liver, lung and gastrointestinal tract by whole exome sequencing. The mutation is at a highly conserved residue within the DNA binding domain. We demonstrated strong expression of *Foxa2* mRNA in the developing hypothalamus, pituitary, pancreas, lungs and oesophagus of mouse embryos using *in situ* hybridization. Expression profiling on human embryos by immunohistochemistry showed strong expression of hFOXA2 in the neural tube, third ventricle, diencephalon and pancreas. Transient transfection of HEK293T cells with Wt(Wild type) hFOXA2 or mutant hFOXA2 showed an impairment in transcriptional reporter activity by the mutant hFOXA2. Further analyses using western blot assays showed that the *FOXA2* p.(S169P) variant is pathogenic resulting in lower expression levels when compared with Wt hFOXA2. Our results show, for the first time, the causative role of *FOXA2* in a complex congenital syndrome with hypopituitarism, hyperinsulinism and endoderm-derived organ abnormalities.

Introduction

The pituitary gland is a master regulator of vital physiological functions such as growth, puberty, lactation, metabolism, stress response and reproduction. The development of the pituitary gland is tightly controlled by signaling molecules and transcription factors that dictate pituitary cell lineage specification, cell proliferation and terminal differentiation into hormone-producing cells (1, 2). Abnormal pituitary development can lead to congenital hypopituitarism (CH) resulting in deficiency in one of more pituitary hormones. CH comprises of a spectrum of disorders with variable phenotypes that can range in severity, from isolated hormone deficiency [isolated growth hormone deficiency being the most common] to combined pituitary hormone deficiency (CPHD) when two or more pituitary hormones are deficient. CH may present as part of a syndrome with abnormalities in structures that share a common embryological origin with the pituitary gland, such as the forebrain and eyes, leading to septo-optic dysplasia (SOD) or holoprosencephaly (HPE) (1). SOD is a rare condition with a prevalence of 1:10,000(3) live births and comprises the following features: optic nerve hypoplasia, midline forebrain defects and hypopituitarism (4, 5). Mutations in transcription factors such as *HESX1* (6), *PROP1* (7), *POU1F1* (8), *LHX3* (9), *LHX4* (10), *PITX1, PITX2* (11), *OTX2* (12), *SOX2* (13) and *SOX3* (14, 15) have been associated with CH in mouse and humans. However, these mutations account only for a small proportion of CH patients with the majority of patients having an unknown genetic cause for their symptoms.

Congenital hyperinsulinism (CHI) is a rare condition with an estimated prevalence of 1 in 50,000 live births, characterized by an inappropriate secretion of insulin from the β-cells of the pancreas during hypoglycemia (16). CHI is the most common cause of severe and persistent hypoglycemia in the neonatal period. The identification and appropriate management of this condition is very important to avoid hypoglycemic episodes and prevent

the consequent neurological impairment. Mutations in genes *ABCC8* (17-21), *KCNJ11* (17-21), *GLUD1* (22), *GCK* (23), *HADH* (24), *UCP2* (25), *HNF4A* (26), *HNF1A* (26), *MCT1* (27), *HK1* (28) and *PGM1* (29) have been associated with genetic forms of CHI (30). However, the genetic cause for many CHI patients remains elusive.

FOXA2 (formerly hepatocyte nuclear factor-3β, *HNF-3β*) belongs to the family of the forkhead class of transcription factors that has an essential role in embryogenesis during the formation of the node, notochord and floorplate (31, 32) which are important for the development of the vertebrate body axis. Thus, *Foxa2* null embryos die during early gestation, at embryonic day 9.5, and fail to form axial mesoderm (31). Later during organogenesis, *Foxa2* co-operates with *Foxa1* and are required for the formation of endoderm-derived organs such as the liver (33), lung (34), pancreas (35) and gastrointestinal tract(36). *Foxa2* has been shown to be important in the development of anterior forebrain structures, which have the same embryonic origin as the pituitary gland(37). Data from murine genetic studies have shown a genetic interaction between *Foxa2* and Sonic Hedgehog (*Shh*) signaling pathway with overlapping expression pattern of *Foxa2* and *Shh* in the notochord and floor plate at E8.5. *Foxa2* can modulate *Shh* signalling, contributing to the specification of ventral motor neuron progenitor identity(38). The secretion of Shh by the notochord and floor plate is an important morphogenetic signal that is required for the development of central nervous system including the pituitary gland(39). In the islet cells of mature pancreas, *Foxa2* has been shown to activate components of insulin secretion, such as sulfonylurea receptor1 [SUR1], encoded by *ABCC8* (40) and the inward rectifier potassium channel member 6.2 [Kir 6.2], encoded by *KCNJ11*(40). In humans, mutations in *ABCC8* and *KCNJ11* (17-21) are the most common causes of genetic forms of CHI. Notably, tissue-specific deletion of *Foxa2* from the pancreatic β-cells ($Foxa2^{loxp/loxp}$; Ins:Cre) has been shown to the development of CHI in mouse(41).

