ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1996, 56: 743-751

Polymorphism of gonadotropin action; molecular mechanisms and clinical implications

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Abstract. Various structural alterations of gonadotropins and their receptors (R) contribute to the polymorphism of gonadotropin action. One reason is the microheterogeneity of gonadotropins due to variations in the degree of their glycosylation. This alters the intrinsic bioactivity of gonadotropins, as reflected by changes in their bioactivity to immunoreactivity ratios in various physiological and clinical conditions. We have reassesses this phenomenon by improved *in vitro* bioassay and immunoassay methods, and it appears that the intrinsic bioactivity of gonadotropins, in particular of LH, is more constant than previously demonstrated.The second part of this chapter deals with a common polymorphism that was recently discovered in the gene of the LH β-subunit. The variant LHβ allele contains two point mutations, both altering the amino acid sequence (Trp8Arg and Ile15Thr), the latter one in addition introduces a new glycosylation signal to the LHβ peptide. The variant seems to represent an evolutionary early form of LH, being structurally closer to hCG than wild-type LH. The LH variant is common world-wide, with the carrier frequency varying from 28% in Finland to 7.5% in North American Hispanics. The LH variant differs functionally from wild-type LH, and it seems to predispose the carriers to mild aberrations of reproductive function.The third section summarizes our findings on the first mutation of the FSHR gene. This inactivating missense mutation is located in the gene sequence encoding the extracellular domain of the FSHR (Ala189Val). The mutated receptor protein is apparently incorrectly folded and devoid of biological activity. The mutation explains about 50% of the hereditary form of hypergonadotropic ovarian dysgenesis in the Finnish population.

Key words: luteinizing hormone, follicle-stimulating hormone, gonadotropin receptors, *in vitro* bioassay, immunofluorometric assay, mutation, polymorphism, microheterogeneity

NEW FINDINGS ON BIOACTIVITY TO IMMUNOREACTIVITY (B/I) RATIOS OF GONADOTROPINS

In addition to immunoassays, also sensitive and specific *in vitro* bioassays can be used for measurement of gonadotropins in peripheral serum (see e.g. Dufau and Veldhuis 1987, Beitins and Padmanabhan 1991, Tsatsoulis et al. 1991, Ulloa-Aguirre et al. 1995). The immunoassays mainly monitor the amount of immunoreactive hormone molecules in the sample, whereas the bioassays take into account the biological activity of the hormone, which does not always correlate directly with the number of hormone molecules. The reason for this discrepancy has been attributed to the microheterogeneity of gonadotropin molecules, due to variations in the degree of their glycosylation. The differently glycosylated gonadotropin molecules are known to have variable intrinsic bioactivities. It is therefore understandable that the concentrations of gonadotropins measured by the two principally different assay systems do not always agree. The ratio of bioactive (B) to immunoreactive (I) LH and FSH in a serum sample, i.e. the bio/immuno (B/I) ratio, has been considered an indicator of the quality of gonadotropins, and it has been shown to vary in predictable manner in various physiological and pathophysiological conditions.

The current *in vitro* bioassay methods for LH are technically easier and more reliable that those for FSH, and therefore the majority of information on the B/I ratio measurements are on LH (reviewed by Dufau and Veldhuis 1978, Beitins and Padmanabhan 1991, Tsatsoulis et al. 1991). Increased B/I ratios of LH have been documented during endogenous and GnRH-stimulated LH pulses (Veldhuis et al. 1987), during pubertal maturation (Lucky et al. 1980, Reiter et al. 1982, Rich et al. 1982) and after orchidectomy (Haavisto et al. 1990b). Low or decreased B/I ratios of LH have been measured in hypogonadotropic hypogonadism (Beitins et al. 1981), during treatments with GnRH agonists (St.Arnaud et al. 1986), estrogen (Veldhuis and Dufau 1987) and androgen (Tsatsoulis et al. 1990), during renal failure (Talbot et al. 1991) and in idiopathic infertility (Bennett et al. 1991). However, the biochemical basis of the altered B/I ratio changes has so far remained elusive in this large number of studies.

