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ORIGINAL ARTICLE ***Reproductive genetics***

A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: a fertility application of whole exome sequencing

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STUDY QUESTION: Can whole exome sequencing (WES) and *in vitro* validation studies be used to find the causative genetic etiology in a patient with primary ovarian failure and infertility?

SUMMARY ANSWER: A novel follicle-stimulating hormone receptor (FSHR) mutation was found by WES and shown, via *in vitro* flow cytometry studies, to affect membrane trafficking.

WHAT IS KNOWN ALREADY: WES may diagnose up to 25–35% of patients with suspected disorders of sex development (DSD). FSHR mutations are an extremely rare cause of 46, XX gonadal dysgenesis with primary amenorrhea due to hypergonadotropic ovarian failure.

STUDY DESIGN, SIZE, DURATION: A WES study was followed by flow cytometry studies of mutant protein function.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study subjects were two Turkish sisters with hypergonadotropic primary amenorrhea, their parents and two unaffected sisters. The affected siblings and both parents were sequenced (trio-WES). Transient transfection of HEK 293T cells was performed with a vector containing wild-type FSHR as well as the novel FSHR variant that was discovered by WES. Cellular localization of FSHR protein as well as FSH-stimulated cyclic AMP (cAMP) production was evaluated using flow cytometry.

MAIN RESULTS AND THE ROLE OF CHANCE: Both affected sisters were homozygous for a previously unreported missense mutation (c.1222G>T, p.Asp408Tyr) in the second transmembrane domain of FSHR. Modeling predicted disrupted secondary structure. Flow cytometry demonstrated an average of 48% reduction in cell-surface signal detection ($P < 0.01$). The mean fluorescent signal for cAMP (second messenger of FSHR), stimulated by FSH, was reduced by 50% in the mutant-transfected cells ($P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: This is an *in vitro* validation. All novel purported genetic variants can be clinically reported only as 'variants of uncertain significance' until more patients with a similar phenotype are discovered with the same variant.

WIDER IMPLICATIONS OF THE FINDINGS: We report the first WES-discovered FSHR mutation, validated by quantitative flow cytometry. WES is a valuable tool for diagnosis of rare genetic diseases, and flow cytometry allows for quantitative characterization of purported variants. WES-assisted diagnosis allows for treatments aimed at the underlying molecular etiology of disease. Future studies should focus on pharmacological and assisted reproductive treatments aimed at the disrupted FSHR, so that patients with FSH resistance can be treated by personalized medicine.

[†] The authors consider that the first two authors should be regarded as joint First Authors.

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Key words: whole exome sequencing / follicle-stimulating hormone receptor / premature ovarian failure / primary amenorrhea / resistant ovary syndrome

Introduction

Normal ovarian folliculogenesis and ovulation depend on an intact hypothalamic–pituitary–gonadal axis. Primary ovarian failure (POF) is characterized by hypergonadotropic hypoestrogenic amenorrhea, typically the end result of premature depletion of the follicular pool. This encompasses a spectrum of pathologies and may occur before menarche, as in the descriptive diagnosis XX gonadal dysgenesis, or any time before age 40. The etiology may be iatrogenic (chemotherapy, radiation or post-surgery), autoimmune or related to karyotypic abnormalities of the X chromosome (most commonly 45, X) or the autosomes. A genetic etiology presently is found in only 25% of cases, and more than 50% of cases are idiopathic (Persani et al., 2010). Single gene etiologies are far more rare than chromosomal etiologies; examples include Fragile X premutation, BMP15, GALT, GDF9, meiotic gene variants, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) resistance (Persani et al., 2010). The rare causative genes have been found by labor-intensive methods, including candidate gene approaches, linkage analysis, homozygosity mapping, mutagenesis screens and copy number variant analysis. Next generation sequencing techniques, including whole exome sequencing (WES), are the newest tools in this arsenal.

Mutations in the follicle-stimulating hormone receptor (FSHR) are an extremely rare cause of POF, with only 11 inactivating mutations reported so far (Aittomaki et al., 1995; Gromoll et al., 1996; Beau et al., 1998; Touraine et al., 1999; Doherty et al., 2002; Allen et al., 2003; Meduri et al., 2003; Nakamura et al., 2008; Kuechler et al., 2010; Katari et al., 2015; Fig. 1). FSHR is a membrane-bound G-protein coupled receptor (GPCR) that produces cyclic AMP (cAMP) as its main second messenger (Jiang et al., 2014). cAMP activates ovarian follicular steroidogenesis, allowing pubertal development and ovulatory menstrual cycles (Channing et al., 1980). We present one of the first applications of WES to find a novel FSHR mutation in a patient with primary amenorrhea and hypergonadotropic gonadal failure, and demonstrate the use of flow cytometry to definitively establish the functional deficit conferred by this mutation.

Materials and Methods

All work was approved by the UCLA Institutional Review Board, and patients provided written informed consent in their native language to affiliate recruiting physicians.

