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Running title: Novel *FSHR* Mutations in Han Chinese POI

Title:

Novel *FSHR* Mutations in Han Chinese Women with Sporadic Premature Ovarian Insufficiency

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Conflicts of interest: none

Capsule: Two novel missense mutations of *FSHR* were identified in Chinese sporadic POI patients, highlighting the contribution of FSHR in the etiology of POI in Han Chinese population.

Abstract

Premature ovarian insufficiency (POI) is characterized by amenorrhea and elevated levels of follicle-stimulating hormone (FSH, usually > 25 IU/L) before 40 years of age. To identify the relationship between FSHR mutations and sporadic POI patients of Han Chinese descent, we performed Sanger sequencing of *FSHR* gene in 192 sporadic POI patients and 192 matched controls of Han Chinese descent. Two heterozygous missense variants, c.793A>G (p.M265V) and c.1789C>A (p.L597I), were identified exclusively in POI patients. Functional studies showed that both mutants were expressed on the cell surface, while p.L597I showed decreased membrane localization compared with wild-type FSHR. Moreover, FSH-induced cAMP production and ERK1/2 phosphorylation were reduced in the cells transfected with p.L597I mutant, but not in the cells transfected with p.M265V mutant. In addition, two single-nucleotide polymorphisms (SNPs), rs1394205 (c.-29G>A) and rs140106399 (c.*111T>C), were identified in both POI group and control group with significantly different genotypic and allelic distributions. These results indicated that dysfunctional FSHR due to mutation or SNPs might explain a fraction of sporadic POI cases in Han Chinese population.

Key words: Premature ovarian insufficiency, *FSHR* gene, mutation, polymorphism, functional characterization

Introduction

Premature ovarian insufficiency (POI), also termed as premature ovarian failure (POF), is characterized by amenorrhea and elevated levels of follicle-stimulating hormone (FSH, usually > 25 IU/L) before 40 years of age. It is a very heterogeneous condition and a wide spectrum of causes including genetic defects, autoimmune disorders, metabolic factors, iatrogenic interventions and environmental factors have been reported (De Vos, et al., 2010). Through candidate gene studies and whole exome sequencing, many causal genes for POI such as *BMP15*, *NOBOX*, *FIGLA*, *STAG3*, *MCM8*, *CSB-PGBD3* and *MSH5*, have been identified (AlAsiri, et al., 2015, Caburet, et al., 2014, Guo, et al., 2017, Jiao, et al., 2018, Qin, et al., 2007, Qin, et al., 2015). However, the etiology of POI is still poorly understood in the majority of sporadic cases.

The pituitary glycoprotein hormone FSH is critical for female reproduction and acts through binding to its receptor (FSHR) which is expressed exclusively on granulosa cells in the ovary (Simoni, et al., 1997). In the classical signaling pathway, FSH-FSHR interaction activates the cAMP-protein kinase A cascade and then increases the phosphorylation of ERK1/2 protein, leading to steroid metabolism via CYP19 induction (Ulloa-Aguirre, et al., 2007). Targeted knockout of the *Fshr* gene in female mice led to a complete arrest of follicular development at the preantral stage (Abel, et al., 2000, Dierich, et al., 1998).

The *FSHR* gene has been identified as the first gene causing nonsyndromic POI (Aittomaki, et al., 1995). Diverse inactivating mutations of the *FSHR* gene have been described, most of which lead to a POI phenotype (Achrekar, et al., 2010, Bramble, et al., 2016, Franca, et al., 2017, Katari, et al., 2015), and patients with different

inactivating mutations in the *FSHR* gene presented with variable phenotypes because of their distinct loss of FSHR function. Those carrying a partial loss-of-function mutation displayed a mild phenotype with secondary or primary amenorrhea, normal puberty and normal-sized ovaries containing antral follicles (Beau, et al., 1998, Woad, et al., 2013), while others with complete loss of FSHR function showed a more severe phenotype with primary amenorrhea, delayed puberty and small ovaries containing small follicles before the secondary stage (Allen, et al., 2003, Kuechler, et al., 2010, Meduri, et al., 2003). Recently, we reported a homozygous nonsense mutation c.175C>T (p.R59X) in the *FSHR* gene in a POI family in which the proband presented with a complete POI phenotype, with elevated FSH level and very low estrogen (Liu, et al., 2017).

The inactivating mutations in the *FSHR* gene have been reported in sporadic POI patients, such as c.566C>T (p.Ala189Val) has mostly been reported in Finnish (Conway, et al., 1999, de la Chesnaye, et al., 2001). While the frequencies of such mutations in Chinese population have remained unknown. This study aimed to determine whether variants in the *FSHR* gene are associated with sporadic POI in Han Chinese population. Here, we identified two novel missense variants as well as two SNPs with significantly different genotypic and allelic distributions in the *FSHR* gene.

