UNIVERSITI PUTRA MALAYSIA

ANALYSIS OF GENETIC POLYMORPHISM AS RISK FACTOR OF DIABETES NEPHROPATHY AMONG TYPE 2 DIABETIC PATIENTS OF A TERTIARY HOSPITAL

MOHD JOKHA BIN YAHYA

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By

MOHD JOKHA BIN YAHYA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

August 2017

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DEDICATION

This thesis is dedicated to my late parents with love

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ANALYSIS OF GENETIC POLYMORPHISM AS RISK FACTOR OF DIABETES NEPHROPATHY AMONG TYPE 2 DIABETIC PATIENTS OF A TERTIARY HOSPITAL

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August 2017

Chairman: Prof. Patimah Ismail, PhD
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Type two diabetes mellitus (T2DM) is one of the most common multifactorial disorders associated with significant risk of nephropathy. Nephropathy is common among the diabetic patients and the prevalence is high and increasing. Unfortunately the genetic aspect behind this was minimally studied in Malaysia. Genetic analysis of the polymorphism and susceptibility provides best apprehension on the pathogenesis elevating to innovative therapeutic approaches in patient management. The aim of this study was to determine the association of Carnosinase (CNDP1-D18S880 and rs2346061), Endothelial nitric oxide synthase (NOS3-rs1799983), Engulfment and cell mortality (ELMO1-rs74130), Manganese superoxide dismutase (MnSOD-rs4880) Methylene tetrahydrofolate reductase (MTHFR-rs1801133), Monocyte Chemoattractant protein-1 (CCL2-rs3917887), Chemokine receptor 5 (CCR5-rs1799987), Matrix Metalloproteinase-9 protein (MMP9-rs17576) and Interleukin-8 (IL8-rs4073) polymorphism to T2DM nephropathy among Malaysian. These nine genes were selected based on their function in the development of nephropathy in T2DM. IL8, CCR5, CCL2 ELMO1 are related to pro-inflammatory effect while CNDP1, NOS3, MnSOD are related to oxidative stress. MMP9 is related to the homeostasis of extracellular matrix and changes of MTHFR enzymatic activity is considered as contributing to development of T2DM nephropathy. More than one thousand diabetic patients were examined and a total of 652 T2DM patients were tested comprising 227 Malays (non-nephrotic=96 and nephrotic=131), 203 Chinese (non-nephrotic=95 and nephrotic=108) and 222 Indians (non-nephrotic=136and nephrotic=86). For the D18S880 of CNDP1, sequencing the gene was the most appropriate approach while the rest are tested by Mass ARRAY to identify the polymorphisms. The alleles and genotypes were tested using 4 genetic models and the best mode of inheritance was chosen. It was found that 7 of the 10 polymorphisms tested were significantly associated with nephropathy in T2DM in this study. The rs2346061 of CNDP1 was significantly associated among the Indians only with OR=1.94 (1.76-3.20) CI=95% fitted best the multiplicative model and D18S880, another polymorphism of CNDP1 was associated among all the three major races with

the Malays having the highest risk with OR=2.46 (1.48-4.10) CI=95%, Chinese OR=2.26 (1.34-3.83) CI=95% and Indians OR=1.77 (1.18-2.65) CI=95%. Meanwhile, the remaining 5 polymorphisms suit best with the additive mode of heritance. MnSOD, rs4880 among the Chinese subjects had OR=2.8 (0.53-14.94) 95% CI, Indians OR=2.4 (0.69-2.84) 95% CI and Malays OR=2.16 (0.54-8.65) 95% CI. Looking at NOS3 rs1799983, the Indians OR=3.16 (0.52-17.56) 95% CI followed by Chinese OR=3.55 (0.36-35.03) and the Malays OR=2.89 (0.29-28.32) 95% CI. Meanwhile in pro-inflammatory gene, CCR5 rs1799987, only the Chinese showed association for this polymorphism, with OR=6.71 (2.55-17.68) 95% CI. The same for IL8 rs4073, only the Indians showed association with nephropathy with OR=1.57 (0.66-3.71) 95% CI. The MMP9 rs17576 variant was found to be significantly associated among the Indians OR=3.91 (1.69-9.03) 95% CI and the Chinese OR=4.38 (1.81-10.59) 95% CI but no association in Malays. In conclusion, this study shows the significant potential of six polymorphisms on the development of T2DM nephropathy. These genes may be considered as risk factors for Malaysian subjects who are predisposed to T2DM nephropathy but differs for different races. Further studies which involve environmental risk factor should be taken into consideration. An individual without any of the polymorphisms may still develop nephropathy due to the, environmental factors such as unhealthy life style.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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ANALISA POLIMORFISME GENETIK SEBAGAI FAKTOR RISIKO NEFROPATI DIABETIS DALAM KALANGAN PENGHIDAP DIABETIS JENIS 2 DI HOSPITAL TERTIARY

