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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 as compared with the *NS* group ($P < 0.05$). At the time of hCG administration, estradiol levels per oocyte retrieved for IVF in the *SS* group were significantly lower as compared with the levels in the *NS* and *NN* groups ($P < 0.05$). 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 adenylate cyclase and phosphorylation of specific proteins (Hsueh *et al*., 1989; Johnson and Dhanasekaran, 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 ^{125}I-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

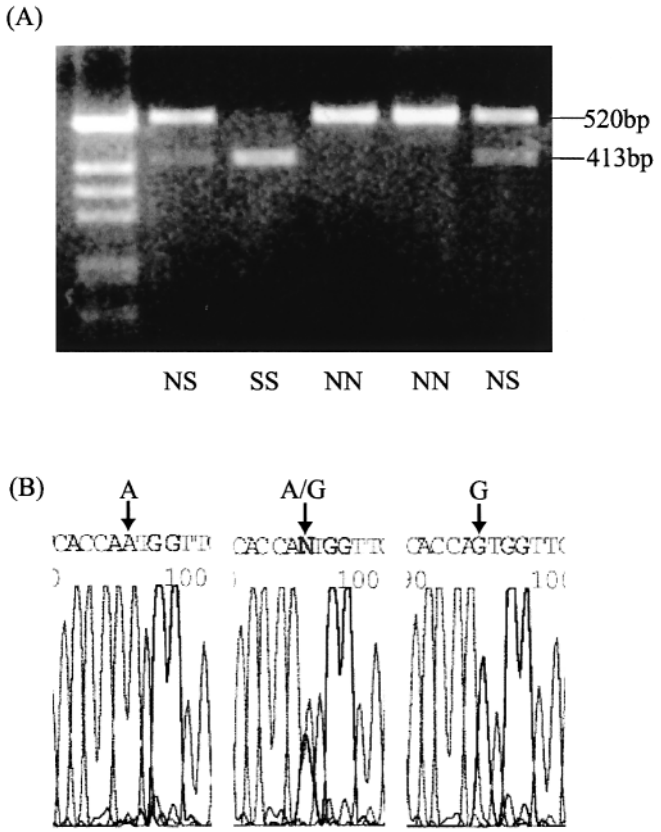

Figure 1. (**A**) Restriction fragment length polymorphism (RFLP) analysis of the Asn680Ser FSH receptor variant. Agarose gel (2.5%) electrophoresis with ethidium bromide staining following *BsrI* digestion of the PCR product is shown. A 520 bp band for *NN*, two bands, 520 and 413 bp, for *NS*, and a 413 bp band for *SS*, are shown. Note that a 107 bp band has run off from the gel. (**B**) DNA sequencing of each variant. PCR products were directly sequenced and points of the nucleotide substitution are indicated by arrows.

University Hospital, and informed consent was obtained. Among them, 168 had conceived spontaneously, or had spontaneous ovulation confirmed by ultrasonography, hormonal examination and/or basal body temperature. Ninety-six of them suffered from amenorrhoea, including hypothalamic primary amenorrhoea, secondary amenorrhoea, hyperprolactinaemia, polycystic ovary (PCO) and premature ovarian failure (POF). The remaining 258 women had regular menstrual cycles without the diseases described above. Patients from this group had not only regular menstrual periods but also ovulatory cycles. All patients with POF had normal 46,XX karyotypes.

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′-TTTGTGGTCATCTGTGGCTGC-3′; and primer-2: 5′-CAAAGGCAAGGACTGAATTATCATT-3′) which amplified the DNA fragment of 520 bp in size. Since the A to G transition creates an endonuclease *BsrI* recognition site, the PCR fragment following *BsrI* digestion and 2.5% agarose gel electrophoresis reveals three different patterns. Based on this RFLP analysis, patients were classified into three groups, *NN* (680Asn/Asn), *NS* (680Asn/Ser) and *SS* (680Ser/Ser) (Figure 1A). Note that the small 107 bp band has run out from the gel. Selected samples were sequenced and their sequence identities were confirmed (Figure 1B).