Clinical and endocrinological evaluation

The proband was the daughter of a consanguineous marriage between first cousins who are of Turkish ethnic background (Fig. 2A). She presented for genetic evaluation at age 22 due to primary amenorrhea. On examination, she had Tanner stage 4 breasts, after exogenous estrogen and progesterone replacement therapy started at age 17; the stage of development prior to estrogen replacement is not known. Ultrasound showed a hypoplastic uterus and small bilateral ovaries with no visible antral follicles. Laboratory evaluation showed the following levels: FSH 67.48 mIU/ml, LH 29.58 mIU/ml, estradiol (E2) 9.54 pg/ml and anti-Mullerian hormone (AMH) 0.59 ng/ml (by Beckman generation II ELISA assay). She had been diagnosed with osteoporosis. The proband also has a sister, age 31 years old at time of evaluation, with primary amenorrhea. She had Tanner stage 2–3 breasts, after exogenous estrogen and progesterone therapy starting at age 19. Her laboratory evaluation revealed levels of: FSH 83.87 mIU/ml, LH 31.03 mIU/ml, E2 12.35 pg/ml and AMH 0.50 ng/ml. Both sisters had normal 46, XX karyotypes and Fragile X trinucleotide repeat lengths less than the premutation threshold. The affected sisters have two unaffected sisters. There is no other family history of primary amenorrhea.

Whole exome sequencing

WES was performed on an Illumina HiSeq 2500 machine as described elsewhere (Lee et al., 2014). Both affected siblings and both parents were sequenced (trio-WES). Regions of homozygosity were evaluated using Linkdatagen (Purcell et al., 2007) and Plink (v1.07) (Smith et al., 2011). Variants were annotated using a custom annotator (Yourshaw et al., 2014) for information regarding known or extrapolated effect on protein function, allele frequency in the general population and prior evidence of disease causality to select likely pathogenic variants. Variants with minor allele frequency $>1\%$ were removed. Variants were then filtered into four categories: *de novo*, homozygous, compound heterozygous and inherited variants. Variants were further filtered by whether they were in genes on a list known to cause disorders of sexual differentiation, gonadal dysgenesis or ovarian insufficiency (Arboleda et al., 2013), but all variants were considered. The suspected variant was confirmed by Sanger sequencing for the affected probands as well as parents and unaffected siblings.

Plasmid construction

A wild-type (WT) FSHR plasmid in the mammalian expression vector pSG5 was previously constructed (Aittomaki et al., 1995), obtained and checked bySanger sequencing. The plasmid differed from the canonical sequence of FSHR (per Genome Reference Consortium GRCh37) at 5 bp. These included c.919G>A (rs6165) and c.2039G>A (rs6166) well-characterized minor alleles causing substitutions with frequencies of 0.49 and 0.41 in the general population that do not have any apparent effect on ovarian function, and c.7C>T, c.864T>C, and c.1149G>A, all of which are silent. Mutagenic primers for the point mutation of interest were designed (5′GTGCAACC TGGCCTTTGCTTATCTCTGCATTGGAATCTAC3′ and reverse complement 5′GTAGATTCCAATGCAGAGATAAGCAAAGGCCAGGTTG CAC3′) and site-directed mutagenesis reactions were performed per protocol with the QuickChange II XL Site-Directed Mutagenesis kit (agilent.com). Successful mutagenesis was confirmed with Sanger sequencing.

FSHR membrane localization assay

HEK 293T cells were seeded at 1.22×10^6 density in a 100 mm cell culture dish, and allowed to grow to 75–80% confluence prior to transfection in standard conditions and complete media (Dulbecco's modified Eagle's medium, 10% fetal bovine serum FBS, 1% penn/strep, 37°C, 5% CO_2). The cells were transfected with 10 μg of mock plasmid, 10 μg of WT FSHR plasmid or 10 μg of the C1222T mutant FSHR plasmid using FuGENE 6 Transfection reagent (promega). The transfected cells were allowed to incubate for a period of 36 h prior to preparation for the flow cytometry membrane localization assay.

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A

Patient genotype	Patient phenotype
• 1. Asp408Tyr	1. Hypergonadotropic primary amenorrhea
• 2. Val221Gly*	2. Hypergonadotropic primary amenorrhea Tanner Stage 3 breasts
• 3. Ile418Ser*	3. Hypergonadotropic primary amenorrhea Tanner Stage 2 breasts
• 4. Asn191Ile†	4. Normal, fertile
○ 5. Pro587His‡	5. Hypergonadotropic primary amenorrhea Tanner Stage 3 breasts Follicular arrest — primordial stage
• 6. Pro348Arg	6. Hypergonadotropic primary amenorrhea Tanner Stage 3 breasts
• 7. Pro519Thr	7. Hypergonadotropic primary amenorrhea Follicular arrest — primary stage
• 8. Ala189Val	8. Hypergonadotropic primary amenorrhea
○ 9. Ala189Val / Ala419Thr	9. Hypergonadotropic primary amenorrhea
○ 10. Arg573Cys / Ile160Thr	10. Oligomenorrhea post menarche Hypergonadotropic secondary amenorrhea Follicular arrest — small antral stage
○ 11. Asp224Val / Leu601Val	11. Hypergonadotropic primary amenorrhea Follicular arrest — small antral stage Normal breast development

*no *in vitro* studies performed

†Heterozygote: Wild-Type/Missense

‡Compound heterozygote: Deletion of exons 9 and 10/Missense

In vitro findings on cell function
- Disrupted cAMP production
- Disrupted membrane localization
- Disrupted FSH binding

