NOTE

Submicroscopic deletion involving the *fibroblast growth factor receptor 1* gene in a patient with combined pituitary hormone deficiency

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Abstract. Combined pituitary hormone deficiency (CPHD), isolated hypogonadotropic hypogonadism (IHH), Kallmann syndrome (KS), and septo-optic dysplasia (SOD) are genetically related conditions caused by abnormal development of the anterior midline in the forebrain. Although mutations in the *fibroblast growth factor receptor 1* (*FGFR1*) gene have been implicated in the development of IHH, KS, and SOD, the relevance of *FGFR1* abnormalities to CPHD remains to be elucidated. Here, we report a Japanese female patient with CPHD and *FGFR1* haploinsufficiency. The patient was identified through copy-number analyses and direct sequencing of *FGFR1* performed for 69 patients with CPHD. The patient presented with a combined deficiency of GH, LH and FSH, and multiple neurological abnormalities. In addition, normal TSH values along with a low free T4 level indicated the presence of central hypothyroidism. Molecular analyses identified a heterozygous ~8.5 Mb deletion involving 56 genes and pseudogenes. None of these genes except *FGFR1* have been associated with brain development. No *FGFR1* abnormalities were identified in the remaining 68 patients, although two patients carried nucleotide substitutions (p.V102I and p.S107L) that were assessed as benign polymorphism by *in vitro* functional assays. These results indicate a possible role of *FGFR1* in anterior pituitary function and the rarity of *FGFR1* abnormalities in patients with CPHD.

Key words: Anterior pituitary, Gonadotropin, Gene deletion, Copy-number, Mutation

FIBROBLAST GROWTH FACTOR (FGF) receptor 1 (*FGFR1*) and its major ligand FGF8 are key molecules for the development of GnRH neurons and the olfactory system [1, 2]. Upon binding of FGF ligands, *FGFR1* dimerizes and activates the Ras-MAPK and phospholipase C γ pathways, thereby controlling cell proliferation and differentiation [1, 2]. Genetic defects in *FGFR1* and *FGF8* have been shown to underlie isolated hypogonadotropic hypogonadism (IHH)

and Kallmann syndrome (KS) [3–5]. To date, several *FGFR1* abnormalities have been identified in patients with IHH/KS mostly as monoallelic mutations, although in a certain percentage of patients, they appeared as digenic or biallelic mutations [5].

It has recently been postulated that IHH, KS, septo-optic dysplasia (SOD), and combined pituitary hormone deficiency (CPHD) are genetically related conditions caused by abnormal development of the anterior

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Abbreviations: CGH, comparative genomic hybridization; CPHD, combined pituitary hormone deficiency; FGF, fibroblast growth factor; FGFR, FGF receptor; FISH, fluorescence *in situ* hybridization; IHH, isolated hypogonadotropic hypogonadism; KS, Kallmann syndrome; MLPA, multiplex ligation-dependent probe amplification; SOD, septo-optic dysplasia; WT, wildtype

midline in the forebrain [6, 7]. Mutations in *FGF8*, *PROKR2*, and *KAL1* have been identified in patients with IHH, KS, SOD, and/or CPHD [6, 7]. Furthermore, *FGFR1* mutations have been detected in a few patients with SOD [7].

Until now, however, *FGFR1* mutations have not been described in patients with CPHD, with the exception of a cytogenetically detectable large deletion involving *FGFR1* that was identified in a single case [8]. Therefore, although a positive role of FGF-FGFR signaling in the proliferation of pituitary cells has been indicated by *in vitro* studies [9, 10], it remains to be determined whether genetic defects in *FGFR1* lead to CPHD. To clarify this, we performed copy-number and mutation analyses of *FGFR1* for patients with CPHD.

