ORIGINAL ARTICLE

Differential Expression of Insulin Resistance Genes in Polycystic Ovary Syndrome

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ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a common hormonal disorder worldwide among women of reproductive age. It is characterized by endocrine, reproductive, and metabolic abnormalities. Insulin resistance (IR) is one of its most important clinical features, which has been associated with metabolic disorders and increased risk of type 2 diabetes (T2D). This study aimed to explore the whole blood gene expression profiling related to IR in PCOS patients compared to controls. **Methods:** Blood RNA was extracted from 5 PCOS and 5 non-PCOS women with matched age and BMI. Homeostasis model assessment (HOMA-IR) was used to estimate the IR. The expression of IR genes was analyzed by Profiler PCR array. **Results:** Both groups have similar levels of HOMA-IR ($p>0.05$). However, differential expression levels were observed between them. Fourteen genes were upregulated and 26 genes were downregulated in PCOS samples. Among the upregulated genes (>2 fold-change, p-value<0.05) are ADIPOQ, ADIPOR1, OLR, IGF-1, and APOE. Downregulated genes (>-2 fold-change, p-value<0.05) include HK-2, IRS1, and SERPINE1. These genes are involved in insulin and adipokines signaling, commonly dysregulated in T2D. They are also involved in innate immunity and inflammatory processes and are important for lipid and carbohydrate metabolism. **Conclusion:** Our finding suggests that despite both groups having no difference in IR level, there are differentially expressed genes involved in the IR pathway.
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INTRODUCTION

In women of reproductive age, polycystic ovary syndrome (PCOS) is the most prevalent hormonal disorder among them (1). PCOS is not only the dominant cause of anovulatory infertility but is also associated with metabolic disorders, among which is impaired glucose metabolism. Insulin resistance (IR) is one of its most important clinical features, which has been associated with metabolic disturbances and increased risk of type 2 diabetes (T2D) (2). IR is a heterogeneous disorder caused by genetic and environmental factors. In IR, cells are less responsive to insulin (3). Additionally, β-cell function early impairment was found in women with PCOS and both prediabetes and T2D are very common in patients with this condition (4,5).

In women with PCOS on the other hand, impaired insulin activity might differ from that seen in T2D without PCOS (6). IR seems to manifest in PCOS due to downstream defects in insulin signaling, which affects the molecular level (7). For example, exercise has been considered the first - choice treatment to balance IR by increasing the insulin - mediated molecular pathway and stimulating GLUT4 translocation via insulin - independent pathways (8,9). However, exercise training was unable to resolve IR in PCOS (10).

We hypothesized that IR may be reflected at a transcriptional level in the blood of PCOS patients. Genes are known to be differentially regulated, and this would indicate the molecular mechanism of IR. These differences in the transcription profiles of insulin sensitivity and insulin resistance could provide markers for the onset of diabetes. This study aimed to explore the gene expression profiling in whole blood related to IR in patients with PCOS and control.

MATERIALS AND METHODS

Ethical approval and consent

This study was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-13-206-15132). Written and informed consent was obtained from all subjects before involvement in the research. The study had been performed following the principles stated in the Declaration of Helsinki.

Subjects

Five PCOS and 5 non-PCOS samples with BMI and age-matched samples were analyzed. All subjects for this study were women aged between 20-40 years and this is a sub-study from the Malay PCOS Prevalence Study, a clinic-based case-control investigation of Malay women (11). PCOS patients were diagnosed according to the Rotterdam Criteria (12).

Biochemical analysis

Blood biochemistry parameters were analyzed by Chemistry Analyser (CS-400 Dirui, China) using Randox reagents (UK). The two-site immunoenzymometric assay kit was used to measure fasting insulin using the TOSOH AIA system analyzer (TOSOH Corporation, Tokyo, Japan). Then, the homeostasis model assessment of IR (HOMA-IR) was calculated.

RNA isolation, PCR array, and bioinformatics analysis

One PCR Array was used for each sample. Total RNA was extracted from whole blood samples using the RNeasy® Mini kit (Qiagen, Germany) according to the manufacturer's protocol. RNA concentration and quality were determined using Multiskan GO Microplate Spectrophotometer with µDrop™ Plate (Thermo Fisher Scientific, USA). Total RNA was used to generate the complementary DNA (cDNA) libraries. After cDNA synthesis, PCR array analyses for endogenous reference gene and target gene were performed with StepOnePlus Real-Time PCR (Applied Biosystems, USA) in 96-well plates (Human IR RT2 Profiler™ PCR array kit, PAHS-156Z, Qiagen, Germany). The data analysis web portal, GeneGlobe, calculated fold change/regulation using the 2^-delta-delta CT formula. Changes in the gene expression level were assessed the for PCOS group in relation to the control group with the gene expression level set up arbitrarily as 1. The differentially expressed genes, with fold regulation greater than ±2, are emphasized in this study.

