RESEARCH ARTICLE

Single-nucleotide polymorphism of *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G* and *CAPN10* genes in the pathogenesis of polycystic ovary syndrome

MAHESWARI THANGAVELU[1], USHA RANI GODLA[2], SOLOMON F. D. PAUL[1] and RAVI MADDALY[1]*

[1]*Faculty of Biomedical Sciences, Sri Ramachandra University, Chennai 600 116, India*
[2]*Department of Obstetrics and Gynecology, Sri Ramachandra Medical Centre, Chennai 600 116, India*

Abstract

Polycystic ovary syndrome (PCOS) is the most common and a complex female endocrine disorder, and is one of the leading cause of female infertility. Here, we aimed to investigate the association of single-nucleotide polymorphism of *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G* and *CAPN10* gene in the pathogenesis of PCOS. A hospital-based, observational case–control study was carried on 169 PCOS and 169 control women in the southern region of India. Genotype was carried out by real-time polymerase chain reaction. A chi-square (χ^2) test was performed and the genotypes were verified to comply with the Hardy–Weinberg equilibrium. Odds ratio and 95% confidence interval were calculated to assess the relative risk. Comparison of clinical characteristics of women with PCOS and controls reveal an increase in body mass index (BMI), luteinizing hormone / follicle stimulating hormone (LH/FSH) ratio, glucose levels, insulin, testosterone, hirsutism and antral follicular count in PCOS women. The variant rs1801278 ($P = 0.002$; OR = 2.88; 95% CI = 1.43, 5.80) show an association with PCOS. In the genotypic ($P = 0.0002$) and allelic models ($P = 0.000$), significance persisted even after Bonferroni correction. The genotypes of SNPs strongly influence BMI, LH, LH/FSH ratio, ovarian volume and antral follicular count in PCOS women. The study results were suggestive of a positive association between Gly972Arg of *IRS1* and PCOS in the south Indian population, while *INS*, *IRS2*, *PPAR-G* and *CAPN10* failed to show any association with PCOS in our studied population. Further studies focussing the role of *IRS1* are warranted to delineate its implication towards PCOS.

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Introduction

Polycystic ovary syndrome (PCOS), the most common and highly complex endocrinopathy affects the female population of reproducing age at an increasing prevalence of about 4–8% (Azziz *et al.* 2004). PCOS, the leading cause of oligo-anovulatory infertility is characterized by insulin resistance, hyperinsulinaemia (found in 50–70% of women diagnosed) (Navaratnarajah *et al.* 2008; De França Neto *et al.* 2010) and many other metabolic abnormalities that include obesity and hyperandrogenism which has resulted in increased risk of diabetes mellitus (DM), dyslipidaemia, atherosclerosis and also endometrial carcinoma (Carmina *et al.* 2006; Orio *et al.* 2006; Giallauria *et al.* 2008). Further, women with PCOS have expressed several interrelated features, including chronic anovulation, polycystic ovaries and oligomenorrhea which are coupled with anomalous androgen and insulin-related parameters irrespective of other standard reproductive factors (Adams *et al.* 2004). Various studies have also suggested that women with PCOS are at a higher risk of gestational diabetes, miscarriages, preeclampsia and preterm labour (Boomsma *et al.* 2006). The genetic basis of the disease is still not clearly understood owing to the difficulties in determining the inheritability of PCOS. The genes that regulate insulin secretion and action, ovarian and adrenal steroidogenesis are considered as candidate genes which determine the expression of several integral phenotypes of PCOS. Insulin gene thought to play a functionally central role in insulin secretion and/or action and also in the signalling pathways. The insulin gene to the 5′-flanking region has variable number of tandem repeats and has shown to influence transcriptional activity of gene *in vitro* (Paquette *et al.* 1998). The insulin receptor gene (*INSR*) is a hetradimeric complex containing 22 exons. Exons 17–21 encode tyrosine kinase domain which is essential for insulin signal transduction. Any polymorphisms in *INSR* gene can introduce

*For correspondence. E-mail: maddalyravi@hotmail.com.

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changes in insulin receptor function and may predispose to the development of PCOS (Siegel *et al.* 2002). Insulin receptor substrate 1 (*IRS1*) and 2 (*IRS2*) involved in the insulin signalling by activating phosphatidylinositol-3 kinase, reported to be associated with insulin resistance, type 2 DM and PCOS (Dilek *et al.* 2005). Peroxisome proliferator receptor gamma (*PPAR-G*) is a nuclear receptor and plays a critical role in carbohydrate, lipid metabolism and adipocyte differentiation by regulating multiple genes (Gonzalez Sanchez *et al.* 2002). The calpain 10 (*CAPN10*) gene is calcium dependent and is expressed at mRNA and protein levels by several tissue types.

