DOI: 10.7860/JCDR/2017/25072.9390

Original Article

Biochemistry Section

Association of +62 G>A Polymorphism in the Resistin Gene with Type 2 Diabetes Mellitus among Thais: Case-Control Study

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ABSTRACT

Introduction: Resistin gene (RETN) polymorphisms in humans may have a role in the pathogenesis of Type 2 Diabetes Mellitus (T2DM) and insulin resistance. There is still lack of evidence on association between +62 G>A polymorphism in the RETN and T2DM among Thais.

Aim: To determine the effect of polymorphisms at +62 G>A of RETN on Thai T2DM.

Materials and Methods: This matched case control study was conducted with a total of 360 samples from all regions of Thailand (180 Thai new T2DM cases and 180 non-T2DM Thais for control) were enrolled. The RETN +62G>A polymorphism were detected using the Polymerase Chain Reaction (PCR) method. Conditional logistic regression was performed to test the association between +62 G>A polymorphism and T2DM.

Results: Among 360 samples that were enrolled, only 350 samples completed molecular analysis. It was found that GA+AA genotype frequencies in T2DM cases was higher than control by 16% (95% CI: 6.0%, 27.0%, p-value=0.002). After adjustments for possible confounders, multivariable analyses by conditional logistic regression showed that the RETN+62 G>A polymorphism was statistically associated with Thai T2DM ($OR_{adjusted}$ =1.84, 95% CI: 1.03, 3.31, p-value=0.04). Other factors such as; low educational attainment ($OR_{adjusted}$=3.87, 95%CI: 1.60, 9.36), hypertension ($OR_{adjusted}$=3.07, 95%CI: 1.56, 6.04), had both obese father and mother ($OR_{adjusted}$=1.94, 95%CI: 1.06, 3.56) and triglyceride≥150 ($OR_{adjusted}$=2.18, 95% CI: 1.18, 4.02) were statistically associated with Thai T2DM (p-value<0.05). While regular consumption of glutinous rice was found to be a protective factor ($OR_{adjusted}$=0.29, 95%CI: 0.13, 0.64).

Conclusion: These findings suggest that RETN polymorphism at position +62 G>A may increase the susceptibility to T2DM in Thais.

Keywords: Metabolic syndrome, Molecular analysis, Risk factors, Thailand

INTRODUCTION

There is global increase of chronic diseases especially Diabetes Mellitus (DM). Worldwide, one in ten adults live with diabetes [1] and 63.0 % of all deaths are caused by chronic diseases [2]. In Thailand, 7.5% of adults aged ≥20 years had DM, with an estimate of 3.2 million people [3].

Among diagnosed cases of diabetes, about 90.0% to 95.0% adult had T2DM. Most of T2DM patients had insulin resistance condition, an abnormity in which the cells do not use insulin suitably [4]. The insulin resistance in type 2 diabetes has been studied extensively. It was found that the fat cell (adipocyte) is a cell that plays an important role in inhibition or stimulates the signal transformation of insulin hormone within the bloods streams [5]. Fat cells produce adipokine and adipocytokine substances to control energy used in the human body, storage and transport of triglyceride in the fat cells, control of insulin function in the metabolism of lipid and glucose. Adipokine substance is secreted from fat cells, with various types such as Tumour Necrosis Factor-alpha (TNF-a), free fatty acid, leptin, Plasminogen Activator Inhibitor-1 (PAI-1), interleukin-6, adiponectin, and resistin hormone [5,6].

RETN gene increased protein level in obesity related insulin resistance in mouse. These genes are inhibiting phosphorylation process of Insulin Receptor Substrate (IRS) of pTy-IRS-1and pTy-IRS-2 and inhibiting signal transformation of insulin in Phosphorylated Adenosine Monophosphate-Activated Protein Kinase (pAMPK) and Phosphorylated Protein Kinase B/Akt (pAkt) resulted in impaired glucose uptake of cells and inhibit glycolysis [7,8]. However, the molecular mechanisms of RETN gene in human and animal studies are still controversial [9-12]. The plasma resistin concentration may be a biomarker for the diagnosis of metabolic syndrome in Asian populations [13,14] as well as Thais [15], and Finnish [16]. The polymorphism of RETN is associated with increased resistin concentrations among such population. However, among Americans, Caucasians, and Italians there were not significant association between resistin levels and RETN polymorphism [17-19].

