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# A Novel Mutation in the FSH Receptor Inhibiting Signal Transduction and Causing Primary Ovarian Failure

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**Inactivating mutations of the FSH receptor (FSHR) are known to cause ovarian failure with amenorrhea and infertility in women. The first mutation identified in the FSHR gene was a missense mutation (566C→T, predicting Ala189Val transition) found in several Finnish patients with primary amenorrhea due to ovarian failure. Only five additional, partially or totally inactivating, mutations of the FSHR have been reported. Here, we report a novel FSHR mutation, 1255G→A, in a Finnish female with primary amenorrhea. The patient was a compound heterozygote for two mutations in the FSHR gene: 566C→T, the Finnish founder mutation, and 1255G→A, a previously unidentified mutation. The new mutation is located in exon 10 in the second transmembrane stretch of the FSHR, and it predicts an Ala419Thr change in the protein structure. In functional testing, the mutation was shown to have minimal effect on ligand binding capacity and affinity, but it almost totally abolished the cAMP second messenger response. Neither of the two FSHR mutations (566C→T or1255G→A) was identified in 40 other Finnish patients with premature ovarian failure. Based on this and previous studies, FSHR mutations remain a rare cause of ovarian failure. (*J Clin Endocrinol Metab* 87: 1151–1155, 2002)**

FSH HAS A pivotal role in the control of female reproduction through binding to the FSH receptor (FSHR) expressed in its target cells, granulosa cells of the ovary. FSHR is a member of the G protein-coupled receptor superfamily and, when activated, cAMP functions as the main second messenger of its signal transduction pathway. The first 349 of the 695 amino acids of the receptor protein constitute the extracellular, the following 264 amino acids the transmembrane, and the last 65 amino acids the carboxy terminal intracellular domain of the FSHR. The homology between different species peaks at 90% in the transmembrane domain (1). FSHR is closely related to the other two glycoprotein hormone receptors, those of LH (LHR) and TSH (TSHR) with 70% homology in the transmembrane domain (1).

In 1969, Jones and De Moraes-Ruehsen (2) suggested that ovarian failure in some patients is caused by ovarian resistance to gonadotropins. Since then, mutations in both FSHR and LHR genes have been identified in women with hypergonadotropic ovarian failure. While numerous activating and inactivating mutations have been found in the LHR gene (3), the number of identified mutations in the FSHR remains low with only six recessively inherited inactivating mutations having been described previously (4–7). The first one of these was the 566C→T mutation in exon 7 of the FSHR gene predicting an Ala189Val change in the protein structure, so far only identified in Finnish patients. The mutation was shown to be inactivating by transfection studies in immortalized mouse Sertoli cells (MSC-1) (4). In northeastern Finland, the carrier frequency of the mutation is as high as 1 in 85 due to enrichment of the founder mutation in this geographical area (8). To date, 29 Finnish women, all homozygous for the mutation, have been found to have primary ovarian failure caused by this mutation. The number of identified males homozygous for the mutation remains five, as reported earlier (9), apparently due to the mild and variable phenotypic expression of FSHR inactivation in men.

The five other inactivating FSHR mutations have all been reported only once. Two mutations, Ile160Thr in exon 6 and Arg573Cys in exon 10, were identified in a patient with secondary amenorrhea, thus contributing to the evolving phenotype of FSH resistance (5). When functionally tested, these mutations were found to cause partial impairment of FSHR function. Another compound heterozygote was a female of Caucasian origin with hypergonadotopic primary ovarian failure. The two mutations this patient was shown to harbor were Asp224Val in exon 9 and Leu601Val in exon 10 (6).The former abolished FSH binding almost totally, whereas the latter showed similar binding affinity with the wild-type receptor but impairment of receptor signaling. Finally, a functionally tested inactivating mutation was identified in a healthy German female who was a heterozygous carrier of an Ile191Leu mutation located in the close vicinity of the Ala189Val mutation (7). Additionally, an activating mutation of the FSH receptor has been identified in a hypophysectomized male; however, the significance of this mutation remains unclear (10).

