The Relevance of Genetic Polymorphism of CYP1A1, ICAM-1, TNF-α and INSR Genes in Women with Polycystic Ovary Syndrome (PCOS)

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***Abstract*—Polycystic Ovarian Syndrome (PCOS) is a complex endocrine disorder commonly seen in about 6.5 - 8% of women of reproductive age. Polygenic trait is common in PCOS and various factors related to the androgenic pathways and the metabolic syndrome have been studied including genes encoding inflammatory cytokines. In this respect we aimed to study the involvement of polymorphisms of four genes; cytochrome P450 1A1 (*CYP1A1*), intercellular adhesion of molecule (*ICAM-1*), tumour necrosis factor alpha (*TNF-α*) and insulin receptor gene (*INSR*). Twelve women fulfilling the criteria of PCOS and 145 controls were recruited. In this study, *TNF-α -1031 (T/C)* (rs1799964) is found to be significantly higher in PCOS group compared to healthy controls (OR = 5.044; CI: 2.139 - 11.899; p-value < 0.05). This suggests *TNF-α -1031 (T/C)* appears to be a potential candidate as a molecular marker in determining PCOS risk. This study also found a strong association between PCOS and obesity (BMI>25); obesity is a major risk factor of PCOS. Studies of association enables clinicians to have a better understanding of the genetic factors for PCOS especially in a multi-ethnic population such as Malaysia, where robust data addressing PCOS are still lacking.**

***Index Terms*—polycystic ovarian syndrome (PCOS), polymorphism, single nucleotide polymorphisms (SNPs)**

I. Introduction

Polycystic ovarian syndrome (PCOS) is a complex endocrine disorder affecting 6.5-8.0% of all women of reproductive age [1].

Genetic predisposition to PCOS is described as a primary factor. Many studies have focused on genes which encode hormone regulating enzymes to elucidate the etiology of PCOS [2]-[7]. Draveka *et al* showed 25% to 50% of first degree female relatives of women with PCOS were reported to have the same disorder [8].

Single nucleotide polymorphisms (SNPs) are described as a difference in a nucleotide sequences and have important roles as biological markers in etiology of disorder. The occurrence of SNPs within genes or regulatory genes may affect gene function.

In this study, we focus on several SNPs on candidate genes which have been reported to be associated with the clinical characteristic of PCOS including cytochrome P450 1A1 (*CYP1A1*), intercellular adhesion of molecule (*ICAM-1*), tumour necrosis factor alpha (*TNF-α*) and insulin receptor gene (*INSR*). All these genes are involved in the regulation of follicular development and steroidogenesis to maintain normal ovarian function.

II. Materials and Methods

A. Recruitment of Subjects

Twelve women fulfilling the criteria of PCOS and 145 controls were recruited from the Gynaecology Department of Sultanah Aminah Hospital, Johor Bahru and National Blood Centre respectively. The assessment for standard criteria diagnosis of PCOS was based on the Rotterdam criteria. Details of the study were explained to the subjects prior obtaining informed consent. Research ethics approval was obtained from International Medical University Ethical Committee of International Medical University and Research Ethics Committee of the Malaysian Ministry of Health (MREC).

B. Clinical Characteristics of Subjects

Subjects were asked to complete a questionnaire regarding demographic information and medical history including specific questions regarding known family history of PCOS.

C. Genotyping

Five milliliter (5 ml) of blood was collected in tubes containing EDTA as anti-clotting factor and stored at -80^{0}C until use. DNA isolation was carried out using commercially available kit which is GF-1 Nucleic Acid Extraction (Vivantis, USA).

DNA concentration and purity were checked using spectrophotometer at wavelength 260 nm and the purity of the nucleic acid was estimated using the ratio of

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readings at 260 nm and 280 nm. The integrity of the genomic DNA was confirmed by electrophoresis.

D. Allele Specific PCR Method

PCR amplification was carried out in a mixture of reagents including 1 x PCR buffer (Solgent, German), 0.20 mM of each dNTPs (Solgent, German), 0.2 µM of reverse primer and 0.2 µM of wild type and variant type primers, 100 to 200 ng (2 µL) genomic DNA as template and 1.0 U DNA Taq polymerase (Vivantis, USA). Reaction mixtures for wild type and mutant primers were prepared separately in a final volume of 25 µL.

PCR amplification was performed with initial denaturation of 95^0C for 5 minutes. The PCR was continued with another 30 cycles of denaturation at 95^0C for 20 seconds, annealing 56^0C for 40 seconds, and elongation at 72^0C for 30 seconds. Finally, the PCR process was hold at 72^0C for 3 minutes. The PCR was performed using Bio-Rad® Thermal cycler System T100 (Bio-Rad Laboratories, Inc., US).

