Genetic and functional analyses of polymorphisms in the human FSH receptor gene

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To determine the influence of FSH receptor variants Thr307-Asn680 (TN) and Ala307-Ser680 (AS) on ovarian function, we investigated the frequency of these gene polymorphisms by using restriction fragment length polymorphism analysis and observed their effects on clinical manifestations. In a population of 522 Japanese women, the overall frequency of TN/TN (NN), TN/AS (NS), and AS/AS (SS) was 41.0, 46.9 and 12.1% respectively. In polycystic ovary patients, the NS population was significantly larger when compared with the spontaneously ovulating group (66.7 versus 43.5%, P < 0.05). In the SS group, a significantly higher (46%) basal level of serum FSH was observed as compared with that in the NS group (P < 0.05). A higher dose of the exogenous gonadotrophin was required to achieve ovulation induction in the SS group (66.7 versus 43.5%, P < 0.05). There were no significant differences in FSH-stimulated cAMP production and PI turnover as well as ligand-binding affinity between the two receptor isoforms when overexpressed in transfected 293T cells. These results suggest that although FSH receptor polymorphisms have no discernible effect on FSH receptor function in vitro, there are associations between the genotype and some aspects of patient status.

Key words: FSH receptor/ovarian function/polycystic ovary/polymorphism/RFLP

Introduction

FSH and its receptor play a major role in the development of follicles and regulation of steroidogenesis in the ovary (Richards, 1980; Hsueh et al., 1989). The interaction of this hormone with its cell surface receptor initiates a chain of intracellular reactions characteristic of G-protein-coupled receptors, including stimulation of adenylyl cyclase and phosphorylation of specific proteins (Hsueh et al., 1989; Johnson and Dhianasekaran, 1989; Richards, 1994).

The FSH receptor is composed of a large N-terminal extracellular domain, and seven-transmembrane domains including three outside and inside loops connecting them, together with an intracellular C-terminal tail. The ligand-binding, large N-terminal extracellular domain is unique to FSH as well as the LH/chorionic gonadotrophin and thyroid-stimulating hormone receptors, all of which belong to the same subfamily of G-protein-coupled seven-transmembrane receptors (Salesse et al., 1991). The C-terminal tail of the FSH receptor, located in the cytoplasm, contains a high proportion of serine and threonine residues which might be potential substrates for receptor phosphorylation (Gromoll et al., 1996b).

Human FSH receptor cDNA was cloned from a testis cDNA library (Tilly et al. 1992) and an ovarian cDNA library (Minegishi et al., 1991). Between these two receptor cDNA, there are two amino acid differences, i.e. Thr307-Asn680 in the extracellular domain and Ala307-Ser680 in the C-terminal region. Although independent expression studies using each receptor cDNA revealed that both recombinant proteins could mediate cyclic AMP (cAMP) formation in transfected cells following FSH stimulation and bind [125I]-labelled FSH with high affinity (Tilly et al., 1992; Minegishi et al., 1994), it is not clear whether these two receptor isoforms are functionally identical.

Recently, Simoni et al. reported that the two polymorphisms are linked and could result in two discrete combinations of FSH receptor isoforms. Furthermore, they investigated the characteristics of these two receptor isoforms, including cAMP production and binding studies, and concluded that these isoforms had similar functional properties. They also showed the frequency and distribution of the FSH receptor allelic variants in 161 men (Simoni et al., 1999).

In the present study, the prevalence of polymorphism of the FSH receptor gene was examined in a large population of Japanese women, and we associated the receptor polymorphism with hormonal profiles and gynaecological diseases. We also tested the in-vitro bioactivity of recombinant FSH receptors containing different polymorphisms at codons 307 and 680.

Materials and methods

Subjects

Polymorphic analysis of the FSH receptor gene was performed in 522 Japanese women consulting the Department of Obstetrics and Gynecology, Hokkaido
The region of nucleotide number 1624 to 2143 in the FSH receptor was amplified by PCR using genomic DNA as templates and a set of primers (primer-1: 5'-CAAAATCTATTTTAAGGCAAGAAAGTTG-3'; and primer-2: 5'-TTTGTGGTCATCTGTGGCTG-3'). 

