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No Association of TCF7L2 and ENPP1 Gene Polymorphisms in Malaysian Type 2 Diabetes Mellitus with or without Hypertension

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Abstract: Several genome-wide association studies were done extensively in many populations in finding the candidate genes predisposing to Type 2 Diabetes Mellitus (T2DM). In that way, Transcription Factor 7-Like 2 (TCF7L2) and Ectoenzyme Nucleotide Pyrophosphate Phosphodiesterase 1 (ENPP1) genetic variants were found to be associated with increased risk of T2DM in various populations. In this cross-sectional study, rs7903146 (C/T) polymorphism of TCF7L2 gene and K121Q polymorphism of ENPP1 gene was analyzed in T2DM with or without hypertension in Malaysian subjects. A total of 165 samples consisting of 50 T2DM without hypertension, 55 T2DM with hypertension and 60 healthy individuals were recruited for this study. Genomic DNA was amplified to determine the genotypes of rs7903146 (C/T) and K121Q polymorphisms using hot start PCR followed by RFLP method. The mean age for patient and control subjects was 57.26±10.02 and 45.65±10.93 years, respectively. There was no significant differences ($p>0.05$) found in genotype and allele frequency for both rs7903146 (C/T) and K121Q polymorphism of TCF7L2 and ENPP1 gene, respectively. This preliminary results show that both polymorphisms was not an independent risk factor to T2DM with or without hypertension in Malaysian subjects. However, replication studies in this population with larger sample size was strongly recommended.

Key words: PCR, RFLP, genetic polymorphism, risk factor, Malaysian population, hypertension

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a polygenic disorder caused by both genetic and environmental factors (O'Rahilly *et al*., 2008). According to National Health and Morbidity Survey III (NHMSIII) in Malaysia, the overall prevalence of diabetes mellitus (known and newly diagnosed) was 11.6%. Among adults (>30 years) the percentage was rose from 8.3% in NHMS II to 14.9% in NHMS III. The overall prevalence of hypertension for subjects aged 15 years was 27.8%. This could be due the dramatic life style changes, less awareness, treatment and control among Malaysians (Rampal *et al*., 2008).

In order to identify the candidate genes in predisposing to T2DM, several genome-wide association studies were done extensively in many populations (Sladek *et al*., 2007). In that way, variants in the Transcription Factor 7-Like 2 (TCF7L2) and Ectoenzyme Nucleotide Pyrophosphate Phosphodiesterase 1 (ENPP1) genes were strongly associated with increased risk of T2DM in many populations.

TCF7L2 gene spans 215.9 kb on chromosome 10q25, a region of replicated linkage to T2DM in Mexican American and Icelandic cohorts (Damcott *et al*., 2006). Recent meta-analysis report shows the association between TCF7L2 gene polymorphisms and susceptibility to T2DM (Tong *et al*., 2009). T allele of a single nucleotide polymorphism (rs7903146, C/T) found in the non-coding part of TCF7L2 gene has been consistently associated with T2DM in individuals of European (Grant *et al*., 2006), Indian (Chandak *et al*., 2007) and Japanese populations (Hayashi *et al*., 2007). However, conflicting results were also found in Han Chinese populations (Ng *et al*., 2007; Chang *et al*., 2007).

The ENPP1 gene is located at 6q22-23, a locus linked to obesity and diabetes (Lyon *et al*., 2006). ENPP1 plays a significant role in the development of insulin resistance and T2DM (Weedon *et al*., 2006). Several variants of ENPP1 have a primary role in mediating insulin resistance and in the development of both obesity and T2DM (Meyre *et al*., 2004). K121Q/rs1044498 (substitution of A base to C base at codon 121) polymorphism is a functional

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missense SNP at exon 4 found in ENPP1 gene causing the changes of amino acid from lysine to glutamine (Keshavarz *et al*., 2006). Q allele of K121Q polymorphism may identify individuals who are at risk of developing insulin resistance, a condition that predispose to T2DM (Abate *et al*., 2005), coronary artery disease (Pizzuti *et al*., 1999), cardiovascular complications in Caucasians from Italy and US populations (Bacci *et al*., 2005). In contrast, there was also some negative association found in Chinese (Chen *et al*., 2006), Japanese (Keshavarz *et al*., 2006) and German Caucasians populations (Gouni *et al*., 2006). Conflicting results have found in various populations in relation with T2DM. These ambiguous findings initiated us to determine the association of TCF7L2 and ENPP1 gene polymorphisms in Malaysian type 2 diabetic subjects with or without hypertension.

