De novo Frameshift Mutation in *Fibroblast Growth Factor 8* in a Male Patient with Gonadotropin Deficiency

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Established Facts

- Missense, nonsense, and splice mutations in *Fibroblast Growth Factor 8* (*FGF8*) have been identified in patients with hypothalamo-pituitary dysfunction and craniofacial anomalies.

Novel Insights

- *FGF8* frameshift mutations account for a part of the etiology of hypothalamo-pituitary dysfunction and craniofacial anomalies.
- Micropenis in patients with *FGF8* mutations can be ascribed to gonadotropin deficiency and impaired outgrowth of the anlage of the penis.

Key Words

Fibroblast Growth Factor 8 · Frameshift mutation · Gonadotropin deficiency · Hypothalamo-pituitary dysfunction

Abstract

Background/Aims: Missense, nonsense, and splice mutations in the *Fibroblast Growth Factor 8* (*FGF8*) have recently been identified in patients with hypothalamo-pituitary dysfunction and craniofacial anomalies. Here, we report a male patient with a frameshift mutation in *FGF8*. **Case Report:** The patient exhibited micropenis, craniofacial anomalies, and ventricular septal defect at birth. Clinical evaluation at 16 years and 8 months of age revealed delayed puberty, hypos-

mia, borderline mental retardation, and mild hearing difficulty. Endocrine findings included gonadotropin deficiency and primary hypothyroidism. **Results:** Molecular analysis identified a de novo heterozygous p.S192fsX204 mutation in the last exon of *FGF8*. RT-PCR analysis of normal human tissues detected *FGF8* expression in the genital skin, and whole-mount in situ hybridization analysis of mouse embryos revealed *Fgf8* expression in the anlage of the penis. **Conclusion:** The results indicate that frameshift mutations in *FGF8* account for a part of the etiology of hypothalamo-pituitary dysfunction. Micropenis in patients with *FGF8* abnormalities appears to be caused by gonadotropin deficiency and defective outgrowth of the anlage of the penis. © 2013 S. Karger AG, Basel

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Introduction

Fibroblast growth factor (FGF) 8 (FGF8, NP_149353.1) is the major ligand of FGF receptor 1 (FGFR1) and plays a critical role in formation of the anterior midline in the forebrain [1–5]. Animal studies have indicated that FGF8 regulates the development of GnRH neurons in a dose-dependent manner [4, 5]. Recently, multiple missense mutations as well as two nonsense and one splice mutation in *FGF8* (NM_033163.3) have been identified in patients with various types of hypothalamo-pituitary dysfunction and craniofacial anomalies [1, 3, 4–9]. The mutation-positive patients invariably manifest gonadotropin deficiency and/or delayed puberty, indicating that GnRH neurons are highly vulnerable to impaired function of FGF8 [1, 3, 4–9]. Furthermore, mutations in several genes involved in the FGF8–FGFR1 network have been shown to underlie gonadotropin deficiency [1].

However, given the small number of reported patients, further studies are necessary to clarify the mutation spectrum and phenotypes of *FGF8* abnormalities. For example, frameshift mutations in *FGF8* have not been identified, and the underlying mechanisms of genital anomalies in patients with *FGF8* mutations have poorly been investigated. Here, we report a male patient with a de novo frameshift mutation in *FGF8*.

Subjects and Methods

Case Report

The male patient was born to non-consanguineous Japanese parents at 38 weeks' gestation. At birth, the patient manifested micropenis, phimosis, and hypoplastic scrotum. He also exhibited cleft lip and palate, strabismus, and ventricular septal defect. Hypospadias and cryptorchidism were absent. From 6 months of age, he underwent surgical interventions for facial and genital abnormalities. He had multiple episodes of convulsions from 12 years of age, and was treated with anticonvulsants. From infancy to early teens, his stature followed the -2.0 SD growth curve for Japanese males (fig. 1a).

At 16 years and 8 months of age, the patient was referred to our clinic for delayed puberty. Clinical assessment revealed a high-pitched voice and pubic and axillary hair of Tanner stage 1–2. His penile length was 1.5 cm (fig. 1b). Bilateral testes of ~ 1 ml were palpable in the scrotum. His stature and weight were 154.0 cm (-2.8 SD) and 52.9 kg (-0.8 SD), respectively. He had mild hearing difficulty and borderline mental retardation with a WISC-III IQ score of 71. Although he showed a response to a smell test using intravenous injection of combined vitamins (Alinamin; Takeda Pharmaceutical Co. Ltd, Osaka, Japan), he was unable to identify various odorants. Thus, he was suspected as having hyposmia. Brain magnetic resonance imaging delineated no structural abnormalities in the hypothalamus, pituitary, or olfactory bulbs (fig. 1b).

The patient manifested no symptoms associated with the heart anomaly, although cardiac evaluation showed a ventricular septal defect of approximately 2 mm. His father, mother and elder brother were clinically normal, and had heights of 164 cm (-1.2 SD), 143 cm (-2.7 SD), and 167 cm (-0.7 SD), respectively.