Thus, *FOXA2* is an important developmental transcription factor required for the formation of ventral midline structures such as the floorplate and forebrain, as well as endoderm-derived organs including the pancreas, and regulating glucose homeostasis in mature pancreatic β-cells. To date, mutations in *FOXA2* causing disorders of glucose homeostasis, endoderm abnormalities and CH have not been described in humans. In this study, we report for the first time, a "*de novo*" heterozygous mutation in *FOXA2* in a patient with unique clinical phenotype of CH, persistent CHI, craniofacial dysmorphism, abnormalities in the liver, heart, lung and the gastrointestinal tract. This finding brings a new insight into the underlying molecular cause of a complex clinical phenotype that will serve as a tool to elucidate the pathogenesis of these congenital malformations and to better understand the development and function of the pituitary gland and the pancreas.

Results

Clinical findings

Our patient, a 5-year-old girl, was born to non-consanguineous Caucasian British parents at 42 weeks' gestation with a birth weight of 4.185 Kg (+1.72 SDS). The pregnancy was normal and the 20-week antenatal scan showed polyhydramnios. The delivery was complicated by shoulder dystocia, needing resuscitation. She was found to be persistently hypoglycemic (blood glucose<2.5mmol/L) requiring a total glucose load of 25 mg/kg/min (normal: 4-6 mg/kg/min) to maintain normoglycemia (plasma glucose>3.5mmol/L). She had low free thyroxine (FT4) (5.3 pmol/L) and suppressed thyroid-stimulating hormone (TSH) (<0.03 mu/L) that persisted even after the phase of acute severe illness. She also had an undetectable adrenocorticotropic hormone (ACTH) (<1.1 pmol/L) with no cortisol response to synacthen stimulation (peak cortisol to synacthen <50 nmol/L). Hydrocortisone replacement (10mg/m2/day) was commenced followed by levothyroxine therapy. The MRI scan of the brain showed a hypoplastic anterior pituitary, absent posterior pituitary, interrupted pituitary

stalk and a thin corpus callosum (Figure 1B). The hypoglycemia persisted and further investigations showed an inappropriately high plasma insulin (200 pmol/L) and c-peptide (1500 pmol/L) with suppressed plasma free fatty acid (<100 µmol/L) and beta hydroxyl butyrate (<100 µmol/L) during hypoglycemia (blood glucose: 1.2mmol/L) confirming the diagnosis of CHI. The GH was undetectable at the time of hypoglycaemia [<0.05µg/L]. A trial of diazoxide (5 mg/kg/day) was commenced along with chlorothiazide (7mg/kg/day). However, the patient suffered from significant fluid retention leading to discontinuation of diazoxide. Commencement of octreotide (10 mcg/kg/day) caused a derangement of liver enzymes and therefore had to be discontinued after which the liver enzymes returned to normal levels. She developed significant feed intolerance due to severe gastroesophageal reflux which persisted despite maximum medical treatment. A gastro-jejonostomy tube was inserted to support feeding. Normoglycemia was maintained by continuous feed via the gastro-jejonostomy tube. Genetic analysis was negative for *ABCC8*, *KCNJ11, HNF4A* and *GCK* mutations. The 18F-DOPA PET-CT scan of the pancreas suggested a diffuse uptake.

The facial dysmorphic features comprise of a single median maxillary central (SMMC) incisor (Figure 1A), congenital nasal pyriform aperture stenosis (CNPAS), which was conservatively managed, and a left choroidal coloboma. She does not have any vision abnormalities. The cardiac echocardiogram revealed pulmonary stenosis which required balloon dilatation. She had a persistent oxygen requirement(0.5-1L via nasal cannulae) of unknown etiology (negative for respiratory infections, normal chest imaging(CT) and bronchoscopy) from birth. At 1.5 years of age she was diagnosed with growth hormone (GH) deficiency (height<-3 SDS, IGF1<3.3 nmol/L and a peak GH of 1.1 µg/L (normal>7 µg/L) to arginine stimulation) and was commenced on rGH (recombinant GH) therapy. She demonstrated a good response to treatment with rGH (25 mcg/kg/day) with an improvement in the height velocity (Figure 1C). She developed persistently elevated liver transaminases

when she was 3 years old, with a negative autoimmune hepatitis and infection screen. The liver biopsy showed dense chronic inflammation with portal-portal bridging fibrosis. The clinical features are summarised in Table 1.

She is currently 5 years old, with persistent CHI, motor, speech and developmental delay and continues to be on rGH, levothyroxine and hydrocortisone replacements.There are no symptoms suggestive of diabetes insipidus and the biochemistry has been completely normal. She has shown response to the reintroduction of diazoxide (5 mg/kg/day) and chlorothiazide without any features of fluid retention, which has enabled her to come off continuous feeds for 6 hours.

Mutation analysis

A novel heterozygous *FOXA2* mutation (c.505T>C, p.S169P) was identified in the affected child but not in the parents by whole exome sequencing. To further validate our results, the mutation was confirmed by Sanger sequencing (Figure 1D). The variant is not present in control databases (ExAc, dbSNP, 1000 genome). Multiple sequence alignment shows that the serine residue at position 169 is highly conserved across different species, from drosophila, human, mouse, chicken to frog (Figure 1E), suggesting that this residue is functionally important and has been maintained throughout evolution in different species. The *FOXA2* mutation (c.505T>C, p.S169P) lies at the DNA binding domain of the transcription factor. In silico analysis using SIFT, PolyPhen, Mutation Taster predict this aminoacid substitution to have deleterious impact on the protein function.

