

ADIPOQ POLYMORPHISMS AMONG THAIS WITH PRE-DIABETES

Surasak Chaikhiandee¹, Benjaluck Phonrat², Anchalee Tungtrongchitr³,
Kanjana Suriyaprom⁴, Somlak Chuengsamarn⁵, Chavit Uttamachai¹
and Rungsun Tungtrongchitr¹

¹Department of Tropical Nutrition and Food Science, ²Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok; ³Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok; ⁴Faculty of Medical Technology, Rangsit University, Pathum Thani; ⁵Division of Endocrinology and Metabolism, Faculty of Medicine, HRH Princess Maha Chakri Sirindhorn Medical Center, Srinakharinwirot University, Nakhon Nayok, Thailand

Abstract. Studies have shown that polymorphisms of adiponectin gene (*ADIPOQ*) are associated with risk of developing type 2 diabetes mellitus (T2DM). However, no studies have investigated the association between genetic variants of *ADIPOQ* and pre-diabetes, a group at higher risk for developing T2DM. A total of 75 pre-diabetes and 130 normal subjects were recruited from volunteers in Bangkok, Thailand. Individuals with pre-diabetes were selected based on American Diabetes Association diagnostic criteria. Six *ADIPOQ* polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism technique. *ADIPOQ* polymorphism rs266729 C>G is significantly associated with pre-diabetes ($p = 0.006$). CG/GG genotypes were found among 60% and 40% of pre-diabetes and normal subjects, respectively. SNP rs266729 C>G was associated with increased pre-diabetes risk (OR = 2.64; 95% CI: 1.18-5.89, $p = 0.018$). No significant differences were found between pre-diabetes and normal subjects for other *ADIPOQ* polymorphisms. However, haplotype analysis revealed that haplotype GGTAAT is significantly associated with pre-diabetes when compared with GCGAAC reference haplotype (OR = 22.31; 95% CI: 1.37-361.93, $p = 0.03$). Our data indicate that *ADIPOQ* rs266729 C>G polymorphism may contribute to the genetic risk of pre-diabetes and provide preliminary data useful in genetic screening for pre-diabetes among Thais.

Keywords: *ADIPOQ*, polymorphism, pre-diabetes, Thais

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex disease characterized by fasting

Correspondence: Rungsun Tungtrongchitr, Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok, 10400, Thailand.

E-mail: rungsun.tun@mahidol.ac.th

or postprandial hyperglycemia (WHO, 1999). Individuals with pre-diabetes, identified as an early stage of T2DM, have an increased risk of developing T2DM (ADA, 2014). Approximately 5-10% of pre-diabetic patients will progress to diabetes each year (de Veegt *et al*, 2001; Diabetes Prevention Program Research Group, 2002).

Changes in adipose tissues cause alterations in metabolic and endocrine

functions to secrete numerous proteins, including adiponectin, which are associated with T2DM (Marseglia *et al*, 2015). Adiponectin is an abundant secretory protein in plasma (0.01% of total protein) encoded by *ADIPOQ* located on chromosome 3q27 (Ouchi *et al*, 2003). The gene (approximately 16 kb) consists of three exons and two introns (Prakash *et al*, 2015). There is strong evidence indicating that adiponectin plays key roles in regulating energy homeostasis and enhancing insulin sensitivity in muscle and liver by suppressing hepatic gluconeogenesis and stimulating fatty acid oxidation in skeletal muscle (Pérez-Martínez *et al*, 2008). Previous studies reported that administration of adiponectin decreases body weight and improves insulin sensitivity and glucose tolerance in experimental animal models (Whitehead *et al*, 2006; Zhang *et al*, 2015). Moreover, *ADIPOQ* was identified as a susceptibility locus for metabolic syndrome and T2DM (Vionnet *et al*, 2000).