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Diabetis mellitus jenis dua (T2DM) merupakan salah satu faktor yang menyebabkan penyakit buah pinggang atau nefropati. Nefropati merupakan gejala yang biasa terdapat dalam kalangan pesakit T2DM dengan kekerapan yang sangat tinggi dan semakin meningkat. Malangnya aspek genetik berkenaan penyakit ini sangat kurang dikaji iaitu pada tahap minimal di Malaysia. Analisa genetik terhadap sesuatu polimorfisme dan gejalanya meningkatkan lagi kefahaman secara lebih mendalam tentang patogenesis dan pendekatan terapeutik yang lebih inovatif dalam pengurusan pesakit. Tujuan ujian ini dilakukan adalah untuk mengenalpasti perkaitan porlimorfisme pada *carnosinase* (CNDP1-D18S880 dan rs2346061), *Endothelial nitric oxide synthase* (NOS3-rs1799983), *Engulfment and cell mortality* (ELMO1-rs74130), *Manganese superoxide dismutase* (MnSOD-rs4880), *Methylene tetrahydrofolate reductase* (MTHFR-rs1801133), *Monocyte Chemoattractant protein-1* (MCP1 or CCL2-rs3917887), *Chemokine receptor 5* (CCR5-rs1799987), *Matrix Metalloproteinase-9 protein* (MMP9-rs17576) dan *Interleukin-8* (IL8-rs4073) dengan penyakit nefropati dalam kalangan pesakit T2DM di Malaysia. Kesemua sembilan gen ini telah dipilih berdasarkan fungsi mereka terhadap pembentukan nefropati pada pesakit T2DM; seperti IL8, CCR5, CCL2, ELMO1 yang berkaitan dengan pro-inflamasi manakala CNDP1, NOS3, MnSOD yang berkaitan dengan tekanan oksidatif. MMP9 terkait dengan homeostasis degradasi matriks luar sel manakala kesan perubahan aktiviti enzim MTHFR boleh menyebabkan pembentukan nefropati. Sejumlah 652 pesakit T2DM telah diuji yang terdiri daripada 227 orang berbangsa Melayu (tanpa nefropati=96, dengan nefropati=131), 203 orang berbangsa Cina (tanpa nefropati=95 dengan nefropati=108) dan 222 orang berbangsa India (tanpa nefropati=136, dengan nefropati=86) untuk mengesan hubungan polimorfisme dengan T2DM nefropati. Untuk D18S880 dari gen CNDP1, penjujukan gene merupakan kaedah yang terbaik untuk tujuan kajian ini manakala untuk polimorfisme yang selebihnya kaedah yang digunakan ialah Mass ARRAY. Alel dan genotip ini telah dianalisa mengguna empat model genetik dan model yang terbaik merujuk cara perwarisan telah dipilih. Daripada sepuluh polismorfisme yang telah diuji tujuh menunjukkan perkaitan ($p<0.05$) dengan