Patients and Methods

Patients

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and was performed after obtaining written informed consent. We studied 69 Japanese patients (37 males and 32 females aged 1–43 years) with combined deficiencies of at least two pituitary hormones and a normal karyotype. Two of the 69 patients had a family history of pituitary dysfunction. The 69 patients invariably manifested GH deficiency. In addition, most patients had deficiencies of TSH and/or prolactin. Apparent ACTH and ADH deficiencies were observed in 24 and 6 patients, respectively. Of 41 patients examined after the pubertal age of normal children, 32 showed compromised FSH/LH secretion. Brain magnetic resonance imaging delineated pituitary hypoplasia in 34 patients, and stalk interruption in seven. Eleven patients, including four with SOD, exhibited eye abnormalities. Seven patients manifested clinically discernible developmental delay. Patients with a history of birth trauma and those carrying protein-altering mutations or copy-number aberrations of *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *OTX2*, and *SOX3* were excluded from this study. DNA samples of 100 healthy Japanese adults were utilized as controls.

Copy-number analyses

Genomic DNA samples were obtained from leukocytes of the patients. Multiplex ligation-dependent probe amplification (MLPA) was carried out using a

commercially available MLPA probe mix (P133 kit; MRC-Holland, Amsterdam, The Netherlands). To confirm a deletion, we performed fluorescence *in situ* hybridization (FISH) analysis on lymphocyte metaphase spreads using a PCR-generated probe. The extent of a deletion was analyzed by an oligoarray-based comparative genomic hybridization (CGH) using 1×244K human catalog array (G4411B; Agilent Technologies, Palo Alto, CA, USA). The size and location of a deletion were analyzed using the UCSC genome browser (<http://genome.ucsc.edu/>).

Mutation analysis

Direct sequence analysis was carried out for the coding exons and their flanking splice sites of *FGFR1*. The primers utilized in this study are shown in Table 1. To confirm a heterozygous nucleotide substitution, the corresponding PCR products were subcloned using a TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, USA), and the two alleles were sequenced separately.

Functional analysis of missense variants

For missense variants, *in vitro* reporter assays were carried out by a previously reported method with modifications [11, 12]. In brief, an expression vector for the wildtype (WT) *FGFR1* was constructed by inserting the PCR-amplified cDNA of human *FGFR1* into a pcDNA3.1⁺ vector (Life Technologies). An expression vector for the *FGFR1* variants was generated by site-directed mutagenesis. A reporter vector was constructed by inserting the rat osteocalcin promoter sequence (from -206 to +38) into the PGL3-basic luciferase vector (Promega Madison, WI, USA). Transfection assays were carried out using rat L6 myoblast cells (CRL-1458, ATCC) that lacked endogenous *Fgfr1* expression. The cells were maintained in DMEM containing 10% fetal bovine serum and seeded into 24-well dishes. Transient transfection was performed using FuGENE 6 Reagent (Roche Diagnostics, Basel, Switzerland) with: (1) empty pcDNA3.1⁺ expression vector (200 ng), (2) WT *FGFR1* expression vector (200 ng), (3) variant *FGFR1* expression vector (200 ng), or (4) variant (100 ng) plus WT (100 ng) *FGFR1* expression vectors, together with the luciferase reporter plasmids (60 ng) and a pRL-null vector (25 ng, Promega) used as an internal control. At 24 hours after transfection, the medium was replaced by fresh medium supplemented with 2.0 nM FGF8 (Wako, Osaka, Japan), 1.0 µg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA), and 0.1% fetal bovine serum. The