Differences in circulating adiponectin levels can be explained by genetic variations in 30-70% of the cases (Comuzzie *et al*, 2001). A number of genome wide association studies (GWAS) showed an association of *ADIPOQ* variants with adiponectin levels (Heid *et al*, 2006; Ling *et al*, 2009). A number of *ADIPOQ* single nucleotide polymorphisms (SNPs) is associated with T2DM in different populations (Hara *et al*, 2002; Yang *et al*, 2008; Biswas *et al*, 2011), suggesting that *ADIPOQ* is a susceptibility gene for T2DM. SNP rs266729 (-11377 C>G), located in *ADIPOQ* promoter, is associated with T2DM in Asian populations (Han, 2011; Chu *et al*, 2013).

In the Thai population, genetic association of *ADIPOQ* rs266729 (-11377 C>G) of which T2DM has been reported (Suriyaprom *et al*, 2010). There is, however, no study to date that has examined

the association between *ADIPOQ* variants and pre-diabetes condition. Thus, this study investigated the association between six *ADIPOQ* polymorphisms and pre-diabetes among Thais. The findings will provide a preliminary database for genetic screening of pre-diabetes among Thais.

MATERIALS AND METHODS

Subjects

This retrospective study used stored DNA and blood specimens derived from volunteers in Bangkok, Thailand. Volunteers were divided into two groups: 75 in pre-diabetes and 130 in normal. Subjects were identified as pre-diabetes according to the American Diabetes Association diagnostic criteria (ADA, 2014), namely, fasting plasma glucose (FPG) of 100 mg/dl (5.6 mM) to 125 mg/dl (6.9 mM) or hemoglobin A1c (HbA1c) of 5.7-6.4%. Normal subjects have FPG < 100 mg/dl (5.6 mM) or HbA1c < 5.7%. Subjects were excluded if they were diagnosed with diabetes (FPG level \geq 126 mg/dl (7.0 mM) or an HbA1c \geq 6.5%).

The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2014-050-02) and prior informed consent was obtained from all participants.

Clinical and biochemical measurements

Clinical parameters, including body weight, height, waist circumference, and systolic and diastolic blood pressure, were measured using standard procedures. FPG, HbA1c and lipid profile [total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)] were assessed using standard enzymatic methods. Insulin level was measured by radioimmunoassay (Millipore, St Charles,

MI) and insulin resistance (IR) by homeostasis model assessment (HOMA-IR), calculated as glucose (mg/dl) x insulin (μ U/ml)/405 (Matthews *et al*, 1985).

ADIPOQ genotyping

DNA was extracted from peripheral leukocytes using Flexi gene DNA kit (Qiagen, Hildren, Germany). Genotyping was carried out by PCR-restriction fragment length polymorphism (RFLP) using Gene Amp PCR system 9700 (Applied Biosystems, Carlsbad, CA). PCR reaction was performed at a total volume of 50 μ l containing 50-100 ng of DNA, 1X PCR buffer, 0.2 mM dNTPs, 1.25 U *Taq* polymerase (Thermo Scientific, Rockford, IL) and 0.4 μ M each primer pair for six *ADIPOQ* SNPs (Suriyaprom *et al*, 2010; Ramya *et al*, 2013). PCR amplification was performed using a thermal cycler (Biometra, Göttingen, Germany) (Table 1). Cycling conditions were performed by initial denaturation at 94°C for 5 minutes; followed by 32 cycles of denaturing at 94°C for 30 seconds at annealing temperature of each specific primer (Table 1), and elongation at 72°C for 30 seconds, then the final extension at 72°C for 7 minutes. Amplicons were digested with 10 μ of restriction enzymes, separated by 3% agarose gel-electrophoresis and the ethidium bromide-stained bands recorded in a documentation system (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM, Armonk, NY). Data are expressed as median and interquartile range (IQR). Categorical variables between the two groups were compared using chi-square test and continuous variables between two groups using Mann-Whitney *U* test. Binary logistic regression analysis was employed to iden-

Table 1
Primers and restriction enzymes used in the study.