In this paper, we report the second FSHR mutation identified in Finnish patients. A young female patient with primary amenorrhea and hypergonadotropic ovarian failure was found to be a compound heterozygote for two inactivating FSHR mutations: the previously identified Finnish founder mutation 566C→T and a novel 1255G→A mutation

Abbreviations: dNTP, Deoxynucleoside triphosphate; FSHR, FSH receptor; LHR, LH receptor; TM, transmembrane; TSHR, TSH receptor.

in the other allele. These mutations, resulting in Ala189Val and Ala419Thr substitutions, were found to be located in the extracellular and the second $\alpha$-helix of the transmembrane domain, respectively.

## Subjects and Methods

### *Case report*

A previously healthy 17-yr-old female patient of Finnish origin presented with primary amenorrhea. She had normal female secondary sexual characteristics, her height was 163 cm, weight 51 kg, and chromosome analysis showed a normal female karyotype 46, XX. In hormonal testing she had high serum gonadotropin concentrations in two consecutive measurements: FSH 91.3 and 78.3 IU/liter (normal values 1–25 IU/liter) and LH 38.7 and 32.6 IU/liter (normal values 1–13 IU/liter outside the LH peak), whereas her serum E2 concentration was 0.06 nmol/liter (normal values for postmenopausal women <0.13 nmol/liter). At presentation transvaginal ultrasound showed very small ovarian follicles. Although she had not had spontaneous menstruation, few scanty menstrual bleedings could be induced with cyclic progestin therapy. However, after 5 months these bleedings could no longer be induced and endometrial thickness, measured by ultrasound, was 4 mm. The patient is the only child of healthy parents. There is no known family history of primary amenorrhea or infertility.

Another 40 Finnish females with primary or secondary amenorrhea due to hypergonadotropic ovarian failure were either ascertained through a previous study on the inheritance of ovarian failure in Finland or had later presented with primary amenorrhea. Of these, 30 had previously been screened with DGGE for possible mutations in the entire coding region of the FSHR gene with negative results (11).

Informed consent was obtained from all individuals before collecting blood samples. The study was approved by the institutional ethics committee.

### *PCR of genomic DNA*

DNA was extracted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech Pty. Ltd., Sydney, Australia) according to manufacturer's protocol. Exons 7/8 and exon 10 of the FSHR gene were amplified using primers described previously (12). For exons 7/8, each PCR contained 1 mM deoxynucleoside triphosphates (dNTPs), 1× Biotech PCR buffer (Biotech International Ltd., Belmont, WA, Australia), 2 mM $MgCl_2$, 250 ng primer, and 1.25 U *Taq* polymerase (Roche Diagnostics, Australia Pty. Ltd., New South Wales, Australia) in 50 $\mu$l distilled $H_2O$. Denaturation at 94 C for 2 min was followed by 35 cycles at 94 C for 30 sec, 58 C for 30 sec, and 72 C for 75 sec followed by a final elongation step at 72 C for 4 min. PCR analysis was performed on an Omne Hybaid thermal cycler (Integrated Sciences Pty. Ltd., Willoughby, Australia). For exon 10 (fragment 'C' which spans 1220–1485bp) each PCR contained 1 mM dNTPs, 1× Biotech PCR buffer, 3 mM $MgCl_2$, 200 ng primer, and 1.25 U *Taq* polymerase (Perkin-Elmer Pty. Ltd., Melbourne, Australia) in 50 $\mu$l total volume. Denaturation at 94 C for 2 min was followed by 35 cycles at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 75 sec followed by a final elongation step at 72 C for 4 min. PCR analysis was performed on a Corbett block heater PCR machine (Corbett Research, Sydney, Australia).