Functional analysis

To further characterize the possible role of *FOXA2* in the observed clinical phenotype, we studied the expression of *Foxa2* at the mRNA level during mouse embryonic development. *Foxa2* mRNA transcripts were detected in the midbrain, ventral forebrain, ventral hindbrain, epithelial structures lining the main bronchus, lungs and the esophagus from embryonic day e11.5 (Figure 2A). Importantly, the expression of *Foxa2* mRNA was detected in the pituitary gland from e13.5 (Figure 2C-C') in the anterior lobe. From e15.5, *Foxa2* mRNA expression was stronger with robust expression in the ventral diencephalon, posterior lobe and anterior lobe of the pituitary gland (Figure 2D-D'). At embryonic day e18.5, *Foxa2* mRNA transcripts were localized in the hypothalamic-pituitary axis, with transcripts strongly expressed in the ventral hypothalamus and anterior pituitary (Figure 2E-E'). Analysis of hFOXA2 expression in human embryos by immunohistochemistry revealed expression of hFOXA2 in the ventral neural tube (Figure 3A') and in the diencephalon (Figure 3A'') at six weeks (Carnegie stage 16), and around the third ventricle at 8 weeks (Carnegie stage 23) (Figure 3B'). hFOXA2 was specifically localized in the cytoplasm of cells scattered in the pancreatic parenchyma at 13 weeks of gestation (Figure 3C-C'-C''). In summary, our expression analysis shows expression of *Foxa2* mRNA in hypothalamic-pituitary axis and lungs during mice embryonic development. hFOXA2 expression was detected in midline neural tube and pancreas.

FOXA2 has been previously shown to bind and activate the human glucose transporter type 2 (*GLUT2*) (42). To determine the pathogenic effect of *FOXA2* p.S169P variant, we performed transcriptional activation assays using the *GLUT2* promoter coupled to luciferase (phGT2-294-promoter-luc). We used transiently transfected HEK293T cells with equal quantities of Wt hFOXA2 or mutant hFOXA2 p.S169P and demonstrated that the hFOXA2 p.S169P significantly impairs the transcriptional activation of the *GLUT2* luciferase reporter (Figure 4A). We also performed quantification of protein expression using western blot and showed

that the mutant hFOXA2 p.S169P results in significantly reduced protein expression levels compared to the Wt hFOXA2 (Figure 4B). Using double immunofluorescence on transiently transfected HEK293T cells, we demonstrated that both the Wt hFOXA2 and mutant hFOXA2 are expressed in the nucleus and the mutation did not result in changes to cellular localization (Figure 5). Together the results indicate that the hFOXA2 p.S169P variant results in lower transcriptional activity due to an effect of the mutation on the FOXA2 protein levels.

Discussion

We have characterized a "*de novo*" heterozygous mutation in the developmental transcription factor *FOXA2* that causes a rare and unique clinical phenotype of hypopituitarism, CHI, dysmorphic features, liver, pancreas, heart and gastrointestinal abnormalities. The c.505T>C, p.S169P genetic variant occurs at the conserved forkhead DNA binding domain of the *FOXA2*. This region binds to the DNA and may provide tissue-specific gene regulation important for the development of multiple organs. Our data has confirmed that the mutation impairs the transcriptional activation of *FOXA2*. Importantly, *FOXA2* is localised at the cytogenetic location 20p11.2 and some studies have linked chromosomal deletions within this region with the clinical phenotype of hypopituitarism, central nervous system (CNS) defects, hypoglycemia, facial dysmorphic features and congenital abnormalities of the heart, liver and gastrointestinal tract (43-47). Chromosomal deletions of the 20p11.2 are rare(46) and recent studies have mapped the minimal critical region which contains 20 genes,(43) including *FOXA2*. All the patients reported to have the 20p11.2 chromosomal deletion have hypopituitarism, CNS abnormalities and facial dysmorphic features as shared features, strongly indicating that a gene or multiple genes within this chromosome region have a key role in CNS, pituitary and facial development. We have identified the gene responsible for the clinical phenotype of hypopituitarism at the 20p11.2 region as *FOXA2* and confirmed the causative role with functional analysis.