Table 1 Primers utilized in the present study

	Forward primer	Reverse primer
<i>FGFR1</i> sequence analysis		
Exon 2	CCTTCTGTTTTTCCTTCCCC	GAGCCTTCCCTGTTGACCA
Exon 3	TCTTCCTCTCTCGCCCCCTT	AAGCAGAGTGGGGGCAGAT
Exon 4 + 5	AAATCTAGGGGTCCCTAGGAG	TCCCCTGTTCCCATTACTCTA
Exon 6	GTCATGGGGCCTGCATTTT	AAGTGCCAATCGCTATCCTGA
Exon 7	ATGGCAAGGTCCCCATGA	AAGCGTGAGGAATGATCCCAT
Exon 8	AGGTCTCATGTCCTGTGCTTG	AGGATTGAGCCCTCAAAGCT
Exon 9	CAGGAGACAGGTGTTGCTTTT	AGCCAGAAAATAAGGCCCAA
Exon 10 + 11	TTGGGAAGCCCTGACTAAGAA	TGTTTGCTTGAATGGGACA
Exon 12	AAAGCAGCCCCTCGACACATA	ACCCAGCTCAGATCTTCTCC
Exon 13	AACCTGCTCACCTGCTGCT	CACAGGCTGGAAGACTAGGG
Exon 14	CAGAGCAGTGTGGCAGAAGTT	ATGAGGGCACAGGTGGGAA
Exon 15	TGAGAACCCACCCCCAGT	GCATGGAGAACAGATCAGCCT
Exon 16	TGGGTAGAGGATTTGTGCTGG	TCAGGGACCGTCTCCTGGAGAT
Exon 17	TAAGCCCCGAGGAGATGTCG	AAAGCTGGGGGAGTACTGGT
Exon 18	CCATCGCTGCTTCCTCTC	TTACAGCTGACGGTGGAGTCT
FISH probe		
LA-PCR <i>FGFR1</i>	AGTAAACAATAGGGTGAGAACTTTTGCT	CCATGTTATAGCATAGAATCAGCACTTT
Reporter vector construction		
Osteocalcin promoter	ACGCGTAGGTCTGGGGCCAT	AGATCTGGTCTGCACCGAGT
Mutagenesis		
V102I	TATGCTTGCATAACCAGCAGCCCCCTC	GGTTATGCAAGCATAGAGGCCGGAGT
S107L	CCCTTGGGCAGTGACACCACCTACTT	GTCAGTCCCAAGGGGCTGCTGGTTA

cells were cultured for an additional 18 hours and then subjected to luciferase analysis using the luciferase reporter assay system (Promega). Relative luciferase activity was measured with Lumat LB9507 (Berthold, Oak Ridge, TN, USA). These experiments were carried out in triplicate within a single experiment and the experiment was repeated three times. The results are expressed as the mean $\pm$ SEM, and statistical significance was determined by the *t*-tests. Values of $p \leq 0.05$ were considered significant.

Results

Identification of a submicroscopic deletion involving *FGFR1*

A heterozygous deletion involving *FGFR1* was identified in one female patient. This deletion was indicated by MLPA and confirmed by FISH (Fig. 1A).

CGH revealed that the deletion was ~8.5 Mb in size and included all exons of *FGFR1* (Fig. 1B). In addition to *FGFR1*, this deletion affected 55 genes and pseudogenes (Table 2). Parental samples of the patient were not available for genetic analysis. This deletion was not detected in the control group.

Identification of two *FGFR1* missense variants

Direct sequence analysis identified two heterozygous variations, p.V102I (c.304 G>A) and p.S107L (c.320 C>T), in two female patients (Fig. 2A). The father of the p.V102I-positive patient also carried this variation, while parental samples of the p.S107L-positive patient were not available for genetic analysis. The p.V102I and p.S107L variants were found in two and one of the 100 control individuals, respectively.

Fig. 1 Submicroscopic deletion involving *FGFR1*
A Deletion analyses. Left: MLPA analysis. The arrows indicate the peaks that correspond to *FGFR1* exons. Reduced peak heights of MLPA probes suggest a heterozygous deletion. Right: FISH analyses on lymphocyte metaphase spreads. The *FGFR1* probe detects only a single red signal (an arrow), whereas the chromosome 8 centromere probe (*D8Z2*) identifies two green signals (arrowheads). **B** Array-based CGH analysis. CGH indicated an ~8.5 Mb deletion at 8p11.1-p12 involving *FGFR1*, together with a small duplication at 8p23.2 that is known as a benign polymorphism (Database of Genomic Variants; <http://projects.tcag.ca/variation/>).

