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Mutation in the Follicle-Stimulating Hormone Receptor Gene Causes Hereditary Hypergonadotropic Ovarian Failure

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Summary

Hypergonadotropic ovarian dysgenesis (ODG) with normal karyotype is a heterogeneous condition that in some cases displays Mendelian recessive inheritance. By systematically searching for linkage in multiplex affected families, we mapped a locus for ODG to chromosome 2p. As the previously cloned follicle-stimulating hormone receptor (*FSHR*) gene had been assigned to 2p, we searched it for mutations. A C566T transition in exon 7 of *FSHR* predicting an Ala to Val substitution at residue 189 in the extracellular ligand-binding domain segregated perfectly with the disease phenotype. Expression of the gene in transfected cells demonstrated a dramatic reduction of binding capacity and signal transduction, but apparently normal ligand-binding affinity of the mutated receptor. We conclude that the mutation causes ODG in these families.

Introduction

Normal gonadal function is critically dependent on the integrity of the pituitary–gonadal axis. A key role in this regulatory circuit is played by the two pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the prepubertal ovary, follicular development is arrested before maturation, resulting in follicular atresia until the onset of gonadotropin stimulation at puberty induces follicular maturation and ovulation. Arrest of ovarian maturation at puberty can occur as a result of failure of gonadotropin secretion or as a lack of ovarian response to the tropic stimulation. Clinically, both conditions are characterized by normal prepubertal development with anatomically normal internal and external genitalia, variably developed secondary sex characteristics, and primary amenorrhea. When the failure of ovarian development is caused by lack of ovarian response, the circulating levels of FSH and LH are elevated, owing to missing gonadal negative feedback (hypergonadotropic hypogonadism). In conjunction with poorly developed streak ovaries, this condition has been labeled gonadal or ovarian dysgenesis (ODG), in spite of the intact sex determination (Simpson et al., 1971; Simpson, 1979). The term is descriptive and does not indicate any causative mechanism.

Ovarian dysgenesis with normal XX karyotype is believed to be rare and mostly sporadic. However, by compiling and analyzing the published data on ovarian dysgenesis with elevated FSH, Simpson et al. (1971) concluded that some cases are likely to be caused by autosomal recessive mutations. Indeed, results of a population-based study of ODG in the Finnish population disclosed as many as 75 cases, allowing the incidence of the condition to be estimated as 1 in 8, 300 females. Moreover, the recessive mode of inheritance was confirmed by segregation analysis, by the existence of several kindreds with two or more affected sisters, and by genealogical studies showing a clear-cut founder effect in an isolated subpopulation of the country (Aittomäki, 1994). The locus was named *ODG1*.

In this paper, we describe a series of investigations undertaken to determine the genetic and molecular basis of ODG. We took advantage of the existence of multiplex families in Finland that allowed us to map a locus to chromosome 2p by a systematic search for linkage. This focused our attention on the FSH receptor (*FSHR*) gene, which had previously been cloned (Sprengel et al., 1990; Minegishi et al., 1991; Kelton et al., 1992) and mapped to 2p16-21 (Rousseau-Merck et al., 1993; Gromoll et al., 1994). By sequencing the coding regions of the gene, we found a missense mutation in the region encoding the extracellular ligand-binding domain of the receptor that segregated perfectly with the phenotype in affected families. Transfection experiments showed that the mutation leads to dramatically reduced binding capacity and cyclic adenosine 3′, 5′-monophosphate (cAMP) production after

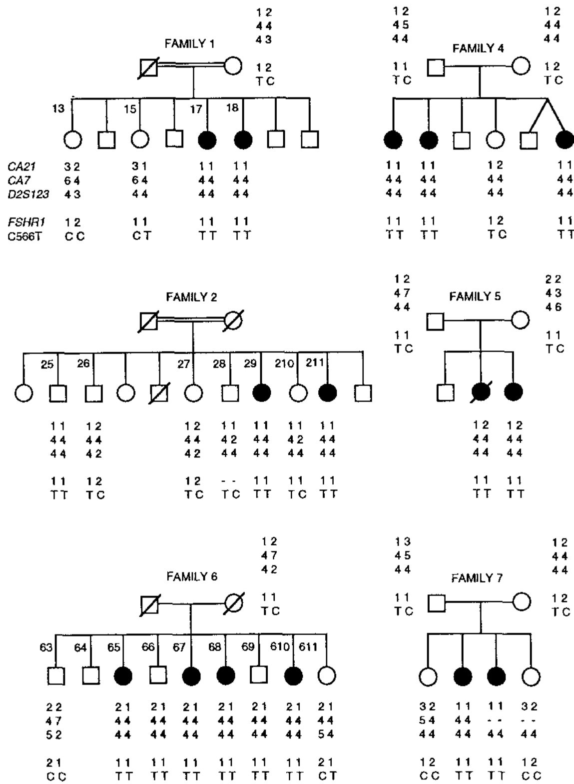

Figure 1. Pedigrees, Alleles, and Haplotypes of Six Multiplex Families
Open squares, unaffected males. Open circles, unaffected females. Closed circles, affected females. Alleles at the polymorphic markers are arranged into most likely haplotypes. The allele at the disease-causing C566T mutation is shown at the bottom of each haplotype.

FSH stimulation, in the face of apparently normal binding affinity in cells expressing the mutated receptor protein. These studies prove that a recessively inherited mutation of the *FSHR* gene causes a hereditary form of hypergonadotropic ovarian failure by possibly interfering with trafficking of the receptor to the cell surface or with its rate of degradation.

