Genetic Polymorphisms of GSTO1 gene and their association with the risk of Polycystic Ovary Syndrome in a sample of Iraqi Women

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Abstract

Polycystic ovary syndrome (PCOS) is the most frequent endocrinological disorder, occurring in young women of reproductive age. The goal of this study was to look at a possible link between GSTO1 A140D (rs4925) polymorphisms and PCOS, investigating the effect of these polymorphisms on the levels of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). This study was carried out in the Laboratories of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies - University of Baghdad during the period from 1 November 2021 until the end of June 2022, The PCOS patients were taken from the Kamal Al-Samarrai infertility treatment Hospital in Baghdad. Women with PCOS (n=50) and apparently healthy subject (n=50), were enrolled . Genotyping of GSTO1 gene SNP exon 4 (C to A; rs4925); was determined using Taqman genotyping assay by RT-PCR. The results showed that the distribution of genotypes and alleles frequencies at rs4925 C>A SNP of GSTO1 gene CC, and CA genotypes indicated no significant differences in frequency percentage which were noted between control subjects and patients with polycystic ovary syndrome. Whereas, the frequency of mutant AA genotype was significantly ($p<0.01$) higher in patients with PCOS than in apparently healthy subjects(28% versus 6%, respectively, $\chi 2=7.117$, OR =1.67)this may be indicating a risk factor for the development of reproductive women to PCOS.

Keywords: GSTO1 gene; Polycystic Ovary; Genetic Polymorphisms

Introduction

PCOS, or polycystic ovarian syndrome, is one of the most common endocrine disorders among women of reproductive age, with a prevalence of 6–15 percent. Chronic anovulation and high levels of androgen may be associated with this disease, and these issues can lead to infertility. Patients with this syndrome may have a higher incidence of obesity, insulin resistance (50–70%), dyslipidemia, endothelial dysfunction, metabolic syndrome, and risk factors for type 2 diabetes mellitus (DM), however, the cause of this heterogeneous syndrome is unknown **[1]**Women with PCOS have at least two of the three requirements, according to the modified criteria stated at the Rotterdam meeting: (1) oligo ovulation or anovulation, (2) higher levels of androgen (male hormones) in blood tests or through signs such as acne and extra hair growth on the face and body, and (3) an excessive number of follicles in ultrasound ovaries, with more than 12 follicles in each ovary measuring 29mm and/or increased ovarian volume (>10 mL) **[2].** Because polycystic ovary syndrome can manifest as a variety of diseases, the Rotterdam criteria divided the disease into four phenotypes: (1) Frank or classic polycystic ovary syndrome, which includes chronic anovulation, hyperandrogenism, and polycystic ovaries, (2) Classic non-polycystic ovary syndrome, which includes chronic anovulation, hyperandrogenism, and normal ovaries, and(3) non-classic ovulatory PCOS, which is distinguished by regular menstrual cycles, hyperandrogenism, and polycystic ovaries. and (4) Non-classic or normoandrogenic PCOS, which involves chronic anovulation, normal androgen levels, and polycystic ovaries [3].

some research suggests that oxidative stress (OS) plays a role in the illness [4].An imbalance in the synthesis and elimination of reactive oxygen and nitrogen species causes oxidative stress (ROS and RNS, respectively). Excessive ROS production can harm lipids, proteins, and cells [5].

Non-enzymatic antioxidants such as vitamin E, ascorbate, and glutathione, as well as enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferases (GSTs), make up an antioxidant defense system [6].

The GSTO1 polymorphism at nucleotide 419 (rs4925) converts alanine to aspartate (A140D),In the last few years, there have been a lot of studies on PCOS

[7] discovered that hormonal abnormalities related to subclinical hypothyroidism include elevated TSH levels and a higher LH-FSH ratio on ultrasonography, as well as polycystic ovaries.

The current study was to look at a possible link between GSTO1 A140D (rs4925) and PCOS, Examine the connection between the prevalence of PCOS in Iraqi women, which is still under investigation.

