

Association of follistatin and cytochrome p450 side-chain cleavage enzyme with polycystic ovarian syndrome

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ABSTRACT

Objectives: Polycystic ovarian syndrome is a complex disorder commonly found in women in their reproductive age around the globe. Due to its higher clinical implications and communicability, this research article describes epidemiological studies and the association of known microsatellite markers with PCOS in the Pakistani population.

Methods: Data were collected from 62 Pakistani women. The study involved pedigree analysis which revealed recessive mode of inheritance in all of them. The mendelian ratio in marriages between non-PCOS (male) and PCOS (female) individuals remained 1:1 while between non-PCOS (male) and non-PCOS (female) remained 3:1, respectively.

Results: The result showed that there was an association of marker D15S519 (CYP11A, 10q24.3) with family 5 with an X2 value of 7.78 ($P < 0.01$) while a strong association of marker D5S822 (FS, 5p14) with an X2 value of 4.89 ($P < 0.05$) was observed in family 2. In the case of family 1, a weak association of allele 1 with marker D19S212 (INSL3, 19p13.1) was observed with an X2 value of 2.25 ($P > 0.10$), While family 6 also presented a very weak association of homozygosity with marker D19S391 (INS receptor, 19p13.2) with X2 value of 3.45 ($P \geq 0.05$).

Conclusion: Findings indicated a higher prevalence of PCOS and highlights the emergence of more interventions to minimize the level of complications in women. Our intrusions intended to describe the genetic basis of PCOS in Pakistani population with special emphasis on large families in current study. Moreover, association of known loci for PCOS with our families.

Keywords: Cytochrome p450 side-chain, endocrine disorder, follistatin microsatellite markers, Pakistan, Polycystic ovarian syndrome

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Introduction

Women with polycystic ovarian syndrome (PCOS) produce an abnormal amount of male hormones known as androgens. Among disorders of the reproductive age group, PCOS requires particular attention^[1] because 5–10% of the pre-menopausal women are affected by PCOS and its major manifestations are chronic anovulation and hyperandrogenism.^[2]

Clinical characteristics and histological features should be analyzed for the symptoms of obesity, bleeding, infertility, as well as miscarriages.^[3,4] Biochemically, PCOS can be described with hyperandrogenism and elevated serum luteinizing hormone (LH) as well as hyperinsulinemia. Hyperinsulinemia has been documented as the cause of hyperandrogenism in PCOS women.^[5-7] Another study suggested that PCOS increases the risk of cardiovascular as well as diabetic events.^[8] The main cause that results in PCOS

is still known; however, insulin resistance, inflammation, and genes are associated with excess androgen production in women suffering from PCOS. PCOS can be explained by different pathways. Those pathways include metabolic as well as regulatory avenues and genes should also be assessed for the identification of candidate genes.^[9] The familial clustering phenomenon of PCOS cases has been studied for the past 30 years and evidence has suggested that PCOS propagates in the family and the risk for the family members increases up to the extent of 50% but the mode of transmission has not been agreed yet.^[10-12,14-16]

The possible candidate genes have been analyzed previously,^[17] involved in the pathways of steroid hormones, metabolism and action, gonadotrophic action, obesity and energy regulation, and insulin action.^[17-19] Primers were designed to amplify these possibly polymorphic sites, that is, short tandem repeat polymorphism (STRPs) and single nucleotide polymorphism

(SNPs), which were located within or adjacent to the candidate genes.^[20]

Power estimates can describe the linkage between markers and disease-susceptibility locus as well as the informativeness of markers. High-density genetic maps have been created which could facilitate positional cloning projects.^[21,22] The CYP11A mRNA was observed to be more abundant in the theca cells of individuals with polycystic ovary syndrome as compared to the theca cells of normal individuals. The potential of developing an association is triggered by different factors, and the medical practitioners should evaluate the locus genotypes for the possible penetration of the disease.^[23] Candidate genes known for PCOS include genes involved in the biosynthesis of androgens, action of insulin, and gonadotrophin.^[24-28] Genes for different receptors have also been reported as PCOS candidate genes. The key genes associated with dysregulation of androgen production in women include CYP11a, CYP21, CYP17, and CYP19. To study the genetic basis of PCOS, linkage and association studies can be a valid tool.^[29] The objective of the present study involves the determination of the Mendelian inheritance of trait, segregation of microsatellite markers for particular loci and determination of the genetic basis of PCOS using linkage analysis and association study strategies.