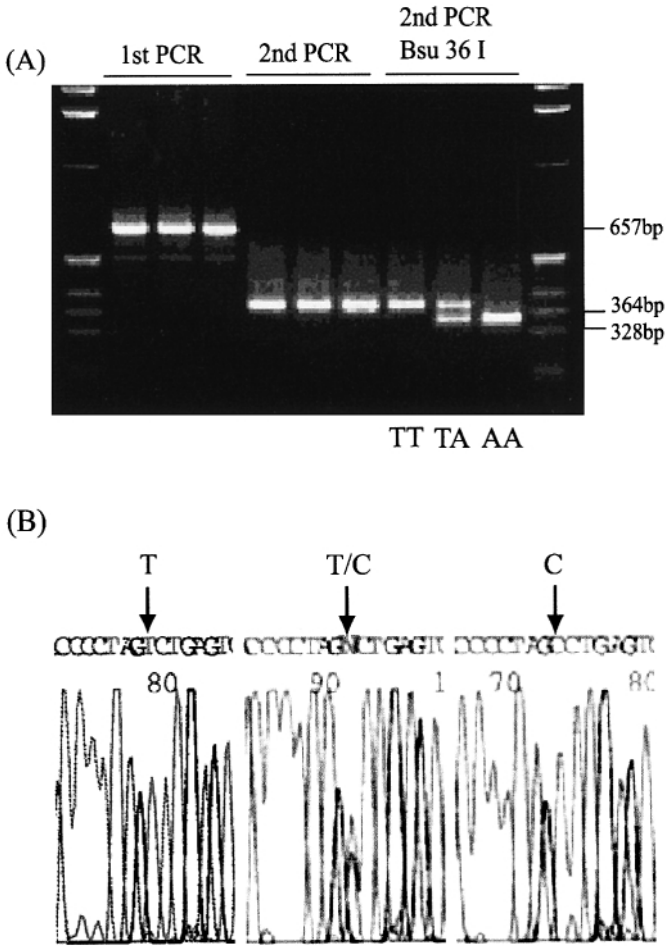

Figure 2. (**A**) Restriction fragment length polymorphism (RFLP) analysis of the Thr307Ala variant. To introduce a *Bsu36I* restriction site for RFLP analysis, the first PCR products (657 bp) were subjected to amplification using a pair of nested primers, one of which had a mismatch sequence. For the 307Ala variant, this mismatch created a new *Bsu36I* restriction site in the second PCR products (364 bp), allowing the detection of polymorphic receptor genotypes. Agarose gel (2.0%) electrophoresis with ethidium bromide staining following *Bsu36I* digestion of PCR product is shown. A 364 bp band for *TT*, two bands, 364 and 328 bp, for *TA*, and a 328 bp band for *AA*, are shown. Note that a 36 bp band has run off from the gel. (**B**) DNA sequencing of each variant. Points of the nucleotide substitution are indicated by arrows. Note that the complementary strand was read in this sequencing.

RFLP analysis of the Thr307Ala variant

Detection of the Thr307Ala variant was performed by the nested PCR–RFLP method. The region of nucleotide number 931 to 1587 in the FSH receptor gene was amplified by PCR using genomic DNA as templates and a set of primers (primer-3: 5′-TCTGAGCTTCATCCAATTTGCA-3′; and primer-4: 5′-GGGAAAGAGGGCA GCTGCAA-3′) which amplified the 657 bp DNA fragment. The PCR product was further amplified by a second PCR using a new set of primers (primer-5: 5′-CAAATCTATTTTAAGGCAAGAAGTTG-ATTATATGCCTCAG-3′; and primer-6: 5′-GTAGATTCCAATGCAGAGA-TCA-3′). In primer-5, a mismatch nucleotide has been induced (indicated by the underline). This mismatch and the A to G transition creates a *Bsu36I* restriction site, so that the second PCR fragment following *Bsu36I* digestion and 2.5% agarose gel electrophoresis reveals three different patterns. Based on this RFLP analysis, patients were classified into three groups, namely, *TT* (307Thr/Thr), *TA* (307Thr/Ala) and *AA* (307Ala/Ala) (Figure 2A). Some selected samples were sequenced and their sequence identities were confirmed (Figure 2B).

