

Adiponectin Gene Polymorphisms and Type 2 Diabetes among Singaporean Chinese Adults

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Abstract

Background: Adiponectin is the most abundant circulating adipokine in human that regulates insulin actions. Association of adiponectin gene variations with type 2 diabetes (T2DM) has been reported albeit predominantly in non-Asian populations. Additionally, proof of variant functionality beyond statistical association is often unavailable. We studied six common (minor allele frequency ≥ 0.05) adiponectin single nucleotide polymorphisms (SNPs) in Singaporean Chinese adults with follow-up functional genetic experiments.

Methods: In a case-control study (N=588), genotyping of six common adiponectin haplotype tagging SNPs [-3964A>G(rs822396), +45T>G(rs2241766), 276C>A(rs1501299), 973G>A(rs3774262), 4551G>C(rs1063539) and 5852G>A(rs6444175)] was performed using Taqman genotyping assay. Allele-dependent differential efficiency of mRNA expression was tested with quantitative real time PCR using human subcutaneous and omental adipose tissues.

Results: Distributions of genotypes for all SNPs among controls were consistent with Hardy-Weinberg Equilibrium. Single locus, genotyped-based analysis suggested borderline significant ($P=0.07$) association between an exon-2 coding-synonymous +45T>G(rs2241766) and T2DM. We demonstrated that the relative mRNA expression of adiponectin gene was ~80% lower among carriers of minor G allele in human subcutaneous adipose tissue (N=43, $p<0.001$). The observed allele-dependent differential expression was replicated (~50% reduction) in an independent sample of human omental adipose tissue (N=52, $p<0.005$).

Conclusions: Our data was indicative of possible association between +45T>G(rs2241766) and T2DM among Singaporean Chinese adults. Functional experiments in both human subcutaneous and omental adipose tissue suggested that polymorphisms in +45T>G(rs2241766) may be associated with differential allelic expression.

Keywords: Adiponectin; Single nucleotide polymorphisms; Type 2 diabetes; Genotype

Introduction

Adiponectin is the most abundant circulating adipocytokine in human that positively regulates insulin actions. Reduced plasma concentration of adiponectin has been well documented in obesity, insulin resistance, T2DM and coronary artery disease [1]. In corollary, low circulating adiponectin has been found to be a strong predictor of T2DM amongst Asian Indians [2]. Experiments on mouse model suggested that adiponectin played a role in energy homeostasis by modulating insulin sensitivity in liver [3]. These data suggested that adiponectin could be an important adipocytokine protective against the development of T2DM and cardiovascular disease [2,4]. Therefore, adiponectin is an attractive candidate gene for T2DM.

The heritability of plasma adiponectin concentration as a quantitative trait was estimated to be approximately 0.46 [5]. This suggested that genetic variations were important determinants of adiponectin level. However, despite many years of intensive studies, whether or not adiponectin gene was associated with plasma adiponectin concentration and T2DM remained controversial [6]. For example, Hara et al. found that +45T>G(rs2241766) and +276C>A(rs1501299) had strong association with T2DM and insulin resistance in Japanese [7]. However, no association was found in Pima Indians [8]. Two other different alleles: -4522C>T and 276C>A were recently reported to be associated in obese (BMI $\geq 25\text{kg/m}^2$) Chinese subjects with T2DM [9].

If adiponectin gene were to be associated with T2DM, little consensus existed on which variant(s) could be the functional (i.e. causal) allele. Amongst the variants studied, +45T>G(rs2241766) appeared to be one of the most promising candidates. For instance,

the G allele has been reported to be associated with higher DNA transcription efficiency and is associated with lower body mass index (BMI) in Taiwanese [10]. Additionally, plasma adiponectin concentration and insulin sensitivity have been reported to vary significantly according to +45T>G(rs2241766) genotype [11]. Nevertheless, given that +45T>G(rs2241766) is a coding synonymous allele, considerable doubt exists on its functionality. Taken together, these data suggested substantial allelic heterogeneity in the association between adiponectin gene and T2DM. Therefore, beyond statistical association, accumulating data based on functional genetic experiments will be helpful in suggesting the exact identity of causal variant [12].

