

BRIEF REPORT

Luteinizing Hormone Beta Mutation and Hypogonadism in Men and Women

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SUMMARY

Selective luteinizing hormone deficiency due to mutations in the luteinizing hormone beta-subunit gene (*LHB*) is a rare cause of hypogonadism. We describe the clinical features of a consanguineous family in which three siblings, two men and one woman, had hypogonadism related to isolated luteinizing hormone deficiency. These subjects have a newly discovered homozygous mutation of a 5' splice site in *LHB*: IVS2+1G→C. This mutation disrupts the splicing of messenger RNA (mRNA), generating a gross abnormality in the processing of the luteinizing hormone beta-subunit mRNA, which abrogates the secretion of luteinizing hormone. We also determined that the female phenotype of this *LHB* mutation is characterized by normal pubertal development, secondary amenorrhea, and infertility.

LUTEINIZING HORMONE PLAYS AN ESSENTIAL ROLE IN NORMAL PUBERTAL development and reproductive function in humans. It consists of two glycosylated, noncovalently linked subunits: a hormone-specific beta subunit and an alpha subunit common to all members of the glycoprotein hormone family. Selective luteinizing hormone deficiency is predicted to compromise reproductive capacity markedly in both sexes.^{1,2}

Inactivating mutations of the human luteinizing hormone beta-subunit gene (*LHB*) were previously described in three men with hypogonadism who had normal genitalia at birth but had no pubertal development and had infertility due to selective luteinizing hormone deficiency.³⁻⁵ To our knowledge, no female phenotype of such inactivating mutations in the *LHB* gene has been described. We describe the clinical and hormonal characteristics of three siblings, two men and a woman, all of whom had hypogonadism associated with a newly discovered mutation of the *LHB* gene.

CASE REPORTS

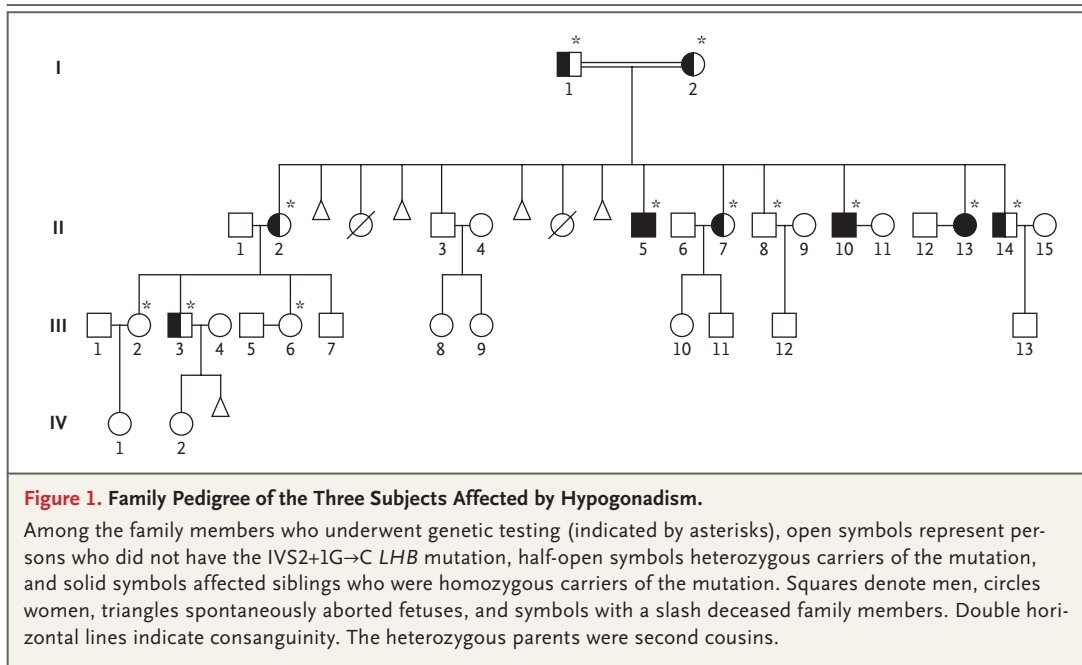
The proband (Subject II-5) (Fig. 1), a 38-year-old Brazilian man born to consanguineous parents, was referred to the Endocrinology Unit of the University Hospital of Brasília for hypogonadism. He presented with a eunuchoid habitus, a juvenile voice, and bilateral gynecomastia, with scant axillary hair and no facial hair (Tanner stage, genitalia 1 and pubic hair 4). He was 181 cm tall, weighed 87 kg, and had an arm span of 193 cm. He had a micropenis (4.5 cm in length; mean [±SD] normal length, 13.3±3.8) and underdeveloped, though descended, testes.

