Cytokine 114 (2019) 86–91

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Evaluating the association of TNF α promoter haplotype with its serum levels and the risk of PCOS: A case control study

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ARTICLE INFO

Keywords:
PCOS
TNF α
Inflammation
Polymorphism
Haplotype
Linkage disequilibrium

ABSTRACT

Poly cystic ovary syndrome is the major cause of anovulatory infertility. TNF α, pro-inflammatory cytokine is associated with obesity, insulin resistance and hyperandrogenism, therefore in present study we tried to find the association between TNF α promoter polymorphisms, TNF α levels and the risk of PCOS. Present case control study was carried on 400 women of age 16–40 years. TNF α levels were measured by ELISA whereas promoter polymorphisms were evaluated by PCR-RFLP. Haplotype and Linkage disequilibrium analysis was also done. TNF α level was significantly higher in PCOS group (13.24 ± 9.78) than control (5.5 ± 3.8). Haplotype analysis revealed that GGTCT, AGTCT, AGCCT and AACCT are the susceptible haplotypes associated with TNF α level. rs361525 and rs1799964 were found to be associated with the risk of PCOS (p = 0.0006, 0.015). GGCCT, AATAT, GATAT (most susceptible), AGCCT, GGTCT and GATCT are the susceptible haplotypes for PCOS. Significant difference between TNF α levels in PCOS and Control group suggest it's one of the promising candidates for the marker of inflammation (sensitivity and specificity 91.23 and 94.56% respectively, with area under the curve 0.907 at 95% CI 0.8723–0.9512). Presence of GGCCT haplotype suggests the susceptibility towards PCOS which needs to be further verified. In addition to this, present study not only provides a pavement for the diagnosis, but also monitoring and management of PCOS too.

1. Introduction

Infertility has emerged out as a new epidemic of young India. According to a survey, it affects one in seven couples and witnessing a continuous increase in its frequency [1]. Poly Cystic Ovary Syndrome (PCOS) being the major clinical factor behind this social stigmatic condition in young women is more than a reproductive disease, associated with a wide range of metabolic disorders, such as glucose intolerance, diabetes, dyslipidemia, hypertension, hepatic steatosis [2–6].

There is wide clinical heterogeneity in the representation of syndrome, but it is well established that PCOS is a chronic inflammatory condition [2]. Women with PCOS are predisposed to increased visceral adiposity. Obese women are prone to have menstrual irregularity and anvolatory infertility than normal-weight women [3]. The presence of increased visceral adipose tissue is associated with insulin resistance, hyperglycemia and dyslipidemia that are co-morbidities associated with PCOS. The excess of macronutrients in the adipose tissues, stimulates them to release inflammatory mediators such as tumor necrosis factor α and interleukin 6, and reduces production of adiponectin, predisposing to a pro-inflammatory state and oxidative stress [5,6].

Studies on the imbalance between pro- and anti-inflammatory cytokines and their associated genetic alterations may help in understanding and management of chronic low grade inflammatory conditions like PCOS [7]. Inconspicuous picture of genetic and epigenetic alterations related to inflammation-related genes somehow contribute to the etiology of PCOS which needs to be explored. TNF α, a major proinflammtory cytokine plays a central role in inflammation and generation of many disorders. TNF α induces hyperandrogenism by down regulating sex hormone binding globulin [8]. On the other hand it also has been reported that TNF α phosphorylates insulin receptor and had a central role in insulin resistance [9]. A number of studies suggest the association of TNF α with obesity, metabolic syndrome and reproductive implications found in PCOS [10].

Levels of TNF α has been studied in a number of disorders [8–12]. Increased level of TNF-α is also linked with conditions such as obesity and insulin resistance [11,12]. Till date, there is no such study carried out in PCOS patients checking the association of all the SNPs of promoter region in TNF α gene and relating them to its levels. In the present analysis, we haven't only compared TNF α levels in PCOS and Controls but also in various phenotypes of PCOS. Genotype distribution

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https://doi.org/10.1016/j.cyto.2018.11.004
Received 29 August 2018; Received in revised form 15 October 2018; Accepted 5 November 2018
Available online 13 November 2018

of TNF α [−238 (G/A; rs361525), −308 (G/A; rs1800629), −857 (C/T; rs1799724), −863 (C/A; rs1800630) and −1031 (T/C; rs1799964)] was also determined to find their association with PCOS susceptibility. In addition to this linkage disequilibrium analysis was also carried out to find the most susceptible haplotype associated with altered TNF α levels and the PCOS risk.

