Analysis of Metallothionein Gene MT1A (rs11076161) and MT2A (rs10636) Polymorphisms as a Molecular Marker in Type 2 Diabetes Mellitus among Malay Population

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I.INTRODUCTION

Abstract-Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder that characterized by the presence of high glucose in blood that cause from insulin resistance and insufficiency due to deterioration β-cell Langerhans functions. T2DM is commonly caused by the combination of inherited genetic variations as well as our own lifestyle. Metallothionein (MT) is a known cysteine-rich protein responsible in helping zinc homeostasis which is important in insulin signaling and secretion as well as protection our body from reactive oxygen species (ROS). MT scavenged ROS and free radicals in our body happen to be one of the reasons of T2DM and its complications. The objective of this study was to investigate the association of MT1A and MT2A polymorphisms between T2DM and control subjects among Malay populations. This study involved 150 T2DM and 120 Healthy individuals of Malay ethnic with mixed genders. The genomic DNA was extracted from buccal cells and amplified for MT1A and MT2A loci; the 347bp and 238bp banding patterns were respectively produced by mean of the Polymerase Chain Reaction (PCR). The PCR products were digested with Mlucl and Tsp451 restriction enzymes respectively and producing fragments lengths of (158/189/347bp) and (103/135/238bp) respectively. The ANOVA test was conducted and it shown that there was a significant difference between diabetic and control subjects for age, BMI, WHR, SBP, FPG, HBA1C, LDL, TG, TC and family history with (P<0.05). While the HDL, CVD risk ratio and DBP does not show any significant difference with (P>0.05). The genotype frequency for AA, AG and GG of MT1A polymorphisms was 72.7%, 22.7% and 4.7% in cases and 15%, 55% and 30% in control respectively. As for MT2A, genotype frequency of GG, GC and CC was 42.7%, 27.3% and 30% in case and 5%, 40% and 55% for control respectively. Both polymorphisms show significant difference between two investigated groups with (P=0.000). The Post hoc test was conducted and shows a significant difference between the genotypes within each polymorphism (P=0. 000). The MT1A and MT2A polymorphisms were believed to be the reliable molecular markers to distinguish the T2DM subjects from healthy individuals in Malay populations.

Keywords—Type 2 Diabetes Mellitus (T2DM), Metallothionein (MT), MT1A (rs11076161), MT2A (rs10636), Malay, Genetic Polymorphism.

Type 2 Diabetes is believed to be the most overwhelming disease in the 21st century (Wild et al., 2004) which generally, becoming a disorder of elevated blood glucose levels and known as a multi-factorial disease (Ganasyam et al., 2012; Tilburg et al., 2001). T2DM arose from the combination of genetic and environmental factors and it is afraid to be irresistible in the current century as the number is rapidly increasing. Up to know, the major reason that leads to T2DM is remain unknown (Arora and Chertow, 2010). However, a range from predominantly insulin secretion due to insulin deficiency is believed to act as the etiology to T2DM (Goldstein, 2003). On the other hand, genomic studies had shown that T2DM may occur from a susceptible gene in a chromosomal region as well (Ganasyam et al., 2012). A study from Doria et al., Stated that suggested susceptibility of certain genes towards T2DM after undergoing a genomic screening was MT1 gene (Ganasyam et al., 2012).

Metallothionein is a protein with low molecular mass and high in cysteine content which presented in many cell types (Skutkova et al., 2012; Cai, 2004 and Chen et al., 2001). MT genes were located on the chromosome 16q13 (Cipriano et al., 2006; Yang et al., 2008; Skutkova et al., 2012 and Chen et al., 2012) It is responsible in metabolic function, detoxification of heavy metals, scavenging of free radicals and metal ion homeostasis (Sato and Kondoh, 2002; Skutkova et al., 2012). Thus, its regulation may give a significant effect on diabetic and its complication. Also, the generation of oxidative stress is believed to be the major route of T2DM too (Yang et al., 2008), this hypothesis was support by the study which was conducted by Chen and coworkers who has proved that MT reduces the level of hyperglycemia as well as β -cell degranulation and necrosis in STZ-induced mice (Chen et al., 2001), the MT have the ability to protect the DNA which is significantly reduced when it is over expressed; subsequently, the DNA cleavage and damaging happening.

