human reproduction

CASE REPORT *Reproductive genetics*

A case of Kallmann syndrome carrying a missense mutation in alternatively spliced exon 8A encoding the immunoglobulin-like domain IIIb of fibroblast growth factor receptor 1

Kiyonori Miura[1],*, Shoko Miura[1], Koh-ichiro Yoshiura[2], Stephanie Seminara[3], Daisuke Hamaguchi[1], Norio Niikawa[4], and Hideaki Masuzaki[1]

[1]Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Japan [2]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan [3]Reproductive Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114, USA [4]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan

*Correspondence address. Tel: +81-95-819-7363; Fax: +81-95-819-7365; E-mail: kiyonori@nagasaki-u.ac.jp

ABSTRACT: Fibroblast growth factor receptor 1 (*FGFR1*) is one of the causative genes for Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism with anosmia/hyposmia. The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing of exons 8A and 8B, respectively. To date, the only mutations to have been identified in D3 of *FGFR1* are in exon 8B. We performed mutation analysis of *FGFR1* in a 23-year-old female patient with KS and found a missense mutation (c.1072C>T) in exon 8A of *FGFR1*. The c.1072C>T mutation was not detected in her family members or in 220 normal Japanese and 100 Caucasian female controls. No mutation in other KS genes, KS 1, prokineticin-2, prokineticin receptor-2 and *FGF-8* was detected in the affected patient or in her family members. Therefore, this is the first case of KS carrying a *de novo* missense mutation in *FGFR1* exon 8A, suggesting that isoform *FGFR1-IIIb*, as well as isoform *FGFR1-IIIc*, plays a crucial role in the pathogenesis of KS.

Key words: Kallmann syndrome / *FGFR1b mutation* / fibroblast growth factor receptor 1 isoform expression

Introduction

Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia, is a clinically and genetically heterogeneous disorder. To date, five causative genes for KS have been reported: KS 1 (*KAL1*, GenBank accession M97 252), prokineticin-2 (*PROK2*, GenBank accession NM 021935), prokineticin receptor-2 (*PROKR2*, GenBank accession NM 144773), fibroblast growth factor-8 (*FGF-8*, GenBank accession NM 033163) and fibroblast growth factor receptor 1 (*FGFR1*, GenBank accession NM 023110.2).

Although sporadic cases of KS are more frequent, families with KS have been reported with X-linked recessive or autosomal dominant or recessive modes of inheritance. Mutations in *KAL1* have been found in familial cases with X-linked recessive inheritance (Franco *et al.*, 1991; Legouis *et al.*, 1991). Mutations in *PROK2* were detected in the

heterozygous state, whereas *PROKR2* mutations were found in the heterozygous, homozygous or compound heterozygous state (Dodé *et al.*, 2006). *PROKR2/PROK2* mutations with true pathogenic potential were found only in the homozygous state (Abreu *et al.*, 2008), and any dominant-negative effect of *PROKR2* mutations was ruled out (Monnier *et al.*, 2009). Mutations in *FGFR1* or *FGF8* underlie an autosomal dominant form with incomplete penetrance. Therefore, KS families harbouring heterozygous *FGFR1* or *FGF8* mutations display variable olfactory phenotypes (Dodé *et al.*, 2003; Falardeau *et al.*, 2008), and a few cases with heterozygous *FGFR1* mutations show a normosmic IHH (Pitteloud *et al.*, 2006a). The *FGFR1* gene, which is located on chromosome 8p12, comprises 18 exons (Ruta *et al.*, 1989), and various mutations, including missense and protein truncation mutations, have been reported (Trarbach *et al.*, 2007). The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing

of exons 8A and 8B, respectively (Johnson et al., 1991). To date, mutations in D3 of *FGFR1* have only been identified in exon 8B, which encodes immunoglobulin domain IIIc, suggesting that isoform FGFR1-IIIc plays a crucial role in the pathogenesis of KS (Pitteloud et al., 2006b; Trarbach et al., 2006; Dodé et al., 2007).

