UNIVERSITI PUTRA MALAYSIA

GENE EXPRESSION PROFILING OF SELECTED GENES (TLR4, PPARY2, TCF7L2 AND IRS1) IN TYPE 2 DIABETES MELLITUS MALAY SUBJECTS AND THEIR FIRST DEGREE RELATIVES

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By

FATEMEH DANAZADEH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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DEDICATION

I dedicated this piece of work to my mother and beloved husband Farshad.
Thank you for all your encouragments and support.
Love you with all my heart.

Abstract of thesis presented to the senate of university Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

GENE EXPRESSION PROFILING OF SELECTED GENES (*TLR4, PPARγ2, TCF7L2 AND IRS1*) IN TYPE 2 DIABETES MELLITUS IN MALAY SUBJECTS AND THEIR FIRST DEGREE RELATIVES

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Chairman: Patimah Ismail, PhD
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Type 2 Diabetes Mellitus (T2DM) is known as a metabolic disorder which characterized by high level of blood sugar due to dysfunction of pancreatic beta cells or insulin resistance in insulin target tissues. International Diabetes Federation (IDF) has predicted that the number of people who suffering from the disease in the world will increase from 382 million in 2013 to 552 million by 2030. According to the National Health Morbidity Survey (NHMS) IV, prevalence of T2DM in Malaysia was 15.2% in 2011, which increased from 14.9% in the third survey despite health campaigns and efforts.

Environmental risk factors such as sedentary lifestyle, dietary factors, smoking, and lack of physical activity in combination with genetic factors play important role in progression of T2DM. First-degree relatives (FDR) of Type 2 diabetic patients are at risk of developing the disease. The risk in offspring rises by two to four-fold with having a parent with T2DM and up to six-fold when both parents are affected. Thus; early diagnosis and prevention programme may lead to decrease the risk of T2DM.

Recently, human genetic studies have reported several candidate genes such as peroxisome proliferator-activated receptor γ (PPARγ) and transcription factor 7-like 2 (*TCF7L2*) which substantially develop the risk of T2DM. In addition, Insulin receptor substrate1(*IRS1*) as another candidate gene is involved in insulin-stimulated signalling pathway in T2DM. Toll-like receptors (TLRs) are innate immune receptors which have showed to play important role in pathogenesis of T2DM, particularly *TLR4*.

The main objective of this study was to determine expression pattern of selected genes (*TLR4*, *PPARγ2*, *TCF7L2* and *IRS1*) among Malay T2DM subjects and their first degree relatives. The candidate genes were selected based on their known role in glucose homeostasis.

A total of 15 T2DM, 15 first degree relatives of Type 2 diabetic patients and 15 healthy subjects as control group were recruited. The RNA was extracted from whole blood specimen by using a commercial extraction kits. Quantitative Real-Time PCR was used to amplify the target cDNA copies of RNA.

Statistical analysis was performed by t-test; crosstabs and general linear model (Anova) through the SPSS statistical software and $P \leq 0.05$ were considered as significant. The gene expression analysis and relative expression in real-time PCR was performed by

using REST software. The anthropomorphic value and the blood biochemical factors were evaluated as supplementary information. The fasting plasma glucose (P=0.000), HA1c (P=0.000) and systolic blood pressure (P=0.003) were significantly different between T2DM and control. Also there was a significant difference between T2DM and healthy subjects in term of HDL (P=0.000) and TG (P=0.000). However, LDL and cholesterol level of T2DM subjects were under control and not significantly different (P=0.201 and P=0.90 respectively) in comparison with control group. Regarding to the relatives of T2DM patients, significant difference was observed in FPG (P=0.000) and SBP (P=0.008). Gene expression pattern was determined in T2DM patients and their first degree relatives. Compared to controls, *TLR4* gene was significantly upregulated in T2DM patients, while it downregulated in their FDR. We also showed that expression of *IRS1* was significantly deceased (down regulated) in patients with T2DM compared with controls whereas altered expression in *PPARγ2* and *TCF7L2* genes were not found among T2DM and FDR compared with healthy individuals. In conclusion, the result from this study demonstrated that *TLR4* and *IRS1* might be involved in pathogenesis of T2DM and also altered expression of *TLR4* in first degree relative of Type 2 diabetes is an important marker showing genetic predisposition to T2DM, and hence could be used as diagnostic tool in the prediction of T2DM in Malay subjects.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMPROFILAN EKSPRESI GEN TERPILIH (*TLR4, PPARγ2, TCF7L2 AND IRS1*) BAGI SUBJEK BERBANGSA MELAYU DAN AHLI KELUARGA DARJAH PERTAMA MEREKA

Oleh

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Diabetes melitus jenis 2 (DMJ2) ialah gangguan metabolik yang memiliki ciri kandungan gula dalam darah yang tinggi. Perkara ini disebabkan oleh disfungsi sel beta pankreas atau kerintangan insulin dalam tisu yang disasarkan oleh insulin. Persekutuan Diabetes Antarabangsa (IDF) meramalkan bilangan pesakit sedunia yang menghidap penyakit ini akan meningkat daripada 382 juta orang pada tahun 2013 kepada 552 juta orang pada tahun 2030.

