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ORIGINAL ARTICLE

The impact of TLR2 Gene variant (RS5743708) on the Genetic Susceptibility to Polycystic Ovary Syndrome: An Analytical Study

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ABSTRACT

Background: Polycystic ovarian syndrome is a common endocrinopathy that involves excess androgen and infertility due to anovulatory factors. This syndrome is also linked to several conditions including obesity and high insulin levels as well as an increased risk of cardiovascular complications.

Aims and Objective: The main objective of this research is to examine the molecular function of the TLR2 gene (rs5743708) polymorphism in Pakistani women who have been recognized as having PCOS based on the Rotterdam criteria. This polymorphism in the TLR2 gene is investigated for its association with PCOS in women.

Methodology: This case-control study recruited 40 patients. Blood samples were collected from 30 patients diagnosed with PCOS and 10 from non-PCOS. The blood samples were placed in an EDTA tube for molecular analysis. After the genomic DNA extraction, the polymerase chain reaction method was used to identify the (rs5743708) polymorphism.

Results: To validate the PCR results, sanger sequencing was performed commercially. The phylogenetic tree was constructed to provide a visual summary of the genetic diversity of the TLR2 gene among PCOS patients from different regions. Our findings indicate that the analysis of the genetic association between the TLR2 gene (rs5743708) polymorphism and PCOS in a Pakistani population showed no significant correlation.

Conclusion: The identification of genetic association may unveil novel therapeutic targets. Targeting the TLR2-related molecular mechanism influenced by this polymorphism could lead to the development of more effective and personalized treatment strategies for PCOS among Pakistani women.

Keywords: Genetic association, POCS, TLR2, Polymorphism, rs5743708

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a complicated reproductive disorder that is characterized by the interaction of genes, hormonal substances, environmental stressors, and a psychiatric condition that affects 5 to 18% of women all over their lives.[1] First time this disease (PCOS) was identified by Stein and Leventhal in 1935. PCOS symptoms include amenorrhea or oligo-amenorrhea, acne, reproductive issues, obesity, hirsutism, infertility, obesity, and androgenic alopecia that can disrupt the endocrine and gynecologic systems. The signs and symptoms of PCOS range from person to person across the lifespan. Reproductive hormone imbalances give rise to PCOS, which causes cysts to grow in the antral follicles of the female ovary. The egg is surrounded by fluid-filled sacs that create a cyst. A functional cyst is described as an egg that transforms into a cyst and further delaysovulation.[2]

The most recent PCOS diagnostic criteria to be utilized globally is the Rotterdam Consensus.[3] The Rotterdam Consensus states that two out of the three main criteria oligo-anovulation (refers to infrequent or irregular periods), hyperandrogenism (elevated level of androgen hormone), and polycystic ovaries (defined as having at least 12 follicles measuring 2-9 mm in diameter and/or an ovarian volume > 10 mL in at least one ovary) must be present for PCOS.[4] The etiology of PCOS described by many studies found the factors that are responsible for its development are hormonal imbalances, insulin resistance, stress, lifestyle factors, and genetic factors. The Genetics, epigenetics, and developmental variables interact in PCOS accounting for around 10% of the heritability of genetic factors.[5] However, the main causes of PCOS are not clear yet.

The global incidence of PCOS has reached 1.55 million cases among women[6]. PCOS is mostly diagnosed in 15-49 years of reproductive-aged women[7,8]. The diagnosis criteria determine the prevalence of PCOS. Using the Rotterdam criterion, incidence rates in identical Caucasian groups were recorded as low as 1.6% and as high as 18%. India has a prevalence of PCOS ranging from 3.7-22.5% due to its immense population size.[9] It has also been claimed that 50-75% of women with polycystic ovaries are unknowing of their condition and left untreated.[10,11] Pakistani women of reproductive age have a reported 52% PCOS prevalence, which is fast-rising in the country.[12] The prevalence of PCOS is significantly higher among South Asian populations, particularly in Pakistan, where 40-50% of infertile femaleshave this disease.[13]

PCOS-afflicted women are more susceptible to developing other diseases like gestational diabetes, preeclampsia, miscarriage, and hypertension during pregnancy. Moreover, 30-40% of females with diabetes or impaired glucose tolerance are affected by PCOS around the age of 40.[14] While in teenage, 66% of females suffering from PCOS have hair growth, pimples, and androgenic alopecia, all of which are signs of clinical hyperandrogenism and the presence of male traits15. PCOS is caused by almost 241 different gene variants[15].These gene variants might be polymorphic or single nucleotide mutations that cause defects in a gene transcriptional function. Those Variants that affect how genes are expressed and how proteins function might impact a person's tendency to have PCOS.[16]

