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Association of SNP *rs*700519 in *CYP19A1* Gene with Polycystic Ovary Syndrome (PCOS) among Females of Quetta, Pakistan

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ABSTRACT Polycystic Ovary Syndrome (PCOS) is endocrine reproductive disorder which causes oligomenorrhea/ amenorrhea, infertility, type II diabetes. The present study aims in *CYP19A1* polymorphism rs700519 (C/T) identification that elevates androgen among PCOS females in Quetta, Pakistan. Cross-sectional study involved enrollment of 100 control and 100 affected females. Blood samples were collected for genetic and hormonal analysis. The samples were amplified via ARMS PCR and analyzed by sequencing. The frequency of CC genotype in control and PCOS group was 48 percent and 33 percent. For CT, it was 52 percent and 67 percent. In control group, the allele frequency for C and T was 0.74 and 0.26. In PCOS group, it was 0.67 and 0.33 for C and T, respectively. The Pearson Chi-square p=0.031 ($p<0.05$) at 95% Confidence Interval inferred a significant difference between the observed genotypes. The study inferred that CT genotype is a risk factor for PCOS progression in the population of Quetta.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is an endocrine heterogeneous disorder (Fauser et al. 2012) characterized by ovary dysfunction that leads to the irregular menstrual cycle, hirsutism, acne (Azziz et al. 2006), infertility, sleep apnea (Deeks et al. 2010), anxiety and obesity that leads to type 2 diabetes due to insulin resistance (Manjunath et al. 2013). The prolong condition of PCOS increased the risk of endometrial cancer (Helvaci et al. 2017). PCOS susceptibility is mainly due to hyperandrogenism that leads to conditions like acne and hirsutism. Whereas type II diabetes appears due to insulin resistance that occurs because of constant hyperandrogenism in females (Saddick 2020). According to NIH, the prevalence rate of PCOS worldwide is 51 percent while as per the Rotterdam criteria it was reported as 83 percent (Amato et al. 2008). PCOS affect females under aged 18-44 (Teede et al. 2010). About 70 percent of females are infertile because of PCOS (PCOS Foundation 2011). The Rotterdam criteria assist in the diagnosis of the PCOS (Rotterdam 2003). PCOS patients have numerous cysts inside the sac of an ovary, there are 12 or more than cysts and of 8mm in size (Futterweit 1999). There is no such treatment to completely cure PCOS, medications and a healthy lifestyle can reduce the severity of this condition. Losing weight up to 5-10 percent reduce the severity (Galletly et al. 1996).

The primary etiology of PCOS is still unknown and there is a combination of factors that are reported. Immunological, biochemical, environmental, and genetic factors are involved in the etiology of PCOS (Deepika et al. 2012). Many candidate genes have known to be responsible for cause of PCOS. Environmental factors include unhealthy diet, toxins reactions, and mediator infections (NHS 2016). Out of many candidate genes aromatase (steroidogenesis enzyme) is also known to be involved in the etiology of PCOS.

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Aromatase genes such as *CYP11A1, CYP11B2, CYP3A7, CYP17A1, CYP1A1, CYP21A2* and *CYP19A1* (Joseph et al. 2015). One of the aromatase genes is *CYP19A1* that converts androgen to estrogen. The chromosomal location of the *CYP19A1* gene is 15q21.2 which spans 123kb which also includes 9 protein-coding exons, an alternative tissue-specific promotor with a large 5' Untranslated Region of 93 kb (Means et al. 1991). *CYP19A1* gene is mainly expressed in the placenta, skin, bone, gonads and adipose (Henderson et al. 1991). The abnormality present in the *CYP19A1* disrupts its functionality and the conversion of androgen into estrogen ceased (Sebastian and Bulun 2001). The polymorphism presents in the exonic region rs700519 (C/T) is known to be responsible for PCOS (Lee et al. 2010; Reddy et al. 2015).

Objectives

The present study aims in the identification of polymorphism in the exonic region rs700519 (C/T) of the *CYP19A1* gene in the females affected with PCOS in the population of Quetta, Balochistan, Pakistan.

MATERIAL AND METHODS

The cross-sectional study involved the enrollment of 100 control and 100 affected females. The females aged 18-44 were selected for the study of polymorphism. The diagnostic criteria were according to the Rotterdam criteria. Patients were selected from the Out-Patient Department (OPD) of Civil hospital Quetta. Their age, marital status, family history and clinical examination were recorded, and the study was approved from Institutional Review Board (IRB) BUITEMS, Quetta.

