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Genotype- Phenotype correlations of Leptin receptor gene in Polycystic ovarian disease (PCOS)

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Abstract :
Introduction: *Polycystic ovarian syndrome (PCOS) is one of the commonest endocrine disorder amongst women of reproductive age group. Polymorphisms in the in the Leptin receptor gene (LEPR) have been associated various related diseases ranging to Obesity, PCOS and infertility. In this context, we evaluated association between PCOS and LEPR 668 A/G polymorphism.*
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 LEPR 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 two fold 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: *The present study has implicated the role of the AG and GG genotypes, and the G allele in contributing towards establishment and progression of PCOS though further studies are warranted to establish the same in the general population.*
Keywords - *PCOS; Rotterdam criteria; TNF-alpha; Cytokines; Infertility*

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I. Introduction

Deficiency of the leptin receptor encoded by *LEPR* gene leads to a variety of disorders. *LEPR* encodes the leptin receptor which has the ability to regulate body weight apart from controlling hunger, thirst, sleep and other functions such as body temperature. Any variation within its sequence has the ability to affect a wide variety of organs where it has been reported to be located. [1-5] Leptin receptor is also responsible for hormone release regulating its activity in the hypothalamus. Alterations and mutations in LEPR gene has been associated with hypogonadotrophic hypogonadism, polycystic ovarian disease (PCOS) and infertility. But, the literature evidence on this LEPR gene and PCOS is inconsistent especially from Indian subcontinent. [6-13] In this context, we conducted this prospective study to look in to this association.

II. Material And Methods

Study design: 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 [The Rotterdam Citeria, 2003].

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. participants age group was in the range from 18-35yrs. The study was approved by the Institutional Ethical Committee. (011 / 022015 IEC / Saveetha University Dated12-02-2015). A written informed consent was collected from all the subjects enrolled in the study. Participants 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^{o}c to isolate the serum and it was stored -20^{o}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

Five ml of whole blood from controls 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.

Reagents

Low salt buffer (TKM1): It is prepared by dissolving 1.576 g of Tris-HCL (pH 7.6), 0.75g of KCl, 2.033g of $MgCl_2$ and 0.748 g of EDTA in one liter of double distilled water. High salt buffer (TKM2): It is prepared by dissolving 1.576 g of Tris-HCL (pH 7.6), 0.75g of KCl, 2.033g of $MgCl_2$, 0.748 g of EDTA and 2.34g of NaCl in one liter of double distilled water.

Procedure

Five ml of blood was collected in EDTA coated vacutainer. Blood was transferred into 15ml centrifuge tube to which, five ml of low salt buffer (TKM1) and 100µl of triton-X was added to lyse the red cells. The contents were mixed well and centrifuged at 2200 rpm for 10 minutes at room temperature in a Beckman table top centrifuge. Then the supernatant was discarded and the nuclear pellet was washed in 5ml of TKM1 and centrifuged as before. The pellet was resuspended in 0.8ml of high salt buffer (TKM2) and to this 50µl of 10% sodium dodecyl sulphate was added. The whole suspension was mixed thoroughly and incubated for 10 minutes at 55^{0}C. After incubation, 0.3ml of 6M NaCl was added to the tube and mixed well. The contents were transferred to an Eppendorf tube and centrifuged at 12000 rpm for 15 minutes in a microfuge. The supernatant was transferred to a 15ml centrifuge tube and 2 volumes of 100% ethanol was added to precipitate the DNA. The precipitated DNA was transferred to a fresh Eppendorf tube containing 1ml of ice cold 70% ethanol and microfuged for 5 minutes at 12000 rpm at 4^{0}C. The pellet was dried and re-suspended in 0.5ml of Tris-EDTA buffer at 65^{0}C for 15 minutes. 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 & quantity can be obtained in the shortest time and with just 500µl of blood sample by following this protocol.

