A common polymorphism in the human aromatase gene alters the risk for polycystic ovary syndrome and modifies aromatase activity in vitro

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ABSTRACT: Aromatase is a key enzyme involved in estradiol and estrone biosynthesis. Given that polymorphisms of the CYP19A1 gene encoding aromatase have been correlated with plasma testosterone levels, CYP19A1 may therefore act as a genetic modifier of the hyperandrogenic phenotype of polycystic ovary syndrome (PCOS). However, no functional CYP19A1 polymorphisms that predict the risk of PCOS have been identified. We explored the role of CYP19A1 genetic variation in a large case-control study involving 1078 samples, in which five common genetic polymorphisms were scored. Human embryonic kidney 293 cells were transiently transfected with a vector encoding either the CYP19A1 wild-type (WT) allele or an Arg264Cys variant to evaluate aromatase activity. Cells were cultured with androstenedione and estrone levels were measured using a specific ELISA. The Arg264Cys variant of CYP19A1 (rs700519) is associated with PCOS (P = 0.004, corrected P = 0.02). In this functional study, when cells were cultured in varying concentrations of androstenedione (100, 400 and 500 nM), transfection with the Arg264Cys variant resulted in increased conversion of androstenedione to estrogen when compared with transfection with the WT construct (P < 0.001). Our data suggest that the common missense polymorphism rs710059 is associated with susceptibility to PCOS and that the Arg264Cys variant may increase aromatase enzymatic activity. Overall, these findings imply that aromatase plays an important role in PCOS.

Key words: PCOS / CYP19A1 / aromatase enzyme activity

Polycystic ovary syndrome (PCOS) is a common endocrine disorder with an estimated prevalence of 5–10% of the general population (Dunaif, 1997). It is a leading cause of female infertility and is associated with polycystic ovaries, hirsutism, obesity and insulin resistance. There is evidence that the hyperandrogenic phenotype of PCOS is a heritable feature of the syndrome (Chang et al., 2005). Although the syndrome’s etiology is still poorly understood, several lines of evidence suggest that PCOS is a complex, multigenic disorder with a high degree of heritability (Ehrmann, 2005).

Aromatase is a key enzyme in estradiol and estrone biosynthesis and acts through the aromatization of testosterone and androstenedione, respectively. It is responsible for maintaining the homeostatic balance between androgens and estrogens in both sexes. Rare loss-of-function mutations in the aromatase gene (CYP19A1) have been observed in cancer and some metabolic diseases (Haiman et al., 2003; Binder et al., 2005; Lin et al., 2007).

The human aromatase gene (CYP19A1) spans ~70 kb of genomic DNA and contains ten exons (Toda et al., 1990). CYP19A1 polymorphisms have been correlated with plasma testosterone levels, indicating that variations in CYP19A1 may act as genetic modifiers of the hyperandrogenic phenotype of PCOS (Xita et al., 2010). In addition, exposure to letrozole, an aromatase inhibitor, has been shown to induce PCOS in rats, which shows striking morphological similarities to human PCOS (Ansari et al., 2004). These findings suggest that CYP19A1 plays an important role in the etiology of PCOS.

Although there is a close relationship between CYP19A1 and PCOS, no functional CYP19A1 polymorphism capable of predicting susceptibility to the disorder has been identified. We therefore hypothesized...
that human genetic variation in CYP19A1 may regulate aromatase activity in vitro, which would thereby suggest that these modifications could increase the risk of PCOS. We explored the role of CYP19A1 genetic variations in a large case-control study involving 1078 individuals. We also characterized the functional activity of CYP19A1 variants associated with PCOS by measuring aromatase activity in vitro using a specific ELISA.

Materials and Methods

Subjects
A total of 374 PCOS patients [mean age (SEM): 27.3 (0.2) years] were recruited from the outpatient clinic of the Xi’an fourth hospital and the Shannxi hospital for women and children. Recruitment was based on the following revised Rotterdam diagnostic criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004): (i) oligo-ovulation and/or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism and (iii) polycystic ovaries. Women who met at least two of these criteria were defined as having PCOS. Among 374 PCOS patients, only five women had both hyperandrogenism and polycystic ovaries, while other women had anovulation and polycystic ovaries. Prior to a definitive diagnosis, other causes of hyperandrogenism or menstrual disorders were excluded. None of the patients had taken hormonal medication, including oral contraceptives, for at least 3 months prior to starting the study. Standardized initial screening was performed on a random cycle day between 09:00 and 11:00 a.m. Fasting blood withdrawal was performed on the third day of the follicular phase between 09:00 and 11:00 a.m. Serum LH, FSH, prolactin, testosterone, estradiol and progesterone levels were measured according to a standard protocol.

