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GENETICS

Homozygous nonsense mutation Trp28X in the *LHB* gene causes male hypogonadism

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Abstract

Purpose The purpose of this study was to investigate a novel mutation in the luteinizing hormone beta-subunit (*LHB*) gene in one male patient with hypogonadism due to selective luteinizing hormone (LH) deficiency.

Methods Sanger sequencing of one 28-year-old man born to consanguineous parents was performed. Treatment with human chorionic gonadotropin (hCG) (2000 IU, twice a week) was initiated for 3 months, followed by 5000 IU weekly to date.

Results We identified a novel c.84G>A[p.W28X] nonsense *LHB* mutation. The W28X mutation produces a truncated LHB peptide of seven amino acids, which prevents the synthesis of intact LH. After 40 days of treatment with hCG, the patient exhibited a few spermatozoa in the semen. Treated for 6 months, the patient exhibited normal seminal parameters.

Conclusions We identified a novel mutation in the *LHB* gene in a male patient with hypogonadism and provided evidence that *LHB* nonsense mutation can cause selective LH deficiency. We reconfirmed hCG treatment may restore male fertility due to *LHB* mutation.

Keywords Primary hypogonadotropic hypogonadism · Luteinizing hormone · Luteinizing hormone beta subunit · Selective luteinizing hormone deficiency

Introduction

Primary hypogonadotropic hypogonadism (HH) is due to a deficiency or dysfunction in gonadotropins caused by either hypothalamic or pituitary diseases [1]. Selective LH deficiency is a rare type of HH that can easily be diagnosed using hormonal assays [2]. This disease is an autosomal recessive syndrome, and several mutations in the *LHB* gene have been identified [3]. Homozygous or compound heterozygous *LHB* gene mutations that abolish the activity of LH have been reported in several men and women [2, 4–7].

LH plays a key role in pubertal development and the regulation of reproductive function. The absence of LH alters the proliferation and maturation of Leydig cells, leading to low levels of serum testosterone [8]. Affected adult may exhibit normal sexual differentiation, delayed puberty, and hypogonadism, whereas three affected women have been reported to have normal pubertal development and menarche [5].

Here, we reported a novel c.84G>A [p.W28X] nonsense *LHB* mutation in a male patient with HH and provided clinical and experimental evidence, confirming the novel mutation in the *LHB* gene causes selective LH deficiency.

Materials and methods

Patient

The proband (Fig. 1, subject IV-2) is a 28-year-old Chinese man born to consanguineous parents who was referred to male infertility. The proband was 170 cm tall, weighed 78 kg, and had an arm span of 174 cm. He exhibited a eunuchoid habitus, bilateral gynecomastia, a juvenile voice, scant distributed

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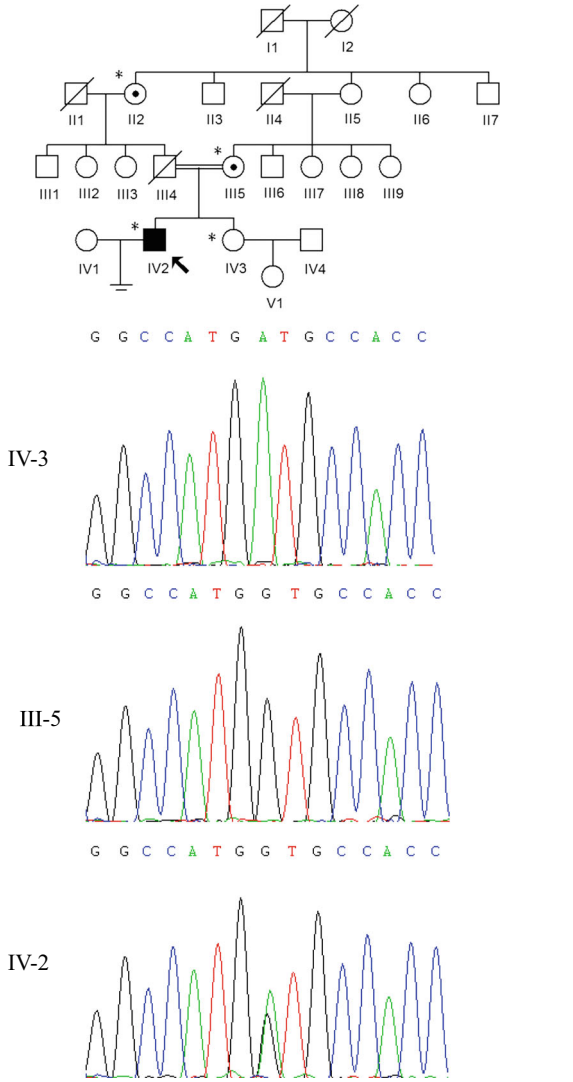

