SHORT COMMUNICATION

Genetic association study of *ERBB4* SNP rs1351592 with polycystic ovary syndrome in Pakistani population

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Abstract

Polycystic ovary syndrome (PCOS) is an oligogenic condition characterised by hyperandrogenism, ovulatory dysfunction and polycystic ovarian morphology. Previously, European and Han Chinese populations identified different susceptibility loci, of which *ERBB4* (rs1351592) was strongly associated with PCOS. Our study aimed to investigate the association of *ERBB4* Single Nucleotide Polymorphism (SNP), rs1351592 with PCOS in Pakistani women of Hazara region. Fifty PCOS patients and 14 healthy women were recruited and SNP was replicated using ARMS-PCR and sequencing. The study showed that Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), and Testosterone (T) were significantly elevated in patients compared to controls ($P < 0.05$). Overall, the frequency of G allele was higher than C allele and the SNP lacked significant association with PCOS.

This is the first study demonstrating the association of *ERBB4* SNP, rs1351592 with PCOS in Pakistani population. Further research using larger population size will help to estimate the role of *ERBB4* SNP as potential biomarker for disease diagnosis.

Keywords: Polycystic ovary syndrome, *ERBB4*, Multifactorial disorder, Pakistani population.

DOI: https://doi.org/10.47391/JPMA.1026

Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial disorder, described as the combination of hyperandrogenism (hirsutism and acne), anovulation (oligomenorrhoea and infertility) and polycystic ovaries at ultrasound.[1] It is a major gynaecological endocrinopathy, affecting 6-10% of women during their reproductive age.[2,3] The precise pathophysiological causes have not yet been established; however, it represents the leading cause of female infertility due to anovulation.[4] Some other risks and complications may be associated with PCOS, including Insulin resistance (IR), obesity, Type 2 diabetes mellitus (T2DM), dyslipidaemia, Metabolic Syndrome, hypertension, cardiovascular ailment, hyperplasia and endometrial carcinoma.

PCOS is genetically heterogeneous, though environmental factors such as diet, geography and socioeconomic status are also involved.[5] Recent studies in European and Han Chinese populations have identified different susceptibility loci, of which *ERBB4* SNP, rs1351592 had shown significant association with PCOS.[6,7] *ERBB4* encodes the transmembrane tyrosine kinase receptor. It plays a crucial role in the regulation of cell growth, survival, proliferation and differentiation through multiple signal transduction pathways.[8] Any type of mutation in the *ERBB4* may lead to aberrant activity of *ERBB4* receptor, which may cause high incidence of tumours (melanoma, lung and colorectal cancer and several neuronal disorders).[9] Based on its role as a disease marker in Europeans and Han Chinese, association of *ERBB4* SNP, rs1351592 was explored in PCOS in Hazara division, Khyber Pakhtunkhwa, Pakistan.

Patients/Methods and Results

The study was conducted from November 2017 to September 2018. A total of 64 female subjects, including 50 PCOS patients and 14 controls were recruited from the Department of Obstetrics and Gynaecology, Ayub Medical Institute and Hospital, Abbottabad, KPK, Pakistan. The study was approved by the institutional review board and the ethical committee of Hazara University Mansehra, according to the declaration of Helsinki. Informed written consent was obtained from all the participants and detailed history was recorded through a well-designed questionnaire. The clinical details included symptoms such as weight, status of the menstrual irregularities, presence of hirsutism, hair fall and acne, along with current pregnancy status, previous pregnancy complications, medical treatment for PCOS and family history. Socioeconomic information included age, education, employment and marital status.

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Table-1: Descriptive statistics and comparison of study variables among cases and controls.