With the advent of more sensitive and specific immunometric assay methods (e.g. the immunofluorometric assay, IFMA, Pettersson and Söderholm 1990) it has become apparent that many, though not all, of the previously detected changes in the B/I ratio of LH are due to a bias introduced into the immunoreactivity measurements by conventional radioimmunoassays (RIA), which often overestimate low hormone concentrations (Chappel 1990, Haavisto et al. 1990a, Jaakkola et al. 1990, Huhtaniemi et al. 1992). The B/I ratios are therefore low at low hormone concentrations and systematically increase whenever the LH levels increase. A higher fold-increase occurs in B-LH resulting in artifactual elevation of the B/I ratio. We have previously demonstrated this type of bias during pulsatile LH secretion (Huhtaniemi et al. 1992), after GnRH-stimulation (Jaakkola et al. 1990), after gonadotropin suppression during GnRH agonist treatment (Jaakkola et al. 1990) and in children with hypogonadotropic hypogonadism (Haavisto et al. 1990a).

One of the widely cited findings on the increased B/I ratio of LH has been that demonstrated during pubertal maturation (Lucky et al. 1980, Reiter et al. 1982, Rich et al. 1982). This information has found its way to textbooks of endocrinology, stating that a crucial event during puberty is, besides reactivation of gonadotropin secretion, an increase in the intrinsic bioactivity of LH. Since the studies reporting this finding have used conventional RIA for the measurement of I-LH, we found it important to reassess this finding by eliminating the bias of RIA with a novel sensitive and specific IFMA (Huhtaniemi et al. 1996). Since also the B-LH levels were expected to be low in the peripubertal serum samples, we adapted a sensitized *in vitro* bioassay, originally described by Debertin and Pomerantz (1992), for measurement on these samples. Depending on the serum dilution used, on average a 10-fold increase

in the sensitivity of the bioassay was achieved, down to 0.05 IU/L (Fig. 1).

Figure 2 shows the mean ± SEM levels of B-LH, I-LH and the B/I ratios at different stages of puberty in healthy boys. I-LH increased between pubertal stages I and IV (according to Tanner) from 0.42 ± 0.13 to 2.24 ± 0.34 IU/L (P <0.01), and B-LH from 1.35 ± 0.49 to 5.04 ± 0.78 IU/L (P <0.01). In contrast, no significant change was observed during the same period in the B/I ratios of LH which varied between 2.58-2.84. Hence, when sensitive IFMA and *in vitro* bioassay methods were used, no alteration could be demonstrated in the intrinsic bioactivity of LH, as reflected by the B/I ratio, during the pubertal maturation of healthy boys.

This study showed yet another physiological condition where a previously demonstrated change in the quality of LH could not be confirmed when more sensitive and specific assay methods were used. It is therefore apparent that the clinical value of *in vitro* bioassays of LH is not as great as previously assumed. The improved immunoassays monitor reliably the LH levels and correlate well with the bioactivity of the hormone. The clinical use of *in vitro* bioassays of LH may therefore be limited to cases where a discrepancy is found between the I-LH level of the patient and the clinical picture. There may be conditions where the immunoassays using monoclonal antibodies are "too specific" and do not detect all LH isoforms, or the hormone of the patient may represent a structural variant. An example of such a condition is described in the next paragraph.