### *Restriction digest to detect 566C→T and 1255G→A mutations*

The 566C→T mutation was analyzed using *BsmI* digestion as previously described (4). To determine the presence of the 1255G→A mutation, 100 ng of amplified DNA (exon 10-fragment 'C' spanning 1220–1485 bp) was digested with *SfaNI* and analyzed in a 3% Nusieve gel. The wild-type allele has two *SfaN1* sites and on digestion the PCR product (265 bp) is cleaved into three fragments of 167, 53, and 45 bp. The mutation destroys one of the *SfaNI* sites creating an extra fragment of 98 bp in heterozygotes.

### *Sequencing*

PCR products were sequenced directly using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit and terminating dideoxyNTPs (Amersham Pharmacia Biotech Pty. Ltd.). For each sequencing reaction, 100 ng of PCR product functioned as the template and 50 ng of primer was added. The conditions for cycle sequencing followed those for the initial PCR.

### *In vitro mutagenesis and transfections*

The 1255A mutation was introduced into a pSG5 expression vector (Stratagene, La Jolla, CA) containing the wild-type FSHR (4) by using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Mutant oligonucleotide primers (sense: 5′-CTACCTGCTGCTCATT*A*CATCAGTTGATATCCATACC-3′, antisense: 5′-GGTATGGATATCAACTGATG-*T*AATGAGCAGCAGGTAG-3′) displaced the wild-type sequence in the double-stranded plasmid as a template using *pfu Turbo* DNA polymerase (Stratagene) in a thermal cycler (DNA Engine Peltier Thermal Cycler, MJ Research, Inc., Watertown, MA). The runs consisted of initial denaturation at 95 C for 30 sec followed by 16 cycles of 95 C for 30 sec, 55 C for 30 sec, and 68 C for 16 min, and a final cooling down to 4 C. The amplification product was digested with endonuclease *Dpn*I, which digested the methylated parental DNA and left the nicked mutated plasmids intact. Supercompetent *Escherichia coli* XL1-Blue cells (Stratagene) were used in the transformation to ensure repair of nicked plasmids. The identity of mutated and wild-type plasmids was verified by restriction endonuclease analysis and sequencing.

COS-7 cells were transiently transfected with expression vectors containing either wild-type receptor or mutated receptor, or the vector only for mock transfections, using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's instructions. Cotransfections with a $\beta$-galactosidase expressing vector were done to control the transfection efficiency.

### *cAMP production of the transfected cells*

Recombinant human FSH (rhFSH; Organon, Oss, The Netherlands) in concentrations of 0, 0.1, 1, 10, 100, and 1000 IU/liter was used to stimulate transfected COS-7 cells in medium containing 0.2 mmol/liter 3-isobutyl-1-methylxanthine (MIX, Aldrich-Chemie, Steinheim, Germany) in quadruplicate using 24-well culture plates. After a stimulation of 2 h, the media were collected and diluted 1:1 with 2 mmol/liter teophylline, heated for 5 min at 100 C, and used for measurement of cAMP by a RIA (13).

### *FSH binding assay*

Recombinant human FSH (Organon) was radioiodinated with Na[$^{125}$I]iodine (IMS 300, Amersham Pharmacia Biotech) with a lactoperoxidase method (14) to specific activity of 3800 cpm/ng. The COS-7 cells transfected with wt or mut FSHR were cultured in 9 cm diameter culture plates for 48 h, washed with PBS and scraped into Dulbecco's PBS containing 0.1% BSA (BSA, Sigma). Triplicate aliquots of cell suspensions, containing 5–20 × $10^4$ cells depending on the experiment, were incubated in the presence of increasing amounts (from 3.3 to 131.6 ng) of radioiodinated recombinant human FSH in a total volume of 300 $\mu$l. Nonspecific binding was determined in the presence of 2.5 IU of recombinant human FSH at each hormone concentration used. Cells were incubated overnight at room temperature, and the radioactivity of the cell pellets was counted in a $\gamma$-spectrometer.

