A single natural loss of function mutation of the follicle stimulating hormone receptor (FSHR) has been described to date. Present in the Finnish population it markedly impairs receptor function, blocking follicle development at the primary stage and presenting as primary amenorrhea with atrophic ovaries. When Western European women with this phenotype were examined for FSHR mutations the result was negative, suggesting that other etiologies corresponding to this clinical pattern are markedly more frequent. We now describe a novel phenotype related to mutations provoking a partial loss of function of the FSHR. A woman with secondary amenorrhea had very high plasma gonadotropin concentrations (especially FSH), contrasting with normal sized ovaries and antral follicles up to 5 mm at ultrasonography. Histological and immunohistochemical examination of the ovaries showed normal follicular development up to the small antral stage and a disruption at further stages. The patient was found to carry compound heterozygotic mutations of the FSHR gene: Ile160Thr and Arg573Cys substitutions located, respectively, in the extracellular domain and in the third intracellular loop of the receptor. The mutated receptors, when expressed in COS-7 cells, showed partial functional impairment, consistent with the clinical and histological observations: the first mutation impaired cell surface expression and the second altered signal transduction of the receptor. This observation suggests that a limited FSH effect is sufficient to promote […]

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A Novel Phenotype Related to Partial Loss of Function Mutations of the Follicle Stimulating Hormone Receptor

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Abstract

A single natural loss of function mutation of the follicle stimulating hormone receptor (FSHR) has been described to date. Present in the Finnish population it markedly impairs receptor function, blocking follicle development at the primary stage and presenting as primary amenorrhea with atrophic ovaries. When Western European women with this phenotype were examined for FSHR mutations the result was negative, suggesting that other etiologies corresponding to this clinical pattern are markedly more frequent.

We now describe a novel phenotype related to mutations provoking a partial loss of function of the FSHR. A woman with secondary amenorrhea had very high plasma gonadotropin concentrations (especially FSH), contrasting with normal sized ovaries and antral follicles up to 5 mm at ultrasonography. Histological and immunohistochemical examination of the ovaries showed normal follicular development up to the small antral stage and a disruption at further stages. The patient was found to carry compound heterozygotic mutations of the FSHR gene: Ile160Thr and Arg573Cys substitutions located, respectively, in the extracellular domain and in the third intracellular loop of the receptor. The mutated receptors, when expressed in COS-7 cells, showed partial functional impairment, consistent with the clinical and histological observations: the first mutation impaired cell surface expression and the second altered signal transduction of the receptor.

This observation suggests that a limited FSH effect is sufficient to promote follicular growth up to the small antral stage. Further development necessitates strong FSH stimulation. The contrast between very high FSH levels and normal sized ovaries with antral follicles may thus be characteristic of such patients. (J. Clin. Invest. 1998, 102:1352–1359.) Key words: follicle stimulating hormone receptor • genetic mutation • premature ovarian failure • infertility

Introduction

The gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) have a central role in the control of follicle growth and function. They act through binding to their cognate receptors belonging to a subgroup of the G protein-coupled receptors (for reviews see references 1 and 2). The particularity of these receptors is to display a large extracellular domain specialized in the binding of the hormone. Genetic defects of the hypothalamo-pituitary-gonadal axis yielding hypogonadism and infertility have been described. At the level of the hypothalamus, Kallmann’s syndrome (3, 4) and the recently described mutations in the gonadotropin releasing hormone receptor gene (5, 6) can cause familial hypogonadotropic hypogonadism. At the level of the pituitary, mutations of FSHβ (7, 8) or LHβ (9) genes have also been detected.

Genetic dysfunctions of gonadotropin receptors have been described. Several cases associated with loss of function mutations of the LH receptor (LHR) have been reported in cases of complete or incomplete male pseudohermaphroditism associated with Leydig cell agenesia or hypoplasia (10–14). Such defects also yield primary amenorrhea in women (12, 15).