Materials and Methods

Ethics statement

The study procedures were approved by the Institutional Review Board of Reproductive Medicine of Shandong University. Written informed consent was obtained from all subjects.

Study population

A total of 192 patients with sporadic POI and 192 control women were recruited from Center for Reproductive Medicine of Shandong University. The POI recruitment criteria included primary or secondary amenorrhea for at least 6 months before 40 years of age, serum FSH>40 IU/L, and no family history of POI. Known causes, such as autoimmune diseases, FMR1 premutation, pelvic surgery, and chemoradiotherapy treatment were excluded. Among these 192 POI patients, 45 presented with primary amenorrhea and 147 presented with secondary amenorrhea. The mean serum FSH level in POI group is (78.7±28.0) IU/L. The 192 age-matched controls were known to be menstruating regularly and had normal FSH levels (7.82±1.71 IU/L). All the participants were of Han Chinese origin with normal karyotype (46, XX).

Sanger sequencing

Genomic DNA was extracted from peripheral blood samples with QIAamp DNA mini kit (QIAGEN) according to the manufacturer's protocol. All exons and exon-intron boundaries of the human *FSHR* gene (NM_000145) was PCR-amplified and directly sequenced on an ABI 3730-Avant Genetic Analyzer (Applied Biosystems). The novel variants were confirmed by three independent PCR runs, followed by sequencing in both forward and reverse directions, and verified in 192 controls. Amino acid sequences from other species were obtained from Uniprot database (http://www.uniprot.org/), and the conservation analysis was conducted by using ClustalW2 website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Plasmid construction and mutagenesis

The wild-type vector was constructed by inserting human FSHR cDNA directly into pSG5 expression vector as described previously (18). The mutant expression vectors for p.M265V and p.L597I were prepared by using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) with wild-type construct as the template.

DNA sequencing was conducted to confirm the desired mutation and exclude other mutations.

Cell culture and transfection

The human embryonic kidney 293T (HEK293T) cells were cultured in high glucose DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37˚C. For functional experiments, cells were transfected with the desired plasmids using lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions, and all assays were carried out at 48 h post transfection. Cells transfected with mock plasmid served as a negative control.

Immunofluorescence

Indirect immunofluorescence was performed as previously described with minor modifications (Allen, et al., 2003). The FSHR antibody (Proteintech) recognizes an epitope located in the extracellular domain (ECD) of the FSHR and enables detection of receptor expression at the cell membrane. At 48 h post transfection, cells grown on cover slips in 24-well plates were chilled at 4˚C and incubated for 1 h in PBS containing 1% bovine serum albumin (BSA) and FSHR antibody (1:50 dilution; Proteintech). The cells were then fixed with 3% paraformaldehyde (PFA) for 20 min at room temperature. After blocking for 1 h with PBS containing 1% BSA, cells were incubated for 1 h with Alexa Fluor 488-goat anti-rabbit immunoglobulin G (1:100 dilution; ZSGB-BI). The cells were washed, counterstained with DAPI, and finally visualized under a fluorescence microscope (BX53, Olympus).

Flow cytometry

Flow cytometry was performed as previously described with minor modifications (22). Cells were seeded in a 6-cm dish for 24 h to grow to 75~80% confluence prior to

transfection with desired plasmids (mock plasmid, wild-type or mutant FSHR plasmids). The cells transfected with mock vector (pSG5) served as a negative control. The transfected cells were maintained for 24 h and then digested into single cell suspensions before staining. Each of the three (WT, M265V, L597I) single cell suspensions were split into two groups, one for non-permeabilized membrane FSHR staining, and the other for internal FSHR staining. For the surface FSHR staining, the FSHR antibody (1:50 dilution, Proteintech) was added to single cell suspension and incubated for 1 h with the addition of 1% BSA for blocking. The cells were then fixed with 4% PFA for 20 min at room temperature, and incubated in DPBS containing 1% BSA and anti-rabbit Alexa Fluor 488-conjugated IgG secondary antibody (1:100 dilution; Proteintech) for 45 min. Finally the cells were washed and stored in DPBS for flow cytometry analysis. For intracellular FSHR staining, the second portion of cell groups were firstly fixed with 4% PFA for 20 min at room temperature, and then permeabilized with 0.3% Triton X-100 for 10 min prior to incubating with the FSHR antibody (1:50 dilution, Proteintech) for 1 h. The other steps were the same as surface staining. Fluorescence signal was measured using FACSCalibur (BD Biosciences). The surface FSHR signal was normalized to the internal FSHR signal for each group, and then the relative FSHR membrane localization of the mutants was obtained by comparing with the value of the wild-type receptor (100%). The experiments were carried out in triplicate.

cAMP Assay

FSH-induced cAMP production was measured by using GloSensor cAMP Assay kit (Promega) according to the manufacturer's instructions. Cells were seeded in a 6-well plate for 24 h to grow to 75~80% confluence and then transfected with 1 μg GloSensor 22-F plasmid and 2 μg desired plasmid (mock vector, wild-type or mutant

plasmids). At 24 h post transfection, cells were seeded into a 96-well plate at a density of 2.0×10^4 cells/well and incubated for another 24 h. Then the cAMP levels were measured in unstimulated cells (basal level) and cells stimulated with human FSH (100 IU/L) for 45 min by using a luminescence counter (PE). In another set of experiments, the cAMP level was measured in cells stimulated with different doses of FSH (0~200 IU/L) for 20 min. Three independent experiments were conducted.

