Journal of Assisted Reproduction and Genetics (2020) 37:1441–1448
https://doi.org/10.1007/s10815-020-01786-7

GENETICS

Clinical and genetic analysis of an isolated follicle-stimulating hormone deficiency female patient

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Received: 21 September 2019 / Accepted: 17 April 2020 / Published online: 5 May 2020

Abstract

Objective To characterize the clinical features of a female patient with isolated follicle-stimulating hormone (FSH) deficiency and to investigate the underlying mechanisms of FSH inactivation.

Methods The proband was a 29-year-old woman with primary amenorrhea, impaired pubertal development, and infertility. Subsequently, reproductive endocrine was screened. DNA sequencing was conducted for the identification of *FSHβ* mutation. RT-PCR, western blots, in vitro immunometric assay, and bioassay were performed to confirm the impact of the mutation on FSH expression and biological activity. Molecular model consisting of FSHα and mutant FSHβ subunit was built for the structural analysis of FSH protein.

Results The evaluation of reproductive endocrine revealed undetectable basal and GnRH-stimulated serum FSH. Sequencing of the *FSHβ* gene identified a homozygous nonsense mutation at codon 97 (Arg97X). RT-PCR and western blot analysis revealed the mutation Arg97X did not affect *FSHβ* mRNA and protein expression. But in vitro immunometric assay and bioassay demonstrated the production of normal bioactive FSH protein was disturbed by the mutation Arg97X. Structural analysis showed the surface structure of the resulting mutant FSH presented with lock-and-key, mosaic binding pattern, while the native structure was an encircling binding mode.

Conclusion The mutation Arg97X could disturb structural stability of the resulting FSH protein consisting of FSHα and mutant FSHβ subunit, which may lead to FSH deficiency.

Keywords Follicle-stimulating hormone · Isolated FSH deficiency · Infertility · *FSHβ* gene · Mutation

Introduction

The hypothalamic-pituitary-gonadal (HPG) axis is important to pubertal development and reproductive functions both in males and females [1]. Generally, gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus regulates the whole function of HPG axis. With the stimulation of GnRH, the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) are released from the pituitary and play essential roles in the regulation of pubertal development and fertility [1, 2]. FSH is a heterodimeric glycoprotein hormone composed of a common α-subunit identical to that of LH, human chorionic gonadotropin (HCG) and thyroid-stimulating hormone (TSH), and a unique β-subunit driving hormone specificity [3, 4]. FSH plays key roles in pubertal development and reproductive functions both in males and females [2, 4–6]. In males, FSH maintains the

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normal proliferation of Sertoli cell and participated in the production of androgen [7, 8], while in females, FSH is involved in the regulation of follicular development and sex steroid production. Generally, all these FSH-related functions are important to fertility in both males and females [2, 3].

The *FSHβ* gene (OMIM 136530) is located at chromosome 11p14.1 and consists of three exons, encoding the FSH β-subunit of 129-amino acids [9]. Up to date, there are several inactivating mutations of *FSHβ* gene that have been identified in 12 patients, including 6 males and 6 females. These females manifested delayed puberty, amenorrhoea, hypogonadism, and infertility [5, 9, 10], and the main manifestations of these males included azoospermia and infertility [6]. However, these rare natural mutations in the *FSHβ* gene provide valuable opportunity to identify the normal processes of gonadotropin-dependent reproductive endocrine.

In the current study, we reported a case of a *FSHβ* mutation in a Chinese female. Also, we investigated the related clinical and biochemical feature. Our results demonstrated that this mutation would encode a truncated FSH protein, causing the impairment of FSH bioactivity.

Materials and methods

Case report

The proband is a 29-year-old Chinese female visited Reproduction Medicine Center of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (HUST, Wuhan, China) because of infertility and primary amenorrhea. She had a height of 160 cm, a weight of 48 kg, and a BMI of 18.75. Physical examination showed Tanner stage III breast development and sparse pubic hair. There was no galactorrhea, dysosmia, hirsutism, or acne. Gynecological examination revealed infantile female external genitalia. The evaluation of reproductive endocrine (Table 1) revealed undetectable basal and GnRH-stimulated serum FSH. Also, the serum level of estradiol (E2) and inhibin B (INHB) were low. However, serum luteinizing hormone (LH) was high and increased after GnRH stimulation. The serum levels of progesterone (P), testosterone (T), anti-Müllerian hormone (AMH), and 17α-hydroxyprogesterone (17OHP) were of normal range. She had a normal karyotype of 46, XX, and normal glucose tolerance. The ultrasound examination of adrenal gland and magnetic resonance imaging (MRI) of pituitary gland showed no abnormalities. There was no family history of infertility or menstrual irregularity, while her parents were cousins with the history of adverse pregnancy outcomes.