Patient genotype
- • Homozygote
- • Heterozygote
- ○ Compound heterozygote

B

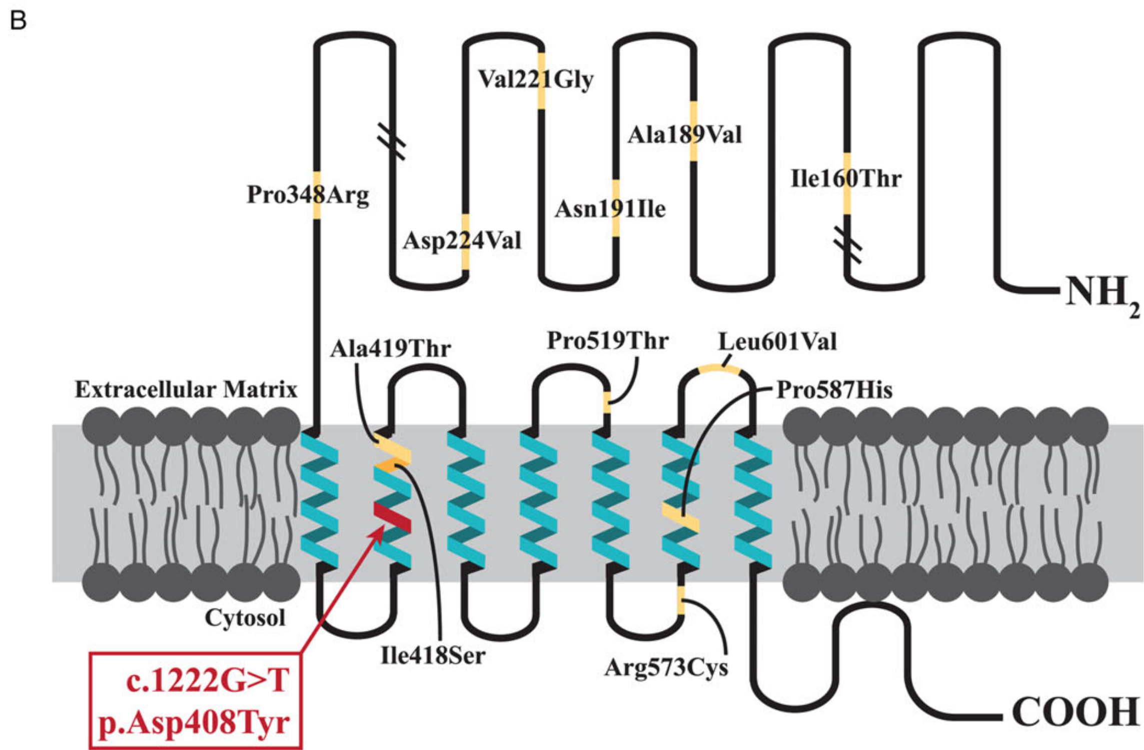

Figure 1 FSHR mutations. (**A**) Table of previously reported FSHR mutations and patient phenotypes. (**B**) FSHR is a 695 amino acid GPCR with a ligand-binding extracellular domain at the amino tail, a transmembrane domain and an intracellular domain at the carboxyl tail. The locations of the novel mutation and the previously reported 11 inactivating missense mutations are demonstrated here.

Flow cytometry preparation

A single cell suspension of the experimental cultures was obtained by aspirating growth media followed by a 1 × Dulbecco's phosphate-buffered saline (DPBS) wash prior to the addition of ACCUTASE cell detachment reagent (Stemcell Technologies). Each of the three (Mock, WT, Mutant) single cell experimental cultures were split into two groups at a density of 2×10^6 cells in 50/50 complete media and DPBS, one for non-permeabilized membrane FSHR staining, and the other for internal FSHR staining.

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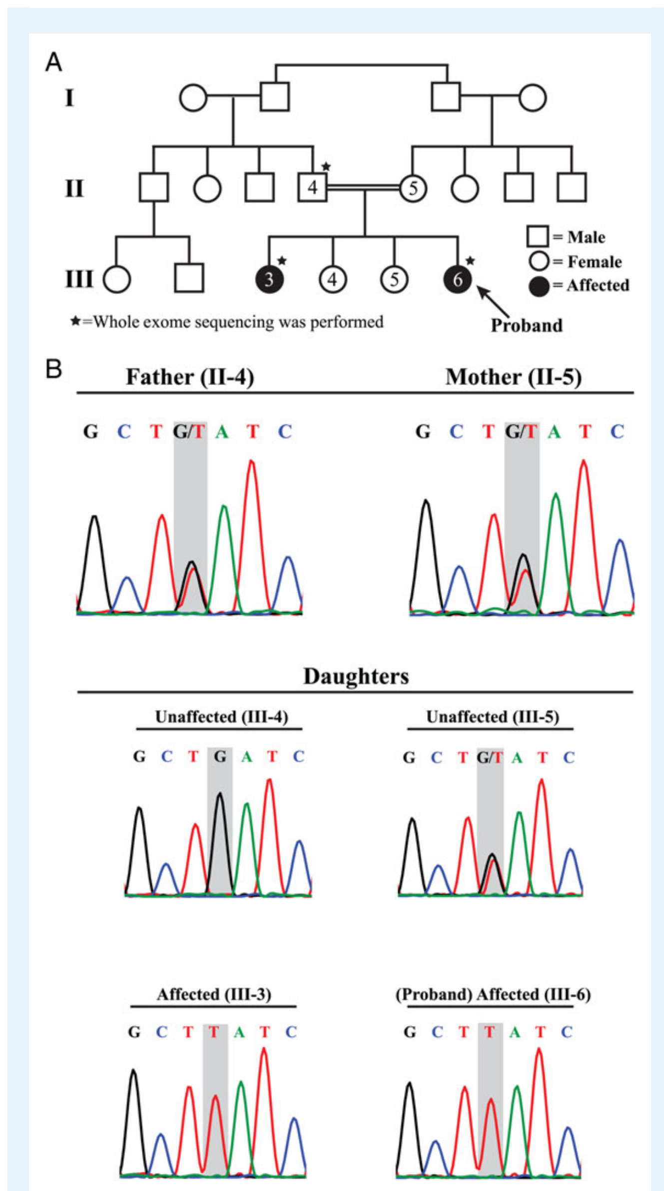

Figure 2 Inheritance of the FSHR mutation. (**A**) A pedigree was obtained demonstrating consanguinity between the parents and no other cases of primary amenorrhea. The proband was patient III-6, and her affected sister was III-3. * indicates WES performed. (**B**) Sanger sequencing confirmed the homozygous variant status of the affected sisters as well as the parents' heterozygous status. One unaffected sister was found to be heterozygous for the variant and the other was WT.