## Results

When studied for the possible presence of the 566C→T mutation, the patient was found to be a heterozygote (Fig. 1A). The substitution was confirmed by sequencing (results not shown). The initial search for a second mutation in this patient focused on exon 10 as sections of this exon share high homology with other receptors indicating important functional domains, in which mutations may be deleterious. Sequencing of exon 10 revealed another heterozygous mutation, 1255G→A (Fig. 1B), which abolishes one of the two *SfaNI* restrictions sites in the fragment spanning 1220–1485 bp of exon 10 (Fig. 1C). The 1255G→A mutation results in the

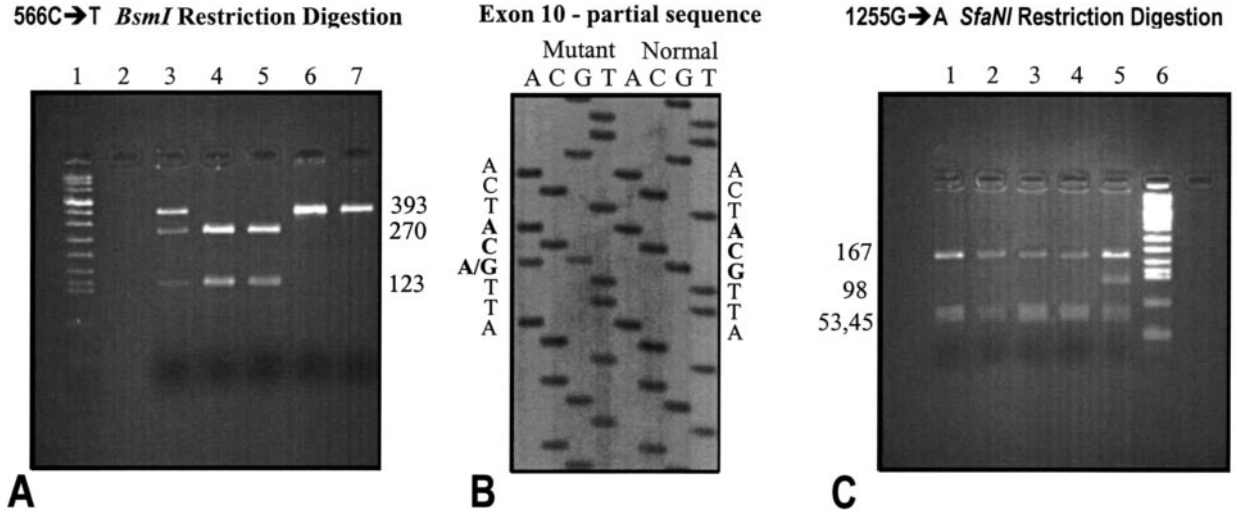


FIG. 1. A, *BsmI* digestion for the 566C→T mutation. Lane 1, Molecular weight marker; lane 2, blank. In wild-type homozygotes, the PCR product is cleaved into two fragments of 123 and 270 bp (lanes 4 and 5), whereas in affected individuals the mutation 566C→T abolishes the restriction site and only one band of 393 bp is observed (lanes 6 and 7). The patient (lane 3) has three bands indicating heterozygosity for the 566C→T mutation. B, Sequencing gel showing the heterozygous mutation, 1255G→A in the affected patient along with a normal control. C, Restriction digest gel for the 1255G→A mutation. PCR of exon 10 (fragment C) was performed on DNA samples from the patient and normal controls. In normal controls (lanes 1–4), there are two *SfaNI* sites and on digestion the PCR product is cleaved into 3 fragments of 167, 53, and 45 bp. The mutation destroys the *SfaNI* site 45bp into the PCR product creating an extra fragment of 98 bp in heterozygotes as seen in the affected patient (lane 5).

substitution of alanine at amino acid 419 for threonine (Fig. 2). Both mutations were subsequently analyzed by digestion with *BsmI* and *SfaNI* in a set of 40 Finnish women with POF. Neither one of these two mutations was observed in these patients (results not shown).