A COMMON GENETIC VARIANT OF LUTEINIZING HORMONE

When we recently tested the suitability of various monoclonal antibody (Mab) combinations for LH measurement by IFMA, we identified a healthy woman with two children, whose LH was not at all detected by a particular Mab combination (Pettersson

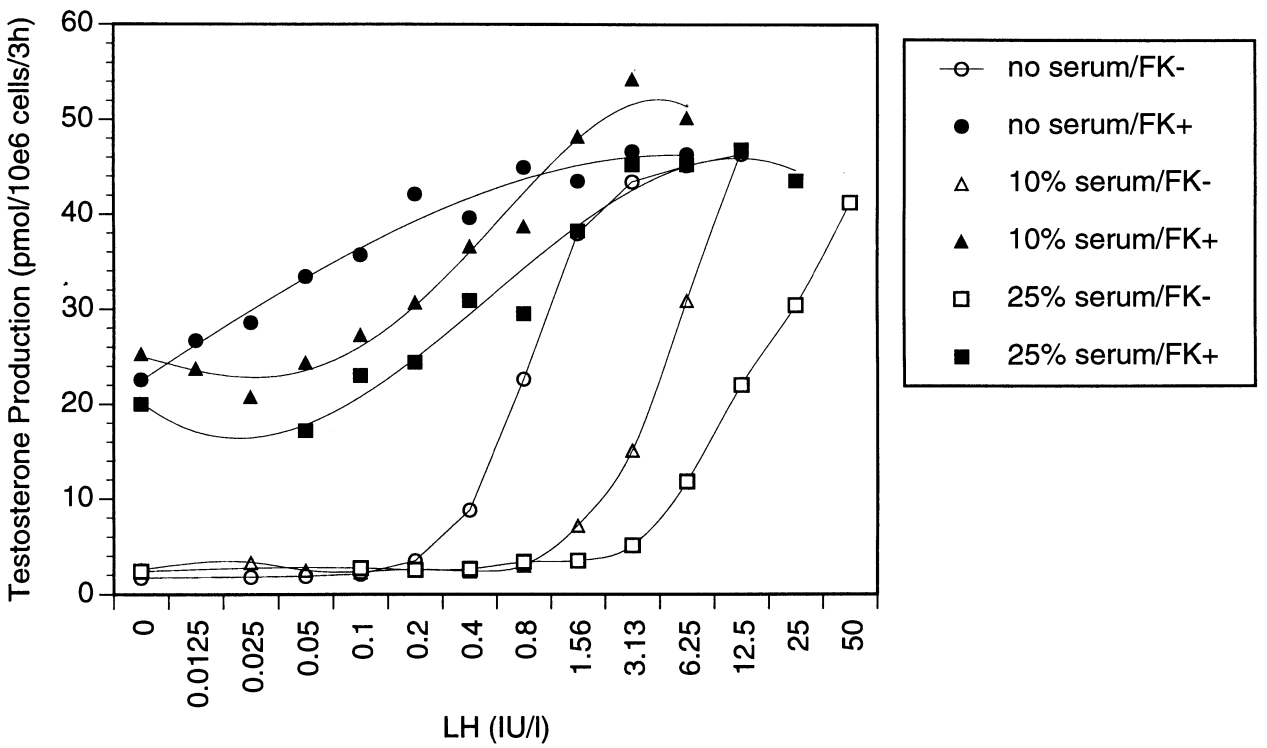

Fig. 1. Standard curves of the *in vitro* bioassay of LH in the presence and absence of 1.5 µmol/L of forskolin (FK), which sensitizes the assay about 10-fold. It is also seen that addition of serum in the assay tubes desensitizes the testosterone response to LH stimulation (Huhtaniemi et al. 1996).