T2DM nefropati iaitu rs2346061 pada CNDP1 dengan nilai OR=1.94 (1.76-3.20) CI 95% hanya dalam kalangan bangsa India dengan model yang paling sesuai iaitu *multiplicative* dan D18S880 mempunyai kaitan dengan ke tiga-tiga bangsa iaitu dengan bangsa Melayu mempunyai risiko yang tertinggi dengan nilai OR=2.46 (1.48-4.10) 95% CI untuk menghidap nefropati diikuti bangsa Cina dengan nilai OR=2.26 (1.34-3.83) 95% CI dan dalam kalangan bangsa India pula nilai OR=1.77 (1.18-2.65). Walau bagaimanapun, didapti baki ke lima-lima polimorfisme mempunyai kaedah perwarisan secara model *additive*. Untuk MnSOD rs4880, bangsa Cina mempunyai nilai OR= 2.80 (0.53-14.94) 95% CI, India OR=2.40 (0.69-2.84) 95% CI and Melayu OR=2.16 (0.54-8.65) 95% CI. Melihat pada NOS3 rs1799983, bangsa India menunjukkan perkaitan yang terkuat terhadap pembentukan nefropati dengan nilai OR=3.16 (0.52-17.56) 95% CI diikuti oleh Cina OR=3.55 (0.36-35.03) dan akhir sekali Melayu OR=2.89 (0.29-28.32) 95% CI. Manakala gen pro-inflamasi pula, untuk CCR5 iaitu rs1799987, hanya bangsa Cina menunjukkan perkaitan dengan polimorfisme ini iaitu dengan nilai OR= 6.71 (1.08-2.999) 95% CI. Perkara yang sama untuk IL8 rs4073, dimana hanya bangsa India menunjukkan perkaitan dengan nefropati dimana nilai OR=1.57 (0.66-3.71) 95% CI. Didapati varian pada MMP9 rs17576 mempunyai perkaitan yang sigfnifikan dalam kalangan bangsa India dengan OR=3.981 (1.69-9.03)95% CI dan bangsa Cina dengan nilai OR=4.38 (1.81-10.59) 95% CI terhadap nefropati dan tiada perkaitan dalam kalangan bangsa Melayu. Di sini dapatlah disimpulkan bahawa terdapat enam polimorfisme yang dikesan mempunyai potensi yang signifikan untuk menjadi risiko penyebab terjadinya nefropati pada pesakit T2DM. Oleh yang demikian, ke enam-enam gen tadi bolehlah dianggap sebagai risiko untuk menghidap nefropati bergantung pada bangsa tertentu. Walau bagaimanapun kajian lanjut yang ingin dilakukan mestilah mengambil kira faktor-faktor risiko persekitaran. Walaupun individu yang tidak mempunyai risiko polymorfisme, faktor luaran seperti gaya hidup yang tidak sihat boleh menyumbang kepada pembentukan nefropati dalam kalangan pesakit diabetis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AER	Albumin excretion rate
AGE	Advance glycation end product
Ang II	Angiotensin II
AP-1	Activator protein-1
AT-1 receptor	Ang II receptor I
AT-2 receptor	Ang II receptor II
C-ATT	Cochran-Armitage's trend test
CCL2	Chemokine Ligand 2
CCR5	Chemokine receptor 5
CI	Confidence interval
CNDP1	Carnosinase gene 1
CTGF	Connective tissue growth factor
DAG	Diacylglycerol
DN	Diabetic nephropathy
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
ELMO1	Engulfment and cell mortality
eNOS	Endothelium nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ESRD	End stage renal disease
ET1	Endothelin 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDPH	Glycerol-3-phosphate dehydrogenase
GFAT	Glutamine fructose-6-phosphate amidotransferase
GFR	Glomerular filtration rate
GLUT1	Glucose transporter 1
HHcy	Hyperhormocsteinaemia
HTJS	Hospital Tuanku Ja'afar Seremban
HWE	Hardy-Weinberg Equilibrium
ICAM1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
IL6	Interlukin-6
IL8	Interleukin-8
iNOS	inducible NOS
MAPK	Mitogen-activated protein Kinase
MCP1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase 9
MnSOD	Manganese superoxide dismutase
MTHFR	Methylene tetrahydrofolate reductase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear transcription factors kappa B
nNOS	neuronal NOS
NO	Nitric oxide
NOS	NO synthase

