Genetic Overlap in Kallmann Syndrome, Combined Pituitary Hormone Deficiency, and Septo-Optic Dysplasia

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Context: Kallmann syndrome (KS), combined pituitary hormone deficiency (CPHD), and septo-optic dysplasia (SOD) all result from development defects of the anterior midline in the human forebrain.

Objective: The objective of the study was to investigate whether KS, CPHD, and SOD have shared genetic origins.

Design and Participants: A total of 103 patients with either CPHD (n = 35) or SOD (n = 68) were investigated for mutations in genes implicated in the etiology of KS (*FGFR1*, *FGF8*, *PROKR2*, *PROK2*, and *KAL1*). Consequences of identified *FGFR1*, *FGF8*, and *PROKR2* mutations were investigated *in vitro*.

Results: Three patients with SOD had heterozygous mutations in *FGFR1*; these were either shown to alter receptor signaling (p.S450F, p.P483S) or predicted to affect splicing (c.336C>T, p.T112T). One patient had a synonymous change in *FGF8* (c.216G>A, p.T72T) that was shown to affect splicing and ligand signaling activity. Four patients with CPHD/SOD were found to harbor heterozygous rare loss-of-function variants in *PROKR2* (p.R85G, p.R85H, p.R268C).

Conclusions: Mutations in *FGFR1*/*FGF8*/*PROKR2* contributed to 7.8% of our patients with CPHD/SOD. These data suggest a significant genetic overlap between conditions affecting the development of anterior midline in the human forebrain. **(*J Clin Endocrinol Metab* 97: E694–E699, 2012)**

In the vertebrate embryo, the preplacodal field arises at the edge of the neural plate adjacent to the neural crest, and its derivatives give rise to neuronal and nonneuronal head structures (1). Cells within the preplacodal field separate into individual placodes, of which the most anterior are the adenohypophyseal, lens, and olfactory placodes (1). The adenohypophyseal placode gives rise to the intermediate and anterior pituitary lobes (2). The olfactory placode gives rise to different cell types, including vomeronasal neurons, support cells, mucous-producing cells, and GnRH neurons (1). These developmental processes are orchestrated by multiple transcription factors and signaling molecules (2).

Mutations in the transcription factors *SOX2, SOX3, HESX1,* and *OTX2* have been implicated in septo-optic dysplasia (SOD), a disorder characterized by pituitary hormone deficiencies, optic nerve hypoplasia, and midline defects including agenesis of the septum pellucidum and/or corpus callosum (3). Furthermore, mutations in transcription factors *PROP1, POU1F1, LHX3,* and *LHX4* underlie combined pituitary hormone deficiency (CPHD) (4). However, such mutations only account for a small percentage of all CPHD/SOD cases.

A different set of genes have been implicated in Kallmann syndrome [KS; defined as idiopathic hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia]. These genes, *KAL1, PROK2, PROKR2, FGFR1,* and *FGF8,* play critical roles in the development of olfactory system and GnRH neuron ontogeny (5). KS manifests as absent or incomplete puberty, sexual immaturity, and infertility, and additional phenotypes include midline defects (5).

FGFR1 and *FGF8* are expressed in Rathke's pouch and in the ventral diencephalon, respectively (6), and murine transcriptome data have identified members of the fibroblast growth factor (FGF)-8 signaling network during pituitary development (7). We therefore hypothesized that mutations in genes underlying KS could also underlie CPHD and/or SOD.

Subjects and Methods

Patients and control subjects

A total of 103 patients with either sporadic CPHD (n = 35) or sporadic SOD (n = 68) were included. Patients were recruited at four medical centers in the United States and the United Kingdom. Patients with SOD exhibited optic nerve hypoplasia, agenesis of the corpus callosum, and/or septum pellucidum on radiologic examination with or without pituitary hormone deficiencies (4). CPHD was diagnosed as a deficiency of at least two pituitary hormones. Unaffected control subjects (n = 268) were also studied. The ethics committees of participating institutions approved the study, and written informed consent was obtained before participation from subjects/parents/guardians.