Currently, World Health Organization (WHO) had reported that T2DM had affected more than 170 million people globally and it is estimating the numbers will rise up to 365 million in the year 2030 (Wild *et al.*, 2004). This alarming rise is believed due to many factors such as population growth, urbanization, age, obesity, family history, hypertension as well as ethnic background (Letchuman *et al.*, 2010; Wild *et al.*, 2010;

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2004 and Van-Tilburg *et al.*, 2001). Based on the survey done by the Malaysian National Health and Morbidity Survey III, an increasing trend of T2DM observed from 1986 to 2006 (8% to 11.6%) respectively among the Malaysian adults (\geq 35 years) (Letchuman *et al.*, 2010). In the United State of America, diabetes is the 6th leading cause of death with more than 1 million victims annually (Riaz, 2010) and despite the numerous available treatments, the trend keeps on increasing.

Prior to our knowledge, no study had been done regarding this gene and its selected loci towards Malaysian T2DM subjects. Hence, it is believed to identifying susceptible genes towards the respective population will come out with a preventive strategies, early diagnosis and target treatment in early future. The main concern in this study was to investigate the correlation of MT1B (rs11076161) and MT2A (rs10636) gene polymorphism, as a genetically risk predictor for T2DM in the Malay population.

II. METHODOLOGY

Ethical approval consents were obtained from National Heart Institute (IJN) Kuala Lumpur [IJNEC/05/10 (02)]. Subjects were being chosen based on their medical records and considering the International Diabetes Federation (IDF) inclusion criteria and those who have cancer, genetic malformations, type 1 diabetes and suspicious to pregnancy were excluded from the study.

A. Study Subjects

A total of 150 case samples that known to have diabetes based on IDF criteria were recruited in the IJN and compared with 120 control samples than known to be healthy and unrelated to T2DM. The questionnaire and consent form were collected from each and every individuals and proceed for their anthropometric measurement and proper specimen collection. For the blood biochemical patterns we refer to the latest medical history of the patients and imported the raw data for further evaluations.

B. Genomic DNA Extraction

Buccal cell samples were collected by the sterile cytobrush (Qiagen Inc., Chatsworth, CA, USA) and the genomic DNA extraction was carried out using the DNA isolation kit (Qiagen Inc., Chatsworth, CA, USA). The purity of extracting DNA was quantified using Quawell Biophotometer. All the genomic analysis was carried out in the Molecular Biology Laboratory of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

C. Genotyping MT1A and MT2A Gene Polymorphisms

In order to determine the genotypes of MT1A and MT2A, amplification was carried out by using the specific primers correspond to the loci by mean of PCR techniques. A total volume of a 25 µL reaction mixture consisting of 10 pmol/µl of each primer, 6 µl of 2X PCR buffer (G-2000, Genet Bio Korea), (50 ng/µl) template DNA and dH₂O was used for amplification. A negative control containing no genomic DNA and a positive control of known genotype was always included in the set of reactions to confirm the accuracy of experiment. All the PCR cycling conditions were carried out by BioRad Thermal cycler. The amplified PCR products for both polymorphisms were separated by 2% to 4% agarose gel (Bioline, London, UK) which was stained with Ultra PowerTM loading dye (BioTek Corporation China) and visualized under UV light (Alpha Innotech, USA). The PCR products of the respective genes were digested with 1-2 units of the respective restriction enzymes (Thermo Fisher Scientific). Table I demonstrated the sequence of the primers used in this study and their specific enzymes.

IABLE I The Primers Specifications. Cycling Condition Foliowed by Proper Restriction Enzymes and Expected Product Size							
Gene Polymorphisms	Forward primer Reverse primer	Restriction Endonuclease Enzymes	PCR Products (bp)	Restriction Fragment Size (bp)			
MT1A (rs111076161)	TTCGGGATTAAGGACATAAAGC GAAATGGATCATTGGCCTACTC	MluCl	347	158/189/347			
MT1B (rs10636)	TCGGACAAGTGCAGCTGCTG CCCTCCCAGTTCAATCCCTC	Tsp451	238	103/135/238			
(Yang et al., 2008)							

D. Statistical Analysis

The observed genotype patterns and biochemical/anthropometric values of the subjects were being analyzed by using SPSS software (Ver. 17). The percentage of wild type alleles, heterozygous and mutant alleles were calculated and subsequently been compared between both control and case samples by using the Chi square test and the values (P<0.05) was considered as statistically significant.