Functional analysis of missense variants

In vitro functional assays were carried out for the p.V102I and p.S107L variants (Fig. 2B and C). The relative luciferase activity of p.S107L did not significantly differ from that of WT ($p = 0.0552$). Furthermore, although p.V102I showed a slightly lower activity compared to WT ($p = 0.0413$), there was no significant difference in activity between WT and p.V102I plus WT (1:1) ($p = 0.0872$).

Clinical features of the deletion-positive patient

The deletion-positive patient presented with primary amenorrhea at 20 years of age. She had no family history of pituitary dysfunction; her non-consanguineous parents were clinically normal.

Clinical examinations revealed delayed pubertal development (breast, Tanner stage 3; pubic hair, stage 2-3; axillary hair, stage 1), in addition to short stature (-2.7 SD) and learning disability. She had several episodes of convulsion from eight months of age and was

Table 2 Genes and pseudogenes involved in the deletion

Gene symbol (Refseq)	Accession number	Description
UNC5D	NM_080872	unc-5 homolog D (C. elegans)
KCNU1	NM_001031836	potassium channel, subfamily U, member 1
ZNF703	NM_025069	zinc finger protein 703
ERLIN2	NM_007175	ER lipid raft associated 2
LOC728024	NR_003671	chromosome X open reading frame 56 pseudogene
PROSC	NM_007198	proline synthetase co-transcribed homolog (bacterial)
GPR124	NM_032777	G protein-coupled receptor 124
BRF2	NM_018310	subunit of RNA polymerase III transcription initiation factor, BRF1-like
RAB11FIP1	NM_025151	RAB11 family interacting protein 1 (class I)
GOT1L1	NM_152413	glutamic-oxaloacetic transaminase 1-like 1
ADRB3	NM_000025	adrenergic, beta-3-, receptor
EIF4EBP1	NM_004095	eukaryotic translation initiation factor 4E binding protein 1
ASH2L	NM_004674	ash2 (absent, small, or homeotic)-like (Drosophila)
STAR	NM_000349	steroidogenic acute regulatory protein
LSM1	NM_014462	LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae)
BAG4	M_004874.3	BCL2-associated athanogene 4
DDHD2	NM_001164234	DDHD domain containing 2
PPAPDC1B	NM_001102559	phosphatidic acid phosphatase type 2 domain containing 1B
WHSC1L1	NM_017778	Wolf-Hirschhorn syndrome candidate 1-like 1
LETM2	NM_144652	leucine zipper-EF-hand containing transmembrane protein 2
FGFR1	NM_023105	fibroblast growth factor receptor 1
C8orf86	NM_207412	chromosome 8 open reading frame 86
RNF5P1	NR_003129	ring finger protein 5, E3 ubiquitin protein ligase pseudogene 1
TACC1	NM_001122824	transforming, acidic coiled-coil containing protein 1
PLEKHA2	NM_021623	pleckstrin homology domain containing, family A member 2
HTRA4	NM_153692	HtrA serine peptidase 4
TM2D2	NM_001024381	TM2 domain containing 2
ADAM9	NR_027639	ADAM metalloproteinase domain 9
ADAM32	NM_145004	ADAM metalloproteinase domain 32
ADAM5P	NR_001448	ADAM metalloproteinase domain 5, pseudogene
ADAM3A	NR_001569	ADAM metalloproteinase domain 3A
LOC100130964	NR_046245	ADAM metalloproteinase domain 3A-like
ADAM18	NM_014237	ADAM metalloproteinase domain 18
ADAM2	NM_001464	ADAM metalloproteinase domain 2
IDO1	NM_002164	indoleamine 2,3-dioxygenase 1
IDO2	NM_194294	indoleamine 2,3-dioxygenase 2
C8orf4	NM_020130	chromosome 8 open reading frame 4
ZMAT4	NM_001135731	zinc finger, matrin-type 4
SFRP1	NM_003012	secreted frizzled-related protein 1
GOLGA7	NM_001174124	golgin A7
GINS4	NM_032336	GINS complex subunit 4
AGPAT6	NM_178819	1-acylglycerol-3-phosphate O-acyltransferase 6
NKX6-3	NM_152568	NK6 homeobox 3
ANK1	NM_000037	ankyrin 1, erythrocytic
MIR486	NR_030161	microRNA 486
KAT6A	NM_001099412	K(lysine) acetyltransferase 6A
AP3M2	NM_006803	adaptor-related protein complex 3, mu 2 subunit
PLAT	NM_033011	plasminogen activator, tissue
IKBKB	NM_001556	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
POLB	NM_002690	polymerase (DNA directed), beta
DKK4	NM_014420	dickkopf homolog 4 (Xenopus laevis)
VDAC3	NM_005662	voltage-dependent anion channel 3
SLC20A2	NM_001257180	solute carrier family 20 (phosphate transporter), member 2
C8orf40	NM_138436	chromosome 8 open reading frame 40
CHRNA3	NM_000749	cholinergic receptor, nicotinic, beta 3
CHRNA6	NM_001199279	cholinergic receptor, nicotinic, alpha 6