| rs number | ADIPOQ position | Location | Primer sequence (5'→3') | Annealing temp (°C) | Enzyme | Amplicon size (bp) | Fragment size (bp) |
|-----------|-----------------|----------|--|---------------------|--------------|--------------------|--------------------|
| rs266729 | -11377 (C>G) | Promoter | ACTTGCCCTGCCCTCTGCTG GCCTGGAGAACTGGAAGCTG | 62 | <i>HhaI</i> | 251 | 137, 114 |
| rs822393 | -4522 (C>T) | Intron 1 | TTCCAAATCGGTGAGCTTTC AGGGCTTGGGAAGATTAGG | 58 | <i>HinfI</i> | 202 | 144, 73 |
| rs822396 | -3971 (A>G) | Intron 1 | CAGGCTGATCGCACCTATTA CATCCTTTTCTGCTGGGAAA | 62 | <i>TruI</i> | 458 | 259, 199 |
| rs2241766 | 45 (T>G) | Exon 2 | GAAGTAGACTCTGCTGAGATGG TATCAGTGTAGGAGGTCGTGTG | 60 | <i>SmaI</i> | 372 | 219, 153 |
| rs1501299 | 276 (G>T) | Intron 2 | TCTCTCCATGGCTGACAGTG AGATGCAGCAAAGCCAAAGT | 55 | <i>BsmI</i> | 468 | 320, 148 |
| rs3774261 | 712 (G>A) | Intron 2 | TTCTGATTCCCTTCTCTGTC ATACATAGGCACCTCTCC | 54 | <i>RsaI</i> | 756 | 531, 225 |

Table 2
Clinical and biochemical characteristics of study groups.

| Characteristic | Normal (n = 130) | Pre-diabetes (n = 75) | p-value |
|---------------------------|-------------------------------|--------------------------|---------|
| Age (years) | 40.0 (29.8-51.0) ^a | 59.0 (52.0-68.0) | <0.001 |
| Weight (kg) | 58.0 (51.0-64.2) | 68.0 (59.0-74.0) | <0.001 |
| Height (cm) | 160.0 (155.0-165.0) | 157.0 (151.0-165.0) | 0.139 |
| Waist circumference (cm) | 78.0 (71.0-84.0) | 91.0 (85.0-97.0) | <0.001 |
| BMI (kg/m ²) | 22.4 (20.4-24.8) | 26.9 (24.4-30.1) | <0.001 |
| Systolic pressure (mmHg) | 112 (102-123) | 132 (121-144) | <0.001 |
| Diastolic pressure (mmHg) | 70 (64-82) | 74 (68-85) | <0.001 |
| Glucose (mg/dl) | 91 (86-95) | 113 (103-120) | <0.001 |
| HbA1c (%) | 5.1 (4.9-5.3) | 5.9 (5.6-6.2) | <0.001 |
| Insulin (μU/ml) | 12.5 (9.7-15.2) | 13.4 (9.1-18.8) | 0.274 |
| HOMA-IR | 2.8 (2.1-3.5) | 3.6 (2.5-5.3) | <0.001 |
| Cholesterol (mg/dl) | 198 (178-222) | 159 (142-184) | <0.001 |
| Triglyceride (mg/dl) | 8 (63-111) | 109 (89-139) | <0.001 |
| LDL-C (mg/dl) | 129 (107-151) | 94 (77-116) | <0.001 |
| HDL-C (mg/dl) | 63 (52-73) | 50.0 (40-60) | <0.001 |

^aData presented as median (interquartile range).

tify association of *ADIPOQ* variants with susceptibility to pre-diabetes. Haplotype frequency and association analysis were estimated using SNPStat web tool (Sole *et al*, 2006). A two-sided *p*-value < 0.05 is considered statistically significant.

RESULTS

Clinical and biochemical parameters between study groups

Distribution of age in pre-diabetes group is significantly (*p* < 0.05) than normal group (Table 2). Weight, waist circumference, BMI, and systolic and diastolic blood pressure are significantly higher in pre-diabetes group compared to normal group (*p* < 0.05). FBG level, HbA1c, HOMA-IR and triglyceride level also are significantly higher in pre-diabetes group (*p* < 0.05). However, pre-diabetes group have significantly lower LDL-C and HDL-C than normal group (*p* < 0.05).