Among different genetic variables, Toll-like receptor 2 (TLR2) is a membrane protein that is expressed on the surface of particular cells. The TLR2 gene, which codes for a protein consisting of 784 amino acid sequences and has a molecular weight of 89.9 kDa, is found on the long arm of chromosome 4q32.[16] It is comprised of 1 coding exon and 2 non-coding exons. TLR2 receptor functions through the myeloid differentiation main response gene 88 signaling cascade, which in turn can trigger other signaling mediators, including activator protein-1, p38, extracellular signal-regulated kinases 1/2, and c-Jun N-terminal kinases.[17] TLR2 was shown to be one of the hub genes that might have been linked to PCOS. To the best of our understanding, the role of TLR2 in PCOS pathophysiology remains unclear, even though multiple studies have implicated its association with PCOS.

This study aimed to investigate the genetic correlation of the TLR2 gene (rs5743708) polymorphism in women suffering from PCOS through a case-control study. The study has explored the

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genetic association between the TLR2 gene polymorphism and PCOS in a Pakistani population, contributing to the broader understanding of the genetic factors.

Influencing this condition. The lack of significant association between the (rs5743708) polymorphism and PCOS suggests that other genetic markers should be investigated to understand the genetic basis of this syndrome. Further, phylogenetic analysis highlighted the genetic relationships between TLR2 gene sequences from different geographical regions which reveal the genetic similarities but also underscored that this specific polymorphism does not contribute to the development of PCOS in Pakistani women. PCOS poses a significant challenge to healthcare systems worldwide. Despite advancements in treatment, patient engagement and self-management remain critical gaps. These findings highlight the intricate nature of PCOS and the necessity for a personalized approach to its diagnosis and treatment. Finally, this research underscores the importance of continued genetic studies in diverse populations to identify potential genetic markers that could inform more effective and tailored treatment options for women with PCOS.

MATERIALS AND METHODS

Ethical Approval: All women who participated in this study have provided their written informed consent. The ethical approval for the implementation of this research was taken from the Ethical Review Committee of the Institute of Molecular Biology and Biotechnology/Center for Research in Molecular Medicine (IMBB/CRIMM), The University of Lahore, issued approval Reference IMBB/BC/24/224 on August 4, 2023. The duration of this study is from 05 August 2023 to 15 August 2024.

Selection of patients: A total of 40 Pakistani women between 18-45 years of age were selected for this study, with 30 cases of PCOS based on the Rotterdam criteria and 10 healthy control or non-PCOS women. Both PCOS and non-PCOS women were selected from Lahore, Punjab, Pakistan. This study was done at the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Lahore, Pakistan.

Inclusion/Exclusion criteria: The inclusion criteria of this study, women with and without PCOS were chosen on a scale ranging from 18 to 45 years old with varying BMI values. PCOS women were selected based on their diagnosis according to the Rotterdam criteria. Non-PCOS women were chosen healthy, based on their regular menstrual cycles and ovulation. Women suffering from other metabolic or infectious diseases, smoker women, non-Pakistanis, and those who refused to sign the written consent form were excluded from this study.

Collection of Blood Sample: A fasted blood sample was collected from 40 women selected for this study. All samples were collected from Lady Willingdon Hospital (LWH) in Lahore, Punjab, Pakistan. Peripheral blood was drawn in a 5 mL EDTA vacutainer. Those vacutainers were labeled with the sample ID number, patient name, and date of sample collection. The samples were placed on ice and transported to the Molecular Biology and CRISPR/CAS Lab 334, The University of Lahore (UOL), and stored at -20℃ until further processing.

Extraction and Quantification of DNA: DNA was isolated from the collected samples of both PCOS and non-PCOS participants using the GeneJET™ Genomic DNA Purification Kit (catalog number K0721). The isolated DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, ND-2000). The purity of the genomic DNA was assessed using a multi-scan microplate reader (Thermo Fisher Scientific). All DNA concentrations were adjusted to 250-700 ng/µL. Additionally, DNA detection was carried out by electrophoresis on a 0.8% agarose gel.

Gel- Electrophoresis: To confirm DNA integrity, a 0.8% agarose gel was prepared using Tris-acetate EDTA (TAE) buffer. Ethidium bromide staining was performed and the DNA samples were visualized through gel electrophoresis.