Materials and Methods

Diagnosis of PCOS

PCOS diagnosis was carried out using ultrasound and biochemical analysis of these families. Women with menstrual problems, hirsutism, obesity, and infertility were designated as polycystic ovarian syndrome (PCOS) women. Phenotype of the six families described in the present study was hirsutism with ferriman gallwey scoring, obesity by body mass index and waist: hip ratio, diagnosis of polycystic ovarian syndrome individual with Rotterdam criteria (2003).

Women diagnosed with polycystic ovarian syndrome (PCOS) at Prof. Dr. Saad Raana Gynecological Advisory Centre, Islamabad were interviewed to record information required for the present study. Extensive pedigrees were drawn^[30] for the genetic studies. Proband was interviewed to trace back the disease in each family. In the context of genetic studies, probands are individuals that initiate the study and research process in a family. The pedigrees were constructed for at least up to five generations depending on how much the proband could recollect about the disease in the members of the family. There were 121 women from six families, of which 17 women died and 42 were not available for interview. A total of 62 women agreed to cooperate. Blood samples were collected from 62 women in sterile containers containing potassium ethylene diamine tetra acetic acid (EDTA) prepared for DNA extraction. Written consent was taken from all the subjects involved in the study.

DNA extraction from whole blood

DNA was extracted from whole blood using standard procedure.^[19]

Polymerase chain Reaction (PCR)^[20]

Microsatellite markers for the known loci of PCOS as described in Table 1 were obtained from research genetics, Inc. (USA). The cytogenetic information was obtained from genome databases including gdb.org and marshmed.org.

The polymerase chain reaction was performed using the following reaction mixture

- Sample DNA 1 µl (microliter)
- 10X PCR buffer 2.5 µl
- MgCl₂ (Magnesium Chloride) 1.5 µl
- Deoxyribonucleotide triphosphate (dNTPs) 0.5 µl
- Forward and reverse primer 0.3 µl
- Taq DNA polymerase 0.3 µl
- PCR water 20.1 µl

A thermo-cycling environment was used for obtaining the mixture as follows:

- 5 min of 90°C for template denaturation
- 40 cycles of amplification each consisting of 3 steps
- 1 min at 90°C for DNA denaturation
- 1 min at 55–57°C for primer annealing
- 1 min at 72°C for extension of complimentary
- DNA strand
- 10 min at 72°C for un-extended strands left
- PCR was performed subsequently.

Agarose gel electrophoresis

PCR products were resolved on 2% agarose gel.

Poly acrylamide gel electrophoresis (PAGE)^[21]

The amplified products were resolved on 8% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 100 volts for 90 min and the gel was stained with ethidium bromide (10 mg/ml) solution for visualization on UV trans-illuminator. The gel was photographed by using a digital camera DC120 (Kodak, USA).

Genetic and primer database analysis

Analysis of microsatellite markers for the known loci as entitled in Table 1 was performed by PCR and gel electrophoresis. Amplified products using microsatellite markers were visualized by placing the ethidium bromide-stained gel on a UV trans-illuminator and genotypes were assigned by visual inspection. Homozygosity and heterozygosity were recorded and alleles were numbered concerning the origin.