Menurut Kajian Morbiditi dan Kesihatan Kebangsaan (NHMS) IV, prevalens DMJ2 di Malaysia ialah 15.2% pada tahun 2011, meningkat daripada 14.9% dalam kajian ketiga walaupun wujud kempen dan usaha kesihatan. Faktor risiko persekitaran seperti gaya hidup yang sedentari, pemakanan, amalan merokok dan kurangnya aktiviti fizikal digabungkan dengan faktor genetik, memainkan peranan penting dalam peningkatan DMJ2.

Ahli keluarga darjah pertama (FDR) pesakit diabetes jenis 2 berisiko untuk mendapat penyakit ini. Risiko bagi zuriat FDR meningkat sebanyak dua hingga empat kali ganda sekiranya salah seorang ibu atau bapa menghidap DMJ2 dan sehingga enam kali ganda apabila kedua-dua ibu bapa menghidap penyakit ini. Oleh itu, diagnosis awal dan program pencegahan boleh mengurangkan risiko DMJ2.

Baru-baru ini, kajian genetik manusia melaporkan beberapa gen calon seperti peroxisome proliferator-activated receptor γ (PPARγ) dan transcription factor 7-like 2 (*TCF7L2*) yang meningkatkan risiko DMJ2. Di samping itu, reseptor insulin substrat-1(*IRS1*) ialah gen calon lain yang terlibat untuk laluan isyarat insulin yang terangsang dalam DMJ2.

Reseptor berupa tol (TLR) ialah reseptor imun semula jadi yang memainkan peranan penting dalam patogenesis DMJ2, terutamanya *TLR4*. Objektif utama kajian ini adalah untuk menentukan corak ekspresi gen yang terpilih (*TLR4*, *PPARγ2*, *TCF7L2* dan *IRS1*) dalam kalangan subjek berbangsa Melayu dan ahli keluarga darjah pertama mereka.

Gen calon dipilih berdasarkan peranan gen yang diketahui dalam homeostasis glukosa. Sebanyak 15 orang pesakit DMJ2, 15 orang ahli keluarga darjah pertama kepada pesakit diabetes jenis 2 dan 15 calon subjek yang sihat sebagai kumpulan kawalan telah dipilih. RNA disari daripada keseluruhan spesimen darah dengan menggunakan kit pengeluaran komersial.

Masa Nyata Kuantitatif PCR digunakan untuk menguatkan salinan cDNA bagi RNA sasaran. Analisis statistik dilakukan dengan ujian-t; manakala penjadualan silang dan model linear am (ANOVA) menggunakan perisian statistik SPSS dan $p \leq 0.05$ dianggap sebagai signifikan. Analisis ekspresi gen dan ekspresi relatif dalam masa nyata PCR dilaksanakan dengan menggunakan perisian REST.

Nilai antropomorfik dan faktor biokimia darah dinilai sebagai maklumat tambahan. Glukosa plasma berpuasa (P=0.000), HA1c (P=0.000) dan SBP (P=0.003) berbeza secara ketara di antara DMJ2 dengan kumpulan kawalan. Terdapat juga perbezaan yang ketara di antara DMJ2 dengan subjek yang sihat dari segi HDL (P=0.000) dan TG (P= 0.000).

Walau bagaimanapun, tahap HDL dan kolesterol bagi subjek DMJ2 adalah terkawal dan tidak jauh beza (P=0.201 dan P=0.90 masing-masing) berbanding dengan kumpulan kawalan. Untuk saudara-mara pesakit DMJ2, perbezaan ketara diperhatikan bagi FPG (P=0.000) dan SBP (P=0.008). Pola ekspresi gen ditentukan pada pesakit T2DM dan ahli keluarga darjah pertama mereka.

Berbanding dengan kumpulan kawalan, ekspresi gen *TLR4* meningkat dengan ketara untuk pesakit T2DM, manakala SMDP mereka menurun. Kami juga menunjukkan bahawa ekspresi gen *IRS1* terbantut dengan ketara (menurun) bagi pesakit DMJ2 berbanding dengan kumpulan kawalan manakala perubahan ekspresi gen *PPARγ2* dan *TCF7L2* tidak ditemui dalam kalangan DMJ2 dan SMDP sekiranya dibandingkan dengan individu yang sihat.

