CASE REPORT

A new *FSHβ* mutation in a 29-year-old woman with primary amenorrhea and isolated FSH deficiency: functional characterization and ovarian response to human recombinant FSH

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Abstract

Context: Mutations of the *FSHβ* gene, causing in women isolated FSH deficiency and hypogonadism, are very rare and only a few have been described.

Objective: To describe the phenotype and response to recombinant human (rh) FSH of a female patient with a novel homozygous loss-of-function mutation of *FSHβ*, and to characterize *in vitro* the molecular mechanisms responsible for the FSH inactivation.

Patient: A 29-year-old woman with primary amenorrhea and impaired pubertal development associated with isolated FSH deficiency.

Methods and results: Sequencing of the *FSHβ* gene revealed a homozygous 1 bp (G) deletion at codon 79 (c.289delG) of exon 3 which produced a frameshift at codon 79 (A79fs108X) and a premature stop codon at codon 109. The wild-type and mutant *FSHβ* cDNAs inserted into expression vector were cotransfected into Chinese hamster ovary cells with the α -subunit. Wild-type FSH was readily detectable in culture medium, whereas no mutant FSH was detectable by either immunoassay or *in vitro* bioassay. Mutant *FSHβ* protein could not be detected in western blot.

In response to a 15-day treatment with rhFSH, sonography revealed multifollicular development in the ovaries. Circulating levels of estradiol and inhibin B were dramatically increased, whereas anti-Müllerian hormone decreased. Serum LH first decreased and then increased, inducing multiovulation associated with supraphysiologic progesterone and inhibin A levels.

Conclusion: A novel *FSHβ* mutation was detected in a hypogonadal woman. rhFSH was effective in ovulation induction in the patient but with signs of ovarian hyperstimulation. The high pretreatment LH levels could contribute to this excessive ovarian response to rhFSH.

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Introduction

The pituitary gonadotropins, LH and FSH regulate the production of sex steroids necessary for the pubertal development and fertility. Inherited genetic defects that cause hypogonadism in females have been identified at multiple levels of the hypothalamic–pituitary–gonadal axis, including GnRH, its receptor, GPR54/KISS1R, TAC3/TACR3, gonadotropin subunits, genes involved in autosomal Kallmann syndrome and CHARGE

syndrome, and gonadotropin receptors (1–17). Inactivating mutations in the *FSHβ* gene have been found to date in five women with delayed puberty and isolated FSH deficiency (8, 10, 11, 16–19). These very rare natural mutations in the *FSHβ* subunit gene are nevertheless interesting models to elucidate several aspects of the gonadotropin-dependent ovarian physiology and physiopathology. In this study, we report and characterize *in vitro* a novel *FSHβ* mutation and demonstrate that the mutant *FSHβ* displays impaired

immunoreactivity and bioactivity *in vitro* and failure of expression at protein level, confirming that this newly described mutation causes the phenotype of isolated FSH deficiency. We also had the opportunity to study in detail the pituitary and ovarian responses in this patient to sustained recombinant human (rh) FSH administration.

Case report

The proband (Subject II-1 in Fig. 1A), a Caucasian woman of Spanish origin, was referred to our department at the age of 29 years for primary amenorrhea. She had undergone incomplete pubarche at the age of 18, but had no evidence of thelarche. At 29 years, she was 165 cm tall, with a bone age of 16 years. Breast development was Tanner stage 2 and pubic hair P4, and there was no hirsutism, acne or seborrhea. There was no family history of delayed puberty or infertility, but her parents were first cousins (Fig. 1A). The patient's karyotype was 46, XX. Initial endocrinological evaluation (Table 1) showed undetectable basal and GnRH-stimulated (100 µg i.v.) levels of serum FSH, measured with three different assays. In contrast, serum LH was high and rose after GnRH stimulation from 49 to 170 IU/l at 30 min, and to 130 IU/l at 60 min. Serum estradiol (E₂) was very low but testosterone,

androstenedione, and DHEAS levels were within the normal range for age. Inhibin B was not detectable, but serum anti-Müllerian hormone (AMH) was in the normal range for age. Prolactin (8.9 µg/l), thyrotropin (2.2 U/l; normal 0.5–4.5 U/l), and free thyroxine levels were normal. Ultrasound examination revealed a small uterus (volume 5.6 cm³) and two small (1.0 and 1.6 ml) ovaries with few follicles of <3 mm diameter. Bone mineral density of the lumbar spine was very low (0.71 g/cm³; Z score –2.6). Magnetic resonance imaging of the brain and pituitary gland revealed no abnormalities.