Fig. 1 Family pedigree of the proband affected by hypogonadism with an *LHB* mutation. Asterisks indicate the family members who underwent DNA sequencing. The black symbols (IV-2) represent the proband who is a homozygous carriers of the *LHB* mutation; the open symbols (IV-3) represent the patient's sister who is normal wild-type sequences of *LHB*; the dotted-circle symbols (II-2, III-5) indicate the patient's mother and grandmother who are heterozygous carriers of the *LHB* mutation

pubic hair, and no facial hair (Tanner stage, genitalia 1 and pubic hair 2). His penile length was 4 cm, and his testicular volume was 5 ml. The patient reported decreased sexuality, normal stimulation of the erect penis, and low semen volume (0.1 ml) without spermatozoa. A semen analysis was performed again after treatment with human chorionic gonadotropin (hCG) (Organon, Roseland, NJ) for 3 days. The volume of semen has become normal (3.0 ml), but no spermatozoa has been found. The proband's sibling was a 33-year-old female who had one daughter. The patient and his relatives provided written informed consent for participation in this study. The study protocol was approved by the local ethics committee of the First Affiliated Hospital of NanJing Medical University.

Hormone assays

A chemiluminescent immunometric system (Immunoassay, Unicel DXI800, Beckman Coulter, Inc., Fullerton, CA) was used to measure the levels of serum LH, follicle-stimulating hormone (FSH), testosterone (T), dehydroepiandrosterone sulfate (DHEAS), prolactin, adrenocorticotropic hormone (ACTH), progesterone (P), estradiol, and cortisol. The mean variation coefficients of the inter- and intra-assay were < 5% (2.5–4.26%) and < 15% (5.19–8.59%), respectively. The levels of inhibin B and anti-Mullerian hormone (AMH) (Kangrun Biotech, Inc., Guangzhou, China) were measured using an enzyme-linked immunosorbent assay. The absence of LH was confirmed twice. A gonadotropin-releasing hormone (GnRH) stimulation test was performed in the affected patient. The serum LH and FSH levels were measured 0, 30, 60, and 90 min after an intravenous administration of 100 μg of GnRH.

Treatment with gonadotropin

The patient was treated with hCG (2000 IU twice a week for 3 months, followed by 5000 IU weekly to date). The hormone levels and semen outcomes after the treatment with hCG were recorded.

Sanger sequencing

Genomic DNA was extracted from the leukocytes using commercially available reagents. DNA was obtained from a normal volunteer and used as a wild-type control. A 1082-bp amplicon containing the complete *LHB* gene was recovered by a polymerase chain reaction (PCR) assay and sequenced in the sense and antisense directions using an automated sequencer. The primer pairs used in this study were as follows: *LHB* forward: GGGAATTCTCTTTGTGGGTGGTGTAC CACGC and *LHB* reverse: GGAGGATCCGGGTG TCAGGGCTCCA. To avoid the co-amplification of the homologous chorionic gonadotropin beta-subunit gene or pseudogenes, the primers contained at least one nucleotide that was mismatched at the 3′ end. The alignments and comparisons of the sequences were performed using two software programs (BestFit and PileUp from the GCG Wisconsin Package, Accelrys).

Plasmid construction

The *CGA* gene encodes the common alpha subunit that combines with the beta subunits of all the glycoprotein hormones. The cDNA of *CGA* gene was cloned into the PXJ40-Flag (BioVector NTCC Inc., Tokyo, Japan) after the Flag epitope. Thus, the alpha subunit and FLAG epitope were co-expressed as a fusion protein. We tested the expression of the alpha

subunit using an anti-Flag antibody. We then introduced the mutation carried by the proband into *LHB* to create a mutant LH beta-subunit construct. The wild-type and mutant *LHB* cDNA were inserted into the pEGFP-C1 vector (Clontech, Pale Alto, CA, USA) immediately to express as a fusion protein. The expression of the LH beta subunit in HEK293 cells can be tested using an anti-GFP antibody. The Trp28 mutation caused a large deletion in the LH beta subunit. However, the expression of the GFP protein was still normal. Therefore, the fusion protein from the *GFP* + wild-type *LHB* exhibited normal GFP plus normal LHB, while another fusion protein from the *GFP* + mutation *LHB* exhibited normal GFP plus the truncated polypeptide (7 AA) of the mutant *LHB*. The sequences of all the promoter fragments were confirmed by dideoxynucleotide sequencing.

