SNP rs2470152 in CYP19 is correlated to aromatase activity in Chinese polycystic ovary syndrome patients

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Abstract. CYP19 encodes aromatase, a key enzyme essential for estrogen biosynthesis. Single nucleotide polymorphism (SNP) rs2470152 in CYP19 is associated with serum estradiol (E2) level and the E2/T (estradiol/testosterone) ratio. A case-control study including 661 individuals [364 polycystic ovary syndrome (PCOS) patients and 297 controls] was conducted to assess the association of SNP rs2470152 with PCOS. The subjects were genotyped using the polymerase chain reaction-restriction fragment length polymorphism method. Hormone levels were analyzed among various genotypes. The genotypic distributions of rs2470152 did not differ in PCOS patients when compared to the controls. However, differences in the E2/T ratio were detected, exhibiting a lower ratio in the heterozygous TC genotype in PCOS patients (p=0.0036) and controls (p=0.000). Testosterone levels also differed between the three genotypes of PCOS patients (p=0.00625), with a higher level in the TC genotype. Therefore, rs2470152 in CYP19 was not a major etiological factor for PCOS; however, the heterozygous TC genotype may inhibit aromatase activity, resulting in hyperandrogenism, particularly in PCOS patients.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most controversial entities in gynecological endocrinology, with a prevalence of 7.4% in women of reproductive age in China (1). PCOS is characterized by the presence of hyperandrogenism, menstrual irregularities and, in a significant portion of patients, insulin resistance (2-4). The familial aggregation of PCOS, hyperandrogenism and associated metabolic abnormalities indicates a genetic origin for PCOS (5). Heterogeneous clinical manifestations influenced by ethnic factors indicate the involvement of environmental factors (6). To date, dozens of genes have been studied for association with PCOS, mostly using ‘case-control study’ and ‘linkage analysis’ methods. Genes investigated are mainly related to the regulation of androgen biosynthesis and function, insulin resistance and chronic inflammation, including CYP19 (7,8), CYP11A1 (9), CYP17 (10), HSD17B6 (11), the insulin gene (12) and TCF7L2 (13); however, currently no single gene has been universally accepted as the fundamental cause of PCOS, partially due to the fact that a globally accepted diagnostic scheme for PCOS is not available.

The enzyme complex aromatase is responsible for the conversion of C19 steroids (androgens) into C18 steroids (estrogens). This enzyme complex is composed of cytochrome P450 aromatase and NADPH cytochrome P450 reductase30. Aromatase is encoded by CYP19 located at 15q21.1 (14). As a key enzyme in the conversion of androgens to estrogens, aromatase may play a potential role in the development of hyperandrogenism. Aromatase deficiency has been reported in a number of hyperandrogenic patients (15). Recently, single nucleotide polymorphism (SNP) rs2414096 in an intron of CYP19 has been reported to be associated with susceptibility to PCOS in Chinese women, and the rs2414096 A allele may be associated with the activity of aromatase (8). These observations indicate that altered regulation of aromatase may be involved in PCOS.

*Contributed equally

Abbreviations: PCOS, polycystic ovary syndrome; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; BMI, body mass index; AAM, age at menarche; T, testosterone; P, progesterone; RIA, radioimmunity assay; PCR, polymerase chain reaction; PCO, polycystic ovarian; GOOD, Gothenburg Osteoporosis and Obesity Determinants; MrOS, Osteoporotic Fractures in Men

Key words: polycystic ovary syndrome, CYP19, single nucleotide polymorphism, rs2470152, aromatase activity
SNP rs2470152 has been reported to be associated with serum estrogen levels and the E2/T (estradiol/testosterone) ratio (16,17). In this study, we investigated whether SNP rs2470152 is associated with susceptibility to PCOS by designing a case-control experiment, including 364 PCOS patients and 297 non-PCOS individuals as controls.

Materials and methods

Subjects. A total of 661 Han Chinese women were included in our study. Among them, 364 were patients with PCOS and 297 were non-PCOS controls. The PCOS patients were diagnosed based on the 2003 Rotterdam Criteria. Controls had normal ovulatory menstrual cycles without hirsutism and other manifestations of hyperandrogenism. None of the controls exhibited obesity or insulin resistance. Peripheral blood samples were collected in Nanjing Drum Tower Hospital, the Affiliated Hospital of Medical School of Nanjing University or at the Department of Obstetrics and Gynecology, Anhui Medical University, between 2004 and 2010. Serum steroid levels of 275 PCOS patients and 235 control women were available for analysis. The study was approved by the Medical School of Nanjing University, and informed consent was obtained from the women prior to inclusion.