DNA isolation

A volume of 300 µl of blood was drawn from each subject with EDTA added as an anticoagulant. Genomic DNA was obtained from peripheral blood leukocytes with the Wizard™ Genomic DNA Purification Kit according to the manufacturer’s instructions (Promega, Madison, WI, USA).

RFLP analysis of the Asn680Ser variant

The presence of the Asn680Ser variant introduces a restriction site that can be used for the PCR–RFLP (PCR–restriction fragment length polymorphism) technique. The region of nucleotide number 1624 to 2143 in the FSH receptor gene was amplified by PCR using genomic DNA as templates and a set of primers (primer-1: 5'-TTTGTGGTCATCTGTGGCTG-3'; and primer-2: 5'-CAAAATCTATTTTAAGGCAAGAAAGTTG-3'). This mismatch and the A to G transition creates a BsrI restriction site, so that the second PCR fragment following BsrI digestion of the FSH receptor is shown. A 520 bp band for NN, two bands, 520 and 413 bp, for NS, and a 413 bp band for SS, are shown. Note that a 107 bp band has run off from the gel.

Clinical parameters

To investigate hormonal profiles in each group, serum FSH levels and FSH stimulation following GnRH analogue administration during the early follicular
phase were analysed. The GnRH test was carried out in 217 patients, excluding the cases with severe hypothalamic amenorrhoea and POF. A total of 500 µg of GnRH was administered i.v. and the serum FSH level was measured at different times after GnRH analogue injection.

Ninety subjects were selected from 168 patients who were confirmed to have ovulatory cycles, and were analysed for serum estradiol (E2) levels and the diameter of a dominant follicle just before ovulation, together with serum levels of E2 and progesterone in the mid-luteal phase.

Furthermore, in 58 patients undergoing their ovarian stimulation cycles with hMG for IVF and embryo transfer, ovarian responses, i.e. the number of follicles of more than 14 mm in diameter, were measured by chemiluminescent immunoassay (Immulyze; DPC, Los Angeles, CA, USA). The diameter of follicles at the ovulatory phase was measured with transvaginal ultrasonography after the LH surge.

Mutagenesis of FSH receptor cDNA
To evaluate the bioactivity of each FSH receptor isoform, cDNA containing either Thr307–Asn680 (TN) or Ala307–Ser680 (AS), were constructed using PCR-based mutagenesis and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA).

Transfection of 293T cells
293T cells derived from human embryonic kidney fibroblast were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l l-glutamine. Before transfection, 2 × 10^6 cells were seeded in 10 cm dishes (Nunc, Naperville, IL, USA). Serum E2 and progesterone were measured by chemiluminescent immunoassay (Immulyze; DPC, Los Angeles, CA, USA). The diameter of follicles at the ovulatory phase was measured with transvaginal ultrasonography after the LH surge.

Analysis of signal transduction
Forty-eight hours after transfection, cells were washed twice with PBS, harvested from culture dishes, and centrifuged at 400 g for 5 min. Cell pellets were then resuspended in DMEM/F12 supplemented with 0.1% bovine serum albumin (BSA). 2 × 10^6 cells in 300 µl were placed on 24-well tissue culture plates (Iwaki Glass, Tokyo, Japan) and preincubated at 37°C for 1 h in the presence of 0.25 mmol/l 3-isobutyl-1-methyl xanthine (IBMX; Sigma Chemical Co., St Louis, MO, USA) before treatment with or without 100 mIU/ml rFSH. Data are shown as mean ± SE.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NN (n = 84)</th>
<th>NS (n = 106)</th>
<th>SS (n = 27)</th>
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<td>FSH (mIU/ml)</td>
<td>9.9 ± 0.7</td>
<td>8.9 ± 0.4a</td>
<td>13.0 ± 2.8a</td>
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Data are shown as mean ± SE. In the SS group, a significantly higher basal level of serum FSH was observed as compared with that in the NS group.

