

Brief Report

DELAYED PUBERTY AND
HYPOGONADISM CAUSED BY
MUTATIONS IN THE FOLLICLE-
STIMULATING HORMONE β -SUBUNIT
GENE

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THE pituitary gonadotropins luteinizing hormone and follicle-stimulating hormone (FSH) regulate the production of sex steroids necessary for pubertal development and fertility. Inherited genetic defects that cause hypogonadism have been identified at multiple levels of the hypothalamic-pituitary-gonadal axis.¹ They include Kallmann's syndrome, which is caused by mutations in the *KAL* gene,² and X-linked adrenal hypoplasia, which is caused by mutations in the *DAX-1* gene.³ Both cause deficiency of hypothalamic gonadotropin-releasing hormone, and *DAX-1* mutations also cause a defect in the production of gonadotropins by the pituitary.⁴ A homozygous mutation in the gene for the β -subunit of luteinizing hormone has been reported as a cause of hypogonadism in a man,⁵ and a homozygous mutation in the gene for the β -subunit of FSH was found in a young woman with delayed puberty.⁶

Abnormalities of ovarian development and function are most often caused by gonadal dysgenesis (Turner's syndrome) and rarely by defects in the syn-

thesis of ovarian steroids.⁷ Mutations that inactivate the receptors for luteinizing hormone and FSH have also been described.^{8,9} The mutation in the luteinizing hormone receptor results in Leydig-cell hypoplasia and undermasculinization in genetic males^{8,10,11} and the FSH-receptor mutation causes primary gonadal failure and hypergonadotropic hypogonadism in females.⁹

In this report, we describe a female with isolated FSH deficiency who presented with delayed puberty. Analysis of the FSH β -subunit gene revealed compound heterozygous mutations, each of which prevented efficient combination of the α - and β -subunits to form intact FSH. The patient's heterozygous relatives were clinically normal. These findings indicate that FSH is required for follicular development and ovarian androgen and estrogen synthesis in females.

METHODS

Subjects

The proband (Subject II-1 in Fig. 1) presented at 15¹/₂ years with primary amenorrhea. She had undergone pubarche at the age of 13, but had no evidence of thelarche. She was 155 cm tall, with a bone age of 14 years. Breast development was Tanner stage 1, and there was no hirsutism. Breast development was induced by treatment with estrogen, and she was then treated with an oral contraceptive. There was no family history of delayed puberty, infertility, or consanguinity (Fig. 1B).

The following laboratory test results were obtained: serum FSH, <0.5 mIU per milliliter (normal, 2.0 to 17.2); luteinizing hormone, 21 mIU per milliliter (normal, 1.6 to 9.3); prolactin, 8.9 ng per milliliter (normal, <18); thyrotropin, 0.71 μ U per milliliter (normal, 0.5 to 4.5); estradiol, <25 pg per milliliter (<92 pmol per liter; normal, 25 to 400 pg per milliliter [92 to 1468 pmol per liter]); and testosterone, 29 ng per deciliter (1 nmol per liter; normal, 23 to 75 ng per deciliter [0.8 to 2.6 nmol per liter]). After the intravenous administration of 100 μ g of gonadotropin-releasing hormone, the serum luteinizing hormone concentration rose from 33 mIU per milliliter to 170 mIU per milliliter at 30 minutes and 130 mIU per milliliter at 60 minutes. In contrast, the serum FSH concentration was 0.6 mIU per milliliter at base line, 0.6 mIU per milliliter at 30 minutes, and 0.8 mIU per milliliter at 60 minutes. Magnetic resonance imaging of the brain and pituitary revealed no abnormalities.

The IM_x microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, Ill.) was used to measure serum FSH. The intraassay coefficient of variation was less than 4 percent and the interassay coefficient of variation was 7 percent for a mean value of 5 mIU per milliliter. The lower limit of detection was 0.2 mIU per milliliter. Luteinizing hormone was also measured by microparticle enzyme immunoassay, and other hormones were measured by radioimmunoassays. Thirty-four normal subjects (18 women and 16 men) served as controls. The study was approved by the institutional review boards at Tufts University and the University of Chicago. Informed consent was obtained from all family members studied.