In the case of the FSH receptor (FSHR), a single homozygous mutation has been reported to date (Ala189Val) and seems to be very frequent in the Finnish population (16, 17). The patients have a phenotype corresponding to ovarian dysgenesis (primary amenorrhea with streak or hypoplastic ovaries). Histological examination of the ovaries showed follicular development blocked at the stage of primary follicles. After this initial report, several groups have searched for FSHR mutations in patients presenting with ovarian dysgenesis. No mutation was found in German (1), English (18), or French (our unpublished results) patients, suggesting the existence of a very peculiar Finnish isolate. In other populations, non–FSHR-related etiologies of ovarian dysgenesis thus seem to be prevalent.

The phenotype observed in the Finnish patients corresponds to a very marked impairment of FSH function. However, in the case of the thyroid stimulating hormone receptor (TSHR) and LHR, which belong to the same family as the FSHR, or in the case of the gonadotropin releasing hormone receptor, it has been suggested (5) that partial loss of function mutations is more frequent than complete inactivating mutations. Therefore, we searched for patients presenting a phenotype possibly related to such a mutation of the FSHR.

1. Abbreviations used in this paper: 3β-HSD, 3β-hydroxysteroid dehydrogenase; FSH, follicle stimulating hormone; FSHR, FSH receptor; LH, luteinizing hormone; LHR, LH receptor; P450c17, 17α hydroxylase cytochrome P450; SHBG, sex hormone binding globulin; TSHR, thyroid stimulating hormone receptor.
In this report, we describe a woman with secondary amenorrhea, very high plasma gonadotropin concentrations (especially FSH), and normal sized ovaries with follicles up to 5 mm at ultrasonography. Histological and immunohistochemical examination of the ovaries showed normal follicular development up to the small antral stage, and a disruption at further stages. The patient was found to carry compound heterozygotic mutations of the FSHR gene. The mutated receptors, when expressed in heterologous cells, showed partial functional impairment, consistent with the clinical and histological observations.

Methods

Patient. The proband was 30 yr old. She was the second daughter of two nonconsanguineous parents of Armenian origin, and was unaware of any reproductive anomaly in her family. Her sister (33 yr old) had a 3-yr-old son. Puberty had occurred at 14.5 yr with normal development of secondary sex characteristics. She had oligomenorrhea (three episodes of bleeding per year) for 1.5 yr and then developed amenorrhea at the age of 16.

At the age of 21, she consulted for infertility. Hormonal evaluations revealed high plasma gonadotropin concentrations: FSH: 108 IU/liter (normal: 1.0–9.0 IU), LH: 80.5 IU/liter (normal: 1.4–12 IU/liter), and relatively low estradiol (E2) levels: 20–40 pg/ml (63–126 pmol/liter) (normal: 20–300 pg/ml, 740–1,000 pmol/liter). She was diagnosed as premature menopause and told that her infertility could not be cured. For the next 8 yr she received intermittent treatment with oral estrogen–progestogen combinations which induced cyclical withdrawal bleedings.

The patient’s height was 156 cm and her weight was 56 kg. She had normal breast development, normal axillary and pelvic hair, and had no morphotypic abnormalities. Plasma hormone concentrations were determined by radioimmunoassay after 2 yr of treatment withdrawal. Plasma FSH and LH levels were elevated: 126.8 and 43.6 IU/liter, respectively. Plasma estradiol levels were relatively low at 21–40 pg/ml, testosterone was 0.6 ng/ml (2 nmol/liter) (normal: 0.2–1.6 ng/ml, 740–1,000 pmol/liter), and dehydroepiandrosterone was 3.9 ng/ml (13.6 nmol/liter) (normal: 2–12 ng/ml) (6.8–40.8 nmol/liter). Sex hormone binding globulin was normal: 60 nmol/liter (normal range: 47.9 ± 12.9 nmol/liter). The plasma concentration of inhibin B was relatively low: 50 pg/ml (19) in contrast to the very high concentration of plasma FSH. Thyroid function and plasma prolactin concentration were normal. No antithyroid or antiovarian autoantibodies could be detected. The karyotype was 46 XX. A progesterone test (chlormadinone acetate: 10 mg/d administered orally during 10 d) was followed by moderate bleeding lasting 4 d.