Reagents

Cell lysis buffer: It is prepared by dissolving 1.57g of Tris HCL (pH 8), 110 g of sucrose, 1.01g of $MgCl_2$ and 10 ml of Triton X 100 in 1 litre of distilled water. Nucleiolysis bufferis prepared by dissolving 1.57g of Tris HCL (pH-8), 10g of SDS, 3.75 g of EDTA and 2.94 g of sodium citrate.

Procedure

To 500µl or 0.5g of blood was transferred 1000µl of Cell lysis buffer was added and the tubes were shaken gently then centrifuged for 3 minutes at 6000 rpm. The supernatant was discarded and again the previous step should be repeated. Then 300µl of Nuclei lysis buffer was added to the microfuge tube and kept at room temperature for 3 minutes to prevent clotting. 100µl of saturated NaCl and 600µl of Chloroform was added to

the microfuge tube followed by gentle and rapid shaking and later centrifuged for 2 minutes at 6000 rpm. 300-500µl of supernatant was transferred to a fresh 1.5ml microfuge tube. 600µl of cold Isopropanol or absolute Ethanol was added and shaken gently and rapidly. The microfuge tube was centrifuged for one minute at 13000 rpm to precipitate DNA. DNA is found as white flakes at the bottom of the tube. After removing the supernatant the samples should be air dried, to this 50-100µl of TE was added for dissolution in a water bath. The dissolved DNA sample was stored at 40^0C or -20^0C for later use.

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 A668G (Q223R) were, initial denaturation at 95^0C for 5 min, denaturation step at 95^0C for 30 sec, annealing at 58^0C for 30 sec, extension at 72^0C for 1 min for 30 cycles. Final extension was carried out at 72^0C for 7 min. The PCR products were subjected to RFLP with *MspI*enzyme *(New England Biolabs)* by incubating overnight at 37^0C which were later checked on 3% Agarosegel **(Fig 1).**

Primer sequences of Leptin receptor gene polymorphism

Polymorphism	Primers	Amplimer (bp)	RFLP
A668G (Q223R)	FP: CCTGCTTTAAAAGCCTAATCCAGTATTT RP: AGCTAGCAAATATTTTTGTAAGCAAT	230	*MspI*

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/cgi-bin/RNAfold.cgi.

III. Results

Deficiency of the leptin receptor encoded by *LEPR* gene leads to a variety of disorders. *LEPR* encodes the leptin receptor which has the ability to regulate body weight apart from controlling hunger, thirst, sleep and other functions such as body temperature. Any variation within its sequence has the ability to affect a wide variety of organs where it has been reported to be located.

Leptin receptor is also responsible for hormone release regulating its activity in the hypothalamus. Deficiency of the leptin receptor could lead to hypogonadotropic hypogonadism, a condition affecting the production of hormones that direct sexual development leading to delayed puberty or absence of puberty in individuals leading to infertility. Hence, the present study was aimed to identify the association between a *LEPR* gene 668A/G polymorphism with PCOS.

The percentage of AA, AG and GG genotypes in patients was 15, 56, 29 while it was 30, 50 and 20 in controls respectively. The allele frequencies of A and G allele are 0.43 and 0.57 for patients while it was 0.55 and 0.45 for controls correspondingly. The genotype frequencies differ significantly between the groups ($p<0.05$) while the allele frequencies did not vary. The patients as well as the controls were following Hardy Weinberg Equilibrium. The genotype and the allele frequencies of the patients and control are given in **Table 1.**

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 two fold 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). The risk estimates of *LEPR 668* A/G genotypes and alleles among the study group are given in **Table 2.**
Secondary structure analysis of the encoded pre-mRNA revealed that the 'A' allele (-74.30 kcal/mol) encoded a stable structure when compared to the variant 'G' allele (-73.60 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 estimatesat 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.

As observed from the **Table 4**, 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 5 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 $p\leq 0.05$ 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.

IV. Discussion

Leptin is involved in energy metabolism and inflammatory processes and is often linked with PCOS. It plays an important role in human physiology, acts through the leptin receptor (LEPR) inturn connects nutrition and immunity. It also regulates neuroendocrine function, energy homeostasis, haematopoiesis, and angiogenesis. Leptin is an important modulator of both the innate and adaptive immune systems. [1-5] Till date several studies had reported an association of BMI with PCOS indicating leptin and its receptor as culprit behind obesity which in turn may progress towards PCOS. [14]

The human leptin is primarily produced by the white adipose tissue and the circulating levels of leptin are directly proportional to the amount of fat in the individual. A study conducted on the leptin/melanocortin pathway that regulates the body energy homeostasis has yielded significant results establishing leptin's role in PCOS and systemic health. Leptin acts via its receptor encoded by the *LEPR* gene, exerting a negative feedback mechanism controlling the amount of food intake and energy metabolism related with the same. [15]

The Leptin receptor protein is a single trans- membrane protein that belongs to the class I cytokine-receptor family and has the ability to mediate the functioning of 20 different cytokines and is of great functional importance. Several LEPR single nucleotide polymorphisms have been examined for their association with that of energy metabolism and of them *LEPR668 A/G* transition leading to a change of Glutamine by Arginine at 223 position.

The present study demonstrated that the AG and GG genotypes posed significant risk towards disease establishment as observed under the codominant and dominant inheritance models. Similarly, the 'G' allele conferred a twofold risk towards PCOS susceptibility. Our results were corresponding with the findings of Li et al, 2013 where they found a significant association of *LEPR* Gln223Arg gene polymorphism with PCOS in a Korean population. [16] Our findings could be explained on the basis of polygenic nature of most human obesity, according to which each individual gene is expected to contribute in a minor way to the phenotypic variation, and combinations of several genes are likely to contribute or predispose to obesity.

Furthermore, the change had led to the replacement of a neutral amino acid with that of a positively charged one affecting the structure of the receptor in turn affecting the cascade of reactions that result due to receptor activation. Change in the structure of the receptor could also lead to improper functioning via the non-binding of the leptin hormone. Mouse obesity model studies (Zucker Mouse Model – Q269P) has led to the belief that this Q223R variation could affect subtle changes in the downstream signaling pathways, thus rendering a leptin-resistant state. [15]

The association of G allele in disease manifestation and progression could be due to haploinsufficiency in the heterozygotes exhibiting a dominant-negative effect forcing the normal protein molecules encoded by the 'A' allele. The pre-mRNA structure prediction led to clear variation due to G allele (indicated by an arrow) and this could be the reason for reduced pre-mRNA molecules leading to a reduction in the overall protein molecules produced, thus, affecting the receptor activity and manifest PCOS. Though the present study had implicated the role of the AG and GG genotypes, and the G allele in poor prognosis, further studies are warranted to establish the same in the general population.

V. Conclusions

The present study has implicated the role of the AG and GG genotypes, and the G allele in contributing towards establishment and progression of PCOS though further studies are warranted to establish the same in the general population.

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Table 1: Genotypic and allelic frequency distribution of the *LEPR* A668G polymorphism in controls and PCOS

SNP	Genotype	Controls n (%)	PCOS n (%)	χ^2 (p value)
A668G	AA	30 (30)	15 (15)	18.44 (0.001)*
	AG	50 (50)	56 (56)	
	GG	20 (20)	29 (29)	
	Allele	Controls n (f)	PCOS n (f)	χ^2 (p value)
	A	110 (0.55)	86 (0.43)	11.75 (0.001)*
	G	90 (0.45)	114 (0.57)	

$\chi 2_T$*= 5.99, $\chi 2_T$**= 3.84, p≤0.05

Table 2: Odds risk estimates of genotypes and alleles in PCOS compared to controls of the *LEPR* A668G polymorphism

SNP (rs1137101)	Model	Geno type	Controls	PCOS	OR (95% CI)	P
A668G	Codominant	AA	30	15	1.00	
		AG	50	56	2.24 (1.02 – 4.96)*	0.04*
		GG	20	29	2.9 (1.15 – 7.38)*	0.02*
	Dominant	AA	30	15	1.00	
		AG+GG	70	85	2.43 (1.15 – 5.17)*	0.018*
	Recessive	AA+AG	80	71	1.00	
		GG	20	29	1.63 (0.81 – 3.30)	0.19
	Over dominant	AA+GG	50	44	1.00	
		AG	50	56	1.27 (0.7 – 2.31)	0.5
		Alleles	Controls	PCOS	OR (95% CI)	P
		A	110	86	1.00	
		G	90	114	1.62 (1.07 – 2.45)*	0.021*

Table 3: Haplotypes in the present study among Controls 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

Table 5: Loss/addition of Transcription factors in A668G polymorphism of LEPR

Type	Transcription factor	Location	Function
Loss	HSTF	234 - 243	Binds to the heat shock elements and promote transcription.
Addition	Ttx	239 - 248	Helps in functional gene activation.
Addition	RAR-alph	240 - 249	Plays a major role in gene activation

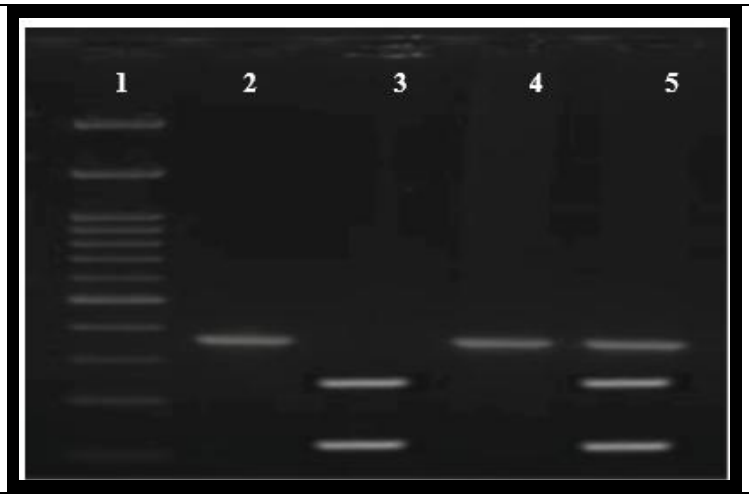

Fig. 1: PCR-RFLP showing genotypes of *LEPR* gene A668G polymorphism
Lane 1 – Marker (100bp), Lane 2 and 4 – AA (367bp), Lane 5 – AG (367+242+125bp)
Lane 3 – GG (242+125bp)

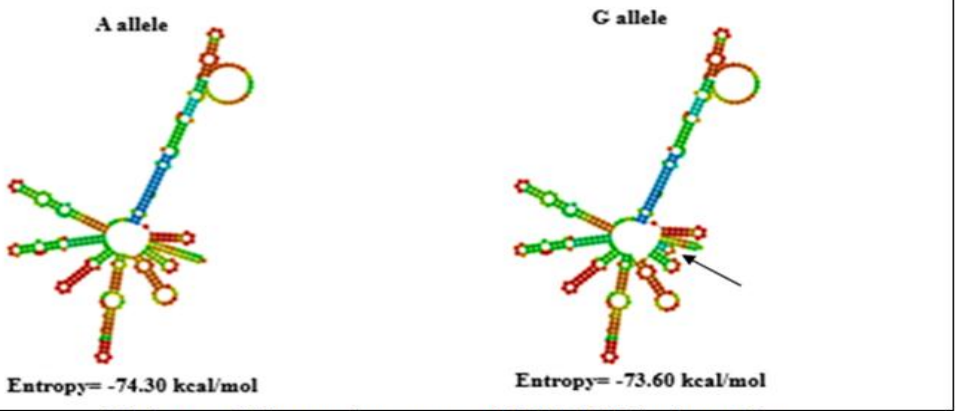

Fig 2 : pre-mRNA secondary structure of *LEPR* A668G polymorphism

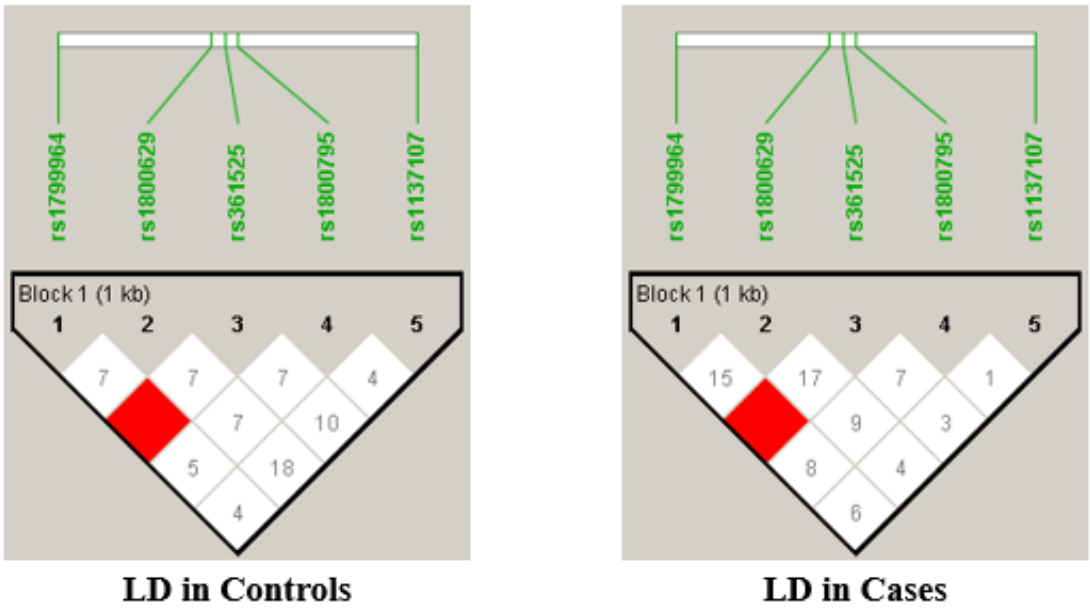

Fig 3: Linkage disequilibrium plot of the variations in PCOS and controls in the present study (D' values were indicated in the respective squares).

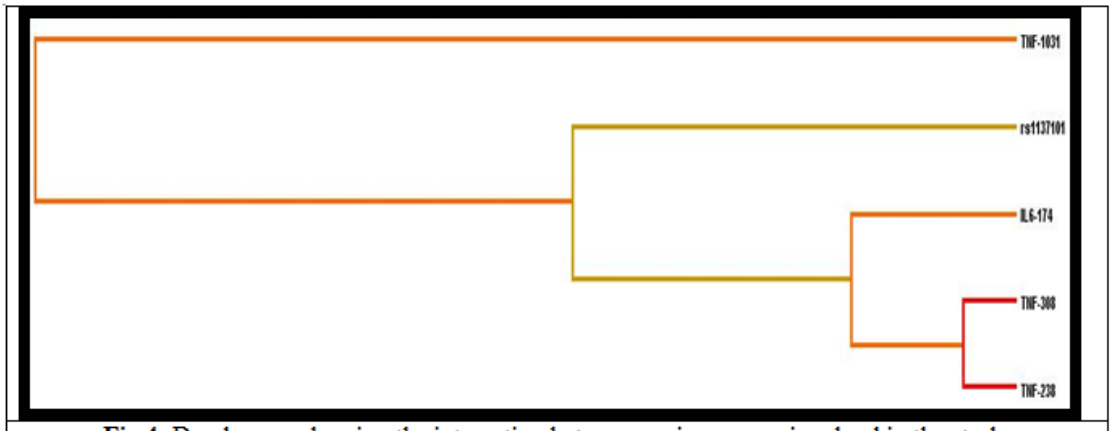

Fig 4: Dendogram showing the interaction between various genes involved in the study

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