UNIVERSITI PUTRA MALAYSIA

ASSOCIATION OF GLUCOKINASE, GLUCOKINASE REGULATOR, GLUCOSE-6-PHOSPHATASE CATALYTIC SUBUNIT 2 AND MELATONIN RECEPTOR 1B GENES POLYMORPHISMS WITH TYPE 2 DIABETIC SUBJECTS

NEDA ANSARI

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By

NEDA ANSARI

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

July 2020

DEDICATION

My Soulmate Iman,

My Beloved Parents

Moreover, also to
My supportive sisters, whom I feel blessed and grateful that I can share this joy with them today. No words can adequately convey the incredible gratitude that I feel for them. They were so supportive through this journey.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ASSOCIATION OF GLUCOKINASE, GLUCOKINASE REGULATOR, GLUCOSE-6-PHOSPHATASE CATALYTIC SUBUNIT 2 AND MELATONIN RECEPTOR 1B GENES POLYMORPHISMS WITH TYPE 2 DIABETIC SUBJECTS

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NEDA ANSARI

July 2020

Chairman : Professor Patimah Ismail, PhD
Faculty : Medicine and Health Sciences

Type 2 Diabetes Mellitus (T2DM) is a complex metabolic disorder characterised by impaired insulin secretion, insulin resistance, and hyperglycemia, caused by the defects in pancreatic b-cells. Although evidence suggests that multiple genetic and environmental factors are involved in the development and progression of T2DM, Still the underlying causes are unknown and have not been fully elucidated. There have been a variety of genetic studies concerning Type 2 Diabetes Mellitus, and some of them showed an association with the occurrence of T2DM. Although there are various candidate genes have been associated with the T2DM in various populations with conflicting results, variations found in Glucokinase (GCK), glucokinase regulatory protein (GCKR), Glucose-6-phosphatase 2(G6PC2) and Melatonin receptor type 1B (MTNR1B) genes are not well studied particularly among Asians. Glucokinase is the key glucose phosphorylation enzyme that regulates glucose-stimulated insulin secretion from pancreatic beta cells and glucose metabolism. A few selected genes polymorphism variant shown to be associated with increased risk of TDM, hyperglycaemia and impaired beta-cell function. The main objective of this study was to determine the candidate genes polymorphism involved in essential Type 2 Diabetes Mellitus among Malay subjects. Since, there have been a variety of genetic association studies of, GCK, GCKR, G6PC2 and MTNR1B conducted on a different population; however, no study was done on Malaysia populations and consistent with Type2DiabetesMellitus.Genetic polymorphism is serving as molecular biomarkers for the detection of the individual at risk of developing the disease. This association study included 200 of subjects without Diabetes as control and 200 of subjects with Diabetes type 2 as a case. Extraction genomic DNA was done all subjects. GCK gene polymorphism was detected using Polymerase Chain reaction (PCR) followed by Restriction Fragment Length Polymorphism (PCR-RFLP). The PCR products were digested with MwoI (Fermentas) restriction enzyme at 60°c for 20 min. The RFLP

products were detected using 2% agarose gel electrophoresis. GCKR, G6PC2, and MTNR1B gene were detected by real-time PCR (RT-qPCR) with Taq-Man probes. Genotype and allele frequencies in case and control samples were compared by using Chi-Square test while characteristic clinical parameters and social-demographic background were analysed using descriptive statics. In this study also the findings have shown that family history of T2DM is high among the subjects (71.3%) compared to the control subjects (54.1%) highlighting the importance of family history assessment in prevention and screening programs and simple measurement of diabetes risk. Body mass index (BMI), fasting blood glucose (FPG), HbA1c, systolic blood pressure (SBP), diastolic blood pressure (DBP) and cholesterol is highly significant ($p<0.05$) between T2DM and control subjects recognizing the risk factors of T2DM among Malay ethnics in Malaysia. However, the other risk factors such as smoking, waist-hip ratio (WHR), high-density lipoprotein (HDL) and triglycerides (TG) did not reveal any significant difference between cases and controls ($p > 0.05$). The results of this study were show that the genotypes and allele frequencies of GCK rs1799884 A/G, GCKR rs780094 T/C, and MTNR1B rs10830963 C/G gene were highly significant risk of developing T2DM Diabetes amongst Malays subjects as compared to the healthy ($p<0.05$), whereas there appears to be no significant association between the genetic polymorphism of G6PC2 rs560887 A/G gene. Hence, this candidate genes as possible genetic biomarker and risk factor for Diabetes Type 2 as a case in Malay subjects.