Because the patient desired to have her fertility assessed, rhFSH treatment was proposed.

Methods

Hormone assays

FSH was measured by three different immunoassays as previously reported (1, 20, 21). LH, inhibin B, and AMH were measured with sensitive immunoradiometric or ELISA assays as previously described (22). Plasma E₂, progesterone, testosterone, androstenedione, and DHEAS were measured by RIA as reported elsewhere (23). Serum inhibin A was measured as reported before (24).

Figure 1 Family pedigree and *FSHβ* new mutation. (A) Family pedigree. Black symbol indicates the affected patient. Half-shaded symbols indicate unaffected heterozygotes. Circles represent females and square male family members. The arrow indicates the proband. (B) Result of automatic sequencing of exon 3 of *FSHβ* of the proband in comparison to the respective wild-type sequence. The deleted guanine at position c.289 is indicated by an arrow on the wild-type sequence. In-frame amino acids are indicated above each sequence. (C) RFLP analysis of the mutated *FSHβ* sequence. The deletion (c.289delG) eliminates a *MscI* restriction site. PCR-amplified fragment of exon 3 in the absence of *MscI* (ND, not digested). After digestion of a PCR-amplified fragment of exon 3, normal PCR product is resolved into two bands of 140 and 250 bp (D, digested). As expected, cleavage of the PCR product was not observed in the proband (individual II.1). We observed three bands after digestion of PCR products of the mother (subject I.1.) and the father (subject I.2.), according to their heterozygous status.

Table 1 Initial hormonal evaluation of the patient.

	Basal	Normal range ^a
FSH IEA (IU/l)	<0.4 ^b	2.8–7.1
FSH IRMA (IU/l)	<0.05 ^b	3.2–8
FSH RIA (IU/l)	<0.1 ^b	2.9–7.8
LH (IU/l)	46–59 ^c	2.8–6.9
α -Subunit (μ g/l)	0.85	0.2–0.6
Estradiol (ng/l)	7	28–76
Progesterone (μ g/l)	<0.4	<0.4
Testosterone (μ g/l)	0.4	0.2–0.6
Androstenedione (μ g/l)	1.5	0.8–1.6
17-Hydroxyprogesterone (μ g/l)	0.32	0.2–0.7
DHEAS (μ g/l)	1887	400–3560
Inhibin B (ng/l)	<10 ^b	60–125
Inhibin A (ng/l)	<10 ^b	<10
AMH (pmol/l)	31	5–52
Prolactin (μ g/l)	8.5	10–20

Conversion to SI units: prolactin: μ g/l $\times$ 27 = mIU/l; DHEAS: μ g/ml $\times$ 2.721 $\times$ 1/1000 = μ mol/l; progesterone: μ g/l $\times$ 3.18 = nmol/l; Δ 4 androstenedione: μ g/l $\times$ 3.49 = nmol/l; testosterone: μ g/l $\times$ 3.467 = nmol/l; estradiol: ng/l $\times$ 3.671 = pmol/l. IEA, immunoassay; AMH, anti-Müllerian hormone.

^aIn early follicular phase in premenopausal women (<35 years).
^bBelow the limit of detection of the assay.
^cNadir and peak during LH pulsatility study.

DNA sequencing and analysis

The propositus and her parents gave their informed consent for genetics analysis (French bioethics law no. 2004-800). Genomic DNA was extracted from white blood cells using standard procedures. The regions spanning exon 2 and exon 3 encoding the FSH β subunit protein were amplified by PCR with primers described in (Supplementary data, Table 1S, see section on supplementary data given at the end of this article). A negative control, containing all reagents except DNA, was included in each PCR. PCR products were sequenced using the GenomeLab Dye Terminator Cycle Sequencing Kit (DTCS, Beckman Coulter, Rassy, France) and analyzed on the CEQ 8000 Genetic Analysis System (Beckman Coulter). In addition, to perform restriction fragment length analysis in the kindred, PCR products were digested with *MscI* (New England BioLabs, Ozyme, France) as recommended by the manufacturer, and the products were resolved on 1.5% agarose gel electrophoresis.