Fig. 2 Missense variations of *FGFR1* identified in the present study

A Electrochromatograms delineating p.V102I and p.S107L variations. Asterisks indicate mutated nucleotides. **B** Luciferase reporter construct utilized in the present study. **C** Representative results of luciferase assays. Relative luciferase activities of the empty expression vector (empty), the wildtype vector (WT), the variant *FGFR1* vectors (p.V102I and p. S107L), and WT plus the variant vectors (1:1) are shown.

Fig. 3 Brain magnetic resonance imaging of the deletion-positive patient. Chiari type I malformation, syringomyelia, and apparently normal pituitary and stalk are shown.

diagnosed as having epilepsy. Brain magnetic resonance imaging delineated Chiari type I malformation and syringomyelia, together with an apparently normal pituitary and stalk (Fig. 3). She had a normal sense of smell, although we did not perform a quantitative examination of olfactory function.

Endocrine evaluation delineated a combined deficiency of pituitary hormones (Table 3). Blood GH lev-

els were normal at the baseline, but did not increase after insulin stimulation. Gonadotropin levels were low at the baseline, and did not respond to GnRH stimulation. Basal and TRH-stimulated values of TSH remained within the reference ranges, while the basal level of free T4 was low. Basal and TRH-stimulated levels of prolactin were normal.

Table 3 Endocrine data of the deletion-positive patient

Hormone	Stimulus (dosage)	Basal value	Peak value	Peak response ^a
GH (ng/mL) ^b	Insulin (0.1 U/ kg) ^c	4.3 (0.2-23.0)	4.4 (> 6.0)	at 90 min.
LH (mIU/mL)	GnRH (100 µg/m ²)	0.1 (0.4-4.1)	0.7 (8.5-15.5)	no peak
FSH (mIU/mL)	GnRH (100 µg/m ²)	0.4 (4.8-10.4)	1.6 (8.3-20.2)	no peak
TSH (µU/mL)	TRH (10 µg/kg)	2.4 (0.9-5.0)	10.8 (5.0-26.5)	at 30 min.
Prolactin (ng/mL)	TRH (10 µg/kg)	7.2 (1.4-14.6)	18.9 (> 13)	at 30 min.
ACTH (pg/mL)	Insulin (0.1 U/ kg) ^c	54.4 (7.4-55.7)	89.0 (> 30)	at 60 min.
Cortisol (µg/dL)	Insulin (0.1 U/ kg) ^c	13.4 (3.9-21.3)	18.9 (> 18.0)	at 60 min.
Estradiol (pg/mL)		14 (36-113)		
Free T4 (µg/dL)		0.4 (0.9-1.7)		
IGF1 (ng/mL)		200 (119-389)		

The conversion factor to the SI unit: GH, 1.0 (µg/L); LH 1.0 (IU/L), FSH 1.0 (IU/L), TSH, 1.0 (mIU/L); ACTH, 0.22 (pmol/L); cortisol, 27.59 (nmol/L); estradiol, 3.671 (pmol/L); free T4, 12.87 (pmol/L) and IGF1, 0.131 (nmol/L). Reference range in age-matched individuals are shown in parenthesis. Hormone values below the reference range are boldfaced.