In this study, our group investigated six common (minor allele frequency, MAF ≥ 0.05) adiponectin SNPs (including rs2241766) widely reported to be associated with various aspects of metabolic phenotype (e.g. T2DM) in Singaporean Chinese - a relatively understudied population. Follow-up functional genetic experiments were conducted using human subcutaneous and omental adipose tissue.

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Material and Methods

Subjects

Using a case-control study design, we investigated the relationship between six adiponectin SNPs and T2DM in 588 Singaporean Chinese adults. The cases (N=300) were individuals with known T2DM diagnosed according to the 1998 World Health Organization criteria. These individuals were recruited from a single secondary care hospital. Controls (N=288) were individuals who has no known history of T2DM, not taking anti-diabetic medications and have fasting plasma glucose (FPG) of < 7.0 mM. Samples of the study were de-identified before analysis. The study was approved by our institutions' Domain Specify (ethics) Review Board and written informed consent was obtained from all participants.

Genomic DNA extraction and genotyping

Blood samples collected from the participants were subjected for routine clinical biochemistry on Cobas Integra 800 Chemistry Analyzer (Roche, Switzerland). Demographic and clinical data were ascertained from existing medical records. Genomic DNA was extracted from peripheral blood leucocytes of all the subjects using standard phenol chloroform method.

Genotyping in adiponectin gene: -3964A>G(rs822396), 973G>A(rs3774262), +45T>G(rs2241766), 276C>A(rs1501299), 4551G>C(rs1063539), and 5852G>A(rs6444175) were performed using Taqman genotyping assays (7300 real time PCR, Applied Biosystems). To verify genotyping error rate, a subset of study population (~30%) was re-genotyped using bi-directional sequencing (Prism 3130 Genetic Analyzer, Applied Biosystems)). Allele assignment was found to be in complete concordance between initial and repeat genotyping experiments.

RNA extraction from human subcutaneous and omental adipose tissue

To understand the potential functionality of candidate variants at molecular level, we studied adiponectin gene transcription efficiency (mRNA copies) using human adipose tissue. Human subcutaneous and omental adipose tissues were obtained during an elective laparoscopic bariatric surgery. Total RNA from known genotype (TT vs. GT and GG) was extracted from the adipose tissue using Trizol (Qiagen). RNA was reverse transcribed according to the manufacturer's recommendation (High capacity reverse transcribe, Applied Biosystems).

Quantitative real time PCR

Allele dependent differential efficiency of mRNA expression was performed using SYBR green quantitative real time PCR (7300 real time PCR, Applied Biosystems). The rarity of minor allele (G) resulted in limited number of subjects having homozygous minor allele genotype, thereby precluded meaningful statistical analysis according to genotype. More importantly, a recent study based on Asian population reported that difference in +45T>G gene function conformed to a dominant genetic model [11]. Therefore, we decided to analyze our functional genetic data based on a dominant genetic model. The primer used for adiponectin were 5'-ATC GGT GAA ACC GGA GTA CC-3' and 5'-GCA TGT TGG GGA TAG TAA CGT AA-3' and β -actin were 5'-CAT GTACGT TGC TAT CCA GGC-3' and 5'-CTC CTT AAT GTC ACG CAC GAT -3'. The expression of adiponectin was normalized against β -actin. Technical duplicate were performed for each genotype.

