Original Research Article

Genotype- phenotype correlations of IL-6 gene in polycystic ovarian disease (PCOS)

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Abstract

Background: Polycystic ovarian syndrome (PCOS) is one of the commonest endocrine disorders amongst women of reproductive age group. Polymorphisms in the in the *IL-6* gene have been associated various related diseases ranging to Obesity, PCOS and infertility. In this context, we evaluated association between promoter polymorphism (-174G/C) and the levels of IL-6 in PCOS. **Material and methods:** This is inter-disciplinary study conducted by collaboration between a tertiary care endocrinology hospital, biochemistry department of a teaching medical institute and genetics lab. In this prospective study involving 100 PCOS patients with 100 age matched controls, we employed 1 set of primer and screened the known single nucleotide polymorphism *IL-6* gene. Apart from qualitative and quantitative evaluation, linkage disequilibrium, multifactor dimensionality reduction analysis and In-silico analysis were performed. **Results:** The percentage of AA, AG and GG genotypes in patients was 15, 56, 29 while it was 30, 50 and 20 in controls respectively. Relative risk analysis of the LEPR 668 A/G polymorphism revealed a threefold risk for the “AG” and “GG” genotypes under the codominant model of inheritance (OR= 2.24, CI= 1.02 – 4.96, p= 0.04 and OR= 2.9, CI= 1.15 – 7.38, p= 0.02 respectively). Similarly, a twofold risk was also observed for the “AG+GG” genotypes under the dominant model of inheritance (OR= 2.43, CI= 1.15 – 5.17, p=0.018). When relative risk for the alleles demonstrated a twofold risk of G allele towards disease establishment (OR= 1.62, CI= 1.07 – 2.45, p= 0.021). **Conclusions:** Based on the observations in the present study could not establish it’s role in incidence and progression of PCOS.

Keywords: PCOS; Rotterdam criteria; IL-6; Cytokines; Infertility

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INTRODUCTION

IL-6 gene encodes a cytokine that functions in inflammation and the maturation of B cells. *IL-6* is a 5 kb gene with five exons and four introns. IL-6 is produced by the T cells, B cells, monocytes, fibrocytes and fat cells[1]. The protein is largely produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor alpha. The IL-6 receptor consists of two heterodimeric subunits, IL6R-a (gp80) and IL6R-b (gp130)[2]. The operation of this gene is linked to a wide variety of inflammation-associated disease states, that including susceptibility to diabetes mellitus and systemic juvenile rheumatoid arthritis. The serum level of *IL-6* is found to be higher in patients with PCOS than in normal controls[3]. Hence, in the present study we have tried to identify the association between a promoter polymorphism (-174G/C) and the levels of IL-6 in PCOS.

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MATERIAL AND METHODS

The study was conducted on 100 PCOS and 100 healthy control women, recruited from patients visiting tertiary care hospital. PCOS participants were selected based on observation of oligoamenorrhea/anovulation, clinical or biochemical evidence of hyperandrogenism and/or polycystic ovaries on ultrasonography[4]. 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. Exclusion criteria were 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. Participant's age group was in the range from 18-35yrs. The study was approved by the Institutional Ethical Committee (011/02/2015 IEC / Saveetha University Dated12-02-2015). A written informed consent was collected from all the subjects enrolled in the study. Participant's history and other anthropometric assessments were carried out. Sample collection and requirement: Blood samples were collected from participants by venipuncture and it was processed within two hours. Then the samples were centrifuged at 3000 rpm for 10 minutes at 20°c to isolate the serum and it was stored -20°C until used. Molecular studies Genotyping study was carried out for modifier genes by Allele Specific-PCR and PCR based Restriction fragment length polymorphism analyses (RFLP).

Genomic DNA Isolation

5 ml of whole blood from controls and subjects was obtained for genomic DNA isolation. In cases/subjects with insufficient amount of blood sample drawn, DNA isolation by Rapid genomic DNA extraction (RGDE) was also carried out. The isolated DNA was considered for mutational screening by PCR based SSCP and for genotyping studies, PCR based RFLP analyses was adopted for the following gene/s. DNA concentration and purity was checked on 0.8% agarose gel or by spectrophotometer. The samples were then stored at -20^{0}C for subsequent analysis. Isolation of DNA following Rapid genomic DNA extraction Genomic DNA of high quality and quantity can be obtained in the shortest time and with just 500µl of blood sample by following this protocol.