Primer design: The online Primer3web version 4.1.0 (https://primer3.ut.ee/) was used to design the primers (forward and reverse). The specificity of the primers which had been ordered from Eurofins Germany was confirmed using BLAST software. The genetic (rs5743708) polymorphism was sequenced using the human (GRCh38/hg38) reference on the University of California, Santa Cruz (UCSC) Genome Browser (https://genome.ucsc.edu/). This browser provided us with a comprehensive genomic location and context of polymorphism aiding in the analysis of sequences. The Sequence amplification and primer optimization were performed using the UCSC In Silico PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr).

PCR amplification: The DNA was amplified by using specific primers designed against TLR2 gene (rs5743708) polymorphism through PCR. After PCR the amplicons were amplified through gel electrophoresis.

Sanger sequencing: The DNA sequencing was done commercially by using dye-labeled dideoxy terminator cycle sequencing on an ABI Prism 3130 XL Genetic Analyzer, provided by (Applied Biosystems, Inc., Foster City, CA). The cycle sequencing product was later purified by Ethanol/EDTA precipitation to remove unincorporated terminators and finally suspended into a buffer HiDi Formamide to be resolved by capillary electrophoresis on SeqStudio Genetic Analyzer 3200 by Applied Biosystems.

Nucleotide Sequence Retrieval: Nucleotide sequences for Homo sapiens toll-like receptor 2 (TLR2), transcript variant 8 (accession number NM_001318796.2), and related sequences from the TLR2 gene family were retrieved from the NCBI nucleotide database for comparative analysis.

Blast Analysis: The query sequence underwent BLAST analysis to identify homologous sequences within the TLR2 family. Aligned sequences in FASTA format were obtained from NCBI, focusing on those with significant similarity for subsequent phylogenetic analysis.

Phylogenetic Tree Construction: A Neighbor-Joining method was used to construct a phylogenetic tree illustrating evolutionary relationships among species, with branch lengths reflecting evolutionary distances calculated using the Maximum Composite Likelihood approach. The analysis utilized 24 nucleotide sequences and MEGA11 for evolutionary studies, encompassing 407 nucleotide positions.

RESULTS

Identification of (rs5743708) polymorphism: For the identification of (rs5743708) polymorphism in the TLR2 gene, site-specific primers were designed. Both primers start at (153705165) but bind at different positions due to the reverse orientation of one primer. The (point of mutation) column represents a (rs5743708) polymorphism mutation position at Forward and reverse position as shown in (Table 1). The PCR successfully amplified the genetic (rs5743708) polymorphism shown in (Figure 2) followed by the Sanger sequencing to retrieve the nucleotide sequence to evaluate the (rs5743708) polymorphism in patients and the control group.

Extraction of DNA from Blood sample: Using the GeneJET™ Genomic DNA purification kit (catalog number K0721) DNA was extracted from 40 blood samples of PCOS and non-PCOS patients.

Table 1: Primer designing

Primers Id	Sequence (5' to 3')	Length(bp)	Tm °C	GC %	Start/Stop	Point of mutation	Differencefrom mutation
rs5743708-Forward	GCTGGAGAACTTCA ATCCC	19	58°C	52%	153705165	153704893	272
rs5743708-Reverse	CCCAACTAGACAAA GACTGG	20	60°C	50%	153705165	153705298	-133

The primers forward and reverse were designed shown in (Table 1) to amplify the region of TLR2 gene polymorphism (rs5743708).

PCR Amplification

Figure 1: PCR Amplification using a primer of (rs5743708) polymorphism

This gel image is labeled as Tm 56 which means that samples underwent PCR amplification with an annealing temperature. While lane (P1-P4) represents a case or PCOS and lane (N1-N2) represents a control or non-PCOS group.

Sanger sequencing results of (rs5743708) polymorphism: The Sanger sequencing result presented as electropherograms showing no mutations in PCOS samples that are illustrated in the following figure 2.

>P4 (patient 4):

TGAACTGGACTTCTCCCATTTCCGTCTTTTTGATGAGAACAAT
GATGCTGCCATTCTCATTCTTCTGGAGCCCATTGAGAAAAAAG
CCATTCCCCAGCGCTTCTGCAAGCTGCGGAAGATAATGAACA
CCAAGACCTACCTGGAGTGGCCCATGGACGAGGCTCAGCGG
GAAGGATTTTGGGTAAATCTGAGAGCTGCGATAAAGTCCTAG
GTTCCCATATTTAAGACCAGTCATTGTCTAGTTGGGA

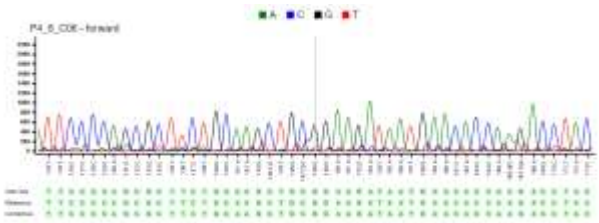

Figure 2: Electropherogram of (rs5743708) polymorphism showing substitution (G) in PCOS sample P4 (Patient 4) instead of (A) in reference gene.