III. RESULTS

A. Demographic Distribution of Subjects

In this study, the T2DM subjects were consist of 60% male and 40% female while in control was 20% and 80% respectively. The age distribution ranged from 20 to 50 years old with a mean of 39 ± 9.4 for control subjects. On the other hand, for T2DM subjects ranged from 30 to 70 years old with a mean of 58 ± 8.0 . The basic characteristic of the subjects were presented in Table II which was significantly different between diabetes and control group in age, Body Mass Index (BMI), Waist Hip Ratio (WHR), Systolic Blood Pressure (SBP), Fasting Plasma Glucose (FPG), H_bA_{1C}, Low Density Lipoprotein (LDL), Triglyceride (TG), total Cholesterol (T-Chol), Cardiovascular Risk factor (CVD%) and family history (P<0.05).

In this study, three banding pattern detected for the MT1A gene, 347 (bp) which represent the Wild Type, 189 and 157 (bp) presence of the Homozygous and the three bands (347,

189 and 157 bp) as the Heterozygous pattern (Fig. 1). The genotype and allele distribution among T2DM and healthy individuals was considered in this study with the effect of MT1A gene (Table III) which was significant among the Malaysian subjects (P= 0.000).

THE CLINICAL CHARACTERISTICS OF T2DM AND CONTROL SUBJECTS							
	T2DM			Control			
	Male	Female	Total	Male	Female	Total	P-Value
Age (years)	59 ± 8.08	58 ± 7.9	58 ± 8.03	38 ± 10.8	39 ± 9.16	$3 \hspace{0.1in} 9 \pm 9.48$	0.000**
BMI (Kg/m ²)	27.24±4.63	7.87±5.24	27.49±4.9	24.89±6.3	2 25.07±6.74	2 25.04±6.67	0.007**
WHR (cm)	0.94±0.05	0.95±0.04	0.94±0.04	0.85±0.02	0.86±0.02	0 0.86±0.02	0.000**
SBP (mm Hg)	140±21.72	136±22.58	138±22.07	122±12.36	128±19.21	127±18.76	0.000**
DBP (mm Hg)	78±8.88	77±9.95	77±9.3	79±10.67	77±12.02	77±11.75	0.779
FPG (mmol/L)	8.09±3.27	8.61±3.14	8.3±3.21	4.06±0.63	4.14±0.67	4.13±0.66	0.000**
H_bA1_C	8.08 ± 1.81	8±2.04	8.05±1.9	5.88 ± 0.38	5.99±0.38	5.97±0.38	0.000**
LDL (mmol/L)	2.45 ± 0.79	2.51±0.81	2.47±0.79	3.15±0.98	3.29±1	3.28±1	0.000**
HDL (mmol/L)	1.33±0.35	1.25±0.38	1.3±0.37	1.3±0.28	1.37±0.34	1.35±0.33	0.220
TG (mmol/L)	1.73±1.77	2.15±2.03	1.9 ± 1.88	1.19±0.67	1.2±0.64	1.2±0.64	0.001**
TChol (mmol/L)	4.56±0.95	4.38±1.04	4.49±0.99	5.23±0.98	5.28±1	5.27±0.99	0.000**
CVD Risk %	4.05±1.44	4.04±1.67	4.04±1.52	4.31±1.68	3.93±1.2	4.01±1.32	0.718
Family History							
No	54 (58.7%)	38 (41.1%)	92(82.7%)	4(21.1%)	15(78.9%)	19(71.1%)	0.006**†
Yes	36 (62.1%)	22(37%)	58(36.5%)	20(19.8%)	81(80.2%)	101(63.5%)	

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**P value indicate that level significance between them P<0.05

*Fisher Exact test



Fig. 1 The PCR product of MT1A polymorphism after digestion with *MluCl* enzyme. Lane 1 and 4 shows wild type producing 1 band, lane 2, 5 and 6 were heterozygous with 3 bands and lane 3 shows homozygous 2 bands. Lane 7 is the PCR product and lane 8 is the negative control followed by DNA Marker (700 to 25 bp)

Gene	Genotypes and Alleles	Case (%)	Control (%)	
MT1A genotypes	Wild type	109 (72.7)	18 (15)	
	Heterozygous	34 (22.7)	66 (55)	
	Homozygous	7 (4.7)	36 (30)	
P-value	0.000**			
Alleles	Α	0.84	0.43	
	G	0.16	0.57	
<i>P</i> -value	0.000**			

TABLE III

Also, the impact of MT1A and MT2A genes were evaluated on biochemical patterns among the T2DM and control subjects which was not significantly different for Diastolic Blood Pressure (DBP), High Density Lipoprotein (HDL), and CVD% risk (Table IV and Table V) respectively.