Table 1: 37 candidate gene panel for PCOS

Marker locus	Gene symbol	Candidate gene	Distance in cM	Chromosome location
Steroid hormone				
DIS514	HSD3III+2	3 β -Hydroxysteroid dehydrogenase, type I and II	<1	1p31.1
D8SI821	STAR	Steroidogenic acute regulatory protein	<2	8_1>_1\2
D9SI809	HSD17B3	17 β -Hydroxysteroid dehydrogenase type III	<1	9q22
DIOS192	CYP17	CYP 17 cytochrome P450 17 α -hydroxylase/17 20 desmolase	<1	10q24.3
D15S519	CYP11A	CYP 11A cytochrome P450 side chain cleavage enzyme	0	15q 23-24 .
DI5S520	CYP II A	CYP 11A cytochrome P450 side chain cleavage enzyme	0	15q 23 to 24
DI7S934	HSD17L.H	17 β -Hydroxysteroid dehydrogenase type I	<2	17q11-21
HSD17B2	HSD17III I	17 β -Hydroxysteroid dehydrogenase type I	0	16q24.2
Gonadotropin action				
D2S2335	ACTRI	Activin receptor I	<1	12q13,12
D2S 1352	LHCGR	Luteinizing hormone/choriogonadotropin receptor	<2	2p21
D2S293	INH11	Inhibin II B	<2	2cen-2q13
D2SI63	INHA	Inhibin A	<1	2q33.34
D3S 1298	ACTR2B	Activin receptor 213	<1	3q22.2
D5S-623	FS	Follistatin	<0.5	5q14
D5S-822	FS	As above	<1	5p14
INHBA	fNHBA	inhibin BA	2	7p13-15
D5S474	FS	Follistatin	<2	5p14
DI2SI691	INHC	Inhibin C	<1	12q13
DI2S347	ACTRI	Activin receptor I	<1	12q13.12
DI7SI353	SHBG	Sex hormone-binding globulin	<1	17p13.2
DI8S474	MADH4	Mothers against decapentaplegic homolog 4	<1	18q21
Obesity and energy regulation				
DI8S64	MC4R	Melanocortin 4 receptor	<3	18q21.32
D7SI875	OB	Leptin	0.2	7q31.3-32.1
DIS198	OBR	Leptin receptor	0.5	1q31
D2SI31	POMC	proopiomelanocortin	<1	2p23
DIIS911	UCP2&3	Uncoupling protein 2 and 3	<4	11q13
Insulin action				
IGF 1	IGFI	Insulin-like growth factor I	0	12q22-23
IGF-IR	IGFIR	Insulin-like growth factor receptor	0	15q25-26
D7S.S19	IGFBP 1&3	Insulin-like growth hormone binding protein 1 and 3	1	7p13-7p1 •
INSR	INSR	Insulin receptor	0	19p13.3
DI9S216	INSR	Insulin receptor	4.2	19p13.3
DI9S884	INSR	Insulin receptor	1.2	19p13.3
DI9S922	INSR	Insulin receptor	1.2	19p13.3
D19S391	INSR	Insulin receptor	3.6	19p13.2
DI9S865	INSR	Insulin receptor	7.2	19p13.2
DI9S906	INSR	Insulin receptor	II	19p13.2
DI9S840	INSR	Insulin receptor	14	19p 1 3.2
DI9S212	INSL3	Leydig Insulin-like protein 3	<1	19p13.1
DI9S410	INSL3	Leydig insulin-like protein 3	<1	19p13.1
D2S2647	IRS1	Insulin receptor substrate 1	0	2q36-37
D3SI263	PPARG	Peroxisome proliferators activated receptor gamma	<0.2	3p25-24.2

Statistical analysis

Association between microsatellite markers for the known loci of PCOS and the disease was carried out using Chi-square analysis.

Results

Pedigree analysis

The six pedigrees proceeded for the present study indicated recessive mode of inheritance. Pedigrees of the strongly associated families, that is, family 2 and family 5 have been described in Figures 1 and 2, respectively. Phenotype of PCOS women in current study exhibit significant hirsutism, obesity acne, acanthosis nigricans and fertility and menstrual issues in 50% moderate hirsutism and acanthosis nigricans in 52.5% women. Family 2 showed strong association with D5S822 at locus ($X^2 = 4.89; P < 0.05$) while family 5 depicted strong association with D15S519 ($X^2 = 7.78; P < 0.01$). Family 1, 3, 4, and 6 showed weak association with different markers [Tables 2 and 3].