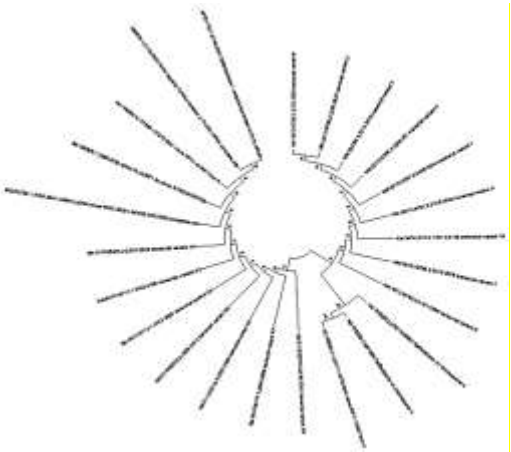

Figure 3: Phylogenetic tree showing the evolutionary relationship among different regions

The mutation site is at position 148: G in the PCOS sample. In this polymorphism (rs5743708) the reference allele G altered in allele A (G>A), but here the allele G did not alter in allele A as shown in (Figure 2) as compared with the normal sample. So, there is no mutation found in PCOS samples.

Phylogenetic tree analysis: To investigate the evolutionary relationships among the retrieved sequences, a MEGA (Molecular Evolutionary Genetics Analysis) software was used to construct a phylogenetic tree. The Neighbor-Joining method was employed to generate the tree which visualized the phylogenetic relationships among TLR2 sequences and their subfamilies from different regions. The tree in (Figure 5) shows the central starting point from which all the sequences diverged. The TLR2 gene and its variants are from different regions around the circular tree with branching points indicating common ancestors. Each branch representing a unique transcript variant of the gene, such as transcript variant 8 of the TLR2 gene which is a target gene containing an accession number (NM001318796.2) helped in tracing their evolutionary divergence. The proximity of this branch having target variant 8 (bordered with a blue line) with other branches indicates the association of PCOS with TLR2 gene sequences from other regions. Clustering with certain clads might indicate a genetic similarity between the TLR2 gene and PCOS from certain regions, reflecting a shared evolutionary pressure and genetic background.

DISCUSSION

Polycystic Ovary Syndrome (PCOS) is a prevalent condition that often develops in females' post-puberty, characterized by the presence of multiple cysts in the ovaries. It commonly manifests through symptoms like acne and hirsutism (excessive hair growth). PCOS is often associated with obesity and insulin resistance, which affects 65-80% of women with the condition. Hyperandrogenism, hyperinsulinemia, and obesity are closely linked, and inflammation plays a key role in increasing adipose tissue through pro-inflammatory processes.[18] The TLR (Toll-like receptor) gene family is involved in chronic inflammation, which is thought to contribute to this mechanism. Specifically, the (rs5743708) polymorphism in the TLR2 gene has been studied for its potential involvement in PCOS. This syndrome significantly affects women's hormonal balance and reproductive health, leading to challenges in managing PCOS within obstetrics and gynecology.

Globally, PCOS affects around 3.4% of women, as reported by the World Health Organization (WHO).[19] However, its prevalence varies due to differences in diagnostic criteria and the influence of geographical location, ethnicity, and race. A systematic review of 27 studies revealed an average prevalence of 21.27%, highlighting regional variations in the condition's impact and the need for standardized diagnostic methods.[20] These disparities underscore the importance of developing accurate approaches to assessing and addressing PCOS across diverse populations.[21]

In this study, 40 women aged 18-45 were recruited, including 30 diagnosed with PCOS and 10 healthy controls. Blood samples were collected from each participant, accompanied by documentation of medical and family histories. Demographic data such as age, weight, height, and BMI were recorded, along with biochemical markers like AST, ALT, TSH, FSH, and LH. The primary aim of this study was to investigate the association between the (rs5743708) polymorphism in the TLR2 gene and PCOS. Various molecular techniques were employed, including DNA extraction, PCR amplification, and Sanger sequencing. Results were validated through bioinformatics analysis using Chromas Software.