The administration of uFSH (Urofollitropin for Injection, Livzon, China) was performed on the proband for the treatment of infertility. Before the uFSH treatment, the study protocol was approved by the Medical Ethics Committee of Tongji Hospital and written informed consents were obtained from the patient. Researches were conducted according to the Declaration of Helsinki for medical research. At first, uFSH was given in a daily dosage of 75 IU i.m. qd. Then, the uFSH dosage was increased to 150 IU/day for the subsequent days. Thirteen days later, 4 follicles were detected via transvaginal ultrasound, with a serum E2 level of 735 pg/ml. HCG (250 mg; Ovidrel; Serono) was given to trigger ovulation, followed by guidanced intercourse. Two weeks later, this female experienced menstrual bleeding.

During uFSH administration, the serum FSH level increased gradually, and so was the E2 level. Then, both serum FSH and E2 fell progressively after discontinuation of the treatment. The high basal level of serum LH underwent a dramatic decrease during administration period (Fig. 1). Transvaginal ultrasonography was performed to assess the effects of uFSH administration on ovarian morphology, which showed that uFSH stimulated multifollicular development and induced an increasing size of ovaries (Fig. 1). What's more, endometrial thickness increased to a maximum of 14.2 mm during the treatment period, providing a solid foundation for implantation.

Table 1 The characteristics of reproductive endocrine under basal state and GnRH stimulation test

Time (min)	FSH (mIU/ml)	LH (mIU/ml)	E2 (pg/ml)	T (nmol/l)	P (ng/ml)	AMH (ng/ml)	INHB (pg/ml)	GH	TSH	ACTH	17-OHP (nmol/l)
0	0.08	49.89	16.62	37.44	0.07	3.06	1.23	N	N	N	1.52
30	< 0.3	> 200	-	-	-	-	-	-	-	-	-
60	< 0.3	> 200	-	-	-	-	-	-	-	-	-
90	< 0.3	> 200	-	-	-	-	-	-	-	-	-
120	< 0.3	> 200	-	-	-	-	-	-	-	-	-
180	< 0.3	185.6	-	-	-	-	-	-	-	-	-

Abbreviations: *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *E2*, estrodiol; *T*, Testosterone; *P*, Progesterone; *AMH*, anti-Müllerian hormone; *INHB*, inhibin B;*GH*, growth hormone; *TSH*, thyroid-stimulating hormone; *ACTH*, adrenocorticotropic hormone; *17OHP*, 17-hydroxyprogesterone