Endocrine examinations indicated multiple hormone deficiencies in the patient (table 1). Blood LH values were low at baseline and poorly responded to GnRH stimulation. FSH values were low-normal. Slightly elevated TSH levels and mildly decreased free T_4 levels indicated primary hypothyroidism. The IGF-1 level was low-normal. Blood levels of prolactin, ACTH, and cortisol were within the normal range. Anti-thyroperoxidase and anti-thyroglobulin antibodies were negative. Thyroid technetium-99m scintigram revealed no abnormalities. After initiation of levothyroxine supplementation therapy (50 μ g/day), TSH and free T_4 values remained within the normal range.

Mutation Analysis

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent. A genomic DNA sample from the patient was analyzed for mutations in 13 genes that have been implicated in gonadotropin deficiency: *FGFR1*, *KAL1*, *FGF8*, *PROK2*, *PROKR2*, *TAC3*, *TACR3*, *KISS1*, *KISS1R*, *GNRHR*, *GNRH1*, *CHD7*, and *NELF* [1, 7, 10, 11]. Mutations were screened by the Haloplex method (Agilent Technologies, Palo Alto, Calif., USA) on a MiSeq next-generation sequencer (Illumina, San Diego, Calif., USA). An *FGF8* mutation indicated by the screening analysis was confirmed by Sanger sequencing with primers, 5'-GCGAGTTGTGAGGGATTAGAGA-3' and 5'-GGGTGCCCTACAGGATGAG-3'. To verify the heterozygous mutation, the PCR product was subcloned into a TOPO TA cloning vector (Life Technologies, Carlsbad, Calif., USA) and the mutant and wild-type alleles were sequenced separately. Genomic DNA samples from the parents and brother were examined for the presence or absence of the *FGF8* mutation.

Expression Analysis for *FGF8*/*Fgf8* in Normal Human Tissues and Mouse Embryos

We investigated mRNA expression of *FGF8* in normal human tissues by PCR. Human cDNA samples were purchased from Clontech (Palo Alto, Calif., USA) or prepared by RT-PCR. PCR analysis of *FGF8* was performed with primers, 5'-AGTCCGAGGAGCCGAGA-3' and 5'-AAGTGGACCTCACGCTGGT-3'. As an internal control, we amplified *GAPDH* with primers, 5'-CCACCCATGGCAAATCCATGGCA-3' and 5'-TCTAGACGCGAGGTCAGGTCCACC-3'.

We also examined the expression of *Fgf8* in the developing external genitalia of mouse embryos by whole-mount in situ hybridization. An antisense cRNA fragment corresponding to nucleotide 1–978 of mouse *Fgf8* (BC048734) was utilized as a probe. The procedures were performed as described previously [12].

Results

Mutation Analysis

The patient carried a heterozygous frameshift mutation in the last exon of *FGF8* (p.S192fsX204, c.574delT)

Fig. 1. Clinical findings of the patient. **a** Growth chart. Actual height of the patient is plotted against the growth curve for Japanese boys (the mean, ± 1.0 SD and ± 2.0 SD). The arrow indicates the midparental height. **b** Upper panel: genital appearance at 16 years and 8 months of age. Lower panels: brain magnetic resonance imaging. No abnormalities are detected in the hypothalamus, pituitary, or olfactory bulbs (arrow).

Table 1. Endocrine data of the patient

	Stimulus (dosage)	Patient		Reference values ¹	
		baseline	peak	baseline	peak
At diagnosis					
LH, mIU/ml	GnRH (100 µg) ²	0.5	4.6	0.8–4.2	18.2–38.0
FSH, mIU/ml	GnRH (100 µg) ²	3.0	9.5	2.9–10.8	5.8–22.3
GH, ng/ml	Insulin (3 U) ²	0.87	3.18 ³	0.6–8.7	>6.0
Prolactin, ng/ml	TRH (350 µg) ²	4.3	14.4	1.1–9.5	–
TSH, µU/ml	TRH (350 µg) ²	8.3	23.0	0.6–5.9	5.8–30.6
IGF-1, ng/ml		301 ⁴	–	250–680	–
ACTH, pg/ml	CRH (100 µg) ²	9.5	50.3	5.3–51.1	17.2–153.3
Cortisol, µg/dl	CRH (100 µg) ²	8.4	25.8	3.9–21.3	13.1–35.6
Free T ₄ , ng/dl		0.79	–	1.0–1.7	–
Free T ₃ , pg/ml		2.8	–	2.1–4.1	–
Testosterone, ng/ml	HCG (4,000 U) ⁵	0.12	0.38	2.8–7.0	11.0–13.1
On levothyroxine treatment ⁶					
TSH, µU/ml		0.68	–	0.6–5.9	–
Free T ₄ , ng/dl		1.14	–	1.0–1.7	–

The conversion factors to the SI unit: LH, 1.0 (IU/l); FSH, 1.0 (IU/l); GH, 1.0 (μ g/l); prolactin, 43.48 (pmol/l); TSH, 1.0 (mIU/l); IGF-I, 0.131 (nmol/l); ACTH, 0.22 (pmol/l); cortisol, 27.59 (nmol/l); free T₄, 12.87 (pmol/l); free T₃, 1.54 (pmol/l), and testosterone, 3.47 (nmol/l). Hormone values above the reference range are italicized and those below the reference range are bold-faced.