^a Blood sampling during the provocation tests: 0, 30, 60, 90, and 120 minutes. ^b GH was measured using the recombinant GH standard, and the peak GH value of 6.0 ng/mL are used as the cutoff value for GH deficiency.

^c Sufficient hypoglycemic stimulations were obtained during all the insulin provocation tests.

Discussion

Copy-number analyses identified a submicroscopic deletion involving *FGFR1* in one of 69 patients with CPHD. The phenotype of the deletion-positive patient, such as brain anomalies and CPHD, is likely be associated with *FGFR1* haploinsufficiency, because none of 55 other genes involved in the deletion are known to play a role in the brain development (NCBI database, <http://www.ncbi.nlm.nih.gov/>; UCSC Genome Browser, <http://genome.ucsc.edu/>). However, we could not exclude the possibility that this deletion is a non-pathogenic variant, because parental samples of the patient were not available for genetic analysis. In this regard, it is worth mentioning that previous studies have shown that *FGFR1* is clearly expressed throughout the neuroepithelium of the developing brain including Rathke's pouch [6], and FGF signaling regulates the proliferation and differentiation of cultured pituitary progenitor cells [9, 10]. In addition, the CPHD phenotype has also been described in a patient with a large interstitial deletion on 8p11.2 affecting *FGFR1* [8]. These results, in conjunction with previous findings that *FGFR1* mutations and deletions are present in a certain part of patients with IHH, KS, and SOD [3, 7], suggest that *FGFR1* abnormalities can be associated with various developmental defects of the anterior midline in the forebrain. Nevertheless, CPHD seems to be a rare manifestation among patients with

FGFR1 abnormalities, because the majority of previously reported patients with mutations or deletions of *FGFR1* had no hormonal abnormalities with the exception of gonadotropin deficiency [3, 13]. These results can be explained by assuming that during development, GnRH neurons are more vulnerable to *FGFR1* abnormalities than the other parts of the anterior midline in the forebrain. Furthermore, the high frequency of gonadotropin deficiency and the rarity of other pituitary hormone deficiencies in patients with *FGFR1* abnormalities are consistent with the results of *in vitro* assays; Norlin *et al.* have suggested that in the developing pituitary, FGFs play a particularly important role in the specification of the *POU1F1*-independent progenitor cells including gonadotrophs [9].

The deletion-positive patient manifested a combined deficiency of GH, LH and FSH. In addition, normal TSH values along with a low free T4 level are indicative of central hypothyroidism [14]. Since endocrine evaluation of this patient was fragmentary, it remains unknown whether the primary lesion of her hormonal defects resides in the hypothalamus or in the pituitary. Further studies, such as GHRH stimulation tests or GnRH stimulation tests after GnRH priming, will clarify the origin of CPHD in patients with *FGFR1* abnormalities.

Mutation analysis identified two heterozygous *FGFR1* variations (p.V102I and p.S107L) in two of 69 patients with CPHD. *In vitro* assays indicated that heterozygosity of the p.V102I and p.S107L vari-

ants resulted in nearly normal transactivating function. Furthermore, p.V102I and p.S107L were found in a small subset of unaffected individuals. Thus, these two substitutions are more likely to be benign polymorphisms rather than disease-causing mutations. These results imply that *FGFR1* intragenic mutations account for only a minor part of the etiology of CPHD, if any. However, given the relatively small number of patients analyzed in the present study, further studies are necessary to clarify the contribution of *FGFR1* mutations to the development of CPHD.

In summary, the present study indicates a possible role of *FGFR1* in anterior pituitary function and the rarity of *FGFR1* abnormalities in patients with CPHD.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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