Inclusion and Exclusion Criteria

The inclusion criteria included all the PCOS patients that were diagnosed based on the Rotterdam criteria and were under the aged of 15-45 years. The females with pregnancy, Gestational Diabetes Mellites, ovaries melanoma, prolactinoma and thyroid dysfunction were excluded from the study.

Genotyping Analysis of CYP19A1 SNP rs700519 (Arg264Cys)

After taking the inform consent from studied group, 6ml peripheral blood was drawn for genomic DNA extraction using Inorganic method and stored at -20 C. 3ml blood was preserved in serum tubes that were sent for total testosterone test using immunoassay method in endocrinology laboratory. The extracted DNA samples were estimated on 2 percent agarose gel and quantified at A_{260} and A_{280} using BIO-RAD Smart Spec Plus Mass Spectrophotometer. Genotype analysis was performed using T100™ thermocycler BIO-RAD USA. Allele-specific tetra primers were used for Amplified Refractory Mutation System (ARMS) PCR. Following are the sequences of tetra primers:

Outer Forward:
5'-GAAGTGTAGGGTCTATGTA-3'
Inner Forward:
5'-CTGATAGCAGAAAAAAGAT-3'
(T allele-specific) Outer Reverse:
5'CTCTGTGATTGACTGTGGAC-3'
Inner Reverse:
5'-CTCTTCTGTGGAAATCCTGCG-3'
(C allele specific)

For the identification of *CYP19A1* polymorphism, the regents used for ARMS PCR were: 10µl Master Mix, 0.3 µl of each primer, 3µl of DNA, 0.2 µ$MgCl_2$ and 5.6µl of PCR H_2O were used in a total 20µl of the reaction mixture. The conditions used for ARMS PCR were: Initial denaturation at 95^0C for 5 minutes. 35 cycles were performed for denaturation, annealing and extension. Denaturation of DNA template was set at 94^0C for 30 seconds, aneling at 58^0C for 1 minute and extension at 72^0C for 1 minute. The fourth step was the final extension which was at 72^0C for 10 minutes. Thermocycler BIO-RAD (USA) remains at 4^0C until the products were safely removed from the machine. 2 percent agarose gel was used for genotyping analysis which was then visualized on Gel Documentation System BIO-RAD (USA). For genotype analysis, bands of 650bp and 251bp indicated wild type homozygous, while 650bp and 439bp indicated mutant homozygous and 3 bands of 650bp, 439bp and 251bp shown heterozygous genotype. Samples with each genotype were se-

quenced at Macrogen South Korea using the Big Dye Terminator sequencing kit (genetic analyzer ABI-USA).

Statistical Analysis

MS Excel 2016 and SPSS version 20 was used for statistical analysis. Graphs on selected parameters for control and PCOS groups were made. The genotype and allelic frequencies were determined using SPSS Version 20 and using Pearson Chi-Square, the statistically significant differences between genotypes of control and PCOS groups were determined. The significant differences were observed at $p<0.05$ at 95% Confidence Interval.

RESULTS

Parameters such as age ratio (Table 1), demographic features (Table 2) such as family history, marital status and clinical examinations were determined for the control and affected group. Following tables displayed the difference of parameters among both groups.

Table 1: Age distribution among PCOS and Non-PCOS females

	Age ratio	
Age range	*Non-PCOS females*	*PCOS females*
15-25 years	47%	29%
26-35 years	20%	31%
36-45 years	33%	40%

Table 2: Demographic characteristics identification of non PCOS and PCOS females

Demographic characteristics	*Non-PCOS females*	*PCOS females*
Family history	29%	71%
Married	37%	62%
Unmarried	63%	38%

The gel images of both control and affected group displayed the observed genotypes. The band for CC genotype was observed at 650bp and 251bp and band for CT was observed at 650 bp and 439bp and 251bp. Figure 1 displayed the gel image of the observed bands of amplified products of both control and PCOS group.

