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Original Research Article

Role of proinflammatory cytokines TNF-α (rs1800629) and IL-6 (rs1800795) in the pathogenesis of polycystic ovarian syndrome in north Indian females

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ABSTRACT

Background: This study aimed to determine whether TNF-α and IL-6 gene polymorphism, their epistatic effects and haplotypes confer pathogenesis of PCOS in north Indian females.
Methods: A case-control study comprising DNA samples of 401 females (200 PCOS cases and 201 controls) of reproductive age. All the subjects were genotyped for TNF-α (-308 G/A) and IL-6 (-174 G/C) genes by tetra-primer ARMS PCR.
Results: There were 41.5% PCOS females revealing hirsutism, 45.5% acne and 36% alopecia. High BMI (p=0.008) and W/H ratio (p=0.0001) was observed among PCOS cases. Frequency of minor allele A for -308 G/A TNF-α was significantly higher in PCOS cases than controls indicating 1.4 fold increased risk for PCOS (p=0.05, OR=1.41, 95% CI=1.00-1.99). -174 G/C IL-6 gene was in association with the decreased risk of PCOS. The epistatic effects of all the possible combinations of both the SNPs shows statistically significant differences (Interaction p value=0.014) indicating modulating effects of TNF-α and IL-6 polymorphism in response to PCOS. Haplotype HT3 AG (p=0.003, OR=2.22, 95% CI=1.31-3.78) was associated with increased risk of PCOS.
Conclusions: The present findings suggest that TNF-α and IL-6 might contribute to pathogenesis of PCOS in north Indian females irrespective of a polymorphism of TNF-α (-308 G/A) and IL-6 (-174 G/C) genes.

Keywords: Epistasis, Haplotype, IL-6, PCOS, Tetra primer-ARMS PCR, TNF-α

INTRODUCTION

Polycystic Ovarian Syndrome (PCOS) emerged as a common endocrine and metabolic disorder in females of childbearing age with a prevalence of 3.7-22.5% in India depending on the population studied and criteria used for diagnosis and approximately 4-21% worldwide.[1,2] The hallmark features of PCOS are hyperandrogenism, obesity, insulin resistance and oxidative stress. Due to the heterogeneity of PCOS, a number of candidate genes have been explored that play a significant role in its progression.[3-5] However, none of them has been accepted as a primary cause.

Chronic inflammatory markers such as TNF-α and IL-6 have been implicated in the pathophysiology of PCOS as they promote insulin resistance and hyperandrogenism.[6] TNF-α gene is an adipocyte cytokine located on chromosome 6p21.3, induces reproductive changes which have close resemblance to those found in PCOS patients.[3] IL-6 encoding gene is located on chromosome 7p21, which induces stimulation of the hypothalamic pituitary-

adrenal axis and modulation of lipid metabolism.[7] 308G/A and -174G/C polymorphisms in the promoter region of TNF-α and IL-6, observed to be associated with obesity, type 2 diabetes mellitus (T2DM), breast cancer, skin psoriasis and ischemic stroke.[8-11] Therefore, the present study aims to analyze the polymorphism association of -308G/A (rs1800629) in TNF-α and -174G/C (rs1800795) in IL-6 with PCOS patients and age-matched healthy controls. Till date there is no such study carried out on TNF-α and IL-6 together in relation to PCOS in North Indian population.

METHODS

Total 200 PCOS cases were recruited from gynaecology OPD of Department of Obstetrics and Gynaecology in government, civil and private hospitals of different regions of Punjab. The present study was conducted over the period of four years starting from February 2017 up to February 2021. The diagnosis of PCOS was made according to modified Rotterdam criteria which include: i) presence of clinical and/or biochemical signs of hyperandrogenism; ii) at least one of the following should be present: oligo- or anovulation and/or polycystic ovaries depending on ultrasound examination, clinical features and laboratory hormonal tests assessed by a gynaecologist.[12] 201 age-matched normally ovulating healthy females belonging to the same geographical area were also recruited as controls from various blood donation camps organized in the region.