Here, we report for the first time a KS case carrying a *de novo* missense mutation in the alternatively spliced exon 8A of *FGFR1-IIIb*.

Materials and Methods

Patient and family

Patient (Subject II-2) was a 23-year-old Japanese woman. When she was 18 years old, she was treated at Nagasaki University Hospital because of primary amenorrhea with anosmia. Her height was 159.2 cm and her weight was 72.0 kg. Her serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E2) were less than 0.5 and 1.5m IU/ml and 10 pg/ml, respectively. Her LH frequent sampling study (sampling performed every 15 min) showed a low-amplitude pattern of LH pulsation (Fig. 1). Her brain magnetic resonance imaging (MRI) examination was negative for tumors and showed no anatomical abnormalities of the hypothalamic−pituitary region and olfactory bulbs. A scratch-and-sniff test (UPSIT, Sensonics, Haddon Hts, NJ, USA) (Doty et al., 1985), which determines ability to smell, indicated anosmia. She was diagnosed as having KS and received hormone replacement therapy for 5 years. Her mother (Subject I-1) was normosmic and had normal puberty and regular menstrual cycles. Her father (Subject I-2), elder brother (Subject II-1) and younger brother (Subject II-3) were also normosmic and had normal puberty (Fig. 2).

Molecular analysis

DNA extraction

Whole blood samples were obtained from the KS patient and from her mother, father, elder and younger brothers. All samples were collected after obtaining written informed consent and the study protocol was approved by the Institutional Review Board of Nagasaki University. Genomic DNA from lymphocytes was extracted using a QIAamp DNA

Figure 1 LH pulsation pattern in a case of KS, assayed using an LH frequent sampling study. LH frequent sampling was performed every 15 min. *Low-amplitude pattern of LH pulse.

blood mini kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions.

Sequence analysis

FGFR1 consists of 18 coding exons. Intragenic mutations were investigated by PCR amplification and sequence analysis using 14 pairs of primers, as previously described (Dodé et al., 2003; Sato et al., 2004). Genomic DNA was PCR amplified using conditions of 95°C for 12 min followed by 95°C for 30 s, 59°C for 30 s and 72°C for 60 s for 35 cycles and a final cycle of 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis, purified with ExoSAP-IT and subjected to sequencing reactions. Sequencing reactions were performed using the BigDye terminator v.3.1 kit and analyzed with an ABI PRISM 3100 Genetic Analyzer™ (Applied Biosystems). The KS patient carrying a mutation in *FGFR1* and her family members were also screened for mutations in the other genes known to be involved in KS [*KAL1*, *PROK2*, *PROKR2* and *FGF8*]. Whether the mutation leads to a change in the protein structure and function was predicted bioinformatically using the ExPASy proteomics server (http://au.expasy.org/) and PolyPhen (http://genetics.bwh.harvard.edu/pph/).

Confirmation of the alternatively spliced exon

Isolation of a full-length murine Fgfr1-IIIb showed that Fgfr1-IIIb was a transmembrane receptor (Beer et al., 2000). Although the mRNA encoding exon IIIb has been found in human (Johnson et al., 1991), the presence of sequences encoding the intracellular domain has not yet been demonstrated. Therefore, to determine the splice site of exon 8A and to detect *FGFR1-IIIb* mRNA encoding the intracellular domain, we performed RT−PCR using specific primers to amplify the splice isoform containing exon 8A. Kal23 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Primer sequences were as follows: kal2: 5′-GACAGAAGGTCGGTTATGTC-3′, Kal23: 5′-CAGATCTTGAAGC ATTCGGG-3′, Kal5: 5′-GGTGGTATTAACTCCAGCAG-3′ and Kal6: 5′-GTACAGGGGCGAGGTCATCA-3′. The BD multiple tissue complementary DNA (cDNA, MTC) panels Human I and Human II (BD Biosciences Clontech, Mountain View, CA, USA) were used to detect the expression of each isoform of *FGFR1*. PCR amplification was performed on cDNAs as follows: 94°C, 30 s; 62°C, 30 s; 72°C, 1 min; 40 cycles. PCR products were analyzed by agarose gel electrophoresis and sequenced using then ABI PRISM 3100 Genetic Analyzer™.