Statistical analysis

The data are presented as means \pm SD. Continuous and categorical

variables were compared using Student's T-test and Chi-square test respectively. Genotype distributions were tested at each polymorphic locus (among controls) for departure from Hardy-Weinberg Equilibrium (all $p>0.05$). Pair-wise linkage disequilibrium coefficients (r^2) were estimated and plotted using the Haploview (version 4.1). Given that most of the SNPs chosen were tag SNP, the pair-wise LD (r^2) was expectedly low, ranging from 0 to 0.66. Allele based (1 df), Cochran Armitage trend test (1 df) and genotype based (2 df) analysis were performed for each SNP. For +45T>G(rs2241766), logistic regression was employed to estimate the relative risk (odds ratio, OR) conferred by GG homozygosity and adjustment of confounders. A two-sided p-value was used and a $p<0.05$ was considered statistically significant. Power of the study was estimated using freeware Quanto version 5.1.

Results

The clinical characteristics of the study groups were summarized in Table 1. The control (N = 288) were 27% male, age 38 ± 12 years, body mass index (BMI) 22.8 ± 3.8 kg/m², systolic blood pressure (SBP) 120 ± 18 mmHg, diastolic blood pressure (DBP) 76 ± 11 mmHg, fasting plasma glucose 5.1 ± 0.5 mM. The cases (N = 300) were 58% male, age 64 ± 10 years, duration of diabetes 16 ± 8 years, BMI 25.5 ± 3.6 kg/m², SBP 139 ± 17 mmHg, DBP 79 ± 9 mmHg, HBA1c 7.8 ± 1.4 %. As expected, SBP, DBP and BMI were higher among cases as compared to control ($p<0.05$).

The genotype distribution and allele frequencies did not differ significantly between cases and control for the five adiponectin haplotype tagging SNPs known as -3964A>G (rs822396), +276C>A (rs1501299), 973G>A (rs3774262), 4551G>C (rs1063539), and 5852G>A (rs6444175) tested individually in our study subjects Table 2. Genotyped based and Cochran Armitage Trend test of these five SNPs were also negative (data not shown). Using Quanto 5.1, we estimated that our study had more than 80% power to detect a relative risk of > 1.4 conferred by any of the minor alleles.

However, the genotype distribution of +45T>G (rs2241766) between cases and controls reached borderline statistical significant ($\chi^2 = 5.3$, df = 2, $p=0.07$) Table 3. The genetic model (addictive or otherwise) of rs2241766 is not well defined; we re-explored the association by assuming a recessive genetic model for this allele. The distribution of homozygous GG genotype among cases (GG = 12%, GT + TT = 88%) was significantly higher than control (GG = 6.6%, GT + TT = 93.4%) ($\chi^2 = 5.06$, df = 1, $p = 0.025$) Table 3. Relative risk (odd ratio) conferred by the GG genotype = 1.89 (95% CI 1.09-3.30, $p = 0.024$) which remained statistically significant after adjusting for differences in BMI.

Given that +45T>G(rs2241766) showed suggestive statistical

	Study groups		
	Controls	Cases	p-value
Number	288	300	
gender (% males)	27	58	
Age (years)	38 ± 12	64 ± 10	< 0.05
SBP (mmHg)	120 ± 18	139 ± 17	< 0.05
DBP (mmHg)	76 ± 11	79 ± 9	< 0.05
BMI (Kg/m2)	22.8 ± 3.8	25.5 ± 3.6	< 0.05
FPG (mM)	5.1 ± 0.5	-	
HBA1c (%)	-	7.8 ± 1.4	
Duration (years)	-	16 ± 8	

Table 1: Clinical characteristics of the study subjects

Study groups				
Polymorphisms Alleles	Controls (%)	Cases (%)	χ^2	p-value
-3964A>G rs822396				
G frequency	43 (15.68)	40 (15.56)	0.00055	0.97
A frequency	230 (84.32)	217 (84.44)		
276C>A rs1501299				
G frequency	165 (77.80)	259 (79.94)	0.14	0.59
T frequency	47 (22.20)	65 (20.06)		
973G>A rs3774262				
G frequency	194 (71.75)	181 (67.29)	0.47	0.34
A frequency	76 (28.25)	88 (32.71)		
4551G>C rs1063539				
G frequency	192 (69.81)	185 (68.61)	0.034	0.8
C frequency	83 (30.19)	85 (31.39)		
5852G>A rs6444175				
G frequency	193 (70.33)	173 (67.65)	0.17	0.57
A frequency	81 (29.67)	82 (32.35)		

Table 2: Genotype and allele frequency distribution of the five adiponectin haplotype tagging SNPs among cases and controls.

