Novel FGF8 Mutations Associated with Recessive Holoprosencephaly, Craniofacial Defects, and Hypothalamo-Pituitary Dysfunction

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Context: Fibroblast growth factor (FGF) 8 is important for GnRH neuronal development with human mutations resulting in Kallmann syndrome. Murine data suggest a role for Fgf8 in hypothalamo-pituitary development; however, its role in the etiology of wider hypothalamo-pituitary dysfunction in humans is unknown.

Objective: The objective of this study was to screen for FGF8 mutations in patients with septo-optic dysplasia (n = 374) or holoprosencephaly (HPE)/midline clefts (n = 47).

Methods: FGF8 was analyzed by PCR and direct sequencing. Ethnically matched controls were then screened for mutated alleles (n = 480–686). Localization of Fgf8/FGF8 expression was analyzed by in situ hybridization in developing murine and human embryos. Finally, Fgf8 hypomorphic mice (Fgf8loxPNeo/−) were analyzed for the presence of forebrain and hypothalamo-pituitary defects.

Results: A homozygous p.R189H mutation was identified in a female patient of consanguineous parentage with semilobar HPE, diabetes insipidus, and TSH and ACTH insufficiency. Second, a heterozygous p.Q216E mutation was identified in a female patient with an absent corpus callosum, hypoplastic optic nerves, and Moebius syndrome. FGF8 was expressed in the ventral diencephalon and anterior commissural plate but not in Rathke’s pouch, strongly suggesting early onset hypothalamic and corpus callosal defects in these patients. This was consolidated by significantly reduced vasopressin and oxytocin staining neurons in the hypothalamus of Fgf8 hypomorphic mice compared with controls along with variable hypothalamo-pituitary defects and HPE.

Conclusion: We implicate FGF8 in the etiology of recessive HPE and potentially septo-optic dysplasia/Moebius syndrome for the first time to our knowledge. Furthermore, FGF8 is important for the development of the ventral diencephalon, hypothalamus, and pituitary. (J Clin Endocrinol Metab 96: E1709–E1718, 2011)

Abbreviations: AVP, Arginine vasopressin; CS, Carnegie stage; FGF, fibroblast growth factor; FT4, free T4; HPE, holoprosencephaly; MRI, magnetic resonance imaging; OT, oxytocin; PN, postnatal day; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDS, SD score; SOD, septo-optic dysplasia; SON, supraoptic nucleus.
Complex midline defects of the forebrain in humans are rare but may be associated with hypopituitarism, which in turn may lead to significant morbidity and mortality. They span a wide spectrum of phenotypes ranging from those which are incompatible with life, to holoprosencephaly (HPE) and cleft palate and septo-optic dysplasia (SOD).

SOD is a highly heterogeneous condition which, although usually sporadic and inclusive of possible environmental (including drug and alcohol induced) pathologies, has also been identified in a number of familial cases involving mutations in an increasing number of early developmental transcription factors including HESX1, SOX2, SOX3, and OTX2 (1–5). These genes are expressed in regions that determine the formation of forebrain and related midline structures such as the hypothalamus and pituitary (6). Consequently, SOD is characterized by variable phenotypes including midline telencephalic abnormalities, optic nerve hypoplasia, and pituitary hypoplasia with variable pituitary hormone deficiencies (7, 8).

HPE is etiologically heterogeneous but is the most frequent developmental forebrain anomaly in humans, with an incidence in liveborns of approximately one in 10,000–20,000 and in conceptuses as high as one in 250 (9). It results from varying degrees of incomplete cleavage of the prosencephalon into the cerebral hemispheres and ventricles. In addition, failure of the frontal and parietal lobes to divide posteriorly results in an absent corpus callosum. Facial features associated with HPE include cyclopia, anophthalmia, midface hypoplasia, hypotelorism, cleft lip and/or palate, and a single central incisor (10).