Results

Linkage

Of the available 37 samples from six multiplex families, 30 were used in the initial search for linkage. As male sibs were not informative for the phenotype, they were not included in the initial search, but in subsequent linkage analyses, six males and one further healthy female sib were included (Figure 1) to contribute haplotype information. The male sibs were coded phenotype unknown. As fathers can be presumed to be heterozygous rather than homozygous for the mutation responsible for a recessive trait, their phenotype was coded unaffected.

After studying 47 markers, we noted preliminary evidence of linkage for the markers *D2S134* and *D2S177*. The linkage was confirmed by using markers *D2S119* and *D2S123*, located between the former markers and flanking an approximately 12 cM genetic region of chromosome 2. This interval was further studied with four additional markers, *D2S391*, *D2S288*, *CA21*, and *CA7*, located in the above defined interval.

The results of the pairwise linkage analyses in the multiplex families are shown in Table 1. There were altogether 46 potentially informative meioses in the six families, and of these, 30 were in affected family members. It is evident from the lod scores shown that there were no recombinations between any of the marker loci and *ODG1*. The highest lod score, Z_{max} = 4.71, was obtained for the marker *D2S391*, while two of the other marker loci, *D2S288* and *CA21*, also gave lod scores exceeding the formal limit of proven linkage ($Z \geq 3.00$).

Haplotype Analysis

Alleles at the most informative closely linked markers were arranged into most likely haplotypes as shown in Figure 1. For markers *CA21*, *CA7*, and *D2S123*, the haplotype 1-4-4 was associated with the disease in most chromosomes. This can be considered the ancestral disease-associated haplotype. The only other disease-associated haplotype, 2-4-4, occurred in the maternal chromosome in family 5 and the paternal chromosome in family 6. As marker *CA21* showed no recombinations with the phenotype, these two chromosomes most likely represent historical crossovers between the disease mutation and marker *CA21*, which thus defines the distal border of the interval in which the mutated gene must be located. The proximal border of the disease gene interval was marker *CA18*, in which a recombination was detected (data not shown). The genetic length of the critical interval (*CA21* to *CA18*) is approximately 10.5 cM (Leach et al., 1994; Gyapay et al., 1994; Hemminki et al., 1994). The detection of an ex-

Table 1. Pairwise Logarithm of Odds (Lod) Scores between *ODG1* and Seven Linked Chromosome 2p Marker Loci in Six Multiplex Families

Recombination	Marker Locus						
Fraction (θ)	*D2S119*	*D2S391*	*D2S288*	*CA21*	*CA7*	*D2S123*	*FSHR1*
0.00	2.41	4.71	3.10	4.20	2.66	2.68	2.72
0.001	2.41	4.70	3.10	4.19	2.66	2.67	2.71
0.01	2.35	4.59	3.03	4.11	2.60	2.62	2.66
0.05	2.12	4.14	2.72	3.74	2.35	2.39	2.44
0.10	1.82	3.56	2.33	3.24	2.03	2.08	2.14
0.20	1.20	2.38	1.52	2.19	1.36	1.44	1.49
0.30	0.62	1.26	0.78	1.16	0.71	0.79	0.82
90% support interval	0–0.17	0–0.09	0–0.13	0–0.10	0–0.16	0–0.16	0–0.16

tended conserved haplotype in all affected chromosomes from the six families suggests that they all share one ancestral disease-causing mutation. This is compatible with previously described genealogical evidence that many of the ancestors originated in a limited subpopulation in the north central region of Finland (Aittomäki, 1994).

FSHR as a Candidate for ODG

Genes that had been assigned to the *ODG1* region in chromosome 2p were the gonadotropin receptor genes, that is, *FSHR* (Rousseau-Merck et al., 1993; Gromoll et al., 1994) and the LH receptor gene (*LHR*) (Rousseau-Merck et al., 1990). Both had already been cloned (Minegishi et al., 1990; Sprengel et al., 1990; Jia et al., 1991; Minegishi et al., 1991; Kelton et al., 1992).

The receptors for FSH and LH, together with the receptor for the third pituitary glycoprotein hormone, the thyroid-stimulating hormone receptor (TSHR), form a subgroup of the large G protein–coupled receptor family. Their protein structure comprises three distinct domains, the extracellular ligand-binding domain, the transmembrane domain consisting of seven membrane-spanning regions with six intervening loops, and finally, the intracellular COOH-terminal domain (Figure 2). A highly conserved structure

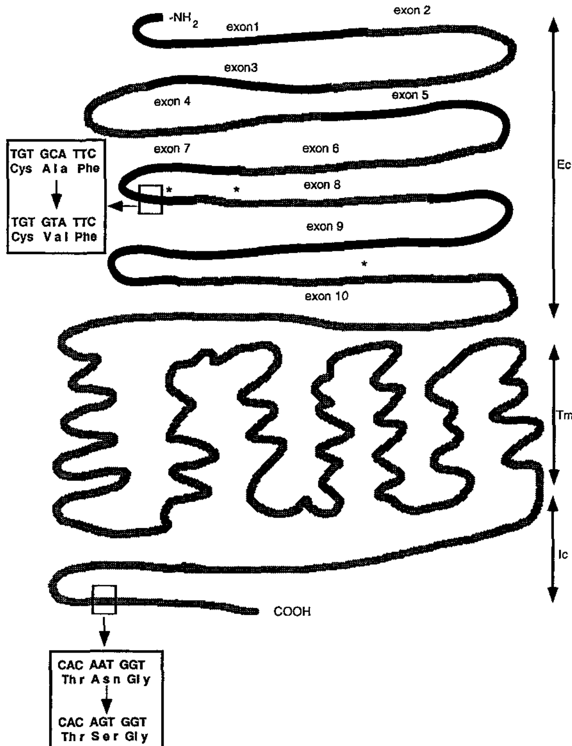

Figure 2. Schematic Representation of the Structure of *FSHR*

Ec, extracellular domain; Tc, transmembrane domain; Ic, intracellular domain. Exons are denoted by alternating black color and stippling. Asterisks denote glycosylation sites. The insert in exon 7 shows the site of the C566T mutation leading to a substitution of Ala-189 to Val. The insert in the part of exon 10 encoding the COOH-terminus shows the G2039A transition polymorphism designated *FSHR1*. Modified from Sprengel et al. (1990).