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Materials and Methods

Patients and samples collection

This study has been achieved at the Kamal Al-Samarrai infertility treatment Hospital in Baghdad during aperriod from November 2021 to January 2022. A total of 100 Iraqi women were separated into two groups for the study. There were 50 PCOS patients and 50 apparently healthy people in the study. All PCOS patients' medical histories were obtained, and the condition was first discovered with an ultrasound exam., If they had oligomenorrhea, amenorrhea, or severely irregular menses, information was noted., Patients range in age from 16 to 42 years old.Blood samples were collected from PCOS patients and healthy women by drawing 6 ml of venous blood from each., during (2 nd –4 th) day of the menstrual cycle; for individuals with a regular cycle, early follicular phase. Depending on the length of the cycle, blood samples were taken from patients with oligomenorrhea or anovulation. Each sample was divided into two tubes, with 2ml of whole blood placed in a tube containing EDTA (Ethylene Diamine Tetracetic Acid) for DNA extraction and genetic section and the remaining 4 ml of whole blood have been placed in a Serum-separating tube; serum was separated and isolated from this 4 ml of blood by centrifuging for 10 minutes at 3000 round per minutes (rpm), for ROS and RNS.

Immunological assay

Detection of ROS and RNS were done by Elissa assay, This test was conducted by manufactures instructions MyBioSource(USA) serum concentration of ROS and RNS for PCOS patient and healthy subject were calculated according to the optical densities of all samples, and standards were read in a microtiter plate with not more than 30 min after adding the stop solution by Elisa reader at 450 nm.

Genomic DNA isolation

Total genomic DNA was extracted from entire fresh blood and stored in EDTA K3-containing tubes for molecular investigations. Geneaid DNA extraction kit (Bioneer, Korea). After obtaining genomic DNA, agarose gel electrophoresis was used to ensure that the extracted DNA was present and intact [8]. and then Estimation of DNA Concentration and Purity A Nanodrop tool is used to determine the concentration of DNA samples. At 260 nm wavelength, 1 µl of extracted DNA was inserted into the lens of an apparatus to determine the concentration in ng/L.

The TaqMan probe contains a reporter dye (FAM and VIC) at the 5´end of the probe and a quencher dye (MGB) at the 3´ end of the probe.

The sequences of TaqMan fluorescent oligonucleotide probes and primers were prepared according to [9], synthesized by Alpha DNA Ltd (Canada), and stored lyophilized at (-23°C). Dilute 10L of primers, probe, and stock solution in 90L of nuclease-free water and store at -23C until use to make a working solution with a concentration of 10M.

Primers and Probes

Table (1): primer and probe used in the study The sequences of the primers and probes utilized in the investigation are listed in Tables(1).	
Primer/probe	Sequence (5' →3' direction)
GSTO1 gene exon4,chromosome 10,NG_023362.1,for rs4925	
Forward	TCTCTGTCTAGGTGCCATCC
Reverse	ACCTCCTCTAGCTTGGTAAATTC
FAM- probe	GACTATGCTGGCCTAAAAGAAGAA
VIC-probe	GACTATGATGGCCTAAAAGAAGAA

Table 2 components of Real-time PCR/ allelic discrimination					
No.	Components	Volume	Final Conc.	Volume (µl)	
1	qPCR Master (PROBE)	10 µl	1X	10	
2	Forward primer	0.2-2.0 µl	0.1-1.0 µM	10 µl	3
3	Reverse primer	0.2-2.0 µl	0.1-1.0 µM	10 µl	
4	Fluorescence Probe	Variable	≤500ng/reaction	20 µl	
5	Template DNA	Variable	-	4	
6	Water, RNase free	Up0 to 20	-	3	

DNA samples from PCOS patients were genotyped for the *GSTO1* gene SNPs (rs4925) with a Taqman SNP genotyping assay using a real-time thermocycler according to the protocol recommended by the manufacturer.

Table (3) Real Time PCR program for *GSTO1* gene SNPs (rs4925)			
Step	Temperature (°C)	Duration	Cycles
Hold 1	50	2 min	1
Hold 2	95	10 min	1
Denature	95	10 sec	25-40
*Anneal	60	45 sec	25-40

* In this step the acquiring Green and Yellow (FAM and VIC) were added.

Statistical Analysis

The Statistical Analysis System- [10] program was used to affect different factors in study parameters. Chi-square test was used for significant comparison between percentage and Least significant difference –the LSD test was used for significant comparison between means in this study, and also odd ratio was used to determine risk factors [10].

Results

This study examines GSTO1 gene polymorphism (rs4925C>A) among Iraqi women with polycystic ovary syndrome (PCOS) and in apparently healthy controls. This variant is located in exon 4 chromosome 10. The genotypes and allele frequency distributions of rs4925 SNP polymorphism are presented in Tables (4).