Inclusion criteria

For PCOS cases: Females of reproductive age belonging to the age group, 15-45 years and showing the signs and symptoms of PCOS i.e. irregular menstrual periods, hirsutism, acne, alopecia, endocrine dysfunction were included in the study. For control: Age matched healthy controls with regular menstrual cycle, non-pregnant, non-lactating, non-smoking, non-alcoholic and also showing no evidence of endocrine dysfunction were included in the study.

Exclusion criteria

For PCOS cases: Subjects having secondary etiologies such as hyperprolactinemia, thyroid disorders, liver or kidney dysfunction (congenital adrenal hyperplasia), androgen-secreting tumors/Cushing's syndrome, type 2 diabetes mellitus and those using medications such as insulin sensitizers, insulin and diuretics were excluded from the study. For control: Females taking any form of oral contraceptives, steroid hormones or any other drugs which affect the ovarian function for at least 3 months were excluded from the study.

Blood sampling

Five ml of venous blood was collected by trained lab technician or nurse after taking informed written consent from each case and control and blood sample was divided into two portions. 2 ml was collected in sterile EDTA coated vials for genomic DNA extraction and the remaining 3ml was collected in sterile vacutainer plane vials which was centrifuged at 4000 rpm for 5 min for serum seperation. The collected serum was stored at -80ºC till further analysis. Detailed lifestyle habits were collected with predesigned data sheet for cases and controls.

Anthropometric measurement

Anthropometric measurements such as height, weight, waist circumference and hip circumference were measured in cases and controls. Body mass index (BMI) was used as a measure of overall adiposity and Waist-hip ratio (WHR) was used as a measure of intra-abdominal or visceral fat.[13]

Genomic DNA isolation

Genomic DNA was isolated from the collected blood samples by using Phenol-Chloroform method and the isolated DNA was stored at -20ºC for future use.[14]

Genotypic analysis

Novel tetra-primer amplification refractory mutation system-polymerase chain reaction (tetra-primer ARMS-PCR) method was performed for the genotypic analysis of -308G/A (rs1800629) and -174G/C (rs1800795) polymorphisms of TNF-α and IL-6, respectively. Set of four primers, specific to each SNP, were designed by Web based allele specific primer designing tool (WASP) and Primer 1, and synthesized from G. Biosciences (Noida, India).[15,16]

Each PCR reaction was carried out in a total volume of 10 µl, containing 100 ng of template DNA, 10 pmol of each inner primer, 20 pmol of each outer primer and optimized concentration of commercially available mastermix (Sigma, G. Biosciences). The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized using Gel documentation system.

Statistical analysis

BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2).[17] Waist-hip ratio (WHR) was calculated by dividing waist circumference (cm) and hip circumference (cm).

Statistical analysis was performed using IBM SPSS statistics software for Windows, version 26.0 (SPSS Inc. Chicago, IL) and Vassar stats-statistical computation website. Characteristics between cases and controls were assessed and their percentage analysis was done.

Continuous variables were expressed as Mean±Standard deviation (Mean±SD).

The Hardy-Weinberg equilibrium test (χ^2-goodness of fit test) was performed for comparison of observed and expected genotype frequencies in the PCOS and control groups using a web-tool Hardy-Weinberg equilibrium calculator.[18]

Chi-square test (χ^2) was performed to assess the association of genotype and allele frequencies of each SNP between the two groups.

Odds ratio (OR) and 95% confidence interval (CI) were calculated using wild type genotypes or alleles as the reference group.

A p value of <0.05 was considered to be statistically significant. Haplotyping, linkage disequilibrium and SNP-SNP interactions were computed using SNPStats software (2006, Institut Catalad' Oncologia).

RESULTS

Age of 200 PCOS cases was found ranging from 15-45 years with mean age of 26.21±6.37 years while in healthy controls, the age ranges from 15-45 years with mean age of 27.39±5.71 years.