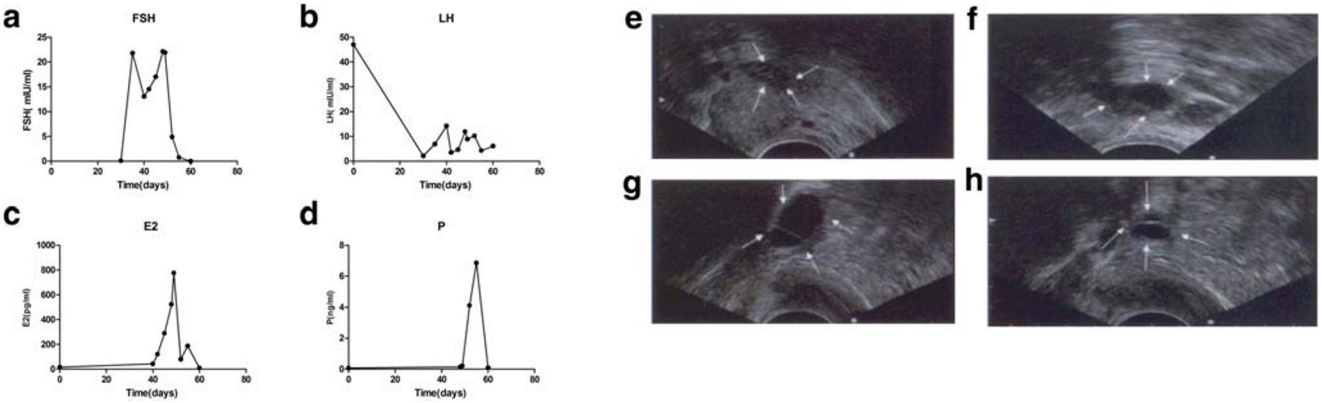

Fig. 1 Characteristics of reproductive endocrine, ovarian morphology, and follicular development during uFSH administration to the patient. **a**–**d** Circulating gonadotropin levels and ovarian hormonal responses during uFSH treatment. **a** FSH. **b** LH. **c** E2. **d** P. **e**–**h** Effect of uFSH treatment on ovarian morphology observed by transvaginal ultrasound. **e** Ovarian sonography of basal state. **f** On day 5. **g** On day 13. **h** After ovulation triggered by HCG administration. FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estrodiol; P, progesterone

DNA sequencing and analysis

Genomic DNA was extracted from peripheral blood using the QG-Mini80 workflow with DB-S kit (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions. PCR primers were designed to amplify and sequence all the exons of *FSHβ* using the Primer Premier 6.0 software and were as follows: exon 1 sense: 5′-TCCT CCATGTCCTGACAAATC-3′; exon 1 antisense: 5′-CCCA ACAAATCCACAAGG-3′; exon 2 sense: 5′-AGCA AAATGTGATTGAGGAGG-3′; exon 2 antisense: 5′-GCTA AAGGACTCATGGCTGTTAC-3′; exon 3 sense: 5′-TGTT AGAGCAAGCAGTATTCAATTTC-3′; exon 3 antisense: 5′-CAGGCTTGGTAGTAATAGCTTGG-3′. The PCR products were directly sequenced with BigDye terminator v3.1 on the 3130 × 1 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Finally, Chromas and DNAMAN program were used to identify mutations by two independent observers. DNA sequences were compared with the reference sequences of NCBI (https://www.ncbi.nlm.nih.gov/gene/2488). NCBI dbSNP database was searched for the confirmation of a SNP and the database of human gene mutation data (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php) was searched for the confirmation of the novel mutation.

In vitro analysis of *FSHβ* mutation

The expression plasmids, pcDNA3.1(+), containing the entire coding region of human *FSHβ* of wild type, mutant type, and that of human FSHα, respectively, were constructed for in vitro analysis. Chinese hamster ovary cells (CHO; American Type Culture Collection, Manassas, VA) were obtained from the American Type Culture Collection and cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum. Then, CHO cells were transiently co-transfected with the pcDNA3.1(+) α-subunit plasmid and either the mutant (mutation (MU) group) or wild-type pcDNA3.1(+)-FSHβ (wild-type (WT) group) construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total RNA was extracted and subjected to RT-PCR. To prevent contamination of genomic DNA, each sample was treated with RNase-free DNase I (Fermentas). Reverse transcription was performed following a standard protocol using PrimeScript reverse transcriptase (TaKaRa, Dalian, China) and random Oligo dT Primer (TaKaRa, Dalian, China). Primers for reverse transcription were as follows: wild-type *FSHβ* sense: 5′-CTCA CCATGCAGATTCCTTG-3′; wild-type *FSHβ* antisense: 5′-ATGATGATGATGATGATGTTCTTTC-3′; mutant FSHβ sense: 5′-CTCACCATGCAGATTCCTTG-3′; mutant *FSHβ* antisense: 5′-TGATGATGATGATGATGCACAG-3′; *FSH α* sense: 5′-GATTACAAGGATGACGACGATAAG-3′; *FSH α* antisense: 5′- GTTTTCCTGTAGCGTGCATTC-3′. The following PCR amplification was performed according to a standard protocol using SYBR Premix Ex Taq (TaKaRa, Dalian, China). Seventy-two hours after transfection, culture medium was collected and stored at − 20 °C for FSH immunoassay and bioassay. Cell lysates were obtained after 72 h of transfection and kept at − 20 °C for western blot analysis.

Immunometric FSH assay and homologous FSH bioassay

Cell-conditioned medium was assayed for immuno-reactive FSH using a FSH ELISA Kit (CUSABIO) according to the manufacturer's instructions with a limit of detection of 1 mIU/mL. The homologous FSH bioassay was performed using COV434 cells expressing FSH receptor. Cells were grown in culture in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum. When percentage of cell confluence reached 70–80%, medium was removed, and cells were placed in cellular media collected from CHO cell after

transfection as described above. Six hours later, culture supernatants were removed and cell lysis solution was collected for the quantification of cAMP using the cAMP ELISA Kit (Genscript). The lower limit of detection of cAMP according to the manufacturer is 1 nmol/L.