In our study we show that *FOXA2* mutation causes the clinical phenotype of hypopituitarism, CHI and facial dysmorphic features that overlaps with the clinical data published in patients with 20p11.2 deletions(43-47). The mutation in hFOXA2 p.S169P is pathogenic, as it results in impaired transcriptional activation of the phGT2-294-promoter-luc reporter and significant reduction in the protein expression compared to wild type hFOXA2. Interestingly, *FOXA2* has been shown to regulate key signaling pathways important in ventral midline, pituitary and CNS development such as *Shh* signaling[38]. Data from in vivo studies using Wnt1:Cre; *Foxa2*$^{flox/flox}$ embryos showed that *Foxa2* has an early role in the initiation of Shh expression. *Foxa2*, in combination with *Foxa1*, downregulates the expression of intracellular transducers and downstream targets of Shh signalling such as Ptch1,Gli1 and Gli2, which regulate the patterning of the ventral midbrain(38). Hence, we hypothesise that hFOXA2 could mediate its role in pituitary development by regulating Shh expression. Moreover, the midline anomalies in our patient including SMMC, CNPAS are often associated with pituitary abnormalities, as described in an extensive literature review by Lo et al (48), where hypopituitarism or growth hormone deficiency were identified in 43-48% of patients with CNPAS or SMMC. This is consistent with the clinical presentation of our patient, who has hypopituitarism along with hypoplastic pituitary, thin corpus callosum and thin pituitary stalk on the MRI.

The detection of *Foxa2* mRNA transcripts from the early stages of mouse pituitary and brain embryonic development suggests a potential role in the development of these structures. Furthermore, the detection of hFOXA2 by immunohistochemistry in human embryos at various developmental stages, along with the biochemical experiments demonstrating that the variant p.S169P mutation in *FOXA2* impairs transcriptional activation and protein expression levels, strongly indicate that *FOXA2* has a pivotal role in hypothalamic-pituitary axis formation in humans.

The co-existence of hypopituitarism along with a persistent form of hyperinsulinism, as encountered in our patient, is extremely uncommon. Hypoglycemia in CHI is caused by unregulated insulin secretion while in hypopituitarism it is due to the lack of counter-regulatory hormonal response due to the deficiency of ACTH and GH. Diagnosis can often be challenging, as the hallmark of CHI is detectable insulin in the presence of hypoketotic hypoglycemia while hypopituitarism causes ketotic hypoglycemia. Almost half of the patients with persistent CHI do not have mutations in the already recognized genes known to cause CHI. Genetic diagnosis is important as it will inform the prognosis, recurrence risk and guide the medical management besides providing valuable insight into β-cell physiology. The negative mutations in the known CHI genes in our patient together with strong biochemical evidence of CHI, makes it highly likely that the CHI in our patient is due to a novel genetic aetiology (*FOXA2*). We have further confirmed this by demonstrating the expression of hFOXA2 in the developing human pancreas.

Glucose-stimulated insulin secretion occurs by the closure of ATP dependent KATP channels situated on the β-cell membrane with the resultant depolarization of the membrane causing the exocytosis of the insulin granules(16, 30). KATP channels consist of 2 subunits, SUR1 and Kir6.2, encoded by *ABCC8* and *KCNJ11* respectively, the mutations of which cause defects in the channels resulting in the most common form of genetic CHI(16, 30). Lantz et.al demonstrated that when SUR1 or Kir6.2 promoter/luciferase reporter was transfected with *Foxa2* expression plasmids, *Foxa2*-Sur1 and *Foxa2*-Kir6.2 promoter constructs showed 6-fold and 4-fold activation respectively demonstrating a vital role of *Foxa2* in the transcriptional activation of the KATP subunits(40). Hence, a *FOXA2* mutation could potentially alter the expression of SUR1 and/or Kir6.2 leading to hyperinsulinism although the precise mechanism is yet to be explored. The other possible mechanism could be linked to *HADH* that encodes L-3-Hydroxyacyl-CoA-dehydrogenase (HADH), an enzyme involved

in the penultimate step of the beta-oxidation pathway(8). Mutations in *HADH* cause CHI in humans. It has been demonstrated in mice that *Foxa2* directly targets *HADH* causing its transcriptional activation(49). Sund et.al demonstrated that *Foxa2* knocked out from the β-cells in mice show a 3-fold downregulation of *Hadh* mRNA leading to severe hyperinsulinaemic hypoglycaemia(41, 42) The third possibility could be linked to *GLUT2*, which is expressed in the plasma membrane of the pancreatic β-cells, liver, kidney and intestine to facilitate insulin secretion by transporting the glucose across the cell membrane (29). Wang et. al showed that *GLUT2* plays an important role in the insulin secretion from the β-cells as its mRNA level is influenced by the plasma concentrations of glucose and insulin(50). Cha et. al demonstrated that *GLUT2* has binding sites for *FOXA2* and showed that the promoter activity of *GLUT2* is synergistically activated by FOXA2 in NIH3T3 cells(42). *FOXA2* also plays a critical role in the tissue specific expression of GLUT2(42). The reduction in the transcriptional activation of the *GLUT2* reporter (phGT2-294-promoter-luc) activity by the mutant hFOXA2 (p.S169P) shown in our transcriptional assay experiment, could imply that the GLUT2 tissue expression is reduced in the pancreatic β-cells of patients with *FOXA2* mutation. However, the precise mechanism by which this leads to hyperinsulinaemic hypoglycaemia is not yet understood.