Clinical findings

Among PCOS cases, the percentage of risk factor scoring such as hirsutism, acne and alopecia was observed to be 41.5%, 45.5% and 36% respectively.

On comparison between the two groups, it was observed that hirsutism score and acne score showed highly significant differences (p<0.00001) between PCOS cases and healthy controls whereas alopecia scoring did not reveal any significant differences between the groups.

Table 1: Anthropometric characteristics of the study sample in relation to PCOS.

Anthropometric characteristics	Total subjects (Mean±S.D)	PCOS cases (Mean±S.D)	Controls (Mean±S.D)	P value
Age (years)	26.80±6.07	26.21±6.37	27.39±5.71	0.050
Weight (kg)	61.61±11.62	63.17±11.52	60.06±11.54	0.007*
Height (m)	1.57±0.06	1.57±0.05	1.57±0.06	1.00
Waist circumference (cm)	69.04±11.96	72.86±12.98	65.24±9.45	0.0001**
Hip circumference (cm)	83.58±12.14	86.11±13.15	81.06±10.50	0.0001**
BMI (kg/m²)	25.07±4.52	25.66±4.53	24.48±4.44	0.008*
W/H ratio	0.83±0.06	0.85±0.07	0.80±0.04	0.0001**

*p value < 0.05 (statistically significant); **p value < 0.001 (highly significant)

Anthropometric measurements such as weight, height, Body Mass Index (BMI), waist circumference (WC), hip circumference (HC) and waist hip ratio (WHR) of PCOS cases were compared with control group in Table 1.

Statistically significant (p=0.0001) differences were observed in the mean waist circumference of PCOS cases (72.86±12.98) and healthy controls (65.24±9.45) as well as mean hip circumference of PCOS cases (86.11±13.15) and healthy controls (81.06±10.50) with p=0.0001. Statistically significant differences were observed for BMI (p=0.008) and W/H ratio (p=0.0001) between PCOS cases and healthy controls. There were more overweight (51.50%) and obese (14.50%) PCOS females as compared to healthy controls (29.36%, overweight and 9.45%, obese).

W/H ratio signifies 74% PCOS cases and 34.83% controls to be under health risk (W/H ratio >0.81). Different genotypes of TNF-α (rs1800629) and IL-6 (rs1800795) revealed statistically significant results under different categories of BMI and W/H ratio (Table 2).

Table 2: BMI and W/H ratio distribution for different genotypes of TNF-α and IL-6 in PCOS cases and controls.

Gen-otype	Subj-ects	BMI					W/H ratio			
		Under-weight <18.5 n (%)	Normal ≤18.5-24.9 n (%)	Over-weight ≤25-29.9 n (%)	Obese ≥30 n (%)	P value	Normal ≤0.80 n (%)	Over-weight 0.81-0.84 n (%)	Obese ≥0.85 n (%)	P value
rs1800629										
G/G	**Case**	7 (3.50)	35 (17.50)	54 (27.00)	18 (9.00)	0.003*	29 (14.50)	28 (14.00)	57 (28.50)	0.00001*
	Control	9 (4.48)	70 (34.83)	41 (20.40)	12 (5.97)		96 (47.76)	14 (6.97)	22 (10.95)	
G/A	**Case**	3 (1.50)	20 (10.00)	47 (23.50)	9 (4.50)	0.00005*	18 (9.00)	15 (7.50)	46 (23.00)	0.003*

Continued.