Molecular docking

FSHα and *FSHβ* monomers were split from FSH structure (Protein Data Bank accession code 1f17). *FSHβ* mutant structure was generated by using Swiss-model (http://www.expasy.org/spdbv/) [11], and was docked to FSHα via HADDOCK server (http://haddock.chem.uu.nl/) [12].

Results

DNA sequence analysis

Genetic analysis of *FSHβ* gene in this proposita revealed a homozygous nonsense mutation at codon 97 (Arg97X), in which the codon CGA (Arg) was changed to a TGA (Stop), leading to a premature at position 97. This mutation was also identified in the proband's parents, elder sister, and maternal grandmother, all of whom were heterozygous. Besides, the proband's grandfather did not carry this mutation (Fig. 2).

RT-PCR and western blot analysis of the mutated *FSHβ* subunit

RT-PCR and western blot were performed on the co-transfected CHO cell lysates for this mutated *FSHβ* subunit analysis. The WT group had detectable FSHα and the wild-type FSHβ on both mRNA and protein level as expected (Fig. 3). Besides, RT-PCR and western blot analysis of MU group also detected FSHα and the mutant FSHβ (Fig. 3). But, neither FSHβ nor FSHα could be detected in the control (CK) group. These results revealed that the mutation Arg97X did not affect the detectable FSHβ protein expression.

Immunometric assay and bioassay of the mutated FSH

In the immunoassay of FSH in cellular medium, detectable FSH was identified in WT group, while no FSH protein was detected in MU group, CK group, or the group with only complete medium (Fig. 3f). Also, in FSH bioassay of cellular medium from transfected CHO cell, only the WT group demonstrated detectable cAMP level, while MU group, CK group, or the group with only complete medium showed unmeasurable levels of cAMP (Fig. 3g). These results corroborated that the mutation Arg97X of FSHβ protein disturbed the production of normal bioactive FSH protein.

