A Novel Compound Heterozygous Mutation of FSHR Causes Primary Ovarian Insufficiency

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Research article

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Abstract

Background: Primary ovarian insufficiency, one of the main causes of female infertility, is a heterogeneous disease when it comes to the phenotype and etiology. Familial cases are observed in approximately 10% of patients which indicates a strong genetic component. However, the underlying cause remains to be identified in most cases of patients.

Methods: Here we studied an un-consanguineous Han Chinese family in which four siblings are primary amenorrhea and hypergonadotropic hypogonadism. Three siblings with POI and one unaffected sibling were exome sequenced. Also, other members in this family were genotyped by Sanger Sequencing. In silicon and in-vitro functional analyses were performed.

Results: Whole exome sequencing identified a shared novel compound heterozygous mutation of FSHR gene in all the affected members. c.1412T>G, the first variant identified in FSHR IL2(intracellular loop2) in POI patients, and another novel mutation c.1090_1091del were the genetic etiology of this family. In-vitro functional analyses showed that cAMP (second messenger of FSHR) producing was abolished by c.1412T>G.

Conclusions: Our study identified two novel FSHR mutations in a compound heterozygous state and gave the evidence that the FSHR IL2 could play a crucial role in FSHR-caused POI.

Introduction

Primary ovarian insufficiency(POI), which affects about 1% women under 40 years old, is a subset of ovarian dysfunction with the cause in the ovary itself [1, 2]. The POI women typically present hypergonadotropic hypoestrogenic amenorrhea, primary or secondary, before the expected menopause age with impaired fertility and health[3]. The mechanisms underlying POI concern with either follicle depletion (e.g. exhaustion of the initial follicle pool, accelerated follicular atresia) or follicle dysfunction (e.g. altered follicular maturation [4, 5]. A wide spectrum of causes may promote such largely unknown mechanisms which include genetic, infectious, iatrogenic, metabolic, and autoimmune factors [6]. Genetic etiology includes chromosomal abnormalities which have a prevalence of 10–13% and monogenetic defects with extreme heterogeneity[7, 8]. A set of variants of X-linked (e.g. BMP15, PGRMC1, FMR1) and autosomal (e.g. HFM1, FSHR, NOBOX, STAG3) genes have been studied to cause isolated or syndromic POI, and dozens of genes have emerged in POI as candidates [8–11].

FSH (Follicle Stimulating Hormone) is essential for genesis of an antrum in the follicle and enabling development to the preovulatory follicle stage. FSH plays a crucial role in the regulation of normal follicle growth and estrogen production via binding to the FSH receptor (FSHR) located at the granulosa cells membrane [12]. FSHR is a G-protein-coupled receptor (GPCR) which consists of the extracellular domain (ECD), the transmembrane domain (TMD), and the intracellular domain (ICD). The TMD comprises seven a-helices, interconnected through three extracellular loops (ELs) and three intracellular loops (ILs). FSH's binding to the ECD induces a series of reactions, ICD couples to a Gs protein bringing up a cascade of

intracellular events and activates the steroidogenesis[13]. As a main second messenger in this process, cyclic AMP (cAMP) could be a target of in vitro functional characterization of FSH-FSHR defects[14]. FSHR variation was the earliest interpreted autosomal molecular cause of POI[15]. There have been over 20 inactivating mutations with effect on FSHR membrane localization, signal transduction, or ligand-binding affinity reported so far. These mutations were found in ECD, TMD, EL1/EL2/EL3, IL3, or ICD. The different mutation location and the nature of substituted amino acid impact FSHR function to different extent [13, 16–31]. However, no inactivating variants in the intracellular loop1 and intracellular loop2 of FSHR have been reported in POI patients.

Here, we studied an un-consanguineous Han Chinese family in which four siblings are affected with POI(Ⅱ-1,Ⅱ-2Ⅱ-3Ⅱ-4) (Fig. 1A). Whole-exome sequencing was used to identify a novel compound heterozygous mutation shared by the affected siblings in gene FSHR, which is potentially the pathogenic variant based on an autosomal recessive inheritance mode.