Prior to genotype screening, preliminary validation was carried out by screening 10 DNA samples. Some samples were randomly chosen and sent for direct sequencing to confirm the validity of the developed method (data not shown).

In this method, allele specific pair of primers were designed to enable the detection of four SNPs mentioned. PCR amplification was performed in two separate tubes where one tube consisted of wild-type primer and while another tube was for variant allele. PCR products from these two tubes were visualized using standard agarose gel electrophoresis using separate lanes for each tube; one lane for wild-type allele and the second lane for variant allele.

E. Statistical Analysis

Statistical test was performed using PASW 18.0 software for Windows (SPSS Inc., USA). Test for normality was performed using Shapiro-Wilk normality test. For non-parametric test, two type of statistic test were used, (1) Mann-Whitney U test is for categorical data with two groups; (2) Kruskal-Wallis test for categorical data with more than two groups. A chi-square test (χ^2) was conducted to examine the data deviation from Hardy Weinberg equilibrium (HWE). The correlation between the presence of a genetic variant with predisposition of PCOS was evaluated by odd ratio (OR) and 95% confident interval. OR and 95% CI values were calculated using MedCalc software available online at http://www.medcalc.org. *P*-value of 0.05 or less is regarded as significant.

III. Results

A. Clinical Characteristic and Demographic Data of Patients and Controls.

The details of demographic data, medical history of parental diagnosed disease and physical activity were recorded as shown in Table I.

In this study, the PCOS group's weight and BMI were significantly higher in comparison with control group ($p<0.001$). Moreover, parent history of diabetes mellitus showed a strong association with PCOS ($p<0.001$), whereas no difference was observed in parental history of hypertension and cardiovascular disease.

TABLE I. Clinical Characteristics of Patients and Controls

Demographic data	PCOS group (n=12)	Control group (n=145)	*p*
Age (years)			
Median ± SD	28.0 ± 3.2	21.6 ± 3.6	-
Range	23 to 35	18 to 35	
Weight (kg)			
Median ± SD	73.6 ± 12.7	57.3 ± 10.5	<0.001*
Range	56-100	44-129	
BMI (kg/m^2)			
Median ± SD	29.2 ± 5.0	24.1 ± 4.0	<0.001*
Range	23-41	17.6-48	
Parental history, n (%)			
Hypertension	4 (33.0%)	33 (22.8%)	0.822
Cardiovascular disease	1 (8.3%)	5 (3.4%)	0.820
Diabetes mellitus	7 (58.3%)	21 (14.5%)	<0.001*
Others:			
Cancer	0	1 (0.7%)	-
Asthma	0	1 (0.7%)	-

* *P-value* less than 0.05 is regarded as significant

B. Distribution of Allele and Genotype Frequencies

The genotype and allele frequency of SNPs of *CYP1A1*2A* (T/C) (rs4646903), *ICAM-1* (G/A) (rs1799969), *INSR* (C/T) (rs1799817) and *TNF-α -1031* (T/C) (rs1799964) for both control and PCOS groups are shown in Table II.

In this study, *TNF-α -1031* (T/C) (rs1799964) is significantly higher in PCOS group compared to control controls group (OR = 5.04; CI: 2.139 to 11.899; *p*-value < 0.05). There was no association found for *CYP1A1*, *ICAM-1* and *INSR* with PCOS (*p*-value > 0.05).

TABLE II. Distribution of Genotype and Allele Frequency

SNP	Genotypes	PCOS (n=12)	Control (n=145)	OR	95% CI	P-value
*CYP1A1*2A* (T/C) rs4646903	**Genotypes**					
	TT (WT)	8 (67%)	100 (69%)	R	R	R
	TC (HT)	4 (33%)	45 (31%)	1.1	0.32 - 3.88	0.8689
	CC (MT)	0	0	-	-	-
	Alleles					
	T	20 (83%)	245 (85%)	1.1	0.36 - 3.34	0.8815
	C	4 (17%)	45 (15%)			