DNA Sequencing and Expression of Mutant Recombinant FSH

DNA was extracted from peripheral-blood leukocytes, and all three exons of the FSH β -subunit gene were amplified by the polymerase chain reaction (PCR) as described previously.¹² The PCR fragments were screened for mutations with denaturing gradient gel electrophoresis. The denaturants were 7 M urea and 40 per-

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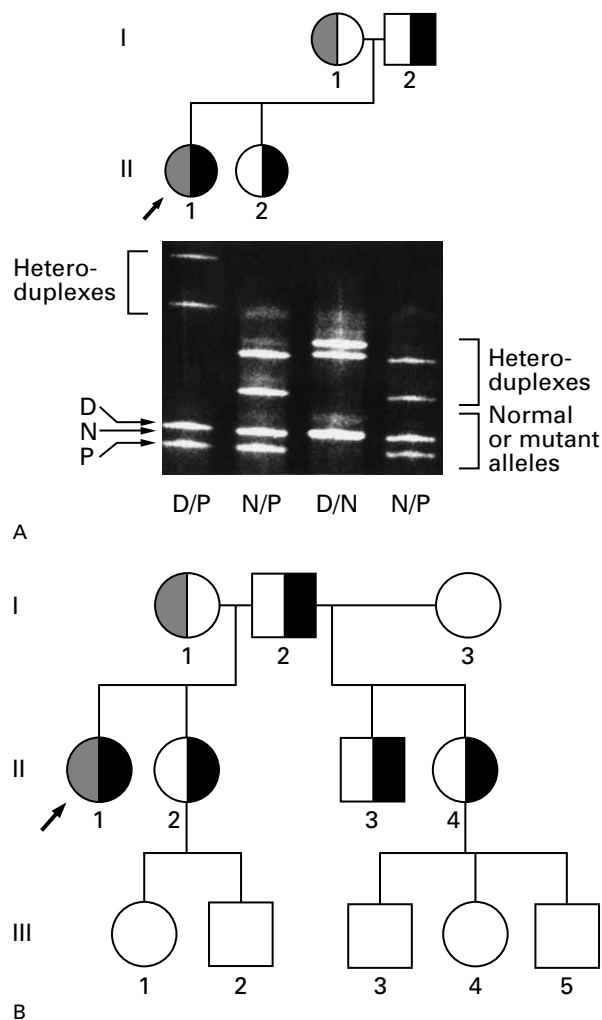


Figure 1. Screening for Mutations in the FSH β -Subunit Gene in the Pedigree with Isolated FSH Deficiency.

A partial pedigree is shown at the top of Panel A. The proband (Subject II-1) is denoted by an arrow and two half-filled symbols (representing compound heterozygosity). Heterozygotes are indicated by half-filled symbols. The results of denaturing gradient gel electrophoretic analysis of exon 3 of the FSH β -subunit gene are shown below the pedigree. Only the allelic variants are shown. The presence of heteroduplexes (the two upper bands in all four lanes), which are formed by the combination of mismatched alleles, confirms heterozygosity. Specific allelic variants — a deletion (D), a point missense mutation (P), and a normal allele (N) — that were determined by DNA sequencing are shown. The genotype is indicated below each lane, with the first letter signifying the upper band and the second letter the lower band. The proband's mother (Subject I-1) is a D/N heterozygote, although she appears to have only one allelic band because the D and N alleles migrate at similar positions on the gel. The mother's heterozygosity was revealed by the heteroduplexes. The pattern of migration of the top band (D) in the first lane (Subject II-1) and the top band (N) in the second lane (Subject II-2) is similar. Panel B shows the complete pedigree, reflecting the fertility of the heterozygotes. Squares denote male family members, and circles female family members.

cent formamide, and gels were run at 60°C. PCR products were subjected to electrophoresis for 1100 volt-hours in gels of 20 to 80 percent (exons 1 and 3) and 800 volt-hours in gels of 0 to 60 percent (exon 2).

Exon 3 fragments were cloned with the TA Cloning Kit (Invitrogen, San Diego, Calif.), and each strand was sequenced at least four times by the [³⁵S]dideoxy technique (SequiTherm Cycle Sequencing kits, Epicentre Technologies, Madison, Wis.).