RESULTS

We compared various metabolic indices between PCOS patients and controls. No significant differences were found in glucose, insulin, and HOMA-IR between both groups although the measurements seem higher in those with PCOS. It is worth noting that the PCOS group had a higher value of IR (2.6 ± 0.58). The baseline overall clinical data are presented in Table I.

We have used the PCR array, to investigate the gene expression in the peripheral blood of 84 genes, related to the biology of IR in those with PCOS relative to healthy individuals. The PCR Array used in this study includes representative genes from various biological pathways: insulin and adipokine signaling, genes commonly dysregulated in non-insulin-dependent diabetes mellitus (NIDDM), enzymes and transporters important for carbohydrate and lipid metabolism, as well as genes involved in innate immunity and inflammatory processes. Fig. 1 shows the scatter plot of the two groups' relative expression levels for each gene. Tables II and III represent the lists of upregulated and downregulated genes.

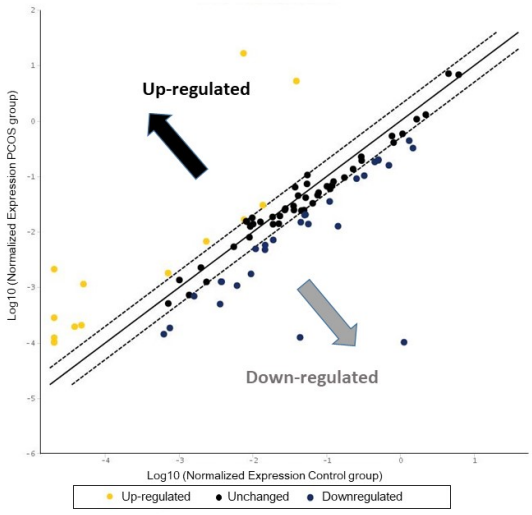

Figure 1 : Scatter Plot PCR array analysis of relative expression levels for each gene in the two samples (PCOS vs Control). The figure shows a log transformation plot of the relative expression level of each gene (2-ΔΔCt) between Normal (x-axis) and PCOS (y-axis). The (yellow) and (blue) dot stands for upregulated and downregulated genes respectively. The dotted lines indicate a two-fold change in the gene expression threshold.

DISCUSSION

Increased risk for developing glucose intolerance and T2D are found in women with PCOS. These PCOS patients with impaired glucose tolerance should be treated with intensive lifestyle modification, which includes weight loss as well as considered for treatment with insulin-sensitizing agents (13). Additionally, the prevalence of metabolic syndrome was significantly higher in women with PCOS as compared to age-matched controls (14). These women show higher IR

Table I : Clinical characteristics of PCOS patients and controls

	PCOS (n=5)	CONTROL (n=5)	p-value
Age (years)	32 ± 4.1	30 ± 3.5	0.884
Weight (kg)	62.1 ± 4.95	64.3 ± 0.60	0.431
BMI (kg/m^2)	27.94 ± 2.82	26.35 ± 7.04	0.343
Waist circumference (cm)[1]	86.0 ± 11.33	80.0 ± 9.51	0.276
SBP (mm Hg)	110.0 ± 5.11	111.0 ±6.88	0.073
DBP (mm Hg)	70.0 ± 10.21	70.0 ± 7.23	0.660
HOMA-IR	2.6 ± 0.58	2.0 ± 0.72	0.751
Serum Insulin (μU/mL)	11.4 ± 1.45	9.8 ± 1.64	0.962
Fasting Glucose (mmol/L)	5.14 ± 0.92	4.93 ± 0.60	0.193
HbA1c (%)	5.3 ± 0.38	5.3 ± 0.32	0.157
Total Cholesterol (mmol/L)	5.3 3 ± 0.31	5.3 ± 0.29	0.157
HDL-C (mmol/L)	5.1 ± 0.62	5.1 ± 0.55	0.330
LDL-C (mmol/L)	1.2 ± 0.22	1.3 ± 0.26	0.270
Serum TG (mmol/L)	1.3 ± 0.55	1.2 ± 0.49	0.910
DHEAS (μg/dL)	118.3 ± 18.6	118.0 ± 20.25	0.504
SHBG (nmol/L)	53.9 ± 2.25	70.6 ± 3.75	< .001
Total Testosterone (nmol/L)	1.7 ± 0.51	1.7 ± 0.66	0.216
Free androgen index	3.0 ± 0.11	2.2 ± 0.24	< .001
Free testosterone (pmol/L)	21.8 ± 0.06	19.1 ± 0.88	0.042