Materials And Methods

Subjects

We enrolled a family in which two members (Ⅱ-1; Ⅱ-2) came to the Reproductive Medicine Center of the First Affiliated Hospital of Zhengzhou University for infertility treatment. Blood from them and another five family members(Ⅱ-1; Ⅱ-2; Ⅱ-3; Ⅱ-4; Ⅱ-5)were obtained before any treatment (Fig. 1A).

All subjects provided their written informed consent. All work was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University.

Whole Exome Sequencing

Three siblings with POI (Ⅱ-1; Ⅱ-2; Ⅱ-4) and one unaffected sibling(Ⅱ-5) were exome sequenced. 500 ng genomic DNA was isolated from peripheral blood for each member using Qiagen DNA Blood Mini Kit (Qiagen) and fragmented with the average fragment size set to 200 bp with Covaris E220 (Covaris). The sample library was prepared for Illumina Multiplex Sequencing with the KAPA Library Preparation Kits (KAPA Biosystems). Exons and flanking intronic regions were enriched by hybridizing the pooled sample library with the SeqCap EZ Library (Roche) and subjected to DNA sequencing on Illumina HiSeq2500. The sequencing reads were aligned to GRCh37.p10 using BWAv0.59[32]. Variants were identified using GATK and annotated by Annovar[33, 34]. The candidate variants were validated by Sanger Sequencing. Also, other members (1 POI daughter and the parents) in this family were genotyped.

To assess the potential functional effects of amino acid substitutions, PolyPhen-2(http://genetics.bwh.harvard.edu/pph2/), SIFT(http://provean.jcvi.org) and Mutation Taster (http://www.mutationtaster.org/) were used. And SWISS-MODEL software(https://swissmodel.expasy.org) was used for structural analysis of the variant.

Plasmid Construction

FSHR cDNA was PCR amplified from FSHR (NM_000145) Human Tagged ORF Clone (Origene RG217736) with primers as follows: 5'-GGGGTACCATGGCCCTGCTCCTGGTCT-3', reverse:5'-CCGCTCGAGTTAGTTTTGGGCTAAATGAC-3'. The product was excised by digestion with XhoI/KpnI and ligated into the Xho I/KpnI restriction site of the pcDNA3.1- Hygro(+) vector to form pcDNA3.1- Hygro(+)-FSHR. And its sequence was checked with commonly used primers CMV-F and BGH-R. Then the mutant plasmid was generated with the mutagenic primers as follows: 5' - TGGCATACCAGCACGCATGCCATGCAGCTG-3', reverse:5' - GGCATGCGTGCTGGTATGCCATCTTTCCAA-3', pcDNA3.1- Hygro(+)-FSHR as the template. The point mutation was confirmed with DNA sequencing using CMV-F and BGH-R (Fig.S1).

cAMP Assay

HEK293 were seeded in 100 mm cell culture dishes to grow to about 80% confluence and transfected with pGloSensor-22F cAMP Plasmid (Promega) and one of the desired plasmids (mock vector, wild-type FSHR, or p.I471S mutant plasmids) using FuGENE HD Transfection Reagent (Promega). The transfected cells were seeded to 96-well plates after 18 h incubation and incubate for another 18 h before preparation for the cAMP measurement. Immediately after adding human FSH (100 IU/L) to the cells, measurement of fluorescence value RLU with a microplate reader (FlexStation3) was performed every 3 min in a 45 min period to record the cAMP levels. Also, FSH of the final concentration of 0, 10, 50, 100, 500, 1000 IU/L was added per well of all three groups and the fluorescence value RLU was measured after a total of 20 minutes. Three independent replications of this assay were performed.