2. Materials and methods

2.1. Study population

A Case control study was carried out in which 200 PCOS and 200 normo- ovulatory women of reproductive age (16–40) were included. Rotterdam criterion was used for the diagnosis of the syndrome, which requires at least two of the following features: (i) oligo-ovulation or chronic anovulation, (ii) clinical and/or biochemical hyperandrogenism, and (iii) ultrasound appearance of polycystic ovaries.

Oligo-ovulation was considered if a woman had less than 8 cycles per year or a prolonged cycle of ≥36 days. Hyperandrogenism was confirmed by the elevation of testosterone level, the presence of acne and/ or hirsutism with a modified Ferriman- Gallway score (more than 8). Ovaries were considered as polycystic ovaries by the presence of more than 12 follicles per ovary with diameter 2–9 mm and increased ovarian volume > 10 ml.

Both PCOS and healthy control were not having any of exclusion criteria such as androgen-producing tumors, hyper prolactinemia, active thyroid disease, Cushing's syndrome. None of the subjects were on any medication known to affect carbohydrate metabolism or endocrine parameters for at least 3 months before entering the study.

Study procedure was approved by the Institutional Ethical Committee and follows the Declaration of Helsinki. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

2.2. Sample collection

Blood sample was used for TNF α levels measurement and polymorphism analysis. Out of 3 ml of blood, 1 ml was used to measure TNF α levels and remaining 2 ml was processed for DNA isolation.

2.3. Clinical–biochemical characterization

After signing of a written informed consent, two blood pressure readings were obtained in sitting position after an interval of 10-min; the mean was determined from two values and was used for further analysis. Weight (in kgs) and height (in cm) were measured. Waist circumference was measured in standing position, at the half of the distance between the lower ribs and the crest of the pelvis. Hip circumference was measured as the widest gluteal circumference.

Basal blood samples for lipid spectrum, glucose, hormonal profiling were processed at PGIMS, Rohtak. Lipid profile along with sugar was done by Auto analyzer whereas LH, FSH, Testosterone, Prolactin was done by chemiluminiscence analyzer.

Number of cysts and ovarian volume was predicted by ultrasonography by expert sonologist at PGIMS, Rohtak.

2.4. Subject classification

Both PCOS and healthy controls were recruited in different subgroups on the basis of their phenotype and BMI. On the basis of Rotterdam criteria, four different phenotypes were assigned i.e.

- Group1 hyperandrogenism with oligo-anovulation (H + O)
- Group2, polycystic ovary with oligo-anovulation (P + O)
- Group3, hyperandrogenism with polycystic ovary (H + P) and
- Group4, presence of all the three features (P + H + O).

Body Mass Index (BMI) was derived from booking weight (kilograms) and height (meters). Based on the revised consensus guidelines for India, patients and their respective controls were categorized as underweight (< 18.5 kg/m^2), normal or lean BMI (18.5–22.9 kg/m^2), overweight (23.0–24.9 kg/m^2) and obese (≥25 kg/m^2).

2.5. Study design

- Measurement of TNF α levels in PCOS and control group
- Detection of TNF α promoter polymorphism in PCOS and control group
- Association of haplotype with TNF α levels
- Association of haplotype with PCOS susceptibility

Serum level of TNF α was measured by Diaclone Human TNFα ELISA kit as per manufacture's protocol. The intra assay and inter assay coefficient of variation was 3.2 and 10.9%, respectively. Five different promoter SNPs (rs361525**,** rs1800629, rs1799724, rs1800630, rs1799964) was studied in the present study. Genomic DNA from blood was isolated by the Genetix MiniPrep DNA isolation kit. DNA was stored in elution buffer supplied in kits and subjected to purity check by taking the absorbance ratio at A260/A280 (NanoDrop™ Thermo Scientific). Extracted DNA was used for genotyping of the restriction variants. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype all five promoter polymorphisms of TNF α gene. The primers used for genotyping, annealing temperature, size of amplicon, restriction enzyme used for RFLP and the

Table 1
Primer, annealing temperature, PCR and digested product Size of TNF α promoter polymorphism.