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The results of the initial laboratory tests are summarized in Table 1. The luteinizing hormone level was not detectable; both the baseline follicle-stimulating hormone level and the peak levels after administration of gonadotropin-releasing hormone were high (see the Methods section). The serum testosterone level was low, the free alpha-subunit level was elevated, and the inhibin B level was normal. The estradiol level was also normal. The proband had azoospermia. Ultrasonography showed heterogeneous, hypoechogenic testes with diffuse microlithiasis and confirmed the clinical finding of reduced testicular volume (right testis, 5.0 cm³; left testis, 4.6 cm³; normal volume, 15.0 to 25.0 cm³). Results of magnetic resonance imaging (MRI) of the brain and pituitary gland were normal. The karyotype was 46,XY.

A diagnosis of hypogonadism due to selective luteinizing hormone deficiency was made. Treatment with intramuscular testosterone (250 mg every 3 weeks) (Durateston, Akzo Organon) was initiated. Over a 12-month period, the testosterone induced virilization, penile growth to 9 cm in length, and an increase in testicular volume to 7.1 cm³ (right testis) and 6.6 cm³ (left testis), a total increase of 42.7%.

The proband's 30-year-old brother (Subject II-10) had been treated with intramuscular testosterone for hypogonadism from the age of 25 years. He reported considerable penile and testicular growth after treatment, but he continued to have

azoospermia. On admission to the endocrine unit, he had normal male genitalia with no gynecomastia; the Tanner stage was genitalia 5 and pubic hair 5, with a penile length of 9 cm. Ultrasonography revealed testicular volumes of 10.7 cm³ (right testis) and 8.2 cm³ (left testis). The hormonal profile was similar to that of his brother (Table 1). A testicular-biopsy specimen showed interstitial fibrous thickening, hypoplastic seminiferous tubules with a predominance of Sertoli cells, spermatogenic arrest, and an absence of Leydig cells.

The proband's 29-year-old sister (Subject II-13) presented with secondary amenorrhea and infertility. She had had spontaneous, normal pubertal development and menarche at the age of 13 years, followed by oligomenorrhea. Her weight was 56 kg, and her height 166 cm. She had normal breast and pubic-hair development (Tanner stage, mammary glands 5 and pubic hair 5). Ultrasonography of the pelvis revealed a normal uterus (volume, 50 cm³; normal range, 30 to 90), with atrophic endometrium (3 mm in thickness). The ovaries were normal in size (right ovary, 10 cm³; left ovary, 7 cm³) and contained multiple antral follicles (up to 13 mm in diameter) not restricted to the periphery (Fig. 2A). Serial ultrasonography of the pelvis over a 2-week period showed no changes in endometrial thickness or ovarian appearance. Repeated measurement of serum estradiol and progesterone showed levels within the low-to-normal range for the follicular phase.

Inhibin B and free alpha-subunit levels were elevated.

The sister also had undetectable luteinizing hormone levels, as measured by two assays (see the Methods section), whereas the follicle-stimulating hormone levels were normal (Table 1). Serum testosterone, androstenedione, dehydroepiandrosterone sulfate, prolactin, and human chorionic gonadotropin levels were all normal, as were the results of thyroid-function tests. After administration of gonadotropin-releasing hormone, luteinizing hormone levels remained undetectable, but the follicle-stimulating hormone level rose from 4.8 IU per liter to 6.8, 8.1, and 8.2 IU per liter after 15, 30, and 60 minutes, respectively (normal baseline range, 2.4 to 9.3; normal peak range after administration of gonadotropin-releasing hormone, 4.6 to 11.7). The pituitary and brain appeared normal on MRI. Therefore, we initiated estrogen-only replacement therapy (0.625 mg of estrogen per day; Premarin, Wyeth) for the first 3 months, followed by combined estrogen-progestagen (Trisequens, Medley). During this period, follow-up ultrasonography showed progressive endometrial thickening and follicle enlargement (Fig. 2B and 2C).