Ligand binding studies
Aliquots of cells used for cAMP measurement were also used for the estimation of cell surface receptor binding as described earlier (Kudo et al., 1994). Cells expressing the allelic variants of the FSH receptor were incubated with 100 000 c.p.m. of [125I]FSH and increasing concentrations of rhFSH (1–1000 mIU/ml) to calculate Kd values based on Scatchard plot analysis. Data from each plate were corrected for the sum of IP1, IP2 and IP3, which were measured by [3H]myo-inositol incorporation.

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| RFLP analysis revealed the complete linkage between 307 and 680 amino acid transition, i.e. there are three genotypes, TN/TN (NN), TN/AS (NS), and AS/AS (SS). Thus, 522 Japanese patients were classified into these three genotype groups and evaluated for association with gynaecological diseases. The overall frequencies of NN, NS and SS were 41.0, 46.9 and 12.1% respectively (Table I). In the spontaneous ovulation group, frequencies were 43.5, 43.5 and 13.0% respectively. When the frequency of the different genotypes in each disease, i.e. hypothalamic primary amenorrhoea, secondary amenorrhoea, and POF, was compared with that of the population of the spontaneous ovulation group, no association of genotypes with gynaecological diseases was observed.

Results
Frequency of the allelic variants of the FSH receptor gene
RFLP analysis revealed the complete linkage between 307 and 680 amino acid transition, i.e. there are three genotypes, TN/TN (NN), TN/AS (NS), and AS/AS (SS). Thus, 522 Japanese patients were classified into these three genotype groups and evaluated for association with gynaecological diseases. The overall frequencies of NN, NS and SS were 41.0, 46.9 and 12.1% respectively (Table I). In the spontaneous ovulation group, frequencies were 43.5, 43.5 and 13.0% respectively.

When the frequency of the different genotypes in each disease, i.e. hypothalamic primary amenorrhoea, secondary amenorrhoea, and POF, was compared with that of the population of the spontaneous ovulation group, no association of genotypes with gynaecological diseases was observed. In terms of PCO, however, the NS population was significantly larger as compared with that in the spontaneous ovulation group (66.7 versus 43.5%, P < 0.05).
FSH receptor polymorphism and endocrinological profile in patients

Since FSH receptor activity may change the serum FSH level, serum FSH was measured before and after GnRH administration. In the SS group, a significantly higher basal level (13.0 ± 2.8 mIU/ml) of serum FSH was observed as compared with the NS group (8.9 ± 0.4 mIU/ml) (mean ± SE; P < 0.05) (Table II). The basal level of serum FSH in the SS group was also higher than that in the NN group; however, this difference was not significant. The fold increases in levels of serum FSH over basal FSH at 15, 30 and 60 min after GnRH administration in each group showed no significant differences (data not shown).

We further examined the relationship of FSH receptor polymorphisms with endocrinological profiles in patients during their follicular and luteal phases based on serum E₂ levels, diameters of the dominant follicle, and serum progesterone levels as parameters. No association between the genotypes and these parameters was observed (data not shown).

The comparison of ovarian responses during ovarian stimulation for IVF demonstrated significant differences between patients with different FSH receptor variants (Table III). In the NS group, lower doses of hMG were required to achieve adequate follicular growth, compared with those in the SS group (P < 0.05). E₂ levels at the time of hCG administration per oocyte retrieved in NN, NS and SS groups were 212.2 ± 29.8, 232.7 ± 20.1 and 127.5 ± 14.3 (pg/ml, mean ± SE) respectively. Thus, the NN and NS groups showed significantly higher levels of serum E₂ than did the SS group (P < 0.05).