Western blotting

HEK293T cells were seeded in 6-well plates and transfected with 2 μg desired plasmids (mock, wild-type or mutant plasmids) at 75~80% confluence. Fourty-eight hours post transfection, cells were serum-starved for 6 h and incubated with human FSH (100 IU/L) for different time (0, 5, 30 and 60 min) at 37℃. Cells were lysed in RIPA buffer (Beyotime) with 1 mM PMSF (Beyotime) and phosphatase inhibitors (Roche). Equivalent amounts of protein were separated by SDS-PAGE. Samples were transferred onto PVDF membranes (Millipore), blocked with 5% nonfat milk and then incubated with a rabbit monoclonal antibody against pERK1/2 (1:5000 dilution, Abcam). The protein levels were measured using the chemiluminescence reader (Bio-Rad), and analyzed by ImageJ software (US National Institutes of Health). To normalize pERK1/2 to total ERK1/2 levels, the same samples were also probed with a rabbit monoclonal antibody against ERK1/2 (1:10000 dilution, Abcam) after stripping the membranes. To compare the change of pERK1/2 level, we quantified the grayscale of western blot bands using Image J software. The grayscale score of pERK1/2 was divided by that of ERK1/2 in each group at specific timepoint to obtain the ratio. The ratio of wild-type group at 0 min was considered as 1, then other groups were compared with it. Independent experiments were conducted three times.

Dominant negative effect assay

To further assess the potential dominant negative effect, the wild-type vector, the p.L597I mutant vector, or the p.L597I mutant vector with wild-type vector (in a 1:1 ratio), were transfected into HEK293T cells. Here, an appropriate amount of mock vector was co-transfected to keep a constant total amount of DNA transfected into each well. At 48 h post transfection, the cAMP levels in response to FSH (100 IU/L) for 45 min, and ERK1/2 phosphorylation in response to FSH (100 IU/L) at 5 min post stimulation were measured by cAMP assay and Western blotting, respectively.

Statistical analysis

SPSS version 21.0 (Armonk, NY, IBM Corp.) was used for statistical analysis. Chi-squared test or Fisher's exact test was used to compare the genotype distribution and allele frequency between patients and controls when appropriate. One-way analysis of variance (ANOVA) followed by Tukey tests was applied to compare the surface signal of FSHR between groups in flow cytometry. Two-way ANOVA with Bonferroni post-hoc test was performed to compare the cAMP activities and ERK1/2 phosphorylation levels between groups. $P < 0.05$ was considered statistically significant.

Results

The novel mutations and polymorphisms identified in the *FSHR* gene

Sequencing analysis revealed three novel variants in three patients with POI (Table 1), all of which were absent in the 192 controls. The prevalence of novel variants in the *FSHR* gene was 1.56% (3 of 192). The phenotype of the patient carrying the missense variant c.1789C>A (p.L597I) was primary amenorrhea; the patient with the missense variant c.793A>G (p.M265V) or the synonymous variant c.1821C>T presented with

secondary amenorrhea. Clinical features of these three POI patients with *FSHR* mutations are shown in Table 2. Mutation p.M265V in exon 9 was located at the ECD, whereas p.L597I in exon 10 resided at the sixth transmembrane domain (TMD; Fig.1A). Leucine at site 597 was highly conserved across species, whereas methionine at site 265 was not (Fig.1B).

In addition, six known SNPs of the *FSHR* gene were identified (Table 1), two of which (rs115030945 and rs75552966) were only found in POI cases. Among the known SNPs, three were nonsynonymous (rs115030945 in exon 1, rs6165 in exon10 and rs6166 in exon10), one was synonymous (rs75552966 in exon 8), and two were located in untranslated region (UTR) including rs1394205 and rs140106399. Remarkably, the two SNPs in UTR showed a significantly different genotypic and allelic distribution between patients and controls.

FSHR membrane localization

Immunofluorescence confirmed that both of the mutant receptors were expressed on cell membrane (Fig.2A). However, the results of quantitative flow cytometry showed that there was 22.0% ($P > 0.05$) and 39.5% ($P < 0.05$) reduction of surface signal in the cells expressing the mutant p.M265V and p.L597I, respectively, compared to the cells expressing wild-type receptor (Fig.2B), suggesting that the mutation p.L597I caused a significant reduction in membrane localization of the FSHR protein.