Results

Sequence analysis of the entire coding region of *FGFR1*, including exon−intron boundary regions, showed that the KS patient had a mutation (c.1072C>T) in exon 8A of *FGFR1-IIIb*, while the other family members did not (Fig. 2). However, the full-length *FGFR1* mRNA that includes exon 8A is not deposited in the full-length cDNA database (GenBank accession no. NM 023110.2). RT−PCR analysis indicated that most transcripts containing exon 8A were spliced to exon 8B in all adult tissues except bone marrow (data not shown). We wished to demonstrate the existence of an alternative transcript, exon 8A which was spliced to exon 9 encoding the transmembrane helix; therefore, RT−PCR products from human fetal brain were cloned and sequenced. In 1 of 27 clones exon 8A was spliced to exon 9 (designated here '*FGFR1-IIIb*', GenBank accession FJ809917, see Fig. 3B), while in the other clones exon 8A was spliced to exon 8B (designated here
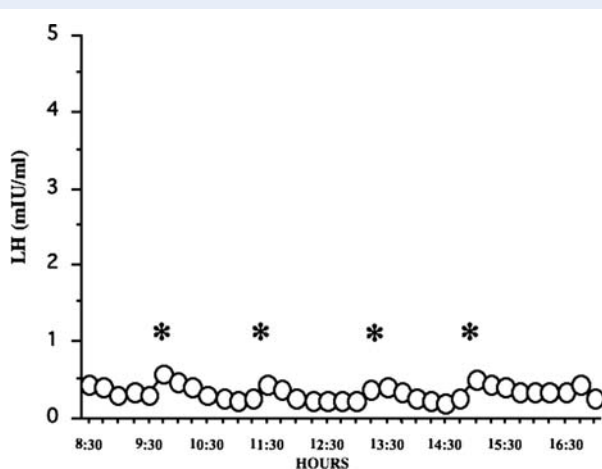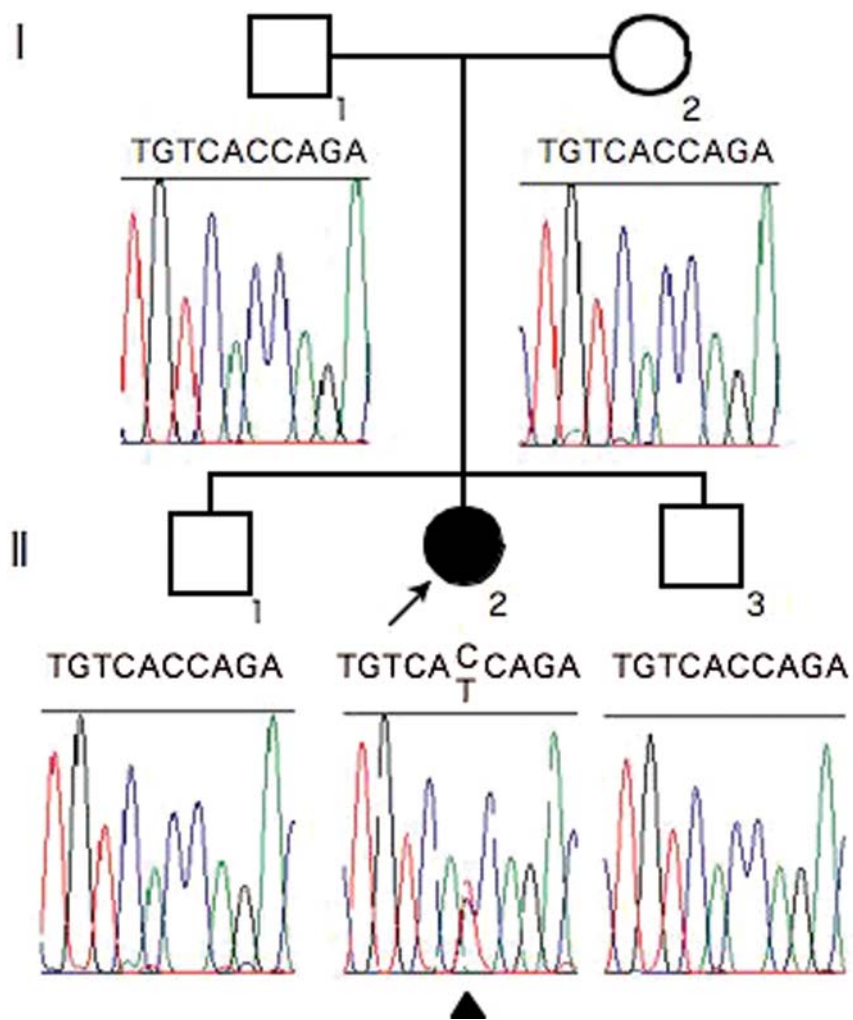
Figure 2 Pedigree of patient's family and the results of fibroblast growth factor receptor 1 (*FGFR1*) sequence analysis. II-2 is a 23-year-old woman with KS. The patient had a mutation in *FGFR1* (C>T) but the other family members did not. The mutation was *de novo* because parentage was assured. Arrowhead under the electropherogram indicates the mutation site.