FSH-stimulated cAMP production and desensitization of the TN and AS variants

To evaluate the bioactivity of FSH receptor isoforms in vitro, FSH-stimulated cAMP production from cells transiently expressing each polymorphic receptor was measured (Figure 3). Both receptors showed dose-dependent cAMP production when stimulated with increasing doses of rhFSH. Because the FSH receptor isoforms have different C-terminal sequences likely involved in receptor desensitization, we further tested differences in their desensitization characteristics. Briefly, 1 h preincubation with or without 100 mIU/ml of rhFSH followed by 90 min incubation of increasing doses of rhFSH showed ~50–70% of suppression of cAMP production, suggesting that desensitization has occurred with both receptor isoforms. No difference, however, was observed in the degree of cAMP suppression determined by enzyme-linked immunosorbent assay.
between the two receptor isoforms. The $ED_{50}$ values of cAMP production in cells expressing each FSH receptor variant for FSH stimulation were 52.41 ± 15.58 and 49.41 ± 3.01 mIU/ml for the TN and AS isoforms respectively (mean ± SD, $n = 3$). Thus, there was no discernible difference in in-vitro bioactivities between these isoforms.

**PI turnover assay of the TN and AS variants**

Increases in IP release after FSH stimulation were observed for cells expressing both the TN and AS receptor isoforms. No significant difference in IP signalling could be found between these two receptors (data not shown).

**Ligand binding studies**

In ligand-binding experiments, the $K_d$ value of the two receptor isoforms, as calculated from displacement curve, did not differ significantly between the TN and AS receptors; the values were 0.70 and 0.71 nmol/l respectively ($n = 3$) (Figure 4).

**Discussion**

FSH is essential for normal reproductive functions. Due to the important roles of FSH on follicular growth and ovarian steroidogenesis in females and spermatogenesis in males, mutations in the FSH receptor gene could affect reproductive ability, especially in women (Conway, 1996; Gromoll et al., 1996a). Aittomaki et al. have reported six Finnish families containing two or more women with gonadal dysgenesis and primary amenorrhoea, and demonstrated that a missense mutation Ala189Val in the FSH receptor was responsible for their infertility (Aittomaki, 1994; Aittomaki et al., 1995). In contrast, an activating mutation of the FSH receptor has been described in a hypophysectomized man who maintained normal testicular volume and fertility despite a complete lack of gonadotrophins (Gromoll et al., 1996). The heterozygous amino acid substitution Ala567Gly led to ligand-independent constitutive activity of the mutant receptor.

The polymorphism at codon 680 was firstly confirmed when Aittomaki et al. identified a loss-of-function mutation of FSH receptor in ovarian dysgenesis due to Ala189Val (Aittomaki et al., 1995). Although this substitution is mentioned as ‘polymorphism’, it was not clear whether this isoform may affect the receptor function and thereby the follicular growth and steroidogenesis in the ovary. In addition, it is also not clear whether this polymorphism is associated with gynaecological diseases. Since the polymorphism is in the C-terminal domain of the receptor, which is important for the coupling to G-proteins and the intracellular phosphorylation followed by desensitization, we designed the present study to examine the frequency of these polymorphisms, analysed the association of polymorphisms with hormonal profiles and gynaecological diseases, and tested receptor function using in-vitro transfection experiments. Although no differences in the in-vitro FSH binding and cAMP responses were found for the different FSH receptor isoforms, we found 46% higher basal serum FSH levels and the requirement for higher hMG doses in ovarian stimulation of patients with the $SS$ genotype as compared with those of the $NS$ genotype.

