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Genetic association study from North India to analyze association of *CYP19A1* and *CYP17A1* with polycystic ovary syndrome

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Abstract

Purpose Polycystic ovary syndrome (PCOS) is a complex multifactorial endocrine disorder affecting approximately 5–10% of women of reproductive age. Affected women have menstrual disturbances due to anovulation, infertility, and hyperandrogenism. Ovarian androgen overproduction is the key physiopathologic feature of PCOS. A number of genes encoding major enzymes of the androgen metabolic pathways, such as HSD17B6, CYP19A1, CYP11A1, CYP17A1, and INSR, have been examined. Very few studies have been done in North India. There is an increasing prevalence of PCOS in women in Punjab and it is the leading cause of female infertility. In view of the strong evidence implicating the importance of CYP19A1 and CYP17A1 in androgen metabolic pathways, we investigated the association of rs700519, rs2414096, and rs743572 (− 34T>C) polymorphisms on susceptibility of developing PCOS, in North India.

Methods A total of 500 subjects (women of reproductive age) including 250 PCOS cases and 250 healthy age-matched controls were included in the present study. DNA was extracted from venous blood for all samples, and association analysis for rs2414096, rs700519, and rs743572 was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Lipid profile was done using a biochemical analyzer and body mass index (BMI) was measured for all cases. Statistical analysis was performed.

Results Significant association of − 34T>C polymorphism of CYP17A1 was found with PCOS ($p = 0.0005$). BMI was statistically different between PCOS cases and controls ($p = 0.000$). Triglycerides were high in PCOS women. Variations of CYP19A1 were not statistically significant with PCOS.

Conclusions These data suggest that − 34T>C polymorphism in CYP17A1 is associated with PCOS in North India. No polymorphism of CYP19A1 was found to be associated.

Keywords CYP17A1 · CYP19A1 · BMI · Polycystic ovary syndrome · Polymorphism · Lipid profile

Introduction

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous disorder of endocrine system among women of reproductive age, affecting approximately 5–10% women worldwide [1]. The main characteristics of PCOS are hyperandrogenism, anovulation, polycystic ovaries, hirsutism, obesity, and hyperinsulinemia [2]. In response to increased luteinizing hormone (LH), ovaries induce high secretion of androgens, leading to suppression of follicular growth and maturation [3]. In a study of 460 girls from south India, 9.13% girls satisfied the Rotterdam's criteria for PCOS [4]. Around 50% of the PCOS women are overweight or obese and mostly having abdominal fat distribution [5]. There is a high prevalence of obesity in Punjab. A study showed that 14.8% of women were overweight and 13.8% were obese. This could be contributory in the increase in the number of cases of PCOS [6].

The exact mode of inheritance of PCOS has not been firmly established yet, although some studies on familial cases of PCOS suggest it to be an autosomal dominant trait [7–9]. Both environmental and genetic factors play a role in the

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pathogenesis of PCOS, and several pathways implicated in its etiology include the metabolic or regulatory pathway of steroid hormone synthesis, regulatory pathways of gonadotropin action, the insulin-signaling pathway, and the regulation of glucose and lipid metabolism pathway [10, 11]. CYP19A1, a key enzyme essential for estrogen biosynthesis, helps in transformation of testosterone to estradiol during steroidogenesis [12, 13]. *CYP19A1* encodes aromatase enzyme and is located at 15p21.13. Studies on several genetic polymorphisms of *CYP19A1* have indicated that aromatase activity might be decreased in PCOS follicles, resulting in abnormal follicle development [14, 15]. Rare loss-of-function mutations in the *CYP19A1* have been identified in women with aromatase deficiency, and these women may develop some features of the PCOS phenotype [16, 17].

CYP17A1 encodes the key enzyme 17-α-hydroxylase/17–20 lyase (P450 17α), which is a qualitative regulator catalyzing the conversion of pregnenolone to 17-hydroxypregnenolone and progesterone to 17-hydroxyprogesterone (17-OHP), which are rate-limiting steps in androgen biosynthesis [18]. CYP17 is predominantly expressed in the adrenal gland, testicular Leydig cells, and ovarian theca cells. Increased activity of this enzyme has been hypothesized to enhance androgen biosynthesis and secretion in PCOS [19]. *CYP17A1* is located on 10q24-q25. A common polymorphism T>C at − 34 base pairs from the translation initiation point in the promoter region has been hypothesized to upregulate the expression of *CYP17A1* by increasing the transcription binding site to a Sp-1 transcription factor, which further results in increased synthesis of androgens and affecting the PCOS phenotype [20].

In view of strong evidence implicating the importance of *CYP17A1* and *CYP19A1* in androgen metabolic pathways, the present study aimed at genotyping rs700519 and rs2414096 polymorphisms of *CYP19A1* and − 34T>C (rs743572) of *CYP17A1*. To best of our knowledge, no study has been conducted to see association of these genes with PCOS in North India; therefore, this study will help in generating baseline data.

Material and methods

Subject selection

After the approval given by the ethics review board of Guru Nanak Dev University, consistent with provisions of the Declaration of Helsinki, the present retrospective case-control study was conducted from the period 2015 to 2017.

The sample size was calculated using the CaTS-Power Calculator to achieve a minimum power of 80% with 95% confidence interval. Assumptions used for calculation were one-stage sample design at 5% significance level ($\alpha = 0.05$), taking worldwide prevalence of 10% and odds ratio of 1.5. The calculated sample size was 250 PCOS cases and 250 controls for analysis of SNPs in the selected candidate genes (*CYP19A1* and *CYP17A1*) [21].

Criteria for cases and controls

Cases were procured from Hartej Hospital, Amritsar, which fulfilled the Rotterdam 2003 criteria [2]. All the cases having 12 or more follicles measuring 2–9 mm in diameter and/or an increased ovarian volume > 10 cm^3 on transvaginal ultrasound scanning, having chronic anovulation, and/or having biochemical or clinical signs of hyperandrogenism were selected. Age-matched healthy women having no signs of PCOS and having normal menstrual cycle formed the control group. Details of their life style and health status were recorded. Voluntary written informed consent was obtained. Detailed information of menstrual and medical history, family history, anthropometric values, and other precipitating factors were recorded on pre-designed proforma from both cases and controls. About 5 ml of venous blood was drawn from cases and controls (3 ml for EDTA vial and 2 ml for serum). The EDTA and serum samples were stored at − 20 °C till further analysis. Genotyping for rs700519, rs2414096 (*CYP19A1*), and rs743572 (*CYP17A1*) polymorphisms was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

Biochemical analysis

Lipid profile including cholesterol, triglycerides, and high-density lipoproteins (HDL) was estimated using specified kits (Erba Mannheim) on biochemical analyzer (Erba Mannheim Chem-7).

Anthropometric measurement

Anthropometric measurements were taken in all cases and controls. Height and weight were recorded. Body mass index was used as a measure of overall adiposity and was defined as weight (kg)/height^2 (cm). The category of body weight (lean, normal, obese, or overweight) was defined as given by the World Health Organization criteria [22].

Genotypic analysis using PCR-RFLP method

Genomic DNA was isolated from 1 ml peripheral blood using the phenol-chloroform method by Adeli and Ogbona (1990) by slight modifications [23]. The DNA was suspended in 100 μl of Tris-HCl and EDTA (TE buffer). DNA was quantified using nanodrop (Thermoscientific 2000 c). For *CYP19A1* rs700519 (C>T), amplification of 173-bp product size was done using forward and reverse primers as mentioned in

Table 1 [24]. PCR product was digested overnight at 37 °C by HpyCH4V (New England Biolabs) restriction enzyme. Digested products were checked on 2.5% agarose gel. CC genotype showed 173-bp band; CT showed 173-, 118-, and 55-bp band; and TT showed 118- and 55-bp band on agarose gel.

PCR amplification for rs2414096 (G>A) was done, using primers as given by Jin et al. [25] CViAII (New England Biolabs) restriction enzyme digested PCR product when treated overnight at 37 °C. After digestion, a band of 189 bp for GG; 189-, 161-, and 28-bp band for GA; and 161- and 28-bp band for AA genotype were seen on 2.5% agarose gel.

Amplification of rs743572 (− 34T>C) was done using a primer sequence [26] given in Table 1. Overnight restriction at 37 °C was done using MSpAI (New England Biolabs) restriction enzyme. A single 414-bp product defined the T homozygotes; 414-, 290-, and 124-bp products defined the TC heterozygotes; and 290- and 124-bp products defined the C homozygotes, on 2% agarose gel.

Statistical analysis

Student's *t* test and Fisher's exact test was used to compare the *CYP19A1* and *CYP17A1* genotype and allele distributions in the case-control study. The relative association between patients and controls for genotype and allele frequencies was assessed by Pearson's χ^2 test. The corresponding odds ratios (ORs) and confidence intervals (95% CIs) were calculated with (SPSS version 17.0, SPSS Chicago, IL, USA). Clinical variables such as cholesterol, triglycerides, HDL, and BMI were compared using one-way analysis of variance (ANOVA). Hardy-Weinberg distribution of genotypes in the PCOS and control groups was assessed. A strong association (significance) was assumed at $p < 0.05$.

Haploview 4.2 [27] was used to determine haplotypes as well as haplotype blocks, using the solid spine of LD algorithm. SNPs and haplotypes were tested for association with PCOS.

Result

In 500 study subjects, the overall mean ± SD age of cases and controls was 24 ± 5.381 and 26.157 ± 5.443. The mean ± SD BMI calculated was 24.523 ± 5.026 and 22.460 ± 3.471 in cases and controls. BMI was found to be statistically different between cases and controls ($p = 0.000$). The overall mean of cholesterol, triglycerides, and HDL in PCOS cases was 159.03, 192.21, and 47.35 but no significant difference was observed among different genotypes. The triglyceride levels were found to be high in cases (Table 2).

Association of − 34T>C (rs743572) was found with PCOS. The genotypic and allelic frequencies were statistically different in cases and controls ($p = 0.0005$) and the genotypic frequencies were in accordance with the Hardy-Weinberg equilibrium ($p = 0.369$) (Table 2).

The genotypic distributions for rs700519 and rs2414096 in *CYP19A1* were not observed to be statistically significant among cases and controls ($p = 0.635$ and $p = 0.614$, respectively). For rs700519, no significant deviation from the Hardy-Weinberg equilibrium was observed ($p = 0.072$); however, for rs2414096, a significant deviation from the Hardy-Weinberg equilibrium was observed ($p = 0.000$) (Table 3).

Haplotype analysis revealed that the studied SNPs (rs700519 and rs2414096) were not in linkage disequilibrium (LD) with each other among PCOS cases and controls ($D' = 0.112$ and $r^2 = 0.0$).

Discussion

The present study is the first to investigate the association of *CYP17A1* (rs743572) and *CYP19A1* (rs700519 and rs2414096) polymorphisms in North Indian women with PCOS (Table 4).

Polycystic ovary syndrome is considered a multifactorial disorder with various genetic, metabolic, endocrine, and environmental abnormalities. It is well documented that PCOS women are more vulnerable to obesity-related health problems like diabetes, hypertension, cardiovascular disorders, anovulation, infertility, difficulties in conception, and adverse pregnancy outcomes [38, 39]. BMI provides the measure of obesity which throws light on associated problems with it. It has been established that Asian population has higher fat deposition at a lower BMI. The socio-economic development in Punjab has led to changes in dietary pattern and reduced

Table 1 PCR primer sequence and amplification condition

Polymorphism	Primers sequence	Annealing temperature	Product size
rs700519	5′-GGCAAATAAATCTGTTTCGCTAGA-3′ 5′-CAACTCAGTGGCAAAGTCCA-3′	66 °C—15 cycles 64 °C—20 cycles	173 bp
rs2414096	5′-TCTGGAAACTTTTGGTTTGAGTG-3′ 5′GATTTAGCTTAAGAGCCTTTTCTTACA-3′	60 °C—35 cycles	189 bp
rs743572	5′-CATTCGCACTCTGGAGTC-3′ 5′-AGGCTCTTGGGGTACTTG-3′	60 °C—15 cycles 59 °C—20 cycles	414 bp

Table 2 Lipid profile according to genotype distribution in PCOS cases

Polymorphism	Cholesterol	Triglyceride	HDL
rs700519			
CC	160.36 ± 39.35	192.21 ± 108.86	49.14 ± 14.47
CT	155.81 ± 37.28	192.22 ± 121.99	44.69 ± 13.02
TT	154.20 ± 22.05	172.93 ± 112.55	48.07 ± 7.84
p value	*0.763*	*0.934*	*0.253*
rs2414096			
GG	160.30 ± 40.37	194.02 ± 116.48	48.65 ± 13.62
GA	154.55 ± 32.59	158.11 ± 60.76	52.79 ± 12.61
AA	143.44 ± 20.21	221.148 ± 140.61	39.01 ± 9.77
p value	*0.592*	*0.514*	*0.323*
rs743572			
TT	158.76 ± 41.08	193.41 ± 119.66	47.82 ± 12.85
TC	157.59 ± 37.73	180.55 ± 111.18	46.70 ± 15.39
CC	166.06 ± 40.23	235.98 ± 97.58	48.65 ± 13.33
p value	*0.741*	*0.217*	*0.880*

HDL high-density lipoprotein

$p < 0.05$ = significant; $p > 0.05$ = non-significant

physical activity, which have contributed towards increasing obesity, among women [40]. Our results have shown a statistically significant association of BMI with PCOS ($p = 0.000$). Our results are in accordance with the study done by Thathapudi et al. in South Indian women which showed BMI to be statistically significant ($p < 0.0001$) [41]. Similar results were observed by Deepika et al. [42] in South Indian women ($p = 0.0001$). Our study is analogous with the study done by Chen et al. showing significant differences in BMI among PCOS subjects ($p = 0.001$) [43]. In the present study, 70% of PCOS patients were overweight (BMI > 25 kg/m2), higher than the reports of Abdulrazak and Al-Tae [44], who found that 63.55, 50, and 35% of women with PCOS were obese or overweighed, respectively. However, Haider et al. [45] and Zhang et al. [46] reported no significant differences in BMI ($p = 0.575$, $p = 0.831$, respectively).

Cholesterol, triglycerides, and HDL levels were not statistically significant among genotypes of the studied polymorphisms. Overall triglyceride levels were high in PCOS cases. Similar to present findings, Macut et al. also reported elevated triglycerides levels in PCOS women [47]. The characteristic dyslipidemic profile [high triglycerides and low high-density lipoprotein-cholesterol (HDL-C)] associated with insulin resistance is the most common metabolic abnormality in PCOS.

In the present study, − 34C>T polymorphism in *CYP17A1* was observed to be associated with PCOS ($p = 0.0005$). Diamanti-Kandarakis also reported a high association of − 34T>C polymorphism with PCOS in Greek population, which is similar to the present study [26]. It has been indicated that high frequency of CC genotype has a significant role in hyperandrogenism in PCOS women. In the present study, we have confirmed the high frequency of CC genotype in PCOS cases as compared with controls. In a study conducted on Thai women, no positive association was found [48]. Subsequent comprehensive studies have also failed to detect significant relation between *CYP17* and PCOS [49, 50]. Our observation of strong association of this polymorphism with PCOS corresponds to the findings of Carey et al. where a strong association of − 34T>C polymorphism with familial PCOS has been reported [28]. However, Gharani et al. reported lesser association of this polymorphism with non-familial PCOS [51]. Studies on *CYP17A1* in Iraqi women showed lack of association between *CYP17A1* and PCOS [34].

Aromatase activity is reduced in PCOS follicular fluid. No significant association was observed between *CYP19* (rs2414096) polymorphism and PCOS in the present

Table 3 Genotypic and allelic frequencies of *CYP19A1* and *CYP17A1* polymorphisms

	Cases (250)		Controls (250)		χ^2 (*p* value)	HWE χ^2 (*p* value)
	Genotype (%)	Allele	Genotype (%)	Allele		
Cyp19A1						
rs700519	CC = 62.18 CT = 35.71 TT = 2.10	C = 80.04 T = 19.96	CC = 66.53 CT = 31.45 TT = 2.016	C = 82.26 T = 17.74	1.02 (0.635)	5.251 (0.072)
rs2414096	GG = 88.23 GA = 7.98 AA = 3.78	G = 92.2 A = 7.8	GG = 88.7 GA = 10.48 AA = 8.06	G = 93.95 A = 6.05	5.57 (0.614)	126.81 (0.000)
CYP17A1						
rs743572	TT = 42.85 TC = 47.05 CC = 10.08	T = 66.3 C = 33.6	TT = 58.46 TC = 37.5 CC = 4.03	T = 77.2 C = 22.7	14.81 (0.0005*)	1.994 (0.369)

*Statistically significant

Table 4 Comparison of present study with other studies

Author	Year	Country/region	Study sample (case/control)	Result (*p* value)
CYP17A1 (rs743572)				
Carey et al. [28]	1994	Caucasian	81/14	0.03*
Diamanti-Kandarakis et al. [26]	1999	Greece	50/50	< 0.05*
Kahsar-Miller et al. [29]	2004	America	259/161	> 0.05
Echiburu et al. [30]	2008	Chile	159/93	0.171
Park et al. [31]	2008	Korea	133/99	0.159
Unsal et al. [32]	2009	Turkey	44/50	0.383
Pusalkar et al. [33]	2009	West India	100/100	< 0.05*
Chua et al. [19]	2012	America	287/187	0.420
Mohammed et al. [34]	2015	Iraq	61/30	> 0.05
Present study	2017	North India	250/250	0.005*
CYP19A1				
rs2414096				
Jin et al. [25]	2009	China	386/298	0.001*
Reddy et al. [35]	2015	South India	249/257	0.03*
Mutib et al. [36]	2015	Iraq	84/65	< 0.05*
Present study	2017	North India	250/250	0.614
rs700519				
Wang et al. [24]	2011	China	374/704	0.02*
Reddy et al. [35]	2015	South India	249/257	0.88
Dou et al. [37]	2017	China	150/143	0.008*
Present study	2017	North India	250/250	0.635

*Significant; $p < 0.05$ = significant; $p > 0.05$ = non-significant

investigated study ($p = 0.614$). The results of present study were not in accordance with the study done by Reddy et al. in South Indian women [35]. But Guo et al. reported significant association of *CYP19* with age at menarche (AAM) in Caucasian females [52]. Jin et al. conducted study on Chinese population and investigated that SNP rs2414096 in *CYP19* is associated with susceptibility to PCOS ($p = 0.001$) [26]. A study by Petry et al. conducted on young women from two different populations including Barcelona women and Oxford women showed that variation in aromatase gene is associated with features of hyperandrogenism [53]. Mutib et al. observed positive association of rs2414096 polymorphism with PCOS hyperandrogenism in Iraqi women [36]. Since, rs2414096 is intronic and therefore does not affect the protein sequence of aromatase and 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 in our ethnicity. Aromatase activity is reduced in PCOS follicular fluid, which might be due to genes involved or when follicular stimulating hormone (FSH) activity is low as compared to LH, and due to reduced levels of FSH, aromatase activity is lowered and the androstenedione and testosterone are not completely aromatized to estrogens.

rs700519 (*CYP19A1*) did not show significant association with PCOS in present study ($p = 0.635$). These results were in contrast with the study on South Indian women, which showed significant difference [35]. Wang et al. [24] showed significance with PCOS in a large case-control study ($p = 0.004$). Urbanek et al. (1999) used linkage analysis to study 37 candidate genes (including *CYP19A1*), but this study found evidence for linkage only with follistatin [54]. Soderlund et al. found no evidence of mutations in *CYP19A1* in patients with PCOS after examining the distribution of the variant in the ovary promoter in 25 PCOS patients and 50 controls [55]. More recently, Nectaria Xita found that a *CYP19A1* polymorphism was associated with serum testosterone concentration [56]. They concluded that *CYP19A1* may not be a major genetic determinant of PCOS but a genetic modifier of the phenotype.

Conclusion

This is the first association study from North India that has reported the association of *CYP17A1* −34T>C (rs743572) polymorphism with polycystic ovary syndrome. This SNP may be a useful marker in determining genetic susceptibility

to the pathogenesis of polycystic ovary syndrome, while rs7005195 and rs2414096 did not show any significant association. However, differences in various populations observed can be attributed to diverse ethnic and geographic differences. Identification of genetic markers through case-control association and further functional studies is needed to analyze potential factors for complex disorders such as polycystic ovary syndrome.

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Compliance with ethical standards

This study was approved by the ethics review board of Guru Nanak Dev University, consistent with provisions of the Declaration of Helsinki. Voluntary written informed consent was obtained.

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