SINGLE NUCLEOTIDE POLYMORPHISMS OF LEPTIN AND ITS RECEPTOR GENE IN TYPE 2 DIABETES PATIENTS AMONG THREE MAJOR MALAYSIA ETHNICS

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a chronic metabolic syndrome that is rapidly increasing across the world, especially in Malaysia. Leptin and its receptor play vital role in the regulation of glucose metabolism and insulin sensitivity. Variations in the *LEP* and *LEPR* genes have been associated with insulin resistance, leptin level, and T2DM across different populations, but have not been extensively reported within the Malaysian population. This study aimed to investigate the genetic impacts of *LEP* and *LEPR* gene polymorphisms (A19G, G2548A, K109R, and Q223R, respectively) on serum leptin levels and insulin resistance among T2DM patients. This case-control study involved 150 T2DM patients and 150 non-diabetic volunteers from ethnic Malays, Chinese and Indians. The genotyping analysis of *LEP* and *LEPR* gene polymorphisms was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by genotyping of a few samples for each SNP by Sanger sequencing method for validation. Serum leptin and insulin levels were determined via Enzyme Linked Immunosorbent Assay (ELISA). A homeostasis model assessment-insulin resistance (HOMA-IR) was calculated. Chi-square test was used to determine the distribution of genotypes and allelic frequencies and ANOVA was used to determine the association of clinical and biochemical parameters with each SNP. The study shows that the frequency of the AG genotype of the *LEPR* Q223R variant was significantly higher in T2DM patients compared to the control group (58.66% vs. 42%, $p = 0.013$). The A allele frequency was significantly higher in patients with T2DM than in non-diabetic individuals (36.66% and 29%, respectively, $P = 0.046$). Besides, T2DM patients with GG genotype had significantly higher serum leptin, insulin, BMI, and HOMA-IR index ($P < 0.05$). The frequency of the AA genotype and the A allele of the *LEP* G2548A variant were significantly ($P < 0.05$) higher in T2DM patients compared to the control groups. Furthermore, elevated serum leptin, insulin levels, and BMI in diabetic patients were found to be associated with the AA genotype of the *LEP* G2548A variant, compared to GG and GA genotypes ($P < 0.05$). However, no statistical significant differences were found in the genotype and allele frequencies of *LEPR* K109R and *LEP* A19G variants between T2DM patients and non-diabetic volunteers ($P > 0.05$). Furthermore, no significant differences in anthropometrical and biochemical parameters were observed between the genotypes of the *LEPR* K109R and *LEP* A19G polymorphisms. The AGAG haplotype combination of four SNPs was significantly different (OR = 0.633, 95% CI: 0.423 – 0.947, $p = 0.025$). Generally, fasting serum leptin levels were significantly ($P < 0.001$) higher in T2DM patients compared to non-diabetic subjects (166.78 pg/ml and 101.94 pg/ml, respectively). This study suggests a significant association between *LEPR* Q223R polymorphism and T2DM patients among Malay and Chinese ethnic groups, and it is significantly correlated with higher serum leptin, insulin, BMI, and HOMA-IR index. *LEP* G2548A polymorphism was significantly associated with T2DM among Malay and Indian ethnics and it is markedly associated with elevated serum leptin, insulin levels, and BMI in diabetic patients. Whereas, the other two SNPs of *LEPR* K109R and *LEP* A19G may not be useful markers for diabetes among Malaysian population but may have synergistic effects on diabetes.