Bone mineral density, determined by dual energy x-ray absorptiometry, revealed osteoporosis according to the WHO classification (20): T-score, −2.58 and −2.60 SD, respectively, for the age of the patient.

Pelvic ultrasonography showed a small uterus (45 × 30 × 20 mm). The ovaries were slightly enlarged: 35 × 27 × 17 for the right and 29 × 16 × 14 mm for the left ovary. Numerous follicles up to 5 mm were detected in both ovaries (Fig. 1) and were interspersed throughout the ovaries.

Coelioscopy showed ovaries of an apparently normal size but with a smooth surface without any maturating follicle or corpus luteum. Two biopsies were performed on each ovary. The ovarian biopsies were performed before any stimulation by FSH.

Ovarian stimulation using recombinant FSH (Gonal-F; Serono Laboratories, Boulogne, France) was performed (21) and monitored by estradiol assays and pelvic ultrasonography. An initial dose of 150 IU was given during 5 d. The dosage was then increased by 75 IU every 5 d. A total amount of 5,625 IU was given in 20 d. There was a clear-cut but very limited response: plasma estradiol concentrations increased from 20 to 65 pg/ml and the maximal size of the follicles at sonography increased from 5 to 8.3 mm. The parents and the sister agreed to give blood samples for DNA studies but did not wish to undergo clinical investigations. The study was approved by the review boards for human research of the different institutions. Informed consent was obtained from the patient.

DNA sequencing. DNA was extracted from peripheral blood leukocytes. The 10 exons of the human FSHR gene were amplified by PCR and sequenced on both strands using primers described previously (16, 22). Automatic genomic sequencing was performed using a Taq dideoxynucleotidyl cycle sequencing kit and a 373 A sequencer (Applied Biosystems, Foster City, CA).

Construction of expression vectors encoding mutated FSHRs. The human FSHR cDNA cloned into the pSG5 expression vector has been described elsewhere (23). The mutations were introduced into the pSG5-FSHR plasmid vector by oligonucleotide-mediated mutagenesis using PCR. The Arg537Cys substitution was engineered with two mutagenic primers: a direct primer A1: GATCGCCAAGTGCACTAGGCCCAT and a reverse primer B1: CCATGCACCTTGGCCATGCTCTTG starting, respectively, at positions 1707 and 1724 of the cDNA sequence (+1 corresponds to the first nucleotide of the initiation codon. The mutated base is underlined). Two other primers were used: primer C1: GCTGCTCATTGCATCAGTTGATATCCATAC and primer D1: GAGGGCAAGTATGTAATGGAAACACACTGG starting, respectively, at positions 1245 and 2036 of the cDNA sequence. A fragment of 821 bp containing the mutation was constructed in two pieces. The first fragment was obtained by PCR using oligonucleotides A1 and D1. The second fragment was obtained using oligonucleotides B1 and C1. The full-length product of 821 bp was obtained by hybridization of the two fragments and PCR amplification using primers C1 and D1. After digestion with PstI (there are two PstI sites located, respectively, at 413 and 298 bases away from the mutation) the fragment was ligated into the pSG5-hFSHR vector previously digested with PstI.

The Ile160Thr substitution was generated using a similar strategy. Two mutagenic primers were used: a direct primer A2: ACATCCA-
CACAACGTGAAAGAAATCTTT CG and a reverse primer B2: GAATTCTTTACACGTTGTTGGATTTATG starting at position 467 and 492 of the cDNA sequence, respectively (the mutated base is underlined). The two other primers were C2: GGACCTG-GAGAAAATAGAGATCTCTCAGAA and D2: GACCCTTAGC-CTGAGTCATAATCAACTT and started at position 210 and 929 of the cDNA sequence, respectively.