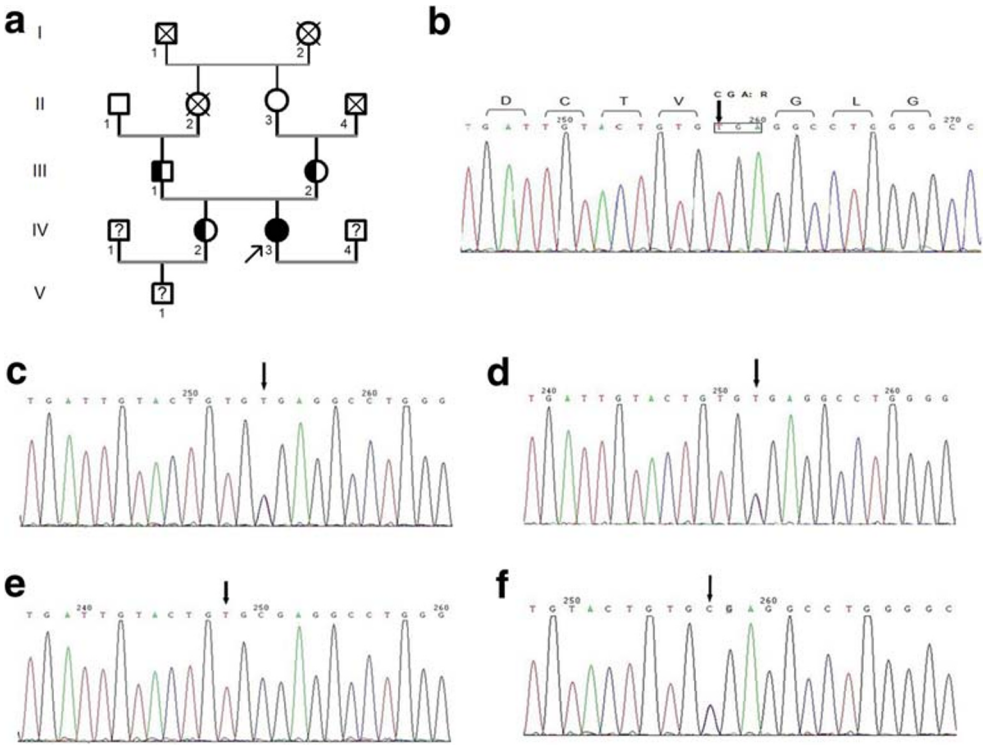

Fig. 2 Family pedigree and FSHβ mutation. **a** Family pedigree. Circles represent females and square male family members. Black symbol indicates the affected patient. Half-shaded symbols indicate unaffected heterozygotes. The arrow indicates the propositus. X mark indicates the family members that have passed away. Question mark indicates the family members who did not be tested. **b** Result of automatic sequencing of exon 3 of FSHβ of the propositus in comparison to the respective wild-type sequence