It is also plausible that *Foxa2* plays a role in the development of the pancreas. *Foxa2* has been shown to regulate *Pdx1*, a homeobox gene essential for pancreatic development(49). *Foxa2* has also been linked to regulating the mRNA levels of pancreatic transcription factors such as *Hnf4a* and *Hnf1a*, mutations of which can cause monogenic forms of diabetes mellitus. However, some studies contradict that *Foxa2* is an upstream regulator of Pdx1,Hnf4a and Hnf1a(50) . While it has been shown that β-cell-specific deletion of *Foxa2* in mice causes a phenotype of hypoglycaemia(41), it also has been demonstrated that it can cause downregulation of *Pdx1* mRNA causing the reduction of PDX-1 protein levels in the

pancreatic islets(51) and a targeted β-cell-specific deletion of *Pdx1* results in diabetes in transgenic mice(52). Thus, *FOXA2* is a crucial transcription factor that controls the expression of multiple genes involved both in glucose sensing and glucose homeostasis and therefore has a potential role in diseases involving insulin secretion and glucose homeostasis.

Diazoxide is used as an effective treatment in majority of patients with CHI except in those with mutations abolishing the KATP channel activity (*ABCC8* or *KCNJ11*) or activating mutations in *GCK*. Our patient has shown response to diazoxide treatment which could potentially imply that the variant p.S169P has not completely abolished the KATP channel activity or increased the *GCK* expression.

Whilst it is difficult to speculate the progression of abnormalities in glucose homeostasis in patients with *FOXA2* mutation, screening more patients with similar phenotype will give further insights into the role of this transcription factor in the insulin secretion and in related diseases like neonatal diabetes mellitus and maturity onset diabetes of the young (MODY). The main limitation of our study is the lack of more patients with similar phenotype. As the combination of the phenotype comprising CHI and hypopituitarism is extremely rare, we were unable to recruit more patients for this study. However we are hopeful that the dissemination of the findings from this study will hopefully alert the researchers from across the world to screen for *FOXA2* mutations in patients with similar phenotype, thereby enabling a better understanding of genotype-phenotype correlations.

In conclusion, we have identified the first disease-causing mutation in *FOXA2* in an individual with an extremely rare complex phenotype of CHI, cranio-facial dysmorphic features, CH, cardiac, liver and gastrointestinal abnormalities. Identification of the genetic cause contributing to such a unique clinical phenotype will help medical management and provide valuable insights into molecular mechanisms underlying pituitary development and β-cell physiology.

Material and Methods

Patient enrolment

The patient was recruited to the 'Whole exome sequencing for rare endocrine disorders' study following written consent from the parents. The study was given favorable ethical opinion by the North West - Liverpool Central Research Ethics Committee (REC Reference: 15/NW/0758) and site study approval was granted by the Clinical Research Business Unit at Alder Hey Children's NHS Foundation Trust, Liverpool, UK.

DNA extraction

DNA was obtained from blood samples of the child and both the biological parents (trio) using the QIAmp DNA blood Midi Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and subjected to whole exome sequencing. The quality and the quantity of the genomic DNA were assessed using the Qubit (ThermoFisher Scientific) and the NanoDrop (ThermoFisher Scientific).

Library preparation, exon capture and sequencing

The samples (3 µg/sample) were sheared with the Picoruptor to a size of approximately 150-200 bp. The samples were cleaned with 1.8x AMPure beads (Agencourt) and end repaired at 20°C for 30 minutes. The products were A tailed by incubation at 37°C for 30 minutes, cleaned with AMPure beads again and ligated to index adapters at 30°C for 10 minutes to make a pre-capture library using the Agilent Sureselect XT target enrichment system for Illumina. Enrichment was achieved by 5 rounds of PCR using Herculase II fusion DNA polymerase. The libraries were checked on an Agilent HS Bioanalyser chip and quantified by Qubit Assay. 750 ng of pre-capture library was used for the hybridization. Samples were lyophilized to attain the required volume. Libraries were then mixed with hybridisation buffer, baits from the Human All Exon 5 kit and incubated overnight (24h) at 65°C. The samples were then mixed with washed streptavidin beads (Dynabeads MyOne Streptavidin

T10) and the captured products were washed and pooled. The quantity and quality of the pool was assessed by Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II, according to manufacturer's instructions. The template DNA was denatured according to the protocol described in the Illumina cBot user guide and loaded at 300 pM concentration. Sequencing was carried out on one lane of an Illumina HiSeq4000 at 2x150 bp paired-end sequencing with v1 chemistry.

Bioinformatics

The sequence data were aligned to the reference genome (GRCh37/hg19). Reads were mapped to the reference sequences using BWA mem version 0.7.5a(53) with default parameters. The mean depth of the coverage was 100x. In order to retain only confidently aligned reads, alignments were filtered to remove reads with a mapping quality lower than 10. The mapped reads were locally re-aligned to improve the alignments around small insertions/deletions (indels) using the Genome Analysis Tool Kit (GATK) version 2.1.13 (54). Base quality scores were recalibrated using GATK Base Quality Score Recalibrator (BQSR). BQSR is a module of GATK to create more accurate base qualities, which in turn improves the accuracy of our variant calls. The variants identified were annotated using SnpEff. The variants present in at least 1% minor allele frequency in 1000 Genomes Project, Exome aggregation consortium (ExAC), dbSNP142, and NHLBI ESP exomes were excluded. The predicted deleterious variants included non-synonymous coding, splice site, frameshift, and stop gain variants. The analysis of variants was performed using the ingenuity variant analysis (Qiagen bioinformatics) software. The identified potential variant segregating with the patient's phenotype was subsequently confirmed by Sanger sequencing.