ABSTRAK

Diabetes mellitus jenis 2 (T2DM) adalah sindrom metabolik kronik yang semakin meningkat di seluruh dunia, terutamanya di Malaysia. Leptin dan reseptornya memainkan peranan penting dalam pengawalan metabolisme glukosa dan kepekaan insulin. Variasi dalam gen *LEP* dan *LEPR* telah dikaitkan dengan ketahanan insulin, paras leptin, dan T2DM dalam pelbagai populasi, tetapi belum dilaporkan secara mendalam dalam populasi Malaysia. Kajian ini bertujuan untuk menyelidik kesan genetik polimorfisme gen *LEP* dan *LEPR* (A19G, G2548A, K109R, dan Q223R) ke atas tahap leptin serum dan rintangan insulin di kalangan pesakit T2DM Malaysia. Kajian kes-kawalan ini melibatkan 150 pesakit T2DM dan 150 sukarelawan bukan diabetes dari etnik Melayu, Cina dan India. Analisis genotip bagi polimorfisme gen *LEP* dan *LEPR* telah dilakukan dengan menggunakan Tindak Balas Berantai Polimerase - Polimorfisme Pemotongan Panjang Cebisan (PCR-RFLP), diikuti dengan penjenisan gen beberapa sampel terpilih bagi setiap SNP dengan menggunakan teknik penjujukan Sanger untuk pengesahan. Tahap leptin serum dan insulin ditentukan melalui ujian Imunojerapan Berpaut Enzim (ELISA). Model homeostasis penilaian-rintangan insulin (HOMA-IR) telah dikira. Ujian Chi-square digunakan untuk menentukan taburan genotip dan frekuensi alel dan ANOVA digunakan untuk menentukan kaitan antara parameter klinikal dan biokimia dengan setiap SNP. Hasil kajian menunjukkan Kekerapan genotip AG daripada varian *LEPR* Q223R lebih tinggi secara signifikan bagi pesakit T2DM berbanding dengan kumpulan kawalan (58.66% berbanding 42%, $p = 0.013$). Kekerapan alel A lebih tinggi secara signifikan bagi pesakit dengan T2DM daripada individu bukan diabetes (36.66% dan 29%, $P = 0.046$). Selain itu, pesakit T2DM dengan genotip GG mempunyai leptin serum, insulin, BMI, dan indeks HOMA-IR yang lebih tinggi ($P < 0.05$). Kekerapan genotip AA dan alel A varian *LEP* G2548A adalah lebih tinggi ($P < 0.05$) secara signifikan dalam pesakit T2DM berbanding dengan kumpulan kawalan. Tambahan pula, peningkatan leptin serum, paras insulin dan BMI dalam pesakit diabetes didapati berkaitan dengan genotip AA bagi varian *LEP* G2548A, berbanding genotip GG dan GA ($P < 0.05$). Walau bagaimanapun, tiada perbezaan yang signifikan secara statistik ditemui dalam frekuensi genotip dan alel bagi varian *LEPR* K109R dan *LEP* A19G antara pesakit T2DM dan sukarelawan bukan diabetes ($P > 0.05$). Tambahan pula, tiada perbezaan yang signifikan dalam parameter antropometri dan biokimia diperhatikan antara genotip polimorfisme *LEPR* K109R dan *LEP* A19G. Kombinasi haplopotip AGAG bagi empat SNPs yang dikaji mempunyai perbezaan yang signifikan (OR = 0.633, 95% CI: 0.423 – 0.947, $p = 0.025$). Secara amnya, tahap leptin serum berpuasa adalah lebih tinggi ($P < 0.001$) secara signifikan dalam pesakit T2DM berbanding subjek bukan diabetes (166.78 pg/ml dan 101.94 pg/ml). Penemuan kajian ini menunjukkan perkaitan yang signifikan antara polimorfisme *LEPR* Q223R dan pesakit T2DM dalam kalangan kumpulan etnik Melayu dan Cina dan ia berkait secara signifikan dengan leptin serum, insulin, BMI dan indeks HOMA-IR yang lebih tinggi. Polimorfisme *LEP* G2548A dikaitkan secara signifikan dengan T2DM dalam kalangan etnik Melayu dan India dan ia berkait rapat dengan peningkatan leptin serum, tahap insulin dan BMI dalam pesakit diabetes. Sementara itu, dua lagi SNP bagi *LEPR* K109R dan *LEP* A19G mungkin bukan penanda berguna untuk diabetes di kalangan penduduk Malaysia tetapi mungkin mempunyai kesan sinergistik terhadap diabetes.

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LIST OF ABBREVIATIONS

A	-	Adenine
BMI	-	Body Mass Index
bp	-	Base pair
C	-	Cytosine
CAD	-	Coronary artery disease
Chr	-	Chromosome
CI	-	Confidence interval
CRC	-	Clinical research centre
CVDs	-	Cardiovascular diseases
CRP	-	C-reactive protein
DBP	-	Diastolic blood pressure
dbSNP	-	Data base of single nucleotide polymorphism
DM	-	Diabetes mellitus
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme linked immunosorbent assay
ESRD	-	End-stage renal disease
FBS	-	Fasting blood sugar
G	-	Guanine
GDM	-	Gestational diabetes mellitus
GWAS	-	Genome wide association studies
HbA1C	-	Hemoglobin A1c
HDL-C	-	High-density lipoprotein- cholesterol
HOMA-IR	-	Homeostasis model assessment of insulin resistance
HRP	-	Avidin-horseradish peroxide
IDDM	-	Insulin-dependent diabetes mellitus
IDF	-	International diabetes federation
IR	-	Insulin resistance
LDL-C	-	Low-density lipoprotein- cholesterol
LD	-	Linkage disequilibrium
Kg	-	Kilogram