Results

Clinical Features

Two probands in the un-consanguineous family were 26 and 27 years old when they came to our institute for artificial reproductive treatment. They were both married and had infertility due to premature ovarian insufficiency characterized by primary amenorrhea. Hormone replacement therapy maintained a period of regular menstruation for them. Physical examinations were both normal with the height of 165;164 cm, weight of 50;65 kg, developed breast and pubic hair of Tanner Stage 4;4. Transvaginal ultrasound revealed hypoplastic uterus and small ovaries (right:18mm × 9 mm, left:17mm × 7 mm;right:16mm × 10 mm, left:16mm × 9 mm) with no visible antral follicles for them. Serum gonadotropin concentration, examined at the time they were going to undergo in vitro fertilization (IVF) with donor oocytes, revealed a menopause FSH level of 80.35;56.98 IU/L, LH:37.5 IU/L;24.71 IU/L, E2:4.88 pg/mL;3.66 pg/mL, T:0.05 ng/dL;0.06 ng/dL, PRL:14.88ug/L;31.13ug/L(Table 1).

Table 1
Clinical features of the siblings in this family

	II-1	II-2
Age	26	27
Height(cm)	165	164
Weight(kg)	50	65
Pubertal development	breast and pubic hair of Tanner Stage 4	breast and pubic hair of Tanner Stage 4
Amenorrhea	Primary amenorrhea	Primary amenorrhea
FSH(UI/l)	80.3	56.9
LH (mIU/mL)	37.5	24.7
E2 (pg/mL)	4.88	3.66
T(ng/dL)	0.05	0.06
PRL(μg/L)	14.88	31.33
Ultrasound imaging	right:18mm × 9 mm, left:17mm × 7 mm; no visible follicles	right:16mm × 10 mm, left:16mm × 9 mm; no visible follicles
Karyotype	46, XX	46, XX
FMR1 premutation	normal	normal

There are another two siblings who were diagnosed with POI for several years, one unaffected sibling with regular menstruation and one normal male sibling who is fertile (Fig. 1A). We performed physical examinations and hormonal studies to evaluate a similar condition of the other two affected patients to their siblings mentioned above, and the normal condition of the unaffected sister. The mother had her menarche at a normal age at 13 years old with regular menstruation until menopause at 54 years old.

All the affected daughters have normal 46, XX karyotype, and normal range of FMR1 repeat length. No autoantibodies were detected in any of them. And, there are no relevant medical findings on the family members.

A novel compound heterozygous mutation in FSHR was identified

Whole exome sequencing was used to screen the potential gene mutation in POI patients. The mean coverage of the reads was about 150X, > 95% of targets were covered at 20X or greater. We focus on homozygous or compound heterozygous functional variants (missense, nonsense, frameshift, splicing)

that had never been reported or had a prevalence below 0.1% in the 1000 Genomes variant database, the NHLBI (National Heart, Lung, and Blood Institute), Exome Sequencing Project database, the Exome Aggregation Consortium and an Inhouse database of ~ 2500 Han Chinese Samples. Then the variant passed the filter if it was present in the three affected siblings and absent in the unaffected sibling. We identified a novel compound heterozygous FSHR mutation consisting of a 2 bp exon6 deletion(c.1090_1091del) resulting in a truncated protein (p. Ile364Profs*29) and an exon10 point mutation c.1412T > G leading to a missense substitution (p.Ile471Ser) (Fig. 1B). The compound heterozygous status of the affected siblings (II-1,2,4) and the homozygous wild type status of the unaffected sibling (II-5) were confirmed by Sanger sequencing. Also, another affected sibling(II-3) was found to carry the compound heterozygous mutation, the mother was found to carry the point mutation in exon9(c.1412T > G) in a heterozygous status, and the father was found to carry the 2 bp deletion mutation in exon6(c.1090_1091del) in a heterozygous mode (Fig. 1C). Surprisingly, we identified the first mutation located in FSHR IL2(intracellular loop2) region.