NOS3	Endothelial nitric oxide synthase
O-GlcNAc	O-linked N-acetylglucosamine
OR	Odd ratio
p38 MAPK	p38 mitogen activated protein kinase
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFB	Platelet-derived growth factor subunit B
PKC	Protein kinase C
PPA-1	Plasminogen activator inhibitor-1
QC	Quality control
RAGE	AGE receptor
RANTES	Regulated on activation normal T cell expressed
ROS	Reactive oxygen species
RPM	Rotation per minute
SAP	Shrimp alkaline phosphatase
SNP	Single nucleotide polymorphism
SOD2	Superoxide dismutase 2
T2DM	Type 2 diabetes mellitus
TGF- β1	Transforming growth factor-β1
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of MMP
TNF-α	Transforming nuclear factor alpha
tPA	Tissue plasminogen activator
UCP-1	Uncoupling protein-1
UDP-N-acetylglucosamine	Uridine diphosphate-N-acetyl glucosamine
UPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth fact

CHAPTER 1

INTRODUCTION

1.1 Study Background

The dramatic prevalence leap for diabetes in Malaysia, from 11.6% in 2006 (Letchuman, 2010) to 22.9% in 2011 (Wan Nazaimon *et al.*, 2013), has alarmingly exceeded the postulated level (13.4%) for the population for 2025; by the World Health Organisation diagnostic criteria (Shaw *et al.*, 2010). On top of that, 91% of diabetic patients are diagnosed or have been diagnosed as diabetic nephropathy (DN) and 50% of dialysis patients are the result of type 2 diabetes complication (Salwa *et al.*, 2010; Mafauzy *et al.*, 2011). The rise of the T2DM is due to the increase of overweight and obesity as the result of unhealthy lifestyle (Zanariah *et* al., 2015).Various strategy have been established by the Malaysian government to overcome the increasing number of diabetes by focusing particularly in healthy lifestyle promotions and screening for diabetes and complications in the population (Zanariah *et al.*, 2015). DN is a syndrome where urine albumin is excreted >300 µg/24 hour or >20 µg/min with urine protein creatinine ratio > 30 µg/mg or loss of glomerular filtration rate (<60mL/min/1.73 m^2) with albuminuria as a micro predominant effect or complication of diabetes that will eventually advance to complete renal failure when patients are not properly managed; especially at the early stages of diabetes. Unfortunately, detecting the early symptoms of nephropathy is almost impossible in diabetic patients particularly when the onset of the diabetes is unknown. Thus, this may result in poor patient management and eventually cause rapid kidney deterioration. Usually, most patients are unaware of being diabetic and nephropathy has already manifested when they are diagnosed. The exact aetiology or biochemical pathology of type 2 diabetes mellitus (T2DM) nephropathy is impossible to be determined immediately with the simple existing tests.

Only about 10% of T2DM patients in Malaysia seem to be protected from nephropathy (Salwa, *et al.*, 2010; Mafauzy *et al.*, 2011). A number of studies in different populations have proven that genetic polymorphisms seem to link very well in increasing the risk for the development and progression in T2DM nephropathy (Krolewski *et al.*, 1987; Quinn *et al.*, 1996; Brownlee, 2005). The progression of nephropathy differs in individuals in T2DM patients. This may be due to the 'protective' factors that individuals may or may not have which can be observed through altered biochemical reactions.