The description of DNA sequencing of *KAL1, FGFR1, FGF8, PROK2,* and *PROKR2* and the assessment of the functional consequences of FGF receptor (FGFR)-1, FGF8, and prokineticin receptor 2 (PROKR2) mutations have been previously reported. Detailed descriptions of these methods as well as a description of Prokr2 promoter reporter gene expression in the mouse pituitary are provided in the Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Results

Among 103 patients with either CPHD (n = 35) or SOD (n = 68), four unrelated probands (3.9%) harbor rare sequence variants in *FGFR1* or *FGF8,* and four (3.9%) have *PROKR2* variants (Table 1). All mutations are heterozygous, and, in most cases, DNA from the parents was not available. The probands' phenotypic data are detailed in Table 1 and case descriptions are provided in the Supplemental Data. Notably, a number of probands exhibited a reproductive phenotype consistent with hypogonadotropic hypogonadism based on their neonatal presentation because they are yet prepubertal.

FGFR1 and *FGF8* mutations in patients with SOD

Three *FGFR1* heterozygous mutations were identified in SOD probands (Table 1). The *FGFR1* variant c.1349C>T, p.S450F maps to the intracellular domain of the receptor upstream of the tyrosine kinase domain and the amino acid (S450) is highly conserved across vertebrates (Supplemental Fig. 1A). The S450F mutant FGFR1 exhibits total protein and receptor cell surface expression levels (Fig. 1A and Supplemental Fig. 1B) similar to wild type (WT), yet downstream signaling is severely compromised (Fig. 1B). The *FGFR1* variant c.1447C>T p.P483S maps to a highly conserved residue in the tyrosine kinase domain. P483S also exhibits normal expression levels (Fig. 1A) but disrupted downstream signaling (Fig. 1B). Of note, the affected amino acid residue is also mutated in a patient with KS (c.1447C>A, p.P483T, Pitteloud, N., unpublished data). Subject 1 harbors a synonymous change (c.336C>T, p.T112T) mapping to the C-terminal end of immunoglobulin-like domain 1 (Supplemental Fig. 1A). This change was not observed in the 268 healthy controls, in the single-nucleotide polymorphism database or in the 1000 genomes data set. The Human Splicing Finder software (8) predicts that this variant generates a new exonic splicing enhancer binding site (TTACTTC) for the SRp40 splicing factor [score 79.46 (0–100)] and/or disrupts an overlapping putative exonic splicing enhancer octamer (CCTACTTC) (score 31.53).

TABLE 1. Phenotypes of SOD and CPHD probands found to harbor gene mutations in either *FGFR1*, *FGF8*, or *PROKR2*

| Patient	SOD ± hormone deficiencies						CPHD	
	1	2	3	5	6	7	4	8
Rare variant	*FGFR1* T112T	*FGFR1* S450F	*FGFR1* P483S	*PROKR2* R85G	*PROKR2* R268C	*PROKR2* R268C	*FGF8* T72T	*PROKR2* R85H *KAL1* H459Y
Gender	Male	Male	Female	Female	Male	Male	Female	Male
Abnormal pituitary MRI	No	No	Yes	No	Yes	Yes	Yes	N/A
Anterior pituitary			Hypoplastic		Hypoplastic			
Posterior pituitary						Ectopic	Prominent	
Infundibular stalk			Undescended			Agenesis		
Hormone deficiencies	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GH	X		X	X		X	X	X
TSH			X	X		X	X	X
ACTH			X			X		X
LH/FSH	X[a]	X[a]	X[a]	X[a]	X[a]	X[a]	X	X[a]
AVP		X						
Midline defects	Yes	Yes	Yes	Yes	Yes	No	No	No
Corpus callosum	Agenesis	Agenesis		Hypoplastic	Hypoplastic			
Septum pellucidum								
Other		1 central incisor	Cleft lip/palate	Cleft lip/palate schizencephaly				
Ocular defects	Yes	No	Yes	Yes	Yes	Yes	No	No
Optic nerves				Agenesis (L)		Hypoplasia		
Optic disc					Hypoplasia	Hypoplasia		
Other	Abnormal eyes		Micropthalmia coloboma					
Reproductive phenotypes	N/A	N/A	N/A	N/A	Yes	Yes	Yes	Yes
Cyrptorchidism					X	X		
Microphallus						X		X
Delayed puberty							X	
Other phenotypes	Seizures	ASD and VSD, brachydactyly, brachycephaly, preauricular skin tags	None	Club foot, syrinx spinal cord	Microcephaly, epilepsy	None	None	None