MATERIALS AND METHODS

Study subjects: The protocol of this study was approved by the Ethical Committee of the Faculty of Medical and Health Sciences, Universiti Putra Malaysia (UPM). A total of 165 subjects were recruited for this study from the period of April 2006-2007. Subjects from 50 T2DM without hypertension and 55 T2DM with hypertension were recruited from UPM Physician Clinic, Hospital Kuala Lumpur, whereas 60 unrelated healthy individuals were collected randomly. Informed written consent was obtained from all the participants of this study.

Physical measurements and DNA preparation: Individual weight and heights were obtained from all the subjects to calculate the Body Mass Index (BMI) using their weight in kilograms divided by the square of height in meters. The fasting blood glucose levels were obtained from the medical records of all the known diabetic subjects. For control subjects, fasting blood glucose level was measured using MediSense Precision-G blood glucose monitor.

All the control subjects were diagnosed according to the WHO criteria (random plasma glucose levels >11.1 mmol L^{-1} or a fasting plasma glucose level >7.0 mmol L^{-1}). Hypertension was defined as ≥140 mmHg of Systolic Blood Pressure (SBP) and ≥90 mmHg of Diastolic Blood Pressure (DBP) or who were receiving the anti-hypertensive therapy. Four-five milliliters of blood samples were collected from the subjects by a qualified phlebotomist. The serum was separated from the blood and the biochemical analysis was measured enzymatically on a Hitachi-912 Autoanalyser (Mannheim, Germany) using kits supplied by Roche Diagnostics (Mannheim, Germany). Genomic DNA extraction was carried out using the DNA isolation kit (BioBasic Inc., Canada). The purity of extracted DNA was quantified using Eppendorf UVette® in Biophotometer (Eppendorf, Hamburg, Germany).

TCF7L2 gene polymorphism: We genotyped the rs7903146 (C/T) polymorphism of TCF7L2 gene using forward primer (5'-AAGAGAAGATTCCTTTTTAAATGGTG-3') and reverse primer (5'-CCTCATACGGCAATTAAATTATACA-3') (Bodhini *et al*., 2007). A master mix (25 μL) for PCR reaction was prepared in each 0.2 mL PCR tube consists of double distilled water, 2 mM $MgCl_2$, 1x PCR buffer, 0.5 μL of BSA, 0.5 μL of 10 mM dNTPs, 20 Pico moles of each forward and reverse primer and the template DNA. The PCR was carried out on iCycler instrument (BioRad Laboratories, Hercules, California, USA) under the following cycling conditions; the initial denaturation was set up for 5 min at 95°C followed by 35 cycles of denaturation at 95°C, annealing at 56.5°C, extension at 72°C for 30 sec, respectively and the final extension was at 72°C for 5 min. The PCR product was stored at 4°C until further use. To avoid the unspecific bands 1 unit of *Taq* DNA polymerase was added to the mixture after the initial denaturation step. The amplified PCR product was separated on agarose gel electrophoresis (Promega, Madison, USA). The PCR products was digested with 2 units of Hpy-CH4III restriction enzyme (New England Biolabs, Beverly, MA, USA) at 37°C for 2 h with 1x NEB buffer 4 in a final volume of 20 μL reaction mixture followed by heat inactivation for 20 min at 80°C.