Genotype and allele frequency of *ADIPOQ* variants in study groups

Of the six *ADIPOQ* SNPs studied (rs1501299 G>T, rs266729 C>G, rs2241766 T>G, rs822396 A>G, rs3774261 G>A, and rs822393 C>T) only the distribution of rs266729 C>G is statistically significant different between the two study groups (*p* = 0.006) with the minor allele frequency significantly higher among pre-diabetes group (*p* = 0.019) (Table 3). Binary logistic regression analysis confirmed the association between pre-diabetes and rs266729 C>G [odds ratio (OR) = 2.64; 95% confidence interval (95% CI) : 1.18-5.89, *p* = 0.018], after adjustment for age, sex and BMI (Table 4).

Haplotype frequency and association analysis in study groups

Only the frequency of GGTAAT haplotype is significantly associated with pre-diabetes when compared to reference

Table 3
Genotype and allele frequency of *ADIPOQ* polymorphism in study groups.

| SNP | Normal (n = 130) | Pre-diabetes (n = 75) | p-value |
|-------------------|----------------------|--------------------------|---------|
| rs1501299 (+276) | | | |
| GG | 73 (61) ^a | 35 (47) | 0.065 |
| GT+TT | 47 (39) | 39 (53) | |
| Allele G | 185 (77) | 102 (69) | 0.075 |
| Allele T | 55 (23) | 46 (31) | |
| rs266729 (-11377) | | | |
| CC | 78 (60) | 30 (40) | 0.006 |
| CG+GG | 52 (40) | 45 (60) | |
| Allele C | 201 (77) | 100 (66) | 0.019 |
| Allele G | 59 (23) | 50 (33) | |
| rs2241766 (+45) | | | |
| TT | 65 (50) | 43 (57) | 0.338 |
| TG+GG | 64 (50) | 32 (43) | |
| Allele T | 183 (71) | 116 (77) | 0.159 |
| Allele G | 75 (29) | 34 (23) | |
| rs822396 (-3971) | | | |
| AA | 93 (72) | 59 (79) | 0.299 |
| AG+GG | 36 (28) | 16 (21) | |
| Allele A | 221 (86) | 133 (89) | 0.387 |
| Allele G | 37 (14) | 17 (11) | |
| rs3774261 (+712) | | | |
| AA | 39 (32) | 22 (30) | 0.715 |
| AG+GG | 82 (68) | 52 (70) | |
| Allele A | 132 (54) | 81 (55) | 0.972 |
| Allele G | 110 (45) | 67 (45) | |
| rs822393 (-4522) | | | |
| CC | 48 (37) | 25 (33) | 0.578 |
| CT+TT | 81 (63) | 50 (67) | |
| Allele C | 157 (61) | 90 (60) | 0.865 |
| Allele T | 101 (39) | 60 (40) | |

^aDistributions of *ADIPOQ* genotype (%).

GCGAAC haplotype (OR = 22.31; 95% CI: 1.37-361.93, $p = 0.03$) (Table 5).

DISCUSSION

Six SNPs found in *ADIPOQ* (rs1501299 G>T, rs266729 C>G, rs2241766 T>G, rs822396 A>G, rs3774261 G>A, and rs822393 C>T) have been reported to be

associated with development of T2DM (Mackawy, 2013; Ramya *et al*, 2013). Our results among a small number of Thai volunteers without clinical evidence of T2DM showed a significant association of rs266729 C>G with pre-diabetes group. Previously Supriyaprom *et al* (2010) reported an association of rs266729 C>G with T2DM in Thai patients who have lower

Table 4
Binary logistic regression analysis with adjustments for age, sex and BMI.

| Variable | OR | 95% CI | p-value |
|----------|------|-----------|---------|
| 11377C>G | 2.64 | 1.18-5.89 | 0.018 |
| Age | 1.12 | 1.08-1.16 | <0.001 |
| BMI | 1.31 | 1.17-1.46 | <0.001 |

Table 5
ADIPOQ haplotype frequency and association analysis in study groups.