shared by the G protein–coupled receptors is the transmembrane domain. The distinctive feature of FSHR, LHR, and TSHR is their large extracellular ligand-binding domain. The *FSHR* gene contains 10 exons. The first nine exons encode the extracellular domain, whereas the transmembrane and intracellular domains are encoded by the terminal exon 10 (Sprengel et al., 1990; Heckert et al., 1992). Disease-causing mutations in the genes encoding glycoprotein hormone receptors have been identified in the well-conserved regions coding for the transmembrane domain, e.g., activating (Kremer et al., 1993; Shenker et al., 1993; Yano et al., 1995) and inactivating mutations of the *LHR* gene (Kremer et al., 1995) and an activating mutation of the *TSHR* gene (Duprez et al., 1994), all leading to distinct disease phenotypes: familial male precocious puberty (FMPP), familial male pseudohermaphroditism, and nonautoimmune autosomal dominant hyperthyroidism, respectively. In view of the central role of FSH in the stimulation of ovarian follicular development, we initiated a search for mutations in *FSHR*.

Screening for Mutations by Denaturing Gradient Gel Electrophoresis: A Polymorphism in *FSHR*

To screen for mutations in the large terminal exon 10 of *FSHR* with denaturing gradient gel electrophoresis (DGGE), this exon was amplified with GC-clamped primers in four different polymerase chain reaction (PCR) reactions with overlapping products covering the entire transmembrane and intracellular domains. A DGGE change was detected in the terminal region of exon 10. The same PCR reaction was repeated with a labeled primer for sequencing. In the sequenced product, a G2039A transition was detected in the region encoding the intracellular domain, predicting a change of Ser-680 to Asn. This change had previously been identified as a difference between the ovarian and testicular sequence for the *FSHR* gene (Kelton et al., 1992). By DGGE analysis, a mother of a large sibship and a healthy female sib were both homozygous for the same allele as were the affected individuals, implying that this was not a disease-causing mutation (Figure 3). Conclusive evidence that this was not a mutation was obtained when the multiplex families were studied for the two alleles by restriction fragment length polymorphism analysis (see Figure 1). This polymorphism (designated *FSHR1*) was subsequently exploited as a marker in the linkage analysis and showed no recombinations with the disease phenotype (Table 1).

Search for Mutations by Sequencing

After excluding mutations in exon 10, we proceeded by screening the other exons by amplifying each exon with flanking intronic primers and sequencing the PCR product. With this method, mutations could be sought and excluded in exons 1, 2, 3, 4, 5, and 9. While working on the remaining exons for which complete sequences of the flanking introns were not available, it turned out that it was possible to amplify mRNA of the *FSHR* gene by using RNA from blood leukocytes as template. It has been reported that a low level of mRNA synthesis occurs in white blood

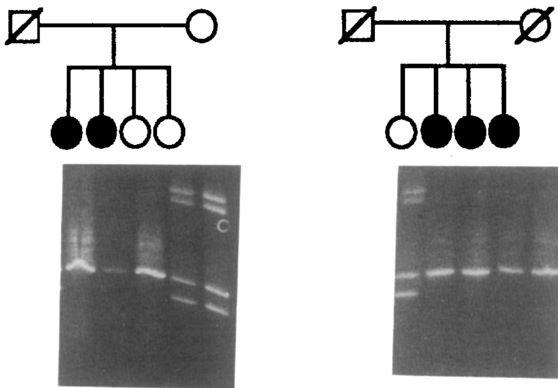

Figure 3. Denaturing Gradient Gel Electrophoresis Showing Segregation of G2039A Polymorphism in Two Families

Pedigree symbols as in Figure 1. In the family on the left, the mother and youngest daughter are heterozygous GA (two heteroduplexes), while the two affected daughters are homozygous GG. In the family on the right, one unaffected daughter is heterozygous GA, while the mother and three other daughters are homozygous GG.

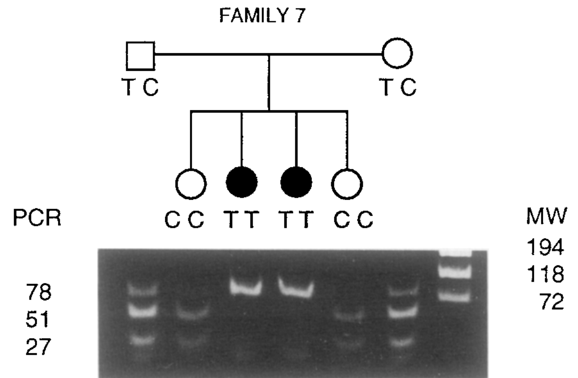

Figure 5. Segregation of C566T Mutation in Family 7 Determined by Restriction Fragment Length Polymorphism Analysis

Pedigree symbols as in Figure 1. A PCR product 78 bp in length comprising nucleotide 566 in exon 7 was digested with BsmI and electrophoresed. In the wild-type situation (CC), the PCR product is cleaved into fragments 51 bp and 27 bp in length (seen in both unaffected sibs). The mutation abolishes the BsmI cleavage site so that both homozygous sibs (TT) show only the 78 bp fragment. The heterozygous parents (CT) show all three fragments.