The missense mutation identified affected a highly conserved amino acid located in the intracellular loop 2 of the FHSR (Fig. 2A). It is predicted to be deleterious in silico by both SIFT and PolyPhen-2 and Mutation Taster (Table 2). The secondary structure modeled by SWISS-MODEL software showed no obvious difference between predicted 3D models of WT and c.1412T > G(Fig. 2C), while the c.1090_1091del mutation resulted in the loss of almost half part of the FSHR (Fig. 2B).

Table 2
Mutation function In silicon prediction

Variation	Location	Amino acid change	SIFT	PolyPhen-2	Mutation Taster
c.1090_1091del	Exon6	p.Ile364Profs*29	-	-	Disease causing
c.1412T > G	Exon9	p.Ile471Ser	Damaging	Probably damaging	Disease causing
-, not available for the 2 bp deletion frameshift mutation					

FSH-induced cAMP was abolished by p.I471S

The FSH induced cAMP levels in HEK293 cells transfected with different plasmids (mock vector, wild-type FSHR, or p.I471S mutant plasmids) were measured. The reaction of cells expressing p.I471S mutant FSHR was most likely the cells transfected with mock vectors but different with cells expressing wild type FSHR. The cAMP level increased once FSH (100 IU/L) was added and reached the highest at 30 min in wild type group while the mutant group almost showed no reaction (Fig. 3A). Similarly, the FSH-induced cAMP level measured at 20 min after FSH added was positively correlated with FSH concentration in wild type group but not in the control group and mutant group (Fig. 3B). These results showed that the point mutation could result in the loss of FSHR function and could lead to the absolute ovary dysfunction when together with the truncated mutation in the compound heterozygous mode.

Discussion

We identified a novel compound heterozygous FSHR mutation in four siblings from an un-consanguineous Han Chinese family, moreover, and firstly identified a mutation located in FSHR IL2 region. The variant c.1090_1091del carried by the father results in a truncated protein with 391 amino acids and chaos of the remaining transmembrane helix and intracellular loop. The variant c.1412T > G carried by the mother leading to a missense substitution (p.Ile471Ser) in the intracellular loop 2 which is predicted to be deleterious in silicon analysis and validated in the cAMP assay. The phenotype separating well with the genotype identified by WES and Sanger sequencing suggests that this compound heterozygous mutation is the disease-associated mutation in this family. Notably, c.1412T > G is the first variant in the FSHR intracellular loop 2 identified in POI patients so far.

During the initiation of follicular growth, FSH-FSHR signaling is not required[35]. FSHR begins to express between the preliminary and antral stage and is essential for later follicular development [36]. FSHR-caused POI is due to the arrest but not depletion of follicles usually with some ovarian reserve as determined by AMH (Anti-Mullerian Hormone) and AFC (antral follicle count). There is a correlation, which could be inferred but not completely reflected by in-vitro functional assay, between the specific mutation with the phenotype[13, 30]. POI patients caused by FSHR mutations identified previously most showed small follicles(3–5 mm) by transvaginal ultrasound or follicular arrest at the small antral stage histologically[18, 19, 29]. In other cases, a block of follicular growth after the primary stage, and no antral follicles was observed in patients with specific mutations[21, 22, 24, 26].

In this study, all the hypergonadotropic patients with primary amenorrhea have partially normal secondary sexual characteristics, after exogenous estrogen and progesterone therapy, and two of them received reproductive treatment and conceived after a donor's oocyte-based IVF treatment indicating that they are not in complete sexual infantilism. However, the transvaginal ultrasound showed a hypoplastic uterus and small bilateral ovaries with no visible antral follicles of the four patients in this study. The phenotype indicated a more severe defect of in vivo FSHR function than that of cases with ultrasound-visible or small antral follicles. It is not difficult to infer that the 2 bp exon6 deletion (c.1090_1091del) resulting in a truncated protein with no TMD and intracellular structure causes a complete loss of function. As to the first IL2 mutation (c.1412T > G) identified in POI patients so far, the in vitro functional assay showed complete loss of the production of FSH induced cAMP. Together with the genotype-phenotype correlation, it suggested that the novel compound heterozygous mutation would remain with no residual FSHR activity. Our patients did not have ovarian histology and AMH tests performed, but we guess some follicles that arrest at different stages ranging from primordial follicles to preantral follicles in their ovaries. The normal male sibling who is fertile (II-7) was not genotyped and examined. However, we could not exclude the possibility that the male sibling also carries the two mutations in a compound heterozygous, for the FSHR defection may not affect male fertility in most case[13].