Clinical features identified in PCOS females are presented in Table 3. In the study groups, the general distribution of genotypes was 48 percent for CC, 52 percent for CT of a control group. The observed genotypes for the PCOS group were measured as 33 percent for CC and 67 percent for CT. The allelic frequencies for control group were calculated as 0.74 for C and 0.26 for T. Whereby the allelic frequencies for a PCOS group were calculated as 0.67 for C and 0.33 for T. Thus, the dissemination of genotypes among both groups was in accordance to Hardy-Weinberg Equilibrium. Table 4 shows the general distribution of observed genotypes of control and PCOS group and their calculated allelic frequencies. Total testosterone values of both the groups being studied are given in Table 5 that were under the normal range 0.1-0.9ng/ml. Table 6 displayed the Pear-

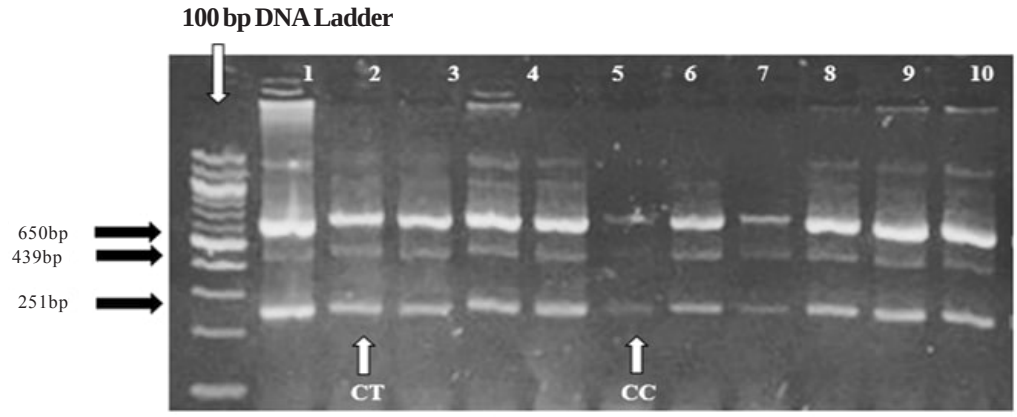

Fig. 1. Gel image of control and affected samples that represents homozygous and heterozygous genotype

Table 3: Clinical features identified in PCOS females for the diagnosis.

Clinical features identified in PCOS female	
Sign & symptoms	*No. of females (In percentage)*
Obesity	17
Infertility	10
Acne	12
Anovulation/Oligoovulation	20
Hirsutism	19
Anxiety/Depression	14
Sleep Apnea	8

Table 4: Genotype and allelic frequencies of control and PCOS group

Groups	*Genotype frequency*			*Allele frequency*	
	CC	*CT*	*TT*	*C*	*T*
Control (n=100)	48%	52%	0	0.74	0.26
PCOS (n=100)	33%	67%	0	0.67	0.33

son Chi-square value, p-value at ($p<0.05$) at a 95 percent Confidence Interval. The value of Chi-square was calculated as 4.669. The p-value calculated was 0.031 ($p<0.05$) and 95 percent Confidence Interval was calculated as 0.038-0.046 which determined significant differences found between the genotypes.

The polymorphism at rs700519 of the *CYP19A1* gene was further confirmed by DNA sequencing. Figure 2 represents the comparison of homozygous and heterozygous genotype. The sequence was analyzed using SeqMan™ II Sequence Analysis Software.

Table 5: Total testosterone test of both study groups shown the normal testosterone level

Groups	*ID*	*Obtained value*
Control Group CC Genotype	PCOC1	0.31
	PCOC2	0.70
	PCOC3	0.40
	PCOC4	0.39
	PCOC5	0.21
	PCOC6	0.41
	PCOC7	0.22
	PCOC8	0.45
	PCOC9	0.32
	PCOC10	0.40
Affected Group CT Genotype	PCOP1	0.22
	PCOP2	0.15
	PCOP3	0.60
	PCOP4	0.71
	PCOP5	0.43
	PCOP6	0.31
	PCOP7	0.22
	PCOP8	0.42
	PCOP9	0.35
	PCOP10	0.45

Table 6: Statistical analysis of control and PCOS group

Groups	*Chi-square*	*Degree of freedom*	*P-value*	*95% confidence interval*
Control VS PCOS Affected group	4.669	1	0.031 P<0.05	0.038-0.046