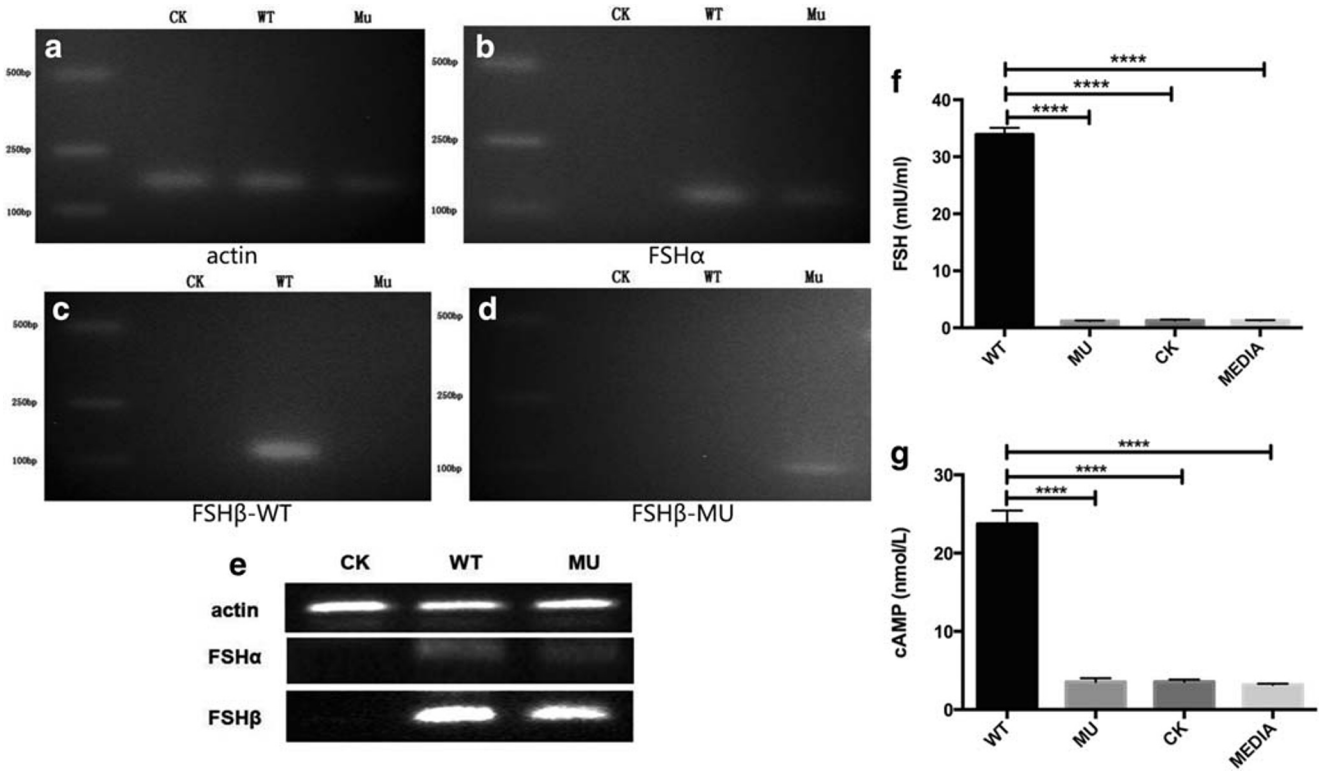

Fig. 3 In vitro studies of mutated FSHβ. FSHα and FSHβ mRNA (**a**–**d**) or protein (**e**) expression in CHO cells co-transfected with the FSHα subunit and either wild-type or mutant FSHβ plasmids. **a** β-actin mRNA was detected in CK, WT, MU groups. **b** FSHα mRNA was detected in WT and MU groups, but not in CK group. **c** Wild-type FSHβ mRNA was detected in WT, but not in CK and MU groups. **d** Mutant FSHβ mRNA was detected in MU, but not in CK and WT groups. **e** The expressions of FSHα and wild-type FSHβ protein were detected in WT group and the expressions of FSHα and mutant FSHβ protein were detected in MU group. Neither FSHα nor FSHβ protein was detected in CK group. Immunoassay (**f**) and in vitro bioassay (**g**) of FSH produced by CHO cells co-expressing wild-type or mutant FSHβ and FSHα-subunit. **f** Wild-type FSH was readily detectable only in culture medium of WT group (****$P < 0.0001$). **g** The cAMP level of WT group was significantly higher than CK, MU, or MEDIA groups (****$P < 0.0001$). WT group: CHO cells co-transfected with the FSHα subunit and wild-type FSHβ plasmids. MU group: CHO cells co-transfected with the FSHα subunit and mutant FSHβ plasmids. CK group: CHO cells transfected with empty vector. MEDIA groups: culture medium without cells

Molecular docking

Structural analysis showed that Arg97X in mutant *FSHβ* subunit caused loss of residues sequence from Arg97 to Glu111 compared with the native. There were important hydrogen bonds between FSHα and *FSHβ* mutant deleted region, including K45 and E108, R42 and Y103. These hydrogen bonds in *FSHβ* mutant commonly deleted region are essential for structural stability of FSH protein consisting of *FSHα* subunit and *FSHβ* subunit. Besides, structural analysis of *FSHβ* subunit revealed that intramolecular hydrogen bond (between E15and E108, E13 and S105) and disulfide linkage (between C20 and C104) were observed in the *FSHβ* mutant losing region, which played important role in maintaining *FSHβ* structure. Moreover, native structure of *FSHα* and *FSHβ* presents an encircling binding mode, while the resulting docking FSH mutant structure shows lock-and-key, mosaic binding pattern **(**Fig. 4d**)**. Binding region and binding pattern of the docking structure changed dramatically compared with the native **(**Fig. 4**)**.

Discussion

FSH is essential for normal pubertal development and reproductive functions both in males and females [1, 2, 13]. Generally, FSH plays pivotal roles in the regulation of Sertoli cell action and spermatogenesis in males. In females, FSH controls the normal follicular development and E2 production [2, 3, 9]. In this family, the proband was a homozygote with a novel mutation Arg97X in *FSHβ* gene, while the mutation was also identified in the proband's parents, elder sister, and maternal grandmother. In vitro analysis of this novel mutation demonstrated that the normal synthesis and function of FSH was disturbed.

This proband with this Arg97X *FSHβ* mutation had representative clinical and laboratory evidence of severe estrogen deficiency, similar to other females diagnosed as isolated FSH deficiency in previous reports (Table 2). The main manifestation of this female included primary amenorrhea, partial breast development, and infertility. In addition, the laboratory evidence also showed similar findings, including undetectable