Mice

All mice were housed with a 12h light/12h dark cycle in a temperature- and humidity-controlled room (21°C, 55% humidity) with constant access to food and water. Timed pregnancies were achieved by mating females and males overnight and, the presence of vaginal plug the following morning, was considered as embryonic day (e) 0.5. All experiments were conducted under the regulations, licenses and local ethical review of the UK Animals (Scientific Procedures) Act 1986.

Immunohistochemistry

Paraffin-embedded human tissue samples at 6, 8 and 13 weeks of gestation were obtained from the Human Developmental Biology Resource (Institute of Genetic Medicine, Newcastle, and Institute of Child Health, London; www.hdbr.org). Immunohistochemistry was performed by deparaffinisation of the sections followed by rehydration through decreasing ethanol dilutions. Heat-induced antigen retrieval was performed with a microwave in 10 mM sodium citrate buffer (pH 6). Samples were left to cool down at room temperature before incubating them for 1hr in blocking buffer [1XPBS, 0.1% Triton X-100, 5% Normal Goat Serum (Vector Laboratories)]. Endogenous hFOXA2 was detected with a primary rabbit monoclonal antibody against hFOXA2 (Thermo Fisher Scientific; 701698; 1:250) followed by a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories; BA-1000; 1:300). Staining was achieved using DAB Peroxidase Substrate Kit (Vector Laboratories; SK-4100). The colorimetric reaction was stopped with washes in water and the sections were counterstained using Haematoxylin (Sigma-Aldrich). Images were acquired using a Leica microscope and figures were done with Adobe Photoshop CS6.

In situ hybridization

Wild type mouse embryos were collected at different embryonic stages of mouse development (e.11.5, e12.5, e13.5, e15.5 and e18.5), fixed with 4% paraformaldehyde (PFA) and washed in PBS before proceeding with paraffin embedding. Paraffin-embedded mouse embryos were sectioned at 7 μm thickness for histochemical evaluation. In situ hybridisation was performed by processing the slides with a pre-hybridisation treatment. Sections were deparaffinised, rehydrated through decreasing ethanol dilutions, fixed with 4% PFA, incubated with proteinase K, fixed again with 4% PFA and finally incubated with 0.1 M triethanolamine, 0.1% acetic anhydride. The mouse Foxa2 gene fragment (1567 bp) plasmid was kindly provided by www.hdbr.org. The digoxigenin-labeled anti-sense probe for mFoxa2 was generated by in vitro transcription using T3 RNA polymerase (Roche). Hybridization with 100 ng of the digoxigenin-labeled probe was carried out overnight at 65°C. Sections were washed in 0.1 M Tris-HCl Buffer (pH = 7.5, 0.15 M Sodium) followed by 1 hour blocking at room temperature and overnight incubation at 4°C with anti-Dig antibody (Sigma-Aldrich). Detection of murine Foxa2 was achieved by colorimetric reaction using 4-Nitro blue tetrazolium chloride solution (NBT, Sigma-Aldrich) and 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP, Sigma-Aldrich). Images were acquired using a Leica microscope and figures were done using Adobe Photoshop CS6.

Plasmids and site-direct Mutagenesis

Full length cDNA of human FOXA2 (GENE Bank RefSeq NM 021784.4) was cloned in ORF mammalian expression vector pCMV3 (pCMV3-hFOXA2, Sino Biological Inc). E.coli DH5α competent cells were transformed with hFOXA2 (cDNA size: 1392 bp). The detected mutation was introduced by site-directed mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer`s instructions (primers used, Forward strand: 5'-AAAGCCGCCCTACCCGTACATCTCGCTC-3'. Reverse

strand: 5'-GAGCGAGATGTACGGGTAGGGCGGCTTTG-3'). Sanger sequencing confirmed the point mutation.

Cell culture and Luciferase assays

HEK293T cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. 2.5 x 10^5 cells/well were seeded in 24-well plates. 200 ng of phGT2-294-promoter-luc reporter (kindly provided by Professor Yong-Ho Ahn) and 100 ng of Renilla SV-40 were transiently co-transfected with either i) equal amounts (50 ng and 75 ng) of Wt or mutant p.S169P hFOXA2 expression plasmids or ii) both Wt and mutant p.S169P hFOXA2 expression plasmid (25 ng or 35.5 ng of each plasmid) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The total amount of DNA transfected was kept constant at 500 ng by adding pBluescript plasmid. The cells were harvested 24h after transfection and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in a BMG LABTECH Microplate reader (Omega, Germany) according to manufacturer's instructions. Firefly luciferase activity was normalised to the Renilla luciferase expression from pRL-SV40 (Promega). The experiments were independently repeated four times in triplicates and statistical analysis was performed using one-way ANOVA.