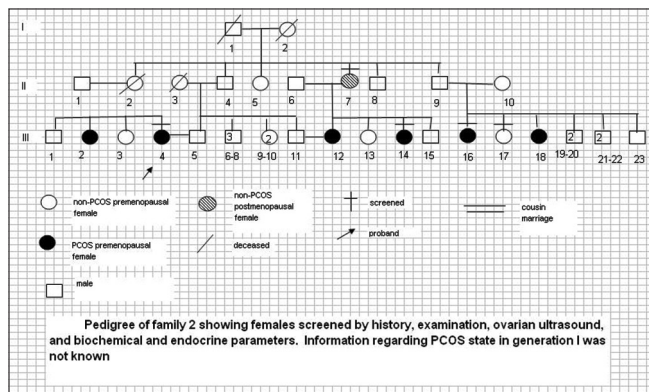


Figure 1: Family 2 Screening

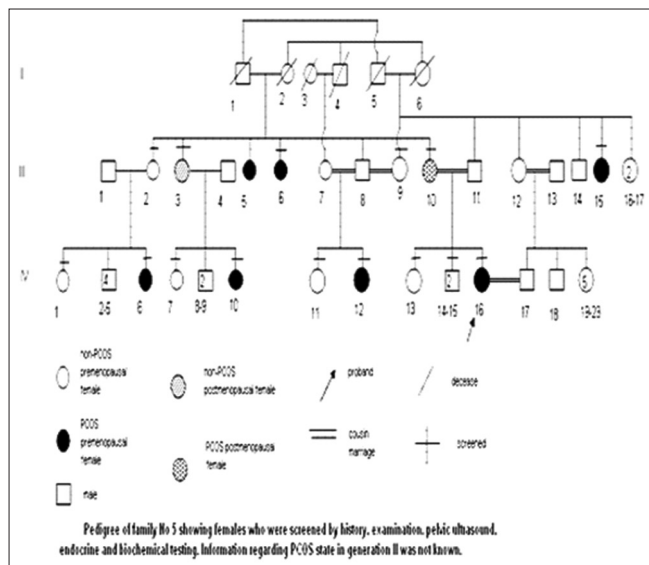


Figure 2: Screening of family 5

Figures 3 and 4 show the Acrylamide gel photographs of markers for chromosomes in different families to investigate the affected, normal and positioning of each individual. It was observed that 7 individuals that were affected ranged from 4 to 9 and 7 normal individuals ranged from 2 to 6. Two types of marriages were observed in these pedigrees (i) a marriage between PCOS and normal and (ii) between normal and non-PCOS. In both marriages, the data was assembled regarding the inheritance of PCOS and non-PCOS conditions in the offspring. Data revealed a 1:1 Mendelian ratio between normal and non-PCOS marriages [Table 4] while a Mendelian ratio of 3:1 has been observed in marriages between normal and PCOS individuals [Table 5].

Association study results

Strong and weak association of different families has been described in Tables 2 and 3.

Family 1

There is no significant association of allele 1 with marker D19S212 but there is the presence of a weak association ($X^2_{(1)} = 2.25, P > 0.10$) in the transmission of allele 1 with marker D19S212 (19p13.1 INSL3, Leydig insulin-like protein 3).

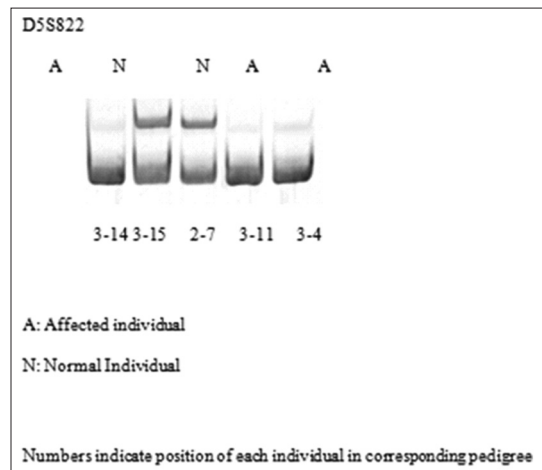


Figure 3: Acrylamide gel Photographs of markers for chromosome no.5 with family 2