The full-length fragment of 719 bp containing the Ile160Thr mutation was digested with AfIII and Bsu36I (the restriction sites were located, respectively, 141 bp upstream and 242 bp downstream from the mutation), purified, and ligated to the pSG5-FSHR vector digested with the same restriction enzymes.

All constructs were verified by double-strand sequencing.

Study of FSH binding to the wild-type and mutated receptors. COS-7 cells were transfected using Superfect (QIAGEN Inc., Chatsworth, CA). 48 h later, cells were incubated for 1 h at 30°C with 400,000 cpm/ml of iodinated FSH (sp act 135 Ci/mg; Amersham, Arlington Heights, IL) as described previously (23) in the absence or in the presence of increasing concentrations of unlabeled recombinant FSH. Nonspecific binding was determined in samples containing an excess (10 μg/ml) of unlabeled FSH and subtracted from the total binding. All experiments were performed twice in triplicate. Transfection efficiencies were estimated by cotransfecting pRSV-β-gal and measuring β-galactosidase activity in the cells. They were in a very similar range when expression vectors encoding either the wild-type or the mutated FSHRs were used.

cAMP assay. cAMP assays were performed as described previously (23) after 45 min of incubation of transfected cells with variable concentrations of FSH (10⁻¹¹–10⁻⁷ M) (Metrodine; Serono Laboratories).

Immunofluorescence and confocal microscopy. Antibody FSHR 323 (23) was used, as described previously (24, 25), to study by indirect immunofluorescence FSHR expression in transfected COS-7 cells. This antibody (5 μg/ml), which recognizes the receptor ectodomain, was incubated with the cells for 1 h at 4°C in PBS containing 1% BSA. The cells were washed and fixed for 15 min in 3% paraformaldehyde. After saturation with PBS, 1% BSA for 1 h the cells were incubated for 1 h with a Cy3-labeled rabbit anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). The cells were washed and mounted with a fluorescence mounting medium (DAKO, Santa Barbara, CA).

In some experiments, the cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.075% saponin in PBS, 1% BSA (26). The cells were then incubated with antibody FSHR 323 for 2 h at room temperature and further processed as described above.

A Zeiss microscope (Axiovert 135M) was used in conjunction with a confocal laser scanning unit (Zeiss LSM410) (24).

Histological and immunocytochemical studies of the ovaries. One biopsy from each ovary was frozen in liquid nitrogen and preserved at −196°C until use. The two other fragments were fixed in buffered 10% formalin and embedded in paraffin.

Gonadotropin receptors could only be studied on frozen sections. LH and FSHRs were immunolabeled as described (23, 27) using antibodies LHR 29 (10 μg/ml), LHR 74 (5 μg/ml), and FSHR 323 (3 μg/ml). Controls omitting primary or secondary antibodies or using antigen-saturated antibodies have been described (27). Steroidogenic enzymes were studied on frozen sections as described (28). Anti-3β-hydroxysteroid dehydrogenase (3β-HSD) (dilution 1:6,000) (29), anti-17α hydroxylase cytochrome P450 (P450c17) (dilution 1:10,000) (30), and anti-aromatase cytochrome P450 (dilution 1:3,000) (31) antibodies were used.

The steroidogenic enzymes were also studied in formalin-fixed paraffin-embedded sections. After deparaffinization and antigen retrieval, the same antibodies were used but at a twofold higher concentration (except for anti-P450c17 which was used at 1:6,000 dilution). In all cases, the bound immunoglobulins were revealed with biotinylated secondary antibodies and peroxidase-labeled streptavidin (LSAB2 immunostaining kit; DAKO) according to the manufacturer’s instructions.

Endogenous peroxidase inhibition was performed with PBS 3% H₂O₂ (Merck, Darmstadt, Germany) for 5 min. Aminoethylcarbozole (Sigma) was used as a chromogen. The sections were lightly counterstained with Meyer’s hematoxylin. Replacement of the specific primary monoclonal antibodies with preimmune mouse immunoglobulins (Sigma) of the same subclass and at the same concentration and concentration of antigen were used to control for nonspecific binding.