cells for at least some and perhaps most tissue-specific genes (Sarkar and Sommer, 1989); this phenomenon has been called illegitimate transcription (Chelly et al., 1989). Since PCR is an extremely sensitive technique, and it is possible to identify mutations by direct sequencing of PCR products, we decided to isolate RNA from blood cells, amplify exons 6–9 by reverse transcription–PCR (RT–PCR), and sequence the product directly. All the affected individuals were found to be homozygous for a C to T transition at position 566, which predicts a change of Ala-189 to Val (Figure 4). It was noticed that the mutation C566T abolishes a restriction site of BsmI. By restriction digestion, the families could now be screened for the mutation. When these data were compared with the linkage results, it was noted that the disease haplotype segregated perfectly with the mutation. All the affected individuals displaying the disease haplotype were homozygous for the mutation, and all the parents (obligatory heterozygotes) that could be studied proved to be heterozygous for the mutation (Figure 5). In every individual studied, the affection status, the result of the haplotype analysis, and the status of the C566T mutation were concordant. This allowed us to conclude tentatively that the C to T transition in exon 7 of the *FSHR* gene causes ODG in the families studied.

Functional Studies of the Mutated Receptor

Recombinant human FSH (rhFSH) induced a 3- to 4-fold dose-dependent stimulation of cAMP production in immortalized mouse Sertoli cells (MSC-1) transfected with wild-type human *FSHR* plasmid (Figure 6). The ED_{50} of the stimulant was approximately 75 IU/liter. In contrast, when cells transfected with the mutated *FSHR* plasmid, or only with pCMV-luci, were studied in the same fashion, only negligible FSH effect on cAMP production was observed.

In the FSH binding assay, Scatchard analysis revealed, after correction for transfection efficiency, a 28-fold higher number of receptors per cell in the MSC-1 cells transfected with the wild-type receptor plasmid than in cells trans-

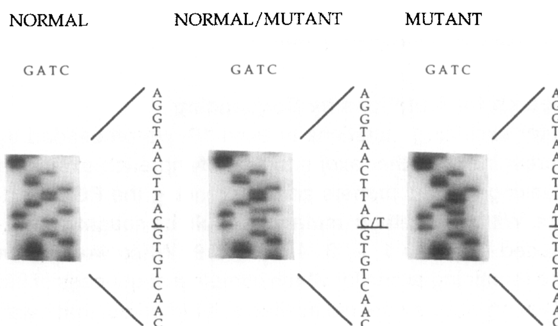

Figure 4. Sequence Analysis of the C566T Mutation

Left, wild-type (CC); middle, heterozygous (CT); right, affected (TT).

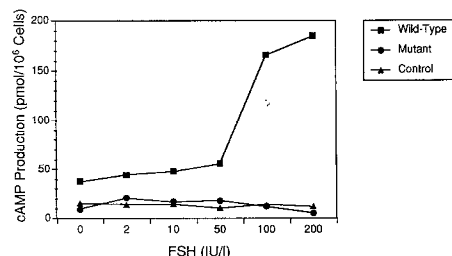

Figure 6. FSH-Stimulated cAMP Production of MSC-1 Cells Transfected with the *FSHR* Expression Constructs

Squares denote wild-type, circles mutant, and triangles mock-transfected controls. Each batch of cells was cotransfected with the pCMV-luci expression plasmid, and the cAMP production was equalized to a constant amount of luciferase expression and calculated per 10^6 cells after a 3 hr incubation. Each point is the mean of duplicate incubations, and one of three similar experiments is presented.

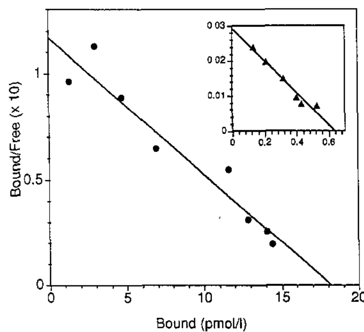

Figure 7. Scatchard Analysis of [^{125}I]Iodo-FSH Binding to Transfected MSC-1 Cells

Circles denote wild-type and triangles (insert) *FSHR* expression constructs. In each case, 2×10^5 transfected cells were incubated in triplicate with increasing concentrations of labeled FSH in a total volume of 250 µl. The cells were cotransfected with the pCMV-luci expression plasmid, and the specific binding was equalized to a constant amount of luciferase activity. After these corrections, the cell suspension transfected with the wild-type *FSHR* construct displayed 18.2 pmol/liter, and that expressing the mutated construct, 0.63 pmol/liter of specific FSH binding. The equilibrium association constants (K_a) of FSH binding in the same cell preparations were 6.7×10^9 mol/liter and 4.8×10^9 mol/liter, respectively. One of two similar experiments is presented.

fected with the plasmid encoding the mutated receptor. The equilibrium association constant (K_a) of FSH binding was 6.7×10^9 mol/liter with the wild-type receptor and very similar, 4.8×10^9 mol/liter, with the mutated receptor (Figure 7). The results agree well with the affinity measured for FSH binding in human testicular homogenate (Wahlström et al., 1983). These studies confirmed the pathogenetic role of the C566T mutation that severely interferes with the signal transduction, but apparently has no effect on affinity of the remaining small number of receptors capable of ligand binding.

Discussion

Hypergonadotropic hypogonadism accounts for up to some 40% of the patients with primary amenorrhea (Reindollar and McDonough, 1984). Many of these cases are due to abnormalities of the sex chromosomes. In the presence of a normal karyotype female, hypergonadotropic hypogonadism is a heterogeneous condition (Reindollar and McDonough, 1984; Aiman and Smentek, 1985; Rebar and Connolly, 1990). We were able to collect 75 females with hypergonadotropic ODG and were able to map the *ODG1* locus to chromosome 2p by linkage. This localization turned out to coincide with the region where *FSHR* and *LHR* had previously been assigned. Both genes were possible candidate genes for ODG. On the basis of data collected by interviewing the female patients, there were no male sibs known to be affected with hypogonadism. This favored the hypothesis that the disease in these families was not due to an *LHR* mutation, which would be likely to cause severe hypogonadism (pseudohermaphroditism) in affected males (Kremer et al., 1995). Subsequently, a mutation was located in exon 7 of the *FSHR* gene and was demonstrated to cause failure of signal transduction of the receptor.