Preparation of expression plasmids

The common α plasmid, PM2 α FSH β (Supplementary Figure 1S, see section on supplementary data given at the end of this article), was kindly provided by Dr Jeffrey Weiss (Northwestern University, Chicago, IL, USA). Human FSH β plasmid, PKR8 (Supplementary Figure 1S), was kindly provided by Dr James A Dias (Wadsworth Center for Laboratories and Research, Albany, NY, USA). Both the plasmids harbored the neomycin resistance gene.

Oligonucleotide-mediated site-directed mutagenesis was performed to create the deletion detected in the mutated FSH β using the QuikChangeII Site-Directed

Mutagenesis Kit (Stratagene, Cambridge, UK), following instructions of the manufacturer. Clones selected by ampicillin were directly sequenced to determine that they contained the correct mutation. The entire sequence of each mutant cDNA was then determined. To confirm that the lack of expression of mutant FSH β protein was due to the specific mutation, the mutated FSH β plasmid was mutated back to the wild-type sequence. To exclude the possibility that the mutant FSH was not recognized by our monoclonal FSH antibody, a FLAG tag was inserted in-frame into the C-terminal coding sequence just before the natural stop codon of wild-type FSH β and a premature stop codon to the mutant FSH β . The insertion of polypeptides at the C-termini of human glycoprotein hormone β -subunits does not affect the α/β heterodimerization (25). Direct sequencing was carried out to confirm fidelity of the recombined sequences.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection and grown in DMEM with F12 nutrient mixture (Invitrogen) supplemented with 10% fetal bovine serum. CHO cells were transiently cotransfected with the pM2 α -subunit plasmid and the mutant or wild-type FSH β construct using Lipofectamine 2000 (Invitrogen). RNA was extracted and subjected to RT-PCR for FSH β and α -subunit mRNA to confirm successful transfection, using appropriate negative controls (tubes without reverse transcriptase). Cellular media was collected 48 h after transfection and stored at -20°C before FSH immunoassay and bioassay. Cell lysates were also collected after 48 h of transfection and stored at -20°C for western blots.

Homologous FSH in vitro bioassay

Immortalized murine granulosa (KK-1) cells (26) stably transfected with the human FSH receptor (KK-1-hFSHR cells) were grown in DMEM with Ham's F12 nutrient mixture (Sigma) supplemented with 10% fetal bovine serum, 200 mM L-glutamine and 300 g/l of geneticin. The KK-1-hFSHR cells were cotransfected with a cAMP-responsive luciferase reporter gene and a Renilla luciferase reporter plasmid. Forty-eight hours after transfection, the cells were treated with diluted FSH standards (WHO71/223) or samples (100 μ l of cell-conditioned medium from wild-type or mutant FSH producing cell lines) for 4.5 h at 37°C . The medium was removed after incubation, and the cells were subjected to the luciferase reporter assay (Luminescence reporter gene assay system, PerkinElmer), by adding 100 μ l of assay buffer, shaking in darkness for 10 min, and measuring luciferase activity using a luminometer (PerkinElmer-Wallac Victor, Turku, Finland). Thereafter, 50 μ l of Renilla mix (5 ml 0.5 M HEPES, 400 μ l 0.5 M EDTA,

10 µl coelenterazine dissolved in 1 mg/ml of methanol) was added to the cells, kept in darkness for 10 min, and measured for luciferase activity in the luminometer. The luciferase/Renilla ratio was used as read-out in the FSH bioassay. The limit of detection was 3.3 IU/l.

Immunoassay

Cell-conditioned medium was assayed for immuno-reactive FSH using a two-site sandwich assay (Abbott Diagnostics) with a limit of detection of 0.5 IU/l.