ENPP1 gene polymorphism: K121Q polymorphism of ENPP1 gene was amplified by PCR (Bacci *et al*., 2005) method using a set of primers (forward, 5'-GCAATTCTGTGTTCACTTTGGA-3' and reverse 5'-GAGCACCTGACCTTGACACA-3'). The PCR reaction mixture was carried out in a final volume of 25 μL consists of both forward and reverse primers, PCR master mix (i-PCR 5X Master Mix, iDNA, Singapore) and the template DNA. After an initial denaturation of 2 min at 94°C, the samples were subjected to 30 cycles for denaturation at 94°C for 1 min, annealing at 55°C for 40 sec and extension at 72°C for 40 sec. The PCR products were digested with *Avall* restriction enzyme (Fermentas) and NEB buffer 4 at 37°C for 2 h. The PCR digested products were separated by electrophoresis at 4% metaphor agarose gel performed in Origins electrophoresis tank (Elchrom Scientific AG, Switzerland) and stained with ethidium bromide. The restricted fragments were visualized in Alpha Imager™ 1220 (Alpha Innotech, San Leandro, CA). All the statistical analysis was carried out by using SPSS (Chicago, IL) software version 14.0 for Microsoft Windows. A level of $p<0.05$ was considered statistically significant.

RESULTS

We have analyzed the ENPP1 and TCF7L2 variants in type 2 diabetic patients with or without hypertension and compared with control subjects. A total of 165 samples consisting of 50 T2DM (30.30%), 55 T2DM with hypertension (33.30%) and 60 samples from unrelated healthy individuals (36.40%) were recruited for this study. The majority of the subjects were included in this study were male 90 as compared to female 75 subjects. Among the 3 major ethnic groups in Malaysia, Malays (68 (41.21%)) were higher compared to Chinese (36 (21.82%)) and Indians (61 (36.97%)). However, Indians comprised most of the samples in T2DM with hypertension (45.50%) when compared to Malays (34.60%) and Chinese (20.00%) subjects. In controls, Malays comprised higher (45.00%) as compared to Chinese (33.30%) followed by Indian subjects (21.70%). Patients were ranged from 31-84 years old, with a mean age of 57.25 years, while the controls were ranged from 27-74 years old with a mean age of 45.92 years.

We investigated the differences in age, blood pressure, BMI and results of clinical parameters according to the genotypes of rs7903146 (C/T) (Table 1) and K121Q polymorphism (Table 2) between the diabetic groups and the control subjects. The genotypes of CT polymorphism of TCF7L2 and K121Q of ENPP1 gene shows no significant difference with respect to age, BMI, SBP, DBP, BGL, LDL, HDL, TG and TC ($p>0.05$). However, the genotypes of K121Q of ENPP1 gene show significant difference in T2DM for SBP and TC when compared with controls.

Based on 4% metaphor agarose gel electrophoresis, the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) products of rs7903146 (C/T) and K121Q polymorphism of TCF7L2 and ENPP1 gene was determined, respectively. For TCF7L2 variant, Hpy-CH4III restriction enzyme has been used to digest the PCR product to yield the homozygous wild type (CC) fragments (112 bp), homozygous mutant type (TT) has no Hpy-CH4III cut which shows 136 and 112 bp represents the heterozygous type (Fig. 1). Genotypes of ENPP1 variant was carried out in 4% metaphor agarose gel electrophoresis using Avall restriction enzyme (Fig. 2). The heterozygous fragments show 208, 155 and 53 bp, while homozygous mutant fragment shows 155 and 53 bp and the wild type fragments represents 208 bp fragments.

Figure 3 shows the genotypic frequency of CT polymorphism of TCF7L2 and K121Q polymorphism of ENPP1 gene in the study subjects. The genotypic frequency of CT polymorphism for CC, CT and TT were 7 (14.00%), 32 (64.00%) and 11 (22.00%), respectively in T2DM ($p = 0.297$), whereas, in T2DM with hypertension was 16 (29.09%), 26 (47.27%) and 13 (23.64%) ($p = 0.63$) were observed, respectively as

Table 1: Association between rs7903146 (C/T) polymorphism and clinical characteristics of the subjects

