Preliminary investigation of follistatin gene mutations in women with polycystic ovary syndrome

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Strong evidence for a link between the follistatin gene and polycystic ovary syndrome (PCOS) has recently been found in a well-designed large-scale study. Follistatin binds to activin and affects its functions, e.g. stimulation of FSH synthesis and secretion. Thus, it may play a role in the functional impairment of the FSH–granulosa cell axis in PCOS. In this study, we screened 64 Chinese patients with PCOS for mutations in the entire coding region (including the region encoding alternative carboxy-terminals) of the follistatin gene using polymerase chain reaction (PCR)-based single-stranded conformational polymorphism (SSCP) and DNA sequencing. However, we could not identify a single mutation of either the activating or inhibiting type, using these techniques. Therefore, it would appear that PCOS in the local Chinese population is not caused by mutations in the coding regions of the follistatin gene.

Key words: follistatin gene/mutation/polycystic ovary syndrome/single-stranded conformational polymorphism

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder of premenopausal women, characterized by the presence of eight or more subcapsular follicular cysts of ≤10 mm in size and increased ovarian stroma (Polson et al., 1988; Franks, 1995). It is a leading cause of female infertility and is associated with androgen excess, hirsutism, obesity and increased risk for developing non-insulin-dependent diabetes mellitus (Dahlgren et al., 1992; Dunaif, 1999). Its prevalence may be up to 10% in women of reproductive age (Waterworth et al., 1997). It is highly prevalent within families, suggesting a genetic basis (Cooper et al., 1968; Carey et al., 1993; Legro et al., 1998), but its precise mode of inheritance and molecular basis remain unclear.

Several genes involved in the biosynthesis of androgen, and action of insulin and gonadotrophin have been examined as candidate genes for PCOS (Moller and Flier, 1988; Carey et al., 1994; Conway et al., 1994; Franks, 1995; Talbot et al., 1996; Gharani et al., 1997; Waterworth et al., 1997; Dunaif et al., 1999). These genes include those for the cholesterol side-chain cleavage enzyme (CYP11A), 17α-hydroxylase/17,20-lyase (CYP17), insulin, insulin receptor, and LH. Two of them, CYP11A and the insulin gene, variable number of tandem repeats (VNTR), have been proposed as predisposing genetic factors contributing to PCOS. However, neither of them has been widely accepted as a major cause for this syndrome.

Recently, in a well-designed large-scale study, Urbanek et al. (1999) tested for linkage and association between 37 candidate genes including those previously studied and PCOS. These genes were carefully selected from those involved in the action of androgen, gonadotrophin and insulin, and also the regulation of obesity and energy. They found evidence for linkage only with the CYP11A and follistatin genes. However, only the linkage with follistatin gene remained significant after correction for multiple testing. Nevertheless, this study apparently confirmed previous findings (Gharani et al., 1997) which demonstrated the linkage of CYP11A gene with PCOS.

Follistatin is a single-chain glycosylated polypeptide that can bind to activin with high affinity and neutralize its biological action of stimulating the secretion of FSH and increasing FSHβ mRNA levels (Carroll et al., 1989; Rivier and Vale, 1991; Shimonaka et al., 1991) and may, therefore, arrest folliculogenesis. Indeed, over-expression of follistatin in the transgenic mice resulted in the suppression of both serum concentrations of FSH and ovarian folliculogenesis (Gou et al., 1998), similar to the clinical features commonly found in PCOS patients.

Follistatin is expressed in numerous tissues including the ovary, pituitary, adrenal cortex, and pancreas (Meunier et al., 1988; Shimasaki et al., 1989; Gospodarowicz and Lau, 1989; Kogawa et al., 1991; Kaiser et al., 1992; Ogawa et al., 1993). The human cDNAs encoding follistatin have been cloned (Shimasaki et al., 1988). There is a single follistatin gene that can generate two mature mRNA transcripts by alternative splicing, thus encoding proteins of 315 (FS-315) and 288 (FS-288) amino acid residues respectively (Shimasaki et al., 1988). The FS-288, a carboxy-truncated variant with increased biological potency (Inouye et al., 1991; Michel et al., 1993), was found to bind strongly to heparin sulphate proteoglycans of the cell membrane, whereas FS-315 had little or no such binding affinity. Furthermore, in the anterior pituitary cells, FS-288 was more potent in suppressing FSH release (Sugino et al., 1993). This cell-associated protein was also found to
The body mass index (BMI = weight/height2) of these 27 patients was 28.62 ± 6.67 (mean ± SD, range 16.48–39.86).

In this study, we screened the follistatin gene in PCOS patients in order to investigate the susceptibility of the follistatin gene to potentially pathogenic mutations.

Materials and methods

Patients

Chinese patients with PCOS (n = 64) were recruited for this study. Their ages ranged from 19 to 38 years (25.6 ± 6.7 years; mean ± SD). The diagnosis of PCOS was based on the clinical features and laboratory examination. They had irregular menses (≤21 or >34 days), hirsutism, acne and obesity, together with multiple ovarian cysts detected by ultrasound. A fasting blood sample for serum FSH, LH, oestradiol, testosterone and progesterone measurements was obtained at 08:00 for all PCOS patients (Table I).

