ORIGINAL ARTICLE

ASSOCIATION OF *Nrf2* GENE POLYMORPHISM WITH POLYCYSTIC OVARY SYNDROME —A CASE-CONTROL STUDY

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Background: Polycystic Ovary Syndrome (PCOS) is considered as one of the most common endocrine disorder in women of reproductive age. Exact cause of PCOS is still unknown. However, evidence for genetic basis has been reported. Reactive oxygen species and antioxidants have been documented as key factors involved in ovarian physiological metabolism. *Nrf2* is a key transcription factor that regulates the expression of antioxidant proteins, therefore provides protection against oxidative stress. Objective of this study was to find the association of *Nrf2* (rs6721961) gene polymorphism with pathogenesis of PCOS among Pakistani population. **Methods:** This case-control study was conducted in Pakistan Railway Hospital, Rawalpindi, from Oct 2020 to Sep 2021. The study included 200 PCOS patients diagnosed according to Rotterdam diagnostic criteria and 200 healthy controls. Blood samples of all participants were collected and DNA was extracted by Chelex Method. Polymerase Chain Reaction (PCR) was performed to find respective allelic frequencies of *Nrf2* (rs6721961) genotype using specific primers. **Results:** The frequency of CC, CA and AA genotypes of rs6721961 polymorphism of *Nrf2* gene were 72.5%, 27.5%, 0% in controls and 56.5%, 39.5%, 4% in cases. Significant association of CA genotype of *Nrf2* gene polymorphism (rs6721961) and allele A were found with PCOS (OR: 0.54, 95% CI: 0.35–0.82, p=0.004), (OR: 0.61, 95% CI: 0.42–0.89, p=0.01) respectively. **Conclusion:** *Nrf2* gene polymorphism (rs6721956) is significantly associated with PCOS among Pakistani population.

Keywords: Polycystic ovary syndrome, *Nrf2* gene, Rotterdam diagnostic criteria

Pak J Physiol 2022;18(3):15–8

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is a common hormonal disorder and one of the most prevalent endocrinopathy in women of reproductive age.[1] This disorder tremendously affects the quality of women's life during reproductive years.[2] Evidence suggests that it is a multifactorial disease caused by genetic[3] and environmental[4] influences. Environmental influences include diet, socioeconomic status, geography and environmental toxins.[4] Moreover, polymorphisms and differential regulation of genes are also believed to affect the pathogenesis of PCOS.[5] The recent studies carried out in different geographical locations in Pakistan showed that prevalence of PCOS is increasing day by day.[6] A significant association has been found between PCOS and genetic factors in various populations all over the world.[7]

Reactive Oxygen Species (ROS) are produced as by-products during ovarian physiological metabolism and antioxidants act as factors that can maintain the balance between ROS production and clearance.[8] Previous work has established that the balance between ROS and antioxidants greatly influences the reproductive activities in female mammalian animals such as endometrial changes in luteal phases, implantation, ovulation and fertilization.[8,9] However, under oxidative stress conditions, compromised reproduction and fertility may be induced including impaired ovarian functions, deteriorated oocyte quantity and infertility.[8] Therefore, antioxidants are crucial for maintaining the redox balance in the ovaries to support normal ovarian function.[10]

Paraoxonase 1 (*PON1)* gene encodes for antioxidant enzymes. Q192R, C108-T, and L55M polymorphisms in *PON1* gene are associated with the risk of developing PCOS.[11] A16V polymorphism in *Superoxide dismutase 2* (*SOD2*) gene was also found to be associated with PCOS.[12] Nuclear factor erythroid 2-related factor 2 (*Nrf2*) is an important transcription factor that regulates the expression of antioxidant proteins, therefore provides protection against oxidative stress.[13] Polymorphism in *Nrf2* gene has been found to be associated with various diseases.[14] A number of genetic studies have established the association of *Nrf2* gene polymorphism (rs6721961) with conditions linked to oxidative stress such as respiratory diseases, cardiovascular diseases and male infertility.[15] However, no research to our knowledge has been done to date to find out association of polymorphism in *Nrf2* gene with PCOS. The objective of this study was to assess the association of *Nrf2* gene polymorphism (rs6721961) with PCOS.