***P*- value was calculated by χ^2 test with 2 x 2 contingency table and

considered P<0.05 as significance

TABLE IV The Clinical Characteristics of T2DM and Control Subjects with the Impact of MT1A Polymorphism

		T2DM			Control		
	Wild (AA)	Hetero (AG)	Homo (GG)	Wild (AA)	Hetero (AG)	Homo (GG)	P-Value
Age (years)	59.21±7.78	57.16±8.77	57.69±8.23	41.02±12.1	39.29±8.78	39.1±9.48	0.000**
BMI (Kg/m ²)	27.78±4.83	26.36±5.12	28.49±4.9	25.66±6.81	24.55±6.01	25.62±7.79	0.012**
WHR (cm)	0.94±0.04	0.95 ± 0.04	0.92 ± 0.03	0.86 ± 0.02	0.86 ± 0.02	0.86 ± 0.02	0.000**
SBP (mm Hg)	138±22.57	137±18.97	150±27.9	126±19.6	125±19.87	130±15.96	0.000**
DBP (mmHg)	78±9.23	77±8.69	76±14.06	74±13.92	79±10.95	76±11.91	0.623
FPG(mmol/L)	7.94±3.17	9.22±3.38	9.48 ± 2.08	4.26±0.67	4.02±0.7	4.26 ± 0.54	0.000**
H_bA1_C	7.9±1.92	8.42±1.89	8.51±1.28	5.85 ± 0.47	5.97±0.35	6.02±0.37	0.000**
LDL (mmol/L)	2.48 ± 0.84	2.48 ± 0.68	2.25±0.53	3.19±0.8	3.31±1.03	3.21±1.04	0.000**
HDL(mmol/L)	1.28±0.35	1.31±0.36	1.54±0.55	1.39±0.39	1.34±0.32	1.36±0.33	0.355
TG (mmol/L)	1.96±1.89	1.8 ± 1.92	1.32 ± 1.64	1.09 ± 0.69	1.23±0.67	1.19 ± 0.57	0.007**
TC (mmol/L)	4.44±1.02	4.63±0.9	4.6±0.89	5.14±0.83	5.3±0.99	5.29±1.07	0.000**
CVD Risk %	4.02±1.61	4.17±1.31	3.82±1.14	4.25±1.29	3.97±1.42	3.95±1.12	0.952
Family history							
No	60.6%	67.6%	42.9%	11.1%	21.2%	8.3%	0.000**†
Yes	39.4%	32.4%	57.1%	88.9%	78.8%	91.7%	

**P value indicate that level significance between them P < 0.05

†Fisher Exact test

TABLE V

	T2DM			Control			
_	Wild (AA)	Hetero (AG)	Homo (GG)	Wild (AA)	Hetero (AG)	Homo (GG)	P-Value
Age (years)	59.5±8.53	58.2±7.12	57.8±8.12	43±10.17	38.9±9.73	38.8±9.31	0.000**
BMI (Kg/m ²)	27.55±4.89	26.43±4.96	28.37±4.77	29.38±10	24±6.97	25.39±5.98	0.002**
WHR (cm)	0.93±0.04	0.95 ± 0.04	0.94±0.04	0.85 ± 0.01	0.86±0.02	0.86 ± 0.02	0.000**
SBP (mm Hg)	139±21.37	141±23.98	136±21.43	122±31.78	128±17.99	126±18.16	0.000**
DBP (mmHg)	77±10.71	77±7.39	78±8.85	76±11.04	76±11.35	78±12.13	0.826
FPG(mmol/L)	7.79±3.62	8.45±2.28	8.88±3.25	4.23±0.52	4.22±0.66	4.05±0.67	0.000**
H_bA1_C	7.92±1.93	8.43±1.58	7.88±2.1	6.21±0.57	5.98±0.38	5.93±0.35	0.000**
LDL (mmol/L)	2.46±0.85	2.43±0.6	2.53±0.87	3.62±0.74	3.19±1.06	3.28±0.97	0.000**
HDL(mmol/L)	1.24±0.39	1.33±0.37	1.34±0.32	1.28 ± 0.28	1.33±0.3	1.38±0.35	0.371
TG (mmol/L)	1.74 ± 2.01	1.81±1.65	2.19±1.9	1.29±0.67	1.1±0.59	1.26±0.67	0.004**
TC (mmol/L)	4.4±0.977	4.65±0.98	4.64±1.02	5.76±1.23	5.36±1.08	5.16±0.89	0.000**
CVD Risk %	4.23±1.74	4.11±1.34	3.71±1.33	3.85±1.88	4.1±1.31	3.95±1.27	0.558
Family history							
No	65.6%	51.2%	64.4%	33.3%	16.7%	13.6%	0.000**†
Yes	34.4%	48.8%	35.6%	66.7%	83.3%	86.4%	