Since the first inactivating mutation p.Ala189Val in the FSHR ECD was reported in Finnish women[16], more variants were identified mostly in ECD, TMD, and ELs[30]. It is well established that the ECD is the

primary hormone-binding domain, whereas the TMD is the signal-transducing domain. ELs also play an essential role in ligand binding and FSHR trafficking in cell membranes[37]. ILs and ICD mutations in POI were relatively uncommon since the first variant in the C-terminal domain(p.Pro688Thr) in POI was just identified recently and only one mutation (p.Arg573Cys) was reported in IL3 so far[18, 31]. Previous studies have provided evidence showing the key role of IL2 in Gs coupling, cAMP production, and also receptor phosphorylation [38, 39], but there was no IL2 variation identified in POI patients before. The missense mutation in this case locates close to the highly conserved class A GPCR ERW motif, which is pivotal for receptor function[40]. Also, it locates at the boundary of the region associated with 14-3-3 tau protein, which is important for ER localization of membrane proteins, interacting activity [39, 41]. These all, with the cAMP assay result, support the c.1412T > G mutation as a variant of certain significance. However, as we didn't perform membrane localization assay, there is still a possibility that the c.1412T > G FSHR protein decreased at cell membrane though no structure change by in silicon prediction. The cAMP induction assay was not necessary for the c.1090_1091del mutation, for the 2 bp deletion frameshift mutation could result in a badly deleterious effect on the receptor structure.

Conclusions

In conclusion, we identified a novel compound heterozygous mutation c.1090_1091del (p. Ile364Profs*29) and c.1412T > G (p. Ile471Ser) in the FSHR gene. And c.1412T > G, as the first FSHR IL2 variation identified in POI patients, provides additional support to the view that the IL2 domain plays an important role in human FSH-FSHR conducted ovarian physiology.

Abbreviations

POI
Primary ovarian insufficiency
FSHR
Follicle stimulating hormone receptor
cAMP
Cyclic adenosine monophosphate
IVF
In vitro fertilization
ECD
Extracellular domain
TMD
Transmembrane domain
ICD
Intracellular domain
ELs
Extracellular loops

ILs
Intracellular loops
AMH
Anti-müllerian hormone
AFC
Antral follicle count

Declarations

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Author contributions

JWX and YPS designed this study. NZ conducted Exom-sequencing, JWX and NZ performed data analysis with help of XB, NZ, WBL and QL finished the mutation function studies with the help of FFZ and DYS. NZ and JWX wrote the first draft and completed the final draft with the assistance of YPS. WBN helped revise the manuscript. All authors agree with the conclusions of our study.

Ethics approval and consent to participate

All blood samples were obtained with written informed consent. All work was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University.

Competing interests

There is no conflict of interest associated with this manuscript.

Consent for publication

Written informed consent for publication of their clinical details was obtained from the patient. A copy of the consent form is available for review by the Editor of this journal.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