Surface staining

Rb-FSHR H-190 antibody (1.5 μg, Santa Cruz biotechnologies) was added to each of the experimental 2.0 × 10^6 single cell solutions, and allowed to bind the antigen for a period of 1 h with the addition of 1% bovine serum albumin (BSA) for blocking. After antibody hybridization, the cells were washed three times in DBPS to remove unbound antibody. The cells were then fixed in 7% formalin solution for a period of 15 min, followed by three DPBS washes to remove fixative reagent. Then 1.5 μg of Alexa-Fluor Rb-488 secondary antibody plus 1% BSA for blocking was added to the antibody-fixed cells, and allowed to incubate for a 45-min period, and this was followed by three DBPS washes to remove unbound secondary antibody. The prepared cells were stored in 1 × DPBS for flow cytometry analysis.

Intracellular staining

Quantities of 2 × 10^6 of each of the prepared experimental cell groups were fixed in 7% formalin solution for a period of 30 min, followed by three DPBS washes. The cells were permeabilized and blocked (in 0.5% TX-100 solution, 1% BSA) for 10 min, followed by the addition of 1.5 μg of Rb-FSHR H-190 (Santa Cruz Biotechnologies) to the permeabilizing/blocking solution for an additional hour. The cells were then washed three times in DPBS to remove unbound primary antibody and 1.5 μg of Alexa-Fluor Rb-488 secondary antibody plus 1% BSA for blocking was added and allowed to incubate for a 45-min period; this was followed by three DBPS washes to remove unbound secondary antibody. The prepared cells were stored in 1 × DPBS for flow cytometry analysis.

Flow cytometry

The control and experimental samples were analyzed on a BD LSR Fortessa Flow Cytometry Analyzer at the UCLA Jonsson Comprehensive Cancer Center Core. Each of the experiments was carried out in separate biological duplicates.

cAMP production assay

The initial cell culture and transfection follows the protocol outlined for the FSHR membrane localization assay.

FSH stimulation

After the 36-h period of growth, the experimental and control cells were stimulated with 25 ng/ml of FSH (human pituitary derived, Sigma-Aldrich) to induce cAMP production via FSHR stimulation. The induction media contained 500 μM of the phosphodiesterase inhibitor, IBMX (Sigma) and 100 μM of the inhibitor of cAMP, phosphodiesterase 4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro20) (Sigma). The cAMP induction via FSH stimulation was carried out for 1.5 h at 37°C.

Flow cytometry preparation

A single cell suspension of the experimental cultures was obtained by aspirating induction growth media followed by a DPBS wash prior to the addition of ACCUTASE cell detachment reagent w/IBMX & Ro20 (Stemcell Technologies; Sigma).

cAMP staining

Quantities of 2 × 10^6 of each of the prepared experimental cell groups were fixed in 7% formalin solution for a period of 30 min, followed by three DPBS washes. The cells were permeabilized and blocked (0.5% TX-100 solution, 1% BSA) for 10 min, followed by the addition of 1.5 μg of Ms mAB-anti-cAMP (Ab Cam) to the permeabilizing/blocking solution for an additional hour. The cells were then washed three times in DPBS to remove unbound primary antibody. Alexa-Fluor Ms-488 secondary antibody (1.5 μg, Life Technologies) plus 1% BSA for blocking was added and allowed to incubate for a 45-min period, followed by three DBPS washes to remove unbound secondary antibody. The prepared cells were stored in 1 × DPBS for flow cytometry analysis, as described above.

Statistical analysis

For cellular localization analysis, proportions were standardized to the level of FSHR in the WT-transfected cells at the location of interest. The significance of the differences was evaluated by *t*-test. For cAMP production analysis, the level of cAMP signal in each genotype was set to be relative to the level in the mock-transfected cells. Differences between groups were evaluated using a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's honest significant difference (HSD).

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Results

Sequencing

The whole exome data showed multiple regions of homozygosity >10 Mbp in both sisters (eight such regions in the proband (9.04%), and 11 in her sister (10.60%)), suggesting consanguinity of first cousin marriage. An average coverage of 95× was achieved across the exome in the proband with 92% of these bases covered at ≥10×. Both affected sisters were found to be homozygous for a previously unreported missense variant in the FSHR, and both parents were heterozygous for this mutation. The variant is a substitution of thymine for guanine at position 1222 in exon 10, causing substitution to tyrosine in place of aspartic acid (c.1222G>T, p.Asp408Tyr) (Fig. 1). This variant is previously unreported according the Exome Aggregation Consortium (exac.-broadinstitute.org). The mutation was predicted to be deleterious by the protein prediction software SIFT and Condel, and 'probably damaging' by PolyPhen. Sanger sequencing confirmed the homozygous status of the affected sisters as well as the parents' heterozygous status. Of the two unaffected sisters, one was found to be heterozygous for the variant and the other was WT (Fig. 2B).