Polymorphisms Alleles	Controls (%)	Cases (%)	χ^2	P-value	P value (Cochrane-Armitage trend test)
Number	288 (100)	300 (100)			
TT	140 (48.6)	143 (47.7)	5.3	0.07	0.238
TG	129 (44.8)	121 (40.3)			
GG	19 (6.6)	36 (12.0)			
T frequency	0.71	0.68	0.64	0.43	-
G frequency	0.29	0.32			
TT & GT	269 (93.4)	264 (88.0)	5.06	0.025	-
GG	19 (6.6)	36 (12.0)			

Table 3: Genotype and allele frequency of SNP +45T>G(rs2241766) among cases and controls.

evidence indicative of an association with T2DM among Singaporean Chinese, we conducted follow-up functional genetic experiments to test for differential allelic expression. In subcutaneous adipose tissue (N=43), transcriptional efficiency of adiponectin was ~80% lower ($p<0.001$) among carriers of minor G allele (Figure 1A). This observation was replicated in an independent sample of human omental adipose tissue (N=52), which showed ~50% reduction ($p<0.005$) in the mRNA expression among the G allele carriers (Figure 1B). When stratified by gender, our results suggested that the allele specific differential gene expression associated with +45T>G(rs2241766) in adipocytes harvested from both subcutaneous and visceral compartment were consistent and independent of gender (supplementary data). Our data suggested that +45T>G(rs2241766), despite being a coding synonymous SNP, may be associated with allele dependent transcriptional efficiency.