PCOS diagnostic criteria and hormone measurements. Patients with PCOS were diagnosed according to the 2003 Rotterdam Criteria (18) (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004), as previously described (19).

Clinical variables, such as body weight and height, were assessed and body mass index (BMI) was calculated as body weight in kilograms divided by the square of the height in meters. Age at menarche (AAM) was obtained through inquiry. Venous blood samples were collected during the 3rd to the 5th day of the menstrual cycle for those who had menstruation and at any time for those who had amenorrhea. Samples were collected between 08:00 and 09:00 after a 12-h overnight fast. Blood samples were immediately centrifuged and serum was separated and frozen at -80˚C until assayed. None of the study participants had been receiving oral contraceptives or drug therapy for the 3 months prior to hormone measurement. Levels of total testosterone (T), estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) in the sera were detected by RIA (Beijing North Institute of Biological Technology of China and the CIS Company of France). Intra- and inter-assay coefficients of variation were <10% for all the assays.

Genotype analysis of polymorphisms. Genomic DNA was extracted from human peripheral blood samples using an SBS UltraPure™ Genome DNA kit (SBS Genetech, Shanghai, China) and stored at -20˚C. All subjects were genotyped using the polymerase chain reaction-restriction fragment length polymorphism method. PCR primers for the fragment containing the SNP were forward 5'-CTG CCT TTG AGG AGC TTA CTG T-3' and reverse 5'-CTT CTC TGG CTT TCC CCT CT-3'. PCR amplification was conducted in a total volume of 25 µl containing 50 ng of genomic DNA, 6.25 pmol of each primer, 2.5 µl short tandem repeat (STR) 10X buffer (STR 10X buffer; Promega, Madison, WI, USA) and 0.75 units of GoTaq DNA polymerase (Promega). The PCR was performed with a TaKaRa PCR Thermal Cycler Dice (TP600; Takara Bio Inc., Japan). The PCR conditions were as follows: 96˚C denaturing for 2 min, followed by 30 cycles consisting of 1 min of denaturation at 94˚C, 1 min of annealing at 60˚C and 1 min of extension at 72˚C; and ending with a single extension of 15 min at 72˚C. The procedure generated a fragment of 278 bp, which was then digested with HpyCH4IV at 37˚C for 16 h. A single 278-bp band corresponds to the wild-type T homozygote; bands of 278, 179 and 99 bp stand for the TC heterozygotes; and 179 and 99 bp for the C homozygote. The DNA fragments were separated by electrophoresis on a 2.2% agarose gel and visualized by staining with ethidium bromide. Digested products (20 µl) were mixed with 4 µl STR 6X loading solution (Promega) and then run for 32 min at 100 V.

Statistical analysis. Frequencies of the three genotypes among PCOS patients and controls were obtained by direct enumeration based on the electrophoresis results. The results of serum hormone levels, age, AAM and BMI were reported as the means ± SD. Between PCOS patients and controls, age and BMI were compared using one way analysis of variance (ANOVA) and serum hormones were assessed using analysis of covariance to correct for age and BMI. Genotypic distributions among individuals with different genotypes were assessed by using ANOVA. The Tukey test was used for further analysis of the differences among the three genotypes. Hardy-Weinberg distribution of genotypes in the PCOS and control groups was assessed using the χ² test. p<0.05 was considered statistically significant. Analyses were performed using SPSS version 17.0.

Results

Marked differences were revealed between PCOS patients and controls, some of which were consistent with the PCOS diagnostic criteria (Table I). The overall CYP19 rs2470152 genotypic distributions in all of the subjects were 0.212 for TT, 0.514 for TC and 0.274 for CC. The genotypic distributions (TT, TC and CC) in women with PCOS (0.228, 0.484 and 0.288, respectively) did not differ from that of the controls (0.192, 0.552 and 0.256, respectively) (p=0.208) (Table II). The rs2470152 T allele frequency in PCOS patients (0.470) was similar to that in controls (0.468) (p=0.949), as was the allele C. The genotypic distributions were in agreement with Hardy-Weinberg equilibrium.