In silico predicted structural alterations conferred by the c.1222G>T SNP

Using the RaptorX protein structure and functional prediction software, we queried whether and how the identified p.Asp408Tyr substitution affected the structure of the FSHR protein, focusing on the second transmembrane helix (Fig. 3). The predicted model of FSHR containing the identified mutation showed a region on the second transmembrane helix with what would be a significant disordering of secondary structure when compared with the predicted model of WT FSHR (Fig. 3). This region of disorder lies upstream of the actual mutation of residue 408. Since secondary structure relies on primary sequence, it is plausible that these disordered regions are generated from steric stress exerted by the substitution of tyrosine at position 408. In addition to using RaptorX modeling, we employed ExPASy protein analysis as a secondary validation of prediction disorder within the same region. ExPASy analysis also determined that there was a drop in helix score within the 400–410 residue regions, indicating again structural instability as a result of the mutation (data not shown). Therefore, the SIFT/Condel/PolyPhen prediction of the mutation as deleterious is borne out by the RaptorX and ExPASy analysis suggesting structural instability (Peng and Xu, 2011a,b; Kallberg *et al.*, 2012; Ma *et al.*, 2013).

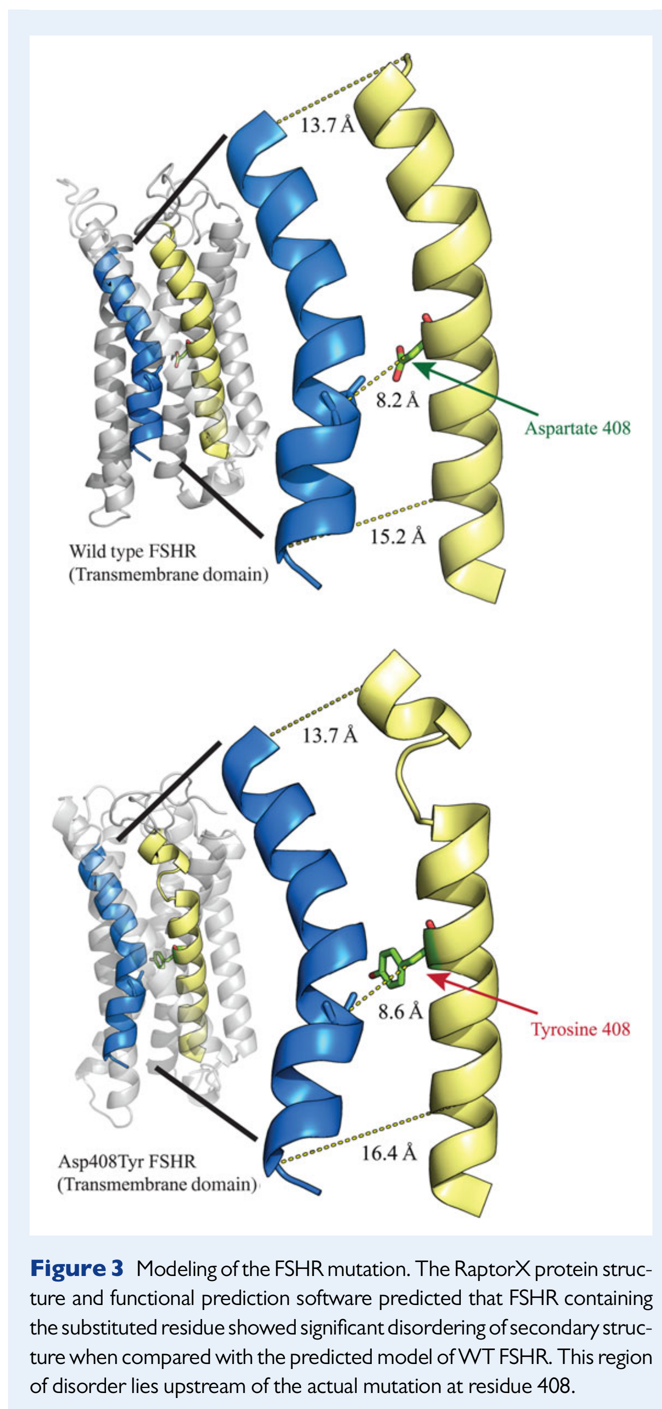

Figure 3 Modeling of the FSHR mutation. The RaptorX protein structure and functional prediction software predicted that FSHR containing the substituted residue showed significant disordering of secondary structure when compared with the predicted model of WT FSHR. This region of disorder lies upstream of the actual mutation at residue 408.

Flow cytometry analysis to assess FSHR membrane localization

Utilizing the location of the exome identified mutation, in addition to the structural abnormality predicted by RaptorX (Fig. 3), we hypothesized that there was a membrane trafficking anomaly accounting for the patient's phenotype. Using transiently transfected HEK 293T cells, coupled with quantitative flow cytometry, we assessed whether the identified mutation caused a reduction in proper membrane surface trafficking of the FSHR protein. In biologically duplicate experiments, we found an average 48.05% reduction of surface signal detection in non-permeabilized cells expressing the mutant p.Asp408Tyr FSHR construct relative to the non-permeabilized cells expressing a WT construct ($P < 0.01$; Fig. 4A). Using permeabilized cells from the same experimental groups, we found an intracellular FSHR signal of 100% for both the p.Asp408Tyr mutant and the WT-transfected cells (Fig. 4A). Having the same intracellular FSHR signal for both constructs indicates that the observed surface membrane reduction was not a transfection efficiency artifact, but rather a cellular trafficking disruption caused by the patient's FSHR mutation.

cAMP production via FSH stimulation

Given that the p.Asp408Tyr mutant caused decreased membrane trafficking (Fig. 4A), an attempt to quantify amounts of cAMP production relative