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from peripheral blood using standard procedures. Specific primers for PCR amplification of the exons are given in Table II. The PCR amplification was carried out in a total volume of 20 μl reaction mixture containing 1.5 mmol/l of MgCl2, 0.2 mmol/l of each dNTP, 50–200 ng of genomic DNA, 10 pmol of each primer, and 1 U of Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD, USA). The cycling profile consisted of denaturation at 94°C for 1 min, annealing at either 52°C or 65°C for 30 s (Table II) and extension at 72°C for 30 s, except for the first cycle when denaturation was extended to 5 min. After PCR, 5 μl of the amplified product was examined for fidelity by electrophoresis on a gel containing 2% agarose.

Single-stranded conformational polymorphism (SSCP) analysis and direct DNA sequencing

The PCR product (10 μl) was used for SSCP analysis. The SSCP was carried out as described previously (Roy et al., 1996) with some modifications: gel concentration, 8–10%; temperature, 4°C, with the presence of 5% glycerol. DNA sequencing was carried out using a DNA sequencer (ABI Prism TM 377, PE Biosystem) with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, Norwalk, Conn., USA).

Results

Patients’ clinical data

Clinical, biochemical and ultrasound parameters were measured in all the subjects studied. Of the 64 PCOS patients, 37 had only irregular menses, five had acne, two hirsutism, 12 obesity, two acne and hirsutism, one acne and obesity, three hirsutism and obesity, and two acne, hirsutism and obesity. The body mass index (BMI = weight/height2) of these 27 patients was 28.62 ± 6.67 (mean ± SD, range 16.48–39.86). The patients’ hormonal parameters are shown in Table II. Higher LH/FSH ratio (>3) was seen in most of the patients.

Screening of the follistatin gene

All the exons of the gene (Figure 1) were successfully amplified by PCR in all patients using the primers with normal sequence. Therefore, no deletion appeared to exist in any of the exons.

### Table I. Patient clinical data

<table>
<thead>
<tr>
<th>FSH (IU/L)</th>
<th>LH (IU/L)</th>
<th>Testosterone (ng/ml)</th>
<th>Oestradiol (pg/ml)</th>
<th>Progesterone (ng/ml)</th>
<th>LH/FSH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>2.37 ± 1.40</td>
<td>10.14 ± 7.98</td>
<td>0.70 ± 0.34</td>
<td>71.5 ± 36.3</td>
<td>3.86 ± 5.25</td>
</tr>
<tr>
<td>Normal range</td>
<td>1.6–6.1</td>
<td>2.0–10.5</td>
<td>0.24–0.89</td>
<td>44–153</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

*aMeasured at day 4.

*bMeasured at day 21.

### Table II. Primers used for PCR and direct DNA sequencing of the human follistatin gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
<th>Position</th>
<th>Hybridization step</th>
<th>Amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FS1: 5’-GCTCCTCGCCCGCGCTG-3’</td>
<td>-27 to -9</td>
<td>65°C, 30s</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>FS2: 5’-TCCTCAAGCAGCCTCCCA-3’</td>
<td>+21 to +40</td>
<td>52°C, 30s</td>
<td>256</td>
</tr>
<tr>
<td>2</td>
<td>FS3: 5’-CCCCCCTGAGCCCTTGTC-3’</td>
<td>-29 to -10</td>
<td>52°C, 30s</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>FS4: 5’-CTCTGAAGTTGGGAAGAAAG-3’</td>
<td>+9 to +28</td>
<td>52°C, 30s</td>
<td>278</td>
</tr>
<tr>
<td>3</td>
<td>FS5: 5’-GGTTTTATACCATGCGT-3’</td>
<td>-32 to -13</td>
<td>52°C, 30s</td>
<td>284</td>
</tr>
<tr>
<td>FS6: 5’-AGTCCTGCTCAACAGGGTAG-3’</td>
<td>+8 to +27</td>
<td>52°C, 30s</td>
<td>284</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FS7: 5’-CAGTTGTTGCTCTGCTTG-3’</td>
<td>-26 to -7</td>
<td>52°C, 30s</td>
<td>137</td>
</tr>
<tr>
<td>FS8: 5’-TTGCTCCTCTCAATCCAGAA-3’</td>
<td>+8 to +27</td>
<td>52°C, 30s</td>
<td>137</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>FS9: 5’-CTCATCACAAGGTATTATA-3’</td>
<td>-26 to -7</td>
<td>52°C, 30s</td>
<td>137</td>
</tr>
<tr>
<td>FS10: 5’-GGCGACAGGTGTTAATAACAACG-3’</td>
<td>+8 to +27</td>
<td>52°C, 30s</td>
<td>137</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>FS11: 5’-ACAACAGCTCCCTGTTATC-3’</td>
<td>-30 to -11</td>
<td>52°C, 30s</td>
<td>137</td>
</tr>
<tr>
<td>FS12: 5’-GAACACTGAAACACTTATAGA-3’</td>
<td>+8 to +27</td>
<td>52°C, 30s</td>
<td>137</td>
<td></td>
</tr>
</tbody>
</table>