Quantification of DNA

DNA was quantified by measuring the absorbance values at 260 and 280nm in a NANOVIEW (GE Healthcare). The ratio of 260/280 nm was observed and the purity of DNA was checked. The isolated genomic DNA was later used for PCR-SSCP and PCR-RFLP analyses. In-vitro amplification of gene of interest by Polymerase chain reaction (PCR) Amplifying a gene of interest was done by PCR using specific primers obtained from published reports under appropriate cycling conditions of denaturation, annealing and extension in a Thermal cycler (Eppendorf, Germany).

PCR-RFLP

PCR-restriction fragment length polymorphism (PCR-RFLP) is one among the various popular techniques utilized for genotyping single nucleotide polymorphisms. The essence of this technique is the exploitation of the fact that SNPs and micro-indels often end up creating or abolishing restriction enzyme recognition sites. The technique involves the amplification of the target sequence containing the variation. The amplified fragment is then treated with an appropriate restriction enzyme. The presence or absence of the restriction site would result in variant fragment sizes which later can be resolved using electrophoresis.

Molecular Analysis

PCR conditions followed for -174 G/C were, initial denaturation at 95^{0}C for 5 min, denaturation step at 95^{0}C for 30 sec, annealing at 58^{0}C for 30 sec, extension at 72^{0}C for 1 min for 30 cycles. Final extension was carried out at 72^{0}C for 7 min. The PCR products were subjected to RFLP with MspI enzyme (New England Biolabs) by incubating overnight at 37^{0}C which were later checked on 3% Agarose gel (Fig 1)

Primer sequences of *IL-6* gene polymorphism

Polymorphism	Primers	Amplicon (bp)	RFLP
-174 G/C	FP: ATGCCAAGTGCTGAGTCACTA RP: CGAGGGCAGAATGAGCCTC	230	*NlaIII*

Statistical Analysis: SPSS 20.0 version was utilized. Following methods were employed.

1. Qualitative and Quantitative variables were described by computation of frequency, mean, Standard Deviation (SD) and Chi square test of association.
2. Hardy-Weinberg equilibrium: Deviations from the Hardy-Weinberg equilibrium were tested for all polymorphisms in cases and controls by comparing observed and expected genotype frequencies and goodness of fit test was adopted (http://www.socscistatistics.com/tests/chisquare/)
3. SNPSTAT software was adopted for the calculation of the Odds ratios, as the estimates of relative risk of disease, with 95% confidence intervals and and ≤0.05 probabilty to determine

dominant/ codominant/ recessive inheritance model for the polymorphisms studied (http://bioinfo.iconcologia.net/snpstats/custom.php).

4. Linkage Disequillibrium test was carried out by Haploview version 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php).
5. Multifactor Dimensionality Reduction MDR analysis was performed by MDR2.0 beta 8.0 software. 6) Online tools for Insilico analysis Secondary RNA structure predictions were carried out by RNA Vienna server – http://rna.tbi.univie.ac.at/cgibin/RNAfold.cgi.

RESULTS

IL-6 gene encodes a cytokine that functions in inflammation and the maturation of B cells. It is produced by the T cells, B cells, monocytes, fibrocytes and fat cells. The protein is largely produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor alpha. The operation of this gene is linked to a wide variety of inflammation-associated diseases such as diabetes mellitus and systemic juvenile rheumatoid arthritis. The serum level of *IL-6* is found to be higher in patients with PCOS than in normal controls (Tarkun *et al.*, 2006). Hence, the present study was aimed to identify the association between a promoter polymorphism (-174G/C) and the levels of IL-6 with PCOS. The percentage of GG, GC and CC genotypes in patients was 24, 58, 18 while it was 22, 50 and 28 in controls respectively. The allele frequencies of G and A allele are 0.53 and 0.47 for patients while it was 0.47 and 0.53 for controls correspondingly. The genotype frequencies differed significantly between the groups whereas the alleles did not differ between the groups. The patients as well as the controls were following Hardy Weinberg Equilibrium. The genotype and the allele frequencies of the patients and controls are given in Table 1. Risk analysis did not reveal any protective or predisposing role towards PCOS. Mendelian effect modelling also did not yield any significant outcome. The risk estimates of *IL-6*-174G/C genotypes and alleles among the study group are given in Table 2. Secondary structure analysis of the pre-mRNA revealed that the structure formed due to the 'G' allele was more stable (-64.60 kcal/mol) when compared to the variant 'C' allele (-59.90 kcal/mol) Fig 2.