The Ala189Val was previously functionally tested (4), and the same testing experiments were performed on the novel mutation. The results of one representative experiment of FSH stimulated cAMP production (A) and binding of radiolabeled FSH (B) in transiently transfected COS-7 cells are shown in Fig. 3. Recombinant human FSH induced a 9- to 19-fold dose-dependent stimulation in cells expressing the wild-type FSHR, whereas in the cells transfected with the mutated receptor there was no cAMP production above the level found in mock-transfected cells (in six individual experiments). In the binding assay, Scatchard analysis showed that the number of receptors was roughly the same both in cells transfected with the wild-type and mutant FSHR ($15.1 \pm 6.7$ fmol/$10^6$ cells and $11.1 \pm 5.8$ fmol/$10^6$ cells, $P > 0.05$), as were also the equilibrium association constants ($K_a$) of FSH binding, *i.e.* $7.5 \pm 1.8 \times 10^9$ mol/liter and $7.4 \pm 1.6 \times 10^9$ mol/liter (n = 4 individual measurements), respectively. These studies showed that although the 1255G→A mutation does not affect expression of the receptor on the cell membrane or ligand binding, it abolishes signal transduction, at least the cAMP-PKA pathway.

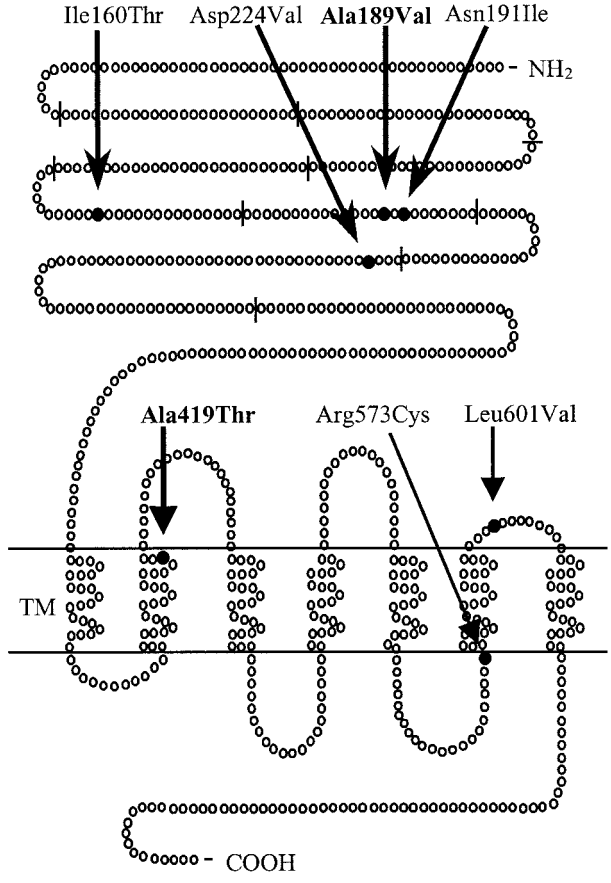


FIG. 2. Schematic presentation of the FSHR with the presently known inactivating mutations. The novel mutation and the Finnish founder mutation are shown in *bold*.

## Discussion

Although it is a decade since the FSHR gene was cloned (15, 16), the number of documented FSHR mutations still remains low, whereas the number of mutations identified in the LHR now amounts to about 30 (3). There is no apparent explanation for this, apart from perhaps the differences in the clinical phenotypes caused by the FSHR and LHR defects.