Gen-otype	Subj-ects	BMI				P value	W/H ratio			P value
		Under-weight <18.5 n (%)	Normal ≤18.5-24.9 n (%)	Over-weight ≤25-29.9 n (%)	Obese ≥30 n (%)		Normal ≤0.80 n (%)	Over-weight 0.81-0.84 n (%)	Obese ≥0.85 n (%)	
	Control	2 (0.99)	42 (20.90)	17 (8.46)	5 (2.48)		32 (15.92)	11 (5.47)	23 (11.44)	
A/A	**Case**	0 (0)	3 (1.50)	2 (1.00)	2 (1.00)	-	5 (2.50)	1 (0.50)	1 (0.50)	-
	Control	0 (0)	0 (0)	1 (0.50)	2 (0.99)		3 (1.49)	0 (0)	0 (0)	
rs1800795										
G/G	**Case**	7 (3.50)	41 (20.50)	53 (26.50)	20 (10.00)	0.0001*	19 (9.50)	23 (11.50)	79 (39.50)	0.00001*
	Control	4 (1.99)	64 (31.84)	22 (10.95)	9 (4.48)		60 (29.85)	15 (7.46)	24 (11.94)	
G/C	**Case**	2 (1.00)	15 (7.50)	45 (22.50)	5 (2.50)	0.0001*	32 (16.00)	15 (7.50)	20 (10.00)	0.001*
	Control	7 (3.48)	46 (22.89)	30 (14.92)	3 (1.49)		64 (31.84)	5 (2.49)	17 (8.46)	
C/C	**Case**	1 (0.50)	2 (1.00)	5 (2.50)	4 (2.00)	-	1 (0.50)	6 (3.00)	5 (2.50)	0.121
	Control	0 (0)	5 (2.49)	4 (1.99)	7 (3.48)		7 (3.48)	5 (2.49)	4 (1.99)	

*p value < 0.05 (statistically significant); **p value < 0.001 (highly significant)

Table 3: Genotypic models and allele frequency distribution of rs1800629 and rs1800795 and association with PCOS.

Genotype		PCOS cases n (%)	Controls n (%)	OR (95% CI)	P value
TNF-α rs1800629 (G/A)	G/G	114 (57.00)	132 (65.67)	Ref.	-
	G/A	79 (39.50)	66 (32.84)	1.37 (0.92-2.09)	0.120
	A/A	7 (3.50)	3 (1.49)	2.70 (0.68-10.69)	0.156
Dominant	G/G	114 (57.00)	132 (65.67)	Ref.	0.075
	G/A+A/A	86 (43.00)	69 (34.33)	1.44 (0.96-2.16)	
Recessive	G/G+G/A	193 (96.50)	198 (98.51)	Ref.	0.210
	A/A	7 (3.50)	3 (1.49)	2.39 (0.61-9.39)	
Over dominant	G/G+A/A	121 (60.50)	135 (67.16)	Ref.	0.165
	G/A	79 (39.50)	66 (32.84)	1.34 (0.89-2.01)	
Allele	G	307 (76.75)	330 (82.29)	Ref.	0.050*
	A	93 (32.25)	71 (17.71)	1.41 (1.00-1.99)	
IL-6 rs1800795 (G/C)	G/G	121 (60.50)	99 (49.25)	Ref.	-
	G/C	67 (33.50)	86 (42.79)	0.64 (0.42-0.97)	0.033*
	C/C	12 (6.00)	16 (7.96)	0.61 (0.28-1.36)	0.228
Dominant	G/G	121 (60.50)	99 (49.25)	Ref.	0.024*
	G/C+C/C	79 (39.50)	102 (50.75)	0.63 (0.43-0.94)	
Recessive	G/G+G/C	188 (94.00)	185 (92.04)	Ref.	0.442
	C/C	12 (6.00)	16 (7.96)	0.74 (0.34-1.60)	
Over dominant	G/G+C/C	133 (66.50)	115 (57.21)	Ref.	0.056
	G/C	67 (33.50)	86 (42.79)	0.67 (0.45-1.01)	
Allele	G	309 (77.25)	284 (70.65)	Ref.	0.033*
	C	91 (22.75)	118 (29.35)	0.71 (0.52-0.98)	

n- number of individuals; OR- Odds Ratio calculated at 95% Confidence Interval (CI); Ref.- Reference group (wildtype genotypes or alleles are taken as reference group); Statistically significant p≤ 0.05