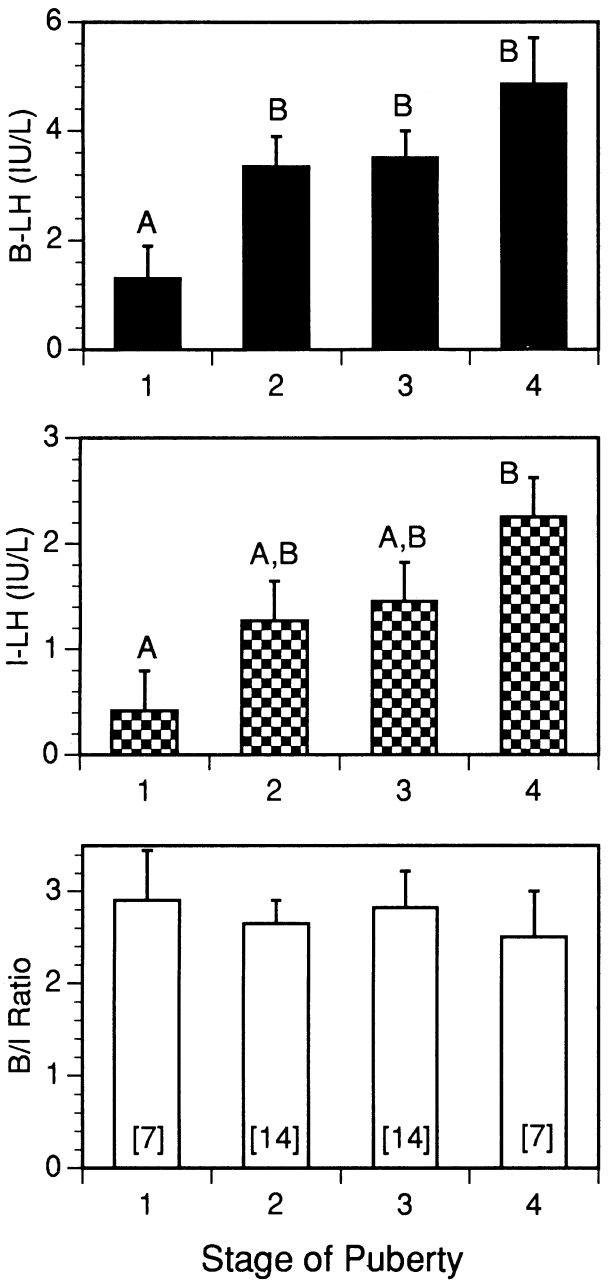

Fig. 2. The mean (± SEM) level of B-LH (top panel), I-LH (middle panel), and the B/I ratio (bottom panel) in 14 perimenopausal boys studied according to their stage of puberty (1-4). The number of subjects analyzed at each pubertal stage is presented in brackets in the bottom panel. When different letters are above the bars these results differ significantly from each other ($P<0.01$) (Huhtaniemi et al. 1996).

et al. 1992). The antibody that did not detect her LH was directed against an epitope in the intact LH αβ dimer (assay 1). Interestingly, all the other Mab combinations tested (α, β and α/β specific) measured normal LH concentrations in her serum. Likewise, her LH bioactivity and B/I ratio (using a subunit-specific IFMA for I-LH measurement, assay 2) were normal, and in accordance with her normal clinical status, including normal fertility. Since the FSH and TSH levels of the subject were normal, we made the assumption that her LHβ subunit was abnormal in structure, probably due to altered gene structure. The LH levels of her family members were also analyzed, and it turned out that her mother had similar nondetectable LH by assay 1, but her father and children displayed LH levels that were by assay 1 about 50% of the levels measured by the reference assay 2, hence the ratio of LH with assay 1/assay 2 was about 0.5. This further strengthened the assumption that the aberrant LH form was due to mutation with Mendelian fashion of inheritance. The subject and her mother were apparent homozygotes, and the father and children heterozygotes with respect to the putative LHβ mutation. A scheme of the molecular alteration of the variant LH and its influence on reactivity of the different epitopes with the Mab's tested is presented in Fig. 3.

When a larger number (n = 249) of serum samples from healthy Finnish volunteers were analyzed (Fig. 4), and the ratio of LH by assay 1/assay 2 was measured, the results fell clearly into three categories: (1) 1.0-2.0, i.e. normal ratio individuals, (2) 0.5-0.75, i.e. low ratio individuals, and (3) the ratio near 0, i.e. zero ratio individuals. The combined frequency of the low (heterozygotes) and zero (homozygotes) ratio LH types in the Finnish population was 28%, and the distribution followed the Hardy-Weinberg equilibrium.