S. No.	SNP ID	Primer	Annealing Temp	Size of PCR product	Restriction enzyme used	Restriction digestion pattern
1	rs361525	CTGTCCCAGGCTTGTCCTGCTAC CTCACACTCCCCATCCTCCC**G**G**A**TC	64.4	376	*Bam*HI	GG:352bp & 24bp GA: 376, 352bp & 24bp AA:376bp
2	rs1800629	GAGGCAATAGGTTTTGAGGG**C**CAT TCTGCTGTCCTTGCTGAGGGA	62	360	*Nco*I	GG: 339bp & 21bp GA:360,339,21bp AA:360bp
3	rs1799724	GCATCTGCACCCTCGATGAAG CCTCTACATGGCCCTGTCTAC	58	325	*Tai*I	CC:306bp & 19bp CT: 325, 306bp & 19bp TT:325bp
4	rs1800630	GCTCAAAGGGAGCAAGAGCTG CTACATGGCCCTGTCTTCGTTACG	67	323	*Tai*I	CC:302bp & 21 bp CA:323, 302bp & 21 bp AA:323bp
5	rs1799964	TATGTGATGGACTCACCAGG CCTCTACATGGCCCTGTCTT	63	264	BbsI	TT:251bp &13bp TC: 264, 251bp &13bp CC:264bp

size of digested product is mentioned in Table 1. Respective fragment having polymorphism polymorphism was amplified by PCR (Applied Biosystems) using 2 μl of genomic DNA with 1 μl of both the forward and reverse primers, 2 μl of 25 mM $MgCl_2$, 2 μl of dNTPs and 1 μl of Taq polymerase with 5 μl of buffer supplied with Taq polymerase and make up to 50 μl by the nuclease free water. PCR conditions used for amplification were initial Denaturation at 95 °C for 5 min followed by 30 cycles of denaturation for 30 s, primer specific annealing temperature at for 30 s and extension for 1 min at 72 °C followed by a final extension for 10 min at 72 °C. Amplified PCR products were visualized on 2% agarose gel and were subjected to RFLP analysis by sequence specific enzyme (Table 1).

Amplified products (10 μl) were digested with 1 U of the corresponding restriction enzyme in a total reaction volume of 30 μl as per the manufacturer's instruction. The digestion products with 50 base pair DNA ladder were resolved on 3% agarose gels stained with ethidium bromide and visualized under Gel Doc™.

2.6. Statistical analysis

Chi square analysis was performed to evaluate the distribution of the genotypes and allele frequencies of TNF α promoter polymorphisms in patients and control subjects using Prism software (Graphpad software Inc; San Diego CA, USA, 2003). P values < 0.01 were considered as statistically significant. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Haplotype analysis was carried out using SNPstat software (http://bioinfo.iconcologia.net/snpstats/start.htm). Linkage disequilibrium plot was constructed by HaploView 4.2.

3. Results

3.1. Study population

Four hundred women were enrolled in the study out of which 200 were having PCOS and 200 were controls. Patients were further segregated on the Rotterdam criteria and categorized into four groups.

Another criterion for the segregation was BMI. Anthropometric data including age, height, weight, blood pressure, physical measurement, personal details along with blood sample was collected. All the biochemical and anthropometric variables are summarized in Table 2. No significant difference was found among subjects in terms of age, height and blood pressure. Glucose and CRP levels were significantly higher in the PCOS group than their controls. Statistical significant difference also exists in terms of lipid profile of both the PCOS and control group. Serum triglyceride and LDL- cholesterol was higher in PCOS group whereas HDL-cholesterol was higher in control group than PCOS group (Table 2).

3.2. TNF α levels

The TNF a level was measured in 200 PCOS patients and 200 controls. Significantly increased serum TNF α levels were found in PCOS patients (13.24 ± 9.78) as compared to controls (5.5 ± 3.8; p < 0.0001). Moreover, when the patient subgroups were analyzed with respect to TNF α levels, patients with all the three features had significantly higher TNF α levels as compared other subgroups (p = 0.014) (Supplemental Fig. S1).

In BMI based groups, obese group was found to have significantly higher levels of TNF α than lean subgroup despite of disease status. Furthermore, hyperandrogenic PCOS subjects were found to have higher levels of TNF α than their non hyperandrogenic counterpart (Supplemental Fig. S1).

Table 2
Anthropometric and biochemical data of PCOS and healthy controls.