MRI, Magnetic resonance imaging; AVP, arginine vasopressin; ASD, atrial septal defect; VSD, ventricular septal defect.

[a] Hypogonadotropic hypogonadism is based on neonatal diagnosis (phenotype: cryptorchidism/micorphallus or low serum gonadotropins or LHRH stimulation test).

A synonymous change in *FGF8* (c.216G>A p.T72T) was identified in a CPHD proband. This variant (Supplemental Table 1) is predicted by the Human Splicing Finder program (8) to compromise an exonic splicing enhancer site for the serine/arginine-rich splicing factor SF2/ASF (9). We generated a minigene expression construct, which includes the entire *FGF8* gene (exons 1a to 3) except for 2.4 kb of intron 1d sequence (Supplemental Fig. 2), to measure relative expression levels of the four FGF8 isoforms in transfected cells using quantitative RT-PCR. Consistent with the software prediction, the e and f isoform transcripts (which incorporate an alternatively spliced exon, 1c) expressed from the mutant construct were significantly elevated compared with WT (Fig. 1C). We further assessed the biological significance of the minigene-induced alterations in gene expression and found that the mutant construct displayed significantly higher activity in a luciferase transcription reporter assay compared with WT (Fig. 1D).

PROKR2 and *KAL1* mutations in patients with SOD or CPHD

Three different *PROKR2* mutations were found in four patients with SOD or CPHD. One Caucasian and one African SOD proband both harbor the identical heterozygous *PROKR2* variant (c.802C>T, p.R268C) (Table 1 and Supplemental Fig. 3A), previously reported in association with both normosmic IHH and KS (10, 11) and shown to be loss of function *in vitro* (11). The other *PROKR2* variant (c.253C>G, p.R85G) found in a SOD proband affects an amino acid conserved across vertebrates (Supplemental Fig. 3A) and is predicted to be loss of function (Supplemental