RETN gene polymorphisms may have a role in the pathogenesis of T2DM and insulin resistance. Previous studies indicated that RETN 3'-Untranslated Region (UTR) Single Nucleotide Polymorphism (SNP) + 62 G>A were associated with T2DM among Chinese [20] but inconclusive for German [21]. SNP at +299 G>A was identified as having relationship with T2DM among Thai people [22]. However, these studies mostly identified the influence of RETN gene polymorphism on T2DM without controlling the effect of behavioural related covariates. Among Thais T2DM, there is still lack of evidence on the roles of RETN at +62 G>A polymorphism and effect of comorbidity. Therefore, this matched case control study was conducted to determine the influence of SNP+62 G>A on Thai T2DM.

MATERIALS AND METHODS

This matched case control study was performed in four districts of each region of Thailand. The data collection was conducted between May to October 2014.

The population in this study were Thai people who were newly diagnosed with T2DM (Case), based on the criteria of the American Diabetes Association (ADA) [23], Fasting Plasma Glucose level (FPG) ≥126 mg/dl and Haemoglobin A1c (HbA1c)≥6.5% and aged ≥35 years. The control were Thai who were not having T2DM based on the ADA criteria, had FPG level <100 mg/dl and HbA1c <5.7 %, had no family history of T2DM, and aged ≥ 35 years. The controls were matched with case based on age (±2 years from case), and sex.

We excluded those who received coronary heart disease treatment, had chronic kidney disease, thyroid, pancreatic disease or severe active inflammatory diseases, receiving insulin, as well as receiving medications that could affect glucose metabolism or weight. The exclusion was performed using primary screening form.

Sample size calculation formula for matched pair study and conditional logistic regression statistic of Ejigou A was applied to estimate the sample size [24]. The study used 1:1 of case: control ratio. A multi stage random sampling was performed to select the case and control in four regions of Thailand (the Tha Wung district in Central, Kut Chap district in Northeast, Phrao district in North and Kanchanadit district in South regions). We selected four provinces from all regions and then selected one district in each province by simple random sampling. The total of 180 T2DM cases were selected based on the inclusion criteria (45 persons per province). We selected 180 persons for control group (45 persons per province), who were living in same community with the case. This study proposal was approved on March 11, 2014 (HE571037) by the Ethics Committee for Human Research of Khon Kaen University, Thailand. All samples gave written consent before data collection.

Data Collection

After screening, a structured questionnaire interview was performed. The questionnaire was developed from instruments of Global Physical Activity Surveillance of World Health Organization (WHO) [25], research of Chanchay S et al., Usay P et al. and Suebsomran P, consisted of six parts as follows: 1) Demographic information; 2) Food consumption habits; 3) Smoking and alcohol consumption behaviours; 4) Physical activities; 5) Stress; 6) Laboratories results. All the interviews were conducted by trained health personnel with interview guideline [26-28].

The demographic, behaviour and life style questions retrieved information regarding the following characteristics: age (in years), marital status, occupation, educational attainment, health insurance schemes, domicile, family history of diabetes, food consumption habits, smoking, drinking alcohol, stress, and level of physical activity which applied the Global Physical Activity Questionnaire (GPAQ) [25] and family history of obesity and comorbidity using medical records.

Physical measurements were obtained using standardized techniques and calibrated equipments. The factors assessed in physical examination were weight, height, waist circumference and blood pressure obtained by medical technicians, nurses and physician. Blood tests among new T2DM cases were: HbA1c, fasting plasma glucose, Total Cholesterol (TC), Triglyceride (TG), High Density Lipoprotein Cholesterol (HDL), and Low Density Lipoprotein Cholesterol (LDL) levels.

Instrument quality control: Content validity was performed by five specialists, who were public health experts, biochemistry specialists and DM specialists. Try out of the instruments were among 30 subjects in Nakhon Ratchasima province in Thailand.