Wild-type and mutant FSH β -subunit genes were expressed in transfected cells to assess the synthesis and secretion of the hormone in vitro. Both FSH β -subunit gene mutations were created with site-directed mutagenesis, confirmed by DNA sequencing, and inserted into a 3.7-kb *HindIII*-*BamHI* genomic fragment containing the entire coding region of the FSH β -subunit gene.¹³ The wild-type and mutant genes were cloned into the *BamHI* site of expression vector pM² α (kindly provided by I. Boime, Washington University, St. Louis), which also expresses the α -subunit gene.¹⁴ Constructs of pM² α -FSH β -subunit were stably transfected into Chinese-hamster-ovary cells (American Type Culture Collection, Rockville, N.Y.) as described previously.⁵ After resistant clones were selected with Geneticin (GIBCO Laboratories, Grand Island, N.Y.), expression of the α - and β -subunit messenger RNA (mRNA) was confirmed by reverse-transcriptase PCR (data not shown). FSH secreted by the cells during a 48-hour culture was measured by an immunoradiometric assay (sensitivity, 0.11 mIU per milliliter) (Diagnostic Systems Laboratories, Webster, Tex.). Bioactive FSH was measured as described previously with a cell line that expresses the FSH receptor (sensitivity, 4 mIU per milliliter).¹⁵

RESULTS

Mutations in the FSH β -Subunit Gene

The FSH β -subunit gene from the proband, unaffected family members, and 34 normal subjects was screened for mutations by denaturing gradient gel electrophoresis. No abnormalities were detected in exons 1 or 2. Analysis of exon 3 revealed a polymorphism at codon 58 (not shown); DNA sequencing revealed the codon to change from TAT to TAC, which did not alter the amino acid (tyrosine) residue. The frequency of the two polymorphic variants among the normal subjects studied followed a normal Hardy-Weinberg distribution, with 17 heterozygous and 7 homozygous for the polymorphism (data not shown) and 10 homozygous for the normal sequence.

When exon 3 fragments from the proband's family were subjected to denaturing gradient gel electrophoresis, additional variant fragments were detected that were not accounted for by the codon 58 polymorphism (Fig. 1A). DNA sequencing revealed that the allele inherited from the mother contained a 2-bp (TG) deletion at codon 61 (Val61X), whereas the allele inherited from the father contained a missense mutation that changed a cysteine (TGT) to glycine (GGT) at codon 51 (Cys51Gly) (Fig. 2A). The proband had both mutant alleles, indicating a compound heterozygous mutation in the FSH β -subunit gene. Her sister (Subject II-2), half-sister (Subject II-4), and half-brother (Subject II-3) were all heterozygous for the paternal missense mutation and a normal allele (Fig. 1B). The proband's full sister had two children, and her half-sister had three

Figure 2. Structure of the FSH β -Subunit Gene, Expression of Mutations, and FSH Concentrations.

Panel A shows the structure of the FSH β -subunit gene along with the locations of the mutations in exon 3 (arrows at codons 51 and 61). The amino acid numbers appear below the gene. The three exons are shown as rectangular boxes. The shaded regions refer to portions of the exons that are translated into protein, whereas the unshaded regions represent untranslated exonic sequences. Panel B shows the expression of the mutant FSH β -subunit genes (FSH β) in vitro. The structure of the expression vector pM $^2\alpha$ -FSH β -subunit gene is shown: the α -subunit and β -subunit genes, the promoter (LTR, or long terminal repeat), the neomycin resistance gene (*Neo^R*), and the *Bam*HI site (H1), which is the restriction site into which the FSH β -subunit gene was cloned. The α -subunit and β -subunit genes are driven by separate promoters. The direction of transcription is clockwise, as indicated by the arrows. Panel C shows the FSH concentrations measured by immunoradiometric assay of the medium from cultures of Chinese-hamster-ovary cells stably transfected with each of the mutant FSH β -subunit genes (Val61X and Cys51Gly) and the wild-type gene. Results are mean (+SD) values from triplicate wells.

children. Her half-brother had had a normal puberty, and the results of his semen analysis were normal (118 million sperm per milliliter, with 80 percent motility), but he has not attempted to father a child.