Study Variables	Study Groups	N	Mean±SD	Minimum	Maximum	p-value
Age (Year)	Control	14	29.64±5.15	22	41	0.16
	Case	50	27.1±6.09	16	40	
Weight (Kg)	Control	11	63.64±14.6	42	99	0.045**
	Case	50	71.9±12.9	50	110	
FSH (mIU/ml)	Control	14	2.63±1.12	0.46	4.29	<0.001***
	Case	50	12.23±17.49	0.98	88.2	
LH (mIU/ml)	Control	14	4.45±1.33	2.1	6.98	0.002***
	Case	50	14.53±21.19	0.17	110.2	
T (ng/ml)	Control	14	1.37±0.71	0.46	12.01	<0.001***
	Case	50	3.40±3.27	0.11	310.2	
PRL (ng/ml)	Control	14	273.57±113.12	98.1	432	0.45
	Case	50	231.96±195.00	0.78	962	

N: Number of subjects; SD: Standard deviation; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; T: Testosterone; PRL: Prolactin; Significance levels: (P<0.05**, P<0.01***).

Table-2: Allelic frequencies in study population (Total, PCOS and Control).

Variables	Frequency (%)			OR (95% CI)	P- value
	Total	PCOS	Control		
Alleles G/C	88(0.71%)/ 36(0.29%)	67(0.68%)/ 31(0.32%)	21(0.81%)/ 5(0.19%)	0.69 (0.32-1.53)	0.37
Exact test for HWE (p-value)	<0.0001	<0.0001	0.026		

OR: odd ratio; CI: confidence Interval; HWE: Hardy Weinberg equilibrium.

Ultrasonographs of ovaries of all participants were analysed by gynaecologists in Ayub Medical Hospital Abbottabad. The diagnosis of PCOS was based on two out of three Rotterdam Criteria including Hyperandrogenism (HA), ultrasonography finding [≥12 follicles with a diameter of 2-9 mm in at least one ovary (PCO) or increased ovarian volume >10 ml and anovulation (OA)].[10] Women in the control group were healthy females and had no menstrual irregularities, HA and PCO. Blood samples were obtained from all the participants in clot/gel activator and Ethylenediaminetetraacetic acid (EDTA) coated tubes for hormonal profiling and DNA extraction respectively.

Blood collected in clot/gel activator tubes were centrifuged to separate plasma. The plasma was then transferred to the Eppendorf tubes and subjected to analyser (ADVIA Centaur XP 1904) for the biochemical measurement of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), Testosterone (T) and Prolactin (PRL).

The genomic DNA was extracted using Phenol-chloroform method. Genotyping of all the subjects was conducted using Amplification Refractory Mutation System (ARMS-PCR). Primers [(outer F 5'-ATTTTTATAAGCATCATCTGGTCCTTAG-3'; outer R 5'-TGAAGGAAGATGTAACAAAGACGTATAA-3') (inner (G) F 5'- TCAATTTCTTTTTAAATCCATCATACAG- 3'; inner (C) R 5'-GGTTTTTAGACAGAGTACATGTTAACACAG-3')] were designed using online software (http://cedar.genetics.soton.ac.uk).

Optimisation of PCR reaction mixture and primer annealing temperature were carried out on BIOER XP Cycler. The reaction was carried out in 25ul mixture

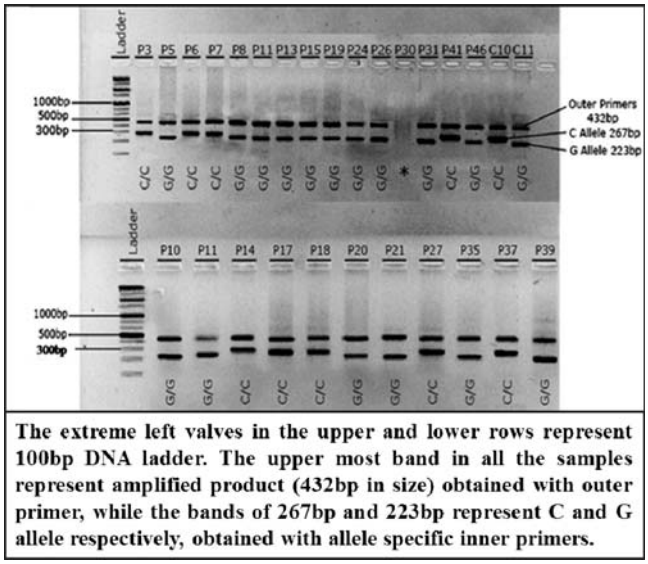

Figure-1: Agarose gel electrophoresis showing the ARMS-PCR results for Patients (represented by P) and Controls (represented by C).