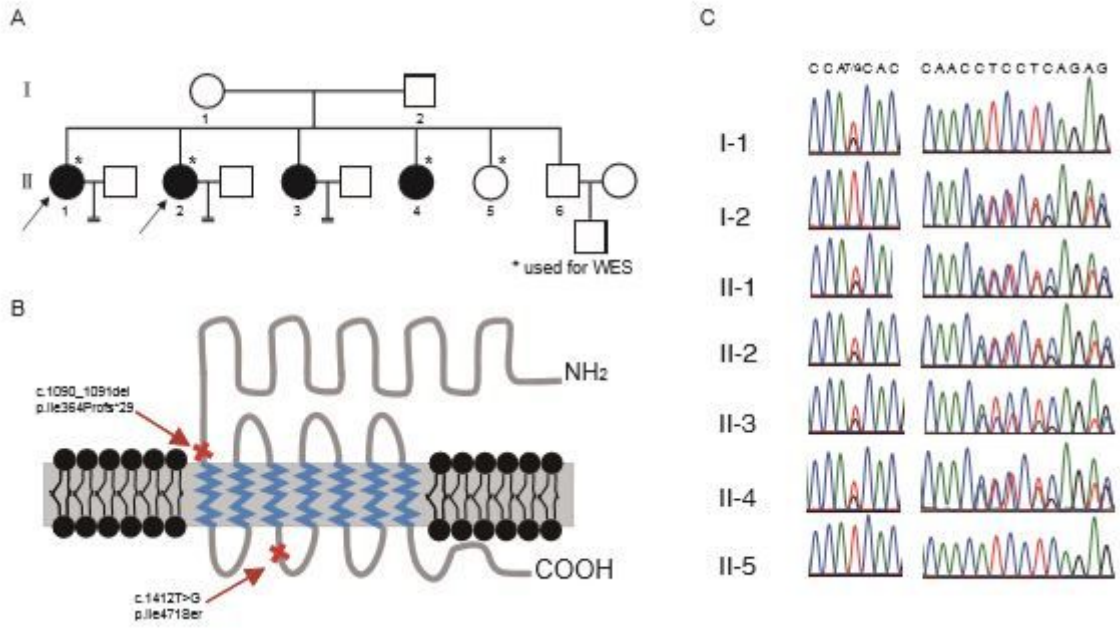

Figure 1

Identification of Mutations in FSHR (A) Family pedigree of women with POI. Squares denote male members, circles denote female members and solid symbols represent affected members. Equal signs indicate infertility, and arrow points to the probands, * indicates WES performed. (B) The overall structure

of FSHR on the cell membrane. A transmembrane domain comprising seven a-helices, interconnected through three extracellular (ELs) and three intracellular loops (ILs); A ligand-binding extracellular domain at the amino tail; An intracellular domain at the carboxyl tail. The locations of the novel compound heterozygous mutation are demonstrated here. (C) Sanger sequencing was used to validate genotypes. The four affected siblings (II-1,2,3,4) had the same compound heterozygous mutations in FSHR: c.1090_1091del (p. Ile364Profs*29) and c.1412T>G (p.Ile471Ser). The parents' heterozygous status of each mutation was confirmed.