Table 1). Western analysis indicates reduced total protein expression suggesting a defect in protein folding and stability (Fig. 1E). Accordingly, cell surface expression is significantly reduced (Fig. 1F) accompanied by a severe decrease in signaling via both calcium (Fig. 1G) and MAPK (Supplemental Fig. 3B) (log
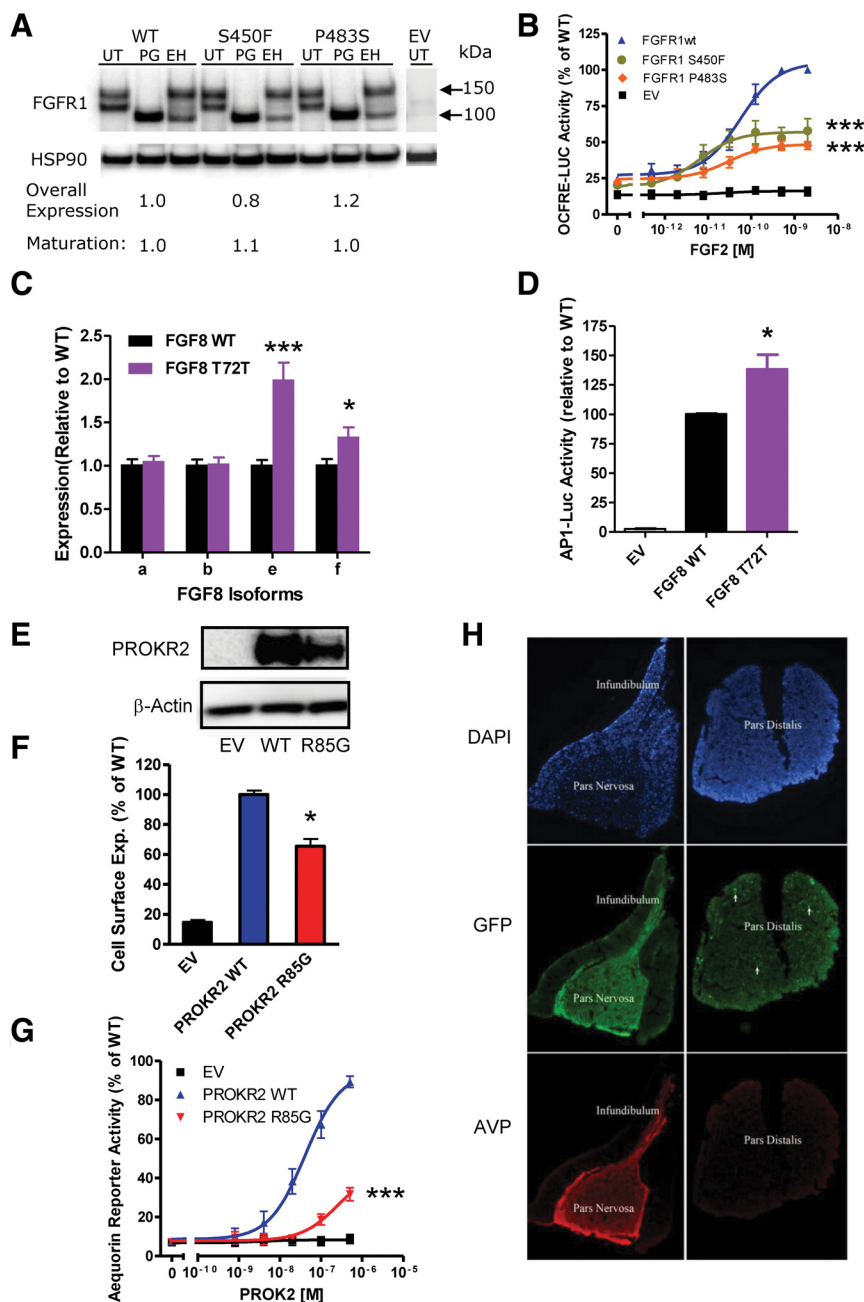
FIG. 1. A, Western blotting analysis showing similar overall expression and maturation levels of mutant FGFR1 compared with WT. FGFR1 was detected using an anti-myc antibody and the blot was reprobed with heat-shock protein 90 to demonstrate equal loading. UT, Untreated; PG, PNGase F treated; EH, endoglycosidase H treated; HSP90, heat-shock protein 90. B, Luciferase reporter assay using the osteocalcin FGF response elements reporter showing decreased signaling activity by FGFR1 S450F and FGFR1 P483S. ***, $P < 0.001$ at maximal dose. C, Quantitative PCR showing that expression of both the *FGF8e* and *FGF8f* isoforms is significantly increased compared with WT. ***, $P < 0.001$; *, $P < 0.05$). D, Representative experiment of a transcriptional reporter (AP1-luciferase) assay of FGF8 WT and T72T mutant minigene showing increased signaling activity by the mutant ligand compared with WT. *, $P < 0.05$ at maximal dose. E, Western analysis showing that overall expression of the PROKR2 mutant (R85G) is reduced compared with WT. F, Expression of the PROKR2 R85G mutant at the cell surface is significantly reduced compared with WT. *, $P < 0.05$ in a radiolabeled antibody binding assay. G, The PROKR2 R85G mutant receptor is loss of function in the aequorin reporter assay. ***, $P < 0.001$. H, Prokr2 is expressed in the adult mouse pituitary. Immunofluorescent staining for GFP in the pituitary of Prokr2-GFP transgenic mice shows immunoreactivity in all pituitary structures and is most pronounced in the pars nervosa. AVP, Arginine vasopressin.