To link polymorphisms and nephropathy, it is best to understand how nephropathy progresses in T2DM patients. Nephropathy in T2DM progresses in five stages. It begins with the silence stage that manifests as normal albuminuria, micro albuminuria, macro albuminuria and end stage renal disease (ESRD). To screen for the first two stages is almost impossible in any small laboratories in Malaysia. The routine practice screening for renal failure is by urinalysis, also known as 'dipsticks' to detect the presence of protein in the urine. Unfortunately, the results will only be distinctively detected positive at stage three or four. The best or the gold standard procedure is to

measure the glomerular filtration rate (GFR) that is determining the iohexal clearance which is an invasive test and may cause anxiety to patients. A simpler biomarker used to classify the stages of nephropathy is by calculating the albumin-creatinine-ratio (ACR) collected at the first urine void in the morning. Another method is by measurement of the albumin in a timed urine collection (24 hours). These biomarkers are cheap and easy to use with good predictor of progressive DN. However, the sensitivity and specificity may be influenced by variation such as hydration, diet and urinary tract infection. Another simple and cheap approach of classifying the DN stages is by estimating the glomerular filtration rate (eGFR) using the modification of diet in renal disease (MDRD) equation. This would require results of blood creatinine level, age, gender and race. Creatinine levels are influenced with muscles mass; men are more masculine then women and older people have decreased muscle mass. Furthermore creatinine is also being removed by the proximal tubular excretion which might not exactly reflect the true GFR. Having all these setbacks; an accurate, precise and non invasive biomarker will be very helpful to pre diagnose nephropathy among high risk T2DM patients. Polymorphisms or variants of related genes are thought to be the next best biomarkers.

The ability to relate the polymorphisms and pathogenesis will personalise T2DM nephropathy patient management. To be able to detect the exact polymorphism which accelerates or causes nephropathy that are usually involve in modification of the enzymes activities or functions, reconfigure receptors function and other biochemical reactions would enable clinicians to understand the aetiology for accurate patient management. Thus, knowing the exact aetiology of the disease may help to manage patients economically, efficiently and eventually might develop novel therapeutic approaches for DN management. The polymorphisms will be the biomarkers with the ability to predict the presence of disease, prognosis of the disorder and progression of the condition (Jha *et al*., 2014).

In order to demonstrate the probability and association of the polymorphism or alleles with nephropathy in T2DM, a proper study needs to be designed. In this study, a case control model is adapted to link the association. The control group is the T2DM patients without nephropathy and the case group is the T2DM patients with nephropathy. The population studied was among the biologically unrelated T2DM patients randomly selected. They were classified in two groups by the inclusion and exclusion criteria. This study involved the three main ethnic groups in Malaysia: Malay, Chinese and Indian. In this study, the polymorphisms to be detected were single nucleotide polymorphism (SNP), repetition of sequence (microsatellite) and insertion-deletion (indel). These were tested on nine candidate genes which carry the risks to develop nephropathy in diabetes. The analytical methods used to detect the polymorphisms were by gene sequencing and Mass Array Polymorphism detection. The genomic deoxyribonucleic acid (DNA) was extracted from whole blood using commercial kit and the gene of interest was then amplified by polymerase chain reaction (PCR). All data were statistically analysed to test for the association between the polymorphism and the disease using four standard models.

1.2 Problem Statement

Approximately 91% of T2DM patients are having or have been diagnosed for nephropathy in Malaysia (Salwa *et al.*, 2010) with 57% of patients with end stage renal disease (ESRD) is caused by diabetes (Lim *et al.* 2008 and Shaza *et al.*2005). Even though the prognosis to develop T2DM nephropathy is mainly base on insufficient glycaemic control but some do not develop nephropathy despite being long time diabetics (Rossing and Krolewski, 1985). It indicates that there is no sensitive and specific biomarker to prognosticate and calculate the risk of an individual to develop diabetic nephropathy and to evaluate pharmacologic responses to assist successful therapeutic interventions at the early stage of nephropathy. The pre-determined variants may provide useful information whether an individual is at higher risk of manifesting nephropathy once diabetes is developed. The variants causing oxidative stress, kidney inflammation, deteriorates ECM homeostasis and endothelial dysfunction degrade the kidney. Enable to determine the responsible variant/s in an individual the aetiology of the nephropathy can be addressed immediately when the current biomarkers could not provide sufficient prognostic information. Diabetes causes renal to fail sooner and progresses more rapidly compare to other diseases. Therefore correct and suitable early intervention can prolong the manifestation of nephropathy. The variants will also provide information regarding the correct prescription of drugs for effective treatment or a therapeutic diet plan could be designed to avoid nephropathy. Patients suffering from diabetes tend to suffer greater or worse than those without diabetes after renal transplant or dialysis. When number of ESRD patients is decreased especially with type 2 diabetes, the financial expenses are also reduced as T2DM ESRD undergoing maintenance dialysis consume significantly more financial resources than those with non-diabetic ESRD. However, the aetiology of diabetic nephropathy (DN) is multifactorial and involves both environment and genetic factors.