In addition to these 3 subjects, we studied 9 asymptomatic family members and 100 unrelated, ethnically matched controls (two alleles from each). Written informed consent was obtained from all participants. The study was approved by the Research Ethics Committee of the Faculty of Medicine, University of Brasília, Brazil.

METHODS

HORMONAL ASSAYS

An immunofluorometric assay (AutoDELFIA, Wallac Oy) was used to measure luteinizing hormone, follicle-stimulating hormone, estradiol, testosterone, progesterone, and free alpha-subunit levels. Serum luteinizing hormone, estradiol, and progesterone levels were confirmed by a chemiluminescent immunometric assay (Immulate 2000, Euro/DPC), which was also used to measure androstenedione and dehydroepiandrosterone sulfate levels, as well as the testosterone levels in female Subject II-13. Inhibin B was detected with the use of an enzyme-linked immunoassay (Active Inhibin B ELISA, Diagnostics Systems Laboratories). The intraassay and interassay variations were less than 8% and less than 10%, respectively, for all hormones. The gonadotropin-releasing hormone stim-

Table 1. Hormonal Data for Three Homozygotes with the IVS2+1G→C Mutation in the Luteinizing Hormone (LH) Beta-Subunit Gene.*

Subject No.†	Sex	Age yr	LH		FSH		Estradiol pg/ml	Progesterone ng/ml	Testosterone ng/ml	Androstenedione µg/liter	DHEAS µg/dl	Free Alpha Subunit ng/liter	Inhibin B pg/ml
			Baseline	Peak‡	Baseline	Peak‡							
II-5§	M	38	ND	ND	20.4	28.7	<13	0.97	1736.9	107	107	1736.9	107
II-10¶	M	30	ND	ND	12.8	18.6	<13	1.88	1192.7	163	163	1192.7	163
II-13	F	29	ND	ND	4.8	8.2	44	0.44	0.27	2.5	303	1010.1	194
Normal range													
M			1.0–8.4	12.0–29.7	0.6–10.5	2.9–7.8	ND–35	2.71–9.65	120.0–790.0	80–300			
F¶¶			2.2–6.8	7.6–31.7	2.4–9.3	4.6–11.7	22–215	0.33–1.20	0–0.99	0.3–3.3	34–430	80.0–604.0	15–90

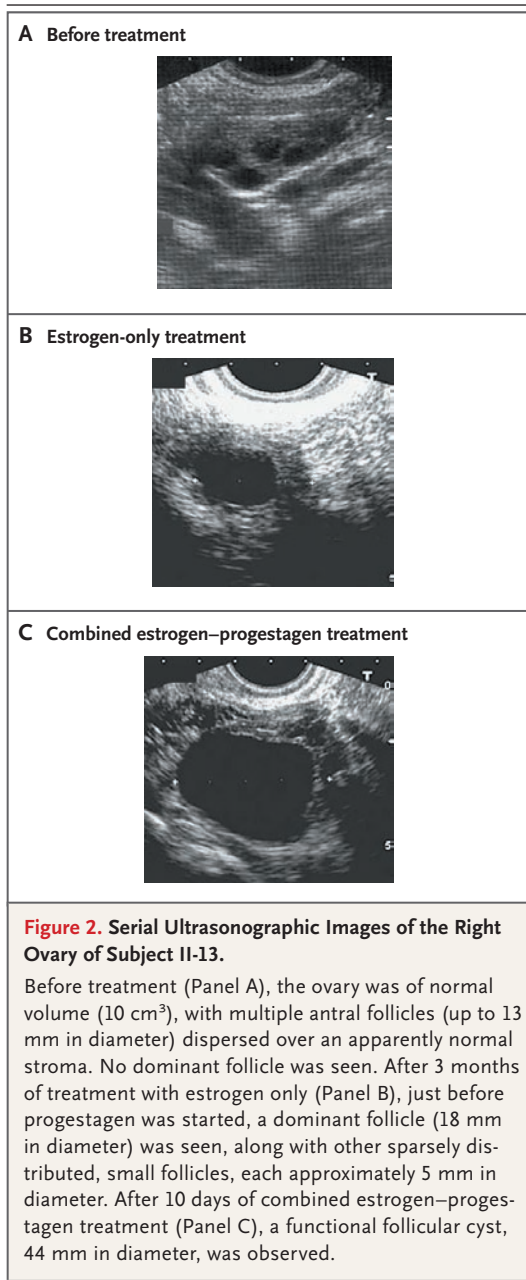
* To convert values for estradiol to picograms per milliliter, divide by 3.671; to convert values for progesterone to nanomoles per milliliter, multiply by 3.180; to convert values for testosterone to nanomoles per liter, multiply by 3.467; and to convert values for androstenedione to nanomoles per liter, multiply by 3.492. LH, follicle-stimulating hormone (FSH), estradiol, testosterone, progesterone, and free alpha-subunit levels were measured with an immunofluorometric assay (except for the testosterone level for the female Subject II-13, which was measured with a chemiluminescent immunometric assay). Androstenedione and dehydroepiandrosterone sulfate (DHEAS) levels were measured — and serum LH, estradiol, and progesterone levels were confirmed — with a chemiluminescent immunometric assay. Inhibin B levels were measured with an enzyme immunoassay. ND denotes not detectable.