Kesimpulannya, keputusan daripada kajian ini menunjukkan bahawa gen *TLR4* dan *IRS1* mungkin terlibat dalam patogenesis DMJ2. Perubahan ekspresi gen *TLR4* yang wujud pada ahli keluarga darjah pertama pesakit diabetes jenis 2 merupakan penanda penting yang menunjukkan kecenderungan genetik terhadap DMJ2 dan penanda tersebut boleh digunakan sebagai alat diagnostik dalam meramal penyakit DMJ2 bagi subjek berbangsa Melayu.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

T2DM	Type 2 Diabetes Mellitus
IDF	International Diabetes Federation
NHMS	National Health Morbidity Survey
FDR	First Degree Relative
PPARG	Peroxisome proliferator-activated receptor Gamma
TCF7L2	Transcription Factor 7-Like 2
IRS1	Insulin Receptor Substrate 1
TLR4	Toll- like Receptor 4
cDNA	Complementary Deoxyribonucleic acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
HbA1c	Glycated Haemoglobin
SBP	Systolic Blood Pressure
DBP	Diastolic Blood Pressure
LDL	Low-Density Lipoprotein
HDL	High-Density Lipoprotein
qPCR	quantitative Polymerase Chain Reaction
T1DM	Type 1 Diabetes Mellitus
GDM	Gestational Diabetes Mellitus
IFG	Impaired Fasting Glucose
CVD	Cardiovascular Disease
NGT	Normal Glucose Tolerance
GWAS	Genome -Wide Association Studies
FTO	Fat mass and obesity-associated protein
KCNJ11	Potassium channel, inwardly rectifying subfamily J, member 11
CAPN10	Calcium-Activated Neutral Proteinase 10
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
HMG	High Mobility Group
GLP-1	Glucagon-like Peptide1
PAMPs	Pathogen Associated Molecular Pattern
LPS	Lipopolysaccharide
FFA	Free Fatty Acid
RXR	Retinoid X Receptor

PPREs	PPAR Response Elements
GLUT2	Glucose Transporter 2
IL-6	Interleukin 6
TNF-α	Tumor Necrosis Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
CHD	Coronary Heart Disease
BMI	Body Mass Index
RIN	RNA Integrity Number
NTC	No Template Control
CT	Cycle Threshold for Real- Time PCR analysis
HTN	Hypertension
IR	Insulin Receptor
VDF	Vancouver Diabetic Fatty
CRP	C - reactive protein
SAA	Serum Amyloid A
IHD	Ischemic Heart Disease
NOTCH2	Neurogenic Locus Notch Homolog Protein 2
HHEX	Hematopoietically-expressed homeobox
JAZF1	Juxtaposed with another zinc finger protein 1
OR	Odds Ratio
RT	Reverse Transcription
TLR	Toll-like Receptor
RR	Relative Risk
bp	Base Pair
ANOVA	Analyse of Variance
Kg	Kilogram
ml	millilitre
ng	nanogram
OD	Optical Density
TG	Triglyceride
SNP	Single Nucleotide Polymorphism
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Diabetes Mellitus (DM) is a metabolic disease (Zaini, 2000) characterized by high level of blood sugar due to dysfunction of pancreatic β-cells or insulin resistance in insulin target tissues such as skeletal muscle, adipose tissue, and liver, resulting in distraction of glucose homeostasis (Barceló *et al.,* 2001). Diabetes Mellitus which is often associated with essential hypertension, obesity and dyslipidemia cause numerous micro and macro vascular complications, including blindness, renal failure and coronary heart disease (CHD) (Mustaffa, 2004). Diabetes Mellitus depends on its etiology includes type 1 and Type 2 Diabetes Mellitus (T2DM). T2DM comprises 90% of the total cases of Diabetes Mellitus (Hariri *et al.,* 2006).

In 1995, population of adult people with diabetes was estimated 135 million in the world and by 2007 this number rose to 248 million and it has been predicted that it will increase to 380 million by 2025 (Zafar *et al.,* 2010). According to the International Diabetes Federation (IDF), there were 382 million people aged 20-79 years living with diabetes in 2013, and this number is expected to rise to 552 million by 2030 (Guariguata *et al.,* 2014).

T2DM prevalence is rising in nearly all countries around world and the greatest health care and economic effect will be appeared in developing countries in Asia (Guariguata *et al.,* 2014). It is reported that more than 60% of all diabetic are living in Asia (Mu *et al.,* 2012). Epidemiological data showed that the prevalence of diabetes is high in the South-East Asia (SEA) Region with number of 72 million persons with diabetes and predict that this number rises in the next few years (IDF diabetes atlas, 2013).