Recent studies have implicated a number of heterozygous genetic missense mutations and deletions in the etiology of HPE; cyogenetically visible abnormalities are estimated to be present in 25% of HPE patients (11). These in turn have led to the identification of a number of causative genes, including SHH, ZIC2, SIX3, and TGF1 with subsequent identification of mutated genes in associated pathways including PTCH1, GLI2, DISP1, TGF1, GAS1, EYA4, and FOXH1 (12–14). However, mutations have been identified in only 17% of cyogenetically normal children with HPE. In recent studies, submicroscopic deletions of a number of loci believed to be implicated in HPE were identified in a number of individuals with HPE (15), suggesting that a number of genetic mutations remain to be described.

Although not previously related to hypopituitarism, Kallmann syndrome is classically defined as the association of hypogonadotropic hypogonadism with anosmia due to hypoplasia of the olfactory bulbs (16). However, the condition is genetically and clinically heterogeneous and may be associated with craniofacial defects such as Moebius syndrome, which is characterized by malformation of the sixth and seventh facial nerves (17, 18). One of the genetic pathways involved in Kallmann syndrome is the ubiquitously expressed fibroblast growth factor (FGF) family of signaling molecules and its receptors (19). Loss-of-function mutations in human FGF8 and FGFRI have been implicated in this condition, and these factors potentially link the disorder to hypopituitarism through the requirement of Fgf8 to maintain anterior pituitary cellular proliferation via Lhx3 in mice (20, 21). Recently a putative role for FGF8 in two patients with HPE has been postulated upon the identification of two heterozygous mutations: 1) a 138-kb deletion at 10q24.3 encompassing FGF8 as well as a number of other genes (15), and 2) a p.T229M substitution associated with incomplete penetrance, which has previously been described in association with isolated hypogonadotrophic hypogonadism (16, 22). Although the latter patient manifested diabetes insipidus, there was no other evidence of endocrine dysfunction in the two pedigrees.

We hypothesized that FGF8 may be essential for the development of the forebrain and pituitary gland in humans and mouse and that mutations in this gene may contribute to the etiology of disorders such as SOD and HPE.

Patients and Methods

Patients

A total of 421 patients with congenital hypopituitarism and midline craniofacial/forebrain defects (SOD or HPE) were recruited between 1998 and 2010; 167 patients (39.7%) were recruited at the London Centre for Pediatric Endocrinology based at Great Ormond Street Hospital for Children and the University College London Hospital, and 157 (37.3%) were referred from national and 97 (23%) from international centers. Ethical committee approval was obtained from the University College London Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics Committee, and informed written consent was obtained from patients and/or parents. Of the 421 patients screened (male to female ratio 1:1), 88.8% (n = 374) had SOD and its variants, whereas 11.2% (n = 47) had HPE or midline clefts.

Mutation analysis

The coding region of human FGF8 (NM_033163) was amplified by PCR on an Eppendorf Thermocycler (Netheler, Germany) over 35 cycles with primers designed using the Primer3 program (available at http://frodo.wi.mit.edu/primer3) flanking each of the coding regions, including the four alternative splice variants of exon 1 (Fig. 1). PCR parameters are available on request. For direct sequencing for mutations, PCR products were treated with MicroClean reagent (Web Scientific, Cheshire, UK; catalog no. 2MCL-10) according to manufacturer’s instructions and then sequenced using BigDye version 1.1 sequencing chemistry (Applied Biosystems, Warrington, UK) and analyzed on a
Upon discovering FGF8 mutations, 480–686 controls were screened at the same allele to provide comparisons.

In situ hybridization and hypomorphic mice

In situ hybridization was performed on paraffin sections from mouse and human embryos as previously described (24). Sections of human embryos were obtained from the Human Developmental Biology Resource (University College London Institute of Child Health, London, UK). The human FGF8 and FGFR1 probes were obtained from the Source BioScience LifeSciences (http://www.lifesciences.sourcebioscience.com/). The Fgf8 hypomorphic mouse embryos (Fgf8loxPNeo\(^{\text{+/−}}\)/H11002) were obtained by crossing the Fgf8\(^{+/+}\)/H11001/ with Fgf8loxPNeo\(^{+/−}\)/H11001 line as has previously been described (25).