Western blot

Whole cell protein extracts were prepared in lysis buffer (50 mM HEPES (pH 7.5–7.9), 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mix (Complete protease inhibitor tablets, Roche Applied Science)). Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories). Selected samples were treated with a 10 µM concentration of the proteasome inhibitor MG-132 for 3 h before harvesting. For western blot analysis, 30 µg protein were resolved on 4–12% gradient gels (NuPAGE Bis-Tris SDS9 gels (Invitrogen)) under denaturing conditions and transferred to PVDF blotting membrane (Invitrogen). The membrane was blocked with 5% milk in TBST (50 mM Tris (pH 7.6), 100 mM NaCl, 0.1% Tween 20) for 1 h, incubated with monoclonal anti-FSHβ antibody 46.3h6.b7 produced in mouse (recognizing the regions demarcated by amino acids 33–53, 49–67, and 66–85 of the β-subunit, kindly provided by Dr James A Dias), monoclonal anti-β-actin antibody produced in mouse (Abcam, Cambridge, UK), or polyclonal anti-FLAG antibody produced in rabbit (Sigma) overnight, followed by washing three times with TBST for 5 min. The membrane was then incubated with the second antibody, i.e. a HRP-conjugated goat anti-mouse antibody or a goat anti-rabbit antibody (1:2000 dilution; DAKO, Cambridgeshire, UK) for 1.5 h and detected with ECL reagent (Amersham) after three final washes with TBST for 5 min. Finally, the membranes were exposed to X-ray film (GE Healthcare, Cambridge, UK).

Ovarian stimulation with rhFSH

Informed consent was obtained from the patient before rhFSH treatment that was requested by the patient in order to assess her fertility potential. The study was approved by the institutional review boards at Bicêtre Hospital/Paris Sud 11 University.

rhFSH (GONAL-f, Laboratoires Merck-Serono) was administered in a daily dose of 150 IU s.c. (20, 27–29) for 5 days, followed by 75 IU/day for the subsequent 10 days. The patient did not receive any exogenous human chorionic gonadotropin. The medication was discontinued according to protocol criteria, because several ovarian follicles were found to exceed the

diameter of 25 mm on day 15. Blood samples were drawn, and transabdominal pelvic and endovaginal pelvic sonography were performed basally on every 5 days during the rhFSH treatment. Pelvic sonography was also performed on 3, 10, and 20 days after discontinuation of the treatment in order to monitor ovarian and follicle sizes. Menses occurred 10 days after interruption of the rhFSH treatment, and a supplementary blood sample was obtained at that time.

Results

Molecular and functional studies

FSHβ mutation Sequencing of exon 3 of *FSHβ* revealed a homozygous 1 bp (G) deletion at codon 79 (c.289delG) (Fig. 1B). This mutation produces a frame-shift at codon 79 (A79fs108X), altering the C-terminal FSHβ subunit amino acid sequence 79–108 and introducing a premature stop codon at position 109. Both parents were heterozygous for the mutation. The deletion eliminates a *MscI* restriction site. Digestion of the PCR-amplified fragment of normal exon 3 (390 bp in size) with *MscI* produces two bands of 140 and 250 bp size (Fig. 1C, control). As expected, the PCR product was not digested in the proband (Fig. 1C, individual II.1). We observed three bands after digestion of the PCR products of the mother (Fig. 1C, individual I.1) and the father (Fig. 1C, individual I.2), according to the heterozygous status for the mutation. The frame-shift mutation was not found in 200 chromosomes from eugonadic Caucasian controls.

Immunological and biological activities of the mutated FSH Both the wild-type and mutant *FSHβ* cDNAs were cotransfected with the pM2α plasmid. Similar levels of mRNAs encoding the α- and β-subunits were confirmed in the cells expressing the wild-type or mutant *FSHβ* by RT-PCR (data not shown). Wild-type FSH was readily detectable in culture medium by immunoassay, whereas no mutant FSH protein was detectable (<0.5 IU/l; Fig. 2A). In bioassays of culture medium with cells expressing the human FSH receptor, wild-type FSH was detected, but mutant FSH was undetectable (<3.3 IU/l; Fig. 2B).