We recruited 704 healthy, non-diabetic female volunteers [mean age (SEM): 29.7 (0.3) years] with regular menstrual cycles as a control group. None of the controls displayed hyperandrogenism or menstrual disorders related to PCOS. All patients and controls were Han Chinese women recruited from the same area. The Fudan University Ethics Review Committee approved the study. Informed consent was obtained from all participants.

Genotyping
Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. Five single-nucleotide polymorphisms (SNPs; rs6493497, rs28892002, rs2236722, rs110046 and rs4646) were selected from the HapMap project database (http://www.hapmap.org) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) to cover a 70 kb region surrounding CYP19A1. All five SNPs were determined using Taqman allelic discrimination assays (assay-on-demand or assay-on-design). Rs6493497: Assay ID_ C_29374681_10; rs28892002: Assay ID_ C_60083234_10; rs2236722: Assay ID_ C_11595498_40; rs110046: Assay ID_ C_8234731_30; rs4646: Assay ID_ C_8234730_1; rs700519 was determined by assay-on-demand.

CYP19A1 Arg264Cys plasmid construction and 293T cell transfection
The full-length wild-type (WT) aromatase cDNA expression plasmid was purchased from Origene (Rockville, MD). This construct was used as the template for site-directed mutagenesis using circular PCR to create Arg264Cys variant constructs using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Constructs were confirmed by sequencing.

Aromatase activity can be evaluated by culturing cells with androstenedione and measuring estrone levels in the culture medium with a specific ELISA (Ohno et al., 2004). Cells were transfected as described earlier. Eighteen hours after transfection, cells were incubated with varying concentrations of androstenedione. Following 24 h of incubation, culture medium was removed from each well and transferred to a 96-well cell culture plate. The estrone concentration in each of these wells was measured using an estrone ELISA kit. The absorbance in each well was measured at 450 nm using an M5 Perkin Elmer plate reader. The results were expressed as estrone quantity per milligram total protein.

Western blot analysis of cells transfected with CYP19A1 Arg264Cys
Aromatase was quantified using 200 µl of cell lysates from the earlier-mentioned aromatase assay. A mouse anti-human aromatase monoclonal antibody (amino acids 376–390) was purchased from Serotec (Raleigh, NC). This antibody has been described elsewhere (Tumer et al., 2002). Equal amounts of total protein were run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 5% nonfat, dry milk powder in a Tris-buffered saline-T solution with 0.2% Tween 20. Membranes were then incubated overnight at 4 °C with anti-myc antibodies (4A6; Millipore, Billerica, MA) diluted 1:1000 in a blocking solution. Following the wash step, membranes were incubated for 2 h at room temperature with horse-radish peroxidase-conjugated goat anti-mouse antibody (GE Healthcare, Buckinghamshire, UK) and diluted 1:20 000 in a blocking solution. Bound secondary antibody was detected using enhanced chemiluminescent reagent (GE Healthcare) and a Chemi-Doc XRS (Bio-Rad, Hercules, CA). Luminescence was quantified using Quantity One (Bio-Rad) according to the manufacturer’s instructions. Serial dilutions of quantified, purified glutathione-S-transferase-aromatase were included to determine protein expression levels. Total protein was measured using the Bradford method with reagents from Bio-Rad.

Statistical analysis
Quantitative variables are expressed as mean ± SEM. The Hardy–Weinberg equilibrium test was performed using the χ² run on Plink (http://pngu.mgh.harvard.edu/~purcell/plink/). Genotype and allele frequencies for the case and control groups were compared using the χ² test as appropriate. Pairwise linkage disequilibrium was tested using Haploview (Barrett et al., 2005). Haplotype frequencies were estimated using SHEsis (Shi and He, 2005). Differences in haplotype frequency distribution between the PCOS and control groups were assessed using the CLUMP program. Biochemical differences between two continuous variables were estimated using the Mann–Whitney U-test or t-test as appropriate. One-way analysis of covariance (ANOVA) was used to determine associations between genotype and clinical features in the PCOS group. Clinical features of interest were chosen as dependent variables, with BMI acting as the covariate. P < 0.05 was considered statistically significant. Unless otherwise noted, statistical data were analyzed using SPSS 11.0 (SPSS, Chicago, IL).
Results

**CYP19A1** polymorphisms and risk of PCOS

The five common genetic variants were scored in 1078 individuals (374 PCOS cases, 704 healthy controls). Clinically characteristic PCOS cases and healthy controls are shown in Table I. Rs710059 was found to be strongly associated with PCOS (P = 0.004, Table II). Compared with healthy controls, PCOS patients had a lower frequency of the T allele and TT genotype. The genotypes of the four polymorphisms were in Hardy–Weinberg equilibrium in both the case and control groups (results not shown). We performed a Bonferroni correction for multiple statistical tests. The correlation found for the rs700519 allele (corrected P = 0.02) was still significant following the correction. In addition, we analyzed linkage disequilibrium for the five genetic variants (Table III) and performed haplotype analyses (Table IV). The GATGG haplotype was associated with PCOS [P = 0.025, odds ratio (OR) = 0.74. 11.8% in PCOS, 15.4% in control].