Mutation Effect on Structure and Function of the Receptor Protein

The *FSHR* gene has 10 exons, which encode three distinct protein domains (Figure 2). The amino acid substitution Ala to Val is located in a region that is highly conserved when compared with the FSHR of the monkey, sheep, and rat (Sprengel et al., 1990; Gromoll et al., 1993; Yarney et al., 1993); therefore, it must be functionally important. Moreover, it belongs to a sequence of five amino acids that are identical in the FSHR, LHR, and TSHR and contain a consensus N-linked glycosylation site (Nagayama et al., 1989; Minegishi et al., 1990, 1991), also pointing to the functional importance of this region. Because of conservation of the mutated region in receptors of the three glycoprotein hormones, it may not be the site of ligand recognition and binding. More likely, it plays a role in transfer of the receptor to the plasma membrane, or its turnover, since the affinity of the low number of mutated receptors expressed upon transfection was normal.

Most previously described disease-causing mutations of the G protein–associated receptor family and the glycoprotein hormone receptor subgroup have been located in the transmembrane region. All activating and inactivating *LHR* mutations so far reported have been in the sixth transmembrane sequence (Shenker et al., 1993; Kremer et al., 1993, 1995; Yano et al., 1995). Interestingly, the only inactivating *LHR* mutation reported (Kremer et al., 1995) caused similar drastic reducton of binding capacity with unaffected affinity as was found here with the *FSHR* mutated in the region encoding the extracellular domain.

The mutation described here is adjacent to a glycosylation site of the FSHR that is essential for FSH binding. At least one of the two essential glycosylation sites has to be glycosylated for the binding of FSH to occur (Davis et al., 1995). It is therefore also possible that the mutation could act by hindering the necessary glycosylation. A computer-simulated structural prediction did not reveal any obvious change of the secondary or tertiary protein structure (data not shown). Hence, the mechanism by which the relatively unremarkable Ala to Val change brings about the dramatic suppression of binding capacity and signal transduction observed upon transient expression of the mutated *FSHR* cDNA remains to be explored. It will be of interest to determine whether the resulting low binding capacity with apparently normal binding affinity is due to deficient translation, intracellular trafficking, ligand-binding, or accelerated receptor degradation.

Physiologic Effects of FSH

The notion that initiation of follicular growth is independent of gonadotropin stimulation is supported by the persistence of initial growth in gonadotropin-deficient mice (Halpin et al., 1986) and by its occurrence in the perinatal rat ovary before the appearance of gonadotropin receptors (Sokka and Huhtaniemi, 1990; Rannikko et al., 1995). At

the time of puberty, the interrupted process of follicular growth is overcome when cyclic ovarian function ensues. The most important hormonal event at that time is the increase in FSH secretion, which rescues a group of follicles from atresia. The receptors for FSH are exclusively present in the granulosa cells of developing follicles (Richards, 1995). When FSH binds to its receptor, it activates the production of estrogen in granulosa cells and also stimulates their growth, initiating a normal ovulatory cycle (LaPolt et al., 1992; Tilly et al., 1992). The ability of FSH to induce follicular development has been demonstrated in women with gonadotropin deficiency (Couzinet et al., 1988). Thus, FSH seems to have a pivotal role in the development of the quiescent prepubertal ovary into a mature, cycling, and hormonally active organ. If this indispensable FSH action does not take place, follicular growth is arrested, atresia continues, and with the lack of normal cyclic ovarian function, it leads to the syndrome of hypergonadotropic primary amenorrhea, that is, ODG as defined here.

Failure of the action of either FSH or its receptor has been proposed to cause a type of hypergonadotropic hypogonadism designated the resistant ovary syndrome (Jones and Moraes-Ruehsen, 1969; van Compenhout et al., 1972; Maxson and Wentz, 1983). In this rare condition, the ovarian histology displays primary follicles with arrested development. A mutation of the β subunit of FSH, leading to the inhibition of the binding of the hormone with its receptor, indeed appeared to cause primary amenorrhea and infertility in a patient (Matthews et al., 1993) with the resistant ovary syndrome. This patient was homozygous for a deletion of two nucleotides in exon 3 of the *FSH* gene. The ovarian histology was not described, but the patient conceived after treatment with FSH. The mother of the patient was an obligatory heterozygote and had been amenorrheic and infertile for six years before the conception of her only child. In contrast, the mutation in the *FSHR* gene described here does not seem to affect ovarian function in the heterozygous state. The mothers who are obligatory and experimentally proven heterozygotes have many children, as demonstrated by the pedigrees in Figure 1.

In the male, FSH is needed for pubertal proliferation of Sertoli cells and for initiation of spermatogenesis, as well as for maintenance of qualitatively normal spermatogenesis of the adult testis. However, normal FSH action is not an absolute requirement for spermatogenesis, which can be maintained at near normal levels with testosterone alone in the absence of FSH (Matsumoto, 1989; Weinbauer and Nieschlag, 1993; Zirkin et al., 1994). What effect might the *FSHR* mutation described here have in men?