LEP	-	Leptin
LEPR	-	Leptin receptor
MAF	-	Minor allele frequency
Mets	-	Metabolic syndrome
MOH	-	Ministry of health Malaysia
MREC	-	Medical research and ethnics committee
NCBI	-	National Center for Biotechnology Information
NIDDM	-	Noninsulin dependent diabetes mellitus
OD	-	Optical density
OGTT	-	Oral glucose tolerance test
PCR	-	Polymerase chain reaction
PVD	-	Peripheral vascular diseases
RFLP	-	Restriction fragment length polymorphism
rpm	-	Revolutions per minute
SBP	-	Systolic blood pressure
SNPs	-	Single nucleotide polymorphisms
T	-	Thymine
TC	-	Total cholesterol
T1DM	-	Type 1 diabetes mellitus
T2DM	-	Type 2 diabetes mellitus
TG	-	Triglycerides
UCSC	-	University California Santa Cruz
UTR	-	Untranslated region
WHO	-	World Health Organization
WHR	-	Waist to hip ratio

LIST OF SYMBOLS

β	-	Beta
ml	-	Millilitre
µl	-	Microliter
nm	-	Nanometre
P^*	-	Average proportion exposed
r	-	Ratio of control to cases
Z_β	-	Standard normal variate for power
$z\alpha/_2$	-	Standard normal variate for level of significance

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Diabetes mellitus (DM) is one of the major healthcare challenges worldwide, increasing more and more mostly in Asian countries (Unnikrishnan et al., 2018; Akhtar et al., 2019; Narayan et al., 2021; IDF Diabetes Atlas, 2021). Asian countries account for more than 60% of the world's diabetic population as the diabetes prevalence rises in these nations (Nanditha et al., 2016). The fact that China, India, and Pakistan are the most populous nations in Asia with the highest incidence of diabetes in the world (141 million, 74.2 million, and 33 million, respectively), these three countries significantly contribute to the global prevalence of diabetes (IDF Diabetes Atlas, 2021).

DM is a chronic medical condition characterized by high blood glucose levels (hyperglycemia) and disruptions in carbohydrate, fat, and protein metabolism caused by insulin resistance, insulin deficiency, or both (Petersmann et al., 2018; Liu et al., 2020). Various organs and tissues such as kidneys, nerves, and eyes, can be damaged by diabetes if untreated for a long time, leading to diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy with the possibility of losing vision, respectively (Zheng et al., 2018). According to Chawla et al. (2019), excessive production of urine, thirst, weight loss, and blurred vision are clinical signs of diabetes mellitus.

Type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the two types of diabetes mellitus. T1DM is characterized by insulin deficiency caused by the inability of pancreatic β-cells to release insulin. Whereas, T2DM is the most common form of diabetes, is caused by peripheral insulin resistance, which is defined as the inability of target tissues like the liver, adipose tissue, and skeletal muscle to respond to insulin (Nolan & Prentki, 2019; Harreiter & Roden, 2019).

In Malaysia, the prevalence of T2DM is increasing and is considered as a major public health problem. Malaysia is a multi-ethnic and multi-cultural country with a population of over thirty million people, and it has one of the highest rates of T2DM prevalence among Asian countries (Akhtar et al., 2022). T2DM prevalence varies by ethnic groups in Malaysia, with Indians having the highest prevalence (26%), followed by Malays (18%) and Chinese having the lowest (14%) (Tee & Yap, 2017). Diabetes has increased significantly throughout the world over the last century due to the change of human's behavior and lifestyle such as dietary habits, habit of smoking, and lack of physical activity (Piccolo et al., 2016; Wang et al., 2020).