'*FGFR1-secr*', GenBank accession FJ809916, see Fig. 3B). The exact acceptor and donor sites of exon 8A in '*FGFR1-IIIb*' mRNA, which produces a membrane-bound FGFR1-containing D3, were determined by sequence analysis of splice isoforms, '*FGFR1-IIIb*' and '*FGFR1-secr*', (Fig. 3). As most full-length *FGFR1* cDNAs in the database were transcripts containing exon 7−exon 8B−exon 9 (designated here '*FGFR1-IIIc*', GenBank accession NM 023110.2, see Fig. 2B) without exon 8A, '*FGFR1-IIIc*' is likely to be the most abundantly expressed human isoform. The EST, CA488712.1, was the only isoform in the EST database corresponding to '*FGFR1-IIIb*'. Although both '*FGFR1-IIIb*' and '*FGFR1-IIIc*' encode membrane-bound *FGFR1*, '*FGFR1-secr*' encodes a secreted form of *FGFR1* because of a sequence frameshift and a termination codon in exon 9.

The exons 8A and 8B of the human FGFR1 isoforms shared the amino acid sequence at 354−357; WLTV. However, exon 8A ends with six extra amino acids at 358−363, TRPVAK, whereas exon 8B ends with only two, LE. These sequences are identical in the mouse Fgfr1 isoforms (Beer *et al.*, 2000). The mutation in exon 8A of

FGFR1-IIIb (GenBank accession no. FJ809917 bankit1193625) is c.1072C>T at the cDNA level and p.T358I at the amino acid level. Bioinformatic analysis shows the mutated amino acid residue to be conserved between human and mouse and to be located in D3 of FGFR1-IIIb, which is a critical region for FGF ligand binding. However, the mutation was not predicted to produce a change in the human protein structure. The c.1072C>T mutation was not detected in 220 normal Japanese women or in 100 normal Caucasian women. The patient had no mutation in any of the other four KS genes.