MATERIAL AND METHODS

In a case-control design, a total of 200 patients with PCOS were recruited from the Gynaecology Department of Pakistan Railway Hospital, Rawalpindi between October 2020 and April 2021. The Hospital caters for patients from all over Pakistan. All cases were diagnosed with PCOS as per Rotterdam diagnostic criteria.

A total of 200 healthy, premenopausal women were recruited as the control group from the local community. All cases and controls had no history of hyperprolactinemia, Cushing's syndrome, thyroid dysfunction, androgen secreting tumour, pregnancy, hormonal therapy including oral contraceptives for at least 3 months prior to the study. Participants provided written informed consent for the study. The study was approved by the Ethical Review Committee of Islamic International Medical College, Rawalpindi (Appl. # Riphah/IRC/20/235. October 19, 2020).

Clinical assessment was done with a questionnaire-based interview regarding socio demographic factors, menstrual cycle and characteristics of PCOS. Detailed physical examination included measurement of height, weight (to determine BMI), waist and hip circumference (waist to hip ratio), systolic and diastolic blood pressure, hirsutism (mFG score), and distribution of acne.

After obtaining written informed consent from the participants, 2–3 ml fasting venous blood samples were collected for serum measurements and genomic DNA isolation. Hormonal evaluation included levels of total testosterone, serum hormone-binding globulin (SHBG), DHEAS, LH, FSH and E2. Fasting glucose and insulin levels were also determined. Genomic DNA extraction from whole blood was done by Chelex Method and stored at -70 °C until use. The rs6721961 polymorphism was identified by multiplex Tetra-primer ARMS-PCR by using specific primers (Table-1).

Table-1: Primer sequence of *Nrf2* gene

Primers	Primer Sequence 5′ to 3′
***Nrf2* FP 1**	CTCCGTTTGCCTTTGACGAC
***Nrf2* RP 1**	GGGGAGATGTGGACAGCG
***Nrf2* FP 2**	GCGAACACGAGCTGCCGGA
***Nrf2* RP 2**	CCCTGATTTGGAGTTGCAGAACC

The PCR reaction was performed in a tube containing forward and reverse primers specific to *Nrf2* gene (rs6721961). The final total volume for PCR reaction was 24.5 µl. PCR reaction mixture constitutes 8.5 µl water by Invitrogen™, 12.5 µl 2× Thermoscientific™ Master Mix which consists of 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). Each primer vial contained 30 nmol of respective primer which was diluted to 0.01 nmol/µl working solution. Primer were supplied by M/S Macrogen™ (Korea). A final volume of 0.5 µl was added to reaction mixture from 4 primers. 3 µl of DNA was added from sample to be genotyped. Extracted DNA was subjected to multiplex tetra ARMS-PCR and respective allelic frequencies were recorded.

PCR was done by DNA denaturation at 96 °C (5 min) accompanied by 35 cycles of 96 °C (1 min), annealing at 63 °C (1 min), extension at 72 °C (1 min) and an ultimate extension step at 72 °C (5 min). Resolution of PCR amplicons was done on two percent agarose gel to envision the DNA bands under ultra violet light in Syngene gel imaging system (Gene Box™).

SPSS-21 was used for data analyses. The differences in demographic and clinical characteristics were compared using independent sample *t*-test between cases and controls. Frequencies and percentages were determined for descriptive statistics. Associations of *Nrf2* genotypes with PCOS were determined by calculating the odds ratio (OR) and 95% confidence intervals (CIs), and $p \leq 0.05$ was considered statistically significant.

RESULTS

Cases and controls were divided into two groups on the basis of age, 16–30 years and 31–60 years. Mean age of PCOS cases was 35.66±11.12 years, which was not significantly different from that of the control group 33.46±10.95 (p=0.04). Anthropometric characteristics revealed significant differences (p<0.01) for BMI (Kg/m^2), waist (inches), hip (inches), LH/FSH, total testosterone (0.004) values between age groups 16–30 years and 31–60 years in PCOS cases. Phenotypic characteristics including acne were also found to be significantly associated with the two age groups in PCOS cases (p=0.01). Fasting glucose level, systolic and diastolic blood pressure were significantly different between the age groups 16–30 years and 31–60 years in cases. Among controls, significant differences (p=0.01) was found for LH/FSH ratio in the two age groups.