Numerous genetic and environmental factors, as well as their interactions, have been reported to implicate the development of T2DM (Abdullah et al., 2017; Prasad & Groop, 2015). Smoking, obesity, lack of exercise, and unhealthy food intake are the environmental factors that play important roles in increasing the risk of developing T2DM (Abdullah et al., 2017). The majority of diabetics suffer from obesity, which is also a major risk factor for cardiovascular disease and T2DM (Wondmkun, 2020). Although environmental factors play roles in diabetes risk, not everyone is affected in the same way. While genetic factors play an important role in diabetes development, the actual genetic variations linked with these inherited risks were not completely understood prior to the development of modern genetic techniques. The progress of human genetics studies in the 1980s finally tried to determine the genetic sites behind this genetic component (Mahrooz et al., 2015).

T2DM has been widely documented to account for the majority of diabetes cases (Petersmann et al., 2018). It affected both sexes proportionately (Shahwan et al., 2019). It is also a polygenic and heterogeneous disease that is associated with a high risk of obesity, hypertension, cardiovascular diseases (CVDs), and dyslipidemia (Henning, 2018; Shahwan et al., 2019; Hong & Choi, 2020). Diabetes risk factors are further increased by a family history of diabetes. Usually, the risk of developing T2DM for an individual with one affected parent is 40% and 70% if both parents are affected. Meanwhile, offspring of diabetic patients have approximately three times the risk of developing diabetes than those with no family history of diabetes (Mahrooz et al., 2015).

Although diabetes can be inherited, and linked with family history and genetics, the actual genetic variants are yet to be discovered. The complex interaction between multiple genes and environmental factors lead to the poor understanding of T2DM underlying mechanism (Kadayifci et al., 2019). In several populations, single nucleotide polymorphisms (SNPs) in leptin and leptin receptor gene have been found to be strongly associated with T2DM, obesity, and insulin resistance (IR) (Dasgupta et al., 2015; Meshkani et al., 2016; Bains et al., 2020).

Leptin (LEP) and leptin receptor (LEPR) have been reported to have a significant role in the control of energy metabolism, body weight, and food intake by their actions (Park & Ahima, 2015; Yang & Niu, 2018). Therefore, leptin is considered as anorexigenic hormone. Numerous studies have also reported the association between high concentrations of leptin with insulin resistance, T2DM development and diabetes complications (Moonishaa et al., 2017; Katsiki et al., 2018).

1.2 Problem statement

In recent years, T2DM has become more common in Malaysians. It was reported that about 3.9 million of the adult Malaysian population complained from diabetes and the prevalence rate has risen from 11% in 2011 to 18.3% in 2019 (Chandran et al., 2019).

The prevalence of the common polymorphisms of *LEP* A19G, *LEP* G2548A, *LEPR* Q223R, and *LEPR* K109R gene present at high frequencies in different populations (Meshkani et al., 2016; Bains et al., 2020; Aljanabi et al., 2021; Ashraf et al., 2022). Moreover, these four SNPs have been studied in various ethnic populations around the world. Findings of these studies revealed that these four SNPs have been associated with insulin resistance and T2DM (Meshkani et al., 2016; Bains et al., 2020; Aljanabi et al., 2021; Ashraf et al., 2022). In addition, *LEP* and *LEPR* gene polymorphisms have also been reported to be associated with high serum leptin levels, metabolic syndrome, and obesity (Ziablitsev et al., 2018; Hastuti et al.,2016; Şahin et al., 2013; Sabi et al., 2022; Ashraf et al., 2022; Bains et al., 2020).

On the other hand, some studies failed to find any associations between these SNPs and T2DM patients among Iranian, Chinese, Thai, Egyptian, and Taiwanese populations (Taghizadeh et al., 2017; Jiang et al., 2014; Yang et al., 2016; Motawi et al., 2015; Suriyaprom et al., 2014; Liao et al., 2012). Conflicting results might be due to different ethnic backgrounds, genetic backgrounds of the study population, different types of samples used such as peripheral blood leukocytes, buccal swab, whole blood samples, different sample sizes of the population, and geographic variation.

Importantly, among these SNPs of *LEP* Q223R, *LEPR* K109R, *LEP* G2548A, and *LEP* A19G have not yet been investigated among Malaysian diabetic subjects. Malaysia is a multi-ethnic country with the Malays as the majority, followed by Chinese and Indians (Department of Statistic Malaysia, 2016). Therefore, the present study was conducted a prospective evaluation for the genetic impacts of *LEPR* Q223R, *LEPR* K109R, *LEP* G2548A, and *LEP* A19G gene polymorphisms on the risk of T2DM among three major ethnic groups in Malaysia; Malays, Chinese, and Indians.