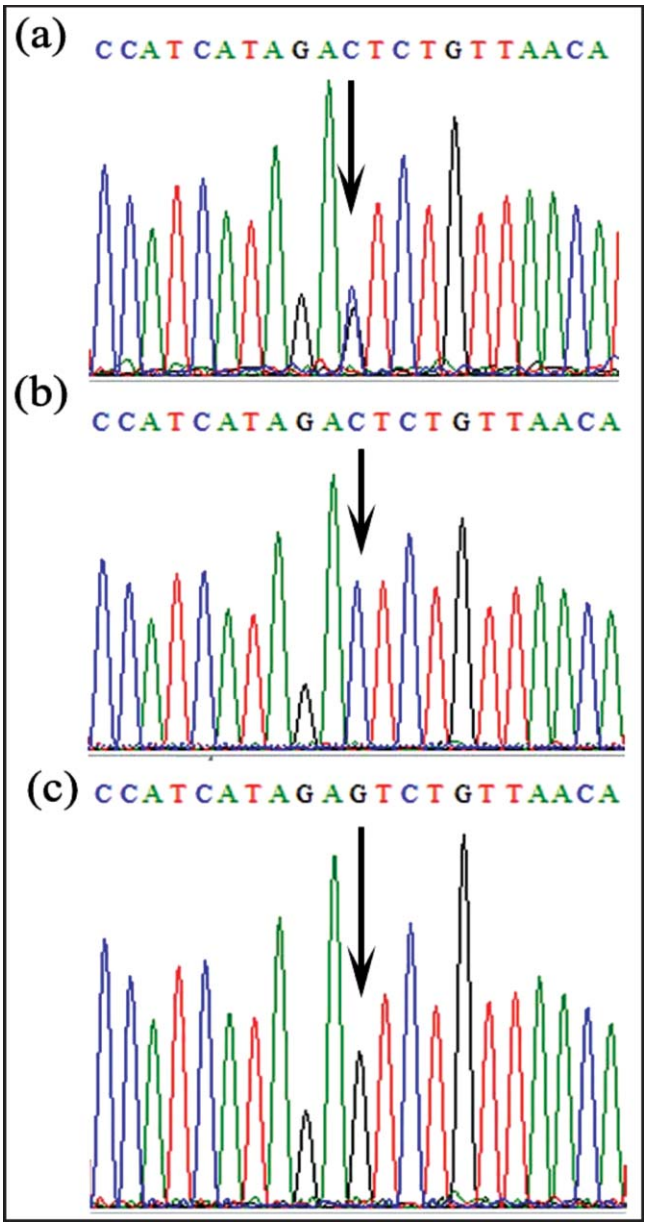

Figure-2: Sequencing chromatograms of SNP rs1351592 (a) Representing the alleles in the heterozygous (GC) condition, (b) Representing the alleles in the homozygous (CC) condition, and (c) Representing the alleles in the homozygous (GG) condition.

containing DNA Template (20ng/µl), 10µl DreamTaq Green PCR Ready Master Mix (Cat# K1081 M/s Thermo Scientific™) and 20pmoles/µl of outer and inner primers in different ratios including 1:1, 1:2, 1:3, 1:4, 1:8, 1:10, 2:5, 3:5, 4:5 and 5:5. The cycling parameters included, initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing temperature (54-62°C) for 30 seconds and extension at 72°C for 45 seconds. Final extension was set at 72°C for 10 minutes at the end of 35 cycles.