Discussion

Mouse *Fgfr1-IIIb* has a low level of expression in a wide variety of adult tissues, but a high level of expression in skin and brain, indicating the existence of specific splicing factors in skin and brain that recognize the relatively weak *Fgfr1-IIIb* splice site (Beer *et al.*, 2000). Consistent with the expression pattern of mouse *Fgfr1-IIIb*, we could isolate human *FGFR1-IIIb* from a fetal brain cDNA library but not from adult
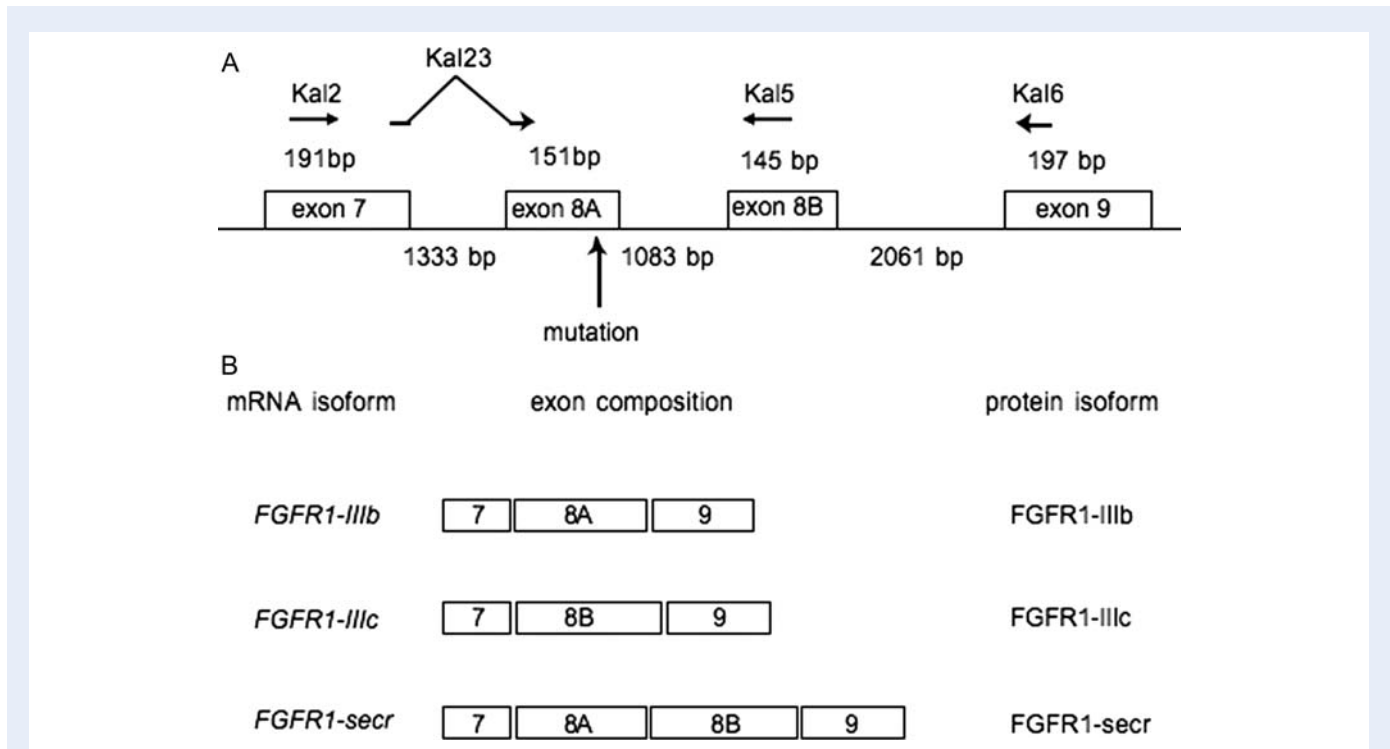
Figure 3 Genomic organization of *FGFR1* around exon 8A. (A) The numbers with bp indicate exon length (over the line) and intron length (under the line). The mutation is located near the end of exon 8A. Horizontal arrows indicate the locations of primers used to perform RT–PCR amplification of the isoform containing exons 7 and 8A. Primer Kal23 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Vertical arrow indicates mutation site. (B) Composition of mRNA isoforms and of putative protein structures. FGFR1-IIIb: membrane-bound form of FGFR1 with immunoglobulin-like domain IIIb encoded by exon 8A, FGFR1-IIIc: membrane-bound form of FGFR1 with immunoglobulin-like domain IIIc encoded by exon 8B, FGFR1-secr: a secreted form of FGFR1.