Cell culture and transfection

HEK293 cells were plated at 3×10^5 cells/well in 12-well plates 1 day before the transfection. Expression vectors were transfected into HEK293 cells with Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA). All transfection experiments were performed in triplicate and repeated at least three times.

Immunoblotting assay

The cells were lysed with RIPA buffer (1 × PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% Sodium Deoxycholate, 1 mM Sodium Orthovanadate, and 1% PMSF) (Beyotime, Jiangsu, China) after the transfection. The lysates were centrifuged at 13,000 rpm for 10 min, the supernatants were collected, and the total proteins were extracted at 4 °C. The total protein concentration in the cell extracts was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). The cell lysates were incubated with either an anti-FLAG monoclonal antibody (SIGMA, MO, USA) or an anti-GFP monoclonal antibody (Abcam, Cambridge, UK) and then co-incubated with Protein A/G agarose (Roche) overnight. The beads were washed with RIPA three times and then denatured at 100 °C for 10 min. The proteins were separated electrophoretically by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% separation gel and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk, incubated with the primary antibodies at 4 °C overnight, and then incubated with the secondary antibodies (goat anti-rabbit IgG horse radish peroxidase conjugated, Cell Signaling Technology, MA, USA) at room temperature for 2 h. The PVDF membrane was washed three times and detected for 5 min with ECL reagent according to the manufacturer's instructions (Cell Signaling Technology, MA, USA). The expression of GAPDH (Cell Signaling Technology, MA, USA) was used as a loading control.

Results

The results of the patient's laboratory tests are shown in Table 1. LH was undetectable and testosterone (0.28 ng/ml) was low. Interestingly, the level of FSH was abnormally higher (40.0 IU/L) than previously reported cases [2, 4–7]. The levels of estradiol (32 pg/ml) and prolactin (7.96 ng/ml) are normal. After 60 min of the administration of GnRH (100 μg, iv), the level of FSH increased to 111 IU/L, whereas the level of LH remained undetectable. The serum T levels increased from 0.28 to 1.95 ng/ml after the 3-day treatment with hCG (4000 IU, im). Magnetic resonance imaging of the brain and pituitary gland showed no abnormalities.

After the 40-day treatment with hCG, the serum T increased to 2.12 ng/ml, estradiol decreased to 1.68 pg/ml, FSH decreased to 22.2 IU/L, LH remained undetectable, and the semen exhibited oligospermia (0.72×10^6 spermatozoa per ejaculate; semen volume, 2 ml) (Table 1). The patient had high-normal levels of cortisol (498 nmol/L; normal range, 170–440), low-normal levels of inhibin B (100.48 pg/ml; normal range, 100–300), and high levels of AMH (15.51 ng/ml; normal range, 1.5–11.8) after treatment. After 6 months of the treatment with hCG, the seminal parameters became normal (60×10^6 spermatozoa per ejaculate; 50% of sperm motility, and 11% of normal morphology rate).

The DNA sequencing of the proband (subject IV-2) revealed a homozygous novel nonsense mutation at codon 28 (p.W28X) in which a TGG (Trp) was changed to a TGA (stop) (Fig. 1). The patient's mother and grandmother were heterozygous for this mutation, but his sister had normal wild-type sequences. His father died years ago but was presumably a heterozygous carrier of the *LHB* mutation since the proband was homozygous.

Six months after the hCG administration, the patient provided informed consent for a testicular biopsy using percutaneous testicular fine needle aspiration. A specimen from the right testicular samples showed heterogeneous seminiferous tubules, and more than half of these tubules exhibited a normal testicular tissue structure. The abundant number of round spermatids and spermatozoa are shown in Fig. 2.