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

HUBUNGKAIT GLUKOKINASE, PENGAWAL GLUKOKINASE, SUBUNIT KATALISIS GLUCOSE -6-PHOSPATASE 2 DAN RESEPTOR MELATONIN 1B GEN POLIMORFISME PADA SUBJEK DIABETIS JENIS 2

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Diabetes Mellitus Jenis 2(T2DM) adalah gangguan metabolik bersifat kompleks yang mempunyai ciri-ciri rembesan insulin, rintangan insulin, dan hiperglisemia, yang disebabkan oleh kecacatan pada sel-b pankreas. Walaupun terdapat bukti yang menunjukkan bahawa pelbagai faktor genetik dan alam sekitar terlibat dalam perkembangan dan pengembangan T2DM, namun dasar punca-punca tersebut masih tidak diketahui dan belum dapat dijelaskan sepenuhnya. Terdapat pelbagai kajian genetik yang berkaitan dengan Diabetes Mellitus Jenis 2 dan sesetengah kajian di antaranya mempunyai kaitan dengan kemunculan T2DM. Walaupun terdapat pelbagai gen calon yang dikaitkan dengan T2DM dalam pelbagai populasi mempunyai percanggahan hasil kajian, variasi bagi Glucokinase (GCK), protein penyelaras glucokinase(GCKR), Glukosa-6-fosfat 2 (G6PC2) dan gen Melatonin reseptor jenis 1B (MTNR1B) tidak dikaji dengan lanjut terutamanya di kalangan orang Asia. Glucokinase adalah enzim fosforilasi glukosa utama yang mengawal rembesan insulin glukosa yang dirangsang daripada sel beta pankreas dan metabolisme glukosa. Beberapa varian polimorfisme gen terpilih ditunjukkan berkaitan dengan peningkatan risiko TDM, hiperglikemia dan gangguan fungsi sel beta. Objektif utama kajian ini adalah untuk menentukan penglibatan calon gen polimorfisme terlibat dalam Diabetes Mellitus Jenis 2 yang utama di kalangan subjek Melayu. Memandangkan, terdapat pelbagai kajian oleh pelbagai persatuan genetik tentang GCK, GCKR, G6PC2 dan MTNR1B yang dijalankan pada populasi yang berlainan, namun tidak ada kajian dilakukan terhadap populasi Malaysia dan berkaitan dengan Diabetes Mellitus Jenis 2. Genetik polimorfisme berfungsi sebagai biomarker molekul untuk mengesan individu yang berisiko untuk menghidap penyakit ini. Kajian persatuan ini mengandungi 200 subjek tanpa Diabetes sebagai kawalan dan 200 subjek dengan Diabetes sebagai kes. DNA dilakukan pada semua subjek. Gen polimorfisme GCK

dikesan menggunakan reaksi berantai polimerase (PCR) diikuti dengan Polimorfisme Panjang Berkas Restriksi (PCR-RFLP). Produk PCR dicerna dengan enzim restriksi MwoI (Fermentas) pada 60 °c selama 20 minit. Produk RFLP dikesan menggunakan elektroforesis gel agarose 2%. Gen GCKR, G6PC2 dan MTNR1B dikesan oleh masa sebenar PCR (RT-qPCR) dengan prob Taq-Man. Frekuensi genotip dan alel dalam sampel kes dan sampel kawalan dibandingkan dengan menggunakan ujian Chi-Square manakala parameter ciri klinikal dan latar belakang sosial-demografi dianalisis dengan menggunakan statik deskriptif. Dalam kajian ini, terdapat juga penemuan menunjukkan bahawa sejarah keluarga untuk T2DM adalah tinggi di kalangan subjek (71.3%) berbanding dengan subjek kawalan (54.1%). Hal ini menunjukkan kepentingan penilaian sejarah keluarga dalam program pencegahan dan pemeriksaan serta pengukuran mudah untuk risiko diabetes. Indeks berat badan (BMI), glukosa darah waktu puasa (FPG), HbA1c, tekanan darah sistolik (SBP), tekanan darah diastolik (DBP) dan kolesterol adalah sangat signifikan ($p < 0.05$) di antara T2DM dan subjek kawalan yang mengenali faktor risiko T2DM antara etnik Melayu di Malaysia. Walau bagaimanapun, risiko faktor lain seperti merokok, rasio pinggang-punggul (WHR), lipoprotein densitas tinggi (HDL) dan trigliserida (TG) tidak menunjukkan perbezaan yang ketara di antara kes dan kawalan ($p > 0.05$). Keputusan kajian ini menunjukkan bahawa frekuensi genotip dan alel GCK rs1799884 A / G, GCKR rs780094 T / C, dan gen MTNR1B rs10830963 C / G mempunyai risiko yang sangat signifikan untuk menghidap Diabetes T2DM di kalangan orang Melayu berbanding dengan yang sihat ($p < 0.05$), malakala tidak terdapat hubungan yang signifikan antara genetik gen polimorfisme G6PC2 rs560887 A / G. Oleh itu, gen calon ini mungkin boleh dijadikan sebagai genetik biomarker dan faktor risiko untuk Diabetes T2DM bagi subjek Melayu.