† The Tanner stage was as follows: for Subject II-5 before treatment, G1P4; for Subject II-10 after prolonged treatment with exogenous testosterone, G5P5; and for Subject II-13 before treatment, M5P5.

‡ The peak value was the maximum level measured within 90 minutes after intravenous administration of 100 µg of gonadotropin-releasing hormone.

§ The hormonal levels shown for the two male subjects were measured 60 days after discontinuation of treatment with exogenous testosterone. The pretreatment hormonal profile was similar in Subject II-5, except that the testosterone level was undetectable with the chemiluminescent immunometric assay.

¶ Normal values for the follicular phase are shown.



ulation test was performed in all three affected siblings. Serum luteinizing hormone and follicle-stimulating hormone levels were measured 0, 15, 30, 60, and 90 minutes after intravenous administration of 100 μ g of gonadotropin-releasing hormone. Each result was compared with established normal values.⁶

DNA SEQUENCING AND ANALYSIS

Genomic DNA samples from subjects and controls were extracted from whole-blood specimens by the

Chelex-100 method.⁷ The region spanning exon 2, intron 2, and exon 3 of the *LHB* gene was amplified with the use of a polymerase-chain-reaction (PCR) assay, with primers designated as LH23F and LH23R, under previously described conditions.⁸ PCR products were sequenced in both the sense and antisense orientations, with the use of an automated sequencer (ABI-377, Perkin-Elmer). The primer LH23R has specific 3'-end mismatches in order to discriminate between *LHB* and the highly homologous human chorionic gonadotropin beta-subunit gene (*hCGB*) and pseudogenes. To investigate whether the identified mutation represented a polymorphism, the same *LHB*-gene fragment from 100 ethnically matched controls was amplified and digested with the *Nco*I restriction enzyme (Fermentas). This procedure, involving PCR and restriction-fragment-length polymorphism assays, was also used to analyze genomic DNA from family members of the three affected subjects.

AMPLIFICATION AND ANALYSIS OF LEUKOCYTE *LHB* mRNA

Since *LHB* mRNA, but not *hCGB* mRNA, was previously shown to be detectable by a reverse-transcriptase-PCR (RT-PCR) assay of unstimulated human blood leukocytes,⁹ we extracted total RNA from a 5-ml buffy-coat sample from each study participant, using Trizol (Invitrogen Life Technologies). To prevent contamination of genomic DNA, each sample was treated with RNase-free DNase I (Fermentas). Synthesis of complementary DNA was performed with the use of the LH23R primer and Moloney murine leukemia virus reverse transcriptase (Promega). The complementary DNA (5- μ l samples) was subsequently amplified in advance of the PCR assay (35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C) with primers (3F5'-GCACCAAGGATGGAGATGCTCCAG-3' and LH23R) and GoTaq DNA Polymerase (Promega). Half-nested PCR was then performed with the preamplification product (1- μ l samples), the internal primer 1F5'-GGCGGGGCATGGGCATCCAG-3', and the LH23R primer, under the preamplification conditions. The sequences and the specificity of the assay were verified by direct sequencing.

RESULTS

Automated DNA sequencing of exon 2, intron 2, and exon 3 of the *LHB* gene in the male proband, his affected brother, and his sister revealed a homozygous G→C substitution at the +1 position of

intron 2 (IVS2+1G→C), a 5' splice-donor site (Fig. 3). The effect of this substitution on splicing of the mRNA transcript is shown in Figure 4.