EC_{50} WT -6.944 ± 0.155, R85G -6.319 ± 0.267, $P < 0.05$) cascades.

A CPHD proband, born with a microphallus (suggesting neonatal GnRH deficiency), harbors a rare variant in *KAL1* (c.1375C>T), inherited from his mother, and a mutation in *PROKR2* (c.254G>A, p.R85H) inherited from his father. The R85H mutation has been reported in KS patients (12) and is loss of function *in vitro* (13). Transgenic mice expressing green fluorescent staining (GFP) under control of the *Prok2* promoter shows GFP immunoreactivity throughout the pituitary structures, and especially in the pars nervosa (Fig. 1H), further supporting a role for PROK2 signaling in the pituitary.

Discussion

We describe eight prepubertal patients with CPHD/SOD carrying a heterozygous mutation in *FGFR1*, *FGF8*, or *PROKR2*, associated with altered function. Thus, mutations in genes generally associated with IHH/KS may also be associated with CPHD/SOD, demonstrating a genetic overlap between these syndromes. Patients with KS often display midline defects such as cleft lip and/or palate and corpus callosum anomalies, and *FGF8* mutations were recently found to be associated with recessive holoprosencephaly, craniofacial defects, and hypothalamo-pituitary dysfunction (6). Additionally, as early as in 1954, de Morsier described a syndrome of dysplasie olfacto-génitale, which included agenesis of the olfactory bulbs, corpus callosum, and the anterior commissure as well as infantile genitalia (14). Although the hypogonadotropic hypogonadism observed in both CPHD and SOD is thought to be of pituitary origin, the verification of hypothalamic GnRH deficiency in these patients is difficult, if not impossible, because of the concomitant presence of a pituitary defect.

As with the *FGFR1* mutations in IHH/KS patients, the mutations identified in SOD probands are also loss of function. In contrast, the *FGF8* mutation in the CPHD proband shows enhanced downstream receptor signaling. The rare synonymous change in *FGF8* leads to differential expression of *FGF8* isoforms and enhanced FGFR1 signaling *in vitro*. Thus far, gain-of-function *FGFR1* mutations have been reported only in osteoglophonic dysplasia and Pfeiffer syndrome. Of note, 40% of patients with Apert syndrome (caused by activating *FGFR2* mutations) have partial or complete absence of the septum pellucidum and 23% have corpus callosum defects (15), phenotypes also observed in SOD. Thus, it remains unclear whether CPHD is caused by increased (and not decreased) FGF signaling or, more broadly, by any significant perturbation of FGF signaling.

There are some precedents of IHH/KS sharing the same genetic basis with another developmental disorder. Mutations in *CHD7* occur in CHARGE syndrome (coloboma, heart defect, choanal atresia, retardation, genital hypoplasia, ear anomalies) and KS patients (16). Similarly, a frameshift mutation in *SOX2* associated with anophthalmia/microphthalmia was also found in an IHH patient (17), suggesting that SOD and IHH share a genetic basis. Furthermore, deletion of *Otx2*, a locus for SOD, targeted to GnRH neurons results in hypogonadotropic hypogonadism in mice (18).

We also evaluated the *PROK2/PROKR2* pathway in patients with CPHD/SOD, identifying three loss-of-function mutations in *PROKR2* in four unrelated CPHD/SOD probands. The *PROKR2* R268C variant has been described in heterozygous state in IHH/KS patients, healthy first-degree relatives of KS probands, and in one of 250 healthy controls (10, 12). We therefore propose that these mutations do not cause major midline defects *per se*, but may act as modifier genes, or contribute to the phenotype through digenic inheritance, as previously demonstrated in IHH/KS (19, 20). Thus, further studies are needed to elucidate the role of PROKR2 signaling in pituitary and midline development. In conclusion, this report identified substantial genetic overlap between syndromes affecting the anterior midline and associated with the primitive placode.

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