As related to CC, and CA genotypes, no significant differences in frequency percentage were noted between apparently healthy subjects and patients with polycystic ovary syndrome. Whereas, the frequency of AA genotype was significantly($p<0.01$) higher in PCOS patients than in apparently healthy subjects (28% versus 6%, respectively, $\chi 2=7.117$, OR =1.67). The AA genotype of rs4925 in GSTO1 gene represents a risk factor for PCOS incidence in Iraqi patients.

The frequencies of C and A alleles were 0.77 and 0.23 in apparently healthy subjects and 0.60 and 0.40 in patients with PCOS, respectively.

Table (4). Genotypes and alleles frequency of rs4925 SNP at GSTO1 gene in apparently healthy subjects *versus* Iraqi polycystic ovary syndrome (PCOS) patients.

Genotype (rs4925 [1])	Patient [2] No (%)	Control [3] No (%)	Chi-Square: χ^2	O.R. (C.I)
Wild: CC	24(48.00%)	30(60.00%)	0.667 NS	Ref.
Heterozygous: CA	12(24.00%)	17(34.00%)	0.862 NS	0.492 (0.22-0.93)
Mutant: AA	14(28.00%)	3(6.00%)	7.117 **	1.67 (0.91-3.06)
Allele	Frequency			
C	0.60	0.77	--	--
A	0.40	0.23	--	

** (P≤0.01), NS: Non-Significant.
[1] SNP in GSTO1 gene; [2] PCOS = polycystic ovary syndrome healthy subjects;
[3] healthy subjects; X^2:chi square; OR: odds ratio; CI: confidence interval. N=50 for each group.

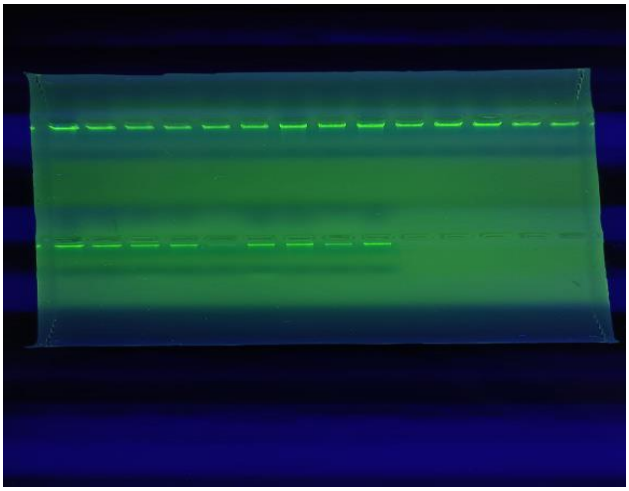

Fig. 2. GSTO1 AS-PCR products on a 2% agarose gel.

ROS and RNS

The mechanism of ROS and RNS in PCOS is clear, but the results of the current study investigated a significant increase in the concentration of both ROS and RNS,. Oxidative stress (OS) occurs when destructive reactive oxygen species (ROS) overwhelm antioxidants, causing DNA damage and/or apoptosis. Furthermore, reactive nitrogen species (RNS), such as nitrogen oxide (NO) with a non-double electron, are also highly reactive and toxic [11].

Table (5): The ROS and RNS concentration in PCOS patients versus apparently healthy subjects

Group	Mean ± SE	
	ROS	RNS
Patients [1]	0.664 ±0.08	0.783 ±0.08
Control [2]	0.452 ±0.05	0.347 ±0.07
T-test	0.210 *	0.228 **
P-value	0.050	0.0008

* (P≤0.05), ** (P≤0.01).
[1] Patient with polycystic ovary syndrome. and [2] healthy subjects. *: Significant at 0.05 level.
** : <0.01 level of highly significance. (ROS**):** reactive oxygen species, (RNS): reactive nitrogen species.

Ethical clearance

The study was approved by the Research Ethics Review of the Institute of Genetic Engineering and Biotechnology for postgraduate studies, University of Baghdad. All participants provided written informed consent.

Source of funding;

This work was done by self-funding, the practical work was done at my college university with the agreement of the university

Conflict of interest:

This work was done by the author only, all the experiments were done at laboratories of my college

and the writing of the paper was done by the author himself

DISCUSSION

This study examined polymorphisms of GSTO1gene (rs 4925 C>A in exon 4) among Iraqi women who suffered from PCOS and apparently healthy as control and tested their association with the phenotype of PCOS at the Kamal Al-Samarrai infertility treatment Hospital in Baghdad.