The asymptomatic parents (Subjects I-1 and I-2) of the affected subjects, two sisters (Subjects II-2 and II-7), one brother (Subject II-14), and one nephew (Subject III-3) were heterozygous for this mutation. All heterozygotes were fertile and had normal basal gonadotropin and sex-steroid levels for their ages, except the mother (Subject I-2), a 66-year-old woman, who had unexpectedly low serum luteinizing hormone levels for her menopausal state (Table S1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org). The IVS2+1G→C mutation was not identified in any of the 200 alleles from the controls.

We detected RT-PCR products of the expected size in both the control and the normal heterozygotes (Fig. 5). In the affected homozygotes, a PCR product approximately 250 bp larger was consistently obtained. Direct sequencing of these amplicons revealed that the observed difference in size was due to the presence of the entire intron 2, containing 236 nucleotides. The larger PCR product was not detected in the heterozygotes (Fig. 5).

Results of the mRNA analysis of the affected siblings clearly suggest that the IVS2+1G→C mutation disrupted the splicing of intron 2 of the *LHB* mRNA, resulting in the insertion of 236 nucleotides in the mutant transcript (Fig. S1 in the Supplementary Appendix).

DISCUSSION

We report a homozygous mutation of the *LHB* gene associated with selective luteinizing hormone deficiency in three siblings, two men and one woman, who were members of a consanguineous Brazilian family. The IVS2+1G→C mutation is located in a noncoding intronic sequence affecting a conserved 5' splice-donor site.

Subject II-13, the woman with a luteinizing hormone deficiency, underwent normal pubertal feminization and thelarche, as have several women with a homozygous inactivating mutation in the luteinizing hormone-receptor gene.^{1,2,10} However, in contrast to those women, Subject II-13 had a uterus of normal size, allowing sporadic menses to occur during the first 14 years after menarche. Although the amount of estradiol produced throughout her life could not be quantified with certainty, it was sufficient for the development of

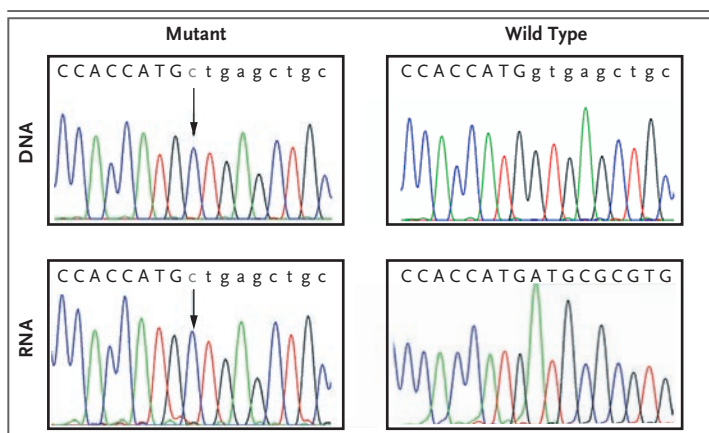


Figure 3. The IVS+1G→C Mutation of the Luteinizing Hormone Beta-Subunit Gene (*LHB*).

Automated sequencing of genomic *LHB* DNA (top) and the messenger RNA (mRNA) transcript of *LHB* (bottom) from a homozygote with the mutation (Subject II-13) and a normal homozygote showed that the IVS+1G→C mutation disrupts the splicing of mRNA. Capital letters indicate exonic sequences, lowercase letters intronic sequences, and gray type and arrows the mutation.