1.3 Significance of Study

In this research, nine strongly associated candidate genes in other research; namely Carnosinase dipeptidase 1 (CNDP1), Endothelial nitric oxide synthase (NOS3), Engulfment and cell mortality (ELMO1), Manganese superoxide dismutase (MnSOD), Methylene tetrahydrofolate reductase (MTHFR), Monocyte Chemoattractant protein 1 (MCP1), Chemokine receptor 5 (CCR5), Matrix Metalloproteinase 9 protein (MMP-9) and Interleukin-8 (IL8) are studied to determine the genetic aetiology of T2DM nephropathy. The polymorphisms affect the function of the proteins coded causing oxidative stress, inflammation, cells maintenance and extracellular cell maintenance. Eventually disturb the architecture and functions of the nephrons. The outcomes of the research may enhance the treatment of diabetic nephropathy by determining the susceptibility and risk of diabetic patients. The variants chosen have the potential to be good biomarkers for diagnosis or prognostic tests for high risk patients.

1.4 Hypothesis

Oxidative related, pro inflammatory, constructive-destructive of ECM and protein and amino acid genes are responsible for nephropathy in T2DM.

1.5 General Objectives

To determine the association between genetic polymorphism in Pro-inflammatory genes, oxidative stress related genes and protein and amino acid genes and the effect of these genes on T2DM developing nephropathy among Malaysian subjects.

1.6 Specific Objectives

1. To determine the allele frequencies and the genotypes of the genetic polymorphisms of the 5CTG repeats or D18S880 , SNP rs2346061 (A>T) of CNDP1, SNP rs1799983 (G>T) of NOS3,SNP rs741301 (G>T) of ELMO1, SNP rs4880 (C>T) of MnSOD , SNP rs1801133 (C>T) of MTHFR, INS/DEL rs3917887 of CCL2, SNP rs1799987 (G>A) of CCR5, SNP rs17576 (G>A) of MMP-9 , SNP rs4073 (T>A) of IL8, in both case and control

2. To determine the association between 5CTG repeats or D18S880, SNP rs2346061 (A>T) of CNDP1 , rs1799983 (G>T) of NOS3, rs741301 (G>T) of ELMO1, SNP rs4880 (C>T) of MnSOD, SNP rs1801133 (C>T) of MTHF, INS/DEL rs3917887 of CCL2, SNP rs1799987 (G>A) of CCR5, SNP rs17576 (G>A) of MMP-9 , SNP rs4073 (T>A) of IL8 and genotype, phenotype correlation among Malaysian T2DM nephrotic subjects.

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LIST OF PUBLICATIONS

Mohd Jokha Yahya, Patimah binti Ismail, Norshariza binti Nordin, Abdah binti Md Akim, Wan Shaariah binti Md. Yusuf, Noor Lita binti Adam, Maryam Jamielah Yusoff. Association of CCL2, CCR5, ELMO1 and IL8 Polymorphism with Diabetic Nephropathy in Malaysian Type 2 Diabetic Patients. *International Journal of Chronic Diseases* 2019 Vol 2019; 13 pages

Mohd Jokha Yahya, Patimah binti Ismail, Norshariza binti Nordin, Abdah binti Md Akim, Wan Shaariah binti Md. Yusuf, Noor Lita binti Adam, Nurul Fasihah Zulkifli. CNDP1, NOS3 and MnSOD Polymorphisms as Risk Factors for Diabetic Nephropathy among Type 2 Diabetic Patients in Malaysia. *Journal of Nutrition and Metabolism* 2019 Vol 2019; 13 pages.

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