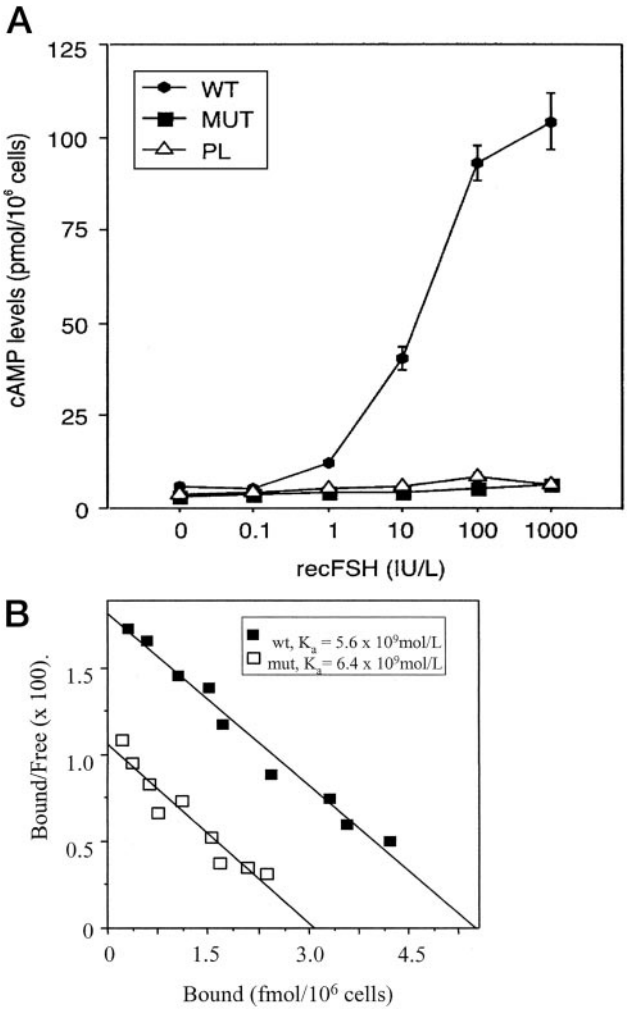


FIG. 3. Functional testing of the mutated FSH receptor. A, FSH-stimulated cAMP production in COS-7 cells transiently transfected with cDNAs encoding the wild-type receptor (wt), mutated receptor (mut), or using empty plasmid (pl). The experiment was repeated six times with similar results, and data of one representative experiment (mean ± SEM on 4 replicates) are presented. B, Scatchard analysis of [$^{125}$I]-iodo-hFSH binding to the same COS-7 cells as in panel A (*same symbols*). The experiment was repeated four times (with three replicates at each hFSH concentration). Of these, the results of one representative experiment are shown. The compiled data showed that the amount of specific FSH binding per $10^6$ cells was similar in those expressing the wt and mut receptor (15.1 ± 6.7 fmol and 11.1 ± 5.8 fmol/$10^6$ cells, respectively, $P > 0.05$ by paired $t$ test). The wide variation is due to differences in absolute levels of binding between individual experiments. Likewise, the equilibrium association constants of hFSH binding were similar: wt 7.5 ± 1.8 × $10^9$ and mut 7.4 ± 1.6 × $10^9$ mol/liter ($P > 0.05$).

Both activating and inactivating LHR defects have a clear male phenotype of familial male-limited precocious puberty and pseudohermaphroditism, respectively. In females, activating mutations do not seem to cause reproductive abnormalities, whereas inactivating mutations have a less severe phenotype with infertility due to anovulation. In contrast, the inactivating FSHR mutations cause a nonspecific phenotype of primary or early secondary amenorrhea and infertility in females and subfertility in males (4, 9). To our knowledge, there are no activating FSHR mutations identified in otherwise healthy individuals. One could therefore postulate that these mutations may not cause reproductive problems or may have unexpected phenotypes, and therefore be more difficult to identify. If this were the case, patients with LHR mutations are more likely to be identified with a known phenotype and hence with an LHR mutation. Furthermore, a founder mutation with a fairly high carrier frequency, such as the Ala189Val in Finland, should "pick up" the more recent and less common mutations such as the one described here. This patient, however, is the first compound heterozygote identified in Finland, which may also reflect the proposed rarity of FSHR mutations. This is also suggested by the paucity of mutations identified in other populations (8, 17–19). As this mutation was not identified in 40 other Finnish patients with ovarian failure, it is likely that it is a recent mutation and may be present in this family only.