RETN gene +62G>A polymorphism Analysis

The four steps of SNP analysis of RETN gene:

Step1. Extraction of Genomic DNA from blood samples: For SNPs test of RETN gene, Polymerase Chain Reaction (PCR) was used to extract the genomic DNA for template. The genomic DNA was extracted from EDTA treated whole blood samples using Standard protocol for purification of genomic DNA from human whole blood [29]. The genomic DNA was resuspended in Tris-EDTA (TE) buffer (pH 8.0) and was ready for direct use or stored at –20ºC until used.

Step2. Amplification of RETN gene by PCR: The DNA fragments of the SNP +62 G>A was amplified using PCR with following primer +62G>AF (3'UTR) forward 5′-AGAGTCCACGCTCCTGTGTT-3′ and +62G>AR reverse 5′-CATCTCCAGGTTTATTTCCAGC-3′ [20]. Each PCR reaction contains 100 ng of genomic DNA templates, 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, and enhancer solution), 2 mM $MgCl_2$, 2.5 mM Deoxynucleotide (dNTP) mix, 0.5 µM of each primer, and 1 unit of *i*-Taq™ DNA polymerase (iNtRON Biotechnology, Korea). The annealing temperatures for PCR were optimized by gradient PCR. The PCR was carried out as follows: preheating at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The condition was repeated for 40 cycles. The 368 bp PCR products were detected by 1.5% agarose gel electrophoresis and then stained with ethidium bromide to confirm the size of SNP.

Step 3. Genotyping of RETN gene SNP by -Restriction Fragment Length Polymorphism (PCR-RFLP) technique: For SNP genotyping, the PCR products (1 µg) of SNP+62 G>A were digested by 2.5 units of *BseR*I restriction enzymes (NEB, United Kingdom) at 37°C for at least three hours. After that, the fragments were separated by electrophoresis on 1.5% agarose gel.

Step 4. Searching DNA sequencing of RETN gene SNP: To confirm the result of SNP detection, DNA sequencing was used from some of resistin gene by using PCR product, which was mentioned in previous steps. Two samples of a positive SNP and two samples of negative SNP were selected for confirmation, using 3 µg of each unpurified PCR product and DNA sequencing by specific primer as mentioned in the PCR step deliver Biobasic Inc. laboratory of Canada for DNA sequencing analysis. It was used to compare the difference of DNA sequence alignment with Genbank data base, and identified position of SNP by BioEdit software.

STATISTICAL ANALYSIS

Data entry and statistical analysis were performed using Stata version 13.0 (Stata Corp, College Station, TX) for Windows. We used descriptive statistics including frequency, percentage, median, mean, Standard Deviation (SD), minimum (min), maximum (max) and inferential statistics including linear regression, bivariate conditional logistic regression testing assumptions; linearity relationship, outlier test (considered p-value ≤0.25 and important variables), Multivariable analysis with backward elimination by conditional logistic regression model, p-value<0.05 of wald test and interested factors that were remained in the model.

RESULTS

The demographic and baseline clinical characteristics of the cases and control are summarized in [Table/Fig-1]. Among 360 samples who were enrolled, their mean ages in cases and control were 50.6±7.2 years and 50.6±7.1 years respectively, the highest proportions of age group was 45 to 54-year-old (41.7% and 43.3% respectively), females (59.4% and 59.4% respectively), married (77.2% and 77.9% respectively), secondary school or lower of educational attainment (87.8% and 69.5% respectively), farmer occupation (54.4% and 45.6% respectively).

Regularly food consumption habits of samples in each region of Thailand: As shown in [Table/Fig-2], majority of the samples (54.7%) ate white rice regularly, and 36.9% ate glutinous rice regularly, of which mostly found in the Northeast followed by the North. The highest proportion of those who regularly ate fatty pork, pig leg, chicken skin was in the Central. The Central and the Southern samples were more likely to have very sweet foods or with coconut milk and fried foods. Sweet drinks were regularly consumed by the Central and the Northeastern samples.

Genotype frequencies of +62 (G>A) polymorphisms among Thais: Since the frequency of the AA genotype was low, we divided the enrolled subjects into two groups: GG and GA+AA (heterozygous combined with homozygous mutant types). As shown in [Table/Fig-3], among 350 samples who completed polymorphism analysis, it was found that the rate of GA+AA genotype frequencies (SNP +62) in T2DM cases was higher than the control's by 16.0% (95% CI: 6.0%, 27.0%, p-value=0.002).