Gonadal dysgenesis has been reported at least twice in a brother of an affected ODG female. Both of these men had small testes, normal serum testosterone, high serum FSH, and azoospermia (Smith et al., 1979; Granat et al., 1983). By direct mutational analysis, it is now possible to determine the mutation status of males and perform a clinical study on those who are homozygous for the mutation. As we have not yet taken this step, for ethical reasons, we do not have conclusive data on the phenotype of the brothers. However, as shown in Figure 1, blood samples were initially obtained from six consenting brothers of probands belonging to the six multiplex families that we studied for linkage. As can be seen in Figure 1, the mutation status has been determined in these males. All three males who are homozygous for the mutation are over 40 years of age. Two of them have two children each, while the third is childless and is said to have sought medical help for infertility. These preliminary data rule out obligatory sterility and tentatively predict that spermatogenesis in the males homozygous for the mutation might vary anywhere from azoospermia to normospermia.

Incidence and Worldwide Distribution

We demonstrate here that hypergonadotropic hypogonadism with normal female karyotype is caused by a mutation in the *FSHR* gene and that the condition is inherited in a regular recessive Mendelian fashion. While this study was conducted on families in which at least two female sibs were affected, it follows that sibships with a single affected female (so-called sporadic cases) must also occur. In our previous study, many such patients were indeed detected, and the segregation ratio was 0.23, close to that expected for a recessive condition (Aittomäki, 1994). Many of the probands were derived from geographically defined subpopulations in Finland by an approach that aimed at as complete an ascertainment as possible. The result allowed the minimum incidence of the condition to be calculated as 1 in 8300 females. This translates into a carrier frequency of 1 in 45. If similar figures occur in other populations, ODG will turn out to be one of the commonest recessively inherited diseases of mankind.

It needs to be determined whether the incidence of this entity is as high in other populations as in Finland. A well-known feature of the Finnish population is a considerable enrichment of mutations for certain recessively inherited disorders (Norio, 1981; de la Chapelle, 1993). The mechanism of enrichment is that of founder effect, population bottlenecks, and genetic drift in the genetically isolated Finnish population. Moreover, regional subisolates in Finland often show even higher gene frequencies than the population as a whole (Nevanlinna, 1972; de la Chapelle, 1993). As this feature is typical of ODG in Finland as well (Aittomäki, 1994), it is possible that a considerable enrichment of the causative ancestral mutation has occurred.

Given the above findings, what proportion of women with primary amenorrhea is likely to have ODG caused by a mutation in the *FSHR* gene? Clearly, it is too early to answer the question, but fortunately a tool is now available to provide the answer. We anticipate that as a first step, mutational analysis of the *FSHR* gene will be practiced in women with otherwise unexplained hypergonadotropic primary amenorrhea. As more data on the mutational spectrum become available, it may be possible to narrow the search to a defined part of the gene. Eventually, simpler methods will undoubtedly be developed for clinical use.

As to the worldwide incidence and distribution of the condition, we hypothesize that it exists in different populations, as shown by the existence of occasional multiplex

families in Europe, North America, and South America (Simpson et al., 1971). We also hypothesize that the incidence of the condition may be seriously underestimated in clinical practice, as seems to have been the case in Finland, where a comprehensive population-wide study led to the discovery of many previously undiagnosed patients (Aittomäki, 1994).

Different Types of Mutations: Genotype-Phenotype Correlations

We look forward to determining whether other mutations of the *FSHR* gene might also cause hypergonadotropic hypogonadism. We shall proceed by testing the sporadic patients diagnosed previously (Aittomäki, 1994). We anticipate finding some of these patients to be homozygous for the C566T mutation, while others may be heterozygous for it. In heterozygous patients, the *FSHR* gene on the other chromosome will be carefully searched for other mutations. These studies will allow the degree of allelic heterogeneity in ODG to be assessed, as well as the proportion of ODG patients that are not due to *FSHR* mutations (genetic heterogeneity) to be determined. Moreover, these data will allow genotype–phenotype correlations to be made. Finally, the effects of the mutation on spermatogenesis need further study.

Experimental Procedures

Patients and Families

To identify patients with ovarian dysgenesis, the registries of hospitals as well as cytogenetic laboratories were screened for patients with amenorrhea, as described in detail previously (Aittomäki, 1994). Among the 75 patients who met the diagnostic criteria described below, 15 belonged to six multiplex families (Aittomäki, 1994). Samples were available from 37 individuals belonging to these six families (Figure 1).

Diagnostic Criteria

The following five criteria were used for final selection: first, primary amenorrhea or secondary amenorrhea before the age of 20 years; second, normal karyotype (46, XX); third, high concentration of serum gonadotropins (FSH at least 40 IU/liter); fourth, date of birth between the years 1950 and 1976 (only when there were multiple affected individuals in the family were all affected individuals included irrespective of age); and fifth, no other known cause of the hypergonadotropic hypogonadism.

Samples

Samples of venous blood (10–20 ml) were collected from each consenting individual. DNA was extracted by standard methods (Sambrook et al., 1989). Total RNA was isolated from fresh leukocytes or lymphoblastoid cell lines established from these patients (Chomczynski and Sacchi, 1987).

Polymorphic Microsatellite Typing

The markers used initially were Généthon microsatellite markers (Weissenbach et al., 1992; Gyapay et al., 1994) that were chosen at approximately 20 cM intervals for a systematic search for linkage. Further markers used were *CA21*, *CA7*, and *CA18* (Leach et al., 1994; Hemminki et al., 1994). Finally, the G2039A change located in exon 10 of the *FSHR* gene that we found was designated *FSHR1* and used as a polymorphic marker to confirm the linkage. The PCR conditions for most of the microsatellite markers were as described before (Sankila et al., 1995).