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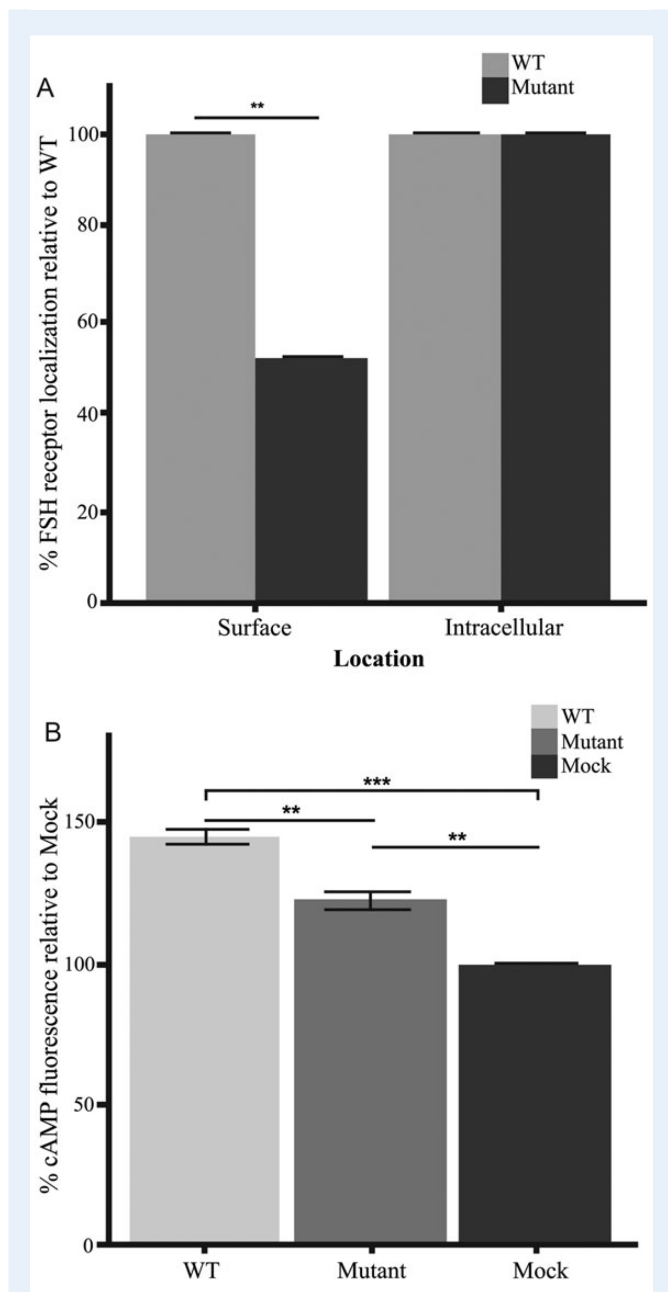

Figure 4 *In vitro* analysis of FSHR function. (**A**) Flow cytometry demonstrated an average 48.05% reduction of surface signal detection in cells expressing the mutant p.Asp408Tyr FSHR construct relative to cells expressing a WT construct (**$P < 0.01$). Permeabilized cells from the same experimental groups showed an intracellular FSHR signal of 100% for both the mutant and the WT-transfected cells. (**B**) After FSH stimulation, cells transfected with p.Asp408Tyr were capable of generating a 22.58% (**$P < 0.001$) increase in the mean cAMP fluorescent signal compared with baseline mock-transfected cells. This was 50.22% lower than the WT FSHR-transfected cells, which produced a 44.96% (***$P < 0.0001$) higher fluorescent mean signal for cAMP relative to the mock transfection (**$P < 0.01$ for difference of means between WT and mutant by ANOVA/Tukey's HSD).

to WT FSHR would inherently be inaccurate, as FSHR at the membrane was not equal between sample groups. Using transiently transfected HEK293T cells coupled again with quantitative flow cytometry, we assessed whether the p.Asp408Tyr mutant variant of FSHR was capable of stimulating any cAMP production. After incubating all experimental groups (Mock, WT, p.Asp408Tyr mutant) for 1.5 h with 25 ng/ml FSH, global levels of cAMP were assessed using quantitative flow cytometry. After FSH stimulation, cells transfected with p.Asp408Tyr were capable of generating a 22.58% ($P < 0.001$ by ANOVA/Tukey's HSD) increase in mean cAMP fluorescent signal compared with baseline mock-transfected cells. This was 50.22% lower than the WT FSHR-transfected cells, which were capable of producing a 44.96% ($P < 0.001$ by ANOVA/Tukey's HSD) higher fluorescent mean signal for cAMP relative to the mock transfection ($P < 0.01$ for difference of means between WT and mutant by ANOVA/Tukey's HSD; Fig. 4B). Taken together, it appears that the mutation causes a decrease in cAMP production directly proportional to the decrease in membrane surface integration.

Discussion

We present a case of familial hypergonadotropic ovarian failure with primary amenorrhea caused by a novel FSHR inactivating mutation c.1222G>T identified by WES, and confirmed by *in vitro* assays. Flow cytometry analysis demonstrated significantly reduced membrane localization as a result of the identified FSHR mutation, which caused a downstream proportional decrease in total second messenger cAMP production. The predicted structural alterations resulting from this missense mutation caused significant disorder within the second transmembrane helix architecture. We propose that, without functional localization of sufficient numbers of FSHR molecules to the granulosa cell surface, the patients' ovarian response to FSH stimulation is disordered and follicles are unable to progress past the primary stage to ovulation, despite high levels of FSH stimulation (Oktay *et al.*, 1997).