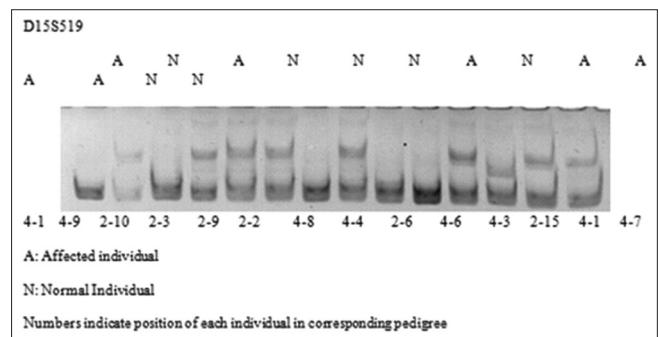


Figure 4: Acrylamide gel photographs of markers for chromosome no. 15 family^[15]

Table 2: Association of microsatellite markers with families of PCOS

Family	Gene and marker	PCOS individuals	Genotype	Homozygosity	Non-PCOS individuals	Genotype	Homozygosity
1	INSLR (D19S212)	III-6, IV-1, IV-7	1-1 (5)	1-1 (5)	IV-3,	1-1 (1)	1-1 (1)
		IV-14, IV-17	2-2 (1)	2-2 (1)	III-2, III-4, IV-9	1-2 (2)	
		V-6	1-2 (1)				
		V-2					
2	FS (D5S822)	II-14, III-16, III-4	1-1 (3)	1-1 (3)	II-17, II-7	1-2 (2)	
4	INHBB (D2S293)	IV-1, IV-9, IV-10	1-2 (3)		III-11, IV-3, IV-8	1-2 (4)	
		IV-4, IV-13	1-1 (2)	1-1 (2)	IV-12	1-1 (1)	1-1 (1)
5	CYP11A (D15S519)	III-15	1-2 (1)		III-2, III-3, III-9	1-3 (7)	
		IV-6	1-3 (1)		IV-1, IV-7, IV-11		
		III-6, III-10, IV-10	1-1 (5)	1-1 (5)	IV-13		
		IV-12, IV-16	1-1 (5)	1-1 (5)			

Numeric numbers indicate the position of individuals in the corresponding pedigree

Table 3: Statistical analysis of associated markers

Family	Marker	X ² value	Probability	Association
1	D19S212	2.25	P>0.10	Weak association
2	D5S822	4.89	P<0.05	Strong association
3	-	-	-	No association
4	D2S293	2.85	P>0.05	Weak association
5	D15S519	7.78	P<0.01	Strong association
6	D19S391	3.45	P≥0.05	Weak association

Family 2

In family 2, PCOS individuals showed homozygosity with allele 1 with marker D5S822, and a strong association was observed at this gene locus ($X^2_{(1)} = 4.89$, $P < 0.05$, Follistatin 5p14).

Family 3

No significant association has been observed among PCOS and non-PCOS individuals of family 3 and allele 1.

Family 4

In family 4, a weak association of allele 1 with D2S293 (2q13) was observed ($X^2_{(1)} = 2.85$, $P > 0.05$, Inhibin β B).

Family 5

In PCOS women, a strong association of homozygosity for allele 1 with marker D15S519 (CYP11A cytochrome P450, 10q24.3) has been observed ($X^2_{(1)} = 7.78$, $P < 0.01$).

Family 6

Genes segregating with different markers showed homozygosity and heterozygosity evenly distributed. A weak association of homozygosity for allele 1 with marker D19S391 (INS receptor 19p 13.2) was observed ($X^2_{(1)} = 3.45$, $P > 0.05$).