Molecular findings

PCOS cases (n=200) and controls (n=201) were genotyped for -308 G/A and -174 G/C polymorphisms of TNF-α and IL-6, respectively. The allele frequencies and genotype frequencies of TNF-α (-308 G/A) rs1800629 and IL-6 (-174 G/C) rs1800795 SNPs in PCOS cases and controls are summarized in Table 3. The genotypes for both the analysed polymorphisms were found to be in Hardy Weinberg equilibrium among PCOS cases and healthy controls. The minor A allele and AA genotype frequency of TNF-α (rs1800629) was found to be higher in PCOS cases as compared to controls (OR=1.41, 95% CI=1.00-1.99; OR=2.70, 95% CI=0.68-10.69) respectively. Frequency distribution of minor allele A was observed to be statistically significant (p=0.05). On the other hand, frequency distribution of genotype AA stands as non-significant (p=0.156). This confirms that AA genotype of TNF-α gene is not associated with the risk of PCOS but minor allele A suggests the increased risk for PCOS. The dominant (OR=1.44, 95% CI=0.96-2.16), over dominant (OR=1.34, 95% CI=0.89-2.01) and recessive (OR=2.39; 95% CI=0.61-9.39) models do not show any association of allele as a risk factor for PCOS. Representative gel picture of TNF-α G/A (rs1800629) gene polymorphism by Tetra primer ARMS PCR with 224 bp (G allele) and 154 bp (A allele) is shown in Figure 1.

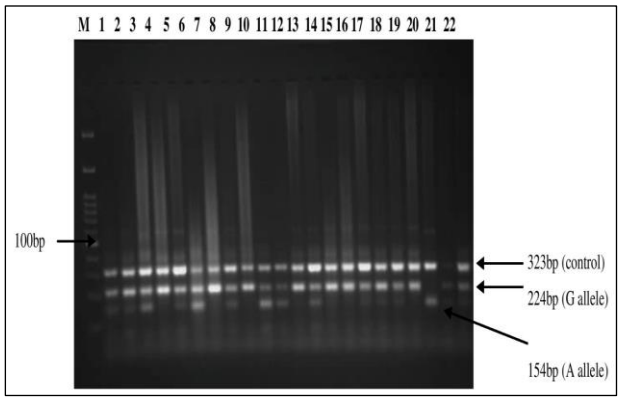

Figure 1: Representative gel picture of TNF-α rs1800629 (G>A) gene polymorphism by tetra-primer ARMS PCR.

The frequency of minor allele C (p=0.033, OR=0.71, 95% CI=0.52-0.98) and heterozygous GC genotype (p=0.033, OR=0.64, 95% CI=0.42-0.97) of IL-6 gene (rs1800795) was found to be higher in controls as compared to PCOS cases with statistically significant differences (p=0.033) were observed.

Computation advocates the association of IL-6 (-174 G/C) with the decreased risk of PCOS as the dominant model (p=0.024, OR=0.63; 95% CI=0.43-0.94) also shows significant association of genotypes with decreased risk of PCOS. However, overdominant (OR=0.67, 95% CI=0.45-1.01) and recessive models (OR=0.74, 95% CI= 0.34-1.60) does not show any association with the risk of PCOS. Representative gel picture of IL-6 (-174 G/C) rs1800795 gene polymorphism by tetra primer ARMS PCR with 205 bp (G allele) and 176 bp (C allele) is shown in Figure 2.