In contrast to inactivating FSH mutations, which result in complete sexual infantilism (Huhtaniemi and Aittomaki, 1998), patients with previously characterized FSHR mutations have shown a range of pubertal development and ovarian histology with some reported breast development occurring even in the absence of *in vitro* cAMP production (Fig. 1; Beau *et al.*, 1998; Touraine *et al.*, 1999; Allen *et al.*, 2003; Nakamura *et al.*, 2008; Kuechler *et al.*, 2010; Katari *et al.*, 2015). This rare form of ovarian dysgenesis, resulting from FSH resistance rather than follicular depletion, has been termed the 'resistant ovary syndrome' (Aittomaki *et al.*, 1996; Huhtaniemi and Aittomaki, 1998). In patients with FSH resistance, follicular maturation is impaired, though most patients show small (3–5 mm) follicles by transvaginal ultrasound and follicular arrest at the small antral stage histologically (Aittomaki *et al.*, 1995; Beau *et al.*, 1998; Touraine *et al.*, 1999; Meduri *et al.*, 2003; Kuechler *et al.*, 2010). During folliculogenesis, the primordial follicles do not express FSHR (Oktay *et al.*, 1997). FSHR begins to be expressed by the granulosa cells as follicles progress from the primary to the secondary (pre-antral) stage, along with LH, estrogen and androgen receptors (Channing *et al.*, 1980; Oktay *et al.*, 1997). During the secondary follicle stage, the theca and granulosa cells begin to synthesize steroid hormones (Channing *et al.*, 1980). Our patients did not have ovarian histology performed,

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but likely their ovaries contain follicles that arrest at the primary or secondary stage due to reduced numbers of FSHR molecules at the membrane and an inability to proceed through the later stages of FSH-dependent follicular maturation. Similar to other patients with inactivating FSHR mutations, our patients' primary or secondary follicles, with a significant reduction of normal FSHR at the membrane, nonetheless likely synthesized sufficient estrogen at puberty to stimulate partial breast development. The low but detectable AMH values in our patients support this conclusion; previous work has shown that patients with FSH resistance have low to normal AMH values, while women with premature ovarian insufficiency due to follicular depletion have very low to undetectable AMH ($P < 0.001$). Our patients' AMH values are within the range of a published series of 12 patients with FSH resistance. Immunohistochemistry performed on the ovaries of two patients with FSH resistance showed AMH expression in the granulosa cells of primary follicles, while no secondary or more advanced follicles were seen (Kallio *et al.*, 2012). In patients with FSH resistance, AMH secreted by small growing follicles remains detectable, with values likely varying with the severity of mutation and the resultant stage of follicular arrest.

WES is a modification of next generation sequencing which targets exon sequences before amplification to limit the output to only the coding regions (1–2% of the genome, 30 Mbp) (Gilissen *et al.*, 2011; Ku *et al.*, 2011; Rabbani *et al.*, 2012). This allows a broad search for variants in patients with puzzling phenotypes. WES has been rarely used to date to find causative gene mutations for patients with sexual development and fertility-related phenotypes, including non-syndromic (Caburet *et al.*, 2014; Le Quesne Stabej *et al.*, 2015) and syndromic POF (Pierce *et al.*, 2013; de Vries *et al.*, 2014; Wood-Trageser *et al.*, 2014; AlAsiri *et al.*, 2015) and LH resistance/empty follicle syndrome(Yariz *et al.*, 2011). The first application of WES to find a novel FSHR mutation has recently been reported (Katari *et al.*, 2015). Even if there is suspicion of a particular causative gene, commercial genetic testing (genetests.org) is not available for many of the genes ultimately found in the above studies (Caburet *et al.*, 2014; de Vries *et al.*, 2014; Wood-Trageser *et al.*, 2014; AlAsiri *et al.*, 2015; Le Quesne Stabej *et al.*, 2015).

WES is thus a promising technology for discovering genetic variants without the limitations of candidate gene or even the newer gene panel approaches. One recent series employing a panel of 70 candidate genes for POF, sequenced using next generation sequencing, found four putative causative variants in 3 (25%) of their 12 patients, although all variants were novel and two were in novel genes with only a theoretical connection to POF (Fonseca *et al.*, 2015). However, none were confirmed by *in vitro* testing. All would have to be reported clinically as 'variants of uncertain significance' (category 3: previously unreported and is of a type which may or may not be causative of the disorder) by American College of Medical Genetics guidelines (Richards *et al.*, 2008). Another study of disorders of sexual differentiation employed next generation sequencing for a panel of 35 genes, and reported making a definitive diagnosis in one (14%) out of seven patients, and a novel 'likely pathogenic' mutation in another (Arboleda *et al.*, 2013). Given the rapid rate of discovery of novel sex development genes as well as the expanding phenotypic spectrum for these genes, the authors subsequently reported that WES allows for greater flexibility in gene lists and is more inclusive for rare and novel genetic diagnosis in the population with disorders of sex development (DSD). A published WES series of 40 patients with 46,XY DSD, most of whom had already had extensive genetic and endocrine testing, generated genetic diagnoses in 35% of cases, with an additional six variants of uncertain significance (Baxter *et al.*, 2015). Other series show comparable yields. A clinical series studied WES ordered by physicians for 250 patients, the majority of whom were children with neurological diseases, and reported a 25% molecular diagnostic rate (Yang *et al.*, 2013). And lastly, our institution published a clinical series of 814 undiagnosed patients with suspected genetic disease, finding an overall molecular diagnosis rate of 26% with a higher rate of 31% for trio-WES (Lee *et al.*, 2014).