We then sequenced the LHβ subunit gene of our original subject with the zero ratio LH. Two point mutations, both resulting in amino acid change, $Trp^{8} \rightarrow Arg^{8}$ (TGG → CGG) and $Ile^{15} \rightarrow Thr^{15}$ (ATC → ACC), were detected (Fig. 5). The latter mutation introduces a new glycosylation signal (Asn-X-Thr) into the LHβ chain, which results in oligosaccharide chain attachment into Asn^{13} (Suganuma et al. 1996). The same tripeptide glycosylation signal is present in the hCG β-chain where

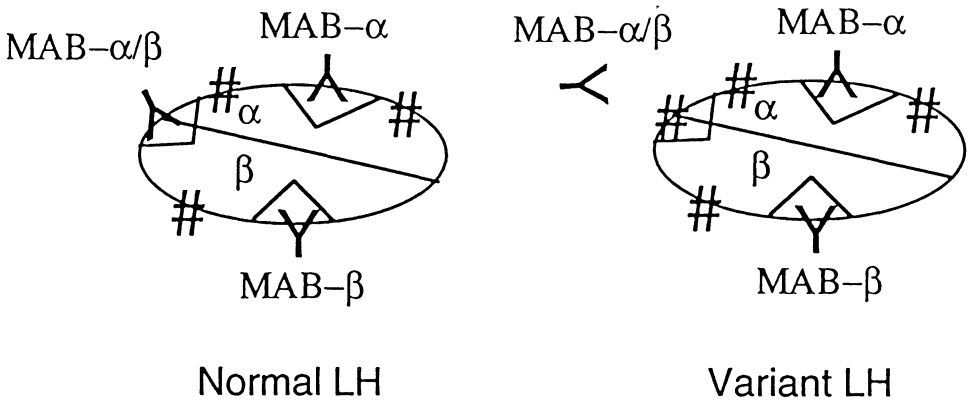

Fig. 3. Schematic presentation of the reason of aberrant immunoreactivity in individuals with the variant form of LH in two-site immunometric assays. The changes in the amino acid sequence and/or an extra carbohydrate chain in the LH β-chain block or eliminate the antigenic epitope present in the α/β dimer. Combination of monoclonal antibody (MAB) α/β and MAB-β was used in assay 1, and MAB-α and MAB-β in assay 2. # = carbohydrate chain.

Asn^{13} is glycosylated. When the structures of the first 20 N-terminal amino acids of wild-type LHβ, variant LHβ and hCGβ are compared (Fig. 5), the variant LHβ differs from hCGβ by two amino acids, whereas wild-type LHβ differs by four amino acids. It seems therefore that the variant represents an earlier form in the evolution of LH, being closer to hCG than wild-type LH. It is intriguing that the frequency of the LH variant varies widely in different populations, from 28% in Finland to 7.5% in North

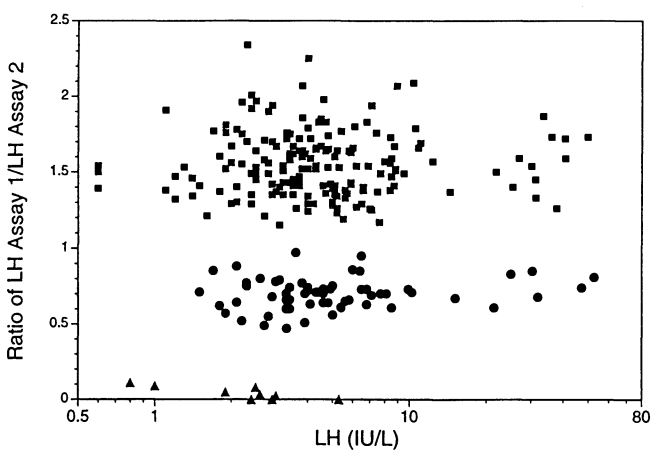

Fig. 4. The distribution of 249 normal Finnish subjects in the normal (■), low (●), and zero (▲) ratio groups according to the results of the ratios of LH measured by assays 1 and 2. The LH level measured by assay 2 is shown on the abscissa. As no sex differences were detected, the male and female data are compiled (Haavisto et al. 1995).