Groups	Genotype	Age (years)	SBP (mm Hg)	DBP (mm Hg)	BMI (kg m^{-2})	BGL (mmol L^{-1})	HDL (mmol L^{-1})	LDL (mmol L^{-1})	TG (mmol L^{-1})	TC (mmol L^{-1})
T2DM	CC (n = 7)	57.00±5.720	127.00±6.950	75.43±5.60	28.21±4.11	9.08±1.21	0.82±0.26	4.58±1.76	2.24±0.73	5.92±1.63
(n = 50)	CT (n = 32)	58.60±10.50	126.47±8.980	73.66±9.02	28.28±3.67	11.89±5.21	0.73±0.22	4.24±1.48	2.04±1.09	5.37±1.59
	TT (n = 11)	54.55±10.58	123.28±12.31	74.64±8.91	26.27±3.05	12.5±6.54	0.68±0.28	3.29±1.18	1.84±0.55	4.42±1.26
	p-value	0.514	0.600	0.864	0.282	0.365	0.491	0.123	0.668	0.103
T2DM	CC (n =0)	56.75±9.180	162.56±21.61	93.13±3.97	27.13±2.510	9.37±2.02	0.86±0.40	3.39±1.17	1.66±0.38	4.86±1.18
+HPT	CT (n =0)	57.96±11.35	162.15±20.99	90.85±7.03	37.73±53.03	11.33±4.61	0.77±0.20	3.87±1.54	1.91±1.19	5.06±1.64
(n = 55)	TT (n =0)	56.62±8.490	155.77±13.73	95.54±6.92	26.74±3.870	11.62±5.53	0.84±0.35	4.04±2.10	2.28±0.88	5.40±2.26
	p-value	0.097	0.582	0.090	0.560	0.275	0.654	0.505	0.219	0.686
Control	CC (n =0)	49.42±9.680	127.67±8.250	78.75±7.77	24.63±3.90	5.01±1.25	1.08±0.41	3.63±1.46	1.55±0.75	5.35±1.68
(n = 60)	CT (n =0)	44.93±11.47	125.57±9.010	76.51±7.02	24.93±4.13	5.41±2.81	1.10±0.42	3.38±1.18	1.86±1.30	5.41±1.52
	TT (n =0)	43.43±9.360	127.71±4.230	82.71±4.39	25.04±3.26	5.06±0.77	0.89±0.27	3.96±1.04	2.17±0.73	5.64±1.11
	p-value	0.395	0.594	0.086	0.967	0.854	0.462	0.467	0.514	0.915

Table 2: Association between K121Q polymorphism and clinical characteristics of the subjects

Groups	Genotype	Age (years)	SBP (mm Hg)	DBP (mm Hg)	BMI (kg m^{-2})	BGL (mmol L^{-1})	HDL (mmol L^{-1})	LDL (mmol L^{-1})	TG (mmol L^{-1})	TC (mmol L^{-1})
T2DM	AA (n = 39)	59.03±10.28	124.44±8.76	74.23±8.04	27.64±4.58	12.68±5.89	0.76±0.20	3.89±1.32	1.89±0.91	5.07±1.36
(n = 50)	AC (n = 10)	55.4±8.71	133±4.22	75.7±7.65	29.31±3.47	9.21±2.11	0.68±0.29	4.32±2.00	2.19±1.20	5.47±2.16
	CC (n = 1)	53.0± 0.00	138.0± 0.00	85.0±0.00	23.87± 0.00	19.7±0.00	0.72±0.00	7.54±0.00	3.83±0.00	9.03±0.00
	p-value	0.521	0.007*	0.382	0.372	0.079	0.598	0.051	0.121	0.043*
T2DM	AA (n = 36)	57.19±10.63	160.28± 21.03	91.56±9.95	34.41±45.16	10.71±4.99	0.90±0.35	3.98±1.86	2.07±1.03	5.51±1.94
+HPT	AC (n = 18)	58.67±7.90	162.39±16.52	92.78±6.60	26.50±3.44	11.21±3.21	0.70±0.23	3.63±0.91	1.79±0.71	4.69±1.05
(n = 55)	CC (n = 1)	56.00±0.00	140.00±0.00	90.00±0.00	32.56±0.00	8.30±0.00	0.55±0.00	2.11±0.00	2.14±0.00	3.04±0.00
	p-value	0.862	0.538	0.875	0.762	0.793	0.059	0.429	0.590	0.120
Control	AA (n = 44)	45.59±10.79	124.89±9.30	76.68±7.38	24.02±4.22	5.50±3.36	1.15±0.44	3.32±1.23	1.79±1.32	5.27±1.50
(n = 60)	AC (n = 15)	44.73±11.02	125.80±8.94	77.53±6.23	23.63±3.26	4.64±0.84	0.89±0.32	3.97±1.30	1.58±0.94	5.51±1.52
	CC (n = 1)	67.00±0.00	135.00±0.00	85.00±0.00	23.01±0.00	5.10±0.00	1.20±0.00	4.14±0.00	1.78±0.00	5.70±0.00
	p-value	0.147	0.541	0.492	0.927	0.622	0.112	0.194	0.846	0.837