S. No.	Parameter	PCOS (n = 200)	Control (n = 200)	P value
Anthropometric data				
	Age	25.45 ± 3.27	25.34 ± 4.53	0.7808
	Height	1.63 ± 0.06	1.62 ± 0.05	0.1179
	BMI (kg/m^2)	23.41 ± 3.39	21.02 ± 3.73	< 0.0001
	BP(systolic)	115.26 ± 8.97	114.89 ± 9.27	0.6852
	BP(dystolic)	74.74 ± 10.52	75.62 ± 11.23	0.4191
Biochemical data				
	Fasting glucose (mmol/dl)	6.55 ± 1.31	5.25 ± 0.45	< 0.0001
	Triglyceride	1.44 ± 0.87	0.82 ± 0.36	< 0.0001
	HDL-C (mmol/dl)	1.82 ± 0.21	1.96 ± 0.29	< 0.0001
	TNF α (pg/ml)	13.24 ± 9.78	5.5 ± 3.8	< 0.0001
	CRP (mg/l)	1.32 ± 0.27	0.49 ± 0.13	< 0.0001
Hormonal data				
	TSH (μIU/ml)	2.62 ± 1.35	2.98 ± 1.49	0.0117
	FSH (mIU/ml)	5.77 ± 2.35	5.00 ± 2.81	0.0031
	LH (mIU/ml)	7.50 ± 0.71	3.49 ± 2.21	< 0.0001
	Free testosterone (nmol/ml)	0.95 ± 0.31	0.35 ± 0.19	< 0.0001
	Prolactin (ng/ml)	15.75 ± 9.81	19.67 ± 11.31	0.00002
	SHBG (nmol/l)	34.9 ± 24.3	59.7 ± 26.7	< 0.001

Abbreviations: BMI body mass index; BP: Blood Pressure; HDL-C: High Density Lipoprotein; CRP: C Reactive Protein; TSH: Thyroid Stimulating Hormone; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; SHBG: Sex Hormone Binding Globulin.

3.3. Association of TNF α promoter polymorphisms with PCOS

Five different restriction sites for (rs361525, rs1800629, rs1800630, rs1799724 and rs1799964) TNF α promoter were amplified and five different amplicon (376,360,325,323,251 bp) were obtained. All the amplified were subjected to restriction digestion and their digested products are given in Table 1. Three different genotypes were assigned on the basis of restriction digestion for the respective SNP (Supplemental Fig. S2 a–e). The genotype and allele frequencies of the investigated TNF α promoter polymorphisms in 200 PCOS patients and 200 controls are summarized in Table 3. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg equilibrium in both patient and control groups (Table 3)). The two promoter polymorphisms (rs361525 and rs1799964) of TNF α were found to be in significant association with PCOS patients (p = 0.0006 and p = 0.015 respectively) when genotypes were compared using chi-squared test.

PCOS patients were segregated on the basis Rotterdam and BMI criteria and genotype frequency was measured in the subgroups also. It has been found that the phenotypic groups differ significantly in genotypic and allelic frequency distribution, however this distribution needs to check in cohort having equal number of subjects (Supplemental Table 1, S2).

3.4. Haplotype analysis and LD (Linkage Disequilibrium) analysis of TNF α promoter polymorphisms

The LD analysis revealed that the five promoter polymorphisms investigated in the TNF α showed variation in LD association in PCOS patients. Considerable association was found in rs1800629, rs1799724 and rs1799964 (Fig. 1). Moderate association was found in rs361525 and rs1799964 with D′ = 0.479 in patients, respectively (Supplemental Table 3). A haplotype evaluation of the five polymorphic sites was performed and the estimated frequencies of the haplotypes were differed significantly between PCOS patients and controls (global p < 0.0001). The susceptible haplotypes: GGCCT, AATAT, GATAT, AGCCT, GGTCT and GATCT were more frequently observed in PCOS patients as compared to controls and were found to increase the risk of

Table 3
Allelic and genotype frequencies of TNF-alpha promoter polymorphism in PCOS and control population.