Both the wild-type and mutant beta subunit of LH were synthesized in HEK 293 cells and detected by Western blotting using a GFP antibody. The alpha subunit was correctly synthesized in the co-transfected HEK293 cells as detected by Western blotting using a Flag antibody (Fig. 3a, b). The cell lysates were immunoprecipitated with the GFP antibody and then subjected to immunoblotting with the Flag antibody. The alpha subunit was only detected in the cells co-transfected with the wild-type beta subunit, indicating that

Table 1 Serum hormone levels in the proband with an LHB gene mutation

Subject no.[a]	Sex	Age (year)	LH (IU/l)		FSH (IU/l)		Testosterone (ng/ml)		Progesterone (ng/ml)	Estradiol (pg/ml)	AMH (ng/ml)	Cortisol (nmol/l)	ACTH (pg/ml)	Prolactin (ng/ml)	Inhibin B (pg/ml)	DHEAS (nmol/l)
			Baseline	Peak[b]	Baseline	Peak[b]	Baseline	Peak[d]								
II-1(proband)		Male 28	ND	ND	40.011		0.28	1.95			32.0				7.96	
II-1(proband)[c]		Male 28	ND		22.2		2.12			1.07	1.68	15.51	498.0	45.58	11.14	100.48
7.38																
Normal range																
Male			1.24–8.62[e]		1.27–19.26[e]		1.75–7.82[e]		0.40–1.10	20.0–47.0	1.5–11.8	170–440	7.2–63.3 [e]	2.64–13.1	100–300	0.7–18.7

ND not detectable

[a] To convert the values of estradiol to picomoles per liter, multiply by 3.671; to convert the values of testosterone to nanomoles per liter, multiply by 3.467; to convert the values of prolactin to mIU/l, multiply by 21.2; to convert the values of progesterone to nanomoles per liter, multiply by 3.18; and to convert the values of ACTH to picomoles per liter, multiply by 0.22.

[b] The peak value was the maximum level measured within 90 min after the intravenous administration of 100 μg gonadotropin-releasing hormone

[c] The hormonal levels shown for the proband were measured 40 days after a continuous treatment with 4000 IU human chorionic gonadotropin twice a week

[d] The peak value was the maximum level measured 72 h after im administration of 4000 IU human chorionic gonadotropin for 3 days

[e] Normal range at 7–10 o' clock in the morning

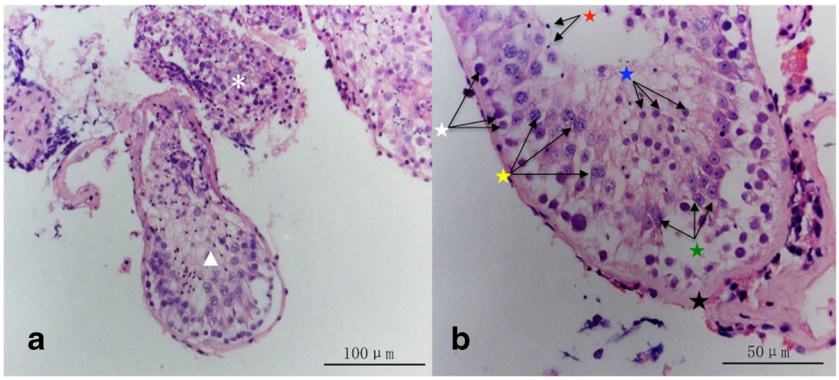

Fig. 2 Histological analysis of the patient's testis after a 6-month hCG treatment using hematoxylin and eosin. Testicular specimens were obtained by testicular fine needle aspiration. **a** Heterogeneous seminiferous tubules; some tubules only exhibited a thickening of the basal membrane (arrowhead), while more than half of the tubules were exhibited a nearly normal testicular structure (asterisk). **b** A section with a seminiferous tubule consisting of mature Sertoli cells and germ cells at all stages of differentiation and a scattered cluster of mature spermatozoa are present. (**a**) magnification, 100; (**b**) magnification, 400. Black star, tunica propria; white star, spermatogonia; green star, Sertoli cells; yellow star, primary spermatocytes; blue star, round spermatids; red star, spermatozoa

heterodimerization had occurred (Fig. 3c). We performed an affirmative experiment in which the cell extracts were immunoprecipitated with a Flag antibody followed by GFP antibody immunodetection. The beta subunit was only detected in the extracts of the cells co-transfected with the wild-type beta subunit but was not detected in the cells co-transfected with the mutant beta subunit or the mock-transfected cells (Fig. 3d).