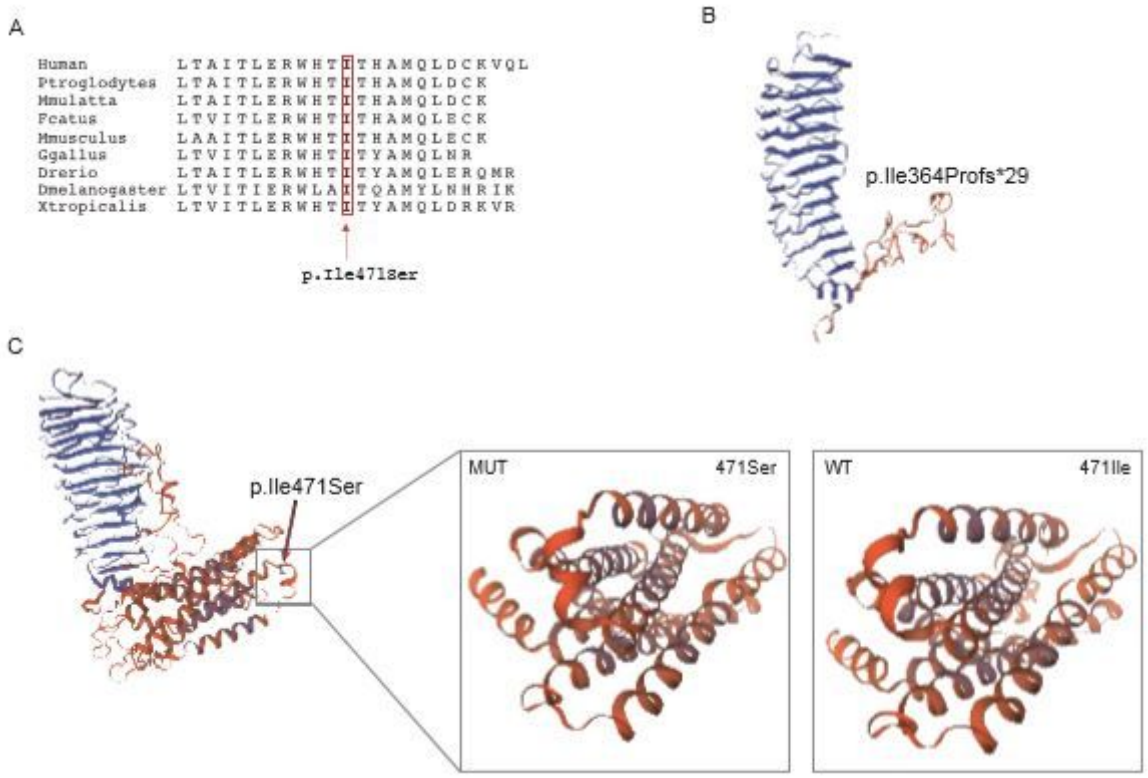

Figure 2

(A) Evolutionary conservation of Ile471 across species. (B) Modeling of the FSHR mutation by SWISS-MODEL. The predicted secondary structure of truncated mutation (c.1090_1091del) showed the absence of TMD and intracellular domains. (C) There is no obvious difference between the predicted secondary structure of missense mutation (c.1412T>G) and wild type.

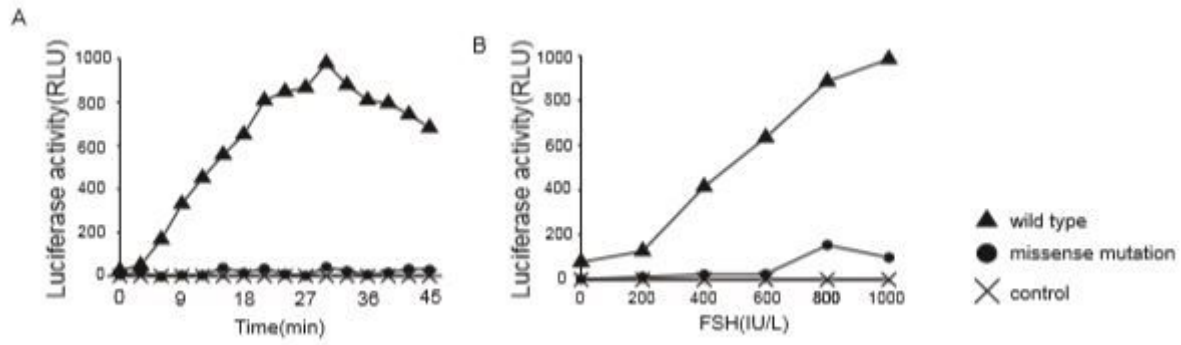

Figure 3

cAMP measurement in 293T cells. The value of all three groups subtracted that of the control group for the plot. Three independent experiments show a similar result. (A) The cells transfected with mock vector, wild-type FSHR, and p.Ile471Ser mutant were stimulated with the use of human FSH (100 IU/L) for 45 minutes, and cAMP was measured every 3min. (B) The cells transfected with mock vector, wild-type FSHR, and p.Ile471Ser mutant were stimulated different doses of FSH (0–1,000 IU/L) and cAMP was measured after 20 minutes.