| Haplotype | Haplotype frequency | | OR | 95% CI | p-value |
|-----------|---------------------|--------------|-------|---------------|---------|
| | Normal | Pre-diabetes | | | |
| GCGAAC | 0.2251 | 0.1620 | 1.00 | Reference | |
| GGTAGT | 0.1538 | 0.2001 | 1.89 | 0.70-5.12 | 0.21 |
| GCTAGC | 0.1426 | 0.1443 | 1.19 | 0.38-3.70 | 0.76 |
| GCTGGC | 0.1119 | 0.0825 | 2.52 | 0.82-7.78 | 0.11 |
| TCTAAC | 0.0849 | 0.1271 | 1.84 | 0.49-6.93 | 0.37 |
| TCTAAT | 0.0773 | 0.0603 | 0.95 | 0.27-3.37 | 0.94 |
| TGTAAT | 0.0599 | 0.0575 | 2.05 | 0.53-7.88 | 0.30 |
| GCGAAT | 0.0437 | 0.0238 | 0.89 | 0.14-5.83 | 0.90 |
| GCTAGT | 0.0419 | 0.0110 | 1.40 | 0.11-18.23 | 0.80 |
| TGTAAC | 0.0191 | NA | 20.28 | 0.87-472.72 | 0.06 |
| GGTAAT | 0.0077 | 0.0244 | 22.31 | 1.37-361.93 | 0.03 |
| GCGGAC | 0 | 0.0244 | 0.07 | 0.00-1,469.89 | 0.60 |

plasma adiponectin level. A number of ADIPOQ SNPs in the proximal promoter region, including rs266729 C>G, are associated with levels of circulating adiponectin and risk of developing T2DM (Hara *et al*, 2002; Vasseur *et al*, 2002; Wassel *et al*, 2010).

Plasma adiponectin level is one of the predictive factors for pre-diabetes (Kim *et al*, 2013). A meta-analysis by Lai *et al* (2015) indicated that adiponectin levels are significantly lower in pre-diabetes patients compared with healthy controls, suggesting that a decrease in the level of circulating adiponectin occurs before progression to T2DM. In an animal study, low

levels of adiponectin occur in early obesity and lead to an increase in IR and development of T2DM (Hotta *et al*, 2001). Onat *et al* (2008) reported lower adiponectin levels may contribute to IR in obese women. The mechanism is thought to be due to adiponectin activation of AMP-activated protein kinase through adiponectin receptor in muscle and liver, which stimulates fatty acid oxidation and inhibits activity of gluconeogenic enzymes resulting in decreasing triglyceride level and increasing insulin sensitivity (Kadowaki and Yamauchi, 2005).

Our study found that CG/GG geno-

types and frequency of the G allele are significantly higher in pre-diabetes than normal subjects, in accordance with a previous study showing that CG and GG genotypes increase the risk of T2DM by two-folds and three-folds, respectively, when compared with the CC genotype after adjustment for age and sex (Kaftan and Hussain, 2015). In addition, the minor G allele of rs266729 was associated with pre-diabetes, in agreement with the meta-analysis report of the G allele being predisposed to T2DM (Li *et al*, 2013). The rs266729 G allele results in high *ADIPOQ* promoter activity due to alteration in SP1 binding site (Wang *et al*, 2009). Other transcription factors also play a role as suppressors of *ADIPOQ* expression (Barth *et al*, 2002).

Previous studies reported that *ADIPOQ* gene polymorphisms and haplotype were associated with increased risk of developing T2DM (Du *et al*, 2011; Ramya *et al*, 2013). Our study also found that the GGTAAT haplotype conferred increased risk of pre-diabetes in Thais.

To the best of our knowledge, this is the first study to demonstrate an association of *ADIPOQ* polymorphisms and pre-diabetes, especially of rs266729. However, this study has a number of limitations. Firstly, adiponectin level was not measured, thus the functional significance of *ADIPOQ* polymorphism discovered could not be determined. Secondly, interactions between genetics and environment factors, such as dietary intake and physical activity, may affect the significance of polymorphisms, which were not evaluated in the study. Further investigations are warranted to examine the relationship between behavioral factors and *ADIPOQ* polymorphisms in T2DM in the Thai population.

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