Linkage Disequilibrium

Linkage disequilibrium (LD) plot for the four variations was constructed with the help of Haploview 4.2 and D' values are given in the Fig.3. As observed from the figure below (Fig.3), a clear linkage between rs1799964 and rs361525 could be observed in both the controls and PCOS group pointing out the fact that both the variations are inherited as a single entity in an individual. No significant D' values could be observed in any other combinations indicating the entities are being inherited as a single unit. The possibility of association between different haplotype combinations of the gene polymorphisms undertaken in the present study in PCOS was tested by performing haplotype analysis. The estimated Chi square values and relative risk estimates at p values ≤0.05 for the 17 haplotypes identified in the present study are given in Table 3. Haplotypes with a frequency of p<0.01 were excluded from the analysis. Seventeen haplotypes have been represented in Table 3. As observed from the below table three combinations of haplotypes were observed to show significant results. As observed from the table the haplotype combination "A-A-C-A-C" and "A-G-T-G-C" conferred protection towards PCOS (OR= 0.321, CI= 0.15 – 0.67, p=0.002 and OR= 0.28, CI= 0.11 – 0.73, p=0.005 respectively) while the haplotype combination "A-G-C-A-C" conferred fourfold risk (OR= 3.38, CI= 1.52 – 7.53, p= 0.0018) towards PCOS establishment. Establishment risk towards establishment and progression of LQTS. The individual SNP involved in the haplotype combinations are: rs1137101 – rs1800629 – rs1799964 – rs361525 – rs1800795.

Multifactor Dimensionality Reduction Analysis

Sequence variants in human genes are being described in remarkable numbers. Determining which variants and which environmental factors are associated with common, complex diseases has become a daunting task. This is partly because the effect of any single genetic variation will likely be dependent on other genetic variations (gene-gene interaction or epistasis) and environmental factors (gene-environment interaction). Detecting and characterizing interactions among multiple factors is both a statistical and a computational challenge. To address this problem, multifactor dimensionality reduction (MDR) method for collapsing high dimensional genetic data into a single dimension has been developed, thus permitting interactions to be detected in relatively small sample sizes. In the present study, the MDR method using a software package for implementing MDR in a case-control design was used to study gene-gene interactions in PCOS. Table 4 represents the results obtained for the number of loci evaluated by MDR software (Version 2). The multi locus model with the best/maximum cross-validation consistency at p≤0.05 is considered to be the best model for disease manifestation and progression. The MDR analysis revealed the four locus model involving A668G, -308G/A, -1.31T/C and -174G/C of *IL-6* with a testing

accuracy of 48% and CV consistency of 9/10 to be the best model in case of PCOS. Even though the table displays loci with a CV consistency of 10/10 for five locus, they were not considered since the testing accuracy was observed to be below the four locus model (Fig 4). As observed from the above entropy dendrogram, a synergistic interaction between -238G/A and -308G/A polymorphisms of the *TNF-α*exists and both together express and appear to protect individuals from PCOS, while the leptin receptor polymorphism (rs1137101) appears to exert its risk in a independent (redundant manner) conferring risk towards PCOS establishment. The Leptin receptor malfunction together with the higher levels of Leptin and other cytokines studied could produce the conducive environment for PCOS establishment and progression in women of reproductive age.