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 (E_2) levels and the diameter of a dominant follicle just before ovulation, together with serum levels of E_2 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 oocytes retrieved and E_2 level at the time of hCG administration, were compared between these three genotype groups. Poor responder and POF patients were excluded from this analysis (Surrey and Schoolcraft, 2000).

Serum FSH and LH were assayed by enzyme-linked immunosorbent assay (ELISA; AIA-600 II, Tohso, Tokyo, Japan). Serum E_2 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.

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). When cells were 70–80% confluent, the medium was replaced with DMEM/F12, and transient transfection was performed using 10 μg of plasmid harbouring each cDNA using the lipofection method (Invitrogen). After 12–16 h of incubation with Opti-MEM I (Invitrogen), cells were washed with Dulbecco's phosphate-buffered saline (PBS). To correct for transfection efficiency, 0.5 μg of RSV-beta-gal plasmid was routinely included in the transfection mixture, and β-galactosidase activity in cell lysate was measured.

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 recombinant human FSH (rhFSH: Org 32489). Medium was then removed and each well was washed once with PBS. Medium containing different amounts of rhFSH (1–1000 mIU/ml) and 0.25 mmol/l IBMX was added to each well to determine cAMP production in transfected cells with or without desensitized FSH receptors. Culture media were collected and measured by a cAMP ELISA kit (Amersham Pharmacia Biotrak, London, UK) (Tilly *et al*., 1992; Valove *et al*., 1994; Kudo *et al*., 1996).

Phosphatidyl inositol (PI) turnover was evaluated in 293T cells by an ion-exchange column method, following 10 IU/ml of rhFSH stimulation (Hirsch *et al*., 1996). Eight hours after transfection, cells were washed twice with DMEM containing 0.1% BSA and labelled for 30 h with 3 μCi [^{3}H]myo-inositol in DMEM/F12 medium supplemented with 5% FBS, penicillin, streptomycin, and L-glutamine. After labelling, cells were washed four times with DMEM supplemented with 0.1% BSA to remove serum and unincorporated [^{3}H]myo-inositol. Aliquots of 4×10^5 cells per tube were preincubated at 37°C in a shaking water bath with 10 mmol/l LiCl added into the incubation to prevent the dephosphorylation of IP. Cells were then incubated for 30 min at 37°C with or without gonadotrophin before termination of the assay by the addition of 500 μl ice-cold 20% trichloroacetic acid. IP were extracted and separated on ion exchange columns. Inositol monophosphate (IP_1), inositol diphosphate (IP_2) and inositol triphosphate (IP_3) were eluted with 1 mol/l ammonium formate/0.1 mol/l formic acid. Radioactivity for the sum of IP_1, IP_2 and IP_3 was measured by β-counting.

Table I. The frequency of gene polymorphisms in 522 patients screened and association with gynaecological disorders

	NN	*NS*	*SS*
Overall	214 (41.0)	245 (46.9)	63 (12.1)
Spontaneous ovulation (+)	73 (43.5)	73 (43.5)[a]	22 (13.0)
Spontaneous ovulation (–)			
Hypothalamic primary amenorrhoea	9 (27.3)	20 (60.6)[a]	4 (12.1)
Secondary amenorrhoea	11 (36.7)	16 (53.3)[a]	3 (10.0)
Polycystic ovaries	3 (16.7)	12 (66.7)[a]	3 (16.7)
Premature ovarian failure	5 (29.4)	9 (52.9)	3 (17.6)

Number of patients are shown with the percentages in parentheses.
For polycystic ovaries, the *NS* population was significantly larger as compared with that in the spontaneous ovulation group (66.7 versus 43.5%, $P < 0.05$).
[a]$P < 0.05$.

Table II. Association between FSH receptor gene polymorphisms and basal FSH levels in patients

Genotype	*NN* (n = 84)	*NS* (n = 106)	*SS* (n = 27)
FSH (mIU/ml)	9.9 ± 0.7	8.9 ± 0.4[a]	13.0 ± 2.8[a]

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.
[a]$P < 0.05$.