FSH-induced cAMP production

The FSH-induced signaling was assessed in the cAMP levels in HEK293T cells transfected with the desired plasmids (mock, wild-type or mutant plasmids). cAMP response was firstly induced using 100 IU/L of human FSH. Both of the mutants displayed low basal cAMP level similar to wild-type receptor. Cells transfected with mock plasmid showed no response to FSH stimulation in the cAMP level, whereas

cells transfected with wild-type or mutant plasmids displayed a dynamic response to FSH. Compared with wild-type construct, cells transfected with p.L597I mutant showed a significantly decreased cAMP level at each time point, whereas cells transfected with p.M265V mutant showed a slight decrease in the cAMP level (Fig.3A). At 20 min post FSH stimulation, cells transfected with p.L597I mutant showed a 44.58% decrease in the cAMP level with respect to wild-type receptor ($P < 0.01$); while p.M265V mutant only exhibited 9.12% decrease in cAMP level with respect to wild-type receptor ($P > 0.05$, Fig.3A).

After stimulation with different doses of FSH (25~200 IU/L), the level of cAMP produced in cells transfected with p.L597I mutant was decreased with respect to wild-type receptor ($P < 0.05$); whereas the level of cAMP produced in cells transfected with p.M265V mutant was slightly decreased with respect to wild-type receptor ($P > 0.05$, Fig.3B).

FSH induced ERK1/2 phosphorylation

Next, the two mutants were evaluated for their effects on FSH-induced ERK1/2 phosphorylation which occurred downstream of cAMP/PKA activation. For wild-type receptor, ERK1/2 phosphorylation reached its peak at approximately 5 min post FSH stimulation and then gradually decreased. For p.L597I mutant, ERK1/2 phosphorylation was significantly lower than that of the wild-type receptor at each time point; whereas p.M265V mutant showed a similar response as the wild-type receptor (Fig.3C-D)

No dominant negative effect of p.L597I mutant

Finally, the dominant negative effect of p.L597I mutant was assessed in cAMP/ PKA signaling pathway. After co-transfection of equal quantities of the wild-type and mutant construct, the response to FSH in both the cAMP levels and ERK1/2

phosphorylation were even higher than that of the wild-type construct alone (Fig.4). Therefore, no dominant negative effect was observed for p.L597I mutant.

Discussion

We screened all 10 exons of the *FSHR* gene in 192 sporadic POI patients of Han Chinese descent, and three novel variants were identified, all of which were absent in the 192 controls. Among the three novel heterozygous variants, one was synonymous (c.1821C>T), while the other two, c.793A>G (p.M265V) and c.1789C>A (p.L597I), were missense mutations that were located respectively in the ECD and the sixth TMD of FSHR.

When tested for functionality, mutation p.L597I was found to cause decreased membrane localization and impairment of receptor signaling as measured by the cAMP level and ERK1/2 phosphorylation, while no such effects were observed for p.M265V mutation. These results suggested the importance of leucine at this site, which was also implied by its high conservation among species. Mutation p.M265V did not significantly affect cell surface expression of FSHR or the signaling pathway as measured by cAMP level and ERK1/2 phosphorylation. These results indicated that the p.M265V substitution was not detrimental, which was consistent with the poor conservation at this site.

In our study, the patient carrying p.M265V mutation experienced her menarche at the age of 15 and amenorrhea at the age of 31, whereas the p.L597I carrier suffered from primary amenorrhea and established menstruation by exogenous estrogen-progesterone therapy at the age of 19. Primary amenorrhea in the patient with p.L597I indicated a severe diminishment in ovarian function compared with secondary amenorrhea in the p.M265V carrier.

In general, the FSHR mutations located in the ECD have been shown to disturb trafficking and cell surface expression of the receptor, but mutations located in the TMD were characterized by the impaired signal transduction without the change in subcellular location of FSHR (Desai, et al., 2013, Siegel, et al., 2013). Interestingly, our study showed that p.L597I mutation caused a decreased membrane expression of FSHR in spite of its location in the sixth TMD. A similar case was the inactivating mutation p.A575V which was also located in the sixth TMD but led to very weak membrane expression of FSHR (Desai, et al., 2015). In addition, another two mutations in the sixth TMD, p.P587H and p.F591S resulted in the complete lack of signal transduction (Kotlar, et al., 1997, Kuechler, et al., 2010). Taken together, these results suggest that the sixth TMD is important for the FSHR function and mutations in this domain very likely affect receptor trafficking and subsequent signaling.