We also analyzed associations between genotype and biochemical hormone concentration in the PCOS group using ANOVA. However, we did not observe associations between polymorphisms and these clinical features.

**Functional analysis of the CYP19A1 Arg<sup>264</sup>Cys variant**

The results described earlier suggest that the CYP19A1 Arg<sup>264</sup>Cys variant can predict the risk of PCOS. To determine whether this variant had an effect on aromatase activity and secretion and/or processing of aromatase, we undertook an aromatase assay and western blot analyses. As shown in Fig. 1, cells transfected with the Arg<sup>264</sup>Cys variant (rs700519) showed increased aromatase activity when treated with 100, 400 and 500 nM concentrations of androstenedione when compared with cells transfected with the WT transcript. We also evaluated the expression of these constructs on protein expression. There was no difference in protein expression between cells transfected with the WT or variant transcripts.

Discussion

**Overview**

In this study, we investigated whether common genetic variations in **CYP19A1** can predict the risk of PCOS. We scored common genetic variations in a large case-control sample (374 PCOS cases, 704 healthy controls) and observed that the variant rs700519 (Arg<sup>264</sup>Cys) contributed to the risk of PCOS. We also found that expression of this variant changes aromatase activity in an in vitro assay. These results indicate that a common functional non-synonymous **CYP19A1** Arg<sup>264</sup>Cys variant affects aromatase enzymatic activity and ultimately alters women’s risk for PCOS.

The role of **CYP19A1** in PCOS

Aromatase is encoded by the **CYP19A1** gene and is a rate-limiting enzyme in estrogen production. In response to stimulation by LH, androstenedione can be either aromatized by the aromatase enzyme to form estrone or converted by 17-β-hydroxysteroid dehydrogenase (17-β-HSD) to form testosterone. Rare loss-of-function mutations in the **CYP19A1** gene have been identified in women with congenital aromatase deficiency. These women develop some of the features of the PCOS phenotype (Ito et al., 1993). Compared with healthy women, aromatase conversion activity is reduced in PCOS follicular fluid (Naessen et al., 2010), and granulosa cells obtained from antral follicles of PCOS patients show little aromatase activity (Magoffin, 2006).

In addition, polycystic ovaries appear in rats following exposure to letrozole, an aromatase inhibitor (Manneras et al., 2007). Fisher et al. (1998) found that targeted disruption of the **CYP19A1** gene in mice (ArKO) induced ovaries to generate numerous follicles with abundant granulosa cells. The authors also found evidence of apparently arrested antrum formation prior to ovulation. ArKO mice became infertile as a consequence of disrupted folliculogenesis and failure to ovulate. Compared with WT mice, ArKO mice showed elevated concentrations of testosterone and LH. Taken together, these findings indicate that **CYP19A1** plays an important role in the development of PCOS.

The non-synonymous, **CYP19A1** Arg<sup>264</sup>Cys variant is associated with PCOS

In a large cohort of 1078 subjects (374 PCOS cases and 704 healthy controls), we found that the non-synonymous Arg<sup>264</sup>Cys variant (rs700519) was associated with PCOS. PCOS patients showed a lower frequency of the T allele and TT homozygocity than healthy controls (allele P = 0.019; genotype P = 0.004, Table II). In addition, the GATGG haplotype was protective against PCOS (OR = 0.74). Compared with healthy controls, PCOS patients showed a lower frequency of the GATGG haplotype (P = 0.025). Because the PCOS group consisted mostly of women with anovulation and polycystic ovaries, the association we found refers to this PCOS subgroup. In addition, no association of the Arg<sup>264</sup>Cys polymorphism was found with biomedical parameters of PCOS. A number of other studies

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**Table I**  Clinical characteristics of healthy women and PCOS patients.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCOS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>704</td>
<td>374</td>
</tr>
<tr>
<td>Age (year)</td>
<td>30.7 ± 0.25</td>
<td>27.3 ± 0.21*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.5 ± 0.21</td>
<td>22.2 ± 0.19*</td>
</tr>
<tr>
<td>LH (MIU/ml)</td>
<td>2.96 ± 0.13</td>
<td>5.14 ± 0.29</td>
</tr>
<tr>
<td>FSH (MIU/ml)</td>
<td>6.13 ± 0.23</td>
<td>6.27 ± 0.23</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>16.3 ± 6.40</td>
<td>27.74 ± 1.05</td>
</tr>
<tr>
<td>P (ng)</td>
<td>1.37 ± 0.32</td>
<td>1.48 ± 0.25</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>0.35 ± 0.01</td>
<td>0.45 ± 0.31*</td>
</tr>
<tr>
<td>E₂ (pg/ml)</td>
<td>43.5 ± 2.39</td>
<td>49.7 ± 2.58</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM. BMI, body mass index.