DISCUSSION

Polycystic Ovarian Syndrome (PCOS) is a complex disorder characterized by hyperandrogenism chronic anovulation and insulin resistance in women of reproductive age. Insulin resistance, in addition to obesity found in majority of young PCOS may also suffer with low-grade inflammation eliciting an overproduction of IL-6 monocytes in IR PCOS subjects[5]. Animal studies on mice demonstrated a vital role of IL-6 alters in follicular maturation and ovarian dysfunction. IL-6 is often considered as a link between anthropometric and metabolic alteration and hyperandrogenemia in PCOS. The IL-6 -174 G/C polymorphism has been reported to influence the rate of transcription of this multifunctional cytokine and is also identified to be involved in the regulation of androgen levels[6]. Higher serum levels of inflammatory markers such as hsCRP, IL-6 and leptin have been reported to be associated with PCOS in various studies and also the production of proinflammatory molecule leptin by white adipose tissue has been reported [7]. Though several studies exist assessing the levels of inflammatory cytokines such as IL-6 and TNF-α and associating them with PCOS, they appear to be inconsistent. Thus the present study's objective was to determine if a correlation exists between IL-6 -174G/C promoter gene polymorphism with PCOS susceptibility[8]. In the present study no association of IL-6 -174G/C polymorphism with PCOS and could be identified and this was similar to the findings of Guo *et al.*, 2015 in Chinese PCOS women[9,10]. A recent meta-analysis by Wang *et al.*, 2015 including four studies containing 351 cases and 464 control also demonstrated that IL-6 –174 G/C polymorphism may be not related to PCOS susceptibility[11]. Our study was contradictory to the findings of Tumu *et al.*, 2013 performed in south Indian and proposed IL-6 as a candidate gene for PCOS since they observed a significant association of IL-6 –174 G/C with PCOS establishment [12,13]. The IL-6 promoter polymorphism was often related to occurrence and metabolic abnormalities and insulin sensitivity seen in PCOS[14, 15, 16].

The pre-mRNA secondary structure demonstrated a lesser stable pre-mRNA coded by 'C' are less stable and affects the overall functioning of IL-6 cytokine. However, no change in transcription factors was observed.

CONCLUSION

Though studies suggesting the elevation of IL-6 levels in women suffering from PCOS exist our study could not point out any specific association between the promoter polymorphism and risk towards PCOS. Nevertheless, further studies based on genome wide association studies are warranted to elucidate the risk factors to PCOS.

Table 1: Genotypic and allelic frequency distribution of the IL-6 -174G/C polymorphism in controls and PCOS

SNP	Genotype	Controls n (%)	PCOS n (%)	χ^2 (p value)
-174G/C	GG	22 (22)	24 (24)	**6.83 (0.033)***
	GC	50 (50)	58 (58)	
	CC	28 (28)	18 (18)	
	Allele	Controls n (f)	PCOS n (f)	χ^2 (p value)
	G	94 (0.47)	106 (0.53)	2.89 (0.089)
	C	106 (0.53)	94 (0.47)	

$\chi 2_T$*= 5.99, $\chi 2_T$**= 3.84, p≤0.05. Odds risk estimates were calculated by comparing the genotypes of PCOS group against controls (Table).

Table 2: Odds risk estimates of genotypes and alleles in PCOS compared to controls of the Il-6 -174G/C polymorphism p≤0.05

SNP (rs1137101)	Model	Geno type	Controls	PCOS	OR (95% CI)	p
	Codominant	GG	22	24	1.00	
		GC	50	58	1.06 (0.5 – 2.25)	1.00
-174G/C		CC	28	18	0.59 (0.24 – 1.46)	0.3

Dominant	GG	22	24	1.00	
	GC+CC	78	76	0.89 (0.44 – 1.82)	0.87
Recessive	GG+GC	72	82	1.00	
	CC	28	18	0.56 (0.27 – 1.16)	0.13
Over dominant	GG+CC	50	42	1.00	
	GC	50	58	1.38 (0.76 – 2.51)	0.32
	Alleles	Controls	PCOS	OR (95% CI)	p
	G	94	106	1.00	
	C	106	94	0.79 (0.52 – 1.187)	0.27

Table 3: Haplotype in the present study among Control and PCOS and their association with PCOS