Western blot analysis of the mutated FSHβ subunit

We investigated the consequence of the mutation for FSHβ protein expression by carrying out western blots on transfected CHO cell lysates. Wild-type *FSHβ* and rhFSH were detected as expected (Fig. 2C). However, mutant *FSHβ* could not be detected, even following treatment with the proteasome inhibitor MG-132 (Fig. 2C). To confirm that the lack of the mutated FSHβ protein was due to the specific mutation, we mutated the construct back to wild-type *FSHβ*. Western blot analysis showed the same band in cells transfected with the back-mutated plasmid as in cells transfected

Figure 2 *In vitro* studies of mutated *FSHβ*. (A) Immunoassay of FSH produced by CHO cells coexpressing mutated (A79fs108X) and wild-type (wt) *FSHβ* with α -subunit. Cells transfected with empty vector were used as negative control. (B) *In vitro* bioassay of FSH produced by CHO cells coexpressing mutated (A79fs108X) and wild-type (wt) *FSHβ* with α -subunit. Cells transfected with empty vector were used as negative control. (C) Lack of FSH β protein expression in cells transfected with A79fs108X mutation of *FSHβ*. Western blot analysis was performed on cell lysates of CHO cells transfected with the α -subunit and either wild-type or mutant *FSHβ* plasmids. Equal amounts of protein were loaded, and an anti- β -actin antibody was used as the loading control. The FSH β MAB 46.3h6.b7 detected a specific band corresponding to *FSHβ* subunit in rhFSH, wild-type, and the back-mutated *FSHβ*, but not in mock-transfected control, mutant, or mutant treated with MG-132. (D) Cells transfected with α -subunit and either wild-type or mutant FLAG-tagged *FSHβ* plasmids. Immunodetection with an anti-FLAG antibody reveals a band of the expected size (about 18 kDa) for the *FSHβ* subunit in wild-type, but not in the mutant or mock-transfected control.

with wild-type *FSHβ* (Fig. 2C). To exclude the possibility that mutant *FSHβ* was expressed but not recognized by our monoclonal FSH antibody, a FLAG tag was inserted in-frame into the C-terminal coding sequence just before the natural stop codon of wild-type *FSHβ* and the premature stop codon of mutant *FSHβ*. Immunodetection with an anti-FLAG antibody showed a band of the expected size (about 18 kDa) for the wild-type *FSHβ* subunit, but not in the mutant or mock-transfected control cells (Fig. 2D). These western blot assays showed that the mutation A79fs108X failed to produce detectable FSH β protein expression.

Patient response to rhFSH administration

Hormonal response of the ovaries Baseline gonadotropin and E₂ levels before rhFSH administration confirmed isolated FSH deficiency of the woman, with undetectable serum FSH and high LH levels (Tables 1 and 2 and Fig. 3). A rise in serum FSH levels was observed during rhFSH administration with a maximum concentration of 11.5 IU/l, slightly above the upper limit normal range, on day 5. The initial increase was followed by a gradual decrease in keeping with the decreased daily dose of rhFSH administered during the

Table 2 Patient's hormonal and endometrial response to recombinant human FSH.

	Basal (D0)	D5	D10	D15	Normal range ^a
FSH IRMA (IU/l)	<0.05 ^b	11.5	8.0	2.5	3.2–8
LH (IU/l)	47	17.5	38.5	5.5	2.8–6.9
Estradiol (ng/l)	7	94	416	1760	28–76
Progesterone (μg/l)	<0.4	<0.4	4.5	68	<0.4
Inhibin B (ng/l)	<10 ^b	276	198	67	60–125
Inhibin A (ng/l)	<10 ^b	<10	12	152	<10
AMH (pmol/l)	26	5.2	9	16	5–52
Endometrial thickness (mm)	2	5	6	8	–

Conversion to SI units: progesterone: μg/l × 3.18 = nmol/l; estradiol: ng/l × 3.671 = pmol/l; AMH, anti-Müllerian hormone; ND, not determined; D, day.

^aIn early follicular phase in premenopausal women (<35 years).

^bBelow the limit of detection of the assay.

Figure 3 Circulating gonadotropin levels and ovarian hormonal responses during rhFSH administration to the patient (see text). (A) FSH, (B) estradiol (note the log scale of the Y axis), (C) LH, (D) inhibin B, (E) anti-Müllerian hormone (AMH), (F) progesterone and inhibin A.

last 10 days of treatment (Fig. 3A and Table 2). FSH fell below the limit of detection (<0.05 IU/l) 2 weeks after discontinuation of the treatment. The barely detectable basal level of plasma E_2 (7 ng/l) showed a progressive but dramatic increase to a maximum of 1760 ng/l on day 15 (day of maximum follicular size; Fig. 3B and Table 2). E_2 progressively decrease after discontinuing rhFSH and returned to undetectable levels (<5 ng/l) 7 days after the onset of menses.