Immunohistochemistry and quantification of hypothalamic arginine vasopressin (AVP)-expressing nuclei

Brains from wild-type, heterozygote, and homozygote Fgf8 hypomorphic mouse embryos (Fgf8\(^{loxPNeo^{+/−}}\)) were collected at postnatal day (PN) 0 and processed for immunohistochemistry on 50-μm frozen sections. Sections were taken from the preoptic area to the mammillary bodies, and neurons were stained for AVP using a rabbit anti-AVP antibody (Millipore, Billerica, MA; catalog no. AB1565) for 48 h at 4 °C. These were then sequentially incubated with a biotinylated donkey antirabbit secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA) and the avidin-biotin complex (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with diaminobenzidine as the chromagen before counterstaining with methyl green.

Quantification of AVP-immunoreactive paraventricular nuclei (PVN) and suprachiasmatic nuclei (SCN) were conducted as recently described (26).

Statistical analysis

Treatments presented (see Fig. 4T) were compared with controls by ANOVA followed by Student Newman-Keuls test. \(P < 0.05\) was used to determine whether results were statistically significant. All statistics were performed using SigmaStat version 3.5 (Systat Software, Inc., San Jose, CA).

Results

Mutation analysis

After direct sequencing of 421 patients, two unrelated female patients were detected with novel mutations in exon 3 of FGF8. These included: 1) a homozygous mutation, c.566G>A, resulting in the substitution of arginine by histidine (p.R189H) (patient 1) and 2) a heterozygous mutation, c.646C>G, resulting in the substitution of glutamine by glutamic acid (p.Q216E) (patient 2). The clinical characteristics of both patients

### TABLE 1. Clinical characteristics of affected patients with FGF8 mutations

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Mutation</th>
<th>Position</th>
<th>Sex</th>
<th>Hypothalamo-pituitary</th>
<th>MRI</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homozygous</td>
<td>c.566G&gt;A, p.R189H</td>
<td>F</td>
<td>DI, ACTHI, evolving TSHD, PRLD</td>
<td>Semilobar HPE, bulky AP, Agenesis of CC, optic nerve hypoplasia, normal pituitary</td>
<td>DD, high arched palate, maxillary hypoplasia, Moebius syndrome, microcephaly, DD, spastic diplegia</td>
</tr>
<tr>
<td>2</td>
<td>Heterozygous</td>
<td>c.646C&gt;G, p.Q216E</td>
<td>F</td>
<td>Borderline GH response to provocation</td>
<td></td>
<td></td>
</tr>
</tbody>
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DI, Diabetes insipidus; ACTHI, ACTH insufficiency; TSHD, TSH deficiency; PRLD, prolactin deficiency; CC, corpus callosum; DD, developmental delay; AP, anterior pituitary; F, female.
are presented in Table 1. None of 480 Caucasian controls screened exhibited either of the mutations. In addition, for patient 1, who was of Pakistani origin, screening a further 206 ethnically matched controls revealed no mutations. Neither patient identified with an FGF8 mutation had mutations in FGFR1, KAL1, PROK2, PROKR2, HESX1, or SHH.

**Patient 1**

A female patient from a consanguineous family of Pakistani origin had been diagnosed antenatally with holoprosencephaly and absent corpus callosum; at birth she was noted to have microcephaly, micrognathia, and a high arched palate. She presented at the age of 7 wk with seizures and hypernatremia and was admitted to intensive care. Although her critical condition did not allow for extensive endocrine investigations, she was diagnosed with diabetes insipidus and ACTH insufficiency and has been treated with desmopressin and hydrocortisone. A brain magnetic resonance imaging (MRI) confirmed semilobar holoprosencephaly with separation of the cerebral hemispheres anteriorly and failure of separation of the basal ganglia structures and the thalami from each other and across the midline. The corpus callosum was absent, and the hypothalamus was abnormal. The anterior pituitary gland was noted to be bulky, and the posterior pituitary was identified at the normal location (Fig. 2F).