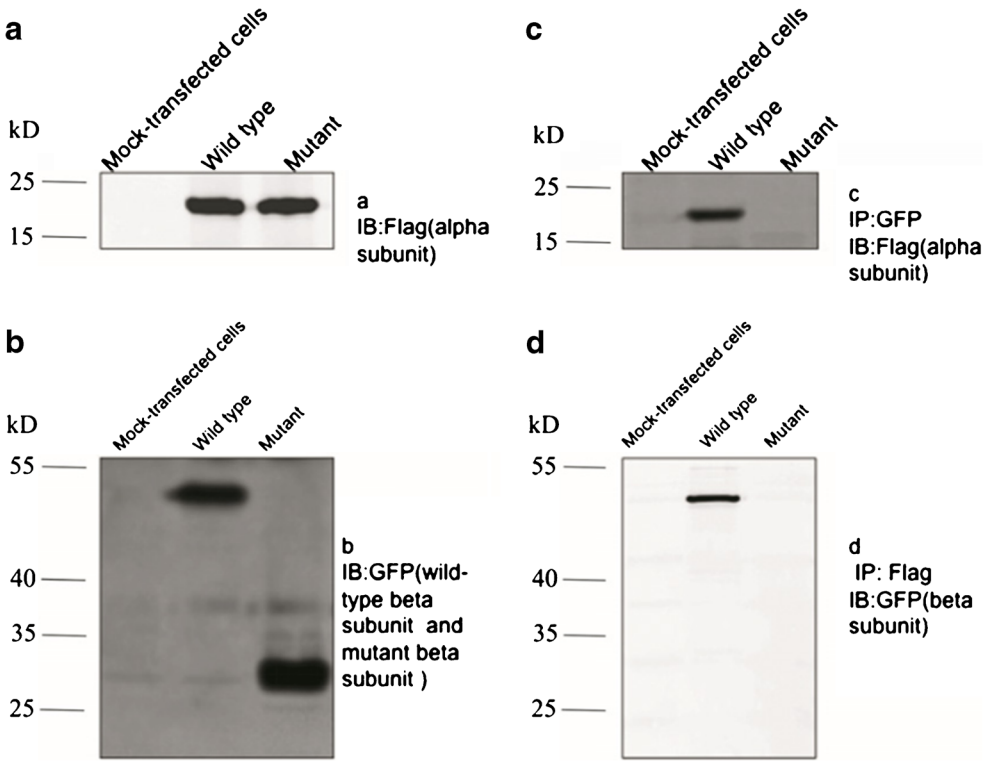

Fig. 3 Interaction of LH alpha subunit and mutant LH beta subunit in HEK 293T transfected cells. (**a**) Corrected production of the alpha subunit (expected size, approximately 22 kD) in co-transfected HEK293 cells as detected by Western blotting with a Flag antibody. (**b**) Wild-type and mutant beta subunits are both correctly synthesized in HEK293 cells transiently transfected with GFP-tagged beta-subunit expression vectors. (**c**) Cell extracts were immunoprecipitated with an anti-GFP antibody and then subjected to immunoblotting with an anti-Flag antibody. The alpha subunit was only detected in cells co-transfected with the wild-type beta subunit. (**d**) Alpha-beta heterodimer formation in HEK293 cells that were co-transfected with the Flag-tagged alpha subunit and either the mutant or wild-type GFP-tagged beta-subunit expression vectors. Immunoprecipitation with an anti-Flag antibody recognizing the Flag-tagged alpha subunit was followed by immunoblotting with an antibody recognizing the GFP-tagged beta subunit. Co-precipitation of the alpha and beta subunits occurred in cells co-transfected with the wild-type, but not the mutant, beta subunit, and the mock-transfected cells

Discussion

An intact LH heterodimer comprises a common alpha subunit and a specific beta subunit, both of which are required for its biological activity [9]. LHB consists of a mature protein of 121 amino acids and a signal peptide of 20 amino acids. The first loss-of-function *LHB* mutation identified in humans, i.e., a homozygous p.Q74R, was identified in a male with selective LH deficiency [2]. The level of LH in this male patient with a Q74R mutation was abnormally high (64.2 IU/L; normal range, 3.0–18.0). The mutation does not affect dimerization or immune reactivity but eliminates the activity of receptor binding. The level of LH in patients with other identified *LHB* mutations is usually undetectable because these mutations impair the synthesis of LH. These mutations include the following: 10-13del [6], 30-32del [10], G56D [7], L72R [11], 118-120del [12], IVS2 + 1G → C [5], and IVS2 + 1G → T [6]. A G52A point mutation in exon 2 of *LHB* has been detected in the heterozygous form in normal DNA samples but has not been reported in the homozygous form thus far [13]. The G52A mutation exhibits normal heterodimerization and signal peptide cleavage.