The high basal concentration of serum LH (47 IU/l) dramatically decreased on day 5 of rhFSH treatment to 17.5 IU/l followed by an increase on day 10 (38.5 IU/l) and another decrease on day 15 to 5.5 IU/l (Fig. 3C and Table 2).

A rapid and dramatic increase in serum inhibin B from undetectable basal (<10 ng/l) levels to 276 ng/l occurred after 5 days of rhFSH administration (Fig. 3D and Table 2). Interestingly, at the same time, serum AMH levels decreased (from 26 ng/l to a nadir of 5.2 ng/l), followed by an increase on days 10 and 15 (Fig. 3E and Table 2). Serum AMH levels returned progressively to high pre-therapeutic levels 1 month later (29 ng/l).

During rhFSH treatment, plasma progesterone concentration increased from undetectable (<0.1 µg/l) to 68 µg/l and serum inhibin A from undetectable (<10 ng/l) to an unphysiological high of 152 ng/l on day 15, indicating the appearance of a multiovulation (Fig. 3F and Table 2). These two ovarian hormones returned to undetectable levels 7 days after the onset of menses (not shown).

Morphological ovarian and endometrial response

Ovarian sonography before rhFSH administration showed small ovaries (1.0 and 1.1 ml right and left respectively) with less than five follicles of a size below 3 mm in each (Figs 4A and 5). rhFSH administration induced an increase in ovarian size and a multifollicular development. After 5 days of treatment, the mean ovarian volume was 4.8 ml, and six and five follicles (sizes between 4 and 7 mm) were observed in the left and right ovary respectively (Figs 4B and 5). On day 10, a further increase in follicles size occurred reaching a diameter ranging between 7 and 18.3 mm

Figure 4 Effect of rhFSH administration on ovarian morphology assessed by transvaginal ultrasound. Ovarian sonography before rhFSH administration (A). After 5 days of rhFSH treatment (B). On day 10 (C), on day 15 (D) and after discontinuation of the treatment (E). The ovarian aspects returned to pre-therapeutic state, 2 months after discontinuation of rhFSH (F). The left ovary (not shown) was similar to the right.

Figure 5 Diameters (mm) of separate follicles as determined by vaginal sonography for both ovaries, before (D0) and during rhFSH treatment (see also text in Results section).

(Figs 4C and 5). Hyperstimulation syndrome with macrocysts appeared on day 15 (Figs 4D and 5), decreasing gradually after discontinuation of the treatment (Fig. 4E). The ovarian aspects returned to pre-therapeutic state, 2 months after discontinuation of rhFSH (Fig. 4F). Endometrial thickness increased by 6 mm during the treatment period (from 2 to 8 mm; Table 2).

Discussion

Normal pubertal development depends on normal secretory patterns of FSH and LH. The functions of FSH include the stimulation of follicular development and E_2 production in females and the regulation of Sertoli cell action and spermatogenesis in males. Both inactivating mutations of *FSHβ* and *FSHR* in humans (16, 17), as well as in knockout mice for the same genes (30–32) have shown that FSH is essential for normal puberty and fertility in females, particularly ovarian follicular development beyond the antral stage. In males, FSH is necessary for qualitatively and quantitatively normal spermatogenesis.

The proband with the novel A79fs108X *FSHβ* mutation presented with clinical and laboratory evidence for severe estrogen deficiency. Her serum LH concentration was appropriately increased, indicating normal maturation of the hypothalamic–pituitary–gonadal axis and an absent negative feedback. The clinical features of the proband reported here are similar to other females with genetically confirmed isolated FSH or *FSHR* deficiency (8, 10, 11, 16–19). Previously described females with *FSHβ* mutations (one homozygote for Val61X, one compound heterozygote Val61X/Cys51Gly, and three homozygotes for Tyr76X) all showed primary amenorrhea, infertility, partial pubarche, and complete or partial lack of breast

development (8, 10, 11, 16–19). The laboratory findings of these patients also showed similar findings with undetectable FSH, low estrogen, and elevated LH. The association of normal androgen levels, low ovarian volume, and presumed low antral follicular sizes in this woman displaying chronically high LH levels is quite remarkable (33) and in agreement with a previous report (34). It emphasizes the crucial role of FSH to achieve the last stages in follicular development necessary for the acquisition of LH receptors and ovarian LH responsiveness, as well as the steroidogenic enzymes involved in androgen synthesis by the theca cells (33).