This gene was recognized as type 2 DM susceptibility loci and is allied to proinsulin processing, insulin secretion and insulin resistance (Baier *et al.* 2000; Horikawa *et al.* 2000). In the present study, we tried to investigate the role of single-nucleotide polymorphisms (SNPs) of *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G* and *CAPN10* genes in Indian population which were previously studied in different populations with consideration of utmost link towards PCOS. Polymorphism of the above said genes in link with PCOS has been reported by several studies in different populations but a very few studies have been reported in the Indian population. *INS*-VNTR in link with PCOS is studied for the first time in Indian population. The SNP rs1805097 of *IRS*2 and rs2975766, rs7607759 of *CAPN10* gene related to PCOS is also studied for the first time in Indian population. This study will shed light on the polymorphic pattern in the selected population and its association towards the syndrome.

Materials and methods

Study subjects

This study was conducted altogether on 338 women, comprising 169 cases (PCOS) and 169 controls. PCOS subjects were selected based on observation of oligoamenorrhea/anovulation, clinical or biochemical evidence of hyperandrogenism and/or polycystic ovaries on ultrasonography (The Rotterdam criteria 2003) and normal, unaffected, age-matched fertile women with regular menstrual cycles (interval of 28–35 days) and with normal ovaries from the same geographical region were included in the study as controls. Women with galactorhea, hyperthyroidism, any systemic disease that affects their reproductive physiology, or any medication which interferes with the normal function of the hypothalamic–pituitary–gonadal axis were excluded from the study. The age of subjects ranged from $\geq$20 to $\leq$40 y. This study was approved by the institutional ethical committee of Sri Ramachandra University. Written informed consent was collected from all the subjects enrolled for the study. Subject's history and other anthropometric assessments were carried out.

Biochemical and hormonal analyses

Blood samples were taken from the subjects after 8-h fast through vein puncture to carry out the biochemical and hormonal analyses on day 2 (D2) or 3 (D3) of the follicular phase. Tests were conducted using Siemens-ADVIA Centaur Automated System and were assayed by chemiluminescent immuno assay (CLIA). Hirsutism was calculated using the modified Ferriman–Gallwey scoring method (Ferriman and Gallwey 1961).Insulin resistance (IR) was assessed using the homeostatic model assessment (HOMA-IR), calculated as (fasting insulin $\times$ fasting glucose)/22.5 (Li *et al.* 2014). Polycystic ovary (PCO) was confirmed by ultrasound assessments by means of a transvaginal ultrasonography with a transvaginal probe of curved array 5.0–2.0 MHz (for ovary) with a frequency of 5.9 MHz using diagnostic ultrasound system, Sonoscape, Guangdong, China; with 12 or more follicles in each ovary, measuring 2–9 mm in diameter and/or increased ovarian volume (10 cm^3).

Molecular analysis

DNA was isolated from peripheral blood using modified salting-out method. Genotyping of SNPs was carried out with real-time polymerase chain reaction technology (Taqman SNP Genotyping Assay, Applied Biosystems, Carlsbad, USA). Each reaction mixture consists of a final volume of 5 μL (2.50 μL of 2$\times$ Taqman Genotyping Master Mix, 0.25 μL of 20$\times$ Taqman Drug Metabolism Genotyping Assay mix and 2.25 μL (3–20 ng of genomic DNA) genomic DNA diluted in distilled water). Thermal Cycle reaction with initial denaturation of 95°C for 10 min followed by 40 cycles of denaturation 95°C for 15 s, annealing/extension of 60°C for 1 min was carried out in a 384 well-optical plate on a 7900-HT fast real time PCR machine. The Taqman Drug Metabolism Genotyping assay mix contains the primers and fluorescent probes. The alleles were labelled with VIC®-dye and FAM™ dye.

Statistical analysis

The continuous variables were expressed as mean $\pm$ standard deviation. All statistical analysis were performed using the SPSS statistical software ver. 9.0. Hardy–Weinberg equilibrium (HWE) tests were performed by comparison of observed and expected genotype frequencies using χ^2 goodness-of-fit test. The genotype and allele frequencies of each polymorphism was compared between subjects with PCOS and the controls by the χ^2 test. The odds ratio and 95% confidence interval were calculated using wild-type genotypes or alleles as the reference group. $P < 0.05$ was considered to be statistically significant. Significant values were further confirmed by multiple testing using Bonferroni correction to address the multiple comparisons problem. Pair-wise linkage disequilibrium (LD) was computed as both D' and r^2 for the *PPAR-G* and *CAPN10* genes using Haploview ver. 4.1. SNP–SNP interactions among variants of *PPAR-G* gene were assessed by nonparametric multifactor dimensionality reduction (MDR) analysis using software ver. 3.0.2.