SNP	Genotypes	PCOS (n=12)	Control (n=145)	OR	95% CI	P-value
ICAM-1 (G241R) rs1799969	**Genotypes**					
	GG (WT)	12 (100%)	112 (77%)	R	R	R
	GA (HT)	0	33 (23%)	0.1	0.01 - 2.33	0.1679
	AA (MT)	0	0	-	-	-
	Alleles					
	G	24 (100%)	257 (89%)	0.2	0.01 - 2.64	0.1984
	A	0	33 (11%)			
INSR (C/T) rs1799817	**Genotypes**					
	CC (WT)	2 (17%)	37 (26%)	R	R	R
	CT (HT)	8 (66%)	96 (66%)	1.5	0.31 - 7.60	0.5948
	TT (MT)	2 (17%)	12 (8%)	3.1	0.39 - 24.32	0.2853
	Alleles					
	C	20 (50%)	170 (59%)	1.4	0.62 - 3.26	0.4128
	T	20 (50%)	120 (41%)			
TNF-α -1031 (T/C) rs1799964	**Genotypes**					
	CC (WT)	3 (25%)	91 (63%)	R	R	R
	CT (HT)	8 (67%)	45 (31%)	5.4	1.37 - 21.31	0.0162*
	TT (MT)	1 (8%)	9 (6%)	3.4	0.32 - 35.86	0.3139
	Alleles					
	C	14 (58%)	227 (78%)	5.0	2.14 - 11.90	0.0002*
	T	10 (41%)	63 (22%)			

*R = reference value

**P-value* less than 0.05 is regarded as significant

IV. Discussion

Polygenic trait is common in PCOS and various factors related to the androgenic pathways and the metabolic syndrome have been studied.

Studies have shown PCOS is a heritable disorder, thus many studies are focusing on searching for candidate genes which could possibly increase risk of PCOS [2]-[5].

In this study, *TNF-α -1031* (T/C) (rs1799964), a promoter region polymorphism is significantly found to be higher in PCOS group compared to healthy controls at 21.7% and 41.7% respectively (p-value < 0.05). Ji-Hyun *et al*. also found strong association between *TNF-α -1031* (T/C) polymorphism in the promoter region of TNF-α and PCOS [9].

Distribution of allele frequency of *TNF-α -1031 (T/C)* was similar to those reported in a Chinese population at 18.2% (p>0.05) [10]. Conversely, there was significant difference between our population with Korean and Tunisian population at 7.6% and 16.4% respectively (p<0.05) [9], [11]. This suggest the distribution of allelic frequency of *TNF-α -1031* (T/C) varies across population due to inter-ethnic differences. Fauser *et al* showed that different phenotypes of severity of PCOS were observed whereby; Asian women demonstrated milder effect of hyperandrogenism as compared to women of Mediterranean origin [12].

In this study, women who carry this allele have 5 times greater risk of developing PCOS than women who do not carry this allele. This suggest *TNF-α -1031* (T/C) appears a potential candidate as a molecular marker in determining PCOS risk.

TNF-α is a proinflammation cytokine and plays an important role in a wide range of reproduction-related disorders. Polymorphism of *TNF-α* has been shown to induce transcriptional activity of the gene which leads to excess production of TNF-α enzyme [12]. This would subsequently lead to increased production of androgen in several tissues including the ovaries resulting in hyperandrogenism. Increased androgen production by ovarian cells is the cause of the classical endocrine phenotype of PCOS. Although the exact mechanism of *TNF-α* in the pathogenesis of hyperandrogenism is still not clear, increased *TNF-α* in adipose and muscle tissue has been proposed to involve insulin resistance in humans [13]-[16]. Insulin resistance and hyperinsulinaemic are common clinical presentations in women with PCOS [17]. This clearly indicates that insulin metabolism impairment in PCOS women [16]. Impairment of insulin will directly effect on follicular development and steroidogenesis which may interfere with physiology of ovarian function.

The current study shows women with parental history of diabetes mellitus was significantly higher in PCOS women compared to control group at 58.3% and 14.5% respectively (p<0.05). This clearly shows the relationship between insulin impairment and genetic predisposition to PCOS.

Our study shows a strong association of body weight and BMI to PCOS (p<0.001). This result is in parallel with other studies whereby 30% to 75% of PCOS women are found to be overweight and obese (obesity is defined as BMI > 25 kg/m^2).

A study by Hector *et al.* showed high serum level of TNF-α was observed in PCOS women as compared to control group [18]. Over-expression of TNF-α in obese individual is well known to be highly associated with insulin resistance. Indirectly, obesity, insulin resistance and PCOS are closely linked.

Our current results highlight the importance and possible impact of genetic polymorphism of *TNF-α* towards predisposition to PCOS. *TNF-α* could be a potential candidate as a molecular marker in determining PCOS risk. Such an approach could identify women at risk of the clinical manifestations of PCOS early in their reproductive life so as enable lifestyle interventions and

perhaps hormonal manipulation. However, future study in a larger sample size may contribute to further our understanding the genetics mechanism of PCOS.

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Currently, Dr Hazwanie and her team are broadly interested in identifying underlying genetic factors as well as clinical characteristic among women diagnosed with polycystic ovary syndrome (PCOS). Our ability to work at the interface of molecular research is strongly supported by the strong research culture and infrastructure at the IMU Research Centre.