*$p<0.005$

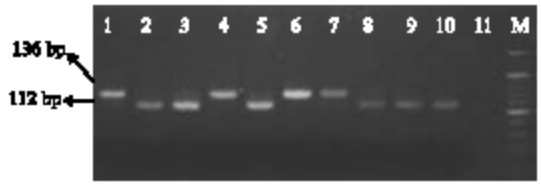

Fig. 1: Restriction fragment of rs7903146 (C/T) polymorphism in 4% metaphor agarose gel electrophoresis. M- 20 bp DNA ladder marker (Fermentas). Lane 1, 4, 6, 7 shows undigested mutant fragment (136 bp) while lane 2, 3, 5, 8, 9, 10 shows wild type (112 bp) and lane 11 was negative control without DNA

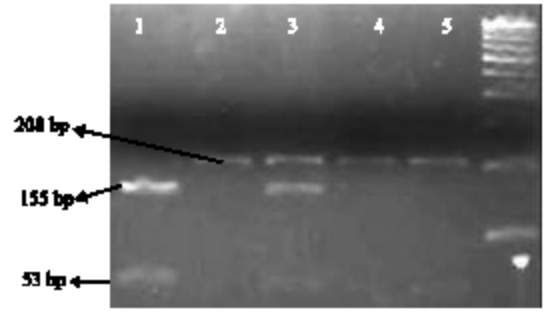

Fig. 2: Restriction enzyme fragments using AvaII of K121Q polymorphism in 4% metaphor agarose gel electrophoresis. Lane M shows the 100 bp DNA ladder (iDNA), Lane 1 shows a mutant fragment (155 and 53 bp), Lane 3 shows the heterozygous fragments (208, 155 and 53 bp) and Lane 2, 4 and 5 shows the wild type fragments (208 bp)

compared to 12 (20.00%), 41 (68.33%) and 7 (11.67%) in control subjects. The derived allele frequency for T allele of TCF7L2 gene was 54.00% in T2DM and 46.00% in T2DM with hypertension as compared to 45.83% of control subjects.

The genotypic frequency in ENPP1 gene for AA, AC and CC were 40 (80.00%), 9 (18.00%) and 1 (2.00%), respectively in T2DM (p = 0.674), whereas in T2DM with hypertension was 37 (67.27%), 17 (30.91%) and 1 (1.82%) (p = 0.774) were observed, respectively as compared to 44 (73.33%), 15 (25.00%) and 1 (1.67%) in control subjects. The derived allele frequency for C allele of K121Q polymorphism of ENPP1 gene was 22.00% in T2DM and 17.27% in T2DM with hypertension as compared to 14.17% of control subjects. No significant difference was observed in both genotypic and allelic frequencies of TCF7L2 and ENPP1 genetic variants among the case and control subjects (p>0.05).

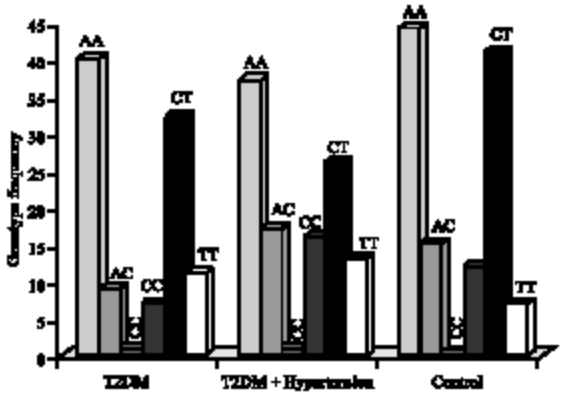

Fig. 3: The genotype frequency in TCF7L2 and ENPP1 gene polymorphism. AA, AC and CC shows the genotypes of K121Q polymorphism, while CC, CT and CC represents the genotypes of rs7903146 (C/T) polymorphism of TCF7L2 gene