tissues. Most full-length *FGFR1* cDNAs in the database represent *FGFR1-IIIc*. *FGFR1-IIIc* is expressed at high levels, but *FGFR1-IIIb* is expressed at very low levels (Johnson *et al.*, 1991). We can amplify only a tiny amount of *FGFR1-IIIb* that has exon 8A spliced to exon 9 by RT–PCR using the primers Kal23 and Kal6. Most of the sequenced RT–PCR products corresponded to *FGFR1-secr*, suggesting that the expression level of the three isoforms is '*FGFR1-IIIc*' ≫ '*FGFR1-IIIsecr*' ≫ '*FGFR1-IIIb*' (Fig. 3B).

Several studies suggested that mutations in exon 8B of isoform *FGFR1-IIIc* are implicated in the pathogenesis of KS (Pitteloud *et al.*, 2006b; Trarbach *et al.*, 2006; Dodé *et al.*, 2007). Mice homozygous for alleles with a stop codon in exon IIIc displayed phenotypes resembling those of embryos homozygous for null alleles, while mice carrying an in-frame stop codon in exon IIIb were viable and fertile (Partanen *et al.*, 1998). Therefore, *Fgfr1-IIIc* is the dominant isoform that carries out the majority of the biological functions of the *Fgfr1* gene, whereas *Fgfr1-IIIb* plays a minor and to some extent redundant role (Partanen *et al.*, 1998). A receptor-binding analysis revealed no difference in the binding specificity between the endogenous *Fgfr1-IIIb* and an artificially created *Fgfr1-IIIb*, which had two different amino acids in the 3′-end of the unique IIIb exon (Beer *et al.*, 2000), suggesting that the carboxyl terminus of D3 may not overtly influence binding specificity. However, being expressed at low levels does not imply that

'*FGFR1-IIIb*' has an unimportant role. The mutation we found, p.T358I, was located in exon 8A of *FGFR1-IIIb*. Therefore, p.T358I may affect ligand binding and cause the KS phenotype, although how this mutation affects the loss of function of *FGFR1-IIIb* is unknown. The spatio-temporal expression of any gene involved in development is key; therefore, the expression and functional involvement of *FGFR1-IIIb* may be important in the early embryonic brain, in particular during GnRH neuronal development. KS missense mutations in *FGFR1* are distributed in the first, second and third immunoglobulin-like domains (D1–D3), in the tyrosine kinase domain and also in the intracellular domain (Dodé *et al.*, 2003; Sato *et al.*, 2004; Albuisson *et al.*, 2005; Pitteloud *et al.*, 2006a; Trarbach *et al.*, 2006; Dodé *et al.*, 2007); therefore, the membrane-bound form of *FGFR1* is probably important for the KS phenotype. The membrane-bound form of *FGFR1-IIIb* could, therefore, be the critical isoform for the KS phenotype.

We present here, for the first time, a case of KS carrying a missense mutation in exon 8A of *FGFR1*, suggesting that the minor isoform '*FGFR1-IIIb*' as well as the major isoform '*FGFR1-IIIc*' has a crucial role in the pathogenesis of KS. Therefore, immunoglobulin-like domain IIIb may have an essential role in GnRH neuronal migration, which is initiated from the nasal placode and runs towards the forebrain following the olfactory sensory neuron axonal connection with the developing olfactory bulb. Further experiments are needed to show that the mutation in

exon 8A causes KS, such as expression of the mutated isoform in trans-fected cells to analyze receptor stability and signaling efficiency. Although *FGFR1* containing immunoglobulin-like domain IIIb has not been analyzed intensively, our mutation report should encourage researchers to analyze immunoglobulin-like domain IIIb function and the spatio-temporal expression of exon 8A in fetal brain development.

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