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LIST OF ABBREVIATIONS

T2DM	Type 2 Diabetes Mellitus
GCK	Glucokinase
GCKR	Glucokinase regulatory protein
(MTNR1B)	Melatonin receptor 1B
G6PC2	Glucose 6 phosphatase catalytic
IDF	International Diabetes Federation
HS	Hospital Serdang
Chol	Cholesterol
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
Hb	Hemoglobin
HbA1c	Hemoglobin A1c
TG	Triglyceride
BMI	Body Mass Index
WHR	Waist Hip Ratio
SBP	Systolic Blood Pressure
DBP	Diastolic Blood Pressure
WT	Wild Type
HET	Heterozygous
HOM	Homozygous
SNPs	Single Nucleotide Polymorphisms
NCDs	Non Communicable Diseases
WHO	World Health Organization

GD	Gestational Diabetes
T1D	Type 1 Diabetes
IGT	Impaired Glucose Tolerance

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Type 2 Diabetes Mellitus (T2DM) is considered as a complex metabolic disorder recognized with impaired insulin secretion, insulin resistance, and hyperglycaemia manifestations caused by the defects in pancreatic b-cells. Although evidence suggests that various environmental and genetic parameters interfere in the development and progression of T2DM, Still the underlying causes are unknown and have not been fully elucidated. There are two types of diabetes; 1) insulin-dependent diabetes known as type 1, caused by functional loss of pancreatic beta cells, and 2) type 2 diabetes which is known as non-insulin dependent and can be due to the decrease in insulin sensitivity within the peripheral tissues (Carpenter, 2008). The most common diabetes, accounting for 90%-95% of all the diabetes cases, is T2DM reported as a life-lasting metabolic syndrome with the high level of blood sugar as well as, the other diabetic complications such as cardiovascular disease, neuropathy and retinopathy threatening the life (Tahir and Cavender, 2018, Deshpande et al., 2008).

The prevalence was 14.9% among adults in 2006 (Third National Health Morbidity Survey in 2006 (Ministry of Health, 2008) and it has been increased to 17.5% in 2015 (Jan Mohamed, et al. 2015). The main reasons for the rapid development of diabetes among Malaysians are inclusive of the lifestyle alteration owing to urbanization and ageing which lead to the increase in obesity and physical inactivity (Letchumanan et al., 2010). Multi-ethnic society includes three major ancestral groups: Malay (~63%); Chinese (~25%); and Indian (~7%). The prevalence of T2D appears to differ among these groups, with Malaysian. However, the Indians having the highest prevalence (25 to 28%), followed by Malays (17 to 19%) and Chinese (9 to 14%) but the major race were Malay (~63%) in Malaysia (Jamal et al., 2014).

T2DM accounts for most cases of diabetes in Malaysia. In a recent report, nearly 1.8 million of people in Malaysia are suffering from T2DM as many of them were unaware of diabetes as they never do the health screening in time (Letchumanan et al., 2010). Although there are several factors involved for the development of T2DM, reports suggested that environmental factors such as smoking, alcohol, physical inactivity, being obese/overweight are the major risk factors characterized in the development of T2DM (Dendup et al., 2018). Apart from environmental factors, genetic factors also play a vital role in the development of T2DM. Studies reported that the genetic variations found in many candidate genes are the risk alleles for the development of many diseases, including T2DM. In favor to that, there are many population studies had been reported the association of genetic polymorphisms and T2DM with significant findings (Murea et al., 2012; Van .A.Hoek et al., 2008).