Until now, most of the causal mutations of the *FSHR* gene have been reported in homozygotes or compound heterozygotes and the heterozygotes seem to be unaffected for many mutations (Siegel, et al., 2013). Although dominant negative effects have been reported for FSHR mutations p.R556A and p.R618A (Zarinan, et al., 2010), our results showed that the p.L597I mutant had no dominant negative effect. However, it has been reported that FSHR haploinsufficiency in mice accelerates oocyte loss, resulting in early reproductive senescence (Danilovich and Sairam, 2002, Yang, et al., 2003), suggesting that the heterozygous mutation might also be involved in the pathogenesis of POI. Similarly, a heterozygous mutation p.V221G was found in a POI patient with primary amenorrhea (Nakamura, et al., 2008) and functional studies indicated that the impaired receptor function due to this mutation might underlie the clinical manifestation (Banerjee, et al., 2017, Li, et al., 2017). Therefore, the decreased cAMP production and diminished ERK1/2 phosphorylation caused by

the p.L597I mutation might, at least partially, explain the POI phenotype in our patient.

Recently, two homozygous FSHR mutations, p.Ll40RfsX16 and p.P504S, were identified in the Chinese patients presenting with resistant ovary syndrome (ROS) (Li, et al., 2017). The presence of the normal-sized ovaries containing an age-appropriate number of antral follicles as well as the normal anti-Müllerian hormone (AMH) level indicated that both patients were affected by ROS rather than POI phenotype. Genetic and bioinformatic analysis suggested that both mutations were pathogenic, but functional studies were not performed. In addition, the first homozygous *FSHR* mutation, c.566C>T (p.A189V), which is prevalent in the Finnish population (Aittomaki, et al., 1995), was not identified in our POI cohort nor in another Chinese POI cohort (Chen, et al., 2006). Together with the reports from many other populations, our findings further suggested that this mutation displayed distinct ethnic specificity within Finland.

Additionally, six known SNPs of the *FSHR* gene were identified in our study. Among them, rs115030945 (c.24G>T, p.L8F) was a rare missense SNP with high conservation at this site, and bioinformatic analysis showed the mutation might not affect the protein function. Moreover, rs1394205 (c.-29G>A) and rs140106399 (c.*111T>C) were identified in both groups with significantly different genotypic and allelic distributions. While the A allele at rs1394205 was positively correlated with POI risk, the C allele at rs140106399 was negatively correlated with genetic susceptibility of POI. These two SNPs located in the 5'-UTR and 3'-UTR, respectively.

Although the genotypic and allelic distribution of the rs140106399 was significantly different between the two groups, its predictive value for POI risk is

limited considering the low minor allele frequency (5.7% in controls and 1.6% in POI, respectively). Future studies in larger cohorts are necessary to establish the role of rs140106399 in POI.

The SNP rs1394205 has been reported to be associated with elevated FSH levels and poor ovarian response (Achrekar, et al., 2009) as well as with diminished ovarian reserve (Ghezelayagh, et al., 2018). Another study found that the expression of FSHR protein on granulosa cells was decreased in carriers with AA genotype of rs1394205 (Desai, et al., 2011). Therefore, the rs1394205 may affect ovarian function by regulating FSHR expression. In addition, there were no significant differences in the distribution of genotypes and alleles of rs6165 (p.A307T) or rs6166 (p.S680N) between the two groups in this study, which is consistent with the previous studies in Chinese POI patients (Du, et al., 2010).

Conclusions

In the study, we identified two novel missense mutations c.793A>G (p.M265V) and c.1789C>A (p.L597I) in the *FSHR* gene in two Chinese patients with sporadic POI, and revealed that dysfunctional FSHR due to mutation or SNP might explain a fraction of sporadic POI cases in Han Chinese population.

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Figure Legends

Figure 1. The novel variants in the *FSHR* gene in patients with POI. (A) Schematic presentation of the FSHR protein and structure. The novel variants, p.M265V and p.L597I, were marked by a red triangle, respectively. (B) Sequence alignment of FSHR among orthologs with arrow heads indicating conservation of amino acid 265 and 597 in mammals. ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain.