American Hispanics (Haavisto et al. 1995, Rajkhowa et al. 1995, Nilsson et al., unpublished observations).

The homozygotes for the LH variant allele that have been detected in Finland are apparently healthy, with no reported infertility. In this respect our findings differ from those of Furui et al. (1994) from Japan, where the same LH variant was related to infertility in three female subjects. We have observed some functional differences between the LH variant as compared to wild-type hormone. Its B/I ratio is higher, but the half-time in circulation shorter (Haavisto et al. 1995). Since the pulse-frequency of the variant LH was normal, the overall action of the variant hormone, although more potent at the receptor site, is shorter in duration *in vivo*. Such a change in the half-time of LH may be of physiological importance. We are gradually accumulating data that this is indeed the case. Men heterozygous for the LH variant have slightly but significantly lower serum concentration of testosterone than men with wild-type LH (our unpublished observation). Women with at least one variant LH allele have higher levels of serum testosterone, estradiol and sex hormone-binding globulin, and the frequency of the variant allele is altered in certain subtypes of polycystic ovarian disease (Rajkhowa et al. 1995). Boys with variant LH have normal age of onset, but slower progression of puberty. During the final

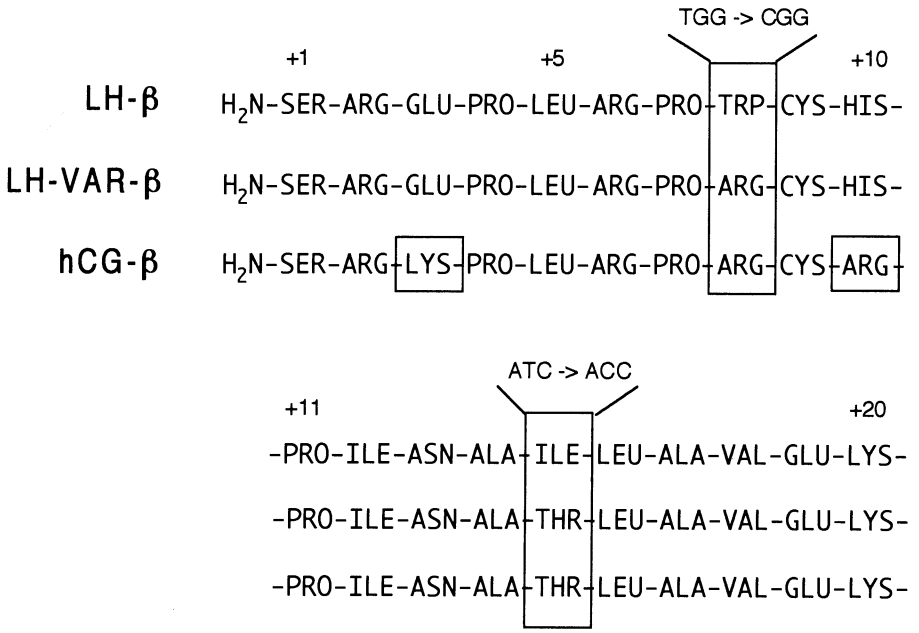

Fig. 5. Sequences of the first 20 amino terminal amino acids in the the β-chains of wild-type LH (LH-β), variant LH (LH-VAR-β) and hCG (hCG-β). The nucleotide changes between wild-type LHβ and the other β-chains are marked above amino acids 8 and 13. In addition, amino acids 3 and 10 in hCG-β are surrounded by boxes to indicate their difference from the other two LH β-chains.

stages of puberty their height, testis weight, plasma IGF-1 binding protein-3 concentration and blood hemoglobin are significantly lower than in matched control boys with normal ratio LH (Raivio et al. 1996).