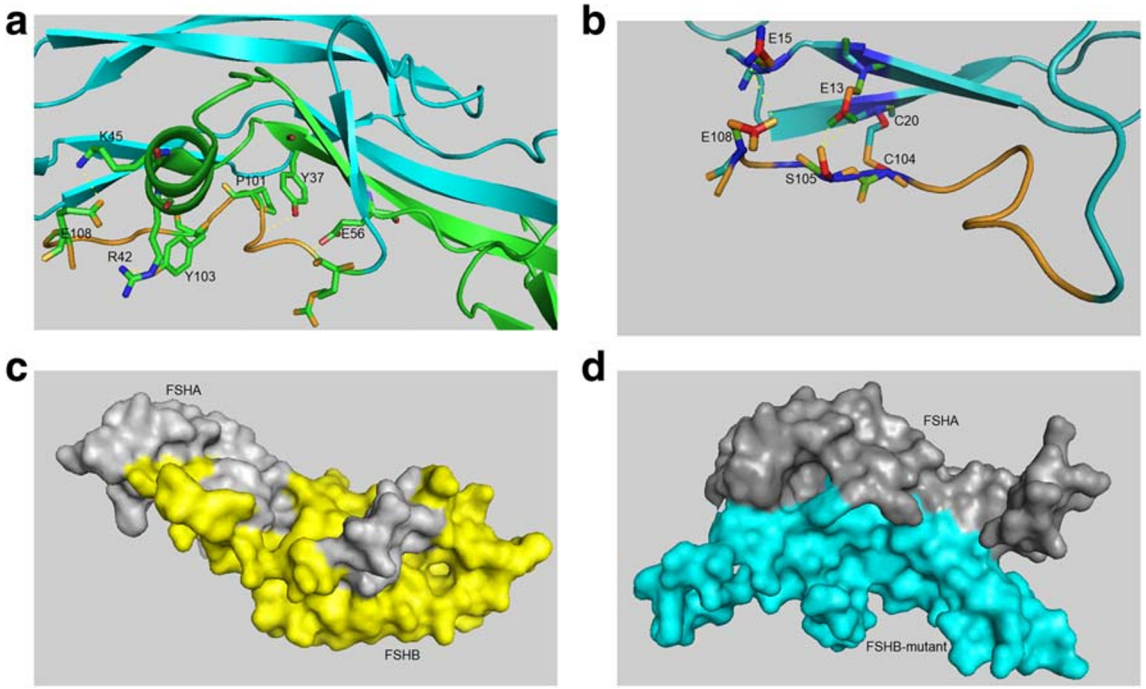

Fig. 4 Molecular docking structures of follicle-stimulating hormone (FSH) protein consisting of FSH-A (FSHα) subunit and wild-type or mutant FSH-B (FSHβ) subunit. **a** Structural analysis of FSH protein revealed the interaction between FSH-A subunit (green part) and wild-type FSH-B subunit (cyan part and saffron yellow region). There is important hydrogen bond between FSH-A and FSH-B mutant commonly deleted region (saffron yellow region), including K45 and E108, R42 and Y103. These hydrogen bond and π-π stacking interaction in FSH-B mutant commonly deleted region (saffron yellow region) are essential for structural stability of FSH protein consisting of FSH-A subunit and FSH-B subunit. **b** Structural analysis of FSH-B subunit revealed that intramolecular hydrogen bond between (E15 and E108, E13 and S105) and disulfide linkage (between C20 and C104) were observed in the FSH-B mutant losing region (saffron yellow region). However, no significant difference was found in FSH-B mutant structure compared with native structure. **c** Surface structure of FSH protein consisting of FSH-A (gray) and wild-type FSH-B (yellow). Native structure of FSH-A and FSH-B presents an encircling binding mode. **d** Surface structure of FSH protein consisting of FSH-A (gray) and mutant FSH-B (cyan). Structural analysis revealed that binding region and binding pattern of the docking structure change greatly compared with the native one. The mutant structure of FSH shows lock-and-key, mosaic binding pattern

FSH, low estrogen, and elevated LH (Table 2). In the two-cell model of ovarian sex steroid hormones synthesis, there is collaborative operation between theca and granulosa cells [5, 15]. LH acts on theca cells to promote the synthesis of androgen [16]. After that, androgen diffuses into adjacent granulosa cells and worked as precursor for synthesis of estrogen. Besides, FSH stimulated granulosa cells for the regulation of follicular development and aromatase expression [17]. Then, the aromatase in granulosa cells would convert the androgen from theca cells to estrogen. Also, FSH is essential to follicular recruitment and development [18]. FSH plays key roles in the achievement of last stages in follicular development, which was significant for the obtaining of LH receptors and ovarian LH responsiveness [9, 19]. Thus, the disturbed FSH production would lead to the precluded synthesis of estrogen. Besides, the serum LH elevation in these females indicated the normal maturation of the hypothalamic-pituitary-gonadal axis, while the negative feedback was attenuated due to reduced estrogen production (Table 2).

Both in vitro immunoassay and bioassay of the Arg97X mutation demonstrated non-measurable FSH. The second messenger cAMP was chosen as the endpoint for FSH bioassay. The results were similar to previous investigations on *FSHβ* mutations [3, 5, 9]. However, both *FSHβ* and *FSHα* could be detected in the Arg97X mutant group, which means that the mutation Arg97X did not affect the detectable *FSHβ* protein expression. Taken together, this mutation Arg97X may impact the normal function and secretion of FSH by the disruption of FSH structural stability.