Western blotting

1.75 x 10^5 cells/well were seeded in 24-well plates and transiently transfected with equal amounts (200 ng) of Wt or mutant p.S169P hFOXA2 expression plasmids using Lipofectamine 2000 according to the manufacturer`s instructions. 300 ng of pBluescript plasmid were added to each transfection mix to maintain the total amount of DNA constant at 500 ng. The cells were harvested 24h after the transfection in a lysis buffer containing 50 mM Tris-Base (pH 7.6), 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Complete Mini, EDTA-free tablets, Roche) at a 1:6 ratio and 1% phosphatase inhibitor Cocktail3

(Sigma-Aldrich). Samples containing 20 µg of total proteins were loaded on 12% polyacrylamide gel. The proteins were transferred on a nitrocellulose membrane and nonspecific binding sites were blocked for 1h with 5% dried skimmed milk in PBS-T (1XPBS, 0.1% Tween 20). The membrane was incubated overnight at 4°C with the primary antibody (rabbit anti-FOXA2; Thermo Fisher Scientific; 701698, 1:5000 dilution in 5% dried skimmed milk in PBS-T), followed by one hour incubation with IRDye 800CW Donkey anti-rabbit antibody (LI-COR Biosciences; 1:5000). Anti-GAPDH (Santa Cruz; 1:5000, rabbit polyclonal) levels were used to normalise the total level of protein. Blots were analysed using Odyssey 2.1 Imaging System (LI-COR Biosciences). The experiments were independently repeated six times and the statistical analysis was performed using one-way ANOVA.

Immunofluorescence

1 x 10^5 cells/well were seeded in 4-well cell culture slide (Millipore, Fisher Scientific) and transiently transfected with 200 ng of Wt or mutant p.S169P hFOXA2 expression plasmids and 300 ng of pBluescript plasmid using Lipofectamine 2000 according to the manufacturer's instructions. 24h after transfection, the cells were fixed in 2% PFA in 1X PBS for 10 min and washed with 1X PBS three times. Samples were permeabilised with 0.1% Triton X-100 in 1X PBS for 30 min and blocked with blocking buffer (5% Normal Goat Serum in 1X PBS) for 30 min. The staining was performed by incubating the samples with α-FOXA2 antibody (Thermo Fisher Scientific; 701698, 2ug/ml) in blocking buffer for 1h, followed by a 30 min incubation with goat α-rabbit Alexa fluor 594 (ThermoFisher Scientific; 1:250) and α-PHALLOIDIN Alexa fluor 488 (Molecular Probes; 1:1000) antibodies. The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using a fluorescence microscope (Leica microsystem, Germany) and processed using Adobe Photoshop CS6.

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Figure legends

Figure 1: Picture of the patient's face showing single central incisor tooth (A, arrowhead). (B) Sagittal view of the MRI scan of the brain: The normal pituitary gland cannot be identified, the sella turcica is shallow and poorly defined with possibly a very hypoplastic anterior pituitary gland (arrowhead). Also, there is no evidence of the normal high signal of the posterior pituitary. There is a very short and thin pituitary stalk in its superior third (arrow) which is suggestive of an interrupted pituitary stalk. The corpus callosum is also noted to be thin (arrowhead). (C) The patient's linear growth curve compared with British contemporary references. Recombinant GH was started at 1.5 years of age when the linear height was -3SDS. A good response to GH treatment is seen subsequently with an improvement in the height SDS. (D) Electropherograms show the wild type (Wt FOXA2 and the presence of the missense mutation (thymine to cytosine) in the patient at the nucleotide position 505. (E) The evolutionary conservation of the amino acid residue serine at position 169 is shown across different species such as drosophila, human, mouse, chicken and frog. Abbreviations: standard deviation score, SDS; wild-type, Wt; growth hormone, GH; drosophila, DROME; frog, XENTR.

Figure 2. mRNA expression of *Foxa2* during mouse embryonic development.

A-D represent sagittal sections, with anterior to the left side, and E is a coronal section. A', C', D', E' show higher-magnification views of the boxed areas in A, C, D, E, respectively. At embryonic day e11.5 (A) Foxa2 mRNA transcripts were expressed within the midbrain (MB) and ventral hindbrain (HB) (arrowheads) and also in a few cells localised in the forebrain (asterisk) (FB). At this stage of development no transcripts were detected in the primordium of the anterior pituitary gland, the Rathke's pouch (RP dotted line in A and A'), or in the infundibulum (Inf). A' shows an enlarged image of the squared area in A, confirming undetectable expression of Foxa2 at this stage in the RP and Inf. At e12.5 (B and B') Foxa2 mRNA transcripts were detected in the epithelial structures lining the main bronchus (MBr) (B, arrowhead) and in the epithelium lining the lung (Lu) and oesophagus (OE) (B', arrowheads). By e13.5, expression of Foxa2 appears localised in the ventral side of the anterior lobe of the developing pituitary gland (AL, arrow) with transcripts localised in the ventral marginal zone (arrowheads in C'). Foxa2 mRNA expression become stronger at e15.5 (D) with robust expression in the ventral diencephalon (VD, asterisk), posterior lobe (PL) and anterior lobe (AL, arrowheads in D') of the pituitary gland. At embryonic day e18.5