In general, genetic variations involves single nucleotide polymorphisms (SNPs) including insertion, deletion, or change of nucleotides in the sequence of that particular gene. Among that, SNPs are responsible for approximately 90% of the sequence variation (exchanges of single base pairs in DNA) within the human genome (Staiger et al., 2009). The variation of SNPs among the candidate genes may alter the insulin sensitivity/signalling and synergistically, and increase the chances of an individual's bias to various complex diseases (Fernandez-Real et al., 2000). Fasting glucose plays a central role in the pathogenesis of diabetes and its complications Several Studies had shown that genetic factors contributed to fasting glucose levels in the population. However, the genes regulating fasting glucose levels are different from the genes affecting either type 1 or type 2 diabetes susceptibility, although fasting glucose is an important component of diabetes diagnosis. Recently, advance was made in identifying genes regulating fasting glucose through genome-wide association studies. (Hu, C., Zhang, R et al., 2010).

Although a variety of candidate genes have been demonstrated to be in association with the development of T2DM in diverse populations, but first wave of discovery of fasting glucose genes identified glucokinase (GCK), glucokinase regulatory protein (GCKR), glucose-6-phosphatase catalytic subunit 2 (G6PC2) and melatonin receptor 1B (MTNR1B) (Hu, C., Zhang, R et al., 2010). these genes are not well studied particularly among Asians. For instance, the secretion of glucose-stimulated insulin from pancreatic beta cells and glucose metabolism has been reported to be regulated by Glucokinase as the main glucose phosphorylation enzyme (Matschinsky,A. 1996). A mutation in the promoter region of GCK gene (230G.A, rs1799884) shown to cause genetic variation predisposing the risk of TDM, hyperglycemia and disruption of beta-cell function (Marz et al., 2014, Rose.A. et al., 2005, Stone et al., 1996).

The concept of candidate gene has had a significant influence on the early development of genetic epidemiology investigating risk alleles by considering their association with clinical traits. Studies on candidate gene have paved the way for further genetic studies involved in identifying risk variants related to a particular disease (Lunetta, 2008). Besides, the similarity of the selection of genes in the development of other diseases made the candidate gene studies economical and quick to perform (Patnala et al., 2013). Most of the association studies used the cross-selection or case-control designs with a group of random and unrelated individuals; these kinds of studies were designed to detect the probable association between the disease and specific alleles. These polymorphisms were presented in both patients and healthy individuals, though with different allelic frequencies (Timberlake et al., 2001).

The enhancement of different laboratory molecular techniques in recent years has allowed the researchers to analyze the DNA more rapidly, particularly through the discovery of Polymerase Chain Reaction (PCR) and its reliability for detection of mutations (Grompe et al., 1993). Restriction Fragment Length Polymorphism (RFLP), for instance, is one of the methods by which the detection of mutation is feasible. In this technique, the specific restriction enzymes will cut the amplicon of homologous DNAs into smaller pieces called restriction fragments; then these fragments will be

compared to detect the differences among the fragments from the homologous DNAs. In addition, RFLP can also help the researchers to understand the status of the disease in individuals (Sing et al., 1992). The polymerisation is known as an amplification method by which the smaller segment of DNA within the sequence of the gene of interest will be intensified. The combination of the PCR and RFLP is known as PCR-RFLP (Sing et al., 1992). Based on the information provided by the International Diabetes Federation (IDF) in 2017, 425 million people had diagnosed to have diabetes over the world. By 2045, this number of diabetic people is assumed to increase to 629 million. In Southeast Asia, the incidence of T2DM alone is expected to show a 71% increase by 2035 (International Diabetes Federation, 2017). According to the latest NHMS reports in the 2015, has shown that the prevalence of diabetes in Malaysia has increased by 31 % in the space of just 5 years, from 11.6% in 2006 to 20.8 % in 2011 and, the latest report was 22.5% in 2015. Considering three major ethnics in Malaysia appears to show a significant variation in the prevalence of Type 2 diabetes among Malaysians. The results have shown that Asian Indians had the highest prevalence rate (25–28%), followed by those of Malay ancestry (17–19%), and Chinese ancestry (9–14%) respectively (Jamal et al., 2014).