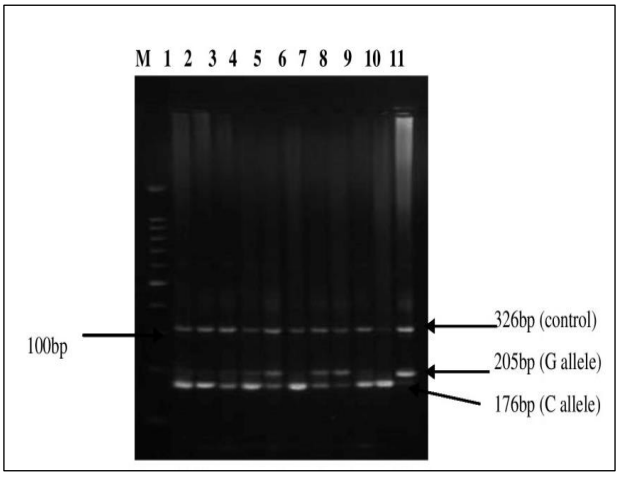

Figure 2: Representative gel picture of IL-6 rs1800795 (G>C) gene polymorphism by tetra-primer ARMS PCR.

Analysis of all possible pair-wise SNP-SNP interactions was done to study the epistatic effects of both the SNPs (Table 4). Statistically significant (Interaction p value=0.014) results for all the possible combinations depicted modulating effects of rs1800629 TNF-α and rs1800795IL-6 with PCOS.

Table 4: SNP-SNP interaction between TNF-α (rs1800629) and IL-6 (rs1800795).

Geno-types	G/G			G/C			C/C		
	PCOS cases n (%)	Controls n (%)	OR (95%CI)	PCOS cases n (%)	Controls n (%)	OR (95% CI)	PCOS cases n (%)	Controls n (%)	OR (95%CI)
G/G	63 (31.50)	74 (36.81)	1.00	44 (22.00)	46 (22.89)	1.12 (0.66-1.91)	7 (3.50)	12 (5.97)	0.69 (0.25-1.85)
G/A	54 (27.00)	24 (11.94)	2.64 (1.47-4.75)	21 (10.50)	39 (19.40)	0.63 (0.34-1.19)	4 (2.00)	3 (1.49)	1.57 (0.34-7.26)
A/A	4 (2.00)	1 (0.50)	4.70 (0.51-43.13)	2 (1.00)	1 (0.50)	2.35 (0.21-26.52)	1 (0.50)	1 (0.50)	1.17 (0.07-19.16)
Interaction p value: 0.014*									

*p value < 0.05 (statistically significant); **p value<0.001 (highly significant)

Table 5: Possible haplotype frequencies of cytokines in PCOS cases and controls.

Haplotype	rs1800629	rs1800795	PCOS cases (%)	Controls (%)	OR (95% CI)	P value
HT 1	G	G	58.10	61.32	1.00	-
HT 2	G	C	18.65	20.77	0.93 (0.63-1.39)	0.73
HT 3	A	G	19.15	9.33	2.22 (1.31-3.78)	0.003*
HT 4	A	C	4.10	8.58	0.55 (0.26-1.14)	0.11
Global haplotype association p value: 0.002*						

Note: *p value < 0.05 (statistically significant); **p value < 0.001 (highly significant).

Haplotype frequencies of PCOS cases and controls are summarized in Table 5. The pairwise LD matrix demonstrated that nearly complete LD (D>0<1) existed between the polymorphism of rs1800629 TNF-α and rs1800795 IL-6 genes. The presence of four haplotypes was observed with the estimated frequency of ≥1. The frequency of HT3 was more in PCOS cases than controls which indicates that haplotype HT3 AG (OR=2.22, 95% CI=1.31-3.78) acts as a risk factor for PCOS and thus confirming its association with increased risk of PCOS.