The above findings, in particular those on pubertal boys and men, suggest that the overall bioactivity of the LH variant is lower than that of the wild-type hormone. Although the final pathophysiological significance of the LH variant remains open, it is likely related to slight alterations of the pituitary-gonadal function. Further studies are needed to delineate the importance of this intriguing phenomenon. Whatever the outcome will be, it is already now clear that the clinical laboratory has to be aware of this common polymorphic form of LH, which behaves aberrantly in several widely applied immunoassay systems.

A NOVEL INACTIVATING MUTATION OF THE FSH RECEPTOR GENE

We recently discovered an inactivating mutation of the FSH receptor gene, which explains the pathogenesis of a part of infertility cases related to hypergonadotropic ovarian dysgenesis (ODG) (Aittomäki et al. 1995). These studies were initiated by Aittomäki (1994) with a population based investigation, where a total of 75 ODG patients were identified in the Finnish population. Segregation analysis, the existence of several kindreds with two or more affected sisters, and genealogical studies showing a clear-cut founder effect in an isolated subpopulation of the country confirmed the recessive mode of inheritance of the disease. The next step of the study was a systematic search for linkage in the multiplex affected families. The ODG locus was mapped to chromosome 2p, with maximum lod score up to 4.71 with chromosome 2p specific markers. Interestingly, two candidate genes were localized to chromosome 2p, i.e. those of FSHR (Rousseau-Merck et al. 1993, Gromoll et al. 1994) and LHR (Rousseau-Merck et al. 1990). Inactivating mutation of either gene could explain ovarian dysgenesis, but due to the critical role of FSH in the early events of ovarian maturation, including follicular development, we considered a mutation in the FSHR gene more likely. Moreover, no male pseudohermaphroditism was apparent in the affected families, which would have been the expected male phenotype in connection with inactivation of the LHR (Kremer et al. 1995).

Since the majority of the previously detected mutations in the TSHR and LHR genes were discovered in the transmembrane region, we first searched for mutations in exon 10, encoding this part of the molecule (Fig. 6). Only a polymorphism which was not related to the disease phenotype was found in this part of the gene. We then screened the first 9 exons of the gene, encoding the extracellular part of the receptor protein, by PCR amplification with intron specific primers and sequencing of the cDNA products. Exons 6-9 were first amplified in this way, and we found that all affected individuals were homozygous for a C to T transition in position 566 of exon 7 of the FSHR, predicting a change in the structure in amino acid 189 from alanine to valine (Fig. 6). When compared with the linkage results, the disease haplotype segregated perfectly with the mutation, and all affected individuals displaying the disease haplotype were homozygous for the mutation. In addition, all parents that could be studied (obligatory heterozygotes) were heterozygous for the mutation.