Haplotype	χ^2	Odds Ratio [95%CI]	p-value
A A C A C	9.782	0.321 [0.153~0.674]	**0.002**
A A C A G	0.973	1.836 [0.539~6.250]	0.324044
A A C G C	0.327	0.530 [0.058~4.853]	0.567683
A A T G C	1.658	2.347 [0.617~8.924]	0.197911
A A T G G	0.890	1.450 [0.668~3.150]	0.345482
A G C A C	9.798	3.380 [1.518~7.527]	**0.001757**
A G C A G	0.020	0.938 [0.386~2.283]	0.888094
A G T G C	7.678	0.280 [0.108~0.726]	**0.005609**
A G T G G	1.114	0.660 [0.304~1.434]	0.291240
G A C A C	1.817	2.216 [0.678~7.246]	0.177705
G A C A G	0.564	0.786 [0.418~1.476]	0.452801
G A T G C	0.080	0.896 [0.420~1.915]	0.777549
G A T G G	0.051	0.909 [0.398~2.079]	0.821932
G G C A C	9.946	0.159 [0.044~0.580]	**0.001621**
G G C A G	7.370	2.961 [1.310~6.689]	**0.006655**
G G T G C	2.920	2.062 [0.886~4.800]	0.087577
G G T G G	0.001	1.020 [0.194~5.360]	0.981362

Table 4: Gene combinations leading to disease manifestation

Model	Training Bal. Acc.	Testing Bal. Acc.	CV consistency
-174G/C	0.55	0.46	6/10
-308, -238	0.61	0.51	5/10
A668G, -308, -174G/C	0.66	0.48	5/10
A668G, -308, -1031, -174G/C	0.74	0.48	9/10
A668G, -308, -1031, -238, -174G/C	0.74	0.47	10/10

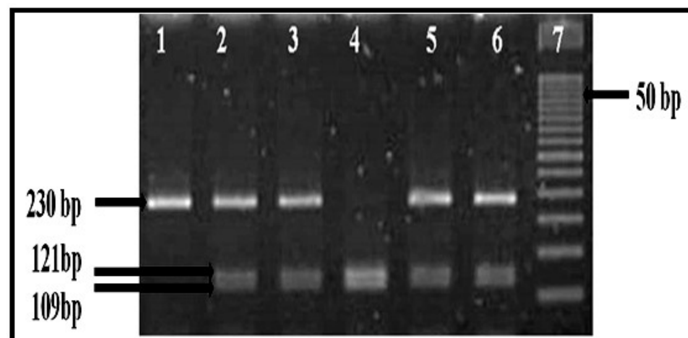

Figure 1: Interlukein-6-174 G/C (rs 1800795) PCR –RFLP using *NlaIII enzyme.* Lane 1: Homozygous (G/G), Lanes 2, 3, 5 and 6: Heterozygotes (G/C). Lane 4: Homozygous (C/C) and Lane 7: 50bp Ladder

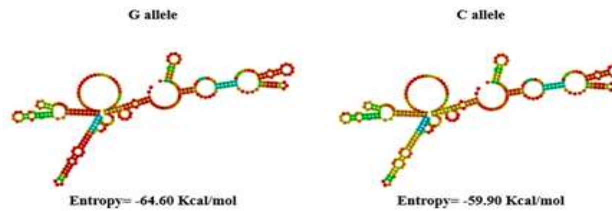

Figure 2: pre-mRNA secondary structure of IL-6 -174G/C polymorphism

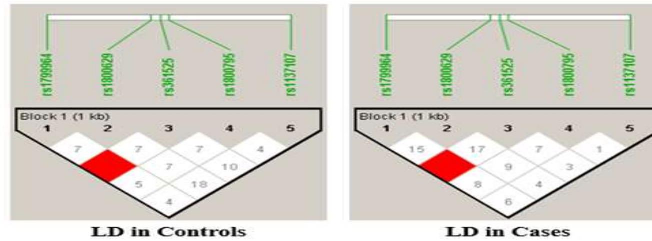

Figure 3: Linkage disequilibrium plot of the variations in PCOS and controls in the present study (D' values were indicated in the respective squares)

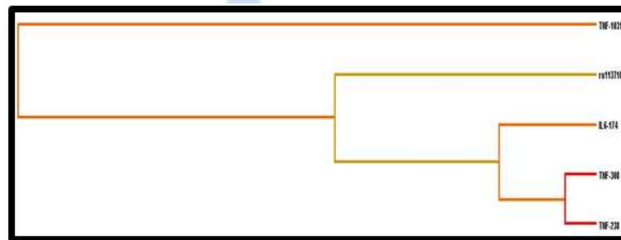

Figure 4: Dendogram showing the interaction between various genes involved in the study

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