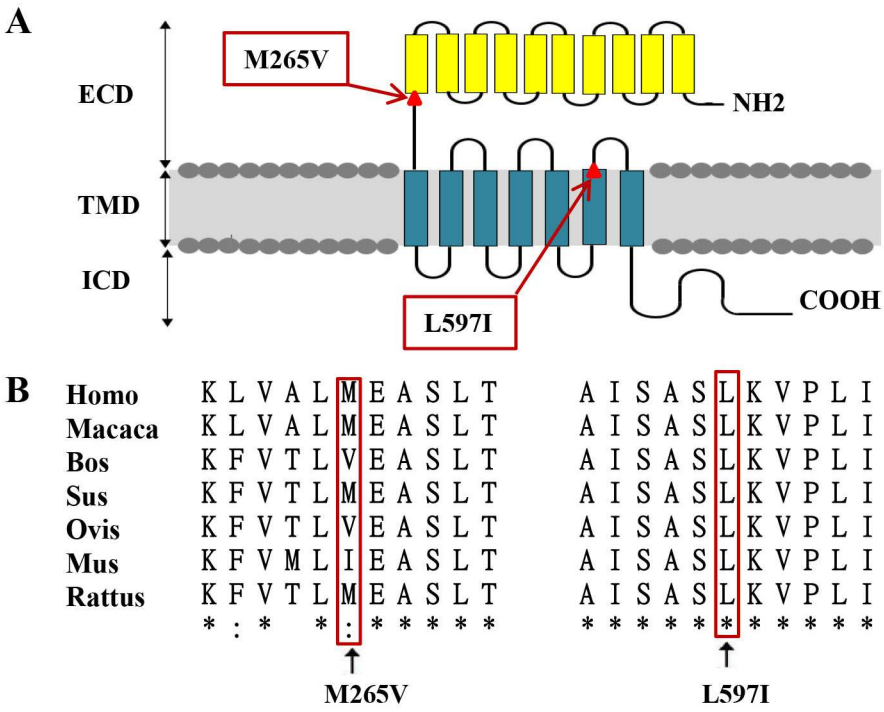

Figure 2. FSHR membrane localization. (A) Immunofluorescence shows both of the mutant FSHR were expressed on cell membrane as well as the wildtype. (Bar=20μm). (B) FSHR membrane localization detected by flow cytometry. The cells were transfected with wild-type receptor or mutants for 48 h before flow cytometry. The surface FSHR signal was normalized to the internal FSHR signal for each group. The histogram represents the mean ± SD of three independent experiments and depicts the relative intensity of the mutants in comparison to wild-type receptor (100%). One-way analysis of variance (ANOVA) followed by Tukey tests was used, *P<0.05.

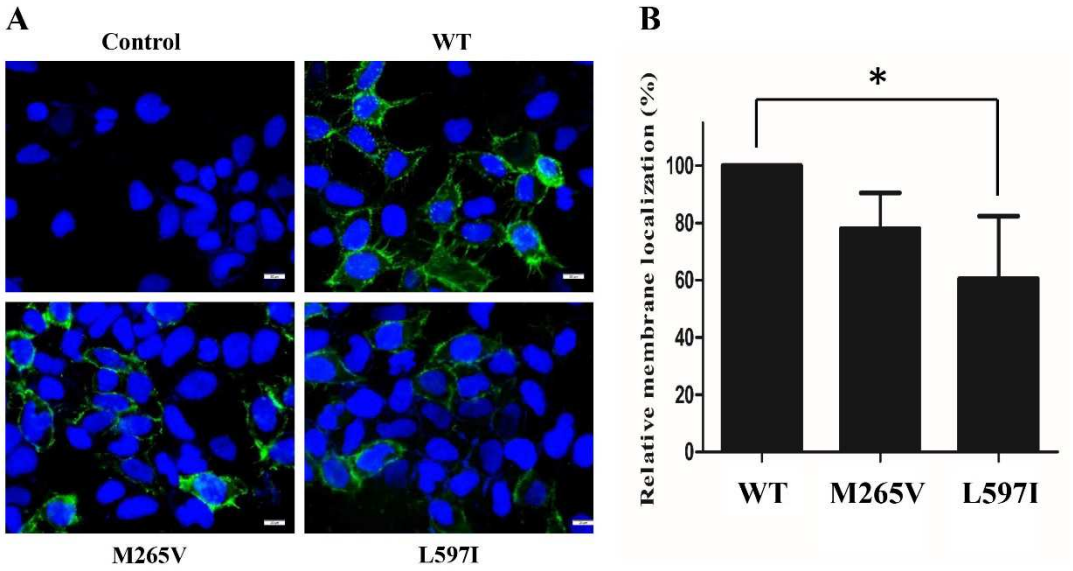

Figure 3. The cAMP production and ERK1/2 phosphorylation stimulated by FSH in HEK293T cells. (A) The cells transfected with mock vector, wild-type receptor or mutants were stimulated with human FSH (100 IU/L) for 45 min and cAMP was measured at each time point. The arrow indicated the start point of FSH stimulation. (B) The maximal cAMP levels stimulated by different doses of FSH (0~200 IU/L). Data expressed are the mean ± SD of three independent experiments. Two-way ANOVA with Bonferroni post-hoc test was performed to compare the cAMP activities, **P*<0.05; ***P*<0.01. (C) ERK1/2 phosphorylation was detected in cells transfected with mock vector, wild-type receptor or mutants and then stimulated with human FSH (100 IU/L) for different time (0~60 min). (D) The relative level of phosphorylated ERK1/2 compared with ERK1/2 according to the results of western blot. **P*<0.05.