In order to find out the possible impact of mutation Arg97X on the structure of FSH protein, molecular modeling was constructed. Arg97X in mutant *FSHβ* subunit caused loss of residues sequence from Arg97 to Glu111 compared with the native one. Although structural analysis showed that no significant difference was found in *FSHβ* mutant structure compared with native structure, there was dramatic change of the structure of the FSH protein consisting of *FSHα* subunit and mutant *FSHβ* subunit. There are important hydrogen bonds between FSHα and *FSHβ* mutant commonly deleted region, including K45 and E108, R42 and Y103. These hydrogen bonds are essential for structural stability of FSH protein consisting of *FSHα* subunit and *FSHβ* subunit. Besides,

Table 2 A review of isolated FSH deficiency caused by inactivating FSHβ gene mutation in female patients

Age	Nucleotide	Protein	Clinical presentation	Homo-/heterozygous	In vitro analysis of function	Ref.
27	GTG → GXX	Val61 → X	Primary amenorrhea, absent breast development, infertility	Homozygous	-	[10]
15	GTG → GXX TGT → GGT	Val61 → X Cys51 → Gly	Primary amenorrhea, absent breast development	Compound homozygous	No detectable FSH	[5]
22	GTG → GXX	Val61 → X	Primary amenorrhea, absent breast development, infertility	Homozygous	-	[14]
32	TAC → TAA	Tyr76 → X	Partial breast development, primary amenorrhea	Homozygous	No detectable FSH	[3]
16	TAC → TAA	Tyr76 → X	Partial breast development, primary amenorrhea	Homozygous	-	[2]
29	c.289delG	Ala79 → X	Primary amenorrhea, partial breast development, infertility	Homozygous	No detectable FSH	[9]
29	CGA → TGA	Arg97 → X	Primary amenorrhea, partial breast development, infertility	Homozygous	No detectable FSH	Presentreport

structural analysis of *FSHβ* subunit revealed that intramolecular hydrogen bond (between E15 and E108, E13 and S105) and disulfide linkage (between C20 and C104) were observed in the losing region of mutant, which are of significance to the stability of *FSHβ* structure [13]. Also, the cysteine knot of *FSHβ* was disrupted due to Arg97X mutation, which was essential to dimer formation and intracellular stability [13, 20]. Moreover, native structure of *FSHα* and *FSHβ* presents an encircling binding mode, while mutant structure shows lock-and-key, mosaic binding pattern. Structural analysis revealed that binding region and binding pattern of the docking structure change greatly compared with the native one, which might lead to structural instability of the resulting FSH protein. This resulting FSH protein could not be secreted to extracellular space, causing FSH dysfunction.

With uFSH replacement, multiple follicular developments were observed by transvaginal sonography, together with a dramatic increase in serum E2 and a remarkable rise of endometrial thickness, which was similar to previous studies [9]. Moreover, there was the report of a female with *FSHβ* mutation, who conceived by the treatment of induced ovulation after adequate exogenous FSH replacement [5]. Therefore, in contrast to those females suffering from combined FSH and LH deficiency in previous reports [21–24], these facts revealed that high LH level was also necessary for collaborative synthesis of estrogen in theca and granulosa cells, while the deficiency of FSH was the dominant cause of ovarian dysfunction in females with *FSHβ* mutations. Taken together, the combination therapy of FSH replacement and assisted reproductive technology (ART) could be effective treatments for infertility in patients with *FSHβ* mutations.

In conclusion, we reported a novel nonsense mutation (Arg97X) in the *FSHβ* gene. In vitro analysis of the Arg97X mutation demonstrated undetectable immuno and bioactive FSH. Also, we described the clinical and laboratory characteristics of the patient with homozygous Arg97X mutation from a consanguineous family. The prevalence of *FSHβ* mutations is rare due to their autosomal recessive transmission and the resulting infertility, while their identification has opened new horizons in the understanding of normal reproductive physiology.

Funding information This work was supported by the research grants from:

1. National Key Research and Development Program (China, 2018YFC1002103)
2. The Chinese Medical Association (16020520668)
3. The Natural Science Foundation of Hubei Province (China, 2017CFB752)

Compliance with ethical standards

Before the uFSH treatment, the study protocol was approved by the Medical Ethics Committee of Tongji Hospital and written informed consents were obtained from the patient. Researches were conducted according to the Declaration of Helsinki for medical research.

Conflict of interest The authors declare that they have no competing interests.

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