The initial search for a second mutation in the patient described here was focused on exon 10 as sections of this exon share high homology with other receptors, thus indicating important functional domains, and indeed several mutations occurring in the transmembrane domain of the G protein-coupled receptors are known to be disease causing (3). The 1255A→G mutation in exon 10 changes the smaller, nonpolar amino acid alanine to the larger, uncharged polar amino acid threonine in the second hydrophobic segment/α-helix of the transmembrane domain. The fact that functional testing of the mutation showed similar results to the mutations previously described in exon 10 supports the pathogeneity of this mutation. The region, in which the mutation is located, and the amino acid itself are highly conserved in the glycoprotein hormone receptor family and between species (Fig. 4). As far as we know, there are no previously reported mutations in the corresponding codon of either LHR or TSHR. The TSHR harbors one reported mutation in the close vicinity of the novel mutation reported here. This inactivating mutation (Thr477Ile) was shown to cause congenital hypothyroidism in a homozygous female (20). In transfection studies, both the expression of the mutant TSHR at the cell surface was low and the signaling of the adenylyl cyclase pathway was impaired.

To date, all identified FSHR mutations are missense mutations. More importantly, the present data also reveal a correlation between the location and the mechanism of the mutation (Fig. 2). Three mutations located in the extracellular, ligand-binding domain of the receptor, namely

| | | 419 |
|---|---|---|
| FSHR | Human | YLLLIASVD |
| | Sheep | YLLLIASVD |
| | Bovine | YLLLIASVD |
| | Pig | YLLLIASVD |
| | Mouse | YLLLIASVD |
| | Rat | YLLLIASVD |
| LHR | Human | YLLLIASVD |
| TSHR | Human | YLLLIASVD |

FIG. 4. Sequence comparison of human FSHR, FSHR of other species, human LHR, and human TSHR around alanine 419.

Ile160Thr, Ala189Val, and Asp224Val, cause a defect in targeting of the protein to the cell surface. This is supported by functional and expression studies which show minimal residual activity, abolished binding and entrapment of an immature protein within the cell (4–6, 21). In contrast, the two previously reported mutations located in the transmembrane (TM) domain of the receptor protein (Arg573Cys, Leu601Val) were expressed on the cell surface, but the signal transduction was impaired. The functional studies performed on the novel mutation, Ala419Thr, are in agreement with the latter and thus fit this pattern. The location, rather than nature on the amino acid change, appears to be critical, as the three mutations located in the extracellular and transmembrane domain both include conservative and nonconservative substitutions.

The location of the mutations also seems to be an important nominator of the residual receptor activity. It was previously shown that the phenotype of the two compound heterozygotes was less severe than the one observed in Finnish patients homozygous for the Ala189Val mutation. The studies by Beau *et al.* (5) and Touraine *et al.* (6) suggest that this may be due to higher residual activity retained by the mutated receptor when the mutation was located in the TM domain. The same seems to apply to the mutation described here. While the patient had primary amenorrhea, she also showed clear signs of endometrial estrogen stimulation in a transvaginal ultrasound examination, had a positive progestin challenge test, and normal development of secondary sex characteristics. This suggests that the phenotype of the compound heterozygote with Ala189Val and Ala419Thr mutations was less severe when compared with patients homozygous for the Ala189Val mutation. However, it is also possible that other modifying factors may affect the phenotype of this single patient, and the previously observed variation of spontaneous pubertal development in Finnish patients could reflect this.

As new mutations of the FSHR will be discovered, the information is gradually emerging that, similar to LHR mutations, there is a correlation between the specific mutation and the phenotype. This is determined by the location of the mutation either in the extracellular or transmembrane domain of the receptor. The present data confirms the pattern previously observed in mutations of the TM domain, namely that normal cell membrane expression is accompanied by impaired signal transduction of the mutated receptor. It also suggests that when the mutation is located in the TM domain, it allows for variable residual activity, either through the cAMP-PKA pathway or through other pathways, such as IP3, which may be less affected by these mutations. Thus, there is likely to be a continuum of clinical phenotypes caused by FSHR mutations. So far, the more severe end of this spectrum has been elucidated; however, with time the opposite end with perhaps several decades of spontaneous menstruation, oligomenorrhea, or anovulation may be characterized.

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