¹ Reference values in age-matched males. ² GnRH, insulin and TRH i.v.; blood sampling at 0, 30, 60, 90, and 120 min. ³ Low GH values of the patient may be due to insufficient hypoglycemic stimulation; blood glucose was 89 mg/dl at 0 min, and 58 mg/dl at 30 min. ⁴ -1.7 SD. ⁵ HCG i.m. for 3 consecutive days; blood sampling on days 1 and 4. ⁶ Levothyroxine 50 μ g/day.

Fig. 2. Mutation analysis of *FGF8* and expression studies of *FGF8/Fgf8*. **a** *FGF8* mutation identified in the patient. The positions of nucleotides and amino acids correspond to NM_033163.3 and NP_149353.1, respectively. Left panel: the genomic and protein structures of *FGF8*. The white and black boxes in genome DNA indicate the non-coding and coding regions, respectively. The blue boxes in the protein depict the receptor interaction site at codons 83–207 and the red box indicates truncated amino acids.

Right panel: chromatograms of the c.574delT mutation. **b** Expression analyses of *FGF8/Fgf8*. Left panel: PCR-based cDNA screening for human *FGF8*. After 40 cycles, PCR products for *FGF8* were detected in all tissues examined. *GAPDH* is utilized as an internal control. Right panel: whole-mount in situ hybridization analysis in mouse embryos. Purple signals indicate expression of mouse *Fgf8*. d.p.c. = Days post-coitum.

(fig. 2a). No pathogenic mutations were identified in other tested genes. The p.S192fsX204 mutation was predicted to truncate the C-terminus of *FGF8* by replacing 53 amino acids with 12 aberrant amino acids (fig. 2a). This mutation affected a part of the receptor interacting site of *FGF8* (codons 83–207). The *FGF8* mutation was not identified in the parents or brother.

Expression Analysis for *FGF8/Fgf8* in Normal Human Tissues and Mouse Embryos

PCR-based cDNA screening indicated that human *FGF8* is expressed in a range of tissues including the hypothalamus, pituitary, thyroid gland, heart, and genital skin (fig. 2b). Whole-mount in situ hybridization indi-

cated that mouse *Fgf8* is expressed in the epithelium of the outmost part of the urogenital sinus before outgrowth of the anlage of the penis (genital tubercle) (10.5 days post-coitum) and in the epithelium of distal urethral plate during development of the genital tubercle (11.5 and 12.5 days post-coitum) (fig. 2b).

Discussion

We identified a de novo *FGF8* frameshift mutation in a Japanese patient with gonadotropin deficiency and multiple complications. The p.S192fsX204 mutation resides in the last exon of *FGF8* and is likely to escape non-

sense-mediated mRNA decay [13]. However, the mutation is predicted to alter the C-terminal structure of the protein and affect the receptor interacting site. In this context, Falardeau et al. [4] indicated that a missense mutation at the 229th codon is sufficient to reduce in vitro activity. Thus, although in vitro functional assays have not been conducted for p.S192fsX204, this mutation appears to markedly impair the function of FGF8. Consistent with this, the patient manifested gonadotropin deficiency, hyposmia, and craniofacial anomalies comparable to the phenotypes of previously reported patients with nonsense, missense, and splice mutations of *FGF8* [1, 3–9]. These data indicate for the first time that frameshift mutations of *FGF8* account for a part of the etiology of hypothalamo-pituitary dysfunction and craniofacial anomalies.

We cannot exclude the possibility that the patient carries additional mutations in other genes involved in hypothalamo-pituitary function. Although *FGF8* abnormalities are known to cause gonadotropin deficiency mostly as monoallelic mutations, they can also appear in an oligogenic condition [1]. Therefore, although our patient has no mutations in the 13 known causative genes for gonadotropin deficiency, he may have mutations in other unexamined genes. Indeed, several genes including *FGF17*, *IL17RD*, *DUSP6*, *SPRY4*, and *FLRT3* have recently been implicated in gonadotropin deficiency [1].

The Japanese patient manifested severe micropenis. This phenotype is consistent with severe gonadotropin deficiency [14]. In addition, defective formation the penis during fetal period may have played a role in the development of micropenis, because outgrowth of the genital tu-

bercle in mouse embryos primarily depends on *Fgf8* signaling [12]. Indeed, we found expression of *FGF8/Fgf8* in human genital skin and in the epithelium of the mouse genital tubercle.

The patient manifested primary hypothyroidism and a congenital heart anomaly, neither of which has been reported in patients with *FGF8* mutations. Although we detected expression of *FGF8* in the human thyroid gland and heart, and several studies have revealed that FGF8 plays an essential role in formation of the cardiovascular system and thyroid gland in mice [15–20], it remains unknown whether thyroid and heart abnormalities of the patient are associated with the *FGF8* mutation.

In summary, we identified the first frameshift *FGF8* mutations in a patient with gonadotropin deficiency. The results indicate molecular diversity of *FGF8* abnormalities.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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