Simoni et al. reported that the 680 codon polymorphism is linked to the 307 codon polymorphism, and resulted in two discrete types of FSH receptor, i.e. Thr307-Asn680 (TN) and Ala307-Ser680 (AS) (Simoni et al., 1999). They showed that these two receptor isoforms had similar functional characteristics. They also examined the proportion of the three genotypes, $NN$, $NS$ and $SS$ (37.2, 45.4 and 17.4% respectively) in 86 proven fathers in the European population. In infertile men, the proportion was 32.0, 48.0 and 20.0% respectively and no significant differences were observed between the normal and infertile populations. Hasbargen et al. described the same FSH receptor isoform distribution to be 22.3, 55.4 and 22.3 respectively, in the studies dealing with twinning (Hasbargen et al., 2001). Furthermore, Perez Mayorga et al. found the proportion to be 29, 45 and 26% in European women (Perez Mayorga et al., 2000). In the present study, we found that the proportion of genotypes is 43.5, 43.5 and 12.1% respectively, in ovulating Japanese women. It is suggested that the Japanese population might have a higher proportion of the $TN$ allele as compared with Caucasian populations.

Our data have shown significantly higher basal levels of serum FSH in the early follicular phase in the $SS$ group as compared with those in the $NS$ group. Although significantly higher basal levels of serum FSH were found in the early follicular phase in the $SS$ patients, other parameters showed no difference between the three groups. It has also been reported that basal FSH levels in women with ovulation are significantly different between the three genotypes (Perez Mayorga et al., 2000). In these patients,
serum FSH levels in the NS and SS groups were significantly higher compared with the NN group. In the present study, a higher basal level of serum FSH was observed in the SS group as compared with the NS group, while that of the NN group did not differ significantly from the NS or SS group. Thus in both studies, the SS group might have a tendency to show higher levels of basal FSH, reflecting the probable difference between the activities of the FSH receptor isoforms and a tuning in the feedback regulation as mentioned by Perez Mayorga et al. However, in the present study, the results for the NN and NS groups did not show a significant difference in basal FSH levels. The different results between these studies might be influenced by various factors, such as different expression and turnover of FSH receptors on the cell surface in vivo, and different inhibin B levels which could regulate the FSH secretion.

In the present study, the NS group made up a significantly larger proportion of PCO patients compared with that of spontaneously ovulating women. The possible relationship between this FSH receptor isoform and PCO pathogenesis is not yet clear. In women, there is no report in which the relationship between the polymorphism and a gynaecological disorder is known. A study aiming at the detection of FSH receptor gene mutations in patients with granulosa cell tumour also showed no correlation of this polymorphism with this type of tumour (Fuller et al., 1998). Recently, it was reported that the AS phenotype was closely associated with repeated twinning, because the AS isoform had higher sensitivity to FSH compared with TN (Al-Hendy et al., 2000). However, a subsequent investigation showed that AS is a common polymorphism not associated with spontaneous human twinning (Hasbargen et al., 2001).

Furthermore, we found that the SS group required higher doses of hMG to achieve mature follicular growth in IVF patients, as shown previously (Perez Mayorga et al., 2000). In this study, the SS group showed the lowest E2 levels at the time of hCG injection per oocyte retrieved after hMG administration. If the receptor function is impaired due to the polymorphism, it is likely that serum FSH levels would be elevated to maintain normal ovarian function. This might be the reason why E2 levels at the time of ovulation demonstrated no difference between the three genotypes in the spontaneous cycles.

In the clinical data analyses, it has been suggested that the FSH receptor function is different according to the allelic variants. We measured as an endpoint of receptor function, however, no functional difference was observed between these two receptors in 293T cells by transfection. When cAMP was stimulating cycle, but also for prediction of ovarian hyperstimulation syndrome. However, we could not identify major differences in signal transduction of overexpressed FSH receptor isoforms in transfected human kidney cell line. It is possible that we underestimated some functional differences between these receptor isoforms, because these experimental conditions cannot allow the detection of subtle functional changes in receptor isoforms. Furthermore, the use of a kidney cell line is not definitely parallel to the natural situation of an FSH target cell, like the Sertoli or granulosa cells, where the impact of the receptor isoforms on ligand binding and signal transduction parameters might be much more pronounced.

Further study will be necessary to elucidate the relationship of this polymorphism with gynaecological diseases and the functional differences between the receptors.

References


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