Results

The anthropometric and clinical parameters between cases and controls are measured and presented in table 1. The mean age of PCOS and control women were 26.92 and 27.52, respectively. BMI between PCOS and control women were more or less similar with mean 25.14 and 24.18, respectively. The mean LH level in controls was 4.68 and a two-fold increase in mean value was observed in PCOS women (9.12). However, a decrease in the FSH levels were observed in PCOS (6.18) compared to controls (7.11). Also the mean LH/FSH ratio was increased among PCOS women (1.62) compared to controls (0.71). A highly significant difference was observed in the mean testosterone levels of PCOS women (71.65) compared to their controls (28.23). Correspondingly, the mean hirsutism scores of PCOS women (4.65) were higher than the controls (1.83). The mean insulin and HOMA-IR levels in PCOS and controls were 13.77, 10.76 and 3.08, 2.28, respectively. Glucose levels were elevated in PCOS women. The unilateral right and left ovarian

Table 1. Descriptive analysis on variables between PCOS cases and control subjects.

	Control			Case			
		95% Confidence interval			95% Confidence interval for mean		
Parameter	Mean	Lower bound	Upper bound	Mean	Lower bound	Upper bound	*P* value
Age	27.52	26.91	28.13	26.92	26.26	27.58	0.190
BMI	24.18	23.67	24.68	25.14	24.45	25.84	0.028*
Waist/hip	0.73	0.72	0.74	0.86	0.85	0.87	<0.001**
LH	4.68	4.32	5.04	9.12	8.37	9.86	<0.001**
FSH	7.11	6.75	7.46	6.18	5.86	6.49	<0.001**
LH-FSH	0.71	0.65	0.77	1.62	1.47	1.77	<0.001**
Estradiol	60.23	56.93	63.53	64.72	60.62	68.84	0.093
T. testosterone	28.23	26.23	30.22	71.65	64.73	78.56	<0.001**
Hirsutism	1.83	1.58	2.06	4.65	3.98	5.31	<0.001**
Insulin	10.76	10.12	11.42	13.77	12.61	14.95	<0.001**
Pg (mg/dL)	85.74	84.47	87.00	89.54	86.94	92.14	0.010*
P-pg (mg/dL)	110.19	108.56	111.84	116.00	111.02	102.98	0.030*
HOMA-IR	2.28	2.13	2.43	3.08	2.77	3.37	<0.001**
OV (right)	4.79	4.18	5.39	10.33	9.32	11.34	<0.001**
OV (left)	4.33	3.71	4.94	9.26	8.52	9.99	<0.001**
AFC (right)	6.07	5.71	6.42	10.43	9.89	10.9	<0.001**
AFC (left)	5.78	5.43	6.13	10.27	9.70	10.85	<0.001**

BMI, body mass index; LH, luteinizing hormone; FSH, follicle stimulating hormone; T. testosterone, total testosterone; Pg, preprandial glucose; P-pg, postprandial glucose, HOMA-IR, homeostatic model assessment of insulin resistance; OV, ovarian volume; AFC, antral follicular count; statistical analysis was performed using the SPSS statistical software ver. 9.0. The continuous variables are expressed as mean ± standard deviation; the significant difference obtained in PCOS subjects and controls were calculated and compared using ANOVA. $P < 0.05$ was considered to be statistically significant; *$P < 0.05$; **$P < 0.01$.

Table 2. Allele and genotype frequencies of *INS* rs689 in PCOS and control groups.

Gene	Genotype	Control ($n = 169$)	Case ($n = 169$)	OR (95% CI)	*P* value
INS	rs689				
	TT	124 (73.37)	131 (77.51)	Reference	0.667
	TA	41 (24.26)	35 (20.71)	0.81 (0.48–1.35)	0.415
	AA	4 (2.37)	3 (1.78)	0.71 (0.16–3.23)	0.656
	TA+AA	45 (26.63)	38 (22.49)	0.81 (0.49–1.31)	0.376
	HW:*P*	0.780	0.711		
	Allele frequency				
	T	289 (85.50)	297 (87.87)	Reference	
	A	49 (14.50)	41 (12.13)	0.81 (0.52–1.31)	0.365
	MAF	0.14	0.12		

A χ^2 test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HW; OR and 95% CI were calculated to assess the relative risk; $P < 0.05$ was considered to be statistically significant.

volume in PCOS and control were 10.33, 9.26 and 4.79, 4.33 and the unilateral right and left AFC in PCOS and control were 10.43, 10.27 and 6.07, 5.78, respectively.