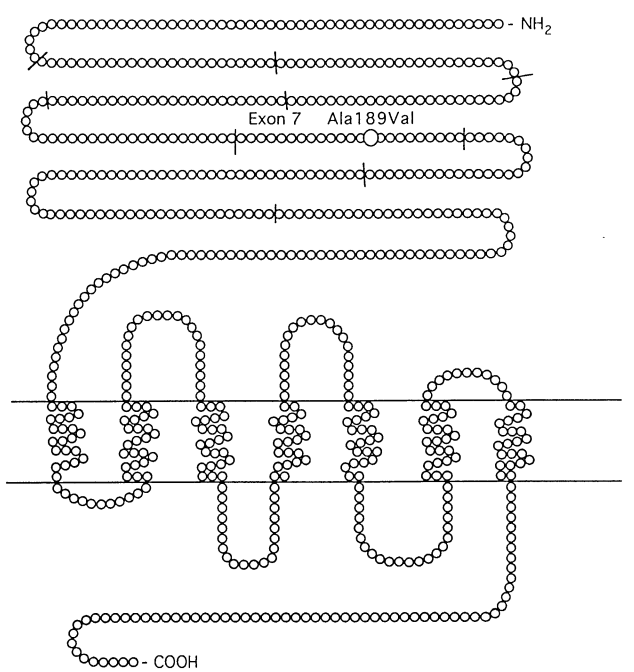

Fig. 6. Shematic presentation of structure of the human FSH receptor gene, and location of the inactivating mutation(o) (Aittomäki et al. 1995). The short lines across the amino acid chain indicate intron-exon junctions.

Due to the conserved nature of the mutation it was important to verify that it affected the function of the mutated FSHR protein. Therefore, the cDNAs encoding the wild-type and mutated human FSHR proteins were transfected into an immortalized murine Sertoli cell line (MSC-1), not expressing the endogenous FSHR gene. A clear 3-4-fold dose dependent stimulation of cAMP production was observed with recombinant human (rh) FSH in cells expressing the wild-type receptor, but no stimulability of cAMP production was observed in those transfected with the mutated cDNA. Appropriate controls were included to control for equal level of transfection. In accordance, when the binding of radioiodinated rhFSH was measured in the same transfected cell lines, those expressing the mutated receptor bound only 3% of that measured in cells expressing the wild-type receptor. Interestingly, the equilibrium association constant (K_a) of binding in both cases was similar (4.8-6.7 x 10^9 liter/mole) and in agreement with the K_a measured for FSH binding in human testicular homogenates (Wahlström et al. 1983). Our recent findings suggest that the mutation does not affect transcription or translation, but merely inhibits proper folding and trafficking of the receptor protein to the cell membrane (Rannikko et al., unpublished observations).

In conclusion, we have identified a mutation in the FSHR gene which partially explains the molecular pathogenesis of ODG. Many of the proband were derived from geographically defined subpopulations in Finland. The minimum frequency of ODG in females for the whole country was calculated as 1 in 8,300, which translates into a carrier frequency of 1 in 45 (Aittomäki 1994). Our preliminary estimate is that about 50% of the ODG cases in Finland are due to the C566T point mutation of the FSHR gene.

The families with the disease are bound to have also males heterozygous and homozygous for the mutated FSHR gene. The phenotype of these men is of particular interest considering the ongoing uncertainty about the necessity of FSH action for male fertility (Zirkin et al. 1994). We have some preliminary data on males homozygous for the C566T mu-

tation, and it seems that men both with normal and suppressed fertility are amongst them (Tapanainen et al., unpublished observations). Since the FSH receptor mutation did not totally abolish FSH binding (about 3% was left with normal affinity in transfected cells), the residual low FSH activity, together with normal testosterone production, appear to be sufficient to maintain spermatogenesis at least in some of the affected men. Such a finding would not be surprising in light of the numerous studies showing that high intratesticular testosterone in the absence of FSH is sufficient for maintenance of spermatogenesis (Bremner et al. 1981, Zirkin et al. 1994). In contrast, in the female, normal FSH secretion is vital for normal follicular development, and the severely suppressed FSH action is thus incompatible with normal ovarian function. Moreover, LH action cannot compensate for the missing FSH action in the female, since the two gonadotropins function in sequential fashion during the process of follicular maturation. It will be interesting to study whether massive doses of FSH could overcome therapeutically the subtotal FSHR failure and induce follicular maturation in the ODG patients. Alternatively, since primordial follicles are present in the ovaries of the affected individuals, *in vitro* culture methods to promote follicular maturation may be another possibility of rescue the fertility of these patients.

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Received 5 April 1996, accepted 15 May 1996