Discussion

A polycystic ovarian syndrome is considered a complex disorder in a clinical setting. The Present study showed autosomal mode of inheritance in all families. Current study describes that there is a strong association of PCOS locus at chromosome 5 with marker D5S822 ($P < 0.05$) in family 2 for allele 1 [Tables 1-5]. A study^[18,22,23] involved families from European descent ($n = 90$), Caribbean descent ($n = 5$), Mexican descent ($n = 2$), African-American descent ($n = 1$), and Asian-Indian descent ($n=1$). The association between PCOS and alleles for four markers (D5S474, D5S822, D5S623, and the SNP in exon 6) was carried out. TDT analysis revealed that 2 markers with the highest X^2 values were the exon 6 variant (allele 1, $X^2 = 5$, $P = 0.025$) and the D5S623 (allele 11, $X^2 = 4.26$, $P = 0.039$). The expression level of follistatin between PCOS and control subjects was compared but no substantial difference was found. The present study highlighted that PCOS are a complex genetic trait requiring immediate medical attention in Pakistan. Current research indicated a very strong association of PCOS locus with marker D15S519 at chromosome 15 for allele1 ($P < 0.01$) in the case of family 5 [Table 2]. This marker is derived from the genetic region of CYP11A which has been involved previously in the etiology of PCOS.^[18] Their association data indicated that allele variants of CYP11A mediate the development of hyperandrogenemia, which, in turn, is associated with PCOS and hirsutism.^[24] Results of family 6 indicated that there is a weak association of PCOS locus at chromosome 19 with marker D19S391 ($P = 0.05$) for allele 1, While a very weak association of PCOS locus at marker D19S212 for allele 1 in the case of family 1 has been observed ($P > 0.01$) [Table 3]. Marker D19S391 has been derived from insulin receptor gene locus while marker D19S212 has been derived from the INSL3 region which represents Leydig insulin-like protein 3. Similar research^[31,32] revealed that insulin receptor gene marker D19S884 was significantly associated with PCOS [$P = 0.001$]. They suggested that the susceptibility gene for PCOS was located

Table 4: The goodness of fit into 1:1 Mendelian ratio in marriages between non-PCOS and PCOS individuals

No of families	Marriage type		Offspring	
	Male X Female		Non-PCOS	PCOS
6	Normal X PCOS	Observed number	19	10
		Phenotypic ratio	1	1
		Expected number	14.5	4.5
		X ²	2.78	p > 0.051

Table 5: The goodness of fit into 3:1 Mendelian ratio in marriages between non-PCOS and PCOS individuals

No of families	Marriage type		Offspring	
	Male X Female		Non-PCOS	PCOS
6	Normal X non- PCOS	Observed number	70	33
		phenotypic ratio	3	1
		Expected number	77.5	25.75
		X ²	2.76	p > 0.05

on chromosome 19p13.3 in the insulin receptor region and insulin-stimulated androgen secretion from the ovarian stroma. INSR function in the ovary is likely involved in the gender susceptibility of PCOS. Another demonstration revealed^[33] that a D19S884 allele shows significant evidence for linkage and association specific to a phenotype of PCOS. A study indicated the involvement of the insulin receptor gene by taking into consideration the tyrosine kinase domain of the insulin receptor gene. They studied 22 patients with PCOS but no abnormalities have been indicated. Molecular scanning of the entire coding region of the gene in 24 hyperinsulinemia subjects was carried out with PCOS but no significant mutation has been detected.^[34] They concluded that mutation of the insulin receptor gene is, therefore, unlikely to be a major cause of insulin resistance in PCOS. A similar opinion was given by another study^[35] in their study. Present study showed secondary amenorrhea in significant number of women which is indicative of anovulation which depicts problem in their steroid and insulin metabolism. Family 2 2 revealed strong association with D5S822 which is follistatin gene marker. Follistatin is involved in autocrine, paracrine and endocrine control of ovarian folliculogenesis.^[29]

Family 5 showed strong association with D15S519 which is involved in steroid hormone control. Candidate genetic loci including CYP11A, CYP17, follistatin, and insulin receptor have been identified by mutation detection, linkage, and case-control association studies. However, traditional linkage analyses have been confounded by extreme phenotypic heterogeneity.^[30] In the search of candidate regions for PCOS, many studies were carried out. Follistatin gene was sequenced but subsequent linkage and association studies yielded negative results.^[22] A vigorous debate is under consideration about whether PCOS is fundamentally a neuroendocrine or ovarian disorder. Evidence is accumulating in favor of the latter. Thus, it seems that PCOS creates genetic complications and causes abnormalities such as steroidogenesis^[25]

It is concluded that different results report the relationship of genes to the risk of PCOS.^[1] The mutations concerning PCOS have not been analyzed extensively particularly its effect on the phenotype. Thus, the future researchers must focus on addressing the mutations of PCOS and its impact on phenotype. The complications in the treatment follow an irregular pattern and they require a patient-centered approach and examination.