DISCUSSION

In relation to TCF7L2 and ENPP1 genetic variants, several reports have been published from Asian populations (Bodhini *et al*., 2007). Our previous study (Ramachandran *et al*., 2008, 2009) observed a strong association ($p<0.05$) in angiotensin converting enzyme and angiotensinogen gene polymorphisms with essential hypertension and T2DM in Malaysian subjects. But, the role of the ENPP1 121Q allele and TCF7L2 T allele on predisposition to T2DM has not been firmly established in Malaysian population. This is the first report on TCF7L2 and ENPP1 genetic variants among type 2 diabetic subjects with or without hypertension in Malaysia. We failed to show the significant association between the TCF7L2 and ENPP1 genetic variants in Malaysian T2DM with or without hypertensive subjects.

TCF7L2 and ENPP1 genetic variants were extensively studied in many populations with conflicting results. The consistency of association evidenced by various replication studies (Zeggini *et al*., 2007; Hayashi *et al*., 2007) suggests that TCF7L2 gene represents an important locus for genetic susceptibility to T2DM compare to other diabetes susceptibility gene polymorphisms. In this study, TCF7L2 and ENPP1 genetic variants were analyzed in 105 diabetic subjects with and without hypertension and 60 unrelated healthy individuals. We did not detect any significant association of TCF7L2 and ENPP1 genetic variants with T2DM in Malaysian subjects. Our study supports to the other studies done in Chinese population (Chen *et al*., 2006), German Caucasians population (Gouni *et al*., 2006), Han Chinese population (Ng *et al*., 2007).

There were also no significance difference was observed for age, SBP, DBP, BMI and the lipid profiles in all the subjects for rs7903146 (C/T) genotypes except for SBP and TC with K121Q polymorphism of ENPP1 gene. This finding is in agreement with earlier studies, which also showed no significance difference in age and BMI (Horikoshi *et al*., 2007) with TCF7L2 variant. Ostaptchouk *et al*. (2007) suggested that BMI does not influence the risk polymorphism in TCF7L2 gene. Based on Fig. 3, there was no significant differences observed in genotype frequencies between T2DM subject and control subject for both CT and K121Q polymorphisms ($p>0.05$). Although, the frequency of T allele of rs7903146 (C/T) polymorphism was higher (64.24%) in T2DM with and without hypertension, it doesn't show any association as compared with control subjects ($p>0.05$). Similar results ($p = 0.390$) was observed in Han Chinese population for rs7903146 (C/T) polymorphism (Chang *et al*., 2007). The rs7903146 (C/T) polymorphism is a non-coding variant found in TCF7L2 gene may effect the findings of this study. However, contradictory results were also observed in many populations for the association of the non-coding variants with T2DM (Hayashi *et al*., 2007; Anuradha *et al*., 2005).

For K121Q polymorphism, no significant difference was found and the results were not similar to the report in South Asian population (Abate *et al*., 2005). However, some negative association was observed in Chinese populations (Chen *et al*., 2006) and German Caucasians population (Gouni *et al*., 2006) was supported to the findings of this study. The frequency of 121Q allele in our study was 14.40% and is lower than South Asian Indians (27.50-34.20%, Bodhini *et al*., 2007). One of the limitations of this study is that it did not address the functional consequences of these genetic variants. Our failure to reproduce associations of 2 selected variants in TCF7L2 and ENPP1 gene could be due to insufficient power and also the population stratification could influence the false association (Campbell *et al*., 2005).

CONCLUSION

This preliminary study failed to show any significant relationship with TCF7L2 and ENPP1 gene polymorphisms with T2DM in Malaysian subjects. However, this study has to further investigate by increasing the sample size at least twice larger than the current study is recommended to detect the association of these genetic variants in T2DM with or without hypertension in Malaysian population.

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