Because FSHR mutations are found in <1% of POF patients (Persani *et al.*, 2010), a candidate gene approach would have been far less efficient than WES for determining the etiology of our patients' primary amenorrhea. As clinicians more widely adopt WES, one important question will arise: when is it sufficient to report a variant of a similar type to previously characterized pathogenic mutations, with the aid of protein prediction software and other bioinformatics tools but without *in vitro* studies, as pathogenic and to counsel and treat accordingly? Clinically, when variants have not been previously reported but are suspected to be pathogenic, they may only be called 'of the type which is expected to cause the disorder' (more certain) or 'of the type which may or may not be causative' (Richards *et al.*, 2008). All novel purported genetic variants may only be clinically reported as 'variants of uncertain significance' unless and until more patients with a similar phenotype are discovered with the same variant. Our variant lies in close proximity to two previously reported pathogenic variants in the second transmembrane domain, p.Ile418Ser (Katari *et al.*, 2015) and p.Ala419Thr (Doherty *et al.*, 2002) (Fig. 1), which adds credibility to its pathogenicity but does not render it clinically reportable.

In contrast, our *in vitro* analysis eliminates the uncertainty; this is the first report of a WES-discovered pathogenic FSHR mutation validated using quantitative experimental methods. We successfully identified that the WES-identified mutation resulted in a significant reduction in membrane localization, and would be the cause for the patients' established phenotype. This validation implemented the use of flow cytometry to assess both membrane trafficking as well as downstream FSH-stimulated cAMP production, a more quantitative methodology than traditionally used for these types of analysis. This approach allowed us to establish that the root cause of the reduction in cAMP production was not a product of FSHR functionality *per se*, but rather caused by the reduction in surface membrane trafficking. The amount of cAMP that can be produced is directly proportional to the fewer surface receptors to trigger the response. Flow cytometry has been reported as a method for studying interactions of GPCRs with their ligands (Sridharan *et al.*, 2014), and for studying surface expression of FSHR (De Leener *et al.*, 2006; Garcia-Velasco *et al.*, 2012), but never, to our knowledge, to study cAMP production. We have shown flow cytometry to be an efficient quantitative method for classifying functional deficits of surface receptors *in vitro*. Under laboratory conditions, with high transfection efficiency and supraphysiologic expression under control of the SV40 promoter, the reduction in mutant FSHR membrane localization was 50%. However, it is possible that under physiologic endogenous regulation of FSHR *in vivo*, the absolute reduction in mutant FSHR surface localization is more profound, leading to the subjects' phenotypes.

The ultimate goal of securing a molecular diagnosis for patients with rare phenotypes is both to end the 'diagnostic odyssey' as well as to provide personalized, targeted therapeutic intervention. For patients with an FSHR mutation, these therapies could theoretically include *in vitro* maturation (IVM) of the oocytes separated from the arrested

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follicles, the use of a molecular chaperone protein to optimize FSHR function, or both. In the USA, IVM is still considered experimental (Practice Committees of the American Society for Reproductive and the Society for Assisted Reproductive, 2013), and optimal protocols have not been established. It remains controversial whether IVM media must contain FSH and/or other gonadotrophins (Chian et al., 2004; Nogueira et al., 2012), especially if the protocol does not include co-culture with cumulus cells (the granulosa cells surrounding the oocyte and the site of FSH signaling). mRNA for FSH and LH receptors has been found in human oocytes, so there may be a role for gonadotrophins in oocyte maturation (Patsoula et al., 2003). It is thus unclear whether IVM may overcome FSH resistance or whether a functional FSHR is mandatory; this has not been studied, though it has been proposed (Chang et al., 2014). Gene therapy has been employed to restore functional FSHR to cells transfected with a c.566C>T inactivating mutation, and can partially restore folliculogenesis and estrogen production to FSHR knockout mice (Ghadami et al., 2008, 2010). One promising line of study is that of 'pharmacoperones' (pharmacological chaperones), small molecules that correct otherwise misfolded proteins and increase expression at the plasma membrane. The molecule Org41841, a thienopyr(im)idine LH agonist, has shown promise at increasing cAMP production *in vitro* in cells transfected with mutant FSHR (variant Ala189Val, causing intracellular sequestration), though not to normal levels (Janovick et al., 2009). Future studies should focus on the pharmacology and efficacy of this molecule *in vivo* as well as a pharmacologic addition to IVM media for patients with inactivating FSH mutations.

In conclusion, we demonstrate the first WES-discovered FSHR mutation also functionally characterized using quantitative flow cytometry. WES is a valuable tool for efficient diagnosis for patients with rare genetic disease. Functional studies targeted toward the protein of interest allow for personalized and quantitative characterization of the functional deficit conferred by a specific variant. Future studies should focus on pharmacological and assisted reproductive treatments aimed at the disrupted FSHR, so that patients with FSH resistance can be treated by personalized medicine.

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Authors' roles

M.S.B. and E.H.G. conceived and designed the study, acquired, analyzed and interpreted the data, and drafted the manuscript. A.L. acquired data and drafted the figures. A.E. and H.B. acquired and interpreted data. T.N. and V.A.A. interpreted data. J.E.G., L.R., N.V. and E.L. acquired data. Z.Y. and M.F. evaluated and received consent from the patients and provided senior oversight for design, analysis and interpretation. D.V. and E.V. provided senior oversight for design, analysis and interpretation. All authors revised the article for critically important content and indicated final approval for publishing.

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Conflict of interest

The authors have no conflicts of interest to declare.

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