The PCR products size for rs6721961 polymorphism were 113 bp for C allele, 205 bp for A allele and 282 bp for control on a 2% agarose gel. The amplicons derived are shown in Figure-1.

The allele and genotype frequencies were compared between cases and controls. We found that the frequency of CA genotype as well as allele A were significantly different in PCOS cases compared to controls, where the heterozygote genotype CA as well as the variant allele A seemed to confer significant protection against developing PCOS (OR: 0.54, 95% CI: 0.35–0.82, p=0.004, (OR: 0.61, 95% CI: 0.42–0.89, p=0.01) respectively. (Table-3).

Table-2: Demographic, clinical and hormonal characteristics of the study population

Variables	PCOS (n=200)		*p*	Controls (n=200)		*p*
Age group (Years)	16–30	31–60		16–30	31–60	
BMI	26.51±4.15	28.48±3.20	<0.001	23.06±1.68	22.96±1.86	0.70
Waist	35.11±2.36	36.57±1.51	<0.001	31.64±2.62	31.79±2.89	0.69
Hip	39.82±3.15	41.28±2.23	<0.001	36.64±2.40	36.88±2.58	0.50
WHR	0.87±0.03	0.88±0.03	0.54	0.85±0.02	0.85±0.03	0.84
mFG	7.69±1.41	7.75±1.89	0.08	3.70±1.81	3.56±1.91	0.60
LH	12.90±4.69	11.64±4.40	0.56	7.14±2.37	7.40±2.48	0.44
FSH	3.38±1.52	3.90±1.66	0.25	4.76±2.42	5.34±2.33	0.88
LH/FSH	4.32±2.14	3.31±1.72	<0.001	1.72±0.66	1.50±0.47	0.01
TT	4.00±0.88	4.43±1.12	0.004	1.64±0.68	1.57±0.69	0.49
SHBG	21.02±17.72	18.86±18.05	0.40	59.39±22.56	63.65±19.84	0.16
Fasting insulin	111.48±45.52	99.66±43.31	0.06	65.72±18.96	64.95±17.26	0.76
Fasting glucose	4.63±0.37	4.82±0.31	<0.001	4.75±0.47	4.64±0.49	0.12
Systolic blood pressure	117.77±8.04	124.48±9.69	<0.001	114.49±10.12	115±9.03	0.44
Diastolic blood pressure	73.39±6.97	79.78±8.65	<0.001	70.96±7.66	72.29±8.38	0.24
Acne	0.17±0.38	0.33±0.47	0.01	0.13±0.33	0.57±0.23	0.08

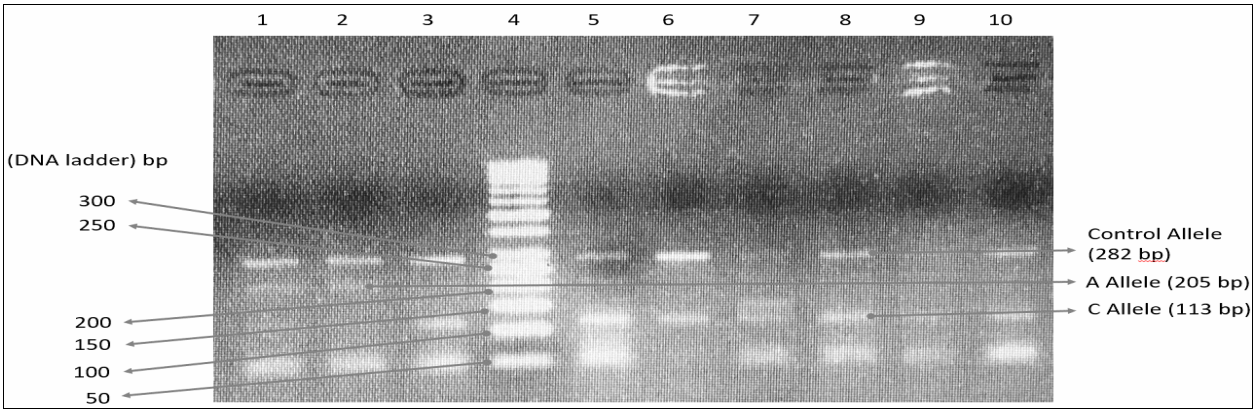

Figure-1: Electrophoretogram on 2% agarose gel showing amplified control, C & A bands separated on electrophoresis