The c.84G>A [p.Trp28X] mutation in the *LHB* gene produces a truncated LHB peptide of only seven amino acids. Alternatively, the mRNA transcripts of this polypeptide that contain a premature termination codon may be destroyed by a nonsense mRNA decay mechanism, also leading to *LHB* insufficiency [14]. In the IP experiments (3c and 3d), which were assessed using a two-way double check, we confirmed that the wild-type LH beta subunit can bind the alpha subunit, but the mutant LH beta subunit cannot bind the alpha subunit. However, detecting the alpha subunit or the intact LH molecule in the medium of transfected HEK293 cells is difficult. The secretion of the fusion protein or free LH alpha subunit from this type of cell may be very limited.

Trp28 in *LHB* has a common variant, i.e., Trp28Arg. The Trp28Arg and Ile35Thr mutations in exon 2 are the most common *LHB* allele variant, and the carrier frequency ranges from 0 to 53% in various populations [15], including 11% in Chinese women [16]. The Trp28Arg and Ile35Thr carrier status does not affect T levels and the sperm parameters in adult males, but LH levels increase [17].

An important question raised by this study is whether this *LHB* gene mutation causes clinical manifestations in heterozygotes. The proband's father was presumably an obligate heterozygote, who had no evidence of hypogonadism. His mother, who is a heterozygous carrier, has normal fertility. We did not perform a testicular biopsy before the treatment due to the lack of indication and informed consent. We postulate that the patient had arrested spermatogenesis in the pachytene spermatocytes or round spermatids because the patient presented with a few spermatozoa in the semen after only 40 days of the treatment with hCG. In humans, spermatogenesis consists of six stages that require approximately 64 days [18]. Several reports have described male patients with *LHB* mutation with an absence or reduced number of mature Leydig cells and arrested spermatogenesis [2, 6]. The patient presented with normal seminal parameters after 6 months of the hCG treatment. Testicular biopsy was performed to assess the histopathology after obtained informed consent. The seminiferous tubules were heterogeneous, some tubules only exhibited a thickening of the basal membrane, while more than half of the tubules were exhibited a nearly normal testicular structure (Fig. 2). Our results are consistent with a previous report [7].

The level of T in the proband before treatment was very low (0.28 ng/ml), which is likely due to the deficiency in the LH activity. Unexpectedly, the level of estradiol during the first examination was 32.0 pg/ml (Table 1). The T level increased to 2.12 ng/ml after the 40-day treatment with hCG likely because of the response of the Leydig cells to the hCG stimulation, whereas the level of estradiol deceased to 1.68 pg/ml (Table 1). These seemingly abnormal results have been reviewed two times in our laboratory. Nevertheless, we did not know the reason clearly. In our clinical practice, patients with Klinefelter syndrome have low levels of T and relatively high levels of estradiol, which may be related to the high aromatization activity.

Testosterone and hCG are recommended treatments for this type of male selective LH deficiency. Both drugs can lead to increased penile length and masculinization, but the testicular function of spermatogenesis is recovered in varying degrees. Therapy with hCG enables more efficient spermatogenesis and maximize the potential for fertility than testosterone alone [19]. Spermatogenesis in humans requires high levels of testicular testosterone, which is ~100-fold higher than that in serum, due to the local production by Leydig cells [20]. Valdes-Soci et al. reported that patients treated with testosterone alone still exhibited azoospermia after 3 months and that, in contrast, the sperm concentration was 1000/ml after treatment with hCG for 1 year [7].

Conclusions

In conclusion, we found a novel homozygous nonsense *LHB* mutation in a male patient with HH and provided evidence that *LHB* mutation can cause selective LH deficiency. This mutation is the first reported nonsense mutation in *LHB*. In addition, this study reconfirmed hCG treatment may restore male fertility due to *LHB* mutation.

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Compliance with ethical standards

Conflict of interest The authors declare that have no conflicts of interest.

Informed consent Informed consent was obtained from all participants included in the study.

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