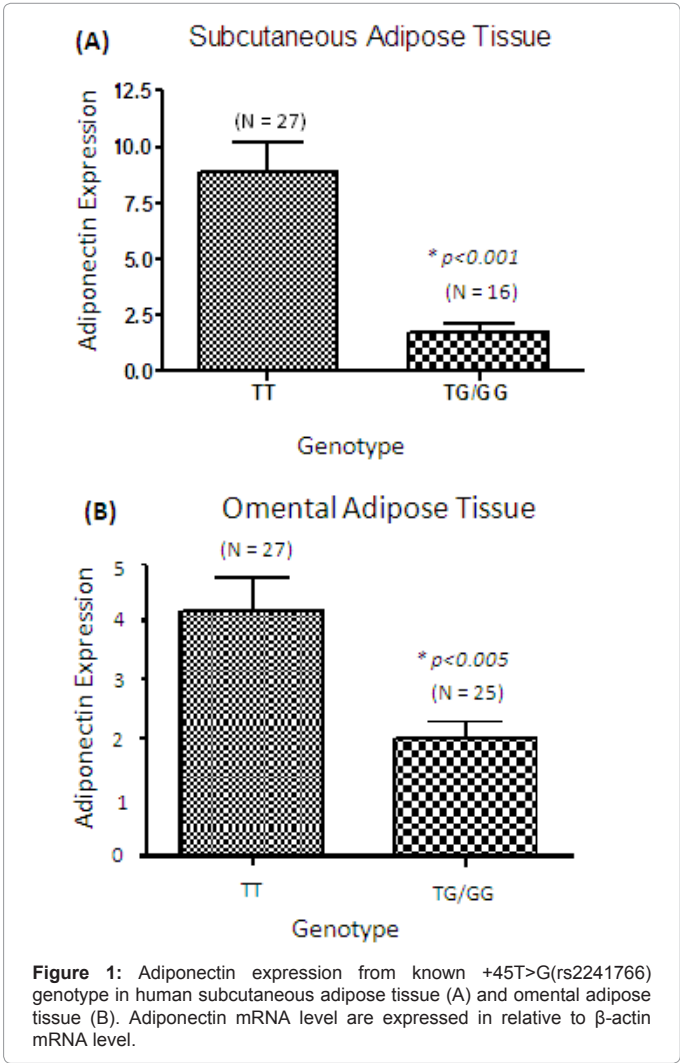
Discussion

In a fairly large Asian case-control study, our data suggested that adiponectin SNP +45T>G(rs2241766) might be associated with increased risk for T2DM. Despite being a coding synonymous SNP, follow-up functional genetic experiments in both human subcutaneous and omental adipose tissue consistently revealed that the minor G allele of +45T>G(rs2241766) might be associated with 50-80% allele-dependent reduction in transcriptional efficiency. Taken together, +45T>G(rs2241766) could be a candidate adiponectin SNP for T2DM among Singapore Chinese.

Among all the adiponectin SNPs examined, the synonymous variation +45T>G (rs2241766) at exon 2 is one of the variants most widely reported to be associated with T2DM. Consistent with these

reports, our data revealed borderline significant ($P=0.07$) association between +45T>G(rs2241766) and T2DM in Singaporean Chinese adult. However, the a priori probability of an association between +45T>G(rs2241766) and T2DM based on existing extensive literature, the relationship could still be plausible [13]. Given that rs2241766 does not change amino acid sequence, it is thus necessary to demonstrate its functionality beyond statistical evidence [14]. Therefore, we conducted functional molecular genetics experiments (stratified by rs2241766 genotype) using human adipose tissues.

Our data suggested that the minor G allele was associated with ~80% reduction in mRNA expression in the subcutaneous adipose tissue. This observation was validated in an independent sample of omental adipose tissue (~50% reduction in gene expression efficiency associated with G allele). Therefore, +45T>G(rs2241766) appeared to demonstrate allele-dependent transcriptional efficiency. This is in keeping with an evolving body of evidence which suggested that up to 50% of human genome might demonstrate differential allelic expression secondary to a network of cis and trans-acting elements [15]. Exactly how a synonymous SNP can influence transcriptional efficiency is however unclear. One purported mechanism is that the variant may alter the transcriptome splicing of the RNA, thus resulting in the instability of the mRNA [16]. Taken together, our data suggested



that +45T>G (rs2241766) could be a causal variant associated with T2DM.

The strength of our paper included studying a fairly large Asian cohort and focusing on common adiponectin variants based on the common disease common variant hypothesis [17]. Besides, public health implication of common allele is important because of its substantial population attributable risk. In addition, beyond providing statistical evidence, we conducted functional genetic study using human adipose tissues from different body compartment. The consistent results obtained from both subcutaneous and omental adipose tissue strengthened the validity of our observations that +45T>G(rs2241766) may exhibit allele-dependent differential efficiency of adiponectin gene expression. There are however several limitations in our study. Firstly, our sample size is only sufficient to detect variant with effect size >1.4. Hence, there is still a possibility of a very weak association between those negative SNPs (rs822396, rs1501299, rs3774262, rs1063539 and rs6444175) and T2DM, which may require a larger sample size to detect its presence in our population. For the same reason, our limited study power might be the reason behind the borderline association ($P=0.07$) observed between +45T>G(rs2241766) and T2DM. Secondly, we may not have thoroughly addressed the issue of confounding by population admixture. However, we have confined our study subjects to Singaporean Chinese who are almost exclusively immigrants from Southern China, thereby limiting the effect of population stratification [18].

In conclusion, our data suggested that +45T>G(rs2241766) may be associated with T2DM among Singaporean Chinese adults. Follow-up functional genetic experiments revealed that the coding synonymous SNP may exhibit allele-dependent differential efficiency of adiponectin gene expression in both human subcutaneous and omental adipose tissue. Our data suggested that +45T>G(rs2241766) could be a causal variant associated with T2DM.

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