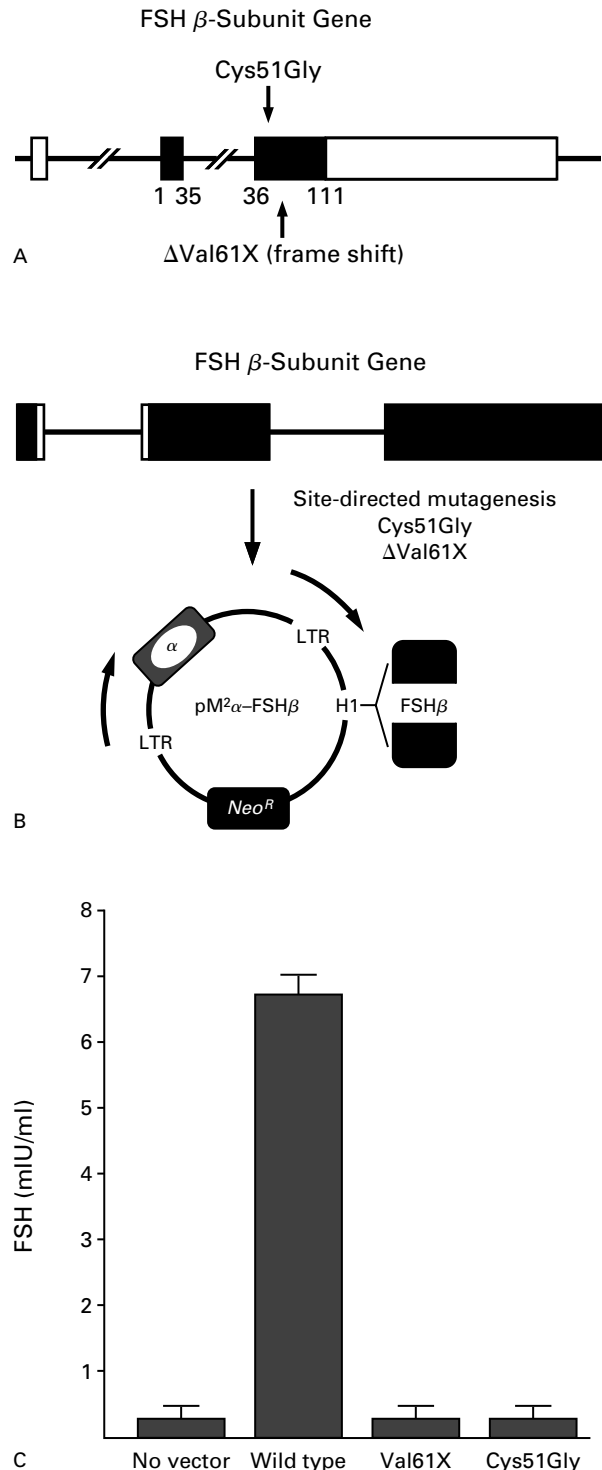
Expression of the Mutant FSH β -Subunit Genes in Vitro

Each of the FSH β -subunit gene mutations was expressed in stably transfected Chinese-hamster-ovary cells. Both normal α -subunit genes and normal or mutant β -subunit genes were placed into one vector to ensure coexpression of the genes. Similar levels of mRNAs encoding the α - and β -subunits in cell lines expressing the wild-type or mutant FSH were confirmed by reverse-transcriptase PCR (data not shown). Wild-type FSH was readily detectable (6.8 mIU per milliliter) in culture medium by immunoradiometric assay, whereas no mutant FSH was detectable (<0.11 mIU per milliliter) (Fig. 2C). In bioassays of culture medium with cells expressing the human FSH receptor,¹⁵ wild-type FSH was detected (5.7 mIU per milliliter), but mutant FSH was not (<4 mIU per milliliter).

DISCUSSION

Mutations in glycoprotein hormone genes rarely cause deficiencies in luteinizing hormone, FSH, or thyrotropin.¹ Nevertheless, these naturally occurring gene “knockouts” are revealing because they help define the physiologic roles of these hormones, and the mutations can delineate important structural domains within the hormones. In the family we describe, the proband was a compound heterozygote for two mutations in the FSH β -subunit gene, each of which precludes effective synthesis and function of FSH, causing an isolated deficiency of the hormone.