The definition of genetic polymorphism between individuals, groups, or population is any dissimilarity in the genomic sequence. Genetic polymorphism is a broad term which is inclusive of different types such as sequence repeats, insertions, deletions, single nucleotide polymorphisms (SNPs), and recombination (Doris, 2002). Understanding the genetic polymorphism along with the involved techniques indicates the variation in the DNA sequence by which the researchers will be able to investigate differences in both clinical manifestations and disease pathways. These include the determination of the differences among individuals in response to a drug, in the rates at which disease develops, and even in the susceptibility to disease (Hanchard & Saunders, 2005). Accounting about 90% of all the genetic mutation that happens in human DNA, the single nucleotide polymorphism is recognised as the most common cause of genetic polymorphism in which the mutation of only a single base occurs (Cargill et al., 1999). Not only can SNP scan the gene of interest to look for the new polymorphisms but also it is used to identify the allele(s) of a recognised polymorphism in the sequences of interest (Kwok & Chen, 2003). There is no wonder that considerable effort would be needed to understand the biology, risk factors and the genetic issues behind the T2DM as it relates with insulin resistance/signaling, the genes and molecules involved in insulin mechanism. In the present study based on the literature, different genes have been evaluated as candidate genes which have close associations with T2DM (Santaniemi et al., 2006).

1.2 Problem Statement

Genetic parameters are one of the risk factors underlying T2DM. Environmental factors affecting T2DM typically are at the roots of genetic susceptibilities. Comprehensive studies have been done to determine the susceptible candidate genes related to T2DM in different populations with contradictory results. Studies have produced various results since genetic varieties exist among inconsistent ethnic populations. However, there are lacks of studies have been reported in relation to

T2DM and susceptibility genes among Malaysian subjects, particularly on Malay ethnics. This great instigated us to conduct a study to assess the association between genetic polymorphisms and the incidence of T2DM among different Malay ethnics.

1.3 Significance of the Study

This case-control study tries to identify the degree at which candidate genes, that might be concerned to the pathogenesis of T2DM, can vary among Malaysian with or without T2DM. Thus, the aim of this study was to make a comprehensive understanding of the identification and ethology of the genes responsible for the development of T2DM through a case-control study among Malay ethnics using Taq Man assay. To our knowledge, there is a paucity of information on the identification of mutations of GCK, GCKR, G6PC2, and MTNR1B genes polymorphisms among the T2DM patients from different Malay ethnics. Besides, the determination of the genese above associated polymorphisms and mutations, along with the evaluation of the genotype-phenotype relationship will contribute substantially to understanding the aetiology of T2DM among Malays. Providing a more practical approach for identification and determination of the genotype/phenotype and their likely correlation is the advantage of analysing the candidate gene leading to the discovery of the contributing genes. The identification of the contributing genes will provide the physicians with tools making the recognition of the weak individuals, classification of the patients into genetic-related subgroups, and the pathogenesis more possible. Hence, the discovery of the novel treatments as an inevitable part of medical science is not possible without identification of the susceptible genes and their genetic effects on the progress of the disease.

1.4 Hypothesis

The null hypotheses (H0) of this study is:

There is no correlation between G6PC, GCKR, MTNR1B, and 2GCK genes' polymorphisms and risk of T2DM among Malay ethnics.

1.5 Alternate Hypothesis (H1)

There is a correlation between G6PC2, GCKR, MTNR1B, and GCK genes' polymorphisms and risk of T2DM among Malay ethnics.

1.6 General Objective

To analyse the association of G6PC2, GCKR, MTNR1B, and GCK gene polymorphisms between type 2 diabetic cases and control subjects.

1.7 Specific Objective

1). To determine the socio-demographic and clinical characteristics between the subjects.
2). To assess the genotype an allele frequency of G6PC2, GCKR, MTNR1B, and GCK genes' polymorphisms between cases and controls.
3). To define the correlation between G6PC2, GCKR, MTNR1B, and GCK genes' polymorphisms between type 2 diabetics and controls.

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