1.3 Scope of the study

The study focused on the T2DM patients of three major ethnic groups of Malaysia (Malays, Chinese, and Indians). This study included two sample groups: T2DM patients and non-diabetic individuals. Weight of two groups were matched and the ages of patients and control groups were more than or equal to 18 years. The genetic impacts of *LEP* A19G, *LEP* G2548A, *LEPR* Q223R, and *LEPR* K109R on serum leptin levels and insulin resistance among Malaysian T2DM patients were investigated in this study.

This study's protocol has been registered under the National Medical Research Registry (NMRR-19-1242-46808) and ethical approval was obtained from the Medical Research and Ethnics Committee (MREC), Ministry of Health Malaysia (MOH) and Clinical Research Centre (CRC), Hospital Serdang (Appendix A). Blood samples were taken from patients diagnosed with T2DM at Hospital Serdang in

Selangor. Meanwhile, blood from healthy individuals was collected as a control group. Sampling collection period was from September 2019 to December 2020. Blood samples were subjected to genotyping and determination of leptin and insulin levels. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR - RFLP) technique was used to study the genetic pattern of the leptin and leptin receptor genes polymorphisms. While, fasting serum leptin and insulin levels were measured using an enzyme linked immunosorbent assay (ELISA).

1.4 Significance of the study

T2DM is a chronic metabolic disease that affects people all over the world. In addition, it is significantly correlated with the increase macro and microvascular complications. As a consequence, the quality of life is reduced through these complications and increased mortality. *LEPR* Q223R, *LEPR* K109R, *LEP* A19G, and *LEP* G2548A are the most common SNPs for leptin and leptin receptor gene being studied in T2DM patients and its association with leptin hormone. In addition, these SNPs have not been studied among Malaysian population, thus providing more information on the linkage T2DM and its effect on leptin hormone. Therefore, the results of this study might be used as a predictive marker for T2DM. The development also might be a future scope to add novel insights that may be introduced to control blood glucose levels in T2DM. Moreover, it could be used as a sign of what is stimulating unregularly of glucose uptake, which will be beneficial for physicians who treating people with diabetes as well as increase awareness among patients with T2DM and reduce the risk of prevalence of diabetes.

1.5 Hypothesis

The present study hypothesized that there is a significant association of leptin and leptin receptor gene polymorphisms (*LEPR* Q223R, *LEPR* K109R, *LEP* A19G, and *LEP* G2548A) among three major ethnic groups in Malaysian T2DM patients.

1.6 Objectives of the study

1.6.1 General objective

The aim of this study is to investigate the genetic impacts of *LEP* and *LEPR* gene polymorphisms (*LEPR* Q223R, *LEPR* K109R, *LEP* A19G, and *LEP* G2548A) among three major ethnics in Malaysians T2DM patients and non-diabetic individuals.

1.6.2 Specific objectives

a) To establish the genotypic and allelic frequencies of these SNPs, as well as their association with T2DM in multi-ethnic Malaysian populations: Malays, Chinese, and Indians.

b) To assess the relationship between these four SNPs and anthropological parameters in Malaysians T2DM patients and controls.

c) To estimate the impact of these polymorphisms on serum leptin levels and insulin resistance among Malaysians T2DM patients.

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Appendix G List of Publications

Indexed Journal

1. **Al-fahham, L. A. A.**, Jemon, K., Latif, N. A., Bakar, S. A., & Alwi, S. S. S. (2022). The association of *LEPR* Q223R polymorphism with type 2 diabetes mellitus in Malaysia. *Human Gene*, 201044. https://doi.org/10.1016/j.humgen.2022.201044. **(Indexed by SCOPUS)**

2. **Ali, L. A.**, Jemon, K., Ab Latif, N., Bakar, S. A., & Alwi, S. S. S. (2022). *LEP* G2548A Polymorphism is Associated with Increased Serum Leptin and Insulin Resistance among T2DM Malaysian Patients. BioMedicine, 12: 3, Article 7. **(Indexed by SCOPUS)**

Non-Indexed Conference Proceedings

Al-fahham, L. A. A., Jemon, K., Latif, N. A., Bakar, S. A., & Alwi, S. S. S. (2021). The association of leptin receptor Q223R polymorphism with type 2 diabetes in Malaysian population. *The Graduate Research Symposium 2021*. Malaysia.