All the genotype frequencies were distributed according to the HWE in both case and control subjects except for rs1801278 of *IRS1* gene (cases only). In the rs689 variant of *INS*, T alleles were more compared to A alleles in both cases and controls. The minor allele frequency was 14% in control and 12% in PCOS case groups (table 2). The genotypic distribution between PCOS and control subjects did not show any significant association. In the *INSR* gene (rs1799817), the frequency of T allele was found to be more (66.27%) than the C allele (33.73%), in both cases and controls (table 3). No significant association was observed at the dominant or allelic model. In the SNP rs1801278 of *IRS1* gene (table 4), the minor allele frequency among cases and controls were 53% and 43%. Significant difference was observed between PCOS and control subjects in genotypes (GA, 0.002; AA, 0.002) of dominant (0.001) and allelic model (<0.001). This significance was maintained after Bonferroni correction for multiple testing (GA, 0.0002; AA, 0.0002; GA+AA, 0.0001). Frequency of A allele (53.25%) was more compared with the G allele (46.75%) in PCOS women and the frequency of G allele (57.40%) was more compared to A allele (42.60%) in control women. Polymorphism of rs1805097 of *IRS2* gene is depicted in table 5. The frequency of homozygous GG was 49.11% and 49.70% in PCOS and control, while the homozygous AA was lesser in both PCOS and control with 8.88 and 11.83%. No significant difference was seen in the genotypic, dominant and allelic models for the variant rs1805097. Table 6 provides explanation for two variants rs1801282 and rs3856806 of *PPAR-G* gene. The genotype distribution frequency for rs1801282 among cases and controls for homozygous GG were higher 76.33 and 73.96% compared to the homozygous CC which was 2.37 and 1.78%. The genotype distribution of rs3856806 for cases and controls for genotypes CC, CT and TT are 72.78, 23.67, 3.55 and 69.23, 28.99, 1.78. The minor allele frequency for cases and control subjects were more or less similar for both the variants. The variants rs7607759 and rs2975766 of *CAPN10* gene are illustrated in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet. No significant difference was observed in the genotype distribution, dominant and allelic models between cases and controls for both

Table 3. Allele and genotype frequencies of *INSR* rs1799817 in PCOS and control groups.

Gene	Genotype	Control ($n = 169$)	Case ($n = 169$)	OR (95% CI)	*P* value
INSR	rs1799817				
	CC	22 (13.02)	19 (11.24)	Reference	0.600
	CT	67 (37.64)	76 (44.97)	1.31 (0.65–2.63)	0.442
	TT	80 (47.34)	74 (43.79)	1.07 (0.53–2.14)	0.845
	CT+TT	147 (86.98)	150 (88.76)	1.18 (0.61–2.27)	0.617
	HW:*P*	0.188	0.938		
	Allele frequency				
	C	111 (32.84)	114 (33.73)	Reference	
	T	227 (67.16)	224 (66.27)	0.96 (0.69–1.32)	0.806
	MAF	0.67	0.66		

A χ^2 test was performed to evaluate the association between SNP and PCOS; the genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; $P < 0.05$ was considered to be statistically significant.

Table 4. Allele and genotype frequencies of *IRS1* rs1801278 in PCOS and control groups.

Gene	Genotype	Control ($n = 169$)	Case ($n = 169$)	OR (95% CI)	*P* value
IRS1	rs1801278				
	GG	50 (29.59)	25 (14.79)	Reference	0.003**
	GA	94 (55.62)	108 (63.91)	2.30 (1.32–4.00)	0.002**
	AA	25 (14.79)	36 (21.30)	2.88 (1.43–5.80)	0.002**
	GA+AA	119 (17.41)	144 (85.21)	2.42 (1.41–4.14)	0.001**
	HW: *P*	0.0742	< 0.001		
	Allele frequency				
	G	194 (57.40)	158 (46.75)	Reference	
	A	114 (42.60)	180 (53.25)	1.94 (1.41–2.66)	< 0.001**
	MAF	0.43	0.53		

A χ^2 test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; $^{**}P < 0.01$; $P < 0.05$ was considered to be statistically significant.

Table 5. Allele and genotype frequencies of *IRS2* rs1805097 in PCOS and control groups.

Gene	Genotype	Control ($n = 169$)	Case ($n = 169$)	OR (95% CI)	*P* value
IRS2	rs1805097				
	GG	84 (49.70)	83 (49.11)	Reference	0.611
	GA	65 (38.46)	71 (42.01)	1.11 (0.70–1.74)	0.664
	AA	20 (11.83)	15 (8.88)	0.76 (0.36–1.58)	0.461
	GA+AA	85 (50.30)	86 (50.89)	1.02 (0.67–1.57)	0.913
	HW:*P*	0.184	0.973		
	Allele frequency				
	G	233 (68.93)	237 (17.12)	Reference	
	A	105 (31.07)	101 (29.88)	0.95 (0.68–1.31)	0.738
	MAF	0.31	0.30		

A χ^2 test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; $P < 0.05$ was considered to be statistically significant.

Table 6. Results of association test with *PPAR-G* gene polymorphisms rs1801282 and rs3856806 in PCOS and control groups.