T2DM is a chronic and largely preventable disease and causes premature death and multiple complications. An estimated around 55% of diabetic persons in the SEA region die by the age of 60 years (Ramachandran *et al.,* 2014). It also imposes great burden on health care system due to outpatient visits, more medications and longer term care compared to health people (Zhang *et al.,* 2010).

T2DM is a multifactorial disease with both genetic and environment etiological factors (Cockram, 2000) and associated with numerous risk factors such as family history, obesity, high blood pressure, high low-density lipoprotein (LDL) and low high- density lipoprotein (HDL) levels (American Diabetes Association, 2013; sterns *et al.,* 2014).

The familial aggregation, higher concordance rate in monozygotic twins compared with dizygotic and the variation of prevalence in different population provide evidences for genetic susceptibility to T2DM. It has been demonstrated that having a parent with T2DM increases by two to four folds an offspring's risk of developing this disorder (Noureddin and Soltanian, 2012). United States has reported that 88-95% of first degree relatives and 70-77% of second relatives of T2DM affected by disease (Hariri *et al.,* 2006).

Recently genome wide association studies (GWAS) identified over 70 loci for Type 2 Diabetes Mellitus (Hara *et al.,* 2014). Genetic studies have also shown that variation in transcription factor 7-like 2 (*TCF7L2*) (Petrie *et al.,* 2011), Insulin receptor substrate 1(*IRS1*) (Rung *et al.,* 2009) and peroxisome proliferator-activated receptor γ (PPARγ) (Altshuler *et al.,* 2000) genes are greatly associated with T2DM. Since inflammatory system may be involved in pathogenesis of T2DM, Toll-like receptors as innate immune receptors has critical role in diabetes and insulin resistance in clinical conditions particularly *TLR4* (Dasu *et al*., 2012).

It has been demonstrated that changing copy number as type of duplication and deletion contribute to human genetic disorder. In recent years, many laboratory techniques have been developed to detect these copy number alteration. The most common method is quantitative PCR (qPCR) (D'haene *et al.,* 2010). qPCR is a choice method for gene expression analysis and has many benefits compared to alternative methods such as high sensitivity, accuracy and fast result. During qPCR, accumulation of the amplified product is measured and followed by an amplification plot.

1.2 Problem Statement

Diabetes is a major health problem in the 21st century. It is considered as the fifth leading cause of death in nearly all countries. In every six seconds, someone dies from diabetes and 5.1 million deaths happened because of the diabetes in 2013. Prevalence of Type 2 diabetes is also increasing and majority of the people with T2DM are living in low- and middle-income countries (Unwin *et al.,* 2010).

Malaysia, as a fast developing nation located in South-East Asia, is not escaping the diabetes epidemic. Based on the Forth National Health Morbidity Survey in Malaysia, prevalence of T2DM was reported 15.2% in 2011 that increased from 14.9% in the third survey (Chew *et al.,* 2011). Since family history is a major risk factor for T2DM, it can be used as an important screening tool to identify those at risk of T2DM and the people with undiagnosed T2DM (Hariri *et al.,* 2006). Additionally, there is a lack of genetic database for selected genes (*PPARγ2*, *TLR4*, *IRS1* and *TCF7L2*) and their impact on T2DM and their first degree relatives among Malay subjects based on Malaysian population.

1.3 Significance of the Study

The case and control study attempt to specify the presence of variation within candidate genes (*PPARγ2*, *TLR4*, *IRS1* and *TCF7L2*) among Malay Type 2 Diabetes Mellitus and their first degree compared to healthy individuals. The candidate gene analysis provides a better approach for identifying the level of expression and their possible correlation. The physicians can recognize the onset of T2DM in high risk individuals which can be prevented or delayed. Identification of the susceptible genes also help physician to control T2DM among Malaysian population.

1.4 Hypothesis

There is a significant difference between *TLR4, TCF7L2, PPARγ2 and IRS1* genes expression in selected T2DM subjects and their offspring/sibling

1.5 General Objective

To evaluate the gene expression profiling of *TLR4, TCF7L2, PPARγ2 and IRS1* genes in Malay T2DM subjects and their first-degree relatives

1.6 Specific objective

a) To identify the gene expression pattern of the selected genes among Malay T2DM subjects and healthy individuals.

b) To determine the gene expression of the selected genes among first degree relatives of diabetic subjects and healthy individuals.

c) To identify gene expression change of candidate genes in T2DM that may be linked to clinical outcome.

d) To identify gene expression change of selected genes in first degree relatives of Type 2 diabetic patients that might be useful in screening programme.

e) To determine whether TLR4, IRS1, TCF7L2 and PPARγ2 are involved in pathogenesis of T2DM.

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