A glucagon stimulation test performed at the age of 5.5 yr [height 107.6 cm (−0.6 sd score [SDS]), weight 16.6 kg (−0.9SDS)] showed a normal GH response (peak GH 29.1 μg/liter) with normal IGF-I (148 ng/ml; normal range 52–297 ng/ml) and IGF binding protein-3 (3.19 mg/liter; normal range 1.3–5.6 mg/liter), but she gradually developed TSH deficiency as shown by persistently low free T4 (FT4) concentrations, with a progressive reduction in prolactin concentrations (Table 2).
Genetic screening revealed that she was homozygous for a novel *FGF8* mutation (c.566G>A, p.R189H) involving a highly conserved region of the gene (Fig. 2, A and B). Both parents were heterozygous and had a normal phenotype with no history of delayed puberty or subfertility and a reported normal sense of smell.

**Patient 2**

A Caucasian female patient with SOD and agenesis of corpus callosum presented at the age of 6 yr for investigation of profound growth deceleration with a height of 99.2 cm (–2.7 SDS) and a weight of 15.8 kg (–1.9 SDS). She was born to unrelated parents, and in addition to SOD, she had microcephaly, global developmental delay involving spastic diplegia, and Moebius syndrome. Endocrine investigations revealed a low normal concentration of IGF-I [107 µg/liter (normal range: 53–302 µg/liter)] with borderline peak GH responses to glucagon and clonidine stimulation (5.13 and 5.5 yr 18 (peak GST on hydrocortisone) 1.00 0.84–1.47 1.1 3.34 3.1–11.1]

**Expression analysis of FGF8 and FGFR1 in the developing human embryo**

To understand the role of FGF8 and FGFR1 in human embryonic development and to bring insights into the pathogenesis of the phenotypes observed in patients harboring mutations in these genes, we performed expression analysis of *FGF8* and *FGFR1* in human embryos.

In situ hybridization analyses on early-staged human embryos, Carnegie stage (CS) 16, revealed *FGF8* mRNA transcripts in the midline commissural plate of the developing telencephalon and telencephalic/diencephalic border and in the ventral diencephalon, but no expression was observed in Rathke’s pouch (the primordium of the anterior pituitary) (Fig. 3A). In mouse, expression of *Fgf8* in the ventral diencephalon is essential for Rathke’s pouch induction and growth (21, 27, 28). In agreement with this notion, expression of *FGFR1*, which is the main receptor mediating FGF8 signaling, was detected in Rathke’s pouch (Fig. 3, B and D) and in a broader domain within the ventral diencephalon (Fig. 3B). At CS 22, *FGFR1* transcripts were present in the neuroepithelium of the developing brain and in Rathke’s pouch (Fig. 3E). *FGF8* and *FGFR1* expression patterns in the human embryo parallel that of the mouse, suggesting a conserved function for *FGF8* and *FGFR1* in the development of these structures.

**Hypothalamic and pituitary defects in mouse embryos carrying a hypomorphic Fgf8 allele**

Mouse embryos carrying a hypomorphic allele of *Fgf8* ([*Fgf8*loxPNeo] over a null allele ([*Fgf8*loxPNeo/]) show severe defects in brain morphogenesis, including midbrain defects, absence of olfactory bulbs and optic chiasm, and HPE with an abnormal corpus callosum [Meyers et al. (25); Storm et al. (29)]. To further understand the pathogenesis of the defects observed in the patients carrying *FGF8* mutations, we analyzed by *in situ* hybridization the differentiation of hormone-producing cells in the pituitary gland and the integrity of the neuroendocrine hypothalamus in *Fgf8* hypomorphic mouse embryos. In addition to HPE, which was observed in two of six mutants analyzed, *Fgf8*loxPNeo/embryos showed severe abnormalities in the pituitary gland and endocrine hypothalamus (Fig. 4). At 17.5 d postcoitum, we observed two different phenotypes: 1) a severe one, characterized by the substantial reduction of anterior pituitary tissue and absence of posterior lobe...
FIG. 3. Expression of FGF8 and FGFR1 during early human development. Expression of FGF8 (A) and FGFR1 (B–E) during human embryonic CS 16 [37 d after fertilization (A and B) and CS 22 (C, D, and E)]. Sagittal (A–D) and coronal sections (E) are shown. A, FGF8 transcripts are localized in the commissural plate of the telencephalon (arrow in A), telencephalon/diencephalic border (asterisk), and the prospective hypothalamus (arrowheads in A) but not in Rathke’s pouch (rp, blue arrow), the primordium of the anterior pituitary gland. B–E, FGFR1 transcripts are widely detected throughout the brain neuroepithelium (C and E) including the prospective hypothalamus (arrows in B, D, and E), hindbrain (arrowheads in B), and the developing Rathke’s pouch (blue arrow in B, rp in D and E). D, An enlarged image of the boxed area in C. Scale bar (B), 200 μm; (C), 2 mm.