Reducing complications of the disease

Polycystic ovarian syndrome which is also known as metabolic syndrome is associated with short-term and long-term complications ranging between obesity hirsutism, menstrual problem, fertility issues and endometrial carcinoma and diabetes mellitus. The reproductive system can be improved by weight loss and a dietary pattern in order to influence insulin resistance. A lifestyle that reduces obesity and IR is the major step to follow by patients with PCOS because even 5% of weight loss can make a significant change. It would also be beneficial to keep track of the situation and provide the patients the safety and therapeutic dietary plans for the recovery procedure. The biosynthesis of CYP11A breaks down the chain of polymerase using the PCR technique which enables the synthesis of accumulated cholesterol and limits a steroid synthetic hormone pathway.^[23,35]

Conclusion

The current study aimed to determine the association of follistatin and cytochrome p450 side-chain cleavage enzyme (CYP11A) in polycystic ovarian syndrome (PCOS). The study targeted the Pakistani population. A sample of 62 participants based on 6 families was collected to obtain data and analyze it in context with CYP11A and PCOS. Furthermore, the findings showed a strong association of polycystic ovarian syndrome (PCOS) locus at chromosome 5 with marker D5S822 in family 2. The PCOS possesses the ability to generate complications in

genetics resulting in abnormalities such as steroidogenesis. The study concluded that treatment and diagnosis complications are a major concern in Pakistan and require a more patient-centered approach to treat the disorder and eliminate the complications. Hence, future medical researchers should pay more attention to addressing the mutations of PCOS and its effect on the phenotype. The study also signifies the sensitivity of the relationship between PCOS and the insulin gene marker (D19S884) that is vitally involved in the gender susceptibility in the ovaries. Multiple genes have been examined for the study of PCOS but none of them seem to play a part in PCOS. However, the candidate genes INS VNTR and CYP11A were observed as the encoders of P450 chain cleavage.

Limitations and future direction

Certain limitations hurdle up while investigating genotypes and phenotypes of the PCOS cases, like, the ongoing need for research on diagnostic methods to the complexities associated with PCOS. Another complication is allocating the visible status of women diagnosed with PCOS and showed inflated responses. There are many clinical and biochemical manifestations advised for the phenotype, primarily irregular menstrual flow, baldness and weight gain, insulin resistance and increased androgens. The minor statistics results were observed in the study due to the smaller size of the sample design of the targeted women population. However, more related studies are required to examine the genotype and phenotype associations of PCOS with specific gene linkages and gene sequencing and development of gene therapy for this syndrome. The knowledge related to humans and genes is escalating more frequently. With the aid of advanced biotechnological techniques and the discovery of novel polymorphisms, there is a possibility of complete mapping of the PCOS locus in the near future. These types of studies will also provide a new way to investigate early PCOS diagnosis in women, screening families with the possibility of PCOS, and measuring complications associated with PCOS in women.

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Authors' Declaration Statements

Ethical approval

The study was carried out after taking ethical approval from the IRB (institutional review board) of Shifa international hospital Islamabad, Pakistan on 29.04.2004 with reference number

IRB# 005-03-04. The study was carried on the 62 individuals and the duration of study was 2 years.

Ethical Consent

No author conducted research using animals. All operations involving human subjects were authorized by the institutional committee ethical standards and in line with the Helsinki Declaration (1964) and its subsequent revisions.

Patient's Consent Statement

Consent was taken before going to conduct this study.

Availability of Data and Material

Data will provide on request.

Competing Interests

There author declares no conflict of interest.

Funding Statement

None

Authors' Contributions

All authors have contributed equally in design, data collection, manuscript preparation, and revision. All authors agreed on the results and therefore approved the final version of the manuscript.

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