**P value indicate that level significance between them P < 0.05

†Fisher Exact test

Also, in this study three banding pattern was observed for the MT2A gene, 238 (bp) which represent the Wild Type, 135 and 103 (bp) presence of the Homozygous and the three bands (238, 135 and 103 bp) as the Heterozygous pattern (Fig. 2). The genotype and allele distribution among T2DM and healthy individuals was considered in this study with the effect of MT2A gene (Table VI) which was significant among the selected Malaysian subjects (P= 0.000).

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Fig. 2 The PCR product of MT2A polymorphism after digestion with *Tsp451* enzyme. Lane F shows Wild Type. Lane A, E, G, J Heterozygous and lane B, C,H and I are Homozygous. Lane D is the negative control and lane K is the PCR product followed by DNA Marker (700 to 25bp)

GENOTYPI	TAE C AND ALLELIC DISTRI	BLE VI bution of MT2A F	OLYMORPHISMS	
Gene	Genotypes a Alleles	nd Case (%)	Control (%)	
MT2A genotypes	Wild type	64 (42.7)	6 (5)	
	Heterozygous	41 (27.3)	48 (40)	
	Homozygous	43 (35)	66 (55)	
P value	0.000**			
Alleles	G	0.56	0.25	
	С	0.44	0.75	
P value	0.000**			

***P*- value was calculated by χ^2 test with 2 x 2 contingency table and considered *P*<0.05 as significant.

IV. DISCUSSION

Based on the finding from this study, there are some clinical characteristics that showed significance difference between T2DM subjects and control category such as age, BMI, WHR, SBP, FPG, HbA1c, LDL, TG, TC, CVD risk ratio and family history with P value less than 0.05. Every significance parameter is reliable to said to be related with T2DM. First, T2DM usually occur in older age people and some studies reported it as adult onset T2DM (Van-Tilburg *et al.*, 2012; Saltiel, 2001).

A supportive fact from Riaz, 2010 highlighted that overweight and obesity happen to be the leading cause to T2DM thus, TChol, TG, BMI and LDL are all related to obese or overweight condition. This appalling state lead to the down regulated activity of insulin due to the accumulation of free fatty acids and tumor necrosis factor from excess adiposity (Van-Tilburg *et al.*, 2012).

Riaz, 2010 also reported that family history known as one of the risk factors in developing T2DM. It is believed that medical history is an important factor for the subjects' genetic makeup as hereditary issues. However, the significant difference between T2DM subjects and control subjects in this study shows that control subjects do have a family history as well which might be due to the lifestyle and environmental factors followed by untraceable/unknown factors. This study also reveals that there was an association between both polymorphisms; MT1A (rs11076161) and MT2A (rs10636) with T2DM subjects among Malay populations by showing significant difference when compared with the control group. For MT1A polymorphism, the genotype frequency accounts for AA, AG and GG correspond to 72.7%, 22.7% and 4.7% in T2DM subjects. It shows significantly different when control group's values were 15%, 55% and 30%. In contrast, a study conducted by Ganasyam *et al.*, in the Indian women population reported that MT1A does not have any association with increased susceptibility to T2DM in that population. However, Yang *et al.* Reported that MT1A were related to diabetes in neuropathy subjects (Yang *et al.*, 2008; Ganasyam *et al.*, 2012).

On the other hand, genotype distribution of MT2A polymorphisms was GG, GC and CC. That accounts for 42.7%, 27.3% and 35% in T2DM subjects compared to 5%, 40% and 55% in control subjects. The significant difference between both subjects declares that MT2A polymorphisms may be subjected to T2DM which was confirmed in neuropathy subjects as well as hyperlipidaemia condition (Yang *et al.*, 2008).

V. CONCLUSION

This study highlighted that there was a significant difference between both polymorphisms with T2DM subjects in comparison with healthy Malaysian subjects. However in the future we need to widen up the study of other ethnic in Malaysia particularly Chinese and Indian. Since the results of these polymorphisms show a significant difference, it could be determined as a genetic marker which has associated with T2DM. It is recommended to investigate the MT gene expression level in T2DM subjects in future with their siblings/offspring to increase our knowledge behind the pathways and proceed for target therapy which save the cost and improve the efficiency of treatment with minimum side effects in early future.

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