Linkage Analysis

Pairwise linkage analyses were carried out with the MLINK subprogram of the LINKAGE program package (Lathrop et al., 1984). Marker allele frequencies were set as estimated in the normal chromosomes by subprogram ILINK. The disease allele frequency was set to 0.01, and the lod scores were computed under a model of equilibrium between the disease locus and each of the marker loci, assuming recessive mode of inheritance with full penetrance in females. Male sibs were assigned affection status unknown.

DGGE

For mutation screening of the transmembrane and intracellular domains of the large exon 10 of the *FSHR* gene, four separate PCR reactions with overlapping PCR products were amplified. Pairs of GC-clamped primers (Table 2, primers 10–13) were optimized for each fragment with the MELT 87 program (Lerman and Silverstein, 1987) and analyzed with DGGE (Myers et al., 1988). A 40 bp GC-clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG) was attached to either end of the primer according to the predicted melting curve. A polyacrylamide denaturing gel was designed for each amplified fragment with a linear concentration gradient (Δ 20%–30%) of the denaturants (formamide and urea) and was run in an aquarium at a stable temperature of 60°C for 16–20 hr in a commercially available apparatus (denaturing gradient gel electrophoresis system, model DGGE-2000, C. B. S. Scientific Company).

Sequencing and Restriction Enzyme Analysis

For sequencing of the observed DGGE change in exon 10 of the *FSHR* gene, a fragment of 326 bp corresponding to nucleotides 1818–2143 (Minegishi et al., 1991; Kelton et al., 1992) was amplified with a different forward primer (Table 2, primers 14) under the same PCR conditions as before and sequenced as described below. A change of G to A was identified in nucleotide 2039, abolishing a restriction site for BbsI. For screening of the two alleles, the amplified DNA fragment was digested with BbsI (New England Biolabs), and the fragments were electrophoresed in a 2% agarose gel.

cDNA Synthesis

Total RNA was isolated from lymphoblasts or leukocytes. RNA (0.8 μg) was used as a template for first-strand cDNA synthesis and primed for reverse transcription by using 40 pmol of a specific reverse primer (Table 2, primer 15.r). Moloney murine leukemia virus (MoMLV) reverse transcriptase buffer (Promega), dNTPs (to 1 mM), 20 U of RNAsin (Promega), and MoMLV reverse transcriptase (200 U) were added in a final volume of 20 μl and the samples incubated at 42°C for 1 hr. The samples were heated to 95°C for 10 min, and a 5 μl aliquot of the resulting product was used as template for PCR as described below.

RT-PCR

cDNAs were amplified by two rounds of PCR using a nesting strategy to enhance yield and specificity. In the first round, the previously used reverse primer and a new forward primer (Table 2, primer 16) were used to produce a 2082 bp product.

For the second round, 5 μl of the first round product was used as template for amplification using two internal primers for exons 6–9 (Table 2, primers 6). In this case, a PCR product of 357 bp was obtained.

PCR Amplification from Genomic DNA for Mutation Detection

To amplify exons 1, 2, 3, 4, 5, 9, and the extracellular region of exon 10 of the *FSHR* gene, primers flanking each exon and the extracellular region of exon 10 were used (Table 2, primers 1–5, 8, and 9). The specific PCR conditions and the cycling parameters for each reaction are available from the authors on request. For the size of each PCR product, see Table 2.

To amplify a suitable segment of the *FSHR* gene containing nucleotide 566 (Kelton et al., 1992), primers flanking the region were used (Table 2, primers 7). A 78 bp product is amplified from genomic DNA by this procedure.

Table 2. Primers Used in the Study

Number	Primer Sequence	Exon	Product Size	Primer Location
1	Forward: 5′-AATTATGGCCTGCTCCTG-3′ Reverse: 5′-ATAGTACGCAATGCACAAATGC-3′	1	215 bp	−4–intronic[a]
2	Forward: 5′-TCTTTGCAGGAGGTTTGTCC-3′ Reverse: 5′-TTGAGGCATTCACTCACAGC-3′	2	118 bp	Intronic[a]
3	Forward: 5′-TCAAGGAGGTAACTGAACTGGC-3′ Reverse: 5′-ATCCCCCAATCTTCTTGCTT-3′	3	146 bp	Intronic[a]
4	Forward: 5′-GCATTCCTTACCATCAAGATGA-3′ Reverse: 5′-GTGGGGGTACCAAACTACATG-3′	4	153 bp	Intronic[a]
5	Forward: 5′-TTCCCAACCTTCAATATCTGTT-3′ Reverse: 5′-AGCCCCACGAAAGAATTTCT-3′	5	148 bp	356–503
6	Forward: 5′-AGAAATTCTTTCGTGGGGCT-3′ Reverse: 5′-GTTTGCAAAGGCACAGCAAT-3′	6–9	357 bp	1484–1840
7	Forward: 5′-GTTATTTCAGATGGCTGAATAAG-3′ Reverse: 5′-GCTCATCTAGTTGGGTTC-3′	7	78 bp	Intronic[a] −592
8	Forward: 5′-GCCTGCTAACCAAGAGCACT-3′ Reverse: 5′-AGGATGGACTCACATTTGCC-3′	9	270 bp	Intronic[a]
9	Forward: 5′-CTACCCTGCACAAAGACAGTG-3′ Reverse: 5′-GCTTAGGGGAGCAGGTCAC-3′	10	272 bp	Intronic[a]–1048
10	Forward: 5′-GC-clamp-GACTTATGCAATGAAGTGGTTG-3′ Reverse: 5′-GTGAAAAAGCCAGCAGCATC-3′	10	341 bp[b]	1006–1346
11	Forward: 5′-GC-clamp-ATTGACTGGCAAACTGGGG-3′ Reverse: 5′-AGAGGAGGACACGATGTTGG-3′	10	396 bp[b]	1300–1695
12	Forward: 5′-GC-clampGGCTGCTATATCCACATCTACC-3′ Reverse: 5′-CAGAACCAGCAGAATCTTTGC-3′	10	201 bp[b]	1639–1839
13	Forward: 5′-GC-clamp-CTTTCTTTGCCATTTCTGCC-3′ Reverse: 5′-CAAAGGCAAGGACTGAATTATC-3′	10	378 bp[b]	1766–2143
14	Forward: 5′-AGCAAAGATTCTGCTGGTTC-3′ Reverse: 5′-13.r	10	326 bp	1818–2143
15	Reverse: 5′-TAGTTTTGGGCTAAATGACTTAGAGGG-3′			
16	Forward: 5′-CCTGCTCCTGGTCTCTTTGCTG-3′			6–2087
17	5′-GAGTGCACCATGGGCGGTGTGAAAT-3′			
18	5′-GGGATTCAAGAAATACACAACTGTGTATTCAATGGAACCC-3′			