Despite thorough analysis, no significant association was found between the (rs5743708) polymorphism in the TLR2 gene and PCOS. This suggests that the TLR2 gene, particularly the (rs5743708) variant, may not play a key role in the genetic predisposition to PCOS. While previous research has implicated

the TLR2 gene in inflammation and immune responses, its role in PCOS remains unclear. This study adds to the growing body of literature, providing evidence that challenges the potential link between TLR2 polymorphisms and PCOS. However, it is essential to note that PCOS has a multifactorial genetic basis, involving various genes and environmental factors. The sample size of 40 participants limits the ability to generalize findings, and further studies with larger populations are needed to explore other genetic markers and pathways.

Globally, previous studies have reported similar findings, showing no significant link between the TLR2 gene and PCOS.[22] Some polymorphisms, such as (rs3804100), (rs4986790), and (rs4986791) are associated with PCOS in Polish women.[23] Furthermore, research on infertile Pakistani women revealed a link between the ERBB4 (rs2178575) variant and reduced infertility risk, while no significant association was found with the DENND1A (rs9696009) variant.[24] A study in India identified Gorilla gorilla as the most suitable model organism for studying PCOS due to its genetic similarity to human genes.[25] This highlights the importance of using primate models to understand the genetic mechanisms of PCOS, which may lead to more effective in vitro studies.

The phylogenetic analysis in this study shows that the TLR2 gene from Pakistani women with PCOS shares genetic similarities with TLR2 genes from other regions. The proximity of these sequences suggests that the genetic background of PCOS may vary across populations, influenced by evolutionary factors. Understanding the genetic landscape of PCOS through phylogenetic analysis can aid in identifying population-specific variations, contributing to personalized medicine and improving treatment outcomes. However, this study's limitations, including a small sample size and reliance on Rotterdam criteria for PCOS diagnosis, may affect the generalizability of the findings. Furthermore, variability in diagnostic criteria across studies can impact the reliability of comparisons and conclusions.

This research was conducted with limited funding, restricting the ability to expand the study and incorporate additional resources. Despite these challenges, the study provides valuable insights into the genetic factors involved in PCOS and underscores the need for further research to explore potential genetic markers that could contribute to the condition's development. Larger sample sizes and more comprehensive studies are essential to better understand the complex genetic underpinnings of PCOS and its variations across different populations.

CONCLUSIONS

Polycystic ovarian syndrome is a common endocrine condition that affects the health of women, particularly in terms of hormonal equilibrium and reproductive processes. Despite its widespread occurrence, particularly among women in Pakistan, the condition remains under-researched, especially regarding its genetic association. This study investigated a potential association between the genetic (rs5743708) polymorphism in the TLR2 gene and PCOS. The study conducted a thorough analysis of 40 women, 30 of whom were diagnosed with PCOS to investigate the genetic variations and their potential link to the condition. However, the findings revealed no significant association between the genetic (rs5743708) polymorphism and PCOS which suggest that this specific polymorphism may not contribute to the genetic predisposition for PCOS in the studied population.

Further, the phylogenetic analysis conducted in this study emphasized the genetic landscape of PCOS and highlighted the evolutionary relationships between TLR2 gene sequences from different geographical regions. The findings obtained from phylogenetic analysis show that certain genetic similarities exist but the genetic (rs5743708) polymorphism does not appear to play a critical role in the development of PCOS in the Pakistani population. This research contributes valuable insights to the ongoing exploration of genetic factors of PCOS which indicates the need for continued investigation into other potential genetic markers and their implications for disease risk and treatment. It is worthwhile to mention here that this study underscores the complexity of the genetic basis of PCOS and the importance of personalized medicine approaches in managing this condition effectively across diverse populations.

Disclosures: Consent was obtained or waived by all participants. The ethics committee for the Institute of Molecular Biology and Biotechnology/Center for Research in Molecular Medicine (IMBB/CRIMM), The University of Lahore, issued approval Reference IMBB/BC/24/224 this research project entitled “The impact of TLR2 Gene variant (rs5743708) on the genetic susceptibility to Polycystic Ovary Syndrome: An analytical study” has been thoroughly reviewed by all participant. This research follows all the ethical guidelines and standards. Written consent from all the human participants has been obtained and necessary precautions have been taken to ensure the confidentiality and safety of all the involved.

Conflict of interest: All the authors declared no conflict of interest in the paper and regarding submission work.

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