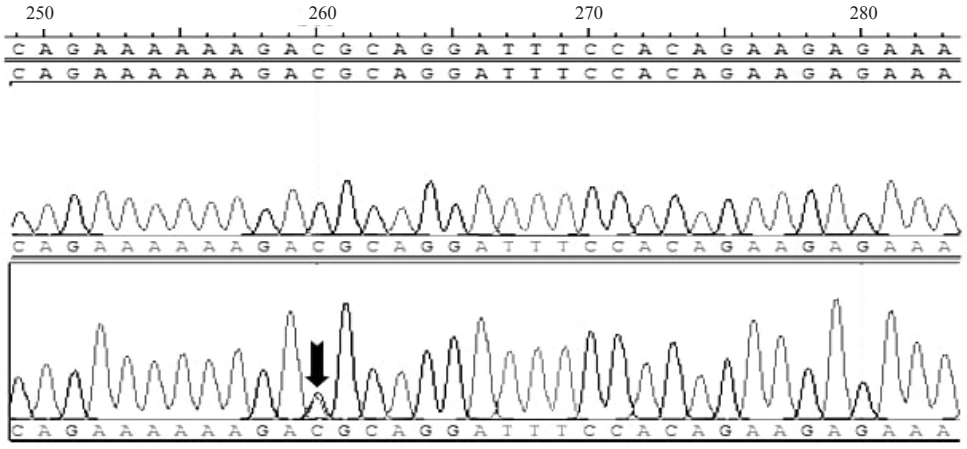

Fig. 2. Chromatogram represents the comparison of homozygous and heterozygous genotype

DISCUSSION

Endocrine metabolic disorder PCOS causes many metabolic anomalies among females (Legro 2003). In a present study, the control group was under age 15-25 years (47%), 26-35 years (20 %), 36-45 years (33%). While the affected group had females under 15-25 years (29%), 26-35 years (31%) and 36-45 years (40%). Different studies conducted on various populations involved females under reproductive age. Hussein conducted a study among females of the Kurdish population who were under age 25-29 years (30.2%) belong to the PCOS group and 35-39 years (24.3%) from a healthy group (Hussein and Alalaf 2013).

Positive family history among affected females was observed as a risk factor in previous studies.A previous study conducted among PCOS females at Birmingham reported positive family history. They had PCOS inherited from their mothers or sisters. The study further suggested that about 35 percent of mothers and 40 percent sister have PCOS or more prone to have PCOS in the future (Azziz and Kashar-Miller 2000). The present study observed a positive family history among PCOS group. Among 100 PCOS individuals, 29 percent of females have a positive family history of PCOS.

Previous findings depicted that PCOS married women have increased risk of infertility. 80 percent of married females affected with PCOS are infertile (Melo et al. 2015). With relevance to this, the current study found that among 100 PCOS women 10 percent of females were infertile due to PCOS in the population of Quetta, Balochistan. In a previous study conducted among females of Qatar population, it was observed that clinical features vary from individual to individual. In current study based on diagnostic criteria about 31.7 percent of females had hirsutism, 30.8 percent had severe acne, 63.3 percent are the females with positive type 2 Diabetes history and 30.8 percent of females had Oligomenorrhea/ Amenorrhea (Sharif et al. 2016).

Different studies conducted on healthy and PCOS individuals inferred that various cytochrome family is involved in the cause of PCOS. A previous study conducted among the females affected with PCOS of South Indian Population has shown that variants at exonic region rs700519 and two intronic regions rs2414096, rs60271534 are involved in the PCOS etiology. The Insilico analysis further displayed the destabilized structure of aromatase in the affected females. The study further suggested identifying the different variants among the different populations (Reddy et al. 2015).

The current study conducted among females of the Quetta population had a significant increase of CT genotype in females affected with PCOS while normal females had a significant increase in CC genotype. Hence it can be inferred that CT genotype is a risk factor in a female affected with PCOS among the population of Quetta, Balochistan and is more significant in the affected group. The statistical analysis on both groups further interpreted that based on of Chi-square ($p<0.05$) there is a significant difference found between the genotypes of control and affected group. Thus the heterozygous genotype CT can be a risk factor in a female affected with PCOS among the population of Quetta.

CONCLUSION

The study conducted in the females affected with PCOS among the population of Quetta, Balochistan inferred that CT genotype is a possible risk factor in the pathogenesis of PCOS.The identified genotype CT in the affected group is recessive heterozygous. There is a significant variation found between the genotypes of control and affected group.

RECOMMENDATIONS

Further variants can be identified from different ethnic groups thereby it is suggested that more research studies must be conducted to find out the best possible variants responsible for PCOS progression and severity.

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