Model		Patients	Controls	P value	P for HWE	ODD ratios(95%CI)
	rs361525					
Co-dominant	G/G	148(74%)	117(58.5%)	0.0006	0.54(P) < 0.0001(C)	0.48 (0.31–0.73)
	G/A	50(25%)	83(41.5%)			
	A/A	2(1%)	00			
Dominant	G/G	148(74%)	117(58.5%)	0.001		0.50(0.320.76)
	G/A- A/A	50(26%)	83(41.5%)			
Recessive	G/G- G/A	200(100%)	198(99%)			
	A/A	0(0%)	2(1%)	0.095		
Over-dominant	G/G- A/A	150(75%)	117(58.5%)	0.0004		0.47 (0.31–0.72)
	G/A	50(25%)	83(41.5%)	0.069		1.40 (0.93–2.11)
	rs1800629					
Co-dominant	G/G	121(60.5%)	135(67.5%)		0.078(P)	
	G/A	79(39.5)	63(31.5)		< 0.0001(C)	
	A/A	00	2(1%)			
Dominant	G/G	121(60.5%)	135(67.5)	0.14		1.36 (0.90–2.04)
	G/A- A/A	79(39.5)	65(32.5)			
Recessive	G/G- G/A	200(100%)	198(99%)	0.095		–
	A/A	0(0%)	2(1)			
Over-dominant	G/G- A/A	121(60.5%)	137(68.5)	0.94		1.42 (0.94–2.14)
	G/A	79(39.5)	63(31.5)			
	rs1799724					
	C/C	120(60)	136(68)	0.095	< 0.0001(P) 0.0032(C)	1.42 (0.94–2.14)
	C/T	80(40)	64(32)			
	rs1800630					
	C/C	128(64)	135(7.5)	0.46	0.0054(P) 0.0031(C)	1.17 (0.77–1.77)
	C/A	72(36)	65(32.5)			
	rs1799964					
Co-dominant	T/T	152(76)	129(64.5)	0.015	0.084(P) 0.031(C)	0.59 (0.38–0.91)
	T/C	48(24)	69(34.5)			
	C/C	0	2(1)			
Dominant	T/T	152(76)	129(64.5)	0.012		0.57 (0.37–0.89)
	T/C- C/C	48(24)	71(35.5)			
Recessive	T/T-T/C	200(1 0 0)	198(99)	0.095		–
	C/C	0	2(1)			
Over-dominant	T/T-C/C	152(76)	131(65.5)	0.021		0.60 (0.39–0.93)
	T/C	48(24)	69(34.5)			

P value: P value for association, HWE: Hardy Weinberg Equilibrium, P: Patient, C: Control.

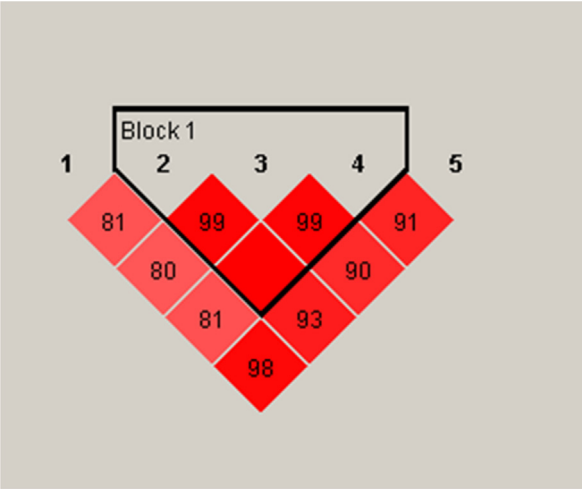

Fig. 1. Linkage Disequilibrium plot for 5 promoter polymorphism of TNF α gene. Block 1 showed that GCC is in close linkage with a score of 0.816.

PCOS as suggested by odds ratio (Supplemental Table 4, S5). However, the non-susceptible haplotypes: GACCC, AACAC were more frequently observed in controls as compared to PCOS patients. GGCCT was found to be most susceptible haplotype associated with PCOS.

3.5. Effect of polymorphisms on TNF α levels

ψ^2 analysis was used to find the association between the genotypes, haplotypes and levels of TNF alpha. TNF α levels were compared with the promoter polymorphisms to reveal any functional association. Genotypes associated with increased TNF α levels were shown in Supplemental Fig. 3. In PCOS patients, the TNF α haplotypes: GGTCT, AGTCT, AGCCT and AACCT were found to increase TNF α levels ($p = 0.013$, $p = 0.009$, $p = 0.002$ and $p = 0.001$ respectively) with susceptible alleles (-238A, -308A, -857T and -1031C) as compared to controls (Supplemental Fig. S3)