Data analysis. The E2/T ratios were significantly different between the three genotypes in PCOS patients and controls (p=0.01036 and p=0.0000, respectively) (Table III). Further analysis (Turkey-test) revealed statistical differences (p<0.05) between the two groups among the various genotypes (Fig. 1A). A significant difference was also found in the testosterone level between the CC and TC genotypes in PCOS patients (Fig. 1B). There were no statistical differences in BMI, AAM or levels of other serum hormones, such as LH, FSH and E2 (Table III), among the three genotypes of CYP19 rs2470152 in PCOS patients and controls.
Discussion

The CYP19 localized at chromosome 15q21.1 encodes aromatase, which is a key enzyme for estrogen biosynthesis (20). CYP19 variations may affect steriodogenesis in the ovary and adrenal glands. As a result, steroid hormone levels may be impacted, which in turn may affect steroid-related phenotypes. Several SNPs in CYP19 have been reported to be associated with aromatase activity. In a 684-sample study Jin et al. reported that rs2414096 in CYP19 was associated with PCOS and that the A allele was positively associated with aromatase activity (p=0.04) (8); Wang et al. conducted a large case-control study involving 1,078 samples and found that the Arg214Cys variant of CYP19 (rs700519) was associated with PCOS (p=0.004). In an additional functional study, they found that 293 embryonic kidney cells transfected with the Arg214Cys variant (minor T allele) resulted in increased conversion of androstenedione to estrogen when compared to WT (major C allele) construct (p<0.001) (21). Meanwhile, Jiang et al. carried out a 1,402-sample study and found that rs2470152 and rs2899470 were associated with aromatase activity (p=0.023 and p<0.001, respectively) (17). These studies indicate that SNPs in CYP19 may change the activity of aromatase, and thus may lead to PCOS.

Table I. Clinical and endocrine characteristics of PCOS patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>FSH (IU/l)</th>
<th>LH (IU/l)</th>
<th>LH/FSH (IU/l)</th>
<th>E2 (pg/ml)</th>
<th>T (nmol/l)</th>
<th>AAM (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>297</td>
<td>31.77±4.70</td>
<td>21.44±3.68</td>
<td>9.76±8.70</td>
<td>5.80±6.86</td>
<td>0.78±1.05</td>
<td>43.43±23.88</td>
<td>1.48±1.56</td>
<td>14.71±1.36</td>
</tr>
<tr>
<td>PCOS</td>
<td>364</td>
<td>25.93±4.78</td>
<td>22.57±3.81</td>
<td>10.20±8.66</td>
<td>17.06±7.02</td>
<td>2.50±1.07</td>
<td>51.19±24.06</td>
<td>4.14±4.38</td>
<td>14.27±1.90</td>
</tr>
</tbody>
</table>

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; AAM, age at menarche.

Table II. Frequency distribution of CYP19 rs2470152 in women with PCOS and controls.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCOS</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alleles, n (%)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>TC</td>
<td>TT</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Control</td>
<td>76 (0.256)</td>
<td>164 (0.552)</td>
<td>57 (0.192)</td>
<td>316 (0.532)</td>
<td>278 (0.468)</td>
</tr>
<tr>
<td>PCOS</td>
<td>105 (0.288)</td>
<td>176 (0.484)</td>
<td>83 (0.228)</td>
<td>386 (0.530)</td>
<td>342 (0.470)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Based on the genotype frequencies vs. control. <sup>b</sup>Based on the allele frequencies vs. control.

Table III. Anthropometric characteristics and serum hormone concentrations in different genotypic women with PCOS and controls.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control p-value</th>
<th>PCOS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>CC</td>
<td>TC</td>
</tr>
<tr>
<td>AAM (years)</td>
<td>32.35±5.12</td>
<td>31.61±4.61</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>14.92±1.85</td>
<td>14.54±1.00</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>21.35±4.30</td>
<td>21.54±4.16</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>8.50±4.66</td>
<td>8.07±2.63</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>4.96±2.62</td>
<td>5.35±3.26</td>
</tr>
<tr>
<td>T (nmol/l)</td>
<td>0.64±0.35</td>
<td>0.70±0.45</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>1.30±1.04</td>
<td>1.22±0.69</td>
</tr>
<tr>
<td>E2/T (ln)</td>
<td>40.87±18.69</td>
<td>45.40±22.66</td>
</tr>
<tr>
<td>PRL (µg/l)</td>
<td>59.10±110.6</td>
<td>16.35±8.64</td>
</tr>
</tbody>
</table>

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; AAM, age at menarche; E2/T, estradiol/testosterone ratio; PRL, prolactin.