Characteristics	Case (n=180)		Control (n=180)	
	number	%	number	%
Gender				
Male	73	40.6	73	40.6
Female	107	59.4	107	59.4
Age				
35 to 44 years	40	22.2	41	22.8
45-54 years	75	41.7	78	43.3
≥ 55 years	65	36.1	61	33.9
	Mean = 50.6, SD. =7.2, Median = 52, Min. = 35, Max. = 62		Mean = 50.6, SD.= 7.1, Median= 52, Min.=35, Max.= 60	
Educational Attainment; Secondary school or lower	158	87.8	135	69.5
Occupation; Farmer	98	54.4	82	45.6
Having history of hypertension	68	37.8	26	14.4
Having history of hyperlipidemia	36	20.0	18	10.0
No Family history of diabetes	116	64.5	180	100.0
Obese father and mother	58	32.2	40	22.2
Never smoker	156	86.7	154	85.6
Never drink alcohol	168	93.3	160	88.9
High level of physical activity; (METs levels)	53	29.4	58	32.2
High stress	35	19.4	25	13.9
BMI/Obesity status				
BMI ≤22.9 (normal)	44	24.4	76	42.2
BMI = 23.0 – 24.9 (overweight)	44	24.5	32	17.8
BMI ≥25.0 (obese)	92	51.1	72	40.0
	Mean = 25.6, SD.= 4.2, Median= 25.2, Min =15.8, Max =40.7		Mean =24.5, SD.= 4.3, Median= 23.9, Min =14.5, Max =41.5	
Abdominal obesity by Waist to hip ratio (WHR)	109	60.6	97	53.9

[Table/Fig-1]: Demographic and baseline clinical characteristics of the samples.

Regularly food consumption habits	North		Northeast		Central		South		Total (%)
	Case (%)	Control (%)	Case (%)	Control (%)	Case (%)	Control (%)	Case (%)	Control (%)	
Glutinous rice	17	30	35	34	1	0	7	9	133
	(37.8)	(66.7)	(77.8)	(75.6)	(2.2)	(0.0)	(15.6)	(20.0)	(36.9)
White rice	27	13	8	5	42	43	26	33	197
	(60.0)	(28.9)	(17.8)	(11.1)	(93.3)	(95.6)	(57.8)	(73.3)	(54.7)
Brown rice	2	2	3	0	4	5	2	6	24
	(4.4)	(4.4)	(6.7)	(0.0)	(8.9)	(11.1)	(4.4)	(13.3)	(6.7)
Fatty pork, pig leg, chicken skin	3	3	2	2	10	10	0	6	36
	(6.7)	(6.7)	(4.4)	(4.4)	(22.2)	(22.2)	(0.0)	(13.3)	(10.0)
Very sweet foods, or with coconut milk	2	3	6	10	15	10	7	11	64
	(4.4)	(6.7)	(3.3)	(22.2)	(33.3)	(22.2)	(15.6)	(24.4)	(17.8)
Fried food	7	7	3	1	20	17	5	11	71
	(15.6)	(15.6)	(6.1)	(2.2)	(44.4)	(37.8)	(11.1)	(24.4)	(19.7)
Sweet drink, sweet fruit drink, tea/coffee with sugar	3	3	12	11	14	18	1	12	74
	(6.7)	(6.7)	(26.7)	(24.4)	(31.1)	(40.0)	(2.2)	(26.7)	(20.6)

[Table/Fig-2]: Regularly food consumption habits of samples in each region of Thailand.

Genotype	T2DM Case (n=175)	Control (n=175)	Rate different of SNP between case and control	95% CI	p-value
GG	88 (50.3%)	116 (66.3%)	16.0%	6.0, 27.0	0.002
GA+AA	87 (49.7%)	59 (33.7%)			

[Table/Fig-3]: Genotype frequencies of +62 G>A polymorphisms among Thais between T2DM case and control.