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*., 1996). Cells expressing the allelic variants of the FSH receptor were incubated with 100 000 c.p.m. of [^{125}I]FSH and increasing concentrations of rhFSH (1–1000 mIU/ml) to calculate K_d values based on Scatchard plot analysis. Non-specific binding was determined in the presence of excess amount of rhFSH (10 IU).

Statistical analysis

Statistical analysis between the groups was performed by Mann–Whitney test and Fisher's exact test. $P < 0.05$ was considered significant.

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%, $P < 0.05$).

Table III. Comparison of clinical parameters of ovarian responses between patients with different FSH receptor gene polymorphisms during ovulation induction by hMG administration for IVF

	NN (n = 19)	*NS* (n = 28)	*SS* (n = 11)
Age (years)	32.9 ± 0.4	31.3 ± 0.6	31.3 ± 1.3
hMG dose (IU)	1890.79 ± 139.0	1757.1 ± 117.0[a]	2195.5 ± 210.5[a]
No. of oocytes retrieved	7.5 ± 0.9	9.6 ± 0.9	9.8 ± 1.5
Estradiol level at the time of hCG administration (pg/ml)	1480.2 ± 190.0	2202.1 ± 334.9	1143.0 ± 194.6
Estradiol level at the time of hCG administration per oocyte (pg/ml)	212.2 ± 29.8[b]	232.7 ± 20.1[c]	127.5 ± 14.3[b,c]

Data are shown as mean ± SE.
In the *SS* group, higher doses of human menopausal gonadotrophin (hMG) were required to achieve mature follicular growth, compared with that in the *NS* group ($P < 0.05$). *NN* and *NS* groups had significantly higher levels of serum estradiol than did the *SS* group ($P < 0.05$).
[a,b,c]$P < 0.05$.

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 E_2 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 ($P < 0.05$). E_2 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 E_2 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

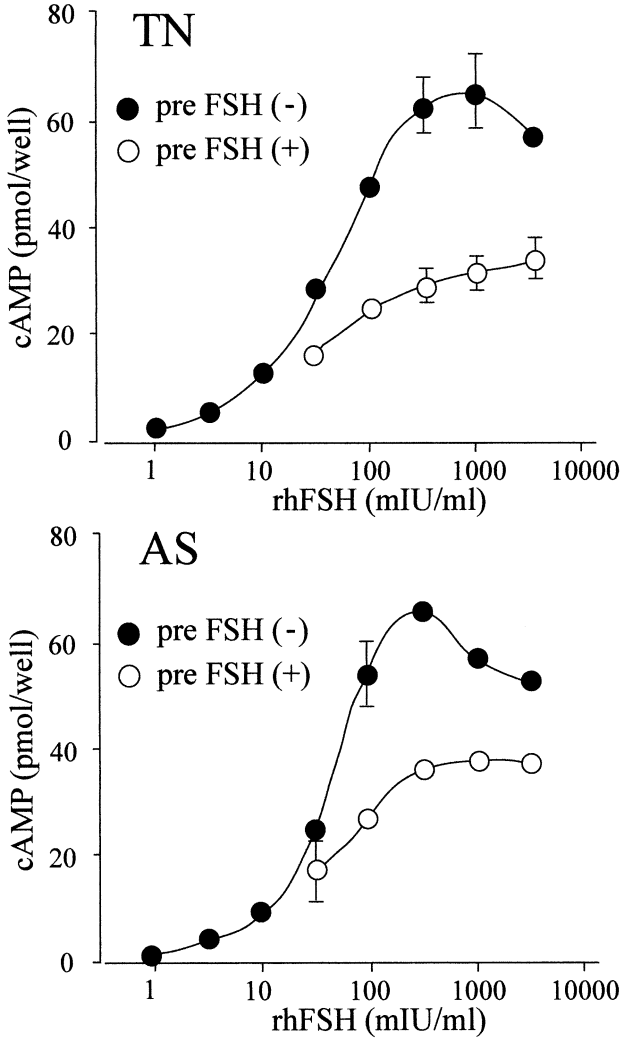

Figure 3. FSH-stimulated cAMP production in 293T cells transiently expressing TN or AS isoforms of cAMP production following increasing concentrations of rhFSH stimulation was determined and shown as mean ± SE. Cells overexpressing each receptor isoform were preincubated with (open circle) or without (closed circle) 100 mIU/ml of rhFSH for 60 min. After washing the cells, increasing doses of rhFSH were added and incubated for a further 90 min. cAMP production in the media was determined by enzyme-linked immunosorbent assay.