The proband had clinical and laboratory evidence of severe estrogen deficiency. Serum luteinizing hor-



mone concentrations were appropriately increased, indicating normal maturation of the hypothalamic–pituitary–gonadal axis. The clinical features of the proband are similar to those of another young woman with a genetically confirmed isolated FSH deficiency.⁶ That woman conceived after the induction of ovulation with exogenous FSH, confirming that ovarian dysfunction was due to a deficiency of FSH. She has a homozygous mutation in the FSH β -subunit gene that was identical to the frame-shift mutation (Val61X) found in one of the mutant alleles of our patient. Although there is no known relationship, these families could be descended from a common founder. In contrast to the members of the family described by Matthews et al.,⁶ none of the five heterozygotes (four with the missense mutation and one with the deletion mutation) in the kindred we described were infertile (Fig. 1B). Other cases of isolated FSH deficiency have been described, although the molecular basis was not known.^{16–19}

Data from mice in which the FSH β -subunit gene has been knocked out²⁰ and from humans with mutations in the FSH β -subunit gene⁶ and the FSH-receptor gene^{9,21} provide evidence that the hormone affects steroidogenesis and gametogenesis differently in males and females. In females, the hormone is required for follicular growth, estrogen production, and oocyte maturation,²² and its absence results in delayed puberty and infertility. Males with isolated FSH deficiency undergo normal virilization because the level of secretion of luteinizing hormone is sufficient to maintain normal serum testosterone concentrations. They have variable degrees of oligospermia, asthenospermia, and teratospermia,^{16–19,23,24} but normospermia and fertility have also been reported.^{17,23} Although no males with isolated FSH deficiency have been studied at the molecular level, male mice in which the FSH β -subunit gene has been knocked out have severe oligospermia but are fertile.²⁰ Female homozygous knockout mice have hypogonadism and infertility similar to those in our patient.²⁰ Similarly, human females who are homozygous for mutations in the FSH receptor present with hypergonadotropic amenorrhea,⁹ and males have oligospermia but are fertile.^{9,21} These findings suggest that the action of FSH is necessary for ovarian function but not for spermatogenesis. The findings in knockout mice also agree with our findings that heterozygosity for mutations in the FSH β -subunit gene does not result in infertility.²⁰

This patient with complete FSH deficiency provides a rare opportunity to evaluate the hormone's actions in normal ovarian physiology. Ovarian synthesis of sex steroids occurs according to a two-cell model involving collaborative actions of theca and granulosa cells.²² Luteinizing hormone acts on theca cells to stimulate the synthesis of androgens, which diffuse into adjacent granulosa cells. FSH acts on

granulosa cells to mediate follicular development and the expression of aromatase, converting androgens derived from theca cells to estrogens. In other conditions involving increased ratios of luteinizing hormone to FSH, such as polycystic ovary syndrome, hyperandrogenism occurs.²⁵ In contrast, our patient had a low serum testosterone concentration despite a high serum luteinizing hormone concentration, suggesting that FSH-induced follicular recruitment and development are necessary for increased androgen production. This finding is consistent with studies showing that FSH enhances the production of androgen by theca cells.²⁶

Naturally occurring mutations in the FSH β -subunit gene also provide useful information about the structure and function of FSH and other glycoprotein hormones. Each mutation (Cys51Gly and Val61X) is predicted to impair the formation of α - β dimers and receptor binding.²⁷ The codon 51 mutation resulting in the substitution of glycine for cysteine, which disrupts the formation of disulfide bonds, prevented efficient synthesis and secretion of FSH in vitro (Fig. 2). The 2-bp deletion at codon 61 causes a frame shift that completely alters amino acids 61 to 86 before leading to a premature stop codon, which means that amino acids 87 to 111 are not translated. This mutation eliminates the last five cysteine residues in the FSH β -subunit and prevents production of FSH in vitro. The Cys20–Cys104 disulfide bond (disrupted by this mutation) forms the “seat belt” that wraps around the α -subunit to stabilize the dimer.²⁸ Because the FSH β -subunits are very unstable in the absence of dimerization with the α -subunit,²⁹ defective dimerization is likely to account in part for defective synthesis.

The prevalence of mutations in the FSH β -subunit gene remains to be determined, but they are probably rare because of their autosomal recessive transmission and because fertility is impaired. However, this diagnosis should be considered in girls with delayed puberty and selective deficiency of FSH.

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