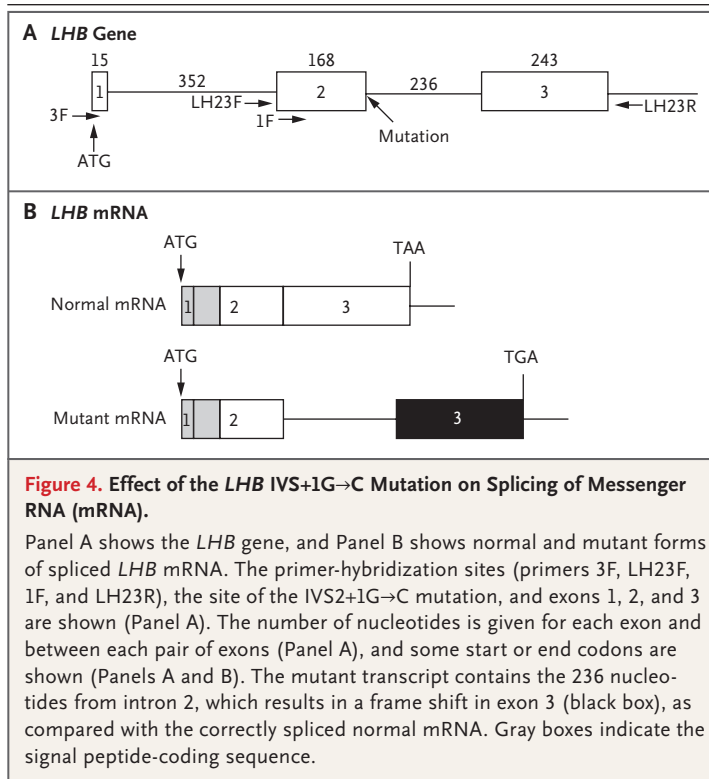
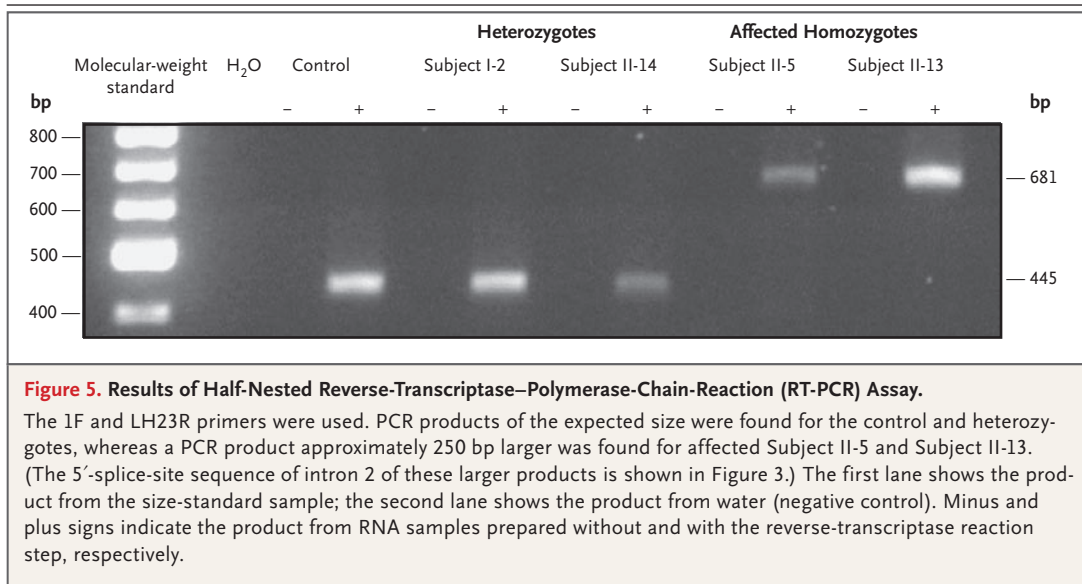


Figure 4. Effect of the *LHB* IVS+1G→C Mutation on Splicing of Messenger RNA (mRNA).

Panel A shows the *LHB* gene, and Panel B shows normal and mutant forms of spliced *LHB* mRNA. The primer-hybridization sites (primers 3F, LH23F, 1F, and LH23R), the site of the IVS2+1G→C mutation, and exons 1, 2, and 3 are shown (Panel A). The number of nucleotides is given for each exon and between each pair of exons (Panel A), and some start or end codons are shown (Panels A and B). The mutant transcript contains the 236 nucleotides from intron 2, which results in a frame shift in exon 3 (black box), as compared with the correctly spliced normal mRNA. Gray boxes indicate the signal peptide-coding sequence.

normal breasts and trophic uterine layers. In the absence of luteinizing hormone activity, the amount of androgenic substrates for aromatization to estradiol in granulosa cells would probably be limited,¹¹ which may have accounted for the low-to-



normal serum estrogen levels and endometrial atrophy in our subject. Accordingly, in mice lacking the luteinizing hormone beta-subunit expression of the majority of steroidogenic enzymes is significantly reduced, although differentiation of the ovarian thecal layer is not impaired.¹²