The results of the present study reveald a non-significant difference in CC wild-type rs4925 gene between PCOS patients and the control group. The Heterozygous CA also non-recorded a significant difference between study groups. While the AA mutant phenotype scored a significant increase in PCOS patients than in the control group at

P≤0.05, this may be indicating a risk factor for the development of reproductive women to PCOS. The development of reproductive women to PCOS increased by 1.67-fold more than the control group. Also, the increased rate of oxidative stress has been suggested as a risk factor for PCOS pathogenesis in reproductive women, although it is mechanism is not clear [4]. The study of [12], their study involved long-term health risks related to polycystic ovary syndrome, and showed the GSTO disorder can cause increased production of oxygen free radicals induced by hyperandrogenism in the early stage of PCOS and may be associated with insulin resistance and other metabolic disorders associated with PCOS women. The Alpha GST-like enzyme is found only in steroidal tissues and has 230-fold higher catalytic efficiency in 3-keto-steroids than 3β-hydroxysteroid dehydrogenase, so the decreased expression of this gene may lead to a decrease in the concentration of steroid hormones [13]. The study of GSTO enzymes, which can form disulfide bonds with GSH, plays important role in cell resistance to oxidative damage. They are also involved in regulating the biosynthesis and transport of hormones within cells. Also, the study of [14], showed that glutathione and its related enzymes have a function in the detoxification and metabolism of ROS and RNS, cytotoxic and carcinogenic compounds. There is ample evidence for the involvement of reactive oxygen species in the physiology and pathology of the reproductive system. So, the defect in the glutathione gene leads to the overproduction of oxidative stress in the reproductive system. GSTOs have been shown to scavenge free radicals in various ways, including downregulation of Docosahexaenoic acid and catalyzing the reduction of inorganic arsenic [15]. A study has been conducted on omega-GST class polymorphisms in malignant diseases. GSTO1 gene polymorphisms are associated with bladder, breast, and ovarian cancer [16]. However, no relationship between polymorphisms of GSTO1 and GSTO2 and PCOS was reported. In addition to its role in infertility disorders, PCOS has been identified as the underlying cause of a variety of reproductive cancers, including endometrial malignancies, and ovarian and breast cancers. A previous Turkish study performed by [17], investigated a relationship between GSTO1 polymorphism (A140D) and an increased risk of cholangiocarcinoma, breast cancer, hepatocellular carcinoma, acute lymphocytic leukemia, and non-small cell lung cancer and urothelial carcinoma,

The current results investigated the wild type and heterozygotes were high recurrent in control than in PCOS, while allele A was a high frequency in PCOS than in control

To the best of our knowledge, this is the first study on the association of GSTO1

polymorphism with PCOS women. The Turkish study performed by [18], and the Italian study of [19], showed that a GST-positive genotype and a GST-null genotype or their combination may be associated with a higher risk of primary open-angle glaucoma in the Turkish population. Our results demonstrated that GSTO1 genotypes can be considered genetic risk factors for developing PCOS. Our results showed that the frequency of the AA genotype is significantly associated with this disease.

The study of [20], recorded the level of ROS and RNS in PCOS women only was higher than in women with PCOS but have a higher concentration of growth hormone, this because GH improved matrix metalloprotease and inhibited Glucocorticoid-induced in patients with PCOS. Matrix metalloproteinases (MMPs) are a major class of zinc-dependent endopeptidases that are secreted as inactive zymogenic substances at neutral pH and which are involved in proteolysis or hydrolysis (collagen and other components such as fibronectin, vitronectin, elastin and proteoglycans) and are the primary contributors to debris removal and thus promoting migration cells and/or the formation of new blood vessels [21].

In addition, oxidative stress plays a major role in female infertility [22], and is associated with poorer fertilization rates and pregnancy outcomes for assisted reproductive procedures [23]. With all of these in mind, it is strongly believed that local and systemic oxidative stress may also be involved in reproductive abnormalities in PCOS[24].Free oxygen radicals and antioxidant factors are actively involved in the physiological processes of female reproduction, such as folliculogenesis, oocyte maturation, steroidogenesis, luteal function, and cholestasis [25].

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