Association of each factor on T2DM susceptibility: As shown in [Table/Fig-4], the bivariate analysis indicated that the factors that were statistically significant (p-value<0.05) associated with T2DM in Thai people were SNP+62 G>A of RETN gene, educational attainment, having history of hypertension, had obese father and mother, and triglyceride.

DISCUSSION

SNP in RETN which is a gene that control the function of insulin could impair insulin function in taking sugar in blood to cell, resulted in hyperglycaemia, insulin resistance and T2DM [5,6]. Our hypothesis was that the RETN gene polymorphism at +62 G>A might have effect on Type 2 DM. Our multivariate analysis observed

Factors	Case	Control	Bivariate Analysis		Multivariate Analysis		
			OR	95%CI	$OR_{adjusted*}$	95%CI	p-value
SNP + 62 (G>A)							0.040
GG	88	116	1		1		
GA/AA	87	59	2.12	1.32, 3.41	1.84	1.03, 3.31	
Educational Attainment							0.003
High school or higher	158	135	1		1		
Secondary school or lower	22	45	2.92	1.51, 5.62	3.87	1.60, 9.36	
Having history of Hypertension							0.001
No	112	154	1		1		
Yes	68	26	3.47	2.02, 5.95	3.07	1.56, 6.04	
Having history of hyperlipidemia							0.074
No	144	162	1		1		
Yes	36	18	2.38	1.24, 4.55	2.20	0.92, 5.27	
Had both obese father and mother							0.031
No	122	140	1		1		
Yes	58	40	1.72	1.05, 2.82	1.94	1.06, 3.56	
Eating glutinous rice regularly							0.002
No	120	107	1		1		
Yes	60	73	0.61	0.35, 1.06	0.29	0.13, 0.64	
Physical activity							
(METs levels)							0.337
High	53	58	1		1		
Moderate & Low	127	122	1.14	0.73, 1.80	1.36	0.72, 2.56	
Stress							0.115
Low	26	34	1		1		
Moderate & high	154	146	1.44	0.79, 2.63	1.90	0.86, 4.20	
BMI							0.223
BMI <25.0	44	76	1		1		
BMI ≥ 25.0	136	104	1.65	1.05, 2.57	1.45	0.80, 2.63	
Triglyceride							0.012
<150	81	112	1		1		
≥150	99	68	2.0	1.30, 3.08	2.18	1.18, 4.02	
Total cholesterol							0.103
<185	53	61	1		1		
≥185	127	119	1.24	0.80, 1.94	1.77	0.89, 3.51	
LDL cholesterol							0.161
≤100	54	38	1		1		
>100	126	142	0.62	0.38, 1.01	0.61	0.31, 1.21	

[Table/Fig-4]: Association of each factor on T2DM susceptibility based on conditional logistic regression.
*Adjusted for baseline measurements and other covariates, including educational attainment, having history of hypertension, having history of hyperlipidemia, had both obese father and mother, eating glutinous rice regularly, physical activity, BMI, stress, TG, TC and LDL.

that SNP+62G>A was significantly raised ($OR_{adjusted}$ 1.84, 95% CI 1.03, 3.31, p-value=0.04) while controlling other covariates. It was similar with a Chinese study that indicated the RETN gene SNP+62 was related to T2DM [20]. However, it was different from a German study which found that SNP+62 had no significant influence on T2DM [21].

When considering the SNPs in other positions of RETN gene in previous studies, we found some positions in this gene are risk factors of DM, such as SNP -420 (C>G) in Japanese [30,31] and SNP +299 (G> A) in Thais [22]. These studies supported the findings of our study that RETN gene had influences on T2DM in Asian people. In addition, a study in Venezuela indicated that the G/A genotype of RETN gene among Metabolic Syndrome group had found SNP+62 G>A more frequently than those with no metabolic alterations [32]. It is similar with a Taiwanese research that indicated allele A in this SNP were higher among ischemic cerebral vascular disease group than the healthy group as well as those with higher fasting plasma glucose had more allele A in this SNP than those who had lower fasting plasma glucose [33].