The heterozygous parents in the proband were fertile, which is consistent with other previous reported heterozygous carriers of *FSHβ* and *FSHR* mutations and in line with a disorder transmitted as an autosomal recessive trait.

In vitro analysis of the A79fs108X mutation demonstrated undetectable FSH by both immunoassay and *in vitro* bioassay, which is similar to previous reports on *FSHβ* mutations (10, 11). The 1 bp deletion in codon 79 caused a frameshift that completely altered amino acids 79–108 and leads to a premature stop codon in codon 109, and amino acids 109–111 not being translated. According to the crystal structure of FSHβ (35), FSH resembles structurally members of the cystine knot growth factor family (36, 37). The β-hairpins of the molecule are stabilized and associated through six important disulfide bridges, and the cystine knot composed of β32–84, β28–82, and β3–51 is the central motif (35). The heterodimer is apparently stabilized by a ‘seatbelt’ structure formed by the disulfide β20–104 wrapping around the α-subunit (16, 25, 38, 39). The current mutant, altering amino acids 79–108, disrupts four disulfide bond pairings of hFSH, including β20–104, β32–84, β28–82, and β87–94, which may cause alteration of the cystine knot and seatbelt configurations. The cystine knot is critical for maximal intracellular stability of the subunit and for mutual recognition and subsequent interaction of the subunits (38). The mutant FSHβ protein could not be detected in the cell lysates suggesting that it is unstable and degrades rapidly. This result is consistent with another study, which demonstrated that a mutation deleting the β28–82 disulfide bond, and disrupting the cystine knot, also resulted in undetectable *FSHβ* (38). The β20–104 disulfide bond disrupted by the current mutation would also interfere with dimer formation. Because free undimerized *FSHβ* are very unstable (8, 40), defective dimerization is likely to account in part for the defective synthesis and subsequent secretion of the mutant FSH. Moreover, the sequence between β93 and β99 is known to belong to the FSH receptor-binding site (41, 42), which is also disrupted by this mutation. Together, the above observations suggest that the A79fs108X mutant of *FSHβ* can be predicted to be unstable, unable to be associated with the α-subunit, unable to be secreted,

and unable to bind to FSH receptor, leading to failure of intact hormone secretion and action. In accordance, our findings showed absence of bioassayable FSH and no FSH immunoreactivity in cells expressing the mutant *FSH β* .

In sharp contrast to the previously reported women with combined FSH and LH deficiency (20, 27–29), we show here in response to rhFSH the development of multiple follicles observed by transvaginal sonography that emerged together with a marked rise in E_2 and a significant increase in endometrial thickness. This indicated that in response to rhFSH, the high spontaneous LH levels are the prerequisite to allow adequate thecal androstenedione synthesis to provide substrate for the FSH-induced aromatase activity in granulosa cells, leading together to substantial E_2 secretion.

Another interesting characteristic in the hormone profile of the woman described here was the undetectable serum inhibin B level associated with normal serum AMH level. It suggests that ovarian inhibin B secretion in humans is mainly FSH-dependent, whereas normal AMH secretion can occur even in the absence of this gonadotropin. Furthermore, we observed a decrease in serum AMH levels in response to rhFSH. This response, also reported in women with polycystic ovarian disease receiving rhFSH (43), could be related to the *in vivo* and *in vitro* data showing that FSH treatment significantly reduces AMH levels in follicular fluid and expression in cultured granulosa cells (44).

Finally, a conspicuous finding was the exaggerated ovarian response to rhFSH in the patient, although the rhFSH doses used were the same as usually administered to female patients displaying gonadotropin deficiency (20, 27–29). The iatrogenic ovarian multifollicular hyperstimulation could therefore be related to the high spontaneous circulating pretherapeutic LH levels which could potentiate in a deleterious way the FSH induced follicular growth (45). Further work is necessary to demonstrate if a decrease in LH levels by GnRH analogs before rhFSH administration could prevent the exaggerated ovarian response in women with isolated FSH deficiency.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-09-0648>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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