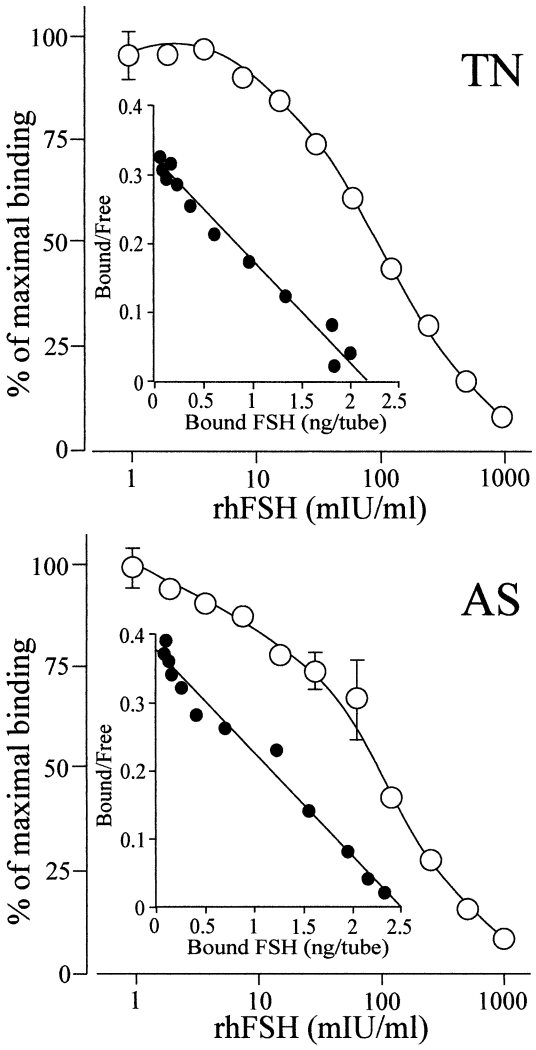

Figure 4. Ligand-binding studies. Cells overexpressing each FSH receptor isoform (the same preparations used for cAMP determination) were incubated with increasing amounts of rhFSH with 100 000 c.p.m. of [^{125}I]FSH at room temperature for 16 h. Non-specific binding was determined using excess amount (10 IU) of rhFSH. The displacement curve and Scatchard plot are shown. In the displacement curve, data are shown as mean ± SE. The K_d values of the TN and AS isoforms were 0.70 and 0.71 nmol/l respectively.

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 E_2 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 E_2 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 speculated that the possible mechanism is a difference of FSH receptor function, i.e. binding activity with FSH, capacity of coupling with G protein, cAMP production, and PI turnover. Therefore, we performed an in-vitro expression study of each receptor protein in 293T cells by transfection. When cAMP was measured as an endpoint of receptor function, however, no functional difference was observed between the two receptors. Our findings are consistent with an earlier report (Simoni *et al*., 1999). Hirsch *et al*. revealed that the TN type of FSH receptor could show a slight increase in IP production during FSH stimulation (Hirsch *et al*., 1996). We tested for differences in IP production between the TN and AS receptors during FSH stimulation. However, there was no difference between these two receptors in PI turnover. It is thought that PI turnover could not explain the clinical differences in ovarian response to hMG administration.

In this study, we have reported the frequency of polymorphisms at residues 307 and 680 in Japanese women, and have indicated the differences in some clinical parameters. Clinically, the difference in ovarian response to hMG among these polymorphisms could be used not only for determination of hMG dose in an ovarian 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.

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