Figure 2. Automatic DNA sequencing (A) and location (B) of the two mutations found in the patient. Pedigree of the family (C). In A the heterozygotic mutations are indicated by arrows. In C the propositus is indicated by an arrow. Solid symbols denote affected subjects, half solid symbols denote unaffected heterozygotes, circles denote female family members and squares are male family members. Members of the family who have been studied are represented by bold symbols.
replacement of rabbit antibodies with rabbit immunoglobulins resulted in absence of staining.

Results

Sequencing of the FSHR gene. Sequencing of the complete coding region of the FSHR gene in the patient revealed two transversions (Fig. 2, A and B) yielding an Ile160Thr substitution in the sixth exon of the receptor (extracellular domain), and an Arg573Cys substitution in the tenth exon of the receptor (third intracellular loop). A control population of 50 subjects did not display these mutated amino acids. The father and the unaffected sister of the patient were heterozygous for the Arg573Cys mutation, while the mother carried only the Ile160Thr mutation (Fig. 2 C).

Expression of the mutated receptors. The mutations were engineered in vitro into expression vectors and were used for transient transfections of COS-7 cells.

Binding of 125I-FSH was studied in the presence or the absence of unlabeled FSH. There was a dramatic reduction in FSH binding of the Ile160Thr mutant, whereas the Arg573Cys FSHR mutant bound FSH with an affinity similar to that of the wild-type receptor (Fig. 3 A).

COS-7 cells transfected with the expression vectors were incubated with increasing concentrations of FSH and the accumulation of cAMP was measured. The biological activities of both mutated receptors were markedly impaired (Fig. 3 B). No stimulation was observed at low concentrations of FSH. At high concentrations of FSH, a limited stimulation of adenylate cyclase was observed for both mutants, the maximal responses being ~30% and ~10% for the Arg573Cys and the Ile160Thr mutants, respectively, when compared with the wild-type receptor.

Cell surface expression of the receptors was then analyzed. The transfected cells were either permeabilized by saponin or not treated and incubated with antireceptor ectodomain antibody (see Methods). Confocal microscopic examination of permeabilized cells showed expression of mutated and wild-type receptors (Fig. 4, a, c, and e). With nonpermeabilized cells, wild-type receptor and the Arg573Cys mutant (Fig. 4, b and f) were observed at the cell surface, whereas the expression at the cell membrane of the Ile160Thr mutant was markedly altered, no receptor molecules being detected with this method at the cell surface (Fig. 4 d).

Histological and immunocytochemical studies of the ovarian biopsies. The surface epithelium and the ovarian stroma of the patient did not differ from those in normal cyclic ovaries. Primordial, primary, and secondary follicles did not exhibit morphological abnormalities and their density in the ovarian cortex was similar to that observed in ovulating women of the same age (Fig. 5 A).

One small antral follicle (~0.5 mm) could be examined in detail. It showed a normal granulosa and theca interna which appeared hypertrophic in places. Immunohistochemistry was performed using monoclonal antibodies directed against the LHRs and FSHRs and polyclonal antibodies against steroidogenic enzymes. Immunostaining of 3β-HSD (Fig. 5 B) and P450c17 (Fig. 5 C) allowed the delineation of the hypertrophic theca interna. No aromatase cytochrome P450 activity could be detected in the granulosa cells (not shown), which is a normal observation for a follicle of 0.5 mm (31–35).

Immunocytochemical analysis of the FSHR (Fig. 5 D) showed strong staining on granulosa cells. This contrasted with the immunostaining of the LHR which was barely visible (Fig. 5 E). In normal women, follicles of a similar size exhibited strong LHR immunolabeling of the theca interna (see Fig. 5 F).