The previous study among Thais found the association between T2DM and SNP+299 G>A of RETN [22]. SNPs at+299G>A and –420C>G were associated with increased resistin levels in Thais. However, the two SNPs were not associated with metabolic syndrome including glucose, lipids, blood pressure and waist circumference among Thais [15]. Some studies had conflicting results in term of the association between resistin levels and metabolic syndrome [34]. Since there were no insulin level measurements in this study, it cannot conclude that resistin is associated with T2DM through insulin resistance mechanism. Therefore, additional studies are needed to identify mechanism of RETN with regard to insulin resistance and also metabolic syndrome.

Our study indicated that those who had SNP+62G>A of RETN gene had 2.12 times higher risks of T2DM in bivariate analysis, quite a strong effect. However, after adjusting for possible confounders, the effect of SNP+62G>A of RETN gene was slightly decreased to 1.84 times. Similarly, BMI and having history of hyperlipidemia showed statistically significant effect on Thai T2DM in bivariate analysis but there was not significant association after adjusting for confounders. It could be explained that some modifiable factors might interact with non modifiable factors for their effect on T2DM.

In addition, our study found that there were some factors that had effect on T2DM such as those who had both obese father and mother had higher risk of having T2DM by two times. Concerning the modifiable factors of Thai T2DM, we could indicate other risk factors, similar with those found in previous studies. These risks factors were hypertension [35], triglyceride [36], and eating glutinous rice indicated that food consumption patterns was associated with T2DM [37].

Our study found that eating glutinous rice regularly can reduce the risk of T2DM among Thais. Most of those who are eating glutinous rice regularly have lived in the North East and the North regions of Thailand. On the other hand, those who have lived in the Central were more likely to eat white rice regularly and also consuming more sweet, carbohydrates and fatty foods than those in other regions. This pattern of food consumptions was similar with the diabetes prevalence survey, that the Central region of Thailand had the highest prevalence of T2DM [3]. In addition, Harvard Health identified that the Glycaemic index of glutinous rice was lower than that of jasmine rice [38], which is the most popular rice among white rice consumed by the Thais. Consistent with the Glycaemic Index Testing and Research of the University of Sydney identified that Glycaemic load of glutinous rice was lower than white rice [39]. Therefore, it is possible that Thais who ate glutinous rice regularly would have lower risk of T2DM than those who ate white or jasmine rice. Even though brown rice could help reducing the risk of T2DM, our study found very few Thais ate this rice.

This study also indicated that those with low educational attainment had three times higher risks of T2DM, which may coincide with the

previous studies that low income have effect on T2DM [40] since those with low educational attainment have lower chances to get good job. Low educational attainment should be identified as a new indicator for T2DM high risk screening. There are also some factors that their association with T2DM were still inconclusive in this study but were found associated in other studies such as alcohol consumption [18], smoking [18,40], BMI [40], waist-to-hip ratio/ abdominal obesity [22,35]. It may be because our study had small proportion of the above factors, if there are bigger sample size these outcome might show their stronger relationships.

The strength of this study is that the samples were recruited from all four regions of Thailand, we controlled some confounding factors which were age and sex through matched cases and controls. In addition, the multivariate analysis which included both modifiable and non modifiable factors with conditional logistic regression analysis process into the final model is also another important measure to control the effect of covariate on the results.

LIMITATION

There are a few limitations of our study. As this is case control study, our sample size was relatively small, although it had enough power to show the association.

CONCLUSION

This study found the RETN gene polymorphism at position +62 G>A is associated with susceptibility to T2DM in Thais. In addition, other covariates including: had obese father and mother, low educational attainment, hypertension, and triglyceride increased the risk of T2DM, whereas eating glutinous rice regularly was a protective factor. Findings from this study should be taken into consideration for proactive prevention strategies for T2DM high risk screening including gene detection.

Financial or Other Competing Interests: This work was financially supported by training center for enhancing quality of life of working age people, Faculty of Nursing, Khon Kaen University, Khon Kaen, Thailand.

ACKNOWLEDGEMENTS

This study was funded by the Training Center for Enhancing Quality of Life of Working Age People, Khon Kaen University. The Faculty of Medicine, Khon Kaen University supported for laboratory tests.

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FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Oct 26, 2016**
Date of Peer Review: **Nov 16, 2016**
Date of Acceptance: **Dec 08, 2016**
Date of Publishing: **Feb 01, 2017**