Note: Data are presented as mean ± standard deviation (SD). Boldface indicates a significantly different versus the control ($p < 0.05$). Abbreviations: BMI Body Mass Index, SBP Systolic Blood Pressure, DBP Diastolic Blood Pressure, HOMA-IR homeostatic model assessment of insulin resistance; HDL-C High Density Lipoprotein-Cholesterol, LDL-C Low Density Lipoprotein-Cholesterol, TG Triglyceride, DHEAS Dehydroepiandrosterone sulfate, SHBG Sex Hormone Binding Globulin.

but also larger insulin secretion to maintain normal glucose homeostasis than BMI-, age- and IR-matched controls (15). Since IR is an important aspect of PCOS, it has been observed not only in obese but also in lean women with PCOS. This condition seems to be an intrinsic part of the syndrome as early impaired β cell function was detected in both lean and obese PCOS patients (4,16,17).

We purposely chose homogenous measurements for both groups. Therefore, between PCOS and controls, we found no difference in glucose and insulin levels as well as the HOMA-IR index. Our results were explained by findings where overweight PCOS and non-PCOS women had comparable HOMA-IR, body fat content, and distribution and hepatic triglyceride content (18).

The increment of expressions for both ADIPOR1 and ADIPOQ agreed with each other, but these results were in contrast with other studies (19,20). There are 2 types of adiponectin, where in PCOS patients, the high-molecular-weight adiponectin is inversely associated with sympathetic activity. Additionally, serum adiponectin (protein of ADIPOQ gene) levels in human PCOS were found to be significantly lower than in controls, implying a role for adiponectin in the pathogenesis of the syndrome (21–23).

Apolipoprotein E (APOE) gene expression increased. APOE*4 binds DNA with a high affinity that includes genes associated with IR (24) and could contribute to the development of metabolic syndrome as it may affect all its components (25). Another gene, ACACA represents novel biomarkers in adipose tissue associated with T2D in obese individuals (26). Up-regulation of the fatty acid-binding protein 4 (FABP4) gene may explain why it may play an

Table II : Table of quantitative real-time PCR analysis (± SD) of the chosen genes. Genes Over-Expressed in PCOS group vs. Control group

	Position	Gene Symbol	Description	Fold Regulation
1	A06	ADIPOR1	Adiponectin receptor 1	136.56
2	C06	IGF1	Insulin-like growth factor 1	105.54
3	E07	OLR1	Oxidized low-density lipoprotein receptor 1	22.75
4	A05	ADIPOQ	Adiponectin, C1Q, And Collagen Domain Containing	14.03
5	A10	APOE	Apolipoprotein E	6.12
6	A12	CCL2	Chemokine (C-C Motif) Ligand 2	5.12
7	A01	ACACA	Acetyl-CoA Carboxylase Alpha	5.07
8	C02	FABP4	Fatty Acid Binding Protein 4, Adipocyte	5.07
9	E11	PDX1	Pancreatic And Duodenal Homeobox 1	5.07
10	B09	CRLF2	Cytokine Receptor-Like Factor 2	4.39
11	C05	IFNG	Interferon, Gamma	2.91
12	G11	UCP1	Uncoupling Protein 1 (Mitochondrial, Proton Carrier)	2.56
13	D02	IL8	Chemokine (C-X-C Motif) Ligand 8 or Interlukin 8	2.25
14	B11	CXCR3	C-X-C motif chemokine receptor 3	2.20

important role in glucose homeostasis and IR (27).

The PCR array produced a few other unexpected results. Individuals with metabolic syndrome also typically have increased plasma levels of plasminogen activator inhibitor-1 (PAI-1), which is the antifibrinolytic factor, or the gene is also known as SERPINE1 (28). Increased SERPINE1 expressions have been linked to the development of obesity and IR (29), yet we found it decreased in PCOS. Another puzzling finding is down-regulated levels of IL-6 which has anti-inflammatory, anti-obesity, and glucose homeostatic roles (30–32). Instead of up-regulation, a list of genes that were downregulated such as insulin receptor substrate 1 (IRS-1), peroxisome proliferator-activated receptor γ (PPARG), PPARG-coactivator 1-α (PPARGC1A), and resistin (RETN). Such finding is intriguing because these genes were found upregulated in other studies (33–35).