A bands at 205 bp are present in 1st, 2nd, 3rd and 5th wells. C bands at 113 bp are present in 3rd, 5th, 6th, 7th, 8th, 9th and 10th wells. *Nrf2* gene bands at 282 bp are present in all samples as internal control. Gene Ruler TM 50 bp ladder is in 4th well.

Table-3: Association of genotypes of *Nrf2* gene polymorphism (rs6721961) with cases and controls

Genotypes	Controls n=200 (%)	Cases n=200 (%)	OR (95% CI)	*p*
CC	145 (72.5)	113 (56.5)	Ref1	0.004
CA	55 (27.5)	79 (39.5)	0.54 (0.35-0.82)	
AA	0 (0)	8 (4)	-	
Alleles				
C	345 (86.25)	305 (79.43)	0.61 (0.42-0.89)	0.01
A	55 (13.75)	79 (20.57)	-	

DISCUSSION

This study was conducted to see the influence of *Nrf2* gene polymorphism (rs6721961) in Pakistani population to see its possible role in aetiology and pathogenesis of PCOS. To our knowledge, this is the first study to report that -617C/A SNP, which is located in the promoter region of the *Nrf2* gene, is associated with PCOS. Individuals with CA heterozygote had a significantly lower risk of PCOS than those with CC homozygotes. This correlates with the study done by Yamaguchi Y *et al*[16] where patients of renal cell carcinoma carrying CA or AA genotype of rs6721961 at the promoter region, showed elevated expression of *Nrf2* protein as compared to CC genotype. In contrast to this study, Shimoyama Y *et al*[17] suggested that individuals carrying the rs6721961 CA genotype showed increased incidence of acute lung injury.

This study suggests that the SNP of rs6721961 in *Nrf2* gene conferred a protective role against PCOS. However, whether this SNP is a direct causal factor or it is a parallel phenomenon due to linkage disequilibrium with other genetic mutations is not known. It is essential to elucidate the mechanisms by which the rs6721961 polymorphism can exert its protective effect in our population. Evidence suggests that this SNP also put forth protective role against other oxidative linked diseases such as diabetes. A study conducted in Mexico showed that rs6721961 (-617 C/A) polymorphism of the gene *Nrf2* was related to diabetes in male subjects, where A carriers have lower risk of developing diabetes.[18] Jiancheng Wang *et al*[15] also found protective effect of -617C/A (rs6721961) polymorphism for diabetic nephropathy in Chinese Han population. This study highlights the importance of early identification of people at risk to undergo genetic testing for *Nrf2* gene polymorphism (rs6721961) and their careful follow up

for early detection and treatment of the disease in our population. The finding of this study can be combined with other diagnostic modalities currently available to reliably diagnose and manage PCOS patients in Pakistan.

LIMITATIONS

We have genotyped only -617C/A SNP variant of *Nrf2* gene promoter region and we could not eliminate the chance that other SNPs in this region might be associated with PCOS. Further studies are needed to see the combined effect of various SNPs of *Nrf2* gene in PCOS.

CONCLUSION

Nrf2 gene polymorphism (rs6721961) is significantly associated with PCOS among Pakistani population.

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Received: 18 Jun 2022 **Reviewed:** 26 Sep 2021 **Accepted:** 29 Sep 2022

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JM: Original idea, sample collection, article writing, Sample processing and results
AR: Proofreading and critical analysis
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MIB: Data analysis and logical reasoning
BK: Data analysis and logical reasoning
TSH: Data analysis and logical reasoning

Conflict of Interest: None
Funding: None to declare