Discussion

The CYP19 localized at chromosome 15q21.1 encodes aromatase, which is a key enzyme for estrogen biosynthesis (20). CYP19 variations may affect steriodogenesis in the ovary and adrenal glands. As a result, steroid hormone levels may be impacted, which in turn may affect steroid-related phenotypes. Several SNPs in CYP19 have been reported to be associated with aromatase activity. In a 684-sample study Jin et al reported that rs2414096 in CYP19 was associated with PCOS and that the A allele was positively associated with aromatase activity (p=0.04) (8); Wang et al conducted a large case-control study involving 1,078 samples and found that the Arg214Cys variant of CYP19 (rs700519) was associated with PCOS (p=0.004). In an additional functional study, they found that 293 embryonic kidney cells transfected with the Arg214Cys variant (minor T allele) resulted in increased conversion of androstenedione to estrogen when compared to WT (major C allele) construct (p<0.001) (21). Meanwhile, Jiang et al carried out a 1,402-sample study and found that rs2470152 and rs2899470 were associated with aromatase activity (p=0.023 and p<0.001, respectively) (17). These studies indicate that SNPs in CYP19 may change the activity of aromatase, and thus may lead to PCOS.
CYP19 rs2470152 is located in intron 2 and thus does not change the amino acid sequences of aromatase. However, variations in the introns may affect regulatory sequences in close proximity. CYP19 is composed of a 30-kb coding region and a 93-kb regulatory region, the latter containing at least 10 distinct tissue-specific promoters. The rs2470152 is located in the region of the L4 promoter (22). Since the conversion of androgens (T) to estrogens (E2) is catalyzed by aromatase, the E2/T ratio may be a marker of aromatase activity with a positive correlation. In our observation, E2/T was significantly different among the three genotypes (Fig. 1A). Notably, in PCOS patients and controls, E2/T was lower in the heterozygous TC genotype than the other two homozygous genotypes, although it was not significantly different between the TT and TC genotypes in PCOS patients (p=0.05477). This may be due to a strong linkage disequilibrium with other functional variants in exons, resulting in the change of protein sequence of aromatase, which may cause a dominant-negative effect of aromatase activity in the heterozygous genotype. Aromatase activity was lower in the heterozygous TC genotype of rs2470152, leading to hyperandrogenism in this genotype (Table III). In our findings, the SNP rs2470152 C/T polymorphisms were correlated to aromatase activity. This may be explained by the hypothesis of Sofe et al that the rs2470152 variation alters a potential binding site for the transcription factor cAMP response element binding protein, which is crucial to the aromatase expression regulation (23).

The estradiol level was not different between the three genotypes in PCOS patients and controls. These may be due to the negative feedback regulation of the hypothalamic-pituitary-ovarian axis. Testosterone levels were higher in the TC genotype than the other two genotypes in PCOS patients, which may be the result of the lower activity of aromatase in the heterozygous TC genotype mentioned above, given that the testosterone level was high enough in the PCOS patients. In the controls, testosterone levels were similar among the three genotypes. The mechanism may be accounted for by the fact that the aromatase activity was high in the CC and TC genotypes, whereas the zymolyte (testosterone) level was not high enough to sustain the conversion rate in controls, resulting in similar testosterone levels in the three genotypes. Therefore, PCOS patients with hyperandrogenism may be a model with which to study the aromatase activity among different genotypes.

In the Gothenburg Osteoporosis and Obesity Determinants (GOOD) project studying young adult men, Eriksson et al demonstrated that subjects with the CC genotype of rs2470152 in CYP19 had 13% higher E2 levels than those with the TT genotype (16). Jiang et al also found that the E2/T ratio was significantly higher in the CC genotype compared to the TT genotype of rs2470152 in Osteoporotic Fractures in Men (MrOS) patients (17). In our study, we failed to demonstrate differences in the E2 levels between the three genotypes of rs2470152, nor did we find any difference in the E2/T ratio between the CC and TT genotypes in PCOS patients and controls. Several reasons may account for the discrepancies. Firstly, the selection criteria for the subjects were different. i) Subjects in the study of Eriksson et al were of Caucasian origin, while in our study they were all Han Chinese; in addition, the allele frequencies of SNP rs2470152 were not the same between the two ethnic groups. ii) Eriksson et al chose adult young men, Jiang et al selected elderly Chinese men with MrOS and we examined young Chinese women with PCOS. The genetic and metabolic factors are different between men and women to a certain extent, as well as among people with different diseases. Secondly, other unknown SNPs in CYP19 or other related genes may have a potential effect on steroidogenesis and they could affect both testosterone and estrogen levels; the allelic frequencies of these SNPs also vary within the two ethnic groups (17).

In conclusion, to our knowledge, SNP rs2470152 in CYP19 was not a major etiological factor for PCOS, but the heterozygous TC genotype may inhibit aromatase activity and thus result in hyperandrogenism, particularly in PCOS patients.

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