Several larger antral follicles (~2–5 mm, still corresponding to a small antral stage) were found on the paraffin-embedded sections and they showed a very characteristic staining pattern (Fig. 5, G and H). The theca interna of these follicles was markedly thickened and consisted of several layers of polygonal cells with a round central nucleus and abundant cytoplasm containing lipid droplets. These cells had the features of luteinized cells. Most of the granulosa cells had disappeared, with only very sparse cells were found in some folds. The latter cells did not express aromatase (not shown). Expression of aromatase in follicles of this size in normal women remains controversial (31–35). Immunocytochemical staining for 3β-HSD (Fig. 5 G)
and P450c17 (Fig. 5 II) confirmed the hypertrophy of the theca interna. A few cells were observed in the periphery of the theca interna which expressed aromatase (not shown). Since follicles of this size were not present in the frozen sections, we could not examine them for FSHR and LHR distribution.

**Discussion**

We report here the case of a women who presented secondary amenorrhea associated with high plasma levels of gonadotropins, especially FSH. The particularity in this context was the
finding of normal sized ovaries and follicular development proceeding up to the small antral stages.

Analysis of the patient’s DNA showed the presence of mutated amino acids in the extracellular domain (Ile160Thr) and in the third extracellular loop (Arg573Cys) of the FSHR. Hydrophobic (Val or Ile) or positively charged (Arg or Lys) residues are constantly found at these positions in FSHR, LHR, and TSHR of different species, suggesting an important func-

**Figure 5.** Conventional histology and immunocytochemical study of the ovaries of the patient. (A) Primordial and primary follicles: conventional histology of a section of paraffin-embedded ovarian tissue (×40). Normal morphology and density of the follicles. (B–E) Small antral follicle (0.6 mm). Frozen sections of the patient’s ovary were immunostained for 3β-HSD (B) (×200), P450c17 (C) (×200), FSHR (D) (×400), and LHR (E) (×400). As a control we show (F) a frozen section of an ovary obtained from a normal woman (hysterectomy for fibroma). A small antral follicle (0.7 mm) was immunostained with anti-LHR antibodies (×400). Note in the patient’s ovary the strong labeling of granulosa cells (G) with the anti-FSHR antibody, the very weak staining of some theca interna (TI) cells with the anti-LHR antibody, and the strong staining of an hypertrophied theca interna with anti-3β-HSD and anti-P450c17 antibodies. In contrast note the strong LHR immunostaining of the control follicle taken from a normal woman. (G and H) Larger antral follicle (∼2 mm). Sections were immunostained for 3β-HSD (G) and P450c17 (H). This follicle has an hypertrophied theca interna (TI) and a few residual granulosa cells. ×70.
cells were observed in the outer layers of the theca interna, have been secreted by the ovary; some aromatase-containing produced by peripheral aromatization of androgens or it may increased plasma estrogen concentration and follicular size.

with an absence of aromatase in the few cells which remained.

with nearly complete disappearance of the granulosa cells and presence of very high concentrations of FSH in blood and on more intense FSH stimulation. Due to receptor inade-

ular selection and preovulatory development are dependent secretion, or residual FSHR function) (34, 35). However, follic-

requires only moderate FSH action (low but not absent FSH mm at sonography). This confirms that basal follicular growth development proceeded up to the stage of a small antrum (5 who had incomplete alteration of FSHR function, follicular

ure revealed that approximately one-third of them had normal-

type have been observed previously (44, 46) but the etiology the small antral stages. Patients presenting with similar pheno-

amenorrhea, subnormal estrogenic activity, and normal sized ovaries including numerous follicles with development up to the small antral stages. Patients presenting with similar pheno-
type have been observed previously (44, 46) but the etiology was unknown. Such cases may not be uncommon since an ultrasonographic study of patients with premature ovarian fail-
ure revealed that approximately one-third of them had normal-sized ovaries with follicular development up to 7.2 mm (47).

Indeed, we have detected recently another patient present-
ing with a similar phenotype. DNA studies showed mutations of the FSHR. This patient is currently under study. It may be important to distinguish such cases from other syndromes such as premature menopause (which was the initial diagnosis for our patient), since the presence of ovarian follicles in these patients may allow the treatment of their infertility in the future (44, 48).

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