Such inconsistencies could be attributable to differences in methods, which is the use of usually conventional real-time quantitative PCR vs. a custom PCR array in which all primers were designed by Qiagen like in this study. In other studies, experiments were carried out on ovary or adipose tissues (36). On the other hand, IR is a heterogeneous disorder caused by a range of environmental and genetic factors It was hypothesized that its etiology varies considerably between individuals (3). Furthermore, the effects of IR on gene expression are modified by cellular context and differentiation state (37).

There have been various studies focused on gene expression in IR and T2D in different metabolic states using microarray technology (38). Altered expression of several genes was found in the effect of insulin treatment on skeletal muscle in T2D (39,40). This factor sets them apart from our insulin resistant but without T2D subject group. As our PCOS subjects had other factors, it is difficult to compare our results to these studies as they often involve T2D or obese subjects. There may be differences in androgen levels between PCOS populations due to differences in BMI and levels of IR and sex hormone-binding globulin, which may explain some of the discrepancies between our report and previous studies. Interestingly, our previous animal model study, found that a Malaysia herb, Labisia pumila improved insulin sensitivity and lipid profile in PCOS rats without affecting body composition (41).

This study is not without limitations. Despite a bigger number enrolled in the original main study,

Table III : Table of quantitative real-time PCR analysis (± SD) of the chosen genes. Genes Under-Expressed in PCOS group vs. Control group

	Position	Gene Symbol	Description	Fold Regulation
1	C04	HK2	Hexokinase 2	-11.15
2	D04	IRS1	Insulin Receptor Substrate 1	-7.14
3	F12	SERPINE1	Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 1	-5.61
4	D01	IL6	Interleukin 6	-5.36
5	D11	LTA4H	Leukotriene A4 Hydrolase	-4.51
6	G07	TLR4	Toll-Like Receptor 4	-4.35
7	F04	PPARGC1A	Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha	-4.29
8	F09	RETN	Resistin	-4.05
9	F03	PPARG	Peroxisome Proliferator-Activated Receptor Gamma	-4.03
10	G10	TNFRSF1B	Tumor Necrosis Factor Receptor Superfamily, Member 1A	-3.09
11	F08	RELA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A	-3.08
12	D12	MAP2K1	Mitogen-activated protein kinase kinase 1	-3.03
13	G01	SLC27A1	Solute carrier family 27 member 1	-2.97
14	C11	IL1R1	Interleukin 1 receptor type 1	-2.95
15	A03	ACSL1	Acyl-CoA synthetase long chain family member 1	-2.95
16	A02	ACACB	Acetyl-CoA carboxylase beta	-2.91
17	C10	IL1B	Interleukin 1 beta	-2.75
18	E10	PDK2	Pyruvate dehydrogenase kinase 2	-2.62
19	A11	CASP1	Caspase 1	-2.54
20	D09	LIPE	Lipase E, hormone sensitive type	-2.52
21	C07	IGF1R	Insulin-like growth factor 1 receptor	-2.50
22	G09	TNFRSF1A	TNF receptor superfamily member 1B	-2.48
23	G03	SOCS3	Suppressor of cytokine signaling 3	-2.47
24	G06	STAT3	Signal transducer and activator of transcription 3	-2.43
25	G12	VLDLR	Very low-density lipoprotein receptor	-2.29
26	G04	SREBF1	Very low-density lipoprotein receptor	-2.19

relatively small numbers of samples were used in this study. However, a low number of samples were used in another study which analyzed mRNA expression using a PCR array as well (42). It is worth noting that even though the results are not statistically significant due to the sample size, differences in some of the participants' features, inclusive of age, ovarian status, BMI, and fat mass, may have influenced the gene responses. Additionally, these data were confined to borderline IR patients, not T2D. In this regard, the

relatively limited number of differentially expressed genes could be partially associated with this condition. Severe IR conditions characterized by disruption of glucose homeostasis (i.e.T2D) could lead to a different pattern of gene expression. Moreover, our PCR array analysis focused on genes related to IR in whole blood, thus, further studies should assess gene expression in other tissues potentially associated with IR in PCOS, especially in adipose and ovary tissues. The importance of the potential of whole blood samples as predictive and reflective markers of disease development have been highlighted in several reports (43).

CONCLUSION

In summary, this study provides evidence regarding the IR genes in the whole blood of PCOS patients. Although blood biochemistry parameters looked similar, gene expression differences were observed. Taken together, our findings suggest that the PCOS condition can disrupt the expression of genes involved in the regulation of insulin and adipokine signaling, inflammation, and metabolic pathways.

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