DISCUSSION

Evidence proved that PCOS is taking lead as a major cause of infertility in females around the world due to its association with hyperandrogenism, insulin resistance and oxidative stress. Clinical manifestations include menstrual disturbances, hirsutism, acne, alopecia, polycystic ovarian morphology, obesity, metabolic disorders, infertility, psychological disorders, etc.[19]

of multiple intrinsic genes with various external environmental factors are supposed to play contributing factors in the pathogenesis of PCOS.[20] Chronic inflammatory markers such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) promote insulin resistance and hyperandrogenism and therefore implicated in the pathophysiology of PCOS.[6,21] TNF-α modulates several biological processes in human ovary such as granulose cell proliferation, follicular development, ovulation and steroidogenesis. Gene expression of TNF-α is regulated at both the transcriptional and post-transcriptional levels.[22] IL-6 plays an important role in reproductive physiology, ovarian steroid production, fertilization and implantation. -174 G/C polymorphism in the promoter region of IL-6 has been shown to affect the gene's transcription rate.[23]

Multivariate logistic regression revealed significant association of BMI and W/H ratio towards PCOS. Statistically significant association was observed in rs1800629 TNF-α and rs1800795 IL-6 with PCOS among different categories of BMI and W/H ratio which reveals its important role in the pathogenesis of the disease.

Punjab has highest obesity rate (29.9%) in India followed by Kerala (28.1%) and Delhi (26.4%).[24] This highlights the issue of concern among obese females of Punjab as they are more prone to obesity related complications such as type 2 diabetes, hypertension, cardiovascular diseases and various metabolic disorders which in turn leads to the development of PCOS, but non-obese are also at risk of having PCOS.[25]

In the present study, prevalence of overweight and obesity is seen more in PCOS females in comparison to healthy controls. Present results are in line with few previous studies related to PCOS in North Indian females which might be due to consumption of high calorie diet, junk food, physical inactivity and sedentary lifestyle occurring as a result of professional and westernized lifestyle of women in different parts of India.[26-29] On the other hand, according to few studies done in Tanzania, China and Pakistan, non-significant differences were observed for overweight and obesity among PCOS cases and controls.[30-32] This might be due to differences in diet and lifestyle in these countries in comparison to North India.

Molecular analysis revealed that minor allele A of -308 G/A TNF-α was associated with increased risk of PCOS (p=0.05, OR=1.41, 95% CI=1.00-1.99). Our results are in accordance with the meta-analysis done by Zhang et al which revealed -308 G/A polymorphism was also found to be significantly associated with PCOS among Asians (allele comparison: OR=1.50, p=0.05).[33] Another study conducted in Hyderabad, India suggested that the TNF-α might contribute to the pathogenesis of hyperandrogenism, obesity and insulin resistance in PCOS females independent of a polymorphism of the TNF-α.[27] While few findings may not be associated with PCOS risk.[22,34-37] 174 G/C polymorphism of IL-6 gene was found to be in association with the decreased risk of PCOS (p=0.033, OR=0.71, 95% CI=0.52-0.98) with only 0.71 risk but significant association of rs1800795, thus indicating the protective role of IL-6 for PCOS.

Our results substantiate the early findings done by Vural et al which indicates lower frequency of IL-6 CC genotype and C allele among PCOS females according to healthy controls and thus conferring the decreased risk to PCOS (OR= 0.66, 95% CI= 0.41-1.05) whereas meta-analyses compiling 5 studies also proves significant association of IL-6 -174 G/C polymorphism with PCOS in Caucasians (recessive comparison: OR=0.58, p=0.04) showing its protective role but pooled analyses did not showed significant association (p>0.05).[33,38] Findings of Azeez et al shows increased risk of -174G/C IL-6 considering CC genotype as a risk factor (OR=1.58, CI=0.16-15.36) with susceptibility to PCOS.[37] To validate the present findings, further studies are required with large sample size.

CONCLUSION

Present study on association of -308G/A (rs1800629) TNF-α and -174G/C (rs1800795) IL-6 gene polymorphism in North Indian females reported that these genes and haplotypes might contribute to the pathogenesis of PCOS. These SNP may act as a potential markers in determining the genetic susceptibility of PCOS. Association of more genetic markers through case-control studies should be done in order to determine the exact effect of these cytokine genes in the progression of a complex disease such as PCOS.

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Ethical approval: The study was approved by the Institutional Ethics Committee of Punjabi University, Patiala vide letter number IEC 2017/10

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