The amenorrhea and repeatedly low progesterone levels in Subject II-13 corroborate the essential roles of luteinizing hormone in ovulatory function. The finding of multicystic ovaries is consistent with follicular growth induced by follicle-stimulating hormone, as reflected by the elevated inhibin B levels, which may represent an increased number of healthy, early antral follicles.^{11,13} Taken together, these findings suggest that granulosa cells fail to maintain sufficient estradiol production, probably in relation to decreased luteinizing hormone–dependent secretion of androgenic precursors by thecal cells, whereas inhibin B production is spared. The high inhibin B levels, in turn, provide negative feedback for the secretion of follicle-stimulating hormone, accounting for the normal follicle-stimulating hormone levels in this subject.¹³

After supplementation with estrogen, the development of a dominant follicle (18 mm in diameter) was evident on ultrasonography, although ovulation remained impaired and no corpus luteum was observed. Given this woman's normal follicle-stimulating hormone level, these findings may indicate that estrogen or estrogen-related factors were the primary determinants of the follicular growth until the early preovulatory stage,

whereas luteinizing hormone activity was not necessary until that stage. We conclude that the *LHB* IVS2+1G→C mutation resulted in impairment of ovulation and corpus luteum function. As expected, this *LHB* mutation did not affect pubertal feminization, thereby permitting normal breast and uterine development.

The two affected men presented with hypogonadism and selective luteinizing hormone deficiency, in association with the absence of mature Leydig cells and spermatogenic arrest, corroborating previous descriptions of hypogonadism.³⁻⁵ This phenotype is similar to that of male *LHB*-knockout mice.¹² Surprisingly, both affected men had an increase in testicular volume after testosterone-replacement therapy. The key determinant of adult testicular size is the number of mature Sertoli cells, though immature cells may persist in patients with pathologic conditions, such as isolated hypogonadotropic hypogonadism.¹⁴ Since both follicle-stimulating hormone and testosterone increase the rate of proliferation and maturation of these cells,¹⁴ the administration of exogenous testosterone may have contributed to the proliferation of Sertoli cells and to testicular growth in these men by acting synergistically with elevated follicle-stimulating hormone levels (a late compensatory mechanism).

The inhibin B levels in the two affected men were inappropriately normal, given the elevated follicle-stimulating hormone levels. In normal adults, inhibin B levels are positively correlated with the function of Sertoli cells and spermatogenesis.

genic status.¹⁵ Although unusual, our findings are in keeping with those in previous studies showing that in boys, during the first pubertal years, basal inhibin B levels increase because of stimulation by follicle-stimulating hormone, in parallel with proliferation of Sertoli cells.¹⁶ Moreover, in patients with hypogonadotropic hypogonadism, baseline inhibin B levels have been shown to increase with increasing testicular volume.¹⁶ Two men previously described as having a luteinizing hormone deficiency also had elevated follicle-stimulating hormone levels,^{3,4} and at least one had normal inhibin B levels.⁴

Previously described gonadotropin mutations are located in the coding region of the genes.^{3-5,17-21} However, mutations in exon-flanking intronic sequences have frequently been associated with disease.²² Our analysis of the *LHB* mRNA isolated from peripheral-blood leukocytes showed that the IVS2+1G→C mutation resulted in the inclusion of the entire intron 2 and, consequently, in disruption of the exon 3 reading frame. Furthermore, the mutation induced a gross abnormality in the *LHB* mRNA (Fig. S1 in the Supplementary Appendix). However, the mechanism accounting for the failure to detect abnormal transcripts in the heterozygotes, but not in the homozygotes, is unknown.

Common variants identified in the *LHB* gene are sometimes associated with pathologic conditions such as the polycystic ovary syndrome and infertility.²³ The absence of the IVS2+1G→C mutation in the 200 control chromosomes reinforces the fact that it is not a polymorphism.

On the basis of the mutant nucleotide sequence, the hypothetical aberrant protein would have an

insertion of 79 residues encoded by intron 2, which would severely affect its tertiary structure. In addition, the exon 3 frame shift results in a lack of domains required for heterodimer stability and receptor binding, such as the conserved “seat belt” region (amino acids 90 to 110) and disulfide bonds from the cysteine knot (Fig. S1 in the Supplementary Appendix).^{3,24,25} These observations strongly suggest that the mutant luteinizing hormone beta subunit would be incapable of correct assembly with the alpha subunit and therefore would not be secreted.

We conclude that the IVS2+1G→C mutation induced a gross abnormality in the processing of *LHB* mRNA, causing familial selective hypogonadotropic hypogonadism. The male phenotype was similar to those described previously, whereas the affected woman underwent apparently normal pubertal development, followed by secondary amenorrhea and chronic anovulation.

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