In each case the exon which is amplified is indicated on the left. The sequence of each pair of primers is given in Table II. The distance of each primer relative to the intron/exon junction is indicated by negative (primer to the junction) or positive (primer located 3’ to the junction) values except the FS1 and FS12 which is 5’ and 3’ to the starting and ending amino acids respectively. The conditions of each hybridization step in PCR and the size of the amplified fragment are indicated for each pair of primers.
The size of PCR products was 137–284 bp (Table II). The PCR products were screened in duplicate for mutations by SSCP analysis. There was no mobility shift in any of the PCR products, suggesting the absence of polymorphisms/mutations in the exons. DNA sequencing was carried out randomly in four to seven samples for each amplified exon with forward and reverse primers to verify the SSCP results, and again no mutations or polymorphisms were detected.

Discussion

Follistatin is a high-affinity binding protein that modulates the bioactivity of activin in vitro and in vivo (Mather et al., 1997; Guo et al., 1998). Activin enhances FSH-induced aromatase activity (Xiao et al., 1990, 1991), LH binding site activity (Hutchinson et al., 1987), and progesterone production (Xiao et al., 1992a,b), and may play a role in preventing premature luteinization of the ovarian follicles. In the rat model, follistatin modifies FSH action on the granulosa cells as evidenced by inhibition of aromatase activity and inhibit production, while it enhances progesterone production (Xiao et al., 1991). It reverses the enhancing effect of activin on the FSH-stimulated steroidogenesis and inhibit production, and inhibits the activin-induced FSH receptor number (Xiao et al., 1992b) and basal inhibit production by granulosa cells (Xiao et al., 1992a).

Thus, follistatin may modulate the granulosa cell function in an autocrine/paracrine fashion through the binding and neutralization of activin action, and this is likely to favour the process of follicular luteinization or atresia. Over-expression of follistatin or an increase in its functional activity would therefore be expected to arrest follicular development, increase ovarian androgen production, and reduce circulating FSH concentrations, these are characteristic features of PCOS. Indeed, when a high amount of follistatin was introduced in the transgenic female mice, some of them became infertile and showed small ovaries due to the blockage of folliculogenesis and suppressed serum FSH concentrations (Guo et al., 1998).

In one study, however, no significant difference in the follicular concentrations of follistatin between PCOS patients and normal subjects was found (Erickson et al., 1995). In another study, follistatin mRNA signal was detected in the granulosa cells of normal follicles but not PCOS follicles, although the cytoplasmic staining of follistatin was observed in both types (Roberts et al., 1994). These observations suggest that an increase in follistatin concentrations in ovarian follicles may not play a key role in the mechanisms of selection, atresia and PCOS in women.

Nevertheless, the recent finding of a substantial evidence for linkage between the follistatin gene and PCOS (Urbanek et al., 1999) sheds some light on the genetic basis of PCOS implicating follistatin gene in the disease process. The focus of this study was therefore to screen the follistatin gene for mutations in PCOS patients for a better understanding of the pathophysiology of PCOS, and for the prevalence of mutations in the follistatin gene contributing to its development.

Primers were designed to amplify the coding regions of follistatin gene (Shimasaki et al., 1988; Figure 1), including the two alternative splicing regions and all the splicing junctions in which any nucleotide transition might result in alternative splicing, affecting relative abundance of the two isoforms of follistatin in particular. However, using the PCR–SSCP technique, we were unable to detect any mutations or polymorphisms in the entire coding regions of the follistatin gene. The SSCP technique can detect 70–90% of mutations in PCR products (Hayashi, 1991; Michaud et al., 1992), and has been successfully used in our laboratory for mutation searching in various other genes (Liao et al., 1996, 1999; Roy et al., 1996; Chen et al., 2000). Furthermore, DNA sequencing results confirmed those of SSCP. Therefore, these results suggest that mutations in the coding regions of follistatin gene are unlikely to be a common cause of PCOS in the women studied. Unfortunately, the regulation region of the gene could not be studied because the sequence is still unknown.

In conclusion, we were unable to detect any mutation of the activating or inhibiting type in the entire coding region of follistatin gene in 64 patients with PCOS. Therefore, mutations in the coding regions of the follistatin gene may not be a common cause of PCOS in the population studied. However, it is possible that mutations may reside in the regulation region of the gene, which should be screened once its sequence is known. Furthermore, it would be of great interest to investigate the presence of mutations in PCOS patients in other ethnic populations, especially of European origin, as it was in this population that the linkage between the follistatin gene and PCOS was established (Urbanek et al., 1999).

References