[a] Unpublished intronic sequence.
[b] Without GC clamp.

DNA Sequencing of PCR Products
To sequence amplified PCR products, the previously described primers were used. Each reaction contained 2 pmol of primer end-labeled with [γ-^{32}P]ATP (specific activity, 5000 Ci/mmol; Amersham International), 15 ng of amplified template DNA, and 1 U of Dynazyme (Finnzymes).

Restriction Enzyme Analysis to Detect the Mutation
Genomic DNA was amplified as described above. To screen individuals for the C566T mutation, 20 U of BsmI (Promega) was used to digest 15 µl of PCR product. The digestion product was electrophoresed through a 10% nondenaturing polyacrylamide gel and DNA visualized with ethidium bromide.

Preparation of the Wild-Type *FSHR* Expression Construct, and Site-Directed Mutagenesis
The human *FSHR* cDNA was obtained by RT–PCR by using testicular poly(A$^+$) RNA, and the resulting cDNA was subcloned into pBluescript SK(−) (Stratagene). The primer and conditions are described elsewhere (Gromoll et al., 1992). The 5′ untranslated region containing a stop codon was deleted, and a perfect consensus sequence for the initiation of translation was constructed (Gudermann et al., 1994; Kozak, 1989). This procedure resulted in a construct comprising 2088 bp of the translated region of the *FSHR* and an additional 5-base 5′ extension and a 92-base 3′ extension. The construct was excised by digestion with SmaI–KpnI and blunt end–ligated into the blunted EcoRI restriction site of the pSG5 vector (Stratagene). Both strands of the cloned cDNA were sequenced by the dideoxy chain termination method applying the primer walk technique (Sambrook et al., 1989).

A plasmid containing the mutated *FSHR* (T566) was created by site-directed mutagenesis using the wild-type *FSHR* cDNA as template. Oligonucleotide-directed mutagenesis was done according to the instructions of the manufacturer (Clontech). The selection primer (Table 2, primer 17) transforms an NdeI site into an NcoI site in the vector sequence. The primer for mutagenesis in the *FSHR* cDNA sequence (Table 2, primer 18) transforms C to T at nucleotide 566. The identity of the plasmids generated (pFSHR(C566), wild type, and pFSHR(T566), mutated) was verified by restriction endonuclease digestion and sequencing.

The cell line used for transfection (MSC-1) was derived from a transgenic mouse Sertoli cell tumor generated by expressing the simian virus 40 (SV40) T antigen under the anti–Müllerian hormone promoter (Peschon et al., 1992). Despite their Sertoli cell origin, the cells do not express endogenous FSHR. Cells in exponential growth phase were transiently transfected in DMEM/F12(1:1) medium by use of lipofectin (GIBCO Life Technologies, Incorporated), as instructed by the manufacturer, using either of the above *FSHR* expression plasmids, or buffer for mock transfection. In addition, a cotransfection with a

luciferase-expressing pCMV-luci plasmid was done to control the transfection efficiency.

cAMP Production of the Transfected MSC-1 Cells

Subsequently, 72 hr after transfection, the cells in the 2 cm culture plates were challenged, in triplicate, with each of the following concentrations of rhFSH (Org 32489, approximately 10,000 IU/mg, Organon International): 0, 2, 10, 50, 100, or 200 IU/liter. After a 3 hr stimulation period, the cells and media were removed from the culture plates and divided into two equal aliquots. One was diluted 1:1 with 2 mmol/liter teophylline, heated at 100°C for 5 min, and subsequently used for measurement of cAMP (see below). Supernatants were used for luciferase assay according to the protocols of Sambrook et al. (1989). The light production was measured with a 1251 luminometer (Bio-Orbit). For cAMP measurement, a radioimmunoassay was used as described before (Harper and Brooker, 1975; Brooker et al., 1979).

FSH Binding Assay

The MSC-1 cells cultured in 9 cm diameter culture plates and transfected with the *FSHR* expression plasmids were used for FSH binding measurements 48 hr following the transfections. The cells were recovered and reconstituted to a concentration of 2×10^6 cells/ml in buffer. rhFSH was radioiodinated with sodium [^{125}I]iodide with a solid-phase lactoperoxidase method (Karonen et al., 1975) to a specific activity of 30 Ci/g and 20% specific binding of radioactivity to an excess of FSHRs, as determined according to the protocols of Catt et al. (1976). Triplicate aliquots of 100 μl of the cell suspensions (containing 2×10^5 cells) were incubated in the presence of 3.13, 6.25, 12.5, 25, 50, or 100 ng of the radioiodinated rhFSH in a total volume of 250 μl. Nonspecific binding at each hormone concentration was determined in the presence of 1.5 IU of rhFSH. After overnight incubation at room temperature, the radioactivity of the cell pellets was counted in a γ-spectrometer.

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