(E), expression was found widely spread in the central nervous system, with strong expression in the lumen surrounding the lateral ventricles (LV, arrowheads) and the third ventricle (TV, asterisks). Enlarged image of the boxed area in E shows mRNA expression localised in the hypothalamic area (Hyp) (E', arroheads) with distinct pattern in the luminal area where the hypothalamic precursors tanycytes reside (arrowheads in E'). mRNA transcripts were also localised in both the posterior (PL) and anterior (AL) lobes of the pituitary gland (arrows in E'). Abbreviations: midbrain, MB; hindbrain, HB; forebrain, FB; Rathke's pouch, RP; infundibulum, Inf; main bronchus, MBr; lung, Lu; oesophagus, OE; ventral diencephalon, VD; pituitary gland posterior lobe, PL; pituitary gland anterior lobe, AL; lateral ventricles, LV; third ventricle, TV; hypothalamus, Hyp. Scale bars represent: 50 μm (A', B, C', D'); 100 μm (B'), 250 μm (A, C, D, E'); 500 μm (E).

Figure 3. Immunohistochemical analysis of human FOXA2 expression during human embryonic development.

A-C represent coronal sections of human embryos at 6 weeks (Carnegie stage 16), 8 weeks (Carnegie stage 23) and 13 weeks of gestation, respectively. A'-C'' show higher-magnification views of the boxed areas in A, B, C, respectively. At 6 weeks of gestation (A) Foxa2 expression was observed in the developing neural tube (NT) (A') and diencephalon (Dc) (arrowheads in A''). At 8 weeks of gestation (B) its expression was localised in the epithelium surrounding the third ventricle (TV) (B') and in the cells lining the diencephalon (Dc) (arrowheads in B''). No expression of Foxa2 was detected in the primordium of the pituitary gland (Rathke's pouch, RP) at CS16 (A''') nor in the developing pituitary gland at CS23 (B'''). In the pancreas at 13 weeks of gestation (C) Foxa2 was specifically localised in the cytoplasm of cells scattered in the pancreatic parenchyma (cells pointed by arrowheads in C' and C''). Abbreviations: neural tube, NT; diencephalon, Dc; Rathke's pouch, RP; pituitary gland, P; third ventricle, TV. Scale bars represent: 50 μm (A', A'', A''', B', C''); 100 μm (B'', B'''), 250 μm (C').

Figure 4: The serine to proline change in position 169 of hFOXA2 results in decreased protein expression levels leading to impairment of transcriptional activation of the human GT2 promoter. Dual luciferase assay (A) in HEK293T cells transiently transfected with 50 ng or 75 ng of Wt hFOXA2 or mutant hFOXA2 p.S169P indicates that Wt hFOXA2 is able to transactivate the human GT2 reporter, whilst the hFOXA2 p.S169P transcriptional activation is impaired (graph represents 4 independent experiments performed in triplicate, * $p<0.05$, one-way ANOVA). 3 independent western blots (B) show that the levels of the variant hFOXA2 p.S169P protein are reduced compared to the Wt hFOXA2, indicating that the mutation is functional and affects protein levels. Graph of the quantification of the western blots (B'') as percentage of Wt hFOXA2 and hFOXA2 p.S169P normalised to GAPDH indicates that hFOXA2 p.S169P variant results in half of the protein expression levels compared to Wt hFOXA2 (results from 6 independent experiments; **** $p< 0.0001$, one-way ANOVA). Abbreviation: NT, non-transfected; Wt, wild-type

Figure 5: The S169P mutation in hFOXA2 does not result in changes in cellular localisation of the protein. Double-immunofluorescence using anti-FOXA2 antibody (red A,B) and anti-PHALLOIDIN (green A',B') performed in HEK293 cells transiently transfected with either 200 ng of Wt hFOXA2 (A-A''') or mutant hFOXA2 p.S169P (B-B''') shows nuclear expression of both Wt and mutant FOXA2 (A,B) which overlaps with the nuclear DNA marker DAPI staining (A'',B'') but not with the cytoskeletal marker phalloidin. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole. Scale bars in A and B represent 10 µm.

Table 1: Summary of clinical features

Face	Single median maxillary central incisor, congenital nasal pyriform aperture stenosis
Eye	Left choroidal coloboma
Heart	Supra-valvular pulmonary stenosis
Gastrointestinal	Feed intolerance, severe gastro-esophageal reflux disease requiring gastro-jejonostomy feeding
Liver	Portal-portal bridging fibrosis, elevated transaminases
Lung	Persistent oxygen requirement of unknown etiology
Pancreas	Persistent form of hyperinsulinism
Pituitary	ACTH,GH and TSH deficiencies Thin pituitary stalk,hypoplastic anterior pituitary Thin corpus callosum
Neuro-developmental	Speech and motor developmental delay

Abbreviations

CH	Congenital hypopituitarism
CHI	Congenital hyperinsulinism
SOD	Septo-optic dysplasia
CPHD	Combined pituitary hormonal deficits
HPE	Holoprosencephaly