Discussion

Novel homozygous mutation in FGF8 in a female with holoprosencephaly

We have identified a novel homozygous mutation, c.566G>A (p.R189H), in exon 3 of FGF8, which resulted in an arginine substitution for histidine in the C-terminal region of the protein, in a patient with semilobar HPE associated with an absent corpus callosum and a bulky anterior pituitary on MRI. The parents of our proband are both unaffected carriers of the mutation and are first cousins. The recessive nature of this mutation, which occurs at a highly conserved residue, and its absence in 686 controls, suggests that FGF8 is critical for normal forebrain development, as indicated in a murine model carrying a hypomorphic Fgf8 allele (25, 29, 30).

The affected region of the protein is conserved across all isoforms and the variably penetrant loss-of-function heterozygous T229M mutation, which lies in the same region, has previously been associated with hypogonadotrophic hypogonadism and HPE (16, 22). However, in vitro ligand receptor binding assays using bacteriological recombinant proteins appear to indicate that this FGF8 region may not be important for receptor binding (30). This suggests that FGF8 activity in vivo may require posttranslational modifications, which were absent in the recombinant proteins used in the previous study, but important for other aspects of FGF8 biology such as intracellular transport, diffusion, or protein half-life. Mouse studies strongly suggest that fine-tuning control of FGF8 signaling through gene dosage and binding to secreted inhibitors is essential for normal development. The assessment of the impact of the p.R189H mutation identified in this study on normal development awaits the development of new mouse models in the future.

Recently a role for FGF8 in the etiology of HPE has been proposed (15). However, because a cause-effect relationship was not established and mutations were found in heterozygosity, the possibility exists that mutations in other genes may underlie the observed defects (digenicity). Although loss-of-function homozygous mutations in FGF8 have been identified or predicted previously in patients with normosmic idiopathic hypogonadotrophic hypogonadism (16) and nonsyndromic cleft lip and palate, respectively (31), our patient is the first with a recessive form of HPE to be associated with an FGF8 mutation; all the other genetic mutations implicated in the disorder have been transmitted in a heterozygous state with variable penetrance. The lack of a phenotype in the heterozygous parents is particularly intriguing, given that homozygous mutations in the gene have previously been associated with Kallmann syndrome.
Novel heterozygous mutation in FGF8 in a female with SOD and Moebius syndrome

The patient with the heterozygous p.Q216E substitution, which lies in the same region as our homozygous R189H mutation, manifested midline defects including an absent corpus callosum and hypoplastic optic nerves, reflecting a variant of SOD. A borderline GH response to stimulation with a low-normal IGF-I was the only endocrine abnormality, although one cannot exclude the possibility of an evolving endocrinopathy, particularly with reference to gonadotropin secretion.