4. Discussion

A number of evidences not only proves the association of obesity [13,14] and circulating levels of TNF α but also with the insulin resistance [9,15] and SHBG [16,17]. TNF α down regulates the sex hormone binding globulin, which leads to increased Testosterone thereby favors hyperandrogenism and generates insulin resistance by phosphorylation of the insulin receptor. In addition to this, obesity and insulin resistance are the co-morbidities associated with the syndrome and TNF α aggravates these conditions, thereby suggesting its role in

the pathophysiology of the syndrome. By our result, we found that circulating levels of TNF α level were higher in PCOS group (13.24 ± 9.78) that differentiates them from their controls (5.5 ± 3.8). Receivers operating curve predicted that TNF α as a better marker of inflammation in study subjects. It has been found that marker has a sensitivity and specificity 91.23 and 94.56% respectively, with area under the curve 0.907 (95% CI 0.8723–0.9512). The TNF a level was higher in the obese group (13.98 ± 10.24) than their lean counterparts (3.19 ± 1.8). TNF α levels were also found to be highest in phenotype having all the three characteristics of syndrome i.e. P + H + O (14.54 ± 11.25) followed by phenotype having O + H (13.78 ± 10.52), H + P (13.08 ± 11.36) and least in O + P (12.86 ± 11.43) associated phenotype. This provides an insight about the severity of syndrome and association of hyperandrogenism with its levels. Hence increased TNF α levels can be used as a susceptibility indicator of hyperandrogenism and suggest that monitoring of TNF α levels may help better management of syndrome.

SNPs associated with promoter region may have the potential to modulate the expression of a gene. The present study was done to find the association of five different promoter polymorphisms with TNF α levels and PCOS risk. Haplotype analysis revealed that GGTCT, AGTCT, AGCCT and AACCT are the susceptible haplotypes associated with TNF α level. A number of evidences in literature are in line with our findings. According to the study of Bayley et al. rs361525 'A' allele was found to increase expression of TNF α in transfected B cells. rs361525 codes for -238 position of TNF α gene which lies in the repressor region [18]. Replacement of native G allele by A allele increase the expression of TNF α gene by 1.4 to 1.8-fold in U937 and Raji cells. Their findings suggest the importance of rs361525 in the regulation of TNF α. Another study proposed rs1800629 role in TNF α regulation as this site was also found to increase the level of TNF α when mutant allele A is present [19].

All the polymorphism except -863 C/A was found to be positively correlated with levels of TNF α whereas -863 C/A was negatively correlated. This can be further supported by the study of Ladha et al which conducted the analysis of TNF α level on both transcription as well as translational levels [19]. The result suggested -238,-308,-857 and -1031 polymorphism increases the transcription of TNF α and resulted increase in TNF α levels, whereas -863 C/A polymorphism decreases the transcription of TNF α and resulted in decrease TNF α levels.

Association of polymorphisms was also checked with the susceptibility of PCOS. Out of five studied promoter polymorphisms, three have been studied in PCOS group. To the best of our knowledge, there is no study exploring the association of rs361525 and rs1799724 till date in PCOS. By our study, we found that rs361525 and rs1799964 are associated with the susceptibility of PCOS whereas no significant association was found in PCOS and rs1800629, rs1800630 and rs1799724. Furthermore, rs361525 and rs1799964 were found to increase the risk of PCOS by 14 and 6 fold respectively (by risk ratios analysis. Our results are in line with other studies done previously. On the other hand remaining three polymorphisms (rs1800629, rs1799724 and rs1800630) was not found to be associated with PCOS. However, no significant deviation was found from the Hardy Weinberg equilibrium in all the promoter sites under study. Haplotype analysis was done to find the most susceptible haplotype associated with the PCOS and it was found that presence of haplotypes GGCCT, AATAC, GATAT, and AGCCT were associated with increased risk of PCOS. GGCCT genotype was found to be most susceptible haplotype for the development of PCOS risk.

5. Conclusion

Presence of higher TNF α levels in PCOS and control group makes one of the promising candidates for the susceptibility marker of PCOS. Difference of TNF α levels in different PCOS Phenotype makes it not only diagnostics, but also a differential screening marker as well. Haplotype analysis also helps to detect the on risk subjects in its native stage. This data not only help to diagnose the syndrome, but also in monitoring and management of the syndrome.

Acknowledgement

The authors acknowledge Centre for Medical Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India and DBT-HRD for providing research work platform. This work was supported by INSPIRE division of the Department of Science and Technology in the form of a research fellowship.

Conflicts of interest

The authors report no conflict of interest.

Details of ethical approval

This work was approved by the Institute Human Ethics Committee of MDU, Rohtak, Haryana, India (IHEC/2016/83) on 17-May-2016.

Funding

No funding for research, writing assistance and peer review

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.11.004.

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