The PCR amplified products were separated by running on 2% agarose gel with the DNA ladder of 100bps and the G and C alleles were assigned by visual inspection. A few randomly selected DNA samples were subjected to Sanger sequencing for the confirmation of PCR based genotyping.

Statistical Package for Social Sciences (SPSS) 20.0 version was used for analysing the results. The t-test was used to compare the hormonal profile of PCOS with the control group. Fisher's exact test was applied to check whether the study population is in Hardy Weinberg Equilibrium (HWE) or not. Co-dominant, Dominant, Recessive and Over-dominant models were used to compare the genotypic frequencies between the cases and the control group. A p-value of ≤ 0.05 was considered statistically significant.

The participants in the current study belonged to different areas of Hazara region with higher frequency 28 (43%) from Abbottabad. According to the data, 31 (62%) of the PCOS women in the study group were married out of which 16 (51%) did not report any pregnancy complications and gave birth to at least one child. The remaining 15 (49%) of the PCOS subjects were infertile either at primary or secondary level, with first or second trimester pregnancy losses and ectopic pregnancies. Analysis of ultrasound reports revealed bilateral polycystic ovaries in majority of the patients, 41(82%) while only 9 (18%) patients had unilateral polycystic aetiology. Among the PCOS, 32 (65%) reported oligomenorrhoea, 9 (19%) had amenorrhoea, while 7 (14%) had polymenorrhoea. Only 23 (46%) PCOS patients were taking medical treatment (Duphastan, Primolute-N, Glucophage, Diane 35, and Clomid). The presence of hirsutism, hair fall and acne problems were found in 11 (22%), 17 (34%) and 32 (64%) patients respectively. Of all the study subjects, 31 (62%) women had family history of PCOS, while the remaining did not report any family history. The control group did not present menstrual irregularities and polycystic ovary/ovaries.

The mean age of control and PCOS study subjects was 29.64±5.15 and 27.1±6.09 years respectively. The PCOS patients weighed more as compared to the control subjects ($p=0.045$) and had significantly raised levels of hormones: FSH ($p<0.001$), LH ($p<0.002$) and T ($p<0.001$). Whereas, PRL did not differ significantly in cases vs controls ($p=0.45$) as shown in Table-1.

In ARMS-PCR, thickest specified DNA bands were obtained with a ratio of 4:5 for outer and inner primers. The best annealing temperature for all the four primers was 56°C. Once the conditions for ARMS-PCR were

optimised, DNA from the study participants was amplified and analysed (Figure-1).

Genotyping and sequencing results (Figure-2) revealed higher frequency of minor allele G in total study population, cases and controls compared to the major C allele shown in Table-2. No significant association of SNP was observed with PCOS (OR= 0.69, 95% CI: 0.32-1.53, p = 0.37). Fisher's exact test showed that the study population did not follow the HWE, both in cases and control subjects. (Table-2)

To check the association of the studied SNP with PCOS, four different types of genetic models were constructed, i.e. Co-dominant, Dominant, Recessive and Over-dominant. None of the genetic model showed significant association with the disease as all p-values were greater than 0.05.

Conclusion

This study has provided an insight for further research and should be extended to larger sample size and include other sub-populations from Pakistan. This will not only confirm the association of SNP with our population but will also help to assess the prevalence of the disease in Pakistan. Additionally, such studies will increase disease awareness and will help to identify potential biomarkers for diagnosis. Ultimately, it will lead to improvement in the health and lifestyle of our women, enabling them to contribute actively in the growth of the country's economy.

Disclaimer: The Manuscript is part of MPhil research of Miss Tayyaba Afzal

Conflict of Interest: None.

Funding Disclosure: Tayyaba Afzal (T.A.) was supported by Higher Education Commission (HEC) under Access to Scientific Instrumentation Programme (ASIP).

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