An unusual finding in this patient was the presence of Moebius syndrome, a rare congenital dysinnervation syndrome characterized by poorly developed abducens (VI)
and facial (VII) nerves (17). The condition has been associated with Kallmann syndrome in five cases (18), with the only midline association reported recently in a child with holoprosencephaly in which discontinuation of the pregnancy had been attempted with misoprostol, an agent previously linked with Moebius syndrome (32, 33). Although FGF8 has never previously been implicated in this disorder, its potential role is highlighted by its expression in the facial primordia and eyes in mice and the presence of facial skeletal defects in heterozygote Fgf8 zebrafish mutants, which resemble several human craniofacial disorders (34, 35). Furthermore, tissue-specific ablation of Fgf8 was associated with abnormal neural crest survival and patterning, which gives rise to the cranial nerves and bone, cartilage, connective tissue, and skin pigmentation (36). In agreement with this finding, the Fgf8loxPNeo/+ embryos analyzed in this study also presented craniofacial defects.

The unaffected mother of the patient was the heterozygous carrier of the FGF8 mutation, suggesting variable penetrance of the mutated allele, or possibly a digenic cause of the disorder, both of which have been associated with Kallmann syndrome due to FGF8 mutations. Cases of the former are not unusual, with variable phenotypic presentations within a given family, making it difficult to predict an expected phenotype based on the genotype alone (37). Variations in environmental exposure may also contribute to phenotypic variations (38). Alternatively, more than one gene could contribute to the condition, particularly in the most severe form (39). Screening for mutations in HESX1 and SHH, as well as other Kallmann syndrome-associated genes, failed to establish a digenic cause, although there may be a contribution from an as-yet-unknown gene, given that no genetic etiology has been identified in the majority of patients with Kallmann syndrome or midline forebrain defects. The association of Moebius syndrome with SOD in our patient, and with Kallmann syndrome and HPE in other patients, implicates an intrinsic link between these disorders in the form of overlapping phenotypes; both our data and those published previously point to associations with mutated FGF8 or related genes.

**Fgf8/FGF8 is expressed in the developing hypothalamus and implicated in normal hypothalmo-pituitary development**

Our expression analysis demonstrates that Fgf8/FGF8 is expressed in the telencephalon and ventral diencephalon (prospective hypothalamus) but not in Rathke’s pouch, which is destined to form the anterior pituitary (39). Expression of FGFR1 colocalizes with FGF8 in the developing hypothalamus and is also found in Rathke’s pouch. The similar localization of Fgf8/FGF8 expression in both mice and humans as presented herein suggests that the disrupted pituitary and hypothalamus in the Fgf8 hypomorph mouse may parallel the endocrine deficits observed in our patients. The patient with the homozygous FGF8 mutation presented a bulky anterior pituitary with the presence of a posterior pituitary despite the association with diabetes insipidus, on MRI, and of note, the murine hypomorphs showed marked variability in the size of the anterior pituitary and the presence or otherwise of the posterior pituitary. Additionally, the reduction in AVP in the magnocellular SON and PVN of the hypomorphs may correlate with the diabetes insipidus observed in our patient, which is due to insufficient vasopressin signaling, a hormone important for maintaining urinary salt/water reabsorption (40). Furthermore, Fgf8 hypomorphs also harbor defects in the parvicellular AVP neurons in the SCN, suggesting that other hypothalamic functions such as circadian output may be dysregulated. Although not quantified in this study, the reduction in OT staining in SON and PVN nuclei is consistent with recently published data, which showed significantly reduced OT immunoreactivity in the same hypomorphic mice as used herein (26). Our data suggest that the hypopituitary phenotypes observed in our patients may be the result of reduced functional FGF8 in the diencephalon, leading to deficiencies in the neuroendocrine hypothalamus.

In conclusion, we report mutations in FGF8 that are associated with complex midline phenotypes, including the first autosomal recessive case of HPE and a potential role in a patient with SOD and Moebius syndrome, the genetic basis of the latter having remained elusive to date. Analysis of our patients and murine hypomorphs, as well as the conserved expression patterns of Fgf8/FGF8 and Fgfr1/FGFR1 in mouse and human, suggests that FGF8 signaling is important for telencephalic and hypothalmo-pituitary axis development and that mutations in this gene play a role in the pathogenesis of midline defects such as HPE and SOD as well as Kallmann syndrome.

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