*P < 0.001 when compared with the control group.
have focused on the genetic variants of CYP19A1 and PCOS. Urbanek et al. (1999) used linkage analysis to study 37 candidate genes (including CYP19A1), but this study found evidence for linkage only with follistatin. Soderlund et al. (2005) found no evidence of mutations in CYP19A1 in patients with PCOS after examining the distribution of the –41 variant in the ovary promoter in 25 PCOS patients and 50 controls. More recently, Nectaria Xita found that a CYP19A1 polymorphism was associated with serum testosterone concentration (Xita et al., 2010). They concluded that CYP19A1 may not be a major genetic determinant of PCOS but a genetic modifier of the phenotype. These linkage and association studies failed to find an association between CYP19A1 and PCOS, most likely because of either their low statistical power or population heterogeneity (frequency distribution of variants is different between Asians and Caucasians).

In a recent paper, Jin et al. (2009) showed that an intronic polymorphism (rs2414096) was associated with PCOS, further providing evidence that there may be a close relationship between genetic variants of CYP19A1 and PCOS. Because rs2414096 is intronic, this polymorphism may not be a direct causal factor and there may be functional variants that are in strong linkage disequilibrium and play a role in PCOS. To our knowledge, this study is the first to indicate that a functional non-synonymous variant in CYP19A1 can alter the risk for PCOS through the regulation of aromatase activity and to show that the GATGG haplotype is protective against PCOS. These findings provide evidence that CYP19A1 plays an important role in the etiology of the PCOS.

The non-synonymous CYP19A1 Arg264Cys variant shows modified aromatase enzymatic activity

We adopted Ken Ohno's method to measure aromatase activity in cells transfected with vectors encoding either the WT or Arg264Cys variant of CYP19A1 (Ohno et al., 2004). Cells expressing the Arg264Cys variant showed increased conversion of androstenedione to estrone when the concentration of androstenedione was increased from 100 to 500 nM (∼P 0.001, Fig. 1). This finding suggests that the Arg264Cys variant can increase aromatase enzymatic activity. Given
that the frequency of this variant is lower in the PCOS group (1.9% TT genotype in the PCOS group versus 3.3% TT genotype in the control group), this suggests that aromatase enzyme activity is lower in the PCOS group. This finding is consistent with Naessen’s report (Naessen et al., 2010).

Androgen biosynthesis is mediated by cytochrome P-450c17, an enzyme with 17-α-hydroxylase and 17β-lyase activities, both of which are required to form androstenedione. Androstenedione is then converted by 17β-HSD to form testosterone or is aromatized by aromatase to form estrone (Ehrmann, 2005). It has been reported that reduced aromatase activity contributes to increased testosterone levels (Naessen et al., 2010). In this study, the Arg<sup>264</sup>Cys variant was shown to increase enzymatic activity. Thus, the T allele of CYP19A1 can increase aromatase enzymatic activity, which in turn affects conversion of androstenedione. This process would contribute to a decrease in the concentration of testosterone and perhaps lower the risk of PCOS.

In a previous study, Watanabe et al. (1997) analyzed separated microsomes and found that the Arg<sup>264</sup>Cys polymorphism did not affect aromatase activity. In this study, however, we used a nonradioactive method to measure aromatase activity, which directly reflects aromatase activity (Ohno et al., 2004). Although protein expression was not changed in cells expressing the Arg<sup>264</sup>Cys polymorphism, it should be borne in mind that the Arg<sup>264</sup>Cys modification of aromatase is located near substrate recognition site 3. As inferred by sequence information of various P450s (Gotoh, 1992), it is likely that this SNP affects binding between the substrate and enzyme and results in a reduction of enzymatic activity.

**Conclusion**

This is the first report demonstrating that the CYP19A1 Arg<sup>264</sup>Cys variant is associated with the risk of PCOS. The in vitro enzyme assays presented here show that the T allele of this gene results in increased androstenedione conversion and therefore increased aromatase activity. Thus, the Arg<sup>264</sup>Cys polymorphism of CYP19A1 is associated with the pathophysiology of PCOS.

**Authors’ roles**


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