Gene	Genotype	Control ($n = 169$)	Case ($n = 169$)	OR (95% CI)	*P* value
PPAR-G	rs1801282				
	CC	3 (1.78)	4 (2.37)	Reference	0.767
	CG	41 (24.26)	36 (21.30)	0.65 (0.13–3.14)	0.598
	GG	125 (73.96)	129 (76.33)	0.77 (0.16–3.52)	0.740
	CG+GG	166 (98.22)	165 (97.63)	0.75 (0.16–3.38)	0.702
	HW:*P*	0.86	0.44		
	Allele frequency				
	C	47 (13.91)	44 (13.02)	Reference	
	G	291 (86.01)	294 (86.98)	1.07 (0.69–1.68)	0.735
	MAF	0.86	0.87		
	rs3856806				
	CC	117 (69.23)	123 (72.78)	Reference	0.357
	CT	49 (28.99)	40 (23.67)	0.78 (0.48–1.27)	0.094
	TT	03 (1.78)	06 (3.55)	1.90 (0.47–7.78)	0.363
	CT+TT	52 (30.77)	46 (27.22)	0.84 (0.53–1.35)	0.471
	HW:*P*	0.405	0.237		
	Allele frequency				
	C	283 (83.73)	286 (84.62)	Reference	
	T	55 (16.27)	52 (15.43)	0.94 (0.62–1.41)	0.751
	MAF	0.16	0.15		

A χ^2 test was performed to evaluate the association between SNP and PCOS; the genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; $P < 0.05$ was considered to be statistically significant.

the SNPs but increased risk was observed in GG genotype ($P = 0.558$; OR = 1.37; CI: 0.47–3.98) of rs7607759 and genotypes GA ($P = 0.121$; OR = 1.83; CI: 0.84–3.98), AA ($P = 0.531$; OR = 2.12; CI: 0.19–23.65), dominant model ($P = 0.099$; OR = 1.85; CI: 0.88–3.91) and allelic model ($P = 0.086$; OR = 1.82; CI: 0.91–3.66) of rs2975766. However neither of these findings remain significant. The clinical and hormonal variables were compared with the genotypes of all the SNPs and are presented in tables 2–9 in electronic supplementary material. The genotypes of rs689 showed increase in waist/hip ratio, LH, LH/FSH and right ovarian volume in PCOS women. Post-hoc test was conducted to find out as which specific groups differed, and the results revealed that the TT/TA combination differed significantly (table 2 in electronic supplementary material). The rs1799817 of *INSR* gene, increase in LH ($P = 0.049$) was observed in PCOS women with CC/CT combination (table 3 in electronic supplementary material). In rs1801278 of *IRS1* gene and rs3856806 of *PPAR-G* gene, the phenotypic variables did not show any significant difference in PCOS group but unilateral left antral follicular count was significantly more in the control groups with AA,GA combinations ($P = 0.036$) in rs1801278 (table 4 in electronic supplementary material) and CC,TT combinations ($P = 0.020$) in rs3856806 (table 7 in electronic supplementary material) showing risk. In *IRS2* gene, an increase in BMI was seen in PCOS women

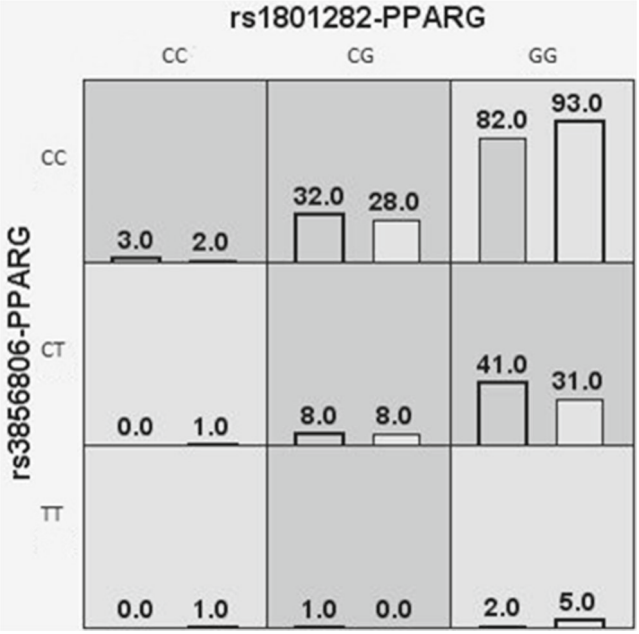

Figure 1. Distribution of high-risk and low-risk genotypes of *PPAR-G* gene between rs1801282 and rs3856806 SNPs in PCOS and control women by MDR analysis. The best two ways SNP–SNP interaction. The high-risk genotype combinations are in dark grey and low-risk genotype combinations in light grey. Higher the intensity of grey shades higher the risk. Each box represent cell and the bars inside the cell represent the number of subjects. The left bar denotes controls and the right bar represent cases.