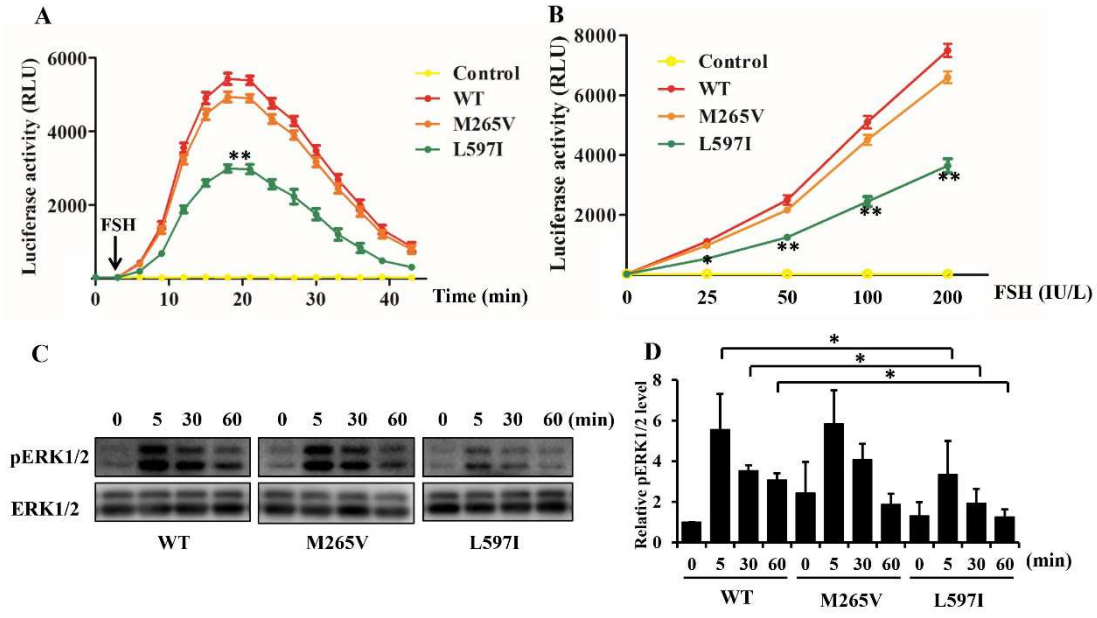

Figure 4. No dominant negative effect of p.L597I mutant. (A) No dominant negative effect at the cAMP level for p.L597I mutant. Data expressed are the mean ± SD of three independent experiments. Two-way ANOVA with Bonferroni post-hoc test was performed to compare the cAMP activities, **P*<0.05. (B) No dominant negative effect at the level of ERK1/2 phosphorylation for p.L597I mutant. ERK1/2 phosphorylation was detected in cells transfected with wild-type receptor, p.L597I mutant or equal quantities of the wild type and p.L597I mutant and then stimulated with human FSH (100 IU/L) for different time (0~60 min). (C) The relative level of phosphorylated ERK1/2 compared with ERK1/2 according to the results of western blot. **P*<0.05, ***P*<0.01.

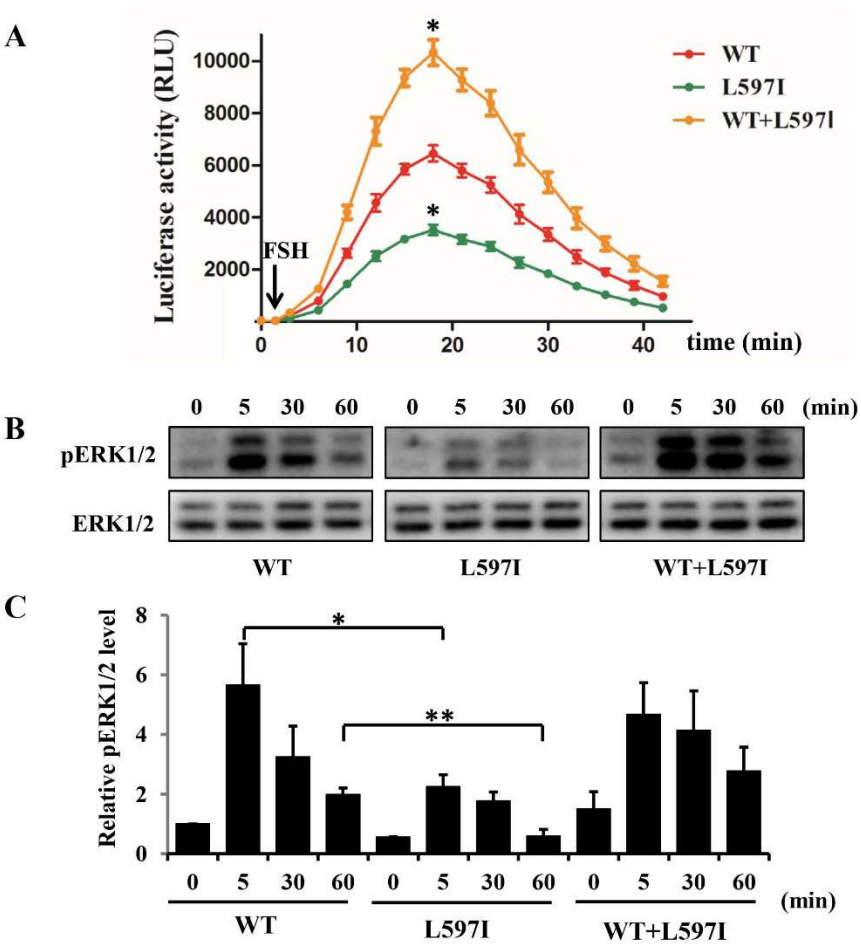

Table 1. Variants identified in the *FSHR* gene in Chinese women with premature ovarian insufficiency.

Location	dbSNP ID	Variation	Genotype	Genotype frequency		*P* value	Allele	Allele frequency		*P* value
				POI (%)	Control (%)			POI (%)	Control (%)	
Exon 9	Novel	c.793A>G	AA	191 (99.5)	-	-	A	383(99.7)	-	-
		p.M265V	AG	1(0.5)	-		G	1(0.3)	-	
			GG	0(0.0)	-					
Exon 10	Novel	c.1789C>A	CC	191(99.5)	-	-	C	383(99.7)	-	-
		p.L597I	AC	1(0.5)	-		A	1(0.3)	-	
			AA	0(0.0)	-					
Exon 10	Novel	c.1821C>T	CC	191(99.5)	-	-	C	383(99.7)	-	-
		Synonymous	CT	1(0.5)	-		T	1(0.3)	-	
			TT	0(0.0)	-					
5’ UTR	rs1394205	c.-29G>A	GG	40(20.8)	54(28.6)	0.02	G	174(45.3)	210(54.7)	0.01
			AG	94(49.0)	102(53.1)		A	210(54.7)	174(45.3)	
			AA	58(30.2)	36(18.8)					
Exon 1	rs115030945	c.24G>T	GG	190(99.5)	192(100)	0.25	G	382(99.5)	384(100)	0.25
		p.L8F	GT	2(1.0)	0(0.0)		T	2(0.5)	0(0.0)	
			TT	0(0.0)	0(0.0)					
Exon 8	rs75552966	c.603C>T	CC	191(99.5)	192(100)	0.49	C	383(99.7)	384(100)	0.49
		Synonymous	CT	1(0.5)	0(0.0)		T	1(0.3)	0(0.0)	
			TT	0(0.0)	0(0.0)					
Exon 10	rs6165	c.919G>A	GG	13(6.8)	20(10.4)	0.40	G	109(28.4)	124(32.3)	0.24

		p.A307T	AG	83(43.2)	84(43.8)		A	275(71.6)	260(67.7)	
			AA	96(50.0)	88(45.9)					
Exon 10	rs6166	c.2039G>A	GG	15(7.8)	16(8.3)	0.77	G	116(30.2)	111(28.9)	0.69
		p.S680N	AG	86(44.8)	79(41.1)		A	268(69.8)	273(71.1)	
			AA	91(47.4)	97(50.5)					
3’ UTR	rs140106399	c. *111T>C	TT	186(96.9)	170(88.5)	0.002	T	378(98.4)	362(94.3)	0.002
			CT	6(3.1)	22(11.5)		C	6(1.6)	22(5.7)	
			CC	0(0.0)	0(0.0)					

Note: POI, premature ovarian insufficiency; -, not applicable.

Table 2. Clinical features of POI patients with FSHR mutation.

Patient No.	*FSHR* mutation	Amenorrhea type	Age of menarche (y)	Age of amenorrhea (y)	FSH (IU/L)	Uterus (cm×cm)	Left ovary (cm×cm)	Right ovary (cm×cm)	Follicles by ultrasound	Breast stage	Pubic hair	BMI	Delivery history
Case 1	c.793A>G p.M265V Heterozygous	SA	15	31	82.34	3.5×2.8	Invisible	Invisible	No	Tanner IV	Thick	29.13	No
Case 2	c.1789C>A p.L597I Heterozygous	PA	-	-	50.69	2.7×1.6	Invisible	Invisible	No	Tanner II	Thin	21.83	No
Case 3	c.1821C>T Synonymous Heterozygous	SA	12	21	59.39	3.5×3.0	0.9×1	1.7×0.9	Yes	Tanner IV	Thick	20.8	No

Highlights

Etiology of premature ovarian insufficiency is heterogenous.

The signal pathway of follicle stimulation hormone plays a crucial role in follicle development by regulating steroid metabolism.

Two novel missense mutations of follicle stimulation hormone receptor were identified in Chinese sporadic patients with premature ovarian insufficiency, which interrupted the activation of cAMP and phosphorylation of ERK1/2 protein.

Dysfunctional follicle stimulation hormone receptor due to mutation or SNPs might explain a fraction of cases diagnosed with premature ovarian insufficiency in Han Chinese population.