($P = 0.024$) and the combination genotypes between AA,GA and AA,GG groups contributed to the difference (table 5 in electronic supplementary material). The SNP rs1801282 of *PPAR-G*, the homozygous GG genotype displayed decrease in levels of waist/hip ratio, LH, LH/FSH, right ovarian volume and right and left antral follicular count compared to the CC and CG genotypes (table 6 in electronic supplementary material). In variant rs7607759 of *CAPN10* gene, genotypes did not show any change in the phenotype except for hirsutism grades in control women ($P = 0.028$) (table 8 in electronic supplementary material). The genotypes rs2975766 revealed relation towards LH and LH/FSH ratio in PCOS women and waist/hip ratio among control subjects (table 9 in electronic supplementary material). Pairwise LD between rs1801282 and rs3856806 of *PPAR-G* ($D' = 0.289$ and $r^2 = 0.22$) and rs7607759 and rs2975766 of *CAPN10* ($D' = 0.211$ and $r^2 = 0.87$) were not strong and significant. Haplotype analysis showed no haplotypes between the SNP's with an estimated frequency more than 5%. SNP–SNP interactions were analysed by MDR. The prediction accuracy of the model was estimated by 10-fold cross-validation. The best model chosen had the highest cross-validation consistency (CVC) of 10/10 and a testing accuracy (TA) of 0.526. The best model consisted of two *PPAR-G* gene variants (rs1801282 and rs3856806) and is shown in table 10 in electronic supplementary material and the combinations of genotype interaction of the two SNPs are depicted in figure 1.

Discussion

Several biochemical and hormonal variables were checked to find the difference in PCOS and control women. Not much difference was observed in our population in the mean values of BMI in PCOS (25.14) and controls (24.18) but waist/hip ratio was observed to be highly significant in PCOS women. Waist/hip ratio could perhaps be a better indicator risk than BMI. LH levels were higher in PCOS women and lower in control and FSH levels were higher in control and lower in PCOS women. A decrease in FSH levels disrupts follicular growth. Also, a significant difference was observed in the testosterone levels of PCOS women compared to that of controls, supporting with the hypothesis that an increase in testosterone level leads to suppression of normal menstruation and ovulation. The augmented GnRH pulse frequency is hugely associated with hyperandrogenism and increased ovarian volume. An increase in testosterone levels above a normal range leads to an unusual increase in hair growth (hirsutism). An increased conversion of testosterone to dihydrotestosterone through the enzyme 5 alpha reductase within the pilosebaceous unit may be one of the root causes for hirsutism. As testosterone increases, a decline in the serum prolactin levels and FSH levels were observed which possibly lead to the suppression of normal menstruation and ovulation. A significant difference in the glucose levels, fasting insulin levels and HOMA-IR index were found between PCOS and control women with an elevation in PCOS women. We also found that the unilateral antral follicular count and ovarian volume of the right ovary showed a marginally higher mean value than that of the left in women with PCOS.

The variable number tandem repeats positioned at –23 bp in the 5′-flanking region of *INS* gene is considered to be a susceptible loci and acts as a surrogate marker. The rs689 polymorphism has shown association with PCOS by the mutant class III alleles also presenting with increased serum insulin levels and BMI in PCOS women (Waterworth *et al.* 1997). The frequency of class III allele (12%) reported in our population was much lower compared to the Japanese, European, Korean and Han Chinese which was 97, 30, 94 and 93.5% accordingly. The homozygous class I alleles in our study were higher in both PCOS and control. This variant rs689 of *INS* gene was not associated with PCOS in the present study. But the heterozygote TA and homozygous AA genotypes showed an increase in waist/hip ratio ($P = 0.020$), increase in luteinizing hormone levels ($P = 0.020$) and right ovarian volume ($P = 0.042$) in PCOS women. Alterations in insulin action and β-cell function may lead to augmented GnRH pulse frequency which increases LH secretion (Li *et al*. 2014) and is largely associated with hyperandrogenism and increased ovarian volume (Kralovicova 2006). Studies carried out on anovulatory women with PCOS selected based on criteria proposed by National Institute of Health (NIH) in Czech women also reported a negative association with OR 1.44 (0.50–4.18; $P = 0.59$) (Vanková *et al.* 2002). Another

study with 216 PCOS and 192 nonPCOS women in Han Chinese population reported no significant difference between cases and control groups either in allele ($P = 0.996$) or genotype ($P = 0.802$) frequencies (Xu *et al.* 2009); also a family based association study reported no excess transmission of alleles of rs689 VNTR polymorphism, regardless of parent of origin (Powell *et al.* 2005).

In our population, the rs1799817 polymorphism of *INSR* gene shows a negative association towards PCOS women but the genotypes influenced the phenotypic expression. Women with TT genotype of His1058His showed an increase in insulin, endometrial thickness, ovarian volume and right antral follicular count and women with CC genotype showed an increase in testosterone, hirsutism grade and preprandial glucose in PCOS women. Insulin resistance might upregulate testosterone production in theca cells, LH secretion in pituitary which may perhaps disturb follicular maturation and lead to PCOS (Le Fur 2006). The rs1799817 polymorphism influenced an increase in LH levels ($P = 0.049$) in PCOS women. The frequencies of CC genotypes (PCOS, 11.24%; control, 13.02%) were much lower in our population. While CC genotype frequency in other population reported: PCOS, 43.9%; control, 52.8%; PCOS, 34%; control, 27.66% (Mukherjee *et al.* 2009; Bagheri *et al.* 2015). Lack of association of His1058His was also reported in other studies (Tehrani *et al.* 2013; Urbanek *et al.* 2005; Bagheri *et al.* 2015). Two GWAS studies by Chen *et al.* (2011) and Shi *et al.* (2012) reported an association of rs1799817 of *INSR* gene in PCOS women. Another study reported an increase in frequency of uncommon T allele in lean PCOS women with body mass index [(BMI) < 27 kg/m^2] compared with lean controls (Siegel *et al.* 2002). Yet another study reported that genetic variation in exon 17 of INSR is associated with insulin resistance and hyperandrogenaemia among lean Indian women with polycystic ovary syndrome but not obese women. The difference in phenotypic expression and association of exon 17 polymorphism towards PCOS in other populations could be due to the frequency of distribution of genotypes TT, CT and CC across population where the effect of genotype influences suppression or increased expression of phenotypic change.

In rs1801278 of *IRS1* gene, 21% PCOS subjects showed glycine to arginine substitution whereas in control 15% had this substitution. The Gly972Arg polymorphism towards susceptibility of PCOS reveal that the heterozygous Gly/Arg genotype may increase the risk of developing PCOS by 2.30 times ($P = 0.002$) and having Arg/Arg genotype which is the mutant in our population might increase a risk 2.88 times in development of PCOS ($P = 0.002$). These findings remained significant even after Bonferroni correction for multiple comparisons ($P = 0.0002$). The dominant model (Gly/Arg+Arg/Arg) did contribute to a relative risk of 2.42 ($P = 0.001$), Bonferroni corrected value ($P = 0.0001$). And the allele Arg showed a risk of 1.94 in developing PCOS ($P < 0.001$), Bonferroni corrected value ($P = 0.0001$).

But Gly972Arg polymorphism did not show a change in the phenotypes except for the left antral follicular count in control subjects ($P = 0.036$). Consistent with our results, a study from the Japanese population reported significantly more IRS-1 972Arg carriers among PCOS women compared to healthy controls (10.6% versus 4.8%, $P = 0.029$), and had a significantly increased risk of developing PCOS (OR: 3.31, 95% CI: 1.49–7.35). They also found that the *IRS1* polymorphism was not associated with its phenotypes such as BMI, insulin resistance or androgen levels in PCOS women (Baba *et al.* 2007). Also, women carrying IRS-1 Gly/Arg had an increased risk of PCOS (OR = 2.49, 95% CI: 1.16–5.37, $P = 0.019$) (Lin *et al.* 2014). Two recent meta-analysis on PCOS women reported that Gly/Arg and Gly/Gly genotypes are significantly associated with risk of developing PCOS (OR = 3.31; 1.49–7.35) which is chiefly mediated by higher levels of fasting insulin (Ruan *et al.* 2012). The IRS-1 variant allele occurs significantly more frequently among PCOS patients than among healthy women (Sir-Petermann *et al.* 2001). On the other hand Spanish, Croatian, Chilean, Taiwanese, Caucasian, Slovak and Iranian populations did not find any association of Gly972Arg towards PCOS. However a study analysing 250 PCOS and 299 controls reported IRS-1 polymorphic alleles having a similar distribution between cases and controls, and seems to have a probable protective role against development of specific PCOS subphenotypes (Dasgupta *et al.* 2012).

In rs1805097 of *IRS2*, we report that Asp was the minor allele in our studied population and the Caucasians and the African Americans also presented Asp as the minor allele (San Millán *et al.* 2004). Nevertheless, Lin *et al.* (2014) reported Gly as the minor allele and association of Asp/Asp genotype with higher fasting insulin and HOMA index (Goodarazi *et al.* 2005). While Ehrmann and his team reported association of Gly/Gly with higher 2h OGTT levels (Ehrmann *et al.* 2002). The variant rs1805097 (Gly1057Asp) of *IRS2* gene was not associated with PCOS in the studied population but this polymorphism may possibly have an effect on weight gain in PCOS women. The nonassociation was confirmed in Spanish and Greek populations as well. *IRS1* and *IRS2* polymorphisms influence glucose homeostasis and could influence obesity, regardless of women presented with PCOS (Taylor 1997). Levels of both IRS-1 and IRS-2 are increased in theca cells of women with PCOS, which leads to proliferation and increased androgen synthesis (Yen *et al.* 2004).

The Ala variant of rs1801282 of *PPAR-G* gene was found to be higher in PCOS and controls. The Ala/Ala variant tend to decrease waist/hip ratio, lower LH levels, LH/FSH ratio, right OV and AFC in PCOS women in our population. Ala variant reduces the risk of PCOS with decline in levels of BMI and fasting insulin (Xu *et al.* 2009).

Polymorphism of rs1801282 was not associated with PCOS in the studied population. Similarly, a negative association was observed in Caucasian and Greek populations and a positive association was seen in Italy and Korea. Earlier

studies have also confirmed that the *PPARG* Pro12Ala variant allele association with higher insulin sensitivity, high-density lipoprotein (HDL) levels (Deeb *et al.* 1998), decreased risk of T2DM (Hara *et al.* 2000) and decreased incidence of cardiovascular disease (CVD) (Doney *et al.* 2004). A meta-analysis reported lower insulin levels in Ala carriers considering PCOS patients and controls as an entire group (San-Millan and Escobar-Morreale 2010). Two additional meta-analyses determined that *PPARG* Pro12Ala variant may result in lower insulin levels but exerts no effect on HOMA-IR in PCOS patients (He *et al.* 2012; Zhang *et al.* 2012). One article published in India found an association towards PCOS at the allelic level (Dasgupta and Reddy 2013) but another study from Mumbai, India showed significant difference in both allelic and genotypic frequency between PCOS and controls with reduced susceptibility to PCOS (Shaikh *et al.* 2013). Therefore, the Ala variant of *PPAR-G* is thought to be associated with reduced transcriptional activity which improves insulin action towards suppression of lipolysis in turn reducing the production of free fatty acids and enables their storage in adipocytes (Taylor *et al.* 1990).

The variant rs3856806 located in exon 6 of *PPAR-G* is a functional synonymous SNP. For rs3856806 of *PPAR-G* (His447His), neither the genotypes nor the alleles show any association towards PCOS but the mutant homozygous TT genotype showed an increase in the right antral follicular count ($P = 0.020$) in control women. Also to see the effect of genotypes of SNPs together, MDR analysis was carried out. The best MDR models for the studied SNP rs1801282 and rs3856806 markers had a testing accuracy (TA) of 0.541 and cross-validation consistency (CVC) of 10/10. However, this model was not significantly associated with PCOS ($P = 0.755$). Each cell is labelled as high risk if the ratio of the affected individual to the unaffected individual exceeds a threshold of 1 and low risk if the threshold does not exceed it. The higher risk group contain combinations of (corresponding to SNP rs3856806 and rs1801282) CC–CC, CC–CG, CT–CG, CT–GG, TT–CG. The homozygous GG of rs180282 signifies no risk and confers protection towards PCOS over the other two genotypes. The pairwise LD values ($D' = 0.289$ and $r^2 = 0.22$) between rs1801282 and rs3856806 revealed that these two SNPs are not in strong LD.

The minor allele frequency of rs7607759 of *CAPN10* gene was 17.75% while Whites, Blacks, Hispanics, Asian/Pacific islanders reported 15.5, 6.73, 11.5 and 9.06% respectively (Wu *et al.* 2000). Even though the frequency is high in Indian population, we found a negative association of rs7607759 genotype and allele frequency of *CAPN10* towards PCOS. We say that the SNP rs7607759 is not a susceptible locus linked to PCOS in our study population. When genotypes of PCOS and control were compared with the phenotypes, we found that the hirsutism grades in control women with presence of AA/AG combination did show any significance and also no phenotypic relation was observed in PCOS women. The genotypes of SNP rs2975766 did not show any association towards PCOS. The mutant homozygous AA genotype was observed in two women with PCOS and one in the control group. This homozygous variant and the heterozygous variant renders risk 2.12 (0.19–23.65); $P = 0.531$ but was not significant enough to show an association. When the genotypes of rs2975766, compared to the biochemical and other hormonal features of PCOS it was revealed that increased levels of LH and LH/FSH ratio was observed in women with AA genotype compared to the other genotypes. The mutant AA genotype may possibly indicate changes at the phenotypic level in PCOS women. Studies from meta-analysis reported no association with the above studied variants of *CAPN10* gene towards PCOS (Shen *et al.* 2013).

The SNP's rs760759 and rs2975766 of *CAPN10* gene are the first of its kind to be studied in the Indian population. The pairwise LD values $D' = 0.211$ and $r^2 = 0.87$, between rs7607759 and rs2975766 of *CAPN10* gene revealed no significant LD between SNPs.

Conclusion

Of all the SNPs studied, Gly972Arg of *IRS1* gene showed an association with PCOS. Polymorphism of Gly972Arg could play a contributory role in the pathophysiology and risk of PCOS. Further analysis of this SNP variant should be evaluated in larger population and the same variant should be checked in different population groups to confirm its significance. Also, additional SNPs of *IRS1* gene has to be studied to confirm the role of insulin receptor substrate 1 gene towards PCOS. Apart from rs1801278, the other SNPs failed to show any association with PCOS in the population we studied. The genotypes of analysed SNPs strongly influenced the change in phenotype by increasing